Elucidating the Neural Mechanism Underlying the Timing of Puberty in the Female Rat

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Elucidating the Neural Mechanism Underlying the Timing of Puberty in the Female Rat

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ABSTRACT

Puberty is a developmental process initiated by maturation of the hypothalamic-pituitary-gonadal (HPG) axis. Kisspeptin plays a critical role in puberty timing and reproduction. In rodents, kisspeptin is located mainly within the hypothalamic anteroventral periventricular (AVPV) and the arcuate (ARC) nuclei. Although distinct roles are ascribed to the two different kisspeptin populations in control of the HPG axis, namely the preovulatory luteinising hormone (LH) surge for AVPV, and the gonadotrophin-releasing hormone (GnRH) pulse generator for ARC, the underlying mechanisms remain to be fully characterised. A moderate knockdown of kisspeptin in the ARC had no effect on pubertal timing, but resulted in abnormal oestrous cyclicity and decreased LH pulse frequency. In addition, it decreased the amplitude but not the incidence of spontaneous LH surges. Knockdown of kisspeptin in the AVPV resulted in a significant delay in puberty, abnormal oestrous cyclicity, and reduced occurrence of spontaneous LH surges, but had no effect on LH pulse frequency. These results suggest a critical importance for AVPV but not ARC kisspeptin neurones in the control of pubertal timing. Despite the classic association of AVPV kisspeptin neurones in LH surge generation, the results of this study suggest that both AVPV and ARC populations are essential for normal LH surges and oestrous cyclicity.

Stress activates the hypothalamic-pituitary-adrenal (HPA) axis and generally
disrupts the HPG axis. The central mechanisms underlying changes in pubertal timing as a consequence of metabolic status or stressful perturbations are not fully understood. A key region of the brain is the amygdala, particularly the posterodorsal subnucleus of the medial nucleus (MePD) and the central nucleus (CeA), noted for their role in modulating reproductive function and response to stress. It has been shown that neurotoxic lesioning of the MePD resulted in advanced puberty, and decreased anxiety-like behaviour during the pubertal developmental period. In addition, chronic infusion of a gamma-amino-butyric acid (GABA) antagonist into the MePD advanced puberty, whilst infusion of a glutamate antagonist delayed puberty. Corticotrophin releasing factor (CRF) in the CeA is a key modulator in adaptation to chronic stress. Over-expression of CRF in the CeA resulted in increased anxiety-like behaviour in peripubertal rats, advanced puberty, disrupted oestrous cyclicity and blocked the spontaneous preovulatory LH surge. Data suggest that these key limbic brain structures may contribute to the complex neural mechanisms mediating the timing of puberty.
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PUBLICATIONS


*co-first author
ABSTRACT PRESENTED AT CONFERENCES


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LIST OF ABBREVIATIONS

ACTH  Adrenocorticotrophic Hormone
aCSF  Artifical cerebrospinal fluid
ARC  Arcuate Nucleus
AVPV  Anteroventral Periventricular Nucleus
BNST  Bed Nucleus of the Stria Terminalis
CeA  Central Nucleus of the Amygdala
CORT  Corticosterone
CRF  Corticotrophin Releasing Factor
D-AP5  d-2-amino-5-phosphonopentanoic acid
Dyn  Dynorphin
E2  17β-Oestradiol
EGFP  Enhanced green fluorescent protein
EPM  Elevated Plus Maze
GABA  Gamma-Amino-Butyric Acid
GABA_A  GABAA Receptor
GnRH  Gonadotrophin Releasing Hormone
HPA  Hypothalamic-Pituitary-Adrenal
HPG  Hypothalamic-Pituitary-Gonadal
ICV  Intracerebroventricular
iv  Intravenous
KiSS1/R  Kisspeptin/Receptor (primates)
Kiss1/r  Kisspeptin/receptor (non-primates)
KNDy  Kisspeptin/Neurokinin B/Dynorphin
LH  Luteinising Hormone
LPS  Lipopolysaccharide
MBH  Mediobasal Hypothalamus
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<thead>
<tr>
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<tr>
<td>ME</td>
<td>Median Eminence</td>
</tr>
<tr>
<td>MeA</td>
<td>Medial Nucleus of the Amygdala</td>
</tr>
<tr>
<td>MePD</td>
<td>Posterodorsal of medial amygdala</td>
</tr>
<tr>
<td>mPOA</td>
<td>Medial Preoptic Area</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Aspartic Acid</td>
</tr>
<tr>
<td>ND</td>
<td>Normal Diet</td>
</tr>
<tr>
<td>NND</td>
<td>Non-Nutritive bulk Diet</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomised</td>
</tr>
<tr>
<td>P&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Progesterone</td>
</tr>
<tr>
<td>pnd</td>
<td>Postnatal Day</td>
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<td>PVN</td>
<td>Paraventricular Nucleus</td>
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CHAPTER 1: GENERAL INTRODUCTION

1.1 Central control of reproductive function

1.1.1 The hypothalamic-pituitary-gonadal axis

The hypothalamic-pituitary-gonadal (HPG) axis is the biological system that regulates reproductive function. The hypothalamic gonadotrophin releasing hormone (GnRH) neuronal population, which is the principal element driving the HPG axis, projects densely to the median eminence (ME) and releases GnRH into the pituitary portal blood. GnRH travels down to the anterior pituitary and acts on the gonadotroph cells, promoting the synthesis and release of luteinising hormone (LH) and follicle stimulating hormone (FSH) into the peripheral circulation. LH and FSH then act on the gonads. In females the action of LH and FSH on the ovaries, promotes the synthesis and release of the gonadal steroids, 17β-oestradiol (E₂) and progesterone (P₄), and stimulates ovulation at the appropriate time. FSH also stimulates folliculogenesis. In males, the action of LH and FSH on the testis leads to spermatogenesis and the synthesis and release of testosterone. In the female, the HPG axis is subject to feedback stimulation or inhibition by these gonadal steroids, varying with the stage of menstrual or oestrous cycle. Additionally, various central neuropeptides and neurotransmitters are known to modulate HPG axis activity, especially at the level of the hypothalamus. Some of these modulators are discussed below.
1.1.1.1 Anatomical localisation of GnRH neurones

GnRH neurones originate outside the central nervous system (CNS), in the olfactory placode (Wray 2010). After migration, during prenatal development, GnRH perikarya scatter within the basal forebrain around the medial preoptic area (mPOA) and mediobasal hypothalamus (MBH) (Schwanzel-Fukuda and Pfaff 1989, Wray et al. 1989), rather than concentrating in a circumscribed region. Disruption of migration results in the absence of GnRH neurones in the CNS, and therefore hypogonadotropism, characterised as Kallmann’s syndrome (Schwanzel-Fukuda and Pfaff 1989). In primates, GnRH perikarya are present in the mPOA, lateral preoptic area, MBH, organum vasculosum of the lamina terminalis and the periventricular area (Silverman et al. 1982). In rats the majority of GnRH perikarya are localised in the mPOA. Small numbers are also identified in the septal nucleus, organum vasculosum of the lamina terminalis and the supraoptic commissure (Witkin et al. 1982), indicating a species difference.

Around 50–70% of GnRH perikarya project to the ME (Merchenthaler et al. 1984, Silverman et al. 1987, Constantin et al. 2013), where terminals are found in close proximity to the capillary bed of the pituitary portal system (Page and Dovey-Hartman 1984). The remaining GnRH neurones, which project to regions throughout the hypothalamus and midbrain, are thought not to be involved in regulating release of pituitary gonadotrophin (King and Anthony 1984, Merchenthaler et al. 1984). It is likely that GnRH may also act as neuromodulators in
other regions of the brain. Indeed this notion is supported by studies showing that GnRH plays a role in reproduction-related processes such as sexual behaviour (Dudley et al. 1983, Sirinathsinghji et al. 1986).

1.1.1.2 The GnRH pulse generator

Development of a radioimmunoassay for LH and FSH allowed the measurement of these hormones in the peripheral circulation (Midgley 1966). Shortly after, Knobil and colleagues first noticed the presence of an episodic pattern of plasma LH levels in the rhesus monkey (Dierschke et al. 1970). Subsequently, a fluctuating pattern of plasma LH levels was also observed in a number of other mammalian species, including rats (Gay and Sheth 1972), ewes (Butler et al. 1972), guinea pigs (Donovan et al. 1977) and pigs (Van de Wiel et al. 1981). The frequency of LH pulses differed in these species; for example, in the ovariectomised (OVX) monkey, LH pulses were observed approximately every hour (Dierschke et al. 1970), whilst it was more frequent, occurring approximately every 20 minutes, in the castrated male and female rat (Gay and Sheth 1972). Moreover, exogenous GnRH administration to OVX rats resulted in the release of LH into the peripheral circulation, indicating that LH release was under GnRH control (Schuiling and Gnodde 1976). In turn, the existence of LH pulsatility led to attempts to characterise the pattern of GnRH release in the pituitary portal circulation. Indeed the pulsatile nature of GnRH in portal blood was observed, in rhesus monkeys (Carmel et al., 1976) and sheep (Clarke and Cummins, 1982), to be of a similar frequency to that of the LH pulses.
Furthermore, the precise and exquisite relationship between GnRH and LH pulses was demonstrated in individual OVX ewes which showed that every LH pulse was produced by one endogenous GnRH pulse (Moenter et al., 1992). Pituitaries from OVX rats showed episodic release of LH following superfusing with episodic administration of GnRH (Osland et al., 1975). Moreover, pulsatile infusion of GnRH into the OVX rat resulted in pulsatile LH secretion (Schuiling and Gnodde, 1976). Therefore, it has been concluded that the pulsatile release of LH from the pituitary is stimulated and controlled by hypothalamic GnRH pulses. A hypothalamic neural construct, thought to be responsible for pulsatile discharge of GnRH into the pituitary portal blood system, is referred as the GnRH pulse generator (Dierschke et al. 1970).

Largely based on in vitro studies, the GnRH pulse generator was thought to be composed of the GnRH neurone operating autonomously. Rhesus monkey GnRH cell cultures were shown to secrete pulsatile GnRH in a voltage and Ca\textsuperscript{2+} dependent manner (Terasawa et al. 1999). GnRH cell lines showed electrically active units which appeared to episodically coordinate, generating an overall network activity correlating with pulses of GnRH (Nunemaker et al. 2001). Whole-cell murine brain slice recordings, using green-fluorescent protein (GFP) labelled GnRH neurones, showed spontaneous action potentials in all recorded neurones and burst firing followed by quiescence in a pattern temporally related to GnRH pulse release (Suter et al. 2000).
As recordings of multi-unit activity (MUA) volleys in the hypothalamic arcuate (ARC) nucleus reliably correlate with LH pulses in rhesus monkeys (O'Byrne and Knobill 1993), rats (Kinsey-Jones et al. 2008) and goats (Ohkura et al. 2009), they have been used as an electrophysiological marker of GnRH pulse generator activity. However, no GnRH neurones have been identified in this region of the rat brain (Merchenthaler et al. 1984). Retrochiasmatic explants from the hypothalamus are able to secrete GnRH in a pulsatile manner in the absence of GnRH cell bodies. This suggests that the GnRH cell bodies may not be required to maintain the pulsatility nature of the decapeptide released in the rat (Purnelle et al. 1997). Additionally, impaired pulsatile LH secretion can be restored in rats with posterior-anterior hypothalamic deafferentation by the transplantation of foetal MBH, which does not contain any GnRH cell bodies (Ohkura et al. 2009). It has also been demonstrated that GnRH is released in an episodic manner from rat isolated ME (Rasmussen 1993). These findings indicate that a group of non-GnRH neurones may, at least in part, constitute the GnRH pulse generator. In line with this, recent in vivo studies using optogenetic approaches have shown that stimulating mouse GnRH neurones to fire continuously at 10 Hz for 2 min was able to generate LH pulses. However, synchronising the exact same population of GnRH neurones to fire together in their normal bursting pattern, 1.9 Hz for 10 s followed by a silent period of 15 s and then 1.9 Hz for 10 s so on for 5 min, failed to generate LH pulses. This observation suggested that burst firing of GnRH neurones by itself may not be required for
pulse generation (Campos and Herbison 2014).

1.1.2 Oestrous cyclicity

The laboratory rat is a non-seasonal, spontaneously ovulating, polyestrous mammal (Levine 2015). The ovarian cycle in rats occurs throughout the year with a mean cycle length of 4-5 days (Goldman et al. 2007). The cycle is characterised by periodic changes in the secretion of the gonadotrophins, LH and FSH, and steroids of ovarian origin (Schwartz 1969). There are four phases in the rat oestrous cycle, namely proestrus, oestrus, metoestrus and dioestrous phase (Marcondes et al. 2002). During the oestrous cycle, prolactin, LH and FSH remain low, principally through the negative feedback actions via the ARC of gonadal steroids (Gallo 1981). Under the influence of a rising tide of E₂, secreted by the ripening follicles (Levine 1997) during metoestrus, E₂ levels reach a peak during proestrus, which induces a preovulatory surge LH via a positive feedback action of E₂ on the AVPV in the afternoon of the proestrous phase, that causes ovulation on the following morning of oestrus (Levine 1997, Kinoshita et al. 2005). The newly formed corpora lutea arise from the ruptured preovulatory follicle that attain their maximum size on dioestrous, and are maintained through to metoestrus of the following cycle and then regress in the dioestrous phase of that cycle. P₄ is secreted from luteinised granulosa cells of the preovulatory follicle and increases to its peak during the late afternoon of proestrus, and then arises from the newly formed corpora lutea to
reach its second peak during metoestrus and dioestrus of that cycle (Levine 2015). Alterations in endocrine relationships among the hypothalamic, pituitary, and ovarian components of the reproductive axis can have marked effects on cyclicity (Goldman et al. 2007). The assessment of oestrous cyclicity in laboratory rats can provide an index for the functional status of the reproductive system (Goldman et al. 2007).

1.1.3 Modulatory effects of the amygdala on reproductive function

The amygdala has been strongly implicated in regulating reproductive processes such as ovulation, oestrous cyclicity, GnRH release, pituitary LH and FSH production, as well as sexual behaviour (Velasco and Taleisnik 1969, Carrillo et al. 1977, Beltramino and Taleisnik 1978, Bagga et al. 1984, Chateau et al. 1984, Salamon et al. 2005). The amygdala complex, a key component of the limbic system, is divided into three major groups (McDonald, 1998): 1) the deep or basolateral group, which includes the lateral, basal and accessory basal nuclei; 2) the superficial or cortical-like group, consisting of the cortical nucleus and the nucleus of the lateral olfactory tract; 3) the striatal or centromedial group, composed of the medial (MeA) and central (CeA) nuclei (Fig.1.1). The centromedial group which projects to the basal forebrain including the bed nucleus of the stria terminalis (BNST), the hypothalamic and brainstem structures, is regarded as the principal output structure of the amygdala (Swanson and Petrovich, 1998).
A recent study has shown that lesioning the whole amygdala in neonatal monkeys advances menarche and first ovulation (Stephens et al. 2015). Stimulation of the MeA in rats induces the lordotic response, whereas lesioning decreases it, (Masco and Carrer 1980) and also reduces the percentage of animals ovulating (Bagga et al. 1984). Furthermore, lesioning the MeA increased plasma levels of LH in adult female rats, (Lawton and Sawyer 1970) and advanced puberty in juvenile rats (Docke et al. 1976), while electrical stimulation of the MeA delayed puberty in female rats (Bar-Sela and Critchlow 1966). These findings suggest that the nucleus of the amygdala plays an inhibitory role on reproductive function. The influence of the MeA on the HPG axis is further supported by neuroanatomical studies which demonstrate the existence of direct projections from the MeA to the GnRH-rich mPOA (Canteras et al. 1995, Hahn et al. 2003). However, the neurochemical phenotype and precise function of these efferents is unknown. More recently, it was shown that a critical site for lesion-induced hyperphagia and obesity is the posterodorsal subnucleus of the medial amygdala (MePD), which may explain the advancement of puberty. Furthermore, the MePD predominantly sends out inhibitory GABAergic projections and is implicated in reproductive behaviour (Choi et al. 2005, Pardo-Bellver et al. 2012). Additionally, glutamate signalling in the amygdala, in particular its NMDA receptor is involved in anxiety-related behaviour (Hegoburu et al. 2014) and intra-MePD administration of NMDA disrupts ovarian cyclicity in the rat (Polston and Erskine 2001). Glutamatergic activity also increases in the MePD.
during puberty without a corresponding GABAergic change, suggesting an overall
activation of this brain region. However, the effect of lesioning specific subnuclei
within the amygdala on puberty has not yet been established and little is known of the
potential impact on pubertal timing of GABAergic or glutamatergic signalling in
individual amygdala subnuclei.

The CeA is also involved in regulating the HPG axis. The CeA contains a major
corticotrophin-releasing factor (CRF) neuronal population (Swanson et al. 1983).
CRF is a key inhibitory neuropeptide of GnRH pulse generator activity (Li et al.
2010). Lentiviral vector-induced over-expression of CRF in the CeA has been shown
to negatively impact the reproductive physiology in female rats by disrupting the
oestrous cycle and decreasing GnRH expression in the mPOA. This indicates that a
constitutive increase of CRF in CeA may be a causal factor in reproductive disorders
(Keen-Rhinehart et al. 2009). The CeA has also been shown to be involved in
maternal (Lubin et al. 2003) and sexual behaviour (Keen-Rhinehart et al., 2009).
Fig. 1.1 Schematic drawings of coronal sections of the adult rat brain showing the central amygdala (CeA) (red), medial amygdala (MeA) (blue) and its subnucleus, the posterodorsal of medial amygdala (MePD) (yellow). Numbers in each drawing indicate the distance (mm) to bregma in accordance with Paxinos and Watson (Paxinos and Watson 1986)
1.2 Control of puberty

1.2.1 Timing of puberty

Puberty is the transitional period between juvenile and adulthood when major hormonal, physical and behavioural changes occur to attain full reproductive capability (Terasawa et al. 2011). The timing of puberty in mammals, including man, is controlled by a multiplicity of complex interactions between genetic and environmental factors. Over the past 30 years it has become clear that initiation of puberty depends on an increased release of GnRH from the hypothalamus (Grumbach 2002, Terasawa et al. 2011). An increase in GnRH pulse generator frequency is a key decisive event for puberty to occur, and is well established in many species including humans (Sisk and Foster 2004), rhesus monkeys (Plant and Zeleznik 2014) and sheep (Foster and Jackson 1994). A gradual acceleration in LH pulse frequency has also been observed in prepubertal female rats (Li et al. 2012).

In rodent models, approximately eight days prior to puberty onset, central excitatory (e.g. glutamate) input to GnRH neurones increases while inhibitory inputs decreases (Terasawa et al. 2011). This results in increased GnRH which acts on the anterior pituitary to increase LH and FSH secretion that drives gonadal development. In females, E₂ and P₄ are released by the developing ovaries. E₂ positively feeds back onto the hypothalamus and pituitary in the late prepubertal period, resulting in further LH release, known as “LH minisurges”. All these events act to further mature
the ovary and lead to increased E$_2$ release. Once the ovaries have sufficient capacity to secrete E$_2$ for an adequate time period, the steroid activates the hypothalamus to bring about the first preovulatory LH surge which is required for ovulation. At the time of puberty rising E$_2$ drives vaginal opening (VO), a key marker of puberty, which is associated with the first ovulation in the female rat and marked by an oestrous vaginal cytology (Ojeda and Urbanski 1994, Chehab et al. 1997, Engelbregt et al. 2001, Sisk et al. 2001). The VO and first day of vaginal oestrus mark the full maturation of the HPG axis. Consequently, both of these occurrences are considered reliable markers for the onset of puberty in rats and usually occur between post-natal day (pnd) 35–55 (Ojeda et al. 1976). In all species, the GnRH pulse generator plays a vital role in producing the surge of gonadotrophin release as it drives the increase in E$_2$ secretion, which in turn acts as the ovarian component of the trigger for the preovulatory LH surge (Plant 2015).

The timing of puberty and subsequent post-pubertal reproductive function are affected by a large number of sensory inputs relating to the internal and external environment. Information on growth, body fat/composition, diet, stress, circadian time, gonadal steroids and olfactory cues all must ultimately reach the mammalian GnRH neuronal system. Thus, a large number of neurotransmitters (e.g. glutamate and GABA) and neuropeptides (e.g. kisspeptin) have been implicated in signalling to GnRH neurones (Ebling 2005). From a neurobiological perspective, the timing of puberty is therefore a function of changes in these neural systems that control
GnRH release. The mechanism of how these factors are transduced by the brain and how they trigger the pubertal increase in GnRH release, in particular pulse frequency, remain to be fully elucidated.

1.2.2 Kisspeptin signalling

1.2.2.1 Discovery of kisspeptin

The kisspeptin (KiSS1) gene, subsequently found to encode a 54-amino-acid peptide known as kisspeptin or KiSS1/Kiss1, was identified in 1996 (Lee et al. 1996). A gene encoding a G protein-coupled receptor, GPR54, with no known function or ligand, was later discovered and found to be expressed in the hypothalamus, amygdala (Lee et al. 1999), pituitary, placenta (Muir et al. 2001), periventricular and ARC nuclei, and dorsomedial hypothalamus (Mikkelsen and Simonneaux 2009). In 2001, Kiss1 was discovered to be the endogenous ligand for Kiss1r (GPR54) (Kotani et al. 2001, Muir et al. 2001, Ohtaki et al. 2001).

In 2003, two groups reported the functional relevance of KiSS1 (de Roux et al. 2003, Seminara et al. 2003). Using linkage analysis and candidate gene screening these teams discovered that the lack of pubertal development and infertility in patients with idiopathic hypogonadotrophic hypogonadism was due to mutations in the KiSS1 receptor, KiSS1R. Transgenic animal models expressing mutated Kiss1r were generated with a homologous phenotype to human idiopathic hypogonadotrophic
hypogonadism. Animals had intact but immature sexual organs, low sex hormone and FSH/LH levels, normal GnRH mRNA levels, typical GnRH neuronal migration and differentiation, but with 60-70% reduced responsiveness to Kiss1 (Seminara et al. 2003).

*Kiss1r* mutations appeared to prevent effective GnRH secretion, inhibiting gonadotrophin driven sexual maturation (Seminara et al. 2003). This established the concept of a Kiss1-GnRH-gonadotrophin axis as being essential for full HPG axis activation. Furthermore *KiSS1R* and *KiSS1* activating mutations, identified in patients with central precocious puberty, supported this theory (Teles et al. 2008, Silveira et al. 2010). Mutations in the GnRH receptor (de Roux et al. 1997, Beranova et al. 2001) and Kiss1 (d'Anglemont de Tassigny et al. 2007) have also been implicated in idiopathic hypogonadotrophic hypogonadism.

1.2.2.2 Location of kisspeptin in the brain

Studies mapping the *Kiss1* gene have identified that the majority of Kiss1 neurones exist in the anteroventral periventricular (AVPV) nucleus in the mPOA and in the ARC nucleus in rodents (Gottsch et al. 2004, Han et al. 2005).

ARC and AVPV Kiss1 neurones express nuclear oestrogen receptor α (ERα) necessary for steroidal feedback (Smith et al. 2005) but with diametric functions. The ARC is posited as the site of E₂ negative feedback to the hypothalamus and the AVPV as the site of positive feedback, triggering the LH surge (Adachi et al. 2007).
OVX increases ARC Kiss1 but decreases AVPV Kiss1 mRNA expression in mice and monkeys, while E2 decreases ARC Kiss1 and increases AVPV Kiss1 mRNA expression (Smith et al. 2005, Alcin et al. 2013). Ablating ARC Kiss1 neurones has been shown to prevent the LH/FSH increase post OVX in rats (Mittelman-Smith et al., 2012).

1.2.2.3 Effect of kisspeptin on GnRH secretion

Central administration of GnRH antagonists inhibit Kiss1-induced gonadotrophin stimulation (Matsui et al., 2004, Irwig et al., 2004), which supports the Kiss1-GnRH-gonadotrophin axis concept. Kiss1 is known to be the most potent stimulator of GnRH, able to stimulate gonadotrophin release in various species including sheep (d'Anglemont de Tassigny et al. 2007), monkeys (Shahab et al. 2005) and humans (Dhillon et al. 2005, Jayasena et al. 2013). Intracerebroventricular (ICV), peripheral and specific intra-ARC and intra-mPOA infusion of Kiss1 dose-dependently stimulates LH release in rats (Thompson et al. 2004, Li et al. 2009b).

Kiss1 may act directly on GnRH neurones, the majority of which express Kiss1r mRNA in rodents (Irwig et al., 2004, Messager et al., 2005) and other mammals (Herbison et al. 2010, Pinilla et al. 2012). Supporting this, Kiss1 causes sustained depolarisation of mouse GnRH neurones in situ (Han et al. 2005) and in brain slice preparations (Pielecka-Fortuna et al. 2008). Furthermore, central and peripheral
Kiss1 exposure induces c-Fos expression in GnRH neurones (Irwig et al. 2004, Matsui et al. 2004). Connections exist between Kiss1 immunoreactive fibres and GnRH neurones in rodents indicating that Kiss1 neurones may contribute to GnRH neurone activation directly (Kinoshita et al. 2005, Clarkson and Herbison 2006).

1.2.2.4 Kisspeptin and the GnRH pulse generator

Although the significance of the GnRH pulse generator has been recognised for many years, its underlying mechanisms and critical neural components remain controversial. A number of studies have indicated that the Kiss1 system may act as a critical player in the control mechanism governing pulsatile GnRH secretion. Kiss1 has been found to co-express with neurokinin B (NKB) and dynorphin (Dyn) (Goodman et al. 2007, Navarro et al. 2009, Wakabayashi et al. 2010), referred to as KNDy (kisspeptin/neurokinin B/dynorphin) neurones (Lehman et al. 2010a). It is hypothesised that Kiss1, NKB and Dyn form a neuronal circuit that facilitates autocrine feedback on the KNDy cell body. NKB exerts a stimulatory effect on KNDy neurones via its receptor, NK3R, to evoke synchronised bursting activities among KNDy neurones. In contrast, Dyn imposes an inhibitory effect on KNDy neurones (Wakabayashi et al. 2010, Goodman et al. 2013, Nakahara et al. 2013). Rhythmic oscillations in the level of NKB/Dyn may result in the pulsed release of Kiss1, which acts on Kiss1r expressed on GnRH neurones at the level of the ME to stimulate pulsatile GnRH secretion (Lehman et al. 2010a). Supporting this model, the dense reciprocal innervation between KNDy neurones that is evident in several
species including the rat and the goat (Burke et al. 2006, Wakabayashi et al. 2010). Furthermore, in monkeys (Ramaswamy et al. 2008), sheep (Franceschini et al. 2006) and rats (Navarro et al. 2009, Wakabayashi et al. 2013) projections have been observed from the ARC nuclei to GnRH neuronal terminals at the ME (Lehman et al. 2010b).

Pharmacological studies support KNDy signalling systems as being a key component of the GnRH pulse generator, as they have shown that administration of Kiss1 can alter the pulsatile release of LH. Human studies have shown an increase in LH pulse frequency following KiSS1 administration. Subcutaneous injection of KiSS1 resulted in a temporary increased LH pulse in women (Jayasena et al., 2013) but this was more sustained following intravenous infusion in men (George et al. 2011). Kiss1 stimulated GnRH release from isolated GnRH nerve terminals (d’Anglemont de Tassigny et al. 2008), while Kiss1 antagonist infused to the ME suppressed GnRH pulses (Roseweir et al. 2009). The ME is a site of GnRH axon and Kiss1 neurone comingling thus axo-axonal interaction may likely occur here (Ramaswamy et al. 2008, True et al. 2011). In line with this, Kiss1 and GnRH are released at the level of ME with a high temporal concordance in rhesus monkeys (Keen et al. 2008). Similar to KiSS1, mutations in NKB and its receptor, NK3R, result in idiopathic hypogonadotrophic hypogonadism in humans (Topaloglu et al. 2009), but with a less severe phenotype and spontaneous activation of the HPG occurring later in life. This suggests that NKB is less fundamental to puberty than
KiSS1 and KiSS1R (Lippincott et al. 2013). As KiSS1 infusion can restore LH pulses in patients with NKB/NK3R mutations, KiSS1 may act downstream of NKB and function independently in controlling GnRH (Young et al. 2013). Unexpectedly, Kiss1 failed to alter the pattern of ARC MUA volley recordings in rats and goats (Kinsey-Jones et al. 2008, Ohkura et al. 2009). However, central administration of Kiss1 antagonists was found to inhibit LH pulses in mice and primates (Roseweir et al. 2009). Intra-ARC Kiss1 antagonist administration dose-dependently reduced LH pulse frequency in rats (Li et al. 2009b).

1.2.2.5 Kisspeptin and the proestrous LH surge

In rodents during the proestrous phase, the rising tide of E2 in plasma triggers the GnRH and LH surge inducing ovulation. The Kiss1 neurones located in the AVPV play a critical role in relaying the positive feedback effects of E2 to GnRH neurones. Kiss1 neurones are critical for surge secretion of GnRH and LH, and therefore for ovulation (Terasawa et al. 2013). The role of the AVPV in the E2-driven LH surge was established by mPOA lesioning studies, which abolished cyclic ovulation in rats (Wiegand et al. 1980). It has also been shown that E2 implanted in the mPOA induced a LH discharge in rats (Goodman 1978). AVPV neurones project onto and directly stimulate the GnRH neurones in the mPOA (Gu and Simerly 1997, Messager et al. 2005). Infusion of a Kiss1 specific monoclonal antibody into the mPOA blocked the LH surge and repressed oestrous cyclicity. This implicates Kiss1, released in the mPOA, as inducing the preovulatory LH surge and regulating
oestrous cyclicity (Kinoshita et al. 2005). It has been shown that the expression of Kiss1 mRNA in the AVPV is dramatically increased by E₂ (Smith et al. 2005, Dungan et al. 2007). Furthermore, AVPV Kiss1 mRNA and c-Fos expression peak in the afternoon of proestrus during the LH surge (Smith et al. 2006). In murine brain slices AVPV neurones show burst and tonic electrical activity, with increased tonic firing on proestrus (Ducret et al. 2010). In line with these studies, a population of rodent ERα-positive Kiss1 neurones has been shown to make direct synaptic contact with GnRH neurones (Terasawa et al. 1980, Smith et al. 2005, Wintermantel et al. 2006). Together, these observations imply that activation of Kiss1 neurones and their signalling to GnRH neurones is a prerequisite for generating the E₂-induced GnRH/LH surge in rodents (Oakley et al. 2009).

1.2.2.6 Changes in kisspeptin during puberty

Many studies have implicated the importance of the Kiss1 signalling system in reproductive function and puberty onset. As described above, Kiss1 neurones are present in the ARC and AVPV nucleus in rodents, the brain structures known to be critical in controlling the negative and positive feedback actions of gonadal steroid hormones on GnRH secretion (Terasawa et al. 2011). Moreover, GnRH neurones express Kiss1r (Messager et al. 2005). Central administration of Kiss1 results in precocious puberty in female rats (Navarro et al. 2004b), whereas central administration of the Kiss1 antagonist delays puberty (Pineda et al. 2010).
Hypothalamic Kiss1 mRNA and immunoreactivity increase during puberty in mice (Han et al. 2005, Clarkson and Herbison 2006) rats (Navarro et al. 2004b) and monkeys (Shahab et al. 2005). There is also an increase in Kiss1r expression on GnRH neurones from 40% at birth to approximately 70% from postnatal day 20 in mice (Herbison et al. 2010). In line with increasing Kiss1 expression, in mice the number of GnRH neurones depolarised by Kiss1 increases from juvenile (27%), to prepubertal (44%) and adult (>90%) (Han et al. 2005), indicating that, in rodents, sensitivity of Kiss1r to Kiss1 may increase prior to puberty.

However, transgenic mice with targeted ablation of whole body Kiss1 or Kiss1r target cells had smaller ovaries although puberty occurred on time and the mice were fertile. This may suggest that Kiss1 is not the gatekeeper for pubertal GnRH activation, or that minimal GnRH neurones are required, as the majority express Kiss1r. However, acute ablation of Kiss1 neurones in adult mice did inhibit fertility, suggesting a compensation for the loss of Kiss1 neurones early in development (Mayer and Boehm 2011, Beale et al. 2013). Although the roles of ARC and AVPV nuclei in the LH surge and pulse generation have been explored, whether it is ARC or AVPV kisspeptin which is essential for puberty onset is unknown; their distinct roles in subsequent oestrous cyclicity have also not been directly investigated.

### 1.2.3 Stress alters puberty timing

Normal pubertal development requires the maturation of the HPG axis, specifically
the increased pulsatile release of GnRH from the hypothalamus. The timing of puberty is affected by numerous intrinsic and extrinsic factors, such as undernutrition, infection, emotional deprivation and stress (Parent et al. 2003, Kinsey-Jones et al. 2010). Evidence suggests that chronic stress suppresses the HPG axis, delays pubertal timing, and disrupts reproductive function in female rats (Kinsey-Jones et al. 2010, Li et al. 2010, Lin et al. 2011). Stress models often used to determine suppression of the GnRH pulse generator include immunological, psychological restraint or isolation, and metabolic challenge (Li et al. 2010). A decrease in the frequency of hypothalamic MUA volleys, which provide an electrophysiological correlate of GnRH pulse generator activity, has been demonstrated following both hypoglycaemic stress in monkeys (Chen et al. 1996) and immunological stress in rats (Yoo et al. 1997). Furthermore, studies have demonstrated stress induced-suppression of pulsatile LH secretion in sheep, monkeys and rats (Clarke et al. 1990, Chen et al. 1992, Li et al. 2006).

There is evidence to show that stress induced a delay of puberty in human. Children who were exposed to physical, psychological and nutritional stressors, showed a delay in puberty and later menarche (Georgopoulos et al. 1999). Furthermore, children who experienced loss of friends and family, general dearth food rationing during wartime conditions (van Noord and Kaaks 1991, Prebeg and Bralic 2000), or natural disasters such as hurricanes (Ponnapakkam and Gensure 2008) also had delayed puberty.
However, stress can also advance puberty in humans. For example, children whose early social environments involve high levels of conflict, absent parents, or poor child-parent relationships tend to show an advancement in puberty (Belsky et al. 1991, Ellis and Garber 2000). Putnam and Trickett (1997) proposed that the trauma of childhood sexual abuse can result in both physiological and psychological consequences for children and also accelerate puberty onset. This has been supported by a number of studies demonstrating that sexually abused girls experienced earlier menarche (Turner et al. 1999, Romans et al. 2003, Wise et al. 2009). However, more recently a study has shown that sexual abuse in children can be associated with both early and late puberty onset (Li et al. 2014a). Thus, stress has the potential to influence the reproductive system by either advancing or delaying pubertal timing. However the underlying mechanism involved remains largely unknown.

1.2.3.1 The hypothalamic-pituitary-adrenal axis

The hypothalamic pituitary adrenal (HPA) axis regulates the production of glucocorticoids from the adrenal cortex, and is the major neuroendocrine system in mammals that provides a rapid response and defence against stress (Spiga et al. 2014). Glucocorticoids (cortisol in humans and corticosterone [CORT] in rodents) are vital hormones that regulate many physiological functions including glucose, fat and protein metabolism (Cherrington 1999, Macfarlane et al. 2008). In addition, glucocorticoids exert anti-inflammatory and immunosuppressive actions and can
affect mood and cognitive function (Chrousos 1995, McEwen 2007). Under basal (nonstress) conditions, the dynamics of the HPA axis is characterised by both a circadian and an ultradian rhythm of hormone secretion (Lightman et al. 2008, Spiga et al. 2014). The activity of the HPA axis increases when the organism is exposed to stress. This stress response is initiated by the activation of several afferent neural pathways, including those originating from limbic and brain-stem structures (Ulrich-Lai and Herman 2009), which in turn induces the release of corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) from the medial parvocellular neurones of the hypothalamic paraventricular nucleus (PVN) (Herman et al. 2005). These neuropeptides are released into the hypothalamic-pituitary portal capillaries in order to access the anterior pituitary corticotrophs, where it stimulates the synthesis and secretion of adrenocorticotrophic hormone (ACTH) into the systemic circulation (Gillies et al. 1982). ACTH stimulates the hypertrophy of cells in the zona fasiculata and zona reticularis of the adrenal gland, and subsequently the release of glucocorticoids (Aguilera 2011, Spiga et al. 2014). Once in the blood, glucocorticoids access target tissues, such as the liver and the heart and vascular tissues, to exert metabolic and cardiovascular effects, respectively. Glucocorticoids that circulate to the brain promote cognitive processes necessary to cope with threatening situations (De Kloet 2004).

1.2.3.2 Stress mediator-CRF

There is overwhelming evidence that suggests a pivotal role for CRF in
stress-induced suppression of GnRH pulse generator frequency. Central or peripheral administration of CRF has been shown to decrease the frequency of MUA volleys and LH pulses in a variety of species including rats and monkeys (Gindoff and Ferin, 1987; Chen et al., 1996; Cates et al., 2004). Cates et al. (2004) demonstrated the role of CRF in mediating the suppressive effect of insulin-induced hypoglycaemic stress on pulsatile LH secretion in the female rat. Exogenous CRF administration in fertile women has also been shown to cause a rapid inhibition of LH secretion during the late follicular and mid luteal phases of the menstrual cycle (Barbarino et al. 1989). Suppression of GnRH release by CRF is further supported by evidence that CRF receptor antagonist administration reverses this inhibition and consequently increases GnRH pulsatility (Tsukahara et al. 1999, Kinsey-Jones et al. 2010). As puberty requires the activation of the GnRH pulse generator, ICV administration of CRF in rats suppresses the GnRH pulse generator and consequently delays the onset of puberty, whereas ICV administration of CRF antagonist advances puberty (Kinsey-Jones et al. 2010). These data suggest there is an endogenous CRF tone which plays a key role in the pubertal timing mechanism in the rat.

Stress activates the HPA axis and suppresses the HPG axis, specifically pulsatile secretion of GnRH, although the mechanism is not completely understood (Li et al. 2005). The inverse relationship between the HPA and HPG axes has led to the suggestion that activation of the HPA axis may underlie stress-induced suppression
of the GnRH pulse generator. Activation of the HPA axis during stress typically coincides with increased CRF release which, in turn, may suppress the GnRH pulse generator and therefore the HPG axis (Li et al. 2010). The exact neuroanatomical pathway of CRF signalling involved in determining the GnRH pulse generator frequency is uncertain. The role of the PVN CRF in controlling LH secretion is controversial since tract-tracing studies have failed to identify CRF neurones projecting from the PVN to the GnRH-rich regions in the mPOA of the female rat (Hahn et al. 2003). Furthermore, although PVN CRF mRNA increases in response to stress, the degree of HPA axis activation is not always parallel to the degree of LH pulse suppression (Li et al. 2003). Moreover, lesioning the PVN does not prevent stress-induced suppression of the GnRH pulse generator (Rivest and Rivier 1991). These observations support the hypothesis of the existence of other CRF producing systems, outside the PVN, that are involved in the interaction between stress and the reproduction function.

1.2.3.3 The role of the amygdala in stress response

The limbic forebrain structures, including the amygdala and hippocampus, are implicated in the processing of stress response (Ulrich-Lai and Herman 2009). These regions receive information from sub-cortical and cortical areas, that are involved in higher-order sensory processing (such as the smell of a predator) and memory, as well as ascending inputs from sites involved in attention and arousal (e.g. the locus coeruleus and raphe nuclei) (Herman et al. 2003). These limbic structures
send projections to the principal stress effectors, the PVN, via relay sites, such as the BNST, allowing downstream integration of stress information (Ulrich-Lai and Herman, 2009).

The amygdala plays a key role in regulating the HPA axis. Numerous studies have demonstrated that amygdala lesioning leads to a decrease in stress-induced release of glucocorticoids (Feldman et al. 1994), whereas stimulation of the amygdala has the opposite effect in several species including the rat (Dunn and Whitener 1986) and the monkey (Weiss et al. 1986). In accordance with the notion that psychogenic and physical stressors activate distinct neuronal pathways, the effect of the amygdala on the HPA axis may also be stressor- and region-specific (Herman et al. 2005). The modulatory role of the amygdala on the HPA system is largely through the MeA and CeA components. The MeA shows a stronger neuronal activation in response to psychogenic stressors, such as restraint, forced swim, noise or predator exposure (Cullinan et al. 1995, Dayas et al. 2001, Figueiredo et al. 2003a), compared with systemic stressors such as interleukin (IL)-1β injection, hypoxia or haemorrhage (Sawchenko et al. 1996, Thrivikraman et al. 1997, Figueiredo et al. 2003a), as measured by c-fos expression. Consistent with this stressor-specific role, selective MeA damage has been shown to block the activation of the hypothalamic PVN CRF cell in response to restraint stress (Dayas et al. 1999), and to increase corticosterone in response to predator odour in rats (Masini et al. 2009). In contrast, the CeA has been shown to be highly responsive to systemic stressors like inflammation or
haemorrhage (Sawchenko et al. 1996, Thrivikraman et al. 1997). Specific lesions of the CeA resulted in an attenuation of the HPA response following IL-1β injection (Xu et al. 1999), but not restraint stress (Dayas et al. 1999). Therefore, the MeA and the CeA play a key role in the regulation of the HPA axis responses to psychogenic and systemic stress, respectively.

Based on the involvement of the amygdala in the stress-response network, and the fact that stress stimulates the HPA axis and suppresses the HPG axis, a key role of this limbic structure in the pubertal timing is postulated. Electrical stimulation of the MeA delays the timing of puberty onset in rats (Bar-Sela and Critchlow 1966). Ablation of the MeA in pre-pubertal rats has been shown to either advance or delay puberty, depending on the age of the rat when lesioned (Docke et al. 1980). It was shown recently that lesioning specifically the MePD resulted in hyperphagia and obesity in adult rats, which may explain the advancement of puberty. However, the role of this specific subnucleus within the amygdala on puberty timing remains to be elucidated. Although there are no comparable ablation or stimulation studies carried out on the CeA to address its role in pubertal timing, it has been shown that lesioning the CeA attenuates lipopolysaccharide (LPS) stress-induced suppression of LH pulses, implicating the CeA in stress-induced suppression of the GnRH pulse generator (Lin et al. 2010). Moreover, the CeA contains a major CRF neuronal population that forms an important component of the limbic brain stress-response network. Over-expression of CRF in the CeA increases anxiety and reduces GnRH
expression in the mPOA in adult rats. This suggests that CRF within the CeA negatively impacts reproductive physiology (Keen-Rhinehart et al. 2009), however its precise role in stress-related change in pubertal timing and reproductive cyclicity remains to be fully established.

1.3 Aims of Thesis

It is well known that the onset of puberty is mainly controlled by the maturation of the HPG axis. This developmental period is affected by various factors including body weight and stress. However, the underlying neural mechanisms remain to be fully established. Kiss1 is critical for pubertal timing, and the roles of AVPV and ARC Kiss1 in LH surge and LH pulse generation have been extensively investigated. However, what remains unknown is whether it is AVPV or ARC Kiss1 that is essential for puberty onset. In addition to the ARC and AVPV, the amygdala is also involved in the regulation of puberty timing and stress response. However, large and unpredictable electrolytic lesions in rats have resulted in both precocious and delayed puberty. Furthermore, the heterogeneous nuclear arrangement of the MeA and its related functions are still unknown. Therefore, the present study was designed to address the following aims:

1. To identify the individual contribution of AVPV and ARC Kiss1 in the timing of puberty onset and subsequent reproductive functions as assessed by VO and first oestrus, LH pulses and surges, and oestrous cyclicity.
a. To determine whether a knockdown of ARC and AVPV Kiss1 would delay puberty onset and impair reproductive function in the post-pubertal period in female rats.

2. To elucidate the underlying mechanisms of stress-induced alteration of puberty timing by investigating the role of CRF in the CeA.
   a. To determine whether CRF over-expression in the CeA would alter puberty timing in female rats.
   b. To determine the effect of over-expression of CRF in the CeA on stress-induced HPA axis activation during the pubertal transition, as well as on LH surge secretion and oestrous cyclicity.

3. To investigate the role of the MePD in regulating the timing of puberty onset and stress responsivity.
   a. To determine the effect of MePD lesion on the timing of puberty onset in female rats.
   b. To determine the effect of MePD lesion on stress-related behaviour during the pubertal timing in female rats
   c. To investigate the role of glutamate and GABA signalling in the MePD in regulating the timing of puberty onset in female rats.
CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Animals

Adult Sprague-Dawley rats obtained from Charles River (Margate, UK) were allocated into breeding pairs. Two weeks after cohabitation with a male rat, female rats were examined every other day. Once female rats were detected of pregnancy, by palpation, they were separated from male rats into individual cages and fed with a breeding diet (12% kcal fat, 27% kcal protein, 61% kcal carbohydrate; RM3; Special Diet Services). The day of birth was considered postnatal day zero (pnd 0). Excess male offspring were culled to reduce litters to maximum 12 pups but at least with 1 male pup per litter on pnd 2. Litters were weaned at pnd 21 and female offspring were housed in groups of four or six per cage under controlled conditions (12-h light, 12-h darkness, with lights on at 07:00 h; controlled ambient temperature 22 ± 2 °C) and provided with food and water ad libitum. Litters were either assigned to normal standard chow diet (ND, 21% kcal fat, 17% kcal protein, 63% kcal carbohydrate; RM1; Special Diet Services), 25% non-nutritional food (NND, the same standard chow to which 25% alphacel non-nutritive bulk [MP Biomedicals, Cambridge, UK] had been added), or the high fat diet (45% kcal fat, 20% kcal protein, 35% kcal carbohydrate; Special Diet Service). Litters were weighed every 3 days after weaning and monitored daily for vaginal opening (VO) from pnd 28. All procedures were conducted in accordance with Animals (Scientific Procedures) Act UK, 1986 and were approved by the King’s College London Ethical Review Panel Committee.
2.2 Surgical Procedures

2.2.1 Anaesthesia and analgesia

All surgical procedures were carried out under a combination of ketamine (Vetalar, 600 mg/kg, Intraperitoneal [ip]; Pfizer, Sandwich, UK) and xylazine (Rompun, 60 mg/kg, ip; Bayer, Newbury, UK); supplementary injections of ketamine (600 mg/kg, ip) were administered to maintain anaesthesia as required. Each rat was injected subcutaneously (s.c.) with 2 ml/kg Duphamox LA antibiotic suspension (200 mg/ml procaine benzylpenicillin, 250 mg/ml dihydrostreptomycin-sulphate; Fort Dodge Animal Health, Southampton, UK) and preoperative analgesia carprofen (5mg/kg; Rimadyl®, Pfizer Animal Health, Tadworth, UK) prior to surgery. Following each surgical procedure, animals were allowed to recover from anaesthesia on a heated pad until fully conscious.

2.2.2 Bilateral lesions of the brain nuclei

Bilateral lesions of the MePD or CeA were accomplished by administrating 0.3µl of 10 µg/µl ibotenic acid (Sigma-Aldrich Ltd, Poole, UK) in sterile 0.1 M sodium phosphate buffer (PBS; pH 7.4). Ibotenic acid, an excitatory neurotoxic amino acid, produces selective lesions of the neuronal cell bodies without destruction of fibres of passage (Guldin and Markowitsch 1981).

The scalp of the anaesthetised animal was shaved and sterilised, after which the
animal was mounted in a David Kopf stereotaxic frame (David Kopf Instruments, Tujunga, California, USA). The surface of the skull was exposed. The surgical procedures were carried out with the aid of a surgical microscope. A stereotaxic alignment tool was used to ensure that the surface of the skull was level with the bregma and lambda sutures in the same horizontal plane. A 25-gauge 1 µl Hamilton injection syringe (Sigma-Aldrich) was mounted in a custom-made clamping devise attached to a micro-manipulator (David Kopf Instruments), which was then fitted onto the stereotaxic frame. Vertical alignment of the syringe needle was checked using a set square. The tip of the syringe needle was then lowered directly above the intersect of the bregma and median sutures to obtain the zero reference anterior-posterior (AP) and medio-lateral (ML) co-ordinates. The needle tip was positioned using the stereotaxic apparatus following the co-ordinates: 3.2 mm lateral and 1.5 mm posterior to bregma for the MePD. The co-ordinates for juvenile rats were determined after careful optimisation in a group of same age pups in a preliminary experiment and used to target the MePD (AP: -3.3 mm; ML: 3.4 mm; DV: 8.6 mm) based on the rat brain atlas of Paxinos and Watson (Paxinos and Watson 1986). The skull was marked to indicate the targeted positions. The micro-manipulator was then moved forward to clean the surface of the skull. A drill with dental burr (FFXC7HP burr; Wright Dental Group Ltd., Brentford, UK) was used to create a hole in the skull, encompassing the areas directly above the MePD and the dorsal sagittal sinus in the same coronal plane. The disc (small piece of bone)
remaining after drilling was carefully removed with forceps. The manipulator arm was then moved back into the calculated AP co-ordinate and the tip of the needle was placed over the midline of the dorsal sagittal sinus to obtain the most accurate ML zero co-ordinate, from which the final ML co-ordinate for the MePD could be calculated. The syringe was lowered, under visualisation using an operating microscope, until the tip of the needle just made contact with the dorsal sagittal sinus. A measurement for the zero reference dorso-ventral (DV) coordinates was taken from this point. The needle tip was then positioned to the final calculated ML coordinate for the MePD. The dura was pierced with a hypodermic needle, allowing entry of the syringe needle without obstacle. The needle tip was then lowered 7.8 mm from the surface of the dura to reach the MePD over a 1-min period. The 0.3 µl of ibotenic acid was slowly infused into the nucleus over a 5-min period, after which the needle was left in place for an additional 5-min to prevent reflux. After being raised up slowly over 1 min, the syringe needle was flushed to ensure successful injection without any debris in the tip. The procedure was repeated to lesion the nucleus on the other side. After being removed from the stereotaxic frame, rats were allowed to recover on a heated pad until fully conscious. The sham-lesioned animals received the vehicle, artificial cerebrospinal fluid (aCSF), following the same procedure.

Unless otherwise stated, all compounds infused into the brain were dissolved in aCSF, which was made up as follows:
100 ml deionised water  0.214 g NaHCO₃

0.724 g NaCl  0.18 g D-Glucose

0.246 g KCl  0.0368 g CaCl₂

0.0163 g KH₂PO₄  0.025 g MgSO₄

Each salt was added to the deionised water until the previous one had fully dissolved. The aCSF solution was then filtered through a sterile filter (Millipore, Watford, UK), aliquotted in batches of 5 ml and stored at -20 ºC.

2.2.3  Bilateral brain nuclei implantation with minipumps

For experiments involving the bilateral chronic infusion of substances into the brain nuclei, rats were fitted with a 28-gauge cannula (Plastics One, Roanoke, VA, USA) directed towards the MePD. The co-ordinates for different brain nuclei are shown in Table 2.1. General surgical procedures were carried out as described in section 2.2.2. The tip of the minipump cannula, held by a manipulator, was positioned directly above the intersect of the median and bregma sutures to get the reference zero AP and ML co-ordinates. The cannula was moved into the correct AP and ML co-ordinates for implantation, such that each cannula was equidistant from the midline, spanning the dorsal sagittal sinus. Pen marks were made on the skull to
indicate the targeted positions. The micro-manipulator was moved clear of the surface of the skull. Three 2 mm diameter holes were drilled to expose the implantation site for the bilateral cannulae and the dorsal sagittal sinus in the same coronal plane. The disk of bone remaining was removed using watch-makers forceps. Additional three small holes were drilled and fitted with stainless steel screws to secure the minipump position. The tip of cannula was then moved back into position, directly over the dorsal sagittal sinus, to obtain final zero ML and zero DV co-ordinates. The cannula was moved to the calculated position. The dura was cut directly below the cannula to allow entry of the cannula. The cannula was carefully lowered through the holes in the dura and on either side of the sinus. The cannula was then cemented in place with the small screws fitted earlier. Once the cannula was well secured in place, it was released from the micro-manipulator. An osmotic minipump (Model 1002; Alza Corp, Mountain View, CA, USA), prefilled with drugs or aCSF, was attached to the cannula with silicone tubing and implanted s.c. in the intrascapular space. Minipumps were used to deliver peptides or vehicle at 0.25 μl/h for a 14-day period. Following minipump implantation skin was sutured around the cannula using 5.0 mercerised silk. Animals were allowed to recover on a heated pad until fully conscious.

2.2.4 Bilateral microinjection into the brain nuclei

A glass pipette was used to microinject virus into the ARC, mPOA or CeA on pnd
10/12 female rats. The glass pipette was made by using a vertical pipette puller (KOPF® instruments, California, US). A 10cm capillary (BF 120-69-10; Linton instrument, UK) tube was placed through the black apparatus at the top of the needle-pulling machine and tightened. The middle of the glass was situated between the top and bottom of the apparatus. When the start button was pressed, the kanthal wire burned red and the glass was pulled to create two pipettes. After the coil had cooled, the newly created pipettes were carefully removed and silicone gel used to block the back of the pipettes. 24hrs later, when the gel was stable, the glass needle could be assembled with the syringe by inserting the syringe needle through the gel.

The anaesthetised rat was placed in a stereotaxic frame and the skull exposed as described above. A stereotaxic alignment tool (David Kopf Instruments) was used to ensure that the surface of the skull was level with the bregma and lambda sutures in the same horizontal plane. A 5μl Hamilton injection syringe (Sigma-Aldrich), attached to a glass pipette preloaded with virus, was mounted on a custom-made clamping devise. Vertical alignment of the glass pipette was checked using a set square, and by the same method as described above to measure zero reference for AP and ML co-ordinates. The needle tip was then positioned using the stereotaxic apparatus following the target co-ordinates (see table 2.1). Small pen marks were made on the skull to indicate the targeted positions. The manipulator was then moved forward to clean the surface of the skull. A disk (FFXC7HP burr; Wright Dental Group Ltd., Brentford, UK) was used to create holes, encompassing the areas
directly above the target position and the dorsal sagittal sinus in the same coronal plane. The small piece of bone remaining after drilling was carefully removed with watchmaker’s forceps. The manipulator arm was then moved back into the calculated AP co-ordinate and the tip of the needle was placed over the midline of the dorsal sagittal sinus to obtain the final ML co-ordinate. The syringe was then lowered until the tip of the needle just made contact with the dorsal sagittal sinus. A measurement for the zero reference DV co-ordinates was taken from this point. The needle tip was then positioned to the final calculated ML co-ordinates using the longitudinal sinus as reference. The dura was pierced with a hypodermic needle to allow entry of the glass pipette without obstacle. The pipette tip was then gradually lowered to the target nuclei within 1 min and left for 1 min before injection (Fig. 2.1 A). The virus was slowly infused into the nucleus over a 10-min period. The pipette was left in place for an additional 5 min to prevent reflux. The needle was then raised over 1 min, and the pipette flushed to remove any debris in the tip. The procedure was repeated to inject the nucleus on the other side. The incision was sutured using 5.0 mercerised silk. After being removed from the stereotaxic frame, the rats were allowed to recover on a heated pad until fully conscious.
Table 2.1 Co-ordinates for bilateral microinjection of the ARC, AVPV, CeA, and the MePD lesion on different pnd in female rats. These measurements (mm) were derived from the rat brain atlas by Paxinos & Watson (1986).

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Anterior-posterior (AP)</th>
<th>Medio-lateral (ML)</th>
<th>Dorso-ventral (DV)</th>
<th>Postnatal day (pnd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC</td>
<td>-2.6</td>
<td>±0.28</td>
<td>-7.4</td>
<td>10</td>
</tr>
<tr>
<td>AVPV</td>
<td>+0.2</td>
<td>±0.35</td>
<td>-6.1</td>
<td>10</td>
</tr>
<tr>
<td>CeA</td>
<td>-2.3</td>
<td>±3.2</td>
<td>-6.6</td>
<td>12</td>
</tr>
<tr>
<td>MePD</td>
<td>-2.5</td>
<td>±3.2</td>
<td>-7.8</td>
<td>21</td>
</tr>
</tbody>
</table>

2.2.5 Head screws for intravenous tether attachment

To allow the anchorage of a metal spring, to protect exteriorised chronically implanted intravenous (iv) catheters used for blood sampling, animals were fitted with a tether screw on the skull. General surgical procedures were carried out as described in section 2.2.2. Three screw holes, two in the occipital bone and a third on the right hand side of the frontal bone, were drilled with a dental bur (RdPC3, Ash Instruments; Dentsply, Addlestone, UK) in a handheld cordless drill (Interfocus, Linton, UK). Three stainless steel cheese head screws (10BA × 3/32 inches; Allscrews, Reading, UK) were screwed into the holes. The screws provided anchorage above that afforded by the otherwise smooth surface of the skull. The slotted screw was held inverted in a micro-manipulator (David Kopf Instruments) on the stereotaxic frame, and was secured in a midline position rostral to the two cheese
head screws in the occipital bone. Dental cement, made from Simplex rapid acrylic powder (Associated Dental Products, Swindon, UK) and mixed with solvent (Associated Dental Products), was applied to the three small screws and the head of the tether screw until all screw heads were completely covered. Once the dental cement had dried, the incision around the shaft of the slotted screw was closed using 5.0 mercerised silk (Pearsalls, Taunton, Somerset, UK). The animal was then removed from the stereotaxic frame and allowed to recover on a heated pad. Once recovered, animals were returned to their individual cages. All experimental surgical procedures were carried out with the aid of a surgical microscope (Carl Zeiss, Welwyn Garden City, UK) and a fibre-optic cold light source (KL 1500 LCD; Schott, Stafford, UK) if required.

2.2.6 Intravenous cannulation

The animals were implanted with intravenous (iv) catheters for collection of blood samples or for systemic injections. Catheters were constructed from 42 cm length PP50 tubing (SIMS Portex Ltd., Hythe, UK) which had a 1.5 cm overlap with a 4 cm Silastic Medical-Grade tubing (Sanitech Corporation) at the proximal end and a 1 cm overlap with 5 cm 0.02 Tygon tubing (Elkay Laboratory Products Ltd.) at the distal end. A 20 cm length of 5.0 mercerised silk was tied to the middle of the overlap of the PP50 and Silastic tubing and held in place using a small amount of Silastic adhesive (Sanitech Corporation). The catheters were left overnight to allow the Silastic
adhesive to dry. The Silastic tubing was cut to a length of 28 mm, measured from the position where the mercerised silk was located. The catheter was flushed with sterile saline and a saline-filled 2 ml syringe was left attached to the distal end of the catheter.

The iv catheterisation was carried out under general anaesthesia, as described in section 2.2.1. The animal was shaved on the ventral surface of the neck and placed on the dorsum on a heat pad with the neck resting on a plasticine support to facilitate iv implantation. The shaved area was sterilised with Betadine surgical scrub followed by Betadine alcohol solution. A 1.5 cm incision was made along the midline over the trachea and the skin retracted with clamps. Blunt dissection was used to clearly expose the left jugular vein; proximal and distal loops of 5.0 mercerised silk were placed around the vessel, lifting the vein slightly to make access easier. The proximal mercerised silk was tightened to close the jugular vein. A small incision was carefully made in the vessel. The Silastic-tipped polythene catheter filled with sterile saline was inserted into the vessel through the incision until the mercerised silk held by Silastic cement reached the vein; at this point the tip of the catheter lay close to the entrance of the right atrium. Blood was then drawn back to check good backflow, indicating correct catheter positioning, and approximately 0.2 ml saline was infused iv. After the blood in the cannula was flushed back to the rat, the catheter position was fixed by the proximal and distal loops of mercerised silk to secure the catheter to the vein. The 5.0 mercerised silk attached to the catheter was sutured to the pectoralis
major muscle to keep the catheter in place. The syringe was removed and replaced with a 0.65 mm diameter stainless steel pin (K.C. Smith Company, UK). The procedure was repeated to implant a catheter into the right jugular vein.

Crocodile forceps were tunnelled from the head tether incision site, under the skin around the neck, to the ventral incision. The free end of the cannula on the same side was held firmly in the crocodile forceps, pulled back under the skin and exteriorised through the head tether incision. This was repeated for the other catheter, and the neck incision was sutured with 5.0 mercerised silk. The catheters were tunnelled through a 30 cm-long protective spring tether (Instec Laboratories Inc.), which was anchored to the head tether screw. A small nut fixed with Loctite adhesive (Henkel Loctite Adhesives Ltd., Welwyn Garden City, UK) was then used to fix the tether to the head screw. Once recovered, the animals were returned to their individual cages, and the end of the spring was attached to a mechanical swivel (Instech Laboratories), which rotated through 360 degrees in a horizontal plane and 180 degrees through a vertical plane, allowing the animals to move freely in their enclosure.

After the animals regained consciousness, catheters were filled with 0.2 ml solution containing 40% polyvinyl pyridilone (Sigma-Aldrich), 2 mg/ml cephalosporin (Eli Lilly and Company, Basingstoke, UK) and 2 mg/ml streptomycin (Sigma-Aldrich) in 200 IU/ml heparinised saline (heparin sodium [mucous]; Holder CP Pharmaceuticals Ltd., Crawford, UK) and re-plugged with stainless steel pins. The solution helped to
maintain cannulae patency, as well as providing anti-bacterial and anti-fungal protection. Animals were left to recover from surgery for three days before initiating experiments.

2.2.7 Ovariectomy

Rats were bilaterally ovariectomised (OVX) to remove fluctuating levels of gonadal steroids. Animals were anaesthetised and an appropriate area in the midline of the back, between the bottom rib and the top of the thigh, was shaved with an electric razor. The shaved area was sterilised by Betadine surgical scrub followed by 75% ethanol as described above. An incision (approximately 2 cm in length) was made along the midline of the back, laterally over the estimated position of the ovaries. Scissors were used to separate the muscle 1 cm left lateral to the spine, and the ovary was retrieved with blunt forceps. The fallopian tube was clamped below the full ovary and the distal end of the uterine horn tied off with a 3.0 mercerised silk suture. The ovary was then removed with a scalpel. The remaining fallopian tube and uterus were replaced in the abdominal cavity and the muscle incision closed with a 3.0 mercerised silk suture. The procedure was repeated to remove the contralateral ovary. Finally, the muscle and skin incision was closed with 3.0 mercerised silk sutures. Once recovered, the animals were returned to their individual cages.
2.3 Experimental procedures

2.3.1 Monitoring vaginal opening, first oestrus and oestrous cyclicity

Rats were inspected daily for vaginal opening (VO) between 09:00-10:00 am from pnd 28. To determine the oestrous cycle phase of each rat, vaginal smears were taken daily for at least consecutive 2 weeks once VO had occurred. Vaginal samples were taken between 09:00-10:00 am. A metal smear loop made from a paper clip was first dipped into saline, then inserted into the vaginal tract to a depth of approximately 4mm and gently rotated. The smear loop was immediately wiped onto a glass slide, which was allowed to air dry, and fixed in 100% ethanol. Slides were examined under a light microscope for the presence of nucleated epithelial cells, cornified epithelial cells, or leukocytes. Oestrous cycle stage was determined using the following criteria: 1) pro-oestrus: predominantly nucleated epithelial cells; 2) oestrus: predominantly cornified anucleated epithelial cells; 3) metoestrus: nucleated epithelial cells, cornified anucleated cells and small leukocytes; 4) dioestrus: predominantly leukocytes, some nucleated epithelial cells and mucus (Marcondes et al. 2002).

A normal oestrous cycle is defined as having at least two consecutive normal cycles, which lasts for 4 to 5 days with 1 to 2 days of oestrus. Cycle length, with three stages observed in the correct order, was determined by the number of days between each occurrence of oestrus (Goldman et al. 2007).
2.3.2 Behavioural observation

Social interaction, play fighting behaviour and the elevated plus maze (EPM) test were used to assess the effect of treatment on anxiety behaviour during the pubertal development period in female rats. Three days before the first behaviour test the rotarod test was used to evaluate whether motor function was impaired by lesion surgery (Rozas et al. 1997). Rats were placed on the accelerating rotarod (4770 Rat Rotarod, Linton Instrumentation, U.K.) and tested at 40 r.p.m. for a maximum of 300 seconds (s). The rat with non-motor deficit was able to stay on the rod at each rotation speed for 300 s.

2.3.2.1 Social interaction behaviour

Before initiating the social interaction test, animals were habituated twice (once per day, 2 days before the day of testing) to the behaviour test room for 15 min. The test was conducted under dim red light during an animal’s light cycle with paired rats (9 am-11 am). The animals are allocated to test partners on the basis of weight; the difference between two tested rats was not greater than 5% so that a large rat was never tested with a small one, as this might change the nature of their interactions, particularly if one rat was clearly dominant over the other. The testing room was dark prior to the entrance of the animals. A pair of rats was placed simultaneously in the 50x50x50 cm arena. Following a 5 min introduction for the paired rats, the duration of social interaction was recorded. The time spent on behavioural events of, snifffing,
chasing, following, grooming and mounting were recorded over a 15 min period by two observers blind to the treatment.

2.3.2.2 Play fighting behaviour

On the day of the experiment, animals were socially isolated in cages for 2–3h prior to testing (Daenen et al. 2002). The test consisted of placing two animals into a novel cage where they were observed under dim red light for a 20-min period between 10:00 am and 11:00 am. Behaviours recorded included: pouncing (one rat lunges at another with its forepaws extended outward: this is initiation of play fighting); wrestling (two rats roll and tumble with each other); boxing (two animals standing upright facing and making pawing attacks at each other); pinning (is the most characteristic posture of play fighting behaviour, where one animals lies with its back on the floor of the cage and the other stands over it, “pinning” it down) (Daenen et al. 2002). Play fighting was defined as a summation of the duration of behaviours listed above, averaged for the four 20 min observation periods. In addition, the frequency of pinning events in the four 20 min observation periods was quantified. Behaviour was analysed by scoring the frequencies of pinning behaviour and the sum duration of play fighting behaviours.

2.3.2.3 Elevated plus maze (EPM) behavioural test

EPM has been described as a simple method for assessing the anxiety response of rodents (Pellow et al. 1985). In this test, the maze consisted of 4 arms: 2 open arms
(51 X 10 cm) and 2 closed arms (51 X 10 cm, with a wall height of 41 cm) and was elevated 72 cm above the floor. Behaviour in this task (i.e., activity in the open arms) reflects a conflict between the rodent’s preference for protected areas (e.g., closed arms). Increased time spent on the open arm reflects an anti-anxiety behaviour. Testing was performed under bright white light. Two days before testing, rats were made to experience handling so as to avoid other stressors altering the EPM results. On the morning of test day, animals were transported to a procedure room from the housing room around 9 am. After a 30 min habituation of the new environment, rats were singly tested in the behavioural testing room. Rats were gently placed in the intersection of the four arms of the EPM and made to face the open arm. The time spent in the open arm was recorded. The test lasted 5 min (Walf and Frye 2007). Between each animal, the EPM was cleaned with 75% ethanol and dried before the next test.

2.3.3 Automated blood sampling for luteinising hormone levels measurement

The computer-controlled automated blood sampling system was based on a previously described system (Clark et al., 1986). The 8-channel system allows intermittent withdrawal of small blood samples (25–35 µl), from eight rats simultaneously, for LH measurement with minimal disturbance to the animal. On the morning of experimentation, one of the two in-dwelling iv catheters was used to connect the animal to the bleeding system via the first channel of a dual-channel
swivel (Instech Laboratories Inc.). Once connected, the animals were left undisturbed for 1 h. Blood sampling commenced at approximately 10:00 h, and samples were collected every 5 min for 5 h or 6 h.

5 cm of size 0.02 Tygon tubing was connected to the distal end of the swivel to the first port on a three-way solenoid valve (Lee Products Ltd., Gerrards Cross, UK). 162 cm of PP10 tubing (SIMS Portex Ltd.) was attached to the second port of the solenoid valve, with the distal end connected to a modified 25G needle (Terumo Ltd) and attached to a 222XL fraction collector (Gilson Inc., Middleton, Wisconsin, USA). Finally, 176.5 cm of PP30 (SIMS Portex Ltd.) tubing was attached to the third port on the solenoid valve and the distal end of the PP30 tubing connected to a bag of heparinised saline (50UI/ml) via a length of peristaltic pump tubing (Gilson Inc.) mounted on an 8-channel peristaltic pump (Gilson Inc.). The heparinised saline served as a reservoir and was made up with 1 ml heparin sodium (25000 IU/ml; Holder CP Pharmaceuticals Ltd.) in a 500 ml bag of 0.9% sterile saline.

A computer-run, custom-made program (D. Layman, King’s College London) controlled the system through a System Interface Module (Gilson Inc.) linked to the peristaltic pump, the fraction collector, and the solenoid valves. The third port on the solenoid, leading to the saline reservoir, was always open whilst the computer could switch between the first port, to the rat, or the second port, to the fraction collector.

Figure 2.1 B provides a schematic representative of the bleeding system. Each blood
sampling cycle lasted 5 min, including a 45 s inactive period. Each cycle started with
the first port on the solenoid valve opening and saline from the reservoir being
delivered to the rat for 4 s. The solenoid then switched to open the second port,
closing the first port towards the animal, and saline was flushed to the fraction
collector for 20 s. Subsequently, the valve to the fraction collector was closed, the
valve to the rat opened, and the direction of the pump reversed to allow the
withdrawal of blood from the rat for 1 min. The solenoid valve was again switched
again to the fraction collector and the pump direction reversed. The pump moved 25
µl of blood through the solenoid into the PP10 tubing leading to the fraction collector
in 10 s. The second port, on the solenoid valve towards the fraction collector, then
closed and the first port opened for 80 s so that the remaining withdrawn blood could
be returned to the animal followed by 25 µl heparinised saline to flush the iv
cannulae and restore fluid lost through blood sampling. The first port was closed and
the second port opened to the fraction collector, and the 25 µl of blood held in the
PP10 tubing was pumped through to the fraction collector and delivered into one of a
series of pre-numbered LP-2 tubes (Thermo Life Sciences, Basingstoke, UK). In
addition to the 25 µl of blood, heparinised saline from the reservoir was pumped
through the fraction collector. This ensured that all the blood was delivered into the
tubes and that the fraction collector tubing was flushed at the end of each cycle. This
saline was also delivered to the LP-2 tubes giving a total volume of 200 µl in each
sample tube (25 µl blood and 175 µl heparinised saline). There was a 45 s pause
before initiation of the next cycle.

After completing all of the sampling cycles, the iv cannulae were disconnected from the bleeding system, flushed with sterile saline, refilled with 0.2 ml polyvinyl pyrolidone solution and re-plugged with stainless steel pins to maintain patency. The animals were removed from the system. The system was then flushed with sterile water followed by 75% ethanol to keep patency and ensure an antibacterial environment. The blood samples were collected and frozen at -20 ºC for later LH assay.
**Fig. 2.1** A. Photograph to show the micro-injection of virus into brain nuclei in pnd 10 rats; B. Components of the automated blood sampling system showing the dual channel swivel leading to the animal connecting to the first port of the solenoid valve, the fraction collector connecting to the second port and the peristaltic pump, and saline reservoir connecting to the third port.
2.3.4 Stress paradigms

2.3.4.1 Restraint stress

Custom-built restraint devices were used for applying restraint stress to rats (Li et al., 2004). This was necessary as commercially available restraint devices were not be suitable for mobile animals with the head tether attachment used for the automated blood sampling in this experiment. The restraint devices were built from strong transparent plastic, in the shape of a cylinder, 15.0 cm in length and 4.8 cm diameter, with a hinged top to allow placement of the rat inside. The head of the rat protruded through a 3.2 cm-diameter collar at one end of the device. At the tail end of the device was a fixture with a 1.5 cm diameter central hole to allow the rat’s tail to extend out from the device. The cylinder was equipped with air holes around the whole tube to avoid overheating. The rat was placed into the open restraint tube and the top half of the tube closed around the rat’s body. The rat was checked to ensure that no skin was caught in the edges of the device and the head could move freely. The rear plate was pushed into the end of the restraint tube with a hole for the rat’s tail. The animal was kept in this device for 1 h.

2.3.4.2 Immunological stress

Typically, after 2 h of blood sampling, lipopolysaccharide (LPS) (25 µg/kg in 0.2 ml saline, Sigma-Aldrich) was administered via the second iv catheter over 1 min; 0.2 ml sterile saline was then flushed through the catheter to ensure complete LPS
delivery. Control animals were administered with 0.2 ml pulse 0.2ml sterile saline.

2.4 Radioimmunoassay

2.4.1 Radioimmunoassay for rat LH

A double-antibody radioimmunoassay supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA) was used to determine LH concentrations in the 25 μl whole blood samples. The reference preparation was rLH-PR-3 and the precipitating antibody was anti-rabbit gamma globulin raised in donkey (IDS, Boldon, UK). The assay sensitivity was 0.093 ng/ml, with inter- and intra-assay variations of 6.8 and 8.0%, respectively. The detailed protocol for LH measurement is attached in the Appendix (section 7.1).

2.4.2 LH pulse analysis

The algorithm ULTRA was used to establish the detection of LH pulses (Van Cauter, 1988). Two intra-assay coefficients of variation of the assay were used as the reference threshold for LH pulse detection. Briefly, ULTRA functions by comparing each data point to the two points before and after it, and determining if a significant rise and fall has occurred in the profile; this is consistent with a pulse. To allow for the fact that a pulse may occur over several data points, the data is ‘cleaned’ so that only absolute troughs and peaks are present; any intermediate points are disregarded. If the difference between any trough and peak is greater that two coefficients of
variation, the peak is considered a pulse.

2.5 Immunocytochemistry

2.5.1 Perfusion

Animals were deeply anaesthetised using either 0.5 ml sodium pentobarbitone (60 mg/ml; Rhone Merieux Ltd, Harlow, UK) administered by iv injection, or 2 ml administered by ip injection. Once unconscious, the rat was immediately prepared for perfusion. The rat was secured with the ventral surface upwards on a perfusion table. A strip of skin and underling musculature extending from the abdomen to the throat was cut, as well as the both side of ribs and diaphragm, to expose the heart clearly. The region around the heart was cleared and the blood flow to the lower body was clamped off at the descending aorta. A blunt 18 gauge needle, attached to Gilson peristaltic pump tubing, was inserted into the apex of the left ventricle and into the aorta where it was secured in place with a clamp. A small cut was made in the right atrium to allow blood and liquid to be leaked from the circulation. Heparinised saline (5 IU/ml) was flushed through the rat using a Gilson peristaltic pump set to deliver approximately 50 ml ice cold saline in 5 min. By this time all blood had been pumped out of the rat and the fluid escaping from the cut in the right atrium was clear. The rat was then perfused for 15 min with 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M PBS until the upper half of the body became stiff. On completion of the perfusion, the brains were carefully removed and placed directly into the solution of
15% sucrose (Sigma-Aldrich) in 4% paraformaldehyde at 4 °C until they sank (approximately 4–6 h). The brains were then transferred to 30% sucrose in 0.1 M PBS solution and left at 4°C until they sank again (approximately 24–36 h). The brains were then frozen in 2-methylbutane solution (Sigma-Aldrich) on dry ice and stored at -80 °C until required.

2.5.2 Brain section

The fixed brain was cut into 30 μm coronal sections using a cryostat (OTF5000; Bright Instrument Co Ltd, Huntingdon, UK). Sections were placed in 0.1 M PBS in a six-well plate. The first section was placed into the first well and each subsequent section was placed into a new well. Once all six wells had a section this was repeated so that every sixth section was in the same well. Once each well contained 10 sections, a new six-well plate was started. The sections of the required specific nucleus were collected in one six well plate, according to coordinates from rat brain atlas (Paxinos and Watson, 1986). Sections were then transferred to antifreeze solution in a 24-well plate, and stored at -20 °C until required for immunocytochemistry.

2.5.3 Immunocytochemical procedure

2.5.3.1 Immunocytochemistry for CRF expression

Every sixth free-floating section throughout the CeA region was used for CRF
immunostaining. After transferring from the antifreeze into a glass vial, the sections were washed three times for 10 min per wash in 0.02 M PBS to remove any sucrose remaining from the antifreeze (in the following processes, if not otherwise specified, 0.02 M PBS was used for washing). The sections were then placed into a 0.3% concentration of triton-X100 in 0.1 M PBS for 45 min to permeabilise the sections. After being washed four times for 15 min each, sections were placed in 2% normal goat serum (NGS) in standard antibody dilution, left for 10 min to minimise non-specific binding, and washed once for 10 min. Sections were then incubated at room temperature for 24 h in 1:2500 rabbit anti-CRF primary antibody (Peninsula Laboratories, Inc., San Carlos, CA) of standard antibody dilution containing 1% NGS. After being washed three times for 20 min per wash to remove the primary antibody, sections were processed with a rhodamine red TM-X goat anti-rabbit IgG (H+L) secondary antibody (Life Technologies Ltd, Inchinnan Business Park, Paisley, UK), at a dilution of 1:500 containing 1% NGS in standard antibody dilution, and left for 2 h at room temperature on a shaker. The sections were washed three times for 10 min per wash to remove the secondary antibody.

The sections were mounted on slides using dapi mounting medium (Vector Laboratories). Omission of the CRF primary antibody resulted in the absence of specific staining. Several brains from each experimental group were processed on the same day to control for inter-batch variability.
2.6 Cresyl violet staining

To establish the extent and location of the neurotoxic lesions or cannula trajectory, every sixth frozen section was stained with modified cresyl fast violet.

2.6.1 Cresyl violet Solution

Cresyl violet solution was prepared by mixing:

0.1g cresyl violet acetate

100 ml ddH₂O

250 μl glacial acetic acid

The cresyl violet solution was stirred overnight at room temperature, filtered through a sterile filter and stored.

2.6.2 Procedure

The frozen fresh brain was cut into 30 μm coronal sections using the cryostat and sections mounted directly on the slides. After fixation in 4% paraformaldehyde and air drying, coronal sections were washed and rehydrated in graded ethanol (100%, 95%, 70% and 50% 3 min each). The slides were counter-stained in pre-warmed cresyl violet solution for 5 min, a stain that colours cells containing endoplasmic reticulum. Sections were then dehydrated in graded ethanol (50%, 70%, 95% and 100% 3 min for each) and were cover-slipped with DPX mountant (Sigma-Aldrich)
after incubating twice in xylene.
CHAPTER 3:

THE RELATIVE IMPORTANCE OF THE ARCUATE AND ANTEROVENTRAL PERIVENTRICULAR KISSPEPTIN NEURONES IN CONTROL OF PUBERTY AND REPRODUCTIVE FUNCTION IN FEMALE RATS

3.1 Introduction

Kiss1 is essential for sexual maturation, as inactivating mutations in Kiss1 (d'Anglemont de Tassigny et al. 2007) or its receptor (Kiss1r) (de Roux et al. 2003, Seminara et al. 2003) result in a lack of puberty and hypogonadotropic hypogonadism, while activating mutations cause precocious puberty (Teles et al. 2008, Silveira et al. 2010). Kiss1 is the most potent upstream stimulator of GnRH in rodents, sheep, primates and humans (Irwig et al. 2004, Matsui et al. 2004, Dhillo et al. 2005, Shahab et al. 2005, d'Anglemont de Tassigny et al. 2007) and is thought to act directly on GnRH neurones. The majority of GnRH neurones express Kiss1r mRNA (Messager et al. 2005, Herbison et al. 2010) and Kiss1 induces GnRH neurone c-Fos expression in rats (Irwig et al. 2004). Signalling through the GnRH neurone Kiss1r has been shown to be essential for the onset of puberty and fertility (Kirilov et al. 2013, Novaira et al. 2014).

In rodents, Kiss1 perikarya are mainly located in the ARC and AVPV hypothalamic nuclei, regions thought to have distinct roles in Kiss1-GnRH function. Unlike GnRH
neurones, both ARC and AVPV Kiss1 neurones express E2 receptor alpha (ERα), acting as relay afferents for estrogentic feedback upon the hypothalamus; positive feedback via the AVPV and negative via the ARC (Smith et al. 2005, Alcin et al. 2013). AVPV neurones project onto and directly stimulate mPOA GnRH neurones (Messager et al. 2005). In rodents, the AVPV has a well-established role in LH surge generation; mPOA infusion of a Kiss1 specific antibody blocks the LH surge and suppresses oestrous cyclicity, and Kiss1 infusion can induce a surge equivalent of LH (Kinoshita et al. 2005). Recently, Kiss1 has been shown to successfully induce an LH surge and egg maturation in humans, with the potential for use in in vitro fertilisation techniques (Jayasena et al. 2014).

ARC Kiss1 neurones are implicated in GnRH pulse generation and are unique in that they co-express NKB and the opioid Dyn, and are, thus, denoted Kiss1-NKB-Dynorphin, or ‘KNDy’, neurones (Wakabayashi et al. 2010). Oscillations of NKB and Dyn are thought to respectively stimulate and inhibit KNDy neurones, driving the pulsed release of Kiss1 which in turn acts to drive GnRH pulses released at the median eminence (Wakabayashi et al. 2010). Axo-axonal interactions may occur between GnRH and Kiss1 neurones at the median eminence (Ramaswamy et al. 2008, True et al. 2011) and Kiss1 and GnRH released in this region have a high temporal concordance in rhesus monkeys (Keen et al. 2008). Kiss1 administration has been shown to increase LH pulse frequency in humans (George et al. 2011, Jayasena et al. 2013), and infusion of a Kiss1 antagonist into the
ME suppresses GnRH pulses in female rhesus monkeys (Roseweir et al. 2009). Intra-ARC administration of a Kiss1 antagonist dose-dependently reduces LH pulse frequency in rats (Li et al. 2009b). We have recently shown that a 27% reduction in ARC Kiss1, using a similar protocol of stereotactic injection of antisense Kiss1 in adult Wistar rats, was sufficient to significantly, albeit modestly, decrease LH pulse frequency without affecting pulse amplitude (Beale et al. 2013). However, humans with severe inactivating mutations in KiSS1 or KiSS1R retain very low amplitude, though normal frequency LH pulses (Tenenbaum-Rakover et al. 2007, Chan et al. 2009). KISS1 may therefore amplify GnRH action, modulating GnRH amplitude.

GnRH pulse amplitude and frequency increase during puberty, stimulating gonadal maturation (Sisk et al. 2001, Harris and Levine 2003). Kiss1 antagonists can delay (Pineda et al. 2010), while agonists can advance (Navarro et al. 2004b) puberty onset in rats. Over the pubertal transition, LH minisurges occur in the afternoon, at approximately the time of the adult surge, increasing in amplitude and frequency until the first mature LH surge proper (Urbanski and Ojeda 1986). E2 dependent maturation of: AVPV Kiss1 gene expression (Gill et al. 2010), AVPV Kiss1 mRNA and immunoreactivity (Navarro et al. 2004a, Han et al. 2005, Shahab et al. 2005), AVPV Kiss1 promoter histone 3 acetylation, recruiting of ERα (Tomikawa et al. 2012), GnRH neurone Kiss1 appositions (Clarkson et al. 2009), Kiss1r expression (Herbison et al. 2010), and GnRH neurone sensitivity to Kiss1 (Han et al. 2005) all occur over puberty.
ARC Kiss1 mRNA increases during puberty in the rat to a much lesser degree than AVPV Kiss1 mRNA (Gill et al. 2010, Li et al. 2012). However, increasing pulse generator frequency is a key pubertal event in the rat (Sisk et al. 2001, Li et al. 2012). Although the roles of ARC and AVPV nuclei in LH surge and pulse generation have been explored, whether it is ARC or AVPV Kiss1 that is essential for puberty onset is unknown. Their distinct roles in subsequent oestrous cyclicity have also not been directly investigated. So far, congenital cell ablation and gene knockout methods have been used to study the necessity of Kiss1 in puberty. Whole body Kiss1 congenital ablation, Kiss1 gene knockout and specific GnRH neurone Kiss1r deletion methods have been previously utilised, with results interpreted as revealing functional compensation, redundant over-production of Kiss1, or the necessity of Kiss1r on the GnRH neurone for puberty, respectively; there have been some conflicting results.

Large-scale gross knockdown methods do not allow discernment of the regional actions of Kiss1. In this study we utilised microinjections of antisense Kiss1 cDNA in a recombinant AAV-associated viral construct (rAAV-kisspeptin-AS) bilaterally into either ARC or AVPV nuclei of female Sprague Dawley rats on pnd 10. The rAAV-encoding enhanced green fluorescent protein (rAAV-EGFP) was used as a control. Unlike global knockdown models, local injections allow discernment of the regional actions of Kiss1 neurones. Some physiological function of Kiss1 is retained which, in combination with injecting the rats at this age, minimises potential
confounding by functional developmental compensation. We were, therefore, able to
investigate the differential role of ARC and AVPV Kiss1 signalling in pubertal
timing, oestrous cyclicity and LH surge and pulse generation.

3.2 Aims

To determine whether Kiss1 knockdown in either APVP or ARC in female juvenile
rats would i) delay puberty onset, ii) affect oestrous cyclicity and spontaneous
proestrus LH surges in the immediate post pubertal period and iii) alter the
frequency of pulsatile LH release.

3.3 Materials and Methods

3.3.1 Antisense kisspeptin construct

Using RT-PCR full length Kiss1 cDNA was isolated and cloned into a pTR-CGW
vector (from Dr Verhaggen, Amsterdam, The Netherlands) with the ampicillin
resistance gene, inverted terminal repeats, human immediate early cytomegalovirus
promoter (CMV) and wood chuk posttranscriptional regulatory element (WPRE).
The CMV promoter was reverse orientation, producing antisense cRNA. The
validity of this construct in inhibiting Kiss1 production was previously tested
whereby transient transfection of RIN 1056a cells with full length antisense Kiss1
significantly reduced Kiss1 secretion compared with control (Beale et al. 2013).
Both rAAV-kisspeptin-AS and rAAV-EGFP were prepared using the adenovirus
free method described previously (Gardiner et al. 2005), and rAAV was produced using the two-plasmid system (plasmid pDG from Dr. Kleinschmidt, Heidelberg, Germany). Dot blot assays were used to establish physical particle titre, and infectious centre assays were utilised to determine infectious particle titre. 1µl of rAAV-kisspeptin-AS or rAAV-EGFP contained 5×10^{12} genome particles.

### 3.3.2 Animals and surgical procedure

Bilateral microinjection of 1µl of virus into the ARC or AVPV nucleus was performed at pnd 10 to allow 3 weeks for maximum expression of kisspeptin-AS to be achieved before puberty onset (Gardiner et al. 2005, Pinilla et al. 2012, Beale et al. 2013). Surgical procedures are described in Chapter 2 section 2.2.4.

Litters were weaned on pnd 21 and housed in groups of 3–4 per cage. 7 days post puberty, the rats were implanted with two indwelling cardiac catheters via the jugular vein (see Chapter 2, section 2.2.6) to enable blood sampling for LH and corticosterone measurement, or infusion of drugs (Li et al. 2012). Following intravenous catheterisation, rats were housed individually and experimentation commenced two days later. When experiments were completed, typically 4–5 weeks after puberty, rats were euthanised and brains collected to evaluate the ARC and AVPV Kiss1, and NKB.

Three weeks after injection of rAAV-EGFP into the ARC or AVPV, a subset of rats...
(n=5 per group) was sacrificed, brains perfused (Chapter 2, section 2.5.1) and harvested and stored at -80°C. Forty-micrometer coronal sections were cut and mounted on poly-lysine slides. The brain sections were rehydrated, cover slipped and viewed by direct fluorescence using a microscope (Nikon A1R Si Confocal, Nikon, Tokyo, Japan).

3.3.3 Quantification of ARC and AVPV kisspeptin, and ARC NKB

Animals were decapitated on pnd 50–60, brains rapidly harvested, snap frozen in isopentane and stored at -80°C. Coronal sections (300 µm) were cut on a cryostat, and punches (1 mm diameter) of these serial sections of the ARC and AVPV were taken from bregma –1.7 to –3.9 mm and +0.2 to –0.4 mm respectively, according to the rat brain atlas (Paxinos and Watson 1986), and based on the micropunch method by Palkovits and Brownstein (Palkovits 1973). The punched tissue was homogenised in an acid-ethanol extraction buffer (0.15% hydrochloric acid in 25% ethanol) and analysed for Kiss1 by specific Kiss1 radioimmunoassay (RIA) using 1.5 x 10^-5 dilution Millipore Kiss1 antibody (Millipore, Watford, UK) and ^125^I-labelled Kiss1-10 (Advanced Biotechnology Centre, Imperial College London, UK) (Beale et al. 2013, Kinsey-Jones et al. 2014). In all assays 350µl of phosphate buffer (pH 7.4) with 0.3% bovine serum albumin and 0.02% Tween 20 was used, and had three days incubation. The smallest detectable limit for the assays was 1 fmol/punch. NKB was quantified in punched ARC tissue from intra-ARC rAAV-kisspeptin-AS and control
rAAV-EGFP injected animals, using an ELISA kit following the manufacturer’s instructions (Peninsula Laboratories LLC).

3.3.4 Experiment One: The effect of knockdown of kisspeptin in the ARC and AVPV on puberty timing in female rats

Animals in all four groups were monitored daily for VO and first oestrus from pnd 28. The body weights of the rat were measured every three days.

3.3.5 Experiment Two: The effect of knockdown of kisspeptin in ARC and AVPV in female rats on cyclicity and the proestrus LH surge

Once vaginal opening occurred, vaginal smears were taken daily for 2–3 consecutive weeks to detect the stage of the oestrous cycles (Chapter 2, section 2.3.1). Approximately 10 days after puberty onset, once confirmed by vaginal smear to be in the proestrus cycle stage in the morning, the rats were connected by one of the two cardiac catheters to a computerised automatic blood sampling system (Chapter 2, section 2.3.3). Half hourly blood sampling began at 1130 h for 8 h. Another set of blood samples (250 µl) were manually collected at 1200 h, via the second catheter, for E$_2$ measurement. Blood samples were frozen at -20 °C for later LH and E$_2$ assays.
3.3.6 Experiment Three: The effect of knockdown of kisspeptin in ARC and AVPV on LH pulse frequency in OVX rats

Following blood sample collection for detection of the spontaneous LH surge, rats were OVX (Chapter 2, section 2.2.7) to facilitate LH pulse detection. After a 10 day recovery period they underwent automated blood sampling for LH pulse frequency, starting at 1000 h, sampling every 5 min for 6h. The animals were then sacrificed and tissues collected.

3.3.7 RIA for LH and oestradiol

A double-antibody radioimmunoassay (RIA) supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD) was used to determine LH concentration in the 25µl whole blood sample. Reference preparation was rLH-RP-3. The sensitivity of the assay was 0.093ng/ml, with an intra-assay variation of 5.3% and an inter-assay variation of 7.5%. A double-antibody RIA (ImmuChem; MP Biomedicals, Orangeburg, NY) was used to estimate the E₂ content of the plasma samples (50µl) following the manufacturer’s protocol. The intra-assay variation was 10.2%. All samples were analysed in a single determination.

3.3.8 Statistical analysis

All data are presented as the mean ± SEM. The effect of rAAV-kisspeptin-AS on LH
surges was determined by analysing the area under the LH profile curve (AUC). The algorithm ULTRA was used to establish the detection of LH pulses (Van Cauter 1988). Two intra-assay coefficients of variation were used as the reference threshold for the pulse detection. The effect of rAAV-kisspeptin-AS on pulsatile LH secretion was analysed by comparing the mean LH pulse interval over the 6-hour sampling period. A Student’s t test was used to analyse Kiss1 immunoreactivity and NKB levels. Fisher’s exact test was used to analyse the percentage of rats spontaneously exhibiting an LH surge in proestrus, and for analysis of oestrous cyclicity. All other results were analysed by one-way ANOVA (Sigmaplot). P < 0.05 was considered significant.

3.4 Results

3.4.1 Anatomical localisation of EGFP after ARC or AVPV injection

After intra-ARC or intra-AVPV rAAV-EGFP injection, EGFP-containing cell bodies and axons were observed at high density within the ARC and AVPV respectively, without spread to surrounding hypothalamic nuclei (Fig. 3.1 A-D). Some slight expression of EGFP was also observed in the injection tracts. Critically, for the intra-ARC or intra-AVPV rAAV-EGFP injected animals, no EGFP-containing neurones were observed in the corresponding AVPV or ARC respectively (Fig. 3.1, A-D).
3.4.2 Effect of rAAV-kisspeptin-AS on ARC and AVPV kisspeptin immunoreactivity (IR) and ARC levels of NKB

Intra-ARC and intra-AVPV rAAV-kisspeptin-AS injections both resulted in significant reductions in Kiss1-IR, validated and quantified by micropunch collection and RIA. Intra-ARC rAAV-kisspeptin-AS caused a 32.0% knockdown in ARC kisspeptin-IR versus control rAAV-EGFP rats (39.4±3.7 [ARC rAAV-kisspeptin-AS; n=7] vs 57.9±4.9 [ARC rAAV-EGFP; n=8] fmol/punch, P<0.05, Fig. 3.1 F. Intra-AVPV rAAV-kisspeptin-AS resulted in a 36.5% reduction in AVPV kisspeptin-IR versus rAAV-EGFP controls (8.1±0.8 [rAAV-kisspeptin-AS; n=7] vs 12.8±1.5 [rAAV-EGFP; n=9] fmol/punch, P<0.05, Fig. 3.1 E. As an additional control for potential cross-contamination between the injection sites in the ARC and AVPV, the effects of intra-ARC or intra-AVPV rAAV-kisspeptin-AS injections, on kisspeptin-IR in the opposite nucleus to where the virus was injected, were examined and shown to have no affect compared with respective rAAV-EGFP controls (ARC content following intra-AVPV rAAV-kisspeptin-AS: 68.3±8.4 fmol/punch, n=6, P>0.05; AVPV content following intra-ARC rAAV-kisspeptin-AS: 16.0±1.2 fmol/punch, n=6, P>0.05). Intra-ARC administration of rAAV-kisspeptin-AS had no significant effect on ARC-NKB content compared with rAAV-EGFP control (31.3±6.5 pg/punch [rAAV-EGFP] vs 27.6±4.8 pg/punch [rAAV-kisspeptin-AS]; n=6-7, P>0.05).
3.4.3 Experiment One: The effect of knockdown of kisspeptin in ARC and AVPV on puberty timing in female rats

Intra-ARC rAAV-kisspeptin-AS did not significantly alter the day of vaginal opening compared with ARC rAAV-EGFP controls (37.2±0.9 [ARC rAAV-kisspeptin-AS; n=7] vs 36.7±0.6 [ARC rAAV-EGFP; n=8] pnd days, P=0.68 Fig. 3.2 A), nor the day of first vaginal oestrus (37.3±0.7 [ARC rAAV-kisspeptin-AS; n=9] vs 37.5±1.0 [ARC rAAV-EGFP; n=7] postnatal days, P=0.86). There was no significant difference between gross body weight gain between ARC rAAV-kisspeptin-AS rats and controls, Fig. 3.2 B.

There was a significant delay in day of vaginal opening following intra-AVPV rAAV-kisspeptin-AS administration compared with AVPV rAAV-EGFP controls (40.2±0.9 [AVPV rAAV-kisspeptin-AS; n=7] vs 36.8±1.0 [AVPV rAAV-EGFP; n=8] postnatal days, P<0.05, Fig. 3.2 C). Intra-AVPV rAAV-kisspeptin-AS also caused a significant delay in day of first vaginal oestrus versus rAAV-EGFP controls (41.0±0.9 [AVPV rAAV-kisspeptin-AS; n=7] vs 37.2±1.0 [AVPV rAAV-EGFP; n=9] postnatal days, P<0.05). Gross body weight gain did not vary significantly between AVPV rAAV-kisspeptin-AS rats and controls, Fig. 3.2 D.
3.4.4 Experiment Two: The effect of knockdown of kisspeptin in ARC and AVPV on cyclicity and proestrus LH surges in female rats

Daily vaginal cytology from puberty onset until the rats were OVX revealed a significant reduction in the percentage of normal oestrous cycles following both ARC and AVPV rAAV-kisspeptin-AS administration. Intra-ARC rAAV-kisspeptin-AS injected rats, compared with rAAV-EGFP controls, had significantly fewer normal oestrous cycles (percentage of normal oestrous cyclicity (28.5% [ARC rAAV-kisspeptin-AS; n=7] vs 87.5% [ARC rAAV-EGFP; n=8]) P<0.05, Fig. 3.3 A). Representative examples of typical oestrous cyclicity are provided, Fig. 3.3 B and C (B: ARC rAAV-EGFP; C: ARC rAAV-kisspeptin-AS). Cycle length was also prolonged (Fig. 3.3 D; P<0.05) with an increase in oestrus and metoestrus phases and a decrease in dioestrous phases, evident in intra-ARC rAAV-kisspeptin-AS treated animals (Fig. 3.3 E; P<0.05). Normal oestrous cyclicity was also significantly reduced in intra-AVPV rAAV-kisspeptin-AS rats compared with rAAV-EGFP controls during the experimental period (percentage of normal oestrous cyclicity (14.2% [AVPV rAAV-kisspeptin-AS; n=7] vs 78.0% [AVPV rAAV-EGFP; n=9], P<0.05, Fig. 3.4 A). Examples of typical oestrous cyclicity are provided, Fig. 3.4 B and C (B: AVPV rAAV-EGFP; C: AVPV rAAV-kisspeptin-AS). Intra-AVPV rAAV-kisspeptin-AS similarly significantly extended cycle length (Fig. 3.4 D; P<0.05) with an increase in proestrus, oestrus and metoestrus and decrease in dioestrous phases evident (Fig. 3.4 E; P<0.05).
To assess LH surge regularity the percentage of rats exhibiting an LH surge on the
day of proestrus was analysed for each experimental group. Spontaneous LH surges
were detected in most control rats on the day of proestrus; no significant reduction
was found in ARC rAAV-kisspeptin-AS animals versus ARC rAAV-EGFP controls
(71.4% [ARC rAAV-kisspeptin-AS; n=7] vs 83.3% [ARC rAAV-EGFP; n=6]
P=0.61, Fig. 3.5 F). However, AUC analysis showed a significant decrease in LH
surge amplitude following ARC rAAV-kisspeptin-AS administration
(2680.84±457.09 [ARC rAAV-kisspeptin-AS; n=5] vs 4814.47±733.10 [ARC
rAAV-EGFP; n=5] ng/ml.min, P<0.05, Fig. 3.5 C). Representative examples of
ARC rAAV-EGFP control, Fig. 3.5 A, and rAAV-kisspeptin-AS, Fig. 3.5 B, LH
surges are included. E2 levels measured on the same proestrous day as detection of
the LH surge were significantly decreased in the ARC rAAV-kisspeptin-AS rats
(23.9±2.4 [ARC rAAV-kisspeptin-AS; n=7] vs 34.1±4.0 [ARC rAAV-EGFP; n=6]
pg/ml, P<0.05).

Representative examples of AVPV rAAV-EGFP, Fig. 3.5 D, and
rAAV-kisspeptin-AS, Fig. 3.5 E, proestrous LH profiles are included. For the AVPV
rAAV-kisspeptin-AS group (Fig. 3.5 E), the representative profile demonstrates an
absence of an LH surge as only one rat out of the seven assessed exhibited a surge on
proestrus. This is reflected in the significant percentage reduction in LH surge
regularity induced following AVPV rAAV-kisspeptin-AS injection compared with
rAAV-EGFP controls (14.3% [AVPV rAAV-kisspeptin-AS; n=7] vs 77.8% [AVPV
AAV-EGFP; n=9] P<0.05, Fig. 3.5 F). There was no significant difference between ARC and AVPV control LH surge AUC analyses, which were comparable in size to the one LH surge exhibited amongst the AVPV rAAV-kisspeptin-AS group. E2 levels measured on the same proestrus day as detection of the LH surge were significantly decreased in the AVPV rAAV-kisspeptin-AS rats (20.8±2.6 [AVPV rAAV-kisspeptin-AS; n=7] vs 38.3±3.5 [AVPV rAAV-EGFP; n=9] pg/ml, P<0.05).

3.4.5 Experiment Three: The effect of kisspeptin knockdown in ARC and AVPV on LH pulse frequency in OVX rats

Regular LH pulses were detected in all OVX experimental groups. Representative LH profiles illustrating the effect of intra-ARC rAAV-EGFP (Fig. 3.6 A), and intra-ARC rAAV-kisspeptin-AS (Fig. 3.6 B) are included. OVX rats administered intra-ARC rAAV-kisspeptin-AS had significantly longer LH pulse intervals compared with ARC rAAV-EGFP controls (27.1±0.6 [ARC rAAV-kisspeptin-AS; n=7] vs 24.9±0.5 [ARC rAAV-EGFP; n=8] min, P<0.05, Fig. 3.6 C). Intra-AVPV rAAV-kisspeptin-AS animals exhibited no significant difference in LH pulse intervals versus controls (26.4±0.8 [AVPV rAAV-kisspeptin-AS; n=7] vs 25.3±0.7 [AVPV rAAV-EGFP; n=9] min, P=0.31, Figure. 3.6F). Representative LH profiles for animals administered intra-AVPV rAAV-EGFP (Fig. 3.6 D) and intra-AVPV rAAV-kisspeptin-AS (Fig. 3.6 E) are included.
Figure 3.1. Effect of intra-ARC and intra-AVPV administration of rAAV-kisspeptin-AS or rAAV-EGFP in female rats. Representative images showing rAAV-EGFP spread in the (A) AVPV and (B) ARC following intra-AVPV administration. Representative images showing rAAV-EGFP spread in the (C) AVPV and (D) ARC following intra-ARC administration. VMH, ventromedial hypothalamus; 3V, third ventricle. (E) and (F), The effect of intra-AVPV or intra-ARC rAAV-EGFP and rAAV-kisspeptin-AS administration (on pnd 10) on kisspeptin-IR in the AVPV and ARC respectively. Quantification of kisspeptin-IR in the AVPV and ARC was carried out between post natal days 50-60 by RIA (n=7-9 per group). Data are shown as means ± SEM; *P < 0.05.
Figure 3.2. The effect of intra-ARC and intra-AVPV rAAV-kisspeptin-AS administration on day of vaginal opening and body weight in rats. (A) No significant delay was observed in day of vaginal opening in intra-ARC rAAV-kisspeptin-AS versus intra-ARC rAAV-EGFP controls. (B) Body weight did not vary significantly between ARC rAAV-kisspeptin-AS and ARC rAAV-EGFP. (C) There was a significant delay in day of vaginal opening following intra-AVPV rAAV-kisspeptin-AS compared with intra-AVPV rAAV-EGFP controls. (D) Body weight did not vary significantly between AVPV rAAV-kisspeptin-AS and AVPV rAAV-EGFP. Results are presented as means ± SEM; *P < 0.05 versus rAAV-EGFP controls; means ± SEM; n=7-9 per group.
Figure 3.3. The effect of intra-ARC rAAV-kisspeptin-AS administration on oestrous cyclicity in juvenile rats. Cyclicity was determined by analysing daily vaginal cytology from day of vaginal opening to ovariectomy. (A) ARC rAAV-kisspeptin-AS resulted in a significant decrease in percentage of normal oestrous cyclicity versus rAAV-EGFP controls. Representative examples of oestrous cyclicity are presented for ARC rAAV-EGFP (B) and ARC rAAV-kisspeptin-AS (C) rats. (D) Cycle length was significantly extended following intra-ARC rAAV-kisspeptin-AS versus intra-ARC rAAV-EGFP controls. (E) Time spent in proestrus (P), oestrus (E), and metoestrus (M) stages was extended, while time spent in dioestrus (D) was decreased by ARC rAAV-kisspeptin-AS compared with controls. Results are presented as means ± SEM; *P < 0.05 versus rAAV-EGFP controls; means ± SEM; n=7-9 per group.
Figure 3.4. The effect of intra-AVPV rAAV-kisspeptin-AS administration on oestrous cyclicity in juvenile rats. (A) AVPV rAAV-kisspeptin-AS resulted in a significant decrease in percentage of normal oestrous cyclicity versus rAAV-EGFP controls. Representative examples of estrous cyclicity are presented for AVPV rAAV-EGFP (B) and AVPV rAAV-kisspeptin-AS (C) rats. (D) Intra-AVPV rAAV-kisspeptin-AS significantly extended cycle length compared with intra-AVPV rAAV-EGFP. (E) Intra-AVPV rAAV-kisspeptin-AS did not significantly extend the percentage of time spent in proestrus (P) compared with AVPV rAAV-EGFP. Both oestrus (E) and metoestrus (M) cycle stages were prolonged following AVPV rAAV-kisspeptin-AS versus AVPV rAAV-EGFP. AVPV rAAV-kisspeptin-AS reduced time spent in dioestrus (D). Results are presented as means ± SEM; *P < 0.05 versus rAAV-EGFP controls; means ± SEM; n=7-9 per group.
Figure 3.5. The effect of intra-ARC and intra-AVPV rAAV-kisspeptin-AS administration on LH surges in rats. Representative LH surges following (A) intra-ARC rAAV-EGFP and (B) intra-ARC rAAV-kisspeptin-AS. (C) AUC analysis of LH surges following intra-ARC rAAV-kisspeptin-AS versus control intra-ARC rAAV-EGFP revealed a significant decrease following ARC kisspeptin knockdown. Representative LH surge for (D) intra-AVPV rAAV-EGFP and (E) the absence of an LH surge following intra-AVPV rAAV-kisspeptin-AS administration. (F), Intra-AVPV rAAV-kisspeptin-AS significantly decreased proestrus LH surge incidence versus intra-AVPV rAAV-EGFP controls; only one rat out of seven exhibiting a normal LH surge occurring on proestrus. There was no significant difference in incidence of LH surges occurring on proestrus following intra-ARC rAAV-kisspeptin-AS versus intra-ARC rAAV-EGFP controls. Results are presented as means ± SEM; *P < 0.05 versus rAAV-EGFP controls; means ± SEM; n=5-9 per group.
Figure 3.6. The effect of intra-ARC and intra-AVPV rAAV-kisspeptin-AS on pulsatile LH release in ovariectomised female rats. Representative LH profiles illustrating the effect of (A) intra-ARC rAAV-EGFP and (B) intra-ARC rAAV-kisspeptin-AS. (C) Intra-ARC rAAV-kisspeptin-AS significantly increased LH pulse interval compared with ARC rAAV-EGFP controls. Representative LH profiles illustrating the effect of (D) intra-AVPV rAAV-EGFP and (E) intra-AVPV rAAV-kisspeptin-AS. (F) LH pulse interval was not significantly altered by intra-AVPV rAAV-kisspeptin-AS compared with rAAV-EGFP controls. Results are presented as means ± SEM; *P < 0.05 versus rAAV-EGFP controls; means ± SEM; n=8-9 per group.
3.5 Discussion

This is the first study in which intra a brain nuclear injections of antisense Kiss1, in juveniles, are used to evaluate the distinct regional roles of ARC versus AVPV Kiss1 in puberty and reproductive function. By knocking down, but not completely ablating, *Kiss1* expression from pnd 10, some physiological function is preserved and the potentially confounding effects of developmental compensation or redundancy are limited. Stereotactically injected rAAV-kisspeptin-AS resulted in a 37% knockdown of AVPV and 32% reduction of ARC Kiss1 content, comparable to a previous study that used this methodology in adult Wistar rats (Beale *et al.* 2013).

AVPV injection of rAAV-kisspeptin-AS delayed the day of vaginal opening and first oestrus by an average of 3.4 and 3.8 days, respectively. There was no effect of ARC Kiss1 knockdown on either measure, despite the close association of puberty with increasing pulse generator frequency in rats (Sisk *et al.* 2001, Li *et al.* 2012). Neither experiment was confounded by changes in body weight, which is a critical factor in pubertal timing in rodents. Although both vaginal opening and first oestrus are surrogate end-point measurements, vaginal opening signifies the rising E₂ levels necessary for puberty initiation and first vaginal oestrus indicates complete HPG axis maturation (Ojeda and Skinner 2006). Given the oestrogenic nature of these markers, the known role of E₂ in the maturation of AVPV Kiss1 over puberty (Navarro *et al.* 2004a, Han *et al.* 2005, Shahab *et al.* 2005, Clarkson *et al.* 2009, Gill *et al.* 2010, Herbison *et al.* 2010, Tomikawa *et al.* 2012), the lack of significant ARC Kiss1
pubertal maturation (Navarro et al. 2004a, Navarro et al. 2004b), and the role of AVPV Kiss1 in E2 positive feedback upon GnRH neurones (Smith et al. 2005), it is perhaps not surprising that it is AVPV and not ARC Kiss1 which appears to be fundamental for puberty onset. It is possible that the ARC knockdown stimulates a greater degree of compensation than AVPV, that AVPV-derived Kiss1 present in the ARC (Clarkson et al. 2009) is significant, or that the residual function remaining after ARC rAAV-kisspeptin-AS is sufficient for maintaining puberty onset, though the abnormal cyclicity and LH pulse data observed following ARC rAAV-kisspeptin-AS administration suggest not.

Whole body congenital ablation of the majority of Kiss1/Kiss1r expressing cells failed to significantly impact puberty timing, oestrous cyclicity, basal LH or subsequent fertility in mice. However, carrying out such an ablation at pnd 20 did have a significant impact on puberty and fertility (Mayer and Boehm 2011). This suggests that if the insult occurs early enough, functional compensatory mechanisms may restore the deficit, safeguarding reproductive function. Whole cell ablation removes all other signalling molecules produced by the cell and may, therefore, trigger a greater level of compensation than other knockout methods. Multiple redundancies may further safeguard fertility. It has been shown that only 34% of GnRH is essential for LH surges and successful ovulation in mice (Herbison et al. 2008). A 95% knockdown of Kiss1 expression did not significantly impact puberty onset, oestrous cyclicity or basal LH in mice, although fecundity was reduced (Popa
et al. 2013). Therefore, either only 5% of murine Kiss1 is necessary for full HPG axis function, or there is compensation, for instance via NKB, glutamate or increasing GnRH neurone sensitivity to the remaining Kiss1 over time (Popa et al. 2013). These results may also reflect the general sensitivity of the Kiss1r to even low levels of Kiss1 (Han et al. 2005). In contrast, in a previous study, Kiss1/Kiss1r mouse gene knockout resulted in delayed vaginal opening, absent oestrous cycles and infertility (Lapatto et al. 2007). The greater degree of gene knockout and the reported heightened GnRH response to Kiss1 could explain the discrepancy of results from this study and support the hypothesised role of compensation. Two studies have recently shown that Kiss1 signalling through Kiss1r on the GnRH neurone is essential for reproductive function. GnRH neurone specific deletion of the Kiss1r resulted in absent puberty over the six month experimental period, no oestrous cyclicity and infertility (Kirilov et al. 2013). A 64% reduction in murine GnRH neurone Kiss1r resulted in delayed day of vaginal opening, abnormal oestrous cyclicity, infertility and reduced basal LH (Novaira et al. 2014). This relationship is causal, as the phenotype was reversed on reinstatement of the Kiss1r (Kirilov et al. 2013).

Puberty halts in ERα knockout mice before first oestrus (Mayer et al. 2010). In agreement with our results, an E2-dependent amplification mechanism may therefore exist, stimulating GnRH neurones, via ERα on AVPV Kiss1 neurones, to reach a mature state. Specific prepubertal ARC E2-inhibition, preventing GnRH pulse
generation and gonadotrophin production in the juvenile period, may be lifted by increasing activation of GnRH neurones via AVPV Kiss1 (Terasawa et al. 2013). The E2/Kiss1-independent trigger driving this activation is unknown; glutamate, GABA and epigenetic regulation of polycomb group genes Eed and Cbx7 are being currently investigated (Clarkson 2013, Lomniczi et al. 2013). Repeats of our methodology using antisense ERα, glutamate, GABA, and regulators such as neuropeptide Y inter alia, may provide information regarding the hierarchy of action, role of critical windows (Clarkson 2013) and the timeline for this process.

There is a general shift from inhibition to stimulation of GnRH neurones over puberty, via changes in GABA, glutamate and Kiss1 signalling, as well as glial cell interactions (Iremonger et al. 2010, Ojeda et al. 2010, Herbison and Moenter 2011). GABA and glutamate are also components of a complex interplay between KNDy neurones and other potential afferents acting upon GnRH dendrons or at the median eminence to stimulate coordinated pulsed secretions of GnRH. Where ARC Kiss1 stimulation sits, and its hierarchical importance within this, is not fully understood. Individually knocking down these alternative regulators using our methodology may also aid in piecing together of the control mechanisms of pulse generation.

Both normal GnRH/gonadotrophin surge (Pineda et al. 2010) and pulse (Li et al. 2009b) generation, supported via Kiss1-Kiss1r signalling in the AVPV and ARC, respectively, are necessary for normal oestrous cyclicity. In accordance with this, we
found abnormal cycles in both rAAV-Kiss1-AS groups. In both ARC and AVPV groups rAAV-Kiss1-AS extended cycle length, in particular by increasing the percentage of time spent in oestrus and metoestrus stages, whereas time spent in dioestrus was approximately halved across both experimental groups. Lapatto et al (Lapatto et al. 2007), Popa et al (Popa et al. 2013), and Mayer and Boehm (Mayer and Boehm 2011) report cycles with some prolonged oestrous phase vaginal cornification, implying estrogentic stimulation has occurred (Chan et al. 2009) at a level insufficient for complete cycles of folliculogenesis and ovulation, reflected in the absent/reduced corpora lutea counts in the former two studies. Kiss1+/−/Kiss1r−/− mice (Chan et al. 2009) and Kiss1 ERα knockout mice (Mayer et al. 2010) move between oestrous and dioestrous phases but remain anovulatory. GnRH knockdown mice that enter puberty, but are incapable of producing an LH surge, are consequently anovulatory and fail to exhibit oestrous cycles (Herbison et al. 2008). Together with our results, these studies suggest that some basal gonadotrophin secretion remains in Kiss1/Kiss1r knockouts, permitting incomplete rounds of folliculogenesis in which the animals get stuck in cornified pre-ovulatory phases as the E2-Kiss1-GnRH stimulus, essential for ovulation, is inhibited (Lapatto et al. 2007, Chan et al. 2009). Our data indicate that a high level of such stimulation is required, as ~35% Kiss1 knockdown in either ARC or AVPV nuclei resulted in Kiss1 levels insufficient for normal cyclicity. The lack of spread of the viral vector between the injection sites in the ARC and AVPV, and the lack of change in kisspeptin-IR in the
opposite nucleus to where the virus was injected suggests that there is unlikely to be any significant cross-contamination between these key Kiss1 containing nuclei.

Our AVPV data are in keeping with the loss of oestrous cyclicity described following mPOA lesioning (Goodman 1978, Wiegand et al. 1980) and mPOA Kiss1 antagonist administration (Kinoshita et al. 2005). Similarly our ARC results correlate with the prolonged oestrous cycles found following ARC lesioning (May et al. 1989), and the disruption in cyclicity we described following rAAV-kisspeptin-AS in adult rat ARC nuclei (Beale et al. 2013). Increased LH inter-pulse interval caused by ARC rAAV-kisspeptin-AS may have impaired tonic release of gonadotrophins and folliculogenesis, and the inhibition of LH surges following both ARC and AVPV rAAV-kisspeptin-AS may have prevented normal ovulation.

LH surges are conventionally associated with AVPV Kiss1 and can be reliably generated using E2 in OVX mice, which stimulates c-fos expression in GnRH and AVPV Kiss1 neurones. This does not occur in Kiss1 and Kiss1r null mice (Clarkson et al. 2008). LH surges were measured in gonadal intact animals, and so are as precise a measurement of spontaneous surge activity as possible. Only one rat in the AVPV rAAV-kisspeptin-AS group exhibited an LH surge correctly occurring on proestrus, which was of normal amplitude. In the ARC knockdown group, all rats had LH surges occurring with normal incidence on proestrus, but with significantly attenuated amplitudes.
AVPV neurones have connections with AVP neurones in the suprachiasmatic nucleus (SCN), implicated in circadian timing (Miller et al. 2004, Vida et al. 2010). In rats the LH surge occurs in the afternoon of proestrus. The circadian rhythm of Kiss1 expression is largely E2-dependent with E2 stimulating both transcription of, and GnRH responsiveness to, Kiss1 whilst GnRH neurones have a time-specific sensitivity to AVP and Kiss1 (Williams et al. 2011). Oestrogenic stimulation, time-gating and additional circadian signalling from the SCN coalesce to control pre-ovulatory LH surge timing (Kriegsfeld 2013, Smarr et al. 2013) triggering ovulation. The lack of normally occurring LH surges induced by AVPV rAAV-kisspeptin-AS supports the role of AVPV Kiss1 in coordinating surge timing. In contrast, the normal temporal occurrence of the AVPV intact, ARC rAAV-kisspeptin-AS LH surges suggests ARC Kiss1 has no influence over this AVPV circadian function, in line with our previous findings (Beale et al. 2013).

As the only LH surge to occur in the AVPV group was of normal amplitude, but the ARC rAAV-Kiss1-AS LH surges were all attenuated, we may infer that ARC Kiss1 has an amplificatory role in surge generation. Rodent ARC KNDy projections extend to GnRH neurones at the ME (Krajewski et al. 2005, Krajewski et al. 2010, True et al. 2011) and perhaps also to an ill-defined point along the dendron projection (Herde et al. 2013). Knocking down ARC Kiss1 may inhibit this amplification and thus the surge amplitude. This may be through a more general disturbance of GnRH neurone activity and hence gonadotrophic hormone support to the ovary. Indeed, selective
ARC KNDy neurone ablation markedly reduced circulating levels of LH in OVX rats (Mittelman-Smith et al. 2012). It is of note that in the present study we did not observe a significant change in ARC-NKB content, following knockdown of Kiss1, thus making it unlikely that the phenotype observed reflects altered action of NKB on gonadotrophin release. Although previously we did not find attenuation of the E2-induced LH surge using this knockdown method in adult OVX rats (Beale et al. 2013), ARC Kiss1 knockdown in the juveniles significantly reduced proestrous E2 levels by approximately 30%. These data raise the question of whether the attenuated LH surge amplitude following ARC Kiss1 knockdown could be related to the lower levels of E2 extant at proestrus in these animals rather than be a direct effect of ARC Kiss1 per se on the GnRH/LH surge process. An area of interest for future work may be to analyse the fos expression of ARC Kiss1 neurones during the LH surge, and to use intra-ARC Kiss1 antagonists to assess if LH surge findings are duplicated, as previous study has tended to focus on the AVPV when analysing LH surges. In addition, a follow-up study using concomitant ARC and AVPV rAAV-kisspeptin-AS, to see if the impact on LH surges and cyclicity is compounded, would also be of interest. Recent general Kiss1 knockdown studies have measured basal LH but not specifically LH surges and pulses (Lapatto et al. 2007, Mayer et al. 2010, Kirilov et al. 2013, Popa et al. 2013, Novaira et al. 2014).

ARC rAAV-kisspeptin-AS modestly but significantly extended the interpulse interval in the OVX adult rats, decreasing LH pulse frequency as described
previously (Beale et al. 2013). Monitoring of LH pulse frequency in the KNDy neurone ablation model of Rance (Mittelman-Smith et al. 2012) would be an intriguing follow-up study. Changes in LH pulse frequency were not observed following AVPV antisense Kiss1, in accordance with the classically distinct role of ARC, and not AVPV Kiss1, in GnRH/LH pulse regulation in various species (O’Byrne and Knobill 1993, Kinsey-Jones et al. 2008, Ohkura et al. 2009). The remaining ARC Kiss1 is insufficient for maintaining folliculogenesis and gonadotropin secretion, and this may underlie the reduced circulating levels of E$_2$ and attenuated LH surges. Kiss1 mRNA and immunoreactivity within the ARC decrease following acute inflammation (Castellano et al. 2010b), during lactation (True et al. 2011) and under stress. This is associated with decreased LH pulse frequency (Kinsey-Jones et al. 2009), which would reduce the likelihood of pregnancy in potentially injurious conditions. Comparable ARC Kiss1 reduction is reported herein and by Beale et al (2013), which may support this theory.

Our data fit well with the developing rodent KNDy-GnRH dendron model which has shown that pulsed ARC KNDy neurone Kiss1, acting through Kiss1r on GnRH dendrons, is crucial for GnRH pulse generation. Although it is probably not the pulse generator per se (Wakabayashi et al. 2010, Herde et al. 2013), as electrical recording of ARC Kiss1/KNDy neuronal activity revealed the majority to be silent or to fire irregularly (Gottsch et al. 2011, de Croft et al. 2012) and no Kiss1 neurones spontaneously burst fire in vivo, that is thought to underlie neuropeptide release
(Kelly et al. 2013). KNDy neurones may instead comprise a major amplifier of GnRH dendron autonomous pulse generation.

In conclusion, this study uses stereotactically injected antisense Kiss1 to knockdown Kiss1 expression within the ARC and AVPV of juvenile female rats, to analyse the subsequent effects on puberty onset and fertility. We have shown that a relatively modest (37%) reduction in AVPV Kiss1 delayed puberty onset and significantly impaired LH surges and oestrous cyclicity. A comparable reduction in ARC Kiss1 did not impact pubertal timing, but significantly impaired LH pulsatility, attenuated LH surge production and impaired oestrous cyclicity. These results suggest a more critical role for AVPV, compared with ARC Kiss1 neurones, in the control of pubertal timing. Our findings support the distinct classical division between ARC and AVPV in the control of LH pulses and surges. Additionally, despite the classical association of ARC Kiss1 with LH pulses and AVPV with LH surges, ARC Kiss1 may have a novel amplificatory role in LH surge production. Both ARC and AVPV Kiss1 appear to be important in maintaining normal oestrous cyclicity. As we have previously concluded (Beale et al. 2013), it seems that Kiss1 expression must be kept within a fairly narrow range so as to maintain normal fertility, and achieve timely puberty.
CHAPTER 4:

CHRONIC OVER-EXPRESSION OF CORTICOTROPHIN-RELEASING FACTOR IN THE CENTRAL NUCLEUS OF THE AMYGDALA INCREASES ANXIETY AND ADVANCES PUBERTY IN FEMALE RATS

4.1 Introduction

The timing of puberty in mammals including man is controlled by a multiplicity of complex interactions between genetic and environmental factors with the latter providing fine tuning to maximise reproductive potential to fit the prevailing or predicted environment. There is unequivocal evidence that chronic stress suppresses the activity of the HPG axis and delays puberty. However, less well recognised is the advancement of puberty by environmental factors such as childhood physical and sexual abuse (Wise et al. 2009, Mendle et al. 2011) or familial stress such as absence of father or parental conflict (Deardorff et al. 2011), with striking parallels in animal models of weak parent-offspring bonding (Cameron et al. 2005).

CRF is heterogeneously distributed throughout the brain, but highly concentrated in the PVN of the hypothalamus and portions of the extended amygdala, including the bed nucleus of the BNST and the CeA. The PVN is the core regulatory component of the HPA axis. Intracerebroventricular administration of CRF or a CRF antagonist delays or advances puberty, respectively (Kinsey-Jones et al. 2010). This suggests
CRF regulation of puberty. We have shown a decline in CRF and CRF receptor type 1 (CRF-R1) and type 2 expression in the PVN across the pubertal transition in female rats (Kinsey-Jones et al. 2010). However, it has been suggested that the CRF containing PVN is not crucial for stress-induced modulation of the HPG axis by PVN lesion studies in adult rats (Rivest and Rivier 1991). However, the site and mechanism of action of the endogenous CRF tone, which plays a critical role in the timing of puberty, remains elusive. An obvious candidate is the CeA because it contains a major CRF neuronal population forming an important component of the limbic brain stress-response network. In addition, CRF expression in the CeA is important in adaptation to chronic stress, during which CRF is upregulated (Shepard et al. 2000, Li et al. 2010). The importance of the amygdala in controlling adult reproductive function is widely recognised, with different sub-nuclei sub-serving stimulatory and/or inhibitory effects on gonadotrophic hormone secretion (Beltramino and Taleisnik 1980, Sanchez and Dominguez 1995). Stimulation and ablation studies in prepubertal rats have demonstrated a critical role of the amygdala in the timing of puberty. Lesions of the MeA have been shown to advance or delay puberty, depending on the developmental age in which the manipulation occurred (Docke et al. 1980), while stimulation has been shown to delay puberty (Bar-Sela and Critchlow 1966). Although similar studies have not been carried out for the CeA, this sub-nucleus plays a critical role in stress-induced suppression of the GnRH pulse generator. We have shown that lesion of the CeA attenuated LPS induced
suppression of pulsatile LH secretion (Lin et al. 2011). Moreover, the use of viral vectors to modulate site-specific CRF expression has enhanced our understanding of the role of CRF in restricted brain nuclei in mediating stress and reproductive related behaviours (Keen-Rhinehart et al. 2009). Over-expression of CRF in the CeA increases anxiety, disrupts oestrous cyclicity, and reduces GnRH expression in the mPOA in adult rats. This strongly suggests that CRF within the CeA negatively impacts reproductive physiology (Keen-Rhinehart et al. 2009).

Puberty is marked by a significant increase in GnRH pulse generator frequency in many species, including rats (Sisk et al. 2001, Li et al. 2012), sheep (Foster and Jackson 2006), monkeys (Watanabe and Terasawa 1989) and humans (Plant and Witchel 2006). It is a time of heightened stress responsivity with earlier onset associated with raised cortisol reactivity (Natsuaki et al. 2009). Although there is clear evidence that the amygdala is involved in facilitating HPA axis activation and regulating the timing of puberty, at least in rodents (Bar-Sela and Critchlow 1966, Docke et al. 1980), its precise role in stress-related change in pubertal timing and reproductive cyclicity remains to be fully established. In the present study, we hypothesise that over-expression of CRF in the CeA in female rats, which mimic the consequences of chronic stress exposure, would alter anxiety behaviour, regulate the HPA axis response to stress, change pubertal timing and disrupt reproduction. To test this hypothesis, we used a lentiviral vector to site-specifically express CRF in the CeA of prepubertal female rats. Lentiviral vectors have proved to be useful for in
vivo studies in the brain because they have a large insert capacity, generate little or no immune response, and maintain expression for the lifetime of the animal (Keen-Rhinehart et al. 2009).

4.2 Aims

To investigate whether continuous CRF over-expression in the CeA in female rats would affect reproductive function, by monitoring puberty timing, oestrous cyclicity and spontaneous LH surge releasing.

To examine the effect of continuous CRF over-expression in the CeA on anxiety behaviour during pubertal development period in female rats.

To investigate whether over-expression CRF specifically in the CeA enhances psychogenic (restraint) stress-induced suppression of pulsatile LH release in the rat, and to compare the response with the commonly used systemic stressor (LPS).

To determine whether CRF over-expression in the CeA affects the pattern of corticosterone in the presence of different types of stressors.

4.3 Materials and Methods

4.3.1 Production of recombinant lentiviral vectors

The cDNA encoding rat CRF was amplified

(5’-CCGCTCGAGCCACCATTGCGGCTGGCTGCTGTGGT-3’ (Xho1) and
5’-CGCAGATCTTTCATTTCCCGATAATCTCC-3’ (BglII)) from rat brain cDNA using Phusion High-Fidelity DNA polymerase (New Enland BioLabs) and cloned into compatible restriction sites of lentiviral vector pRRL.SIN.CPPT.CMV.IRES-GFP.WPRE (LV-CRF). A lentiviral vector expressing GFP (pRRL.SIN.CPPT.CMV.GFP.WPRE) (LV-GFP) was used as control (Addgene Cambridge, MA). Lentiviral vectors were propagated in Stbl3-competent cells and plasmid constructs were purified using the PureLink HiPure Plasmid Filter Maxiprep kit (Invitrogen). Viruses were generated by transient transfection of the transfer vector together with three packaging plasmids (pMDLg/Prre, Prsv-Rev, PMD2.G; Addgene) into HEK293T cells using the calcium phosphate method (Panyasrivanit et al. 2011). Culture supernatant containing lentivirus was collected 48 h and 72 h after transfection, cell debris was removed by centrifugation, and the supernatant filtered through a 0.45μm filter (catalog #430770, Corning). High-titre lentiviruses were produced by centrifugation at 6000 × g for 16 h (400 ml), followed by ultracentrifugation of the resuspended pellet (10 ml of PBS) for 1.5 h at 50,000 × g. The viral pellet was resuspended in 150 μl of prewarmed PBS and stored in 5 μl aliquots at -80 °C. Viral titres were determined by counting GFP-positive cells at day 3 following infection of HEK293T cells (Greenwood et al. 2014). All viruses in the present study had a titre of >3.5 × 10⁹ IU/ml.
4.3.2 Animals and surgical procedures

Under general anaesthesia, rats received bilateral microinjection of Lenti-CMV-CRF-Ires-GFP (LV-CRF) into the CeA (see Chapter 2, section 2.2.4) on pnd 12 (n=25). Controls were carried out using the same procedure but with LV-GFP on same day (n=26). Litters were weaned on pnd 21 and housed in groups of 3–4 per cage. Ten days post puberty, the rats were implanted with two indwelling cardiac catheters via the jugular veins (see Chapter 2, section 2.2.6) to enable blood sampling for LH and corticosterone measurement or infusion of drugs (Li et al. 2012). After intravenous catheterisation, rats were housed individually and experimentation commenced two days later. Following completion of experimentation, typically 4–5 weeks after puberty, the rats were euthanised. Brains were perfused (Chapter 2, section 2.5.1) and collected to evaluate the LV-CRF OR LV-GFP injection sites under a fluorescent microscope for GFP visualisation. Rats that showed GFP at the target injection site were used for CRF immunostaining to quantify positively labelled CRF cells (Chapter 2, section 2.5.3.1). Rats that did not show GFP at the CeA injection site were excluded from data analysis. Ovaries were harvested, wax-embedded, sectioned into 4μm, and the number of corpora lutea in each tenth section averaged.
4.3.3 Experiment One: The effect of over-expression of amygdala CRF on puberty timing

Animals were monitored daily for vaginal opening and first oestrus from pnd 28. The body weight of the rats was measured every three days.

4.3.4 Experiment Two: The effect of over-expression of amygdala CRF on anxiety behaviour

Two days before the first behavioural test was initiated, the rotarod test was used to evaluate motor function (Rozas et al. 1997). The EPM and social interaction tests were performed, as previously described (Chapter 2, section 2.3.2.3) to assess anxiety. Ten days after microinjection of the lentivirus into the CeA, the EPM tests were performed at 5–6 day intervals until approximately 5 days post puberty. Social interaction was tested only once on pnd 30 (Chapter 2, section 2.3.2.1).

4.3.5 Experiment Three: The effect of over-expression of amygdala CRF on oestrous cyclicity and prooestrous LH surge

Once vaginal opening occurred, vaginal smears were taken daily for 2–3 consecutive weeks to detect the stage of the oestrous cycle (Chapter 2, section 2.3.1). Once the prooestrus vaginal cytology was detected by the daily smears carried out in the morning, rats were attached via one of the two cardiac catheters to the automated blood sampling system. Once connected, animals were left undisturbed for 1 h before
sampling commenced. Blood sampling commenced at 1200 h, and samples were collected every 30 min for 8 h for LH surge analysis.

4.3.6 Experiment Four: The effect of over-expression of amygdala CRF in CeA on restraint, LPS stress-induced suppression of pulsatile LH secretion

Following blood sample collection for the detection of spontaneous LH surge, rats were OVX (Li et al. 2005) to facilitate LH pulse detection. After about 7 days, blood samples were collected every 5 min for 6 h for the measurement of LH. After 2 h of controlled blood sampling for baseline LH pulses, animals were exposed to stressors. For restraint stress, the animals were placed in a restraint device (Chapter 2, section 2.3.4.1) for 60 min, with continuous automated blood sampling during restraint and 3-h post-restraint period. On a subsequent occasion, at least 4 days later, the animals were exposed to an immunological stressor, LPS, 2 h into the 6 h blood sampling procedure. LPS (20 µg/kg in 0.2 ml saline, Sigma-Aldrich Ltd., Poole, UK) was injected via the second cardiac catheter (Chapter 2, section 2.3.4.2). Manual blood samples (25μl) were collected via the second catheter immediately before and at 30, 60, 120 and 240 min after restraint or onset of LPS stress. Blood samples were frozen at -20 C for later assay to determine LH and corticosterone concentrations.
4.3.7 Statistical analysis

Comparisons between LV-GFP and LV-CRF groups with respect to body weight, day of vaginal opening, and behavioural parameters were made using a one-way analysis of variance (ANOVA) followed by Dunnett’s test. The percentage of normal oestrous cycles was compared using the $x^2$ Test. Comparison of CRF neurone number and the presence of LH surges between groups were analysed using the Mann–Whitney Rank Sum test. Additionally, to evaluate LH surges, AUC of the LH profile was calculated during the 8-h sampling period and compared using ANOVA. The algorithm ULTRA was used to establish the detection of LH pulses (Van Cauter 1988). Two intra-assay coefficients of variation of the assay were used as the reference threshold for the pulse detection. The inhibitory effects of restraint or LPS stress on LH pulses in the treatment and control groups were calculated by comparing the mean LH pulse interval before stress with the first prolonged interval after stress onset. For the restraint stress the first prolonged interval corresponded to the first LH pulse interval after stress onset. In the case of LPS stress, the first prolonged LH pulse interval was delayed by approximately 25 min post LPS injection. The post stress recovery period was defined as the remaining observation period after the first prolonged LH pulse interval. Statistical significance of LH pulse frequencies and amplitudes, and mean plasma levels of corticosterone at different time points relative to stress between treatment and control rats was tested using ANOVA. $P < 0.05$ was considered statistically significant. Data are presented as
the mean ± SEM.

4.4 Results

4.4.1 Verification of LV-CRF injection

Seventeen out of 26 rats injected with LV-CRF and 15 out of 25 rats treated with LV-GFP virus, verified by correct bilateral detection of GFP in the CeA, were included in the data analysis. Infusion of LV-CRF virus into the CeA resulted in CRF over-expression in the CeA (Fig. 4.1, C, D), compared with control animals that were injected with a LV-GFP (Fig. 4.1, A, B). The CRF over-expression was also limited to the CeA (Fig. 4.1, C) The number of CRF neurones was significantly higher in the treated rats compared with controls (Fig. 4.1, F; P < 0.05).

4.4.2 Experiment One: The effect of over-expression of amygdala CRF on puberty timing

Bilateral administration of LV-CRF virus into the CeA on pnd 12 resulted in a significant advancement in puberty onset compared with controls (vaginal opening: 36.08±0.69 versus 38.44±0.93 days, respectively; Fig. 4.2, A; P < 0.05; first oestrus: 36.30±0.61 versus 38.62±0.93 days, respectively, mean ± SEM). However, there was no significant difference in body weight gain (Fig. 4.2, B). Thus, the advancement of puberty was not induced by body weight change.
4.4.3 Experiment Two: The effect of over-expression of amygdala CRF on anxiety

The EPM was performed once every 5–6 days starting 10 days after microinjection of LV-CRF or LV-GFP and ending about 5 days after puberty. In control animals (LV-GFP), the percentage of time spent in the open arms decreased with approaching puberty, reaching a nadir at 9–5 days before vaginal opening, and followed by a dramatic increase after puberty (1–5 days) (Fig. 4.3, A; P < 0.05). In animals with CRF over-expression in the CeA (LV-CRF), there was a more dramatic and significant decrease in the percentage of time spent in the open arms with puberty development compared with controls (Fig. 4.3, A; P < 0.05). Although the percentage of time spent in the open arms increased post puberty, this was significantly attenuated in the LV-CRF treated group (Fig. 4.3, A; P < 0.05). Rotarod analysis showed that there was no difference between treatment groups in the time the rats stayed on the rotating rod, indicating an absence of impaired locomotion.

The social interaction testing was performed in the prepubertal period on pnd 30. There was a major impact of CRF over-expression in the CeA with a significant decrease in the cumulative mean time spent engaged in social interaction behaviour (including, sniffing, chasing, following, grooming and mounting) between the LV-GFP control (120.25±35.13) and LV-CRF (30.14±3.41) treatment groups (Fig
4.4.4 Experiment Three: The effect of over-expression of amygdala CRF on oestrous cyclicity and proestrous surge

Microinjection with LV-CRF disrupted oestrous cyclicity in the post pubertal period (Fig. 4.4, A; P < 0.05). The majority (76%) of LV-GFP control rats showed normal 4–5 days oestrous cycles post puberty, while only 32% of the LV-CRF treated animals showed normal cyclicity (Fig. 4.4, A; P < 0.05). Representative examples of oestrous cycles from each group are illustrated in figure 4.4 B and C. Cycle length was also prolonged (Fig. 4.4, D; P<0.05) and a predominance of the oestrus phase was evident (Fig. 4.4, E; P<0.05) in LV-CRF treated animals. The number of corpora lutea was significantly less in the LV-CRF compared with LV-GFP treated animals (number per ovarian section: 3.91±0.83 versus 10.38±0.61, respectively; mean±SEM; n=8-9 per group; P<0.05).

Once the animal was in the proestrous phase, the spontaneous LH surge was detected. Low basal levels of LH were seen between 1200 and 1400 h on the day of proestrus in all experimental rats. Seven out of 9 rats from the control group expressed the LH surge on proestrous, typically starting at 1500 h with high LH levels maintained until 1800 h (Fig. 4.5, A). However, all 6 rats from the LV-CRF group failed to
show an LH surge and maintained low levels of LH throughout the 8-h sampling period on the day of proestrus (Fig. 4.5, B). The AUC of the LH profile calculated for the 8-h sampling period quantified the LH surge (2171.97±511.16 vs 937.96±240.91 ng/ml/h; LV-GFP vs LV-CRF group, respectively; P < 0.05).

4.4.5 Experiment Four: The effect of over-expression of amygdala CRF in CeA on restraint or LPS stress-induced suppression of pulsatile LH secretion

Regular pulsatile LH secretion, representing normal GnRH pulse generator activity, was observed during the 2-h baseline blood sampling period, with no significant difference in LH pulse interval between the LV-GFP control (n = 12) and LV-CRF treatment (n = 11) groups (Fig. 4.6, A-C). Restraint stress induced an immediate suppression of pulsatile LH secretion. However, there was no significant difference between treatment groups (Fig. 4.6 A-C). There was also no significant difference in LH pulse amplitudes before (4.17±0.58 vs 4.20±0.59;ng/ml) or after (4.32±0.62 vs 4.20±0.45 ng/ml; mean±SEM) restraint stress between the LV-CRF and LV-GFP groups, respectively.

Similarly, there were no significant differences in LH pulse interval before immunological stress onset between the LV-GFP control (n = 12) and LV-CRF treatment (n = 10) groups (Fig. 4.6, D-F). A prolongation of LH interpulse interval was detected in all animals treated with LPS (Fig. 4.6 D-F). However, the animals
with LV-CRF showed a significantly shorter prolongation of LH pulse interval compared with the control group in response to LPS (Fig. 4.6 D-F; P<0.05). The LH pulse interval in the post-recovery period was comparable between groups (Fig. 4.6 D-F). There was no difference in LH pulse amplitude before LPS stress (3.94±0.56 vs 3.81±0.46 ng/ml) or during the recovery period (4.01±0.61 vs 4.25±0.63 ng/ml; mean±SEM) between the LV-CRF and LV-GFP groups, respectively.

To evaluate whether changes in CRF expression in the CeA modified HPA axis activity, corticosterone levels, under basal conditions and in response to restraint or LPS stress, were determined. Rats had low basal plasma levels of corticosterone prior to restraint or LPS stress and no significant difference was detected under these non-stress condition between the LV-CRF and LV-GFP groups (Fig. 4.7, A and B). Both restraint and LPS increased plasma levels of corticosterone (Fig. 4.7, A and B) as described previously (Li et al. 2006). However, there was no significant difference in the corticosterone response to either stressor between the CRF over-expression and control groups.
Figure 4.1. Lenti-CMV-CRF-Ires-GFP (LV-CRF) microinjection into the central nucleus of the amygdala (CeA) on postnatal day 12 significantly increased corticotrophin-releasing factor (CRF) protein production site-specifically. A, Representative section used to quantify the number of CRF immunopositive staining neurones in the CeA from a rat injected with the control virus, expressing GFP (LV-GFP). B, Enlargement of the CeA region corresponding to the control virus injection site illustrated in A. C, Representative section showing the effects of LV-CRF injection into the CeA on the number of positive labelled CRF neurons. D, Enlargement of the CeA region corresponding to the LV-CRF injection site illustrated in C. E, Schematic representation of site of injection adapted from Paxinos and Watson rat brain atlas. F, Summary of number of positive labelled CRF cells in the CeA of LV-GFP and LV-CRF treated rats determined by immunocytochemistry. *P < 0.05 versus LV-GFP control; mean ± SEM; n = 15-17 per group.
Figure 4.2. Lenti-CMV-CRF-Ires-GFP (LV-CRF) microinjection into the central nucleus of the amygdala on postnatal day 12 advanced puberty in female rats. A, Data are presented as day of vaginal opening (puberty) in rats injected with LV-CRF compared with control virus, Lenti-CMV-GFP (LV-GFP). B, There was no significant difference in body weight gain between LV-CRF and LV-GFP. *P < 0.05 versus LV-GFP control; mean ± SEM; n = 15-17 per group.
Figure 4.3. Effects of over-expression of CRF in the central nucleus of the amygdala (CeA) on anxiety-like behavior evaluated using the elevated plus maze (EPM) across pubertal development and social interaction behaviour in the female rat. A, Lenti-CMV-CRF-Ires-GFP (LV-CRF) injected animals spent significantly less time in the open arms of the EPM (more anxious) compared with control virus (LV-GFP) injected rats. Data are aligned to the day of puberty (vaginal opening, day 0). B, LV-CRF treated animals spent significantly less time in social interaction behaviour compared with controls. *P < 0.05 vs controls at same time point; #P < 0.05 vs -(19–15) days time point in same treatment group; $P < 0.05 vs all other time points in same treatment group; †P < 0.05 vs control LV-GFP group at -(19–15) and -(14–10) days’ time points in same treatment group; n = 15-17 per group.
Figure 4.4. Lenti-CMV-CRF-Ires-GFP (LV-CRF) microinjection into the central nucleus of the amygdala on postnatal day 12 disrupted oestrous cyclicity in female rats. A, The percentage of normal oestrous cycles was significantly decreased immediately after puberty in LV-CRF treated compared with LV-GFP control rats. B, C, Representative examples of oestrous cyclicity are illustrated: a normal control rat (B) and a rat injected with LV-CRF (C). D, Length of oestrous cycles was significantly increased by LV-CRF treated compared with LV-GFP. E, The effect of treatment on the percentage of time spent within each oestrous cycle phase is shown, P, proestrus; E, oestrus; M, metoestrus; D, dioestrus.; LV-CRF primarily increased oestrus and decreased dioestrus. *P < 0.05 versus LV-GFP control; mean ± SEM; n = 15-17 per group.
Figure 4.5. The effects of CRF over-expression in the central nucleus of the amygdala (CeA) on the spontaneous preovulatory luteinising hormone (LH) surge. A, Representative example of an LH surge on the day of proestrus in a rat microinjected with control virus, Lenti-CMV-GFP. B, An example showing no proestrus LH surge from a rat injected with Lenti-CMV-CRF-Ires-GFP (LV-CRF). Blood samples were collected between 1200 and 2000 h on the day of proestrus for the measurement of LH.
Figure 4.6. Effect of Lenti-CMV-CRF-Ires-GFP (LV-CRF) microinjected into the central nucleus of the amygdala (CeA) prepubertally (postnatal day 12) on stress-induced suppression of pulsatile LH secretion in rats subsequently ovariectomised postpubertally to facilitate LH pulse detection. Representative examples illustrating the effect of restraint (1 h) stress on pulsatile LH secretion in control virus (Lenti-CMV-GFP: LV-GFP) (A) or Lenti-CMV-CRF-Ires-GFP (LV-CRF) (B) microinjected animals. Representative examples illustrating the effect of LPS (20 µg / kg iv) stress on pulsatile LH secretion in control virus (LV-GFP) (D) or LV-CRF (E) microinjected animals. Summary showing that treatment with LV-CRF to overexpress CRF in the CeA attenuates the inhibitory effects of LPS (F), but not restraint (C) stress on pulsatile LH secretion. *P < 0.05 versus pre-stress control period within the same group. #P < 0.05 versus LV-CRF group at the same time point; mean ± SEM; n = 10-12 per group.
Figure 4.7. The response of the hypothalamic-pituitary-adrenal axis to stress is not modified by over-expression of CRF in the central nucleus of the amygdala (CeA). The increased plasma levels of corticosterone in response to LPS (20 µg / kg iv) (A) or restraint (1 h) (B) stress were not significantly different between the Lent-CMV-CRF-Ires-GFP (LV-CRF) and control virus (LV-GFP) group. Values are expressed as the mean ± SEM; n = 10-12 per group. CORT: corticosterone
4.5 Discussion

The present study demonstrates increased anxiety-like behaviour, advancement of puberty onset, followed by irregular oestrous cycles with CRF over-expression in the CeA. These results demonstrate for the first time a novel role for the classical stress neuropeptide, CRF, within the CeA on the timing of puberty. The CeA, which contains one of the largest populations of CRF neurones outside of the hypothalamus and is a key integrator of autonomic, behavioural and neuroendocrine responses to stress (Flandreau et al. 2013) has not been studied. We have previously shown that an endogenous CRF tone plays a role in modulating the timing of puberty (Kinsey-Jones et al. 2010). A reduction in CRF and CRF-R1/R2 expression in the PVN was detected across the pubertal transition in female rats (Kinsey-Jones et al. 2010). However, the CRF containing PVN may not be crucial for control of pulsatile LH secretion (Rivest and Rivier 1991), which is critical for puberty onset (Watanabe and Terasawa 1989, Sisk et al. 2001). In the amygdala, CRF receptors demonstrate age- and sex-specific changes in binding across puberty (Weathington and Cooke 2012). Although CRF receptor binding was lowest in the CeA compared with other amygdaloid subnuclei, and CRF-R1 was unchanged, CRF-R2 binding increased substantially more in male than in female rats across puberty (Weathington and Cooke 2012). Exposure to peripubertal stress, however, enhanced CRF-R1 but not CRF-R2 expression in the CeA, which was associated with increased anxiety (Veenit et al. 2014). Whether these changes in the CeA were linked to pubertal
timing was not studied.

In the Japanese quail, genetic selection for high or low HPA axis stress responsivity delays or advances puberty, respectively (Satterlee et al. 2002). Mice over-expressing CRF are hyper-corticosteronemic, stress hyper-responsive and display reduced fertility (Stenzel-Poore et al. 1992). Juvenile female rats receiving intracerebroventricular infusion of CRF had delayed puberty, although corticosterone levels were unchanged (Kinsey-Jones et al. 2010). Furthermore, central administration of a non-selective CRF receptor antagonist advanced puberty, indicating an endogenous CRF tone might be a key component of the pubertal timing mechanism (Kinsey-Jones et al. 2010). CRF and various stressors downregulate hypothalamic mPOA and ARC kisspeptin in adult rats (Kinsey-Jones et al. 2009, Castellano et al. 2010a). Moreover, early life stress, such as neonatal exposure to LPS, which delays puberty, also downregulates kisspeptin, a major regulator of puberty (Kinsey-Jones et al. 2010). This suggests that an action of CRF on kisspeptin signalling might underlie stress-induced delay of puberty.

Contrary to our expectation, in the present study, puberty was advanced in rats over-expressing CRF in the CeA. However, there are several situations where stressful environmental conditions are associated with advanced puberty. Girls encountering familial conditions that are unfavourable, such as family stress and poor parent-child relations were shown to have early menarche (Reardon et al. 2009,
Absence of a biological father advanced puberty in girls (Deardorff et al. 2011). In addition, childhood physical and sexual abuse has been shown to more robustly advance puberty and is accompanied by increased anxiety (Reardon et al. 2009, Wise et al. 2009, Mendle et al. 2011). There is considerable evidence that parental care serves as a mediator for the effects of environmental adversity on development (Cameron et al. 2005). In animal models of weak parent-offspring bonding, such as Meaney’s low licking and grooming rats, the offspring from such dams are not only more fearful and show increased HPA responses to stress, but have raised CRF expression in the CeA, and reported to have advanced puberty (Cameron et al. 2005). In general, advancement of puberty in high-risk environments, where survival is potentially less certain, may be seen as an adaptive response presumably increasing capacity to reproduce, which could have an evolutionary advantage (Hochberg and Belsky 2013).

The advancement of puberty in rats over-expressing CRF in the CeA may result from mechanisms of habituation that reduce the inhibitory influence of daily life stress on the GnRH pulse generator. Indeed, in the present study we have shown an attenuation of LPS stress-induced suppression of LH pulses in these animals. Although CeA lesions do not affect LH pulse frequency under stress-free conditions (Lin et al. 2011), we have previously shown that LPS stress-induced suppression of LH pulses was blocked by CeA lesions (Lin et al. 2011). Therefore, over-expression of CRF in the CeA might be expected to negatively impact on the GnRH pulse
generator. Nevertheless, neither basal corticosterone levels, nor the corticosterone response to stress, indicated an upregulation of the HPA axis in the CeA CRF over-expression animals. This may support the notion of habituation or adaptation under chronic stress conditions.

In the present study, irregular oestrous cyclicity accompanied by lack of LH surges was observed in the post-pubertal period of rats over-expressing CRF in the CeA, consistent with previous reports showing the involvement of CRF in the CeA in reproductive dysfunction in adult rats (Keen-Rhinehart et al. 2009). Specifically, continuous over-expression of CRF in the CeA lengthened the oestrous cycle or induced acyclic and significantly decreased GnRH levels in the mPOA (Keen-Rhinehart et al. 2009). Psychosocial stress associated with subordinate status reduces fertility, secondary to an increased incidence of anovulation in rhesus monkeys (Adams et al. 1985). Rats subjected to chronic mild stress showed reduced oestrous cyclicity (Baker et al. 2006). Cynomolgus monkeys that exhibit immediate suppression of menstrual cycles and anovulation upon stress onset, termed ‘stress sensitive’, have a greater density of CRF fibres in the CeA compared with stress resilient conspecifics that continue to cycle normally (Centeno et al. 2007). Therefore, it is suggested that long-term increase of CRF in the CeA may be a causal factor in stress-related reproductive disorders, including functional hypothalamic amenorrhea, and may help to explain the loss of normal reproductive function following early puberty in the present study.
It is well established that the amygdala is involved in the control of ovulation in rodents. Lesioning of the MeA blocked ovulation (Bagga et al. 1984), while stimulation advanced the time of the LH surge (Beltramino and Taleisnik 1978). Stimulation of the basolateral amygdaloid nucleus or CeA blocked or delayed the LH surge, respectively (Beltramino and Taleisnik 1978). Thus, the amygdala exerts a dual effect on the preovulatory LH surge; the basolateral and CeA being inhibitory and the MeA facilitatory. However, the mechanism by which CRF in the CeA is involved in the control of the LH surge or ovulation, as alluded to in the present study, remains to be elucidated.

Although there are extensive projections from the MeA to the GnRH-rich region of the mPOA (Usunoff et al. 2009), there are very few (Berk and Finkelstein 1981) or no (Prewitt and Herman 1998, Hahn et al. 2003) neuronal projections from the CeA to the GnRH-rich mPOA or kisspeptin-rich AVPV that control ovulation. Nevertheless, the CeA is known to densely innervate the BNST and locus coeruleus, primarily with CRF containing neurones (Swanson et al. 1983, Sakanaka et al. 1986), which in turn project to the ARC (Dong et al. 2001, Iqbal et al. 2005) and mPOA (Dong and Swanson 2006, Campbell and Herbison 2007). Moreover, the dorsolateral part of the anterior BNST, stimulation of which blocks the LH surge (Beltramino and Taleisnik 1980), is preferentially innervated by the CeA (Dong et al. 2001) and thus provides an indirect functional route for amygdaloid influence on kisspeptin and gonadotropic hormone secretion. However, we cannot rule out the possibility that the
absence of an LH surge in the CRF over-expressing groups is due to an inadequate $E_2$
positive feedback signal; the measurement of circulating levels of the steroid is
needed in future studies. An additional caveat is the possibility of a delay in the
timing of the LH surge beyond 2000h, when we stopped sampling. Nevertheless,
reduced ovulatory efficiency is evident with a significant decrease (62%) in the
number of corpora lutea and a complete absence in 2 of the 8 animals
over-expressing CRF. The present study suggests that the CeA is a key component in
the forebrain stress neural circuitry impacting ovulation. Why over-expression of
CRF in the CeA promotes early activation of the HPG axis, but suppresses its activity
in adulthood, is a conundrum. The neural mechanisms underlying puberty onset
versus maintenance of ovarian cyclicity might be differentially regulated by the CeA
and requires further study. However, parallels are seen, for example obesogenic diets
which advance puberty (Castellano et al. 2011, Li et al. 2012) can also induce
irregular oestrous cycles in adult rodents (Balasubramanian et al. 2012, Lie et al.
2013). Although advancement of puberty following overnutrition is associated with
upregulation of hypothalamic kisspeptin (Castellano et al. 2011, Li et al. 2012), the
role of kisspeptin in the disruption of ovarian cyclicity, following overnutrition, has
not been fully characterised (Lie et al. 2013). The dynamics of kisspeptin signalling
remain to be examined in the model of CeA CRF over-expression.

Over-expression of CRF in the CeA is thought to recapitulate many of the effects of
chronic stress, including hyper-activation of the HPA axis. However, in the present
study, there is no evidence of up-regulation of the HPA axis both in terms of basal or stress-induced corticosterone release. The CeA plays a major role in modulating the HPA axis and anxiety behaviours triggered by stress. Lesioning of the CeA attenuates corticosterone release, evoked by cytokines (Xu et al. 1999) or chronic stress, and decreases stress-induced anxiety (Ventura-Silva et al. 2013). It has been reported that over-expression of CRF in the CeA increases HPA axis reactivity, enhances the ACTH and corticosterone response to a dexamethasone/CRF test, increases the expression of both CRF and AVP in PVN, and reduces glucocorticoid negative feedback (Keen-Rhinehart et al. 2009). Resistance to glucocorticoid feedback inhibition is present in many situations associated with chronic stress, including anxiety disorders and functional hypothalamic amenorrhea, and is thought to reflect decreases in central glucocorticoid receptor (GR) number, binding affinity or activation (de Kloet et al. 2005). However, other studies have failed to observe changes in the HPA axis following CeA CRF over-expression (Regev et al. 2011). The present study showed that basal levels of corticosterone were not altered by CRF over-expression in the CeA, which is in agreement with previous publications using site specific over-expression of CRF in the CeA (Keen-Rhinehart et al. 2009, Regev et al. 2011, Regev et al. 2012, Flandreau et al. 2013). It has even been reported that there is a significant increase in basal corticosterone levels in the CeA-CRF knockdown mouse (Regev et al. 2012). The corticosterone response to restraint stress was not modified by prolonged (4 months) CRF over-expression in the CeA in
mice (Regev et al. 2011). Chronic stress, which is known to upregulate CRF in CeA, attenuates the stress response through the habituation of the HPA axis (de Kloet et al. 2005). The increase in AVP in PVN is a well established consequence of chronic stress (Ma et al. 1999). However, CRF expression has been shown to remain at basal levels (Ma et al. 1999). Although tonic elevation of plasma corticosterone due to chronic stress may inhibit the HPA axis via negative feedback mechanism, the HPA axis usually retains responsiveness to new acutely applied stressors (Ma et al. 1999). The peripubertal rats used in the present study may have greater neural plasticity and therefore did not show a significant change in the corticosterone response to LPS or restraint stress despite the prolonged CRF over-expression in the CeA. This may be crucial for the individual’s survival and health, whereby limiting the stress response and preventing pathologies associated with excess CRF levels in CeA such as in chronic stress condition.

We demonstrated that over-expression of CRF in the CeA decreased open arm exploration in the EPM and social interaction in the peripubertal rats. This further confirms previous reports suggesting a central role for CRF in the limbic brain region in control of anxiety-like behaviours in adult animals (Flandreau et al. 2012). However, Flandreau et al (Flandreau et al. 2013), unlike the present study, did not show any changes in social interaction following over-expression of CRF in the CeA (for 8 weeks), but they did observe increased anxiety-like behaviour in the defensive withdrawal test in the rat. Although, Regev et al (Regev et al. 2011) reported that
CeA CRF over-expression did not increase basal anxiety-like behaviour, they did show augmented behavioural responses to acute stress exposure indicative of enhanced anxiogenic effects in mice. These results may suggest a threshold for the anxiogenic effect of CRF, below which CRF in the CeA does not affect anxiety-like behaviours. However, it is of note that knockdown of CRF in the CeA resulted in a decrease in anxiety-like behaviour as measured using the EPM (Regev et al. 2011). Although a decrease in anxiety behaviour is seen in late adolescent rats (Lynn and Brown 2009), the dramatic and sudden nature of the decrease observed immediately post puberty in the present study is novel. The adolescent brain undergoes dramatic developmental change (Spear 2000), controlled in part by gonadal steroids (Sisk and Zehr 2005), however, it remains to be established if they underlie this phenomenon.

In conclusion, over-expression of limbic brain CRF with concomitant enhanced anxiety may provide a novel mechanism for early puberty associated with early life adversity. Additionally, continued longer-term hyper-activation of CRF in the CeA might contribute to disruption to ovarian cyclicity and ovulation and hence infertility in adulthood.
CHAPTER 5:
THE ROLE OF THE POSTERODORSAL MEDIAL AMYGDALA IN PUBERTAL TIMING IN FEMALE RATS

5.1 Introduction

The onset of puberty is heralded by the maturation of the HPG axis which is determined by a multiplicity of inter-related processes, although its mechanism remains unknown. Psychosocial stress can either delay or advance puberty both in humans and experimental animals (Wise et al. 2009, Wilson et al. 2013, Li et al. 2014a, Li et al. 2014b). The changes in the timing of menarche can result from both environmental and social contexts. Parental divorce or childhood sexual or physical abuse accelerates menarche (Wise et al. 2009, Li et al. 2014a), while social context has been shown to result in earlier menarche in higher ranking monkeys (Wilson et al. 2013) and later ovulation in lower ranking females (Wilson et al. 2013, Stephens et al. 2015). We have also shown that mimicking stress by the overexpression of CRF in the CeA results both in anxiety and advancement of puberty in female rats (Li et al. 2014b). Numerous brain regions have been implicated in these stress-related neural circuits, with a prominent role suggested for the amygdala (Rauch et al. 2003). The amygdala is also strongly implicated in social behaviours, especially play behaviour and playing fight (e.g. pinning), which are the earliest forms of non-mother-directed social behaviours and associated with pre-pubertal rats (Hole 1988).
Amygdala lesions affect both fear (Raper et al. 2014) and timing of puberty in female monkeys (Stephens et al. 2015). Such lesions have been shown to alter the timing of puberty in rats from studies that date back to the 1960s (Docke et al. 1983, King et al. 1996a). Neurotoxic lesioning of whole amygdala at one month of age advanced puberty in female rhesus monkeys (Stephens et al. 2015), while lesions at 10–13 months had no effect on menarche, but delayed first ovulation in some monkeys (Norman and Spies 1981). There are also controversies relating to the effects of amygdala lesions on pubertal timing in rats (Docke 1974, Docke et al. 1976, King et al. 1996a). Results from the large and unpredictable electrolytic lesions used in the rat studies may confound our understanding of amygdaloid organisation, because they do not take into account its heterogeneous nuclear arrangement and their related functions. For example, GABA is associated with distinct roles in different subnuclei; aggression and anxiety with CeA (Sanders and Shekhar 1995, Haller et al. 2005), fear extinction with basolateral amygdala (Li et al. 2009a), and social behaviours with MeA (Trezza and Campolongo 2009). However, the effect of lesioning specific subnuclei within the amygdala on puberty has not yet been established, and little is known of the potential impact on pubertal timing of GABAergic or glutamatergic signalling in individual amygdala subnuclei.

The MeA is strongly implicated in ovulation, oestrous cyclicity, gonadotrophin secretion and sexual behaviour (Beltramino and Taleisnik 1978, Salamon et al. 2005). Gonadotrophins increased after MeA lesion (Lawton and Sawyer 1970),
while electrical stimulation delayed puberty in rats (Bar-Sela and Critchlow 1966), suggesting that the MeA exerts an inhibitory influence on reproduction. The MeA, in particular its MePD, receives olfactory information (Choi et al. 2005), predominantly sends out inhibitory GABAergic projections, and is implicated in reproductive behaviour (Choi et al. 2005, Pardo-Bellver et al. 2012). GABA is strongly implicated in fear and anxiety with GABA$_A$ receptors (GABA$_A$R) playing a major role (Zarrindast et al. 2001, Tasan et al. 2011, Smith et al. 2012). Stressors also alter expression of GABA (Gilabert-Juan et al. 2011) and GABA$_A$R in the amygdala (Jacobson-Pick and Richter-Levin 2012). Additionally, glutamate signalling in the amygdala, in particular via its NMDA receptor, is involved in anxiety-related behaviour (Hegoburu et al. 2014), and intra-MePD administration of NMDA disrupts ovarian cyclicity in the rat (Polston and Erskine 2001). Whether NMDA or GABA$_A$R signalling in the MePD is involved in puberty timing has not been investigated.

Decrease in GABAergic and increase in glutamatergic tone in the hypothalamus are critical for initiating GnRH release essential for puberty (Maffucci and Gore 2009, Kurian et al. 2012). What is unknown, however, is the mechanism that regulates this hypothalamic tone. The MePD sends direct efferents to the GnRH-rich mPOA (Canteras et al. 1995, Hahn et al. 2003), which controls gonadotrophin release. Therefore, the MePD is ideally situated to process environmental and sexual cues and bring about appropriate alterations in the reproductive axis. The MePD has the
highest density of sex steroid receptors of the amygdala. It undergoes dramatic change during puberty including increased glutamatergic activity, without a corresponding GABAergic change, suggesting an activation of this area at the onset of puberty (Cooke 2011). The MePD is also a critical site for lesion-induced hyperphagia and obesity in rats (King 2006), which may explain altered pubertal timing by way of an earlier attainment of critical body mass (Frisch 1983). The MeA is implicated in psychological stress-induced suppression of LH secretion in rats (Lin et al. 2011), which is of interest because psychological stress alters pubertal timing (Wise et al. 2009, Li et al. 2014a).

5.2 Aims

To test the hypothesis that the MePD is critical for timing of puberty and investigate whether this is independent of its regulatory role on weight gain.

To examine the effect of MePD lesions on oestrous cyclicity following puberty.

To determine whether the advancement of pubertal timing is correlated with reduced anxiety-like behaviour in MePD lesioned female rats.

To investigate the role of the MePD GABA<sub>A</sub>R and the glutamatergic NMDA receptor in mediating pubertal timing and behaviour.
5.3 Materials and Methods

5.3.1 Animals and surgical procedures

5.3.1.1 Bilateral lesions of the MePD

Under general anaesthesia, rats received bilateral injection of ibotenic acid into the MePD (see Chapter 2, section 2.2.2) on pnd 21 (n=28). Controls were carried out using the same procedure but with aCSF on same day (n=17). Litters were weaned on pnd 21 and housed in groups of 3–4 per cage. On pnd 31, rats were implanted with two indwelling cardiac catheters via the jugular veins (see Chapter 2, section 2.2.6) to enable blood sampling for LH and corticosterone measurement or infusion of drugs (Li et al. 2012). After intravenous catheterisation, rats were housed individually and experimentation commenced one day later. Following completion of experimentation, typically 4–5 weeks after puberty, the rats were euthanised. Brains were collected and stained to evaluate the injection sites using a microscope for visualisation. Rats with lesions outside of the desired structure were excluded from subsequent analysis.

5.3.1.2 Osmotic minipump implantation with GABA and glutamate antagonist administration

On pnd 21, standard chow diet fed female rats were implanted bilaterally with an osmotic minipump prefilled with antagonists or aCSF (Chapter 2 section 2.2.3). A glutamate antagonist, D-2-amino-5-phosphonovalerate (D-AP5, NMDA antagonist,
Tocris, Bristol, UK) (n = 10), GABA\textsubscript{A} antagonist, bicuculline (n=12) (Sigma-Aldrich, UK), or aCSF (n = 7-10 per group) were delivered via the osmotic minipump at 0.25 μl/h for 14 days. The concentrations of D-AP5 and bicuculline used were 15 nmol/μl and 2.26 pmol/μl, respectively. Following completion of experimentation, the rats were euthanised. Brains were collected and stained to evaluate the injection sites under a microscope for visualisation. Rats with cannulae outside of the desired structure were excluded from subsequent analysis.

5.3.2 Experiment One: The role of the MePD in pubertal timing in different diet fed female rats

To determine whether the advancement of puberty seen in the lesion group could be explained by a change in body weight gain, as previous reported (King 2006), litters were either assigned to normal standard chow diet (ND) (RM1; Special Diet Services, Witham, UK) or a non-nutritive bulk diet (NND) [the same standard chow to which 25% alphacel non-nutritive bulk (MP Biomedicals, Cambridge, UK) had been added] immediately after being weaned. Litters were monitored daily for vaginal opening and first vaginal oestrus from pnd 28. The body weight of rats was determined every three days.
5.3.3 Experiment Two: The effect of lesioning the MePD on LH pulse frequency in pre-pubertal female rats

On pnd 33, rats were attached via one of the two cardiac catheters to the automated blood sampling system (Chapter 2, section 2.3.3). Blood sampling commenced at 1200 h, and samples were collected every 5 min for 6 h for LH pulse analysis. Blood samples were frozen at -20 °C for later assay to determine LH.

5.3.4 Experiment Three: The effect of lesioning the MePD on oestrous cyclicity after puberty in female rats

Once vaginal opening occurred, vaginal smears of each rat were taken daily for 2–3 consecutive weeks to detect the stage of the oestrous cycle (Chapter 2, section 2.3.1).

5.3.5 Experiment Four: The effect of lesioning the MePD on anxiety behaviour in female rats

Three days before the first social interaction or play fighting behaviour test was initiated, the rotarod test was used to evaluate motor function (Rozas et al. 1997). The social interaction (Chapter 2, section 2.3.2.1) and play fighting behaviour (Chapter 2, section 2.3.2.2) (Daenen et al. 2002) tests were performed as described previously to assess anxiety. This procedure was carried out on pnd 27, 29 and 31 in the MePD lesion study. Each pair of tested animals was from an identical treatment group (lesion against lesion, sham against sham) and on the same diet regime.
5.3.6 Experiment Five: The effect of endogenous glutamatergic and GABAergic signaling in the MePD in pubertal timing and anxiety behaviour

After implantation of osmotic minipumps (Chapter 2, section 2.2.3) on pnd 21, rats were monitored daily for vaginal opening and first vaginal oestrus from pnd 28. The body weight of rats was determined every three days. The social interaction (Chapter 2, section 2.3.2.1) and play fighting behaviour (Chapter 2, section 2.3.2.2) (Daenen et al. 2002) tests were performed on pnd 29 and 31 as described previously to assess anxiety.

5.3.7 Statistical analysis

Comparisons between rats that received ibotenic acid and sham lesions, GABA or glutamate antagonist and their vehicle controls in the MePD with respect to body weight, day of vaginal opening and first oestrus and/or behavioural parameters were made using analysis of variance (ANOVA) followed by a post hoc Tukey–Kramer test, where appropriate. Percentage of normal oestrous cycles was compared using Fisher’s Exact Test. Data are presented as mean ± SEM and $p < 0.05$ was considered statistically significant. All other results were analysed by one-way ANOVA followed by post hoc Tukey–Kramer test. Data are presented as mean ± SEM and $p <$
0.05 was considered statistically significant.

5.4 Results

5.4.1 MePD lesion verification

The extent and location of the lesions were confirmed by microscopic histological inspection, using cresyl-violet staining (Chapter 2, section 2.6). The presence of extensive neuronal loss within the MePD was used as a parameter to determine the existence of significant lesions. Only bilateral lesions which destroyed the MePD while leaving surrounding brain tissue largely intact were included in the study (Fig. 5.1, A, C and D). Animals were excluded from the analysis as a result of unsuccessful lesioning, unilateral damage, or damage extending outside of the MePD. Of the 28 rats that underwent neurotoxic lesion surgery, 19 were verified with correct bilateral lesions in the MePD and included in the analysis. The remaining 9 were excluded. Of the 19 animals with correct lesions, 8 were fed with the non-nutritive bulk diet and 11 with standard diet. The sham lesion group contained 7 fed with non-nutritive bulk food and 10 with normal diet. All vehicle injected rats sustained no obvious damage to the MePD (Fig. 5.1 E and F).

5.4.2 Experiment One: The role of the MePD in pubertal timing in different diet fed female rats

Although there was no significant difference in body weight (Fig. 5.2 A and B) at
weaning among the experimental groups, MePD lesioning on pnd 21 increased body weight gain in animals fed normal standard diet, which was evident from pnd 27 onwards (Fig. 5.2 A; $p < 0.05$). Unsurprisingly, this was associated with a significant advancement of puberty onset compared with sham lesioned controls (vaginal opening: $34.4 \pm 0.5$ vs $37.8 \pm 0.7$ days, respectively; Fig. 2C; n = 10-11 per group; $p < 0.05$; first oestrus: $34.6 \pm 0.7$ vs $37.9 \pm 0.7$ days, respectively, mean ± SEM; $p < 0.05$).

Feeding with a 25% non-nutritive bulk diet completely blocked the MePD lesion-induced increase in body weight (Fig. 5.2 B), but failed to prevent the advancement of puberty onset (vaginal opening: $34.9 \pm 0.7$ vs $39.0 \pm 0.9$ days, respectively; Fig. 5.2 D; n = 7-8 per group; $p < 0.05$; first oestrus: $35.5 \pm 0.7$ vs $39.4 \pm 0.8$ days, respectively, mean ± SEM; $p < 0.05$).

5.4.3 Experiment Two: The effect of lesioning the MePD on LH pulse frequency in pre-pubertal female rats

There were considerable individual variations in LH pulse frequency between and within groups. Figure 5.3, A, B, D and E show LH profiles of representative rats in different diet groups. On pnd 30, one or no LH pulse was detected in the 4 h sampling period in the sham lesioned controls fed with ND (n=6) or NND diet (n=7) (Fig. 5.3 B and E). On pnd 30, all of the lesioned rats fed with ND showed one or two pulse during a 4 h observation period (Fig. 5.3. A). Similarly, four out of five lesioned rats
on NND showed one or two LH pulses during the 4 h blood sampling period (Fig. 5.3 D). The mean number of LH pulses during the 4 h sampling period was significantly greater in the lesioned rats on the same pnd day (Fig. 5.3 C and F) in both the ND and NND groups compared with their controls (P<0.05). However, there was no significant difference in means of the LH pulse amplitudes in rats fed with both diets between control and lesion groups (ND: 1.75±0.35 vs 2.07±0.73, n=6-5 per group, respectively; NND: 1.6±0.44 vs 1.8±0.68, n=7-5 per group, respectively). There was also no significant difference between lesion and sham lesion rats on the basal levels of LH in both diet groups between sham lesioned controls and lesioned rats neither (ND: 1.12±0.03 vs. 0.99±0.06; n=6-5 per group, respectively; NND: 0.98±0.10 vs. 0.94±0.06; n=7-6 per group, respectively).

5.4.4 Experiment Three: The effect of lesioning the MePD on oestrous cyclicity after puberty in female rats

Oestrous cyclicity was disrupted in the immediate post pubertal period of the MePD lesioned rats fed either ND or NND (Fig. 5.4-5.5; p < 0.05). The majority of the control rats showed normal 4–5 days oestrous cycles (80% of the ND group, n = 10; 75% of the NND group, n = 7), while only 38% of the ND group (n = 11) or 29% of the NND group (n = 8), in the MePD lesioned animals showed normal oestrous cycles (Fig. 5.4 A and 5.5 A; p < 0.05). Representative examples of oestrous cycles from each group are shown in Fig. 5.4 B, C and Fig 5.5 B, C. Cycle length was
prolonged (Fig. 5.4 D and Fig 5.5 D; \( p < 0.05 \)) with an increase in metoestrus and decrease in dioestrous phases evident in MePD lesioned rats fed with ND or NND (Fig. 5.4 E and Fig 5.5 E; \( p < 0.05 \)).

5.4.5 Experiment Four: The effect of lesioning the MePD on anxiety behaviour in female rats

Rotarod analysis revealed no difference between MePD lesioned animals and their sham lesioned controls in the time the rats stayed on the rotating rod, indicating an absence of impaired motor function.

*Social interaction:* The social interaction test was performed on pnd 30. There was a marked impact of lesioning the MePD on social behaviour, with a significant increase in the cumulative mean time spent engaged in social interaction behaviour, including sniffing, chasing, following, grooming and mounting in the MePD lesioned compared with sham lesioned controls, irrespective of diet (ND: 73.2 ± 6.9 vs 116.5 ± 17.6 sec, control vs lesion, \( n = 10-11 \) per group, \( p < 0.05 \); NND: 87.4 ± 8.3 vs 138.3 ± 1.7 sec, control vs lesion, \( n = 7-8 \) per group, mean ± SEM, \( p < 0.05 \); Fig. 5.6 A).

*Play fighting behaviour:* The frequency of pinning events was significantly decreased in the lesion animals in both the ND and NND fed groups compared with their respective sham lesioned controls (\( p < 0.01 \)). The average number of pinning
events recorded during the observation periods between pnd 27 and 31 in the sham lesion groups fed ND and NND was 3.4 ± 0.5 and 3.2 ± 0.8 per 20-min, respectively (mean ± SEM) (Fig. 5.6 B). This decreased dramatically in the lesion animals fed ND and NND to 0.4 ± 0.1 and 0.8 ± 0.3 per 20-min, respectively (Fig. 5.6 B). In lesioned animals, the duration of play fighting behaviour was remarkably decreased compared with sham lesioned rats in both dietary food groups (ND: 109.6 ± 19.4 vs 39.3 ± 14.5 sec, control vs lesion, n = 10-11 per group, p < 0.05; NND: 109.1 ± 24.3 vs 53.4 ± 19.9 sec, control vs lesion, n = 7-8 per group, mean ± SEM, p < 0.05; Fig. 5.6 C).

5.4.6 Experiment Five: The effect of endogenous glutamatergic and GABAergic signalling in the MePD on pubertal timing and anxiety behaviour in female rats

Correct intra-MePD placement of cannulae connected to the osmotic minipumps filled with GABA or glutamate antagonists was verified with reference to the atlas of Paxinos and Watson (Paxinos and Watson 1986) and histological analysis is displayed in figure 5.7 and figure 5.8. A representative example is shown in figure 5.7 A. Schematic diagrams of coronal sections of the rat brain identifying the location of the cannulae tips for all rats that received intra-MePD infusions of D-AP5 (Fig. 5.8 A), bicuculline (Fig. 5.8 B), or their respective aCSF control (Fig. 5.8 C and D) are shown. Chronic infusion of a glutamate antagonist, D-AP5 (90 nmol/6μl/day,
bilaterally), into the MePD started on pnd 21 for 14 days did not affect body weight gain (Fig. 5.9 A), but resulted in a significant delay of puberty (Fig. 5.9 C). The mean age of vaginal opening in the D-AP5 treatment group was significantly later than those controls infused with aCSF (42.7 ± 1.3 vs 38.0 ± 0.9 days, respectively, mean ± SEM; n = 5-7 per group; p < 0.05). However, bilateral chronic infusion of the GABA<sub>A</sub> antagonist, bicuculline (13.56 pmol/6μl/day, bilaterally), into the MePD from pnd 21 to 35 caused a significant advancement of puberty (Fig. 5.9 D), without a change in body weight gain (Fig. 5.9 B). The mean age of vaginal opening in the bicuculline treatment group was significantly earlier compared with those animals treated with aCSF (35.9 ± 0.7 vs 38.1 ± 0.8 days, respectively, mean ± SEM; n = 7-8 per group; p < 0.05; Fig. 6D).

The behavioural effect of intra-MePD administration of GABA and glutamate antagonists is shown in figure 5.10.

Social interaction: The social interaction testing was performed on pnd 30. There was a marked impact of intra-MePD administration of D-AP5, with a significant decrease in the cumulative mean time (61.2 ± 7.8 sec, n = 7) spent engaged in social interaction behaviour compared with the control (97.7 ± 11.6 sec, n = 5, mean ± SEM, p < 0.05; Fig. 5.10 A). On the contrary, chronic infusion of bicuculline into the MePD significantly increased the mean time spent engaged in social interaction behaviour (bicuculline treatment vs control, 124.8 ± 5.5 vs 89.5 ± 6.3 sec, n = 7-8
per group, mean ± SEM, *p* < 0.05; Fig. 5.10 A).

*Play fighting behaviour:* There was no significant effect of intra-MePD administration of D-AP5 administration on play fighting behaviour (Fig. 5.10 B and C). However, the frequency of pinning events was significantly decreased by bicuculline (*p* < 0.05). The average number of pinning events recorded during the observation periods on pnd 29 and 31 in the control and bicuculline treatment groups was 4.1 ± 1.1 and 1.0 ± 0.3 per 20-min respectively (mean ± SEM) (Fig. 5.10 B). In the bicuculline treated animals, the duration of play fighting behaviour was also decreased compared with controls (133.8 ± 23.1 vs 79.2 ± 12.0 sec, aCSF vs bicuculline, *n* = 7-8 per group, mean ± SEM, *p* < 0.05; Fig. 5.10 C).
Figure 5.1. Coronal sections through the rat brain at the level of the posterodorsal medial amygdala (MePD) showing its spatial relation with the surrounding nuclei and ibotenate lesion resulting in specific neuronal cell loss. A, Section without staining showing the lesion area localised to the MePD. B, Schematic representation of site of lesion in the MePD (black filled) adapted from rat brain atlas. C, Representative example of a coronal brain section stained with cresyl violet illustrating substantial neurone loss in the MePD following ibotenic acid lesioning. The dotted line indicates approximate border of MePD. E, Representative photomicrograph from a MePD sham-lesioned rat. D and F, Magnified images of the areas enclosed by the squares in C and E respectively and illustrating the significant neuron loss to the MePD (D). opt, optic track.
Figure 5.2. The effect of posterodorsal medial amygdala (MePD) lesions on body weight gain and day of vaginal opening in rats. A, Cumulative body weight gain was significantly greater from postnatal day (pnd) 27 onward in the lesioned group compared with the sham lesioned female rats fed with normal chow diet. B, In contrast, there is no significant difference in the body weight gain between lesioned and sham lesioned animals fed the 25% non-nutritive bulk diet. The MePD lesion resulted in a significant advancement of pubertal onset (vaginal opening) in both normal (C) and non-nutritive bulk diet (D) fed rats. *P < 0.05 vs sham lesion control. Results represent mean ± SEM, n = 7-11 per group.
Figure 5.3. Effects of posterodorsal medial amygdala (MePD) lesions from weaning (pnd 21) on pulsatile LH secretion in prepubertal female rats. A and B, Representative examples showing LH pulse frequency in MePD lesioned rats and sham lesioned rats on pnd 30 in rats fed normal chow diet (ND); C, Summary showing effects of lesioning the MePD on pulsatile secretion in ND fed rats; D and E, representative examples illustrating the effect of lesioning the MePD on LH pulse frequency on pnd 30 in 25% non-nutritive bulk diet (NND) rats. F, Summary showing that lesioning the MePD significantly increased the pulsatile LH frequency on pnd 30 compared with controls in female rats fed with NND. *P < 0.05 versus controls; means ± SEM; n= 5-4 per group.
Figure 5.4. The effect of posterodorsal medial amygdala (MePD) lesions on oestrous cyclicity in female rats fed with normal chow diet. Cyclicity was determined by analysing daily vaginal cytology for 12 consecutive days from the day of vaginal opening. A, MePD lesion resulted in a significant decrease in percentage of normal oestrous cycle vs sham-lesioned controls. Representative examples of oestrous cycle are shown for sham lesioned (B) and lesioned (C) rats. D, Cycle length was significantly extended following MePD lesion vs controls. E, Time spent in the metoestrus (M) stage was extended, whereas time spent in dioestrous (D) was decreased by MePD lesion compared with controls. P, proestrous; E, oestrous. Results are presented as means ± SEM. *P < 0.05 vs controls (means ± SEM; n = 7-10 per group).
Figure 5.5. The effect of posterodorsal medial amygdala (MePD) lesions on oestrous cyclicity in female rats fed with 25% non-nutritive bulk diet (NND). Cyclicity was determined by analysing daily vaginal cytology for 12 consecutive days from the day of vaginal opening. A, MePD lesion resulted in a significant decrease in percentage of normal oestrous cycle vs sham-lesioned controls. Representative examples of oestrous cycle are presented for sham lesioned (B) and lesioned (C) rats. D, Cycle length was significantly extended following MePD lesion vs controls. E, Time spent in metoestrus (M) stage was extended, whereas time spent in dioestrus (D) was decreased by MePD lesion compared with controls. P, prooestrus; E, oestrous. Results are presented as means ± SEM. *P < 0.05 vs controls (means ± SEM; n = 8-11 per group).
Figure 5.6. The effect of posterodorsal medial amygdala (MePD) lesions on behavioural activities monitored between postnatal days (pnd) 27 and 31 in female rats fed with normal chow diet (ND) or 25% non-nutritive bulk diet (NND). A, In the 15-min social interaction (SI) test, the MePD lesion significantly increased the time of behavioural interaction (sniffing, chasing, following, grooming and mounting) in both the ND and NND groups. For the 20-min play fighting behavioural test, the lesion significantly decreased the frequency of pinning events per 20-min observation period (Pin frequency) (B) and the duration of play fighting behaviour (pouncing, wrestling, boxing and pinning) (C) in both ND and NND fed rats. *P < 0.05 vs sham lesion control. Results represent mean ± SEM, averaged for behavioural tests carried out on pnd 27, 29, 31 and 33, n = 7-11 per group.
Figure 5.7. Photomicrograph and schematic illustrations of the osmotic minipump microinfusion sites targeted to the posterodorsal medial amygdala (MePD). A, Photomicrograph of a coronal brain section in a representative animal implanted with bilateral cannulae in the MePD. Arrows indicate the site corresponding to the tip of the cannulae. B, Schematic illustration showing the target site for bilateral cannulation of the MePD at bregma (AP) -3.3 mm, mediolateral (ML) ±3.4 mm and dorsoventral (DV) -8.6 mm according to the rat brain atlas of Paxinos and Watson (Paxinos and Watson 1986). Arrows point to the location of the cannulae tips. 3V, third ventricle.
Figure 5.8. Schematic illustrations of the osmotic minipump microinfusion sites targeted to the posterodorsal medial amygdala (MePD). A and B, Schematic drawings of the MePD illustrating the individual sites of chronic infusion of the D-2-amino-5-phosphonovalerate (D-AP5) or bicuculline, respectively. C and D, Schematic drawing of the MePD illustrating the individual sites of chronic infusion of artificial cerebrospinal fluid (aCSF) as control for D-AP5 or bicuculline group, respectively. Open circles show the injection sites. Numbers in each drawing indicate the distance (mm) to bregma in accordance with Paxinos and Watson (PAXINOS Gand WATSON 1986). 3V, third ventricle.
Figure 5.9. The effect of bilateral chronic microinfusion of D-2-amino-5-phosphonovalerate (D-AP5), bicuculline (BIC) or artificial cerebrospinal fluid (a-CSF: 6μl/day) in the posterodorsal medial amygdala (MePD) for 14 days starting on postnatal day (pnd) 21 on body weight gain and the day of vaginal opening in rats. A and B, There was no significant differences in cumulative body weight gain between the D-AP5 (90 nmol/6μl/day, bilaterally, for 14 days) or BIC (13.56 pmol/6μl/day, bilaterally, for 14 days) treatment groups and their corresponding a-CSF controls. C, Chronic infusion of D-AP5 resulted in a significant delay of puberty onset. D, Chronic infusion of BIC in the MePD resulted in a significant advancement of puberty onset in female rats. *P < 0.05 vs a-CSF control. Results represent mean ± SEM, n = 5-8 per group.
Figure 5.10. The effect of bilateral chronic micro-infusion of D-2-amino-5-phosphonovalerate (D-AP5), bicuculline (BIC) or artificial cerebrospinal fluid (aCSF) in the posterodorsal medial amygdala (MePD) on behavioural activities monitored between postnatal days (pnd) 29 and 31 in female rats. A, In the 15-min social interaction (SI) test carried out on pnd 30, chronic infusion of D-AP5 (90 nmol/6μl/day, bilaterally, for 14 days starting on pnd 21) significantly decreased the time of behavioural interaction, whereas chronic infusion of BIC (13.56 pmol/6μl/day, bilaterally, for 14 days starting on pnd 21) increased the interaction time significantly compared with controls (aCSF: 6μl/day, bilaterally, for 14 days starting on pnd 21). B, There was no significant difference in the frequency of pinning events per 20-min observation period between the D-AP5 and aCSF control treated rats, whereas chronic infusion of BIC into the MePD results in a significant reduction of pin frequency. C, There was no significant difference in the duration of play fighting behaviour in D-AP5 treated rats, whereas BIC administration significantly decreased the duration of play fighting behaviour compared with controls. *P< 0.05 vs aCSF control. Results represent mean ± SEM, averaged for play fighting behavioural test carried out on pnd 29 and 31, n = 5-8 per group. Treatment: D-AP5 or BIC
5.5 Discussion

We have shown for the first time that neurotoxic lesions specific to the MePD markedly advanced the timing of puberty onset in female rats and resulted in an average 17% weight gain by pnd 39. Previous studies in adult animals have also shown lesions of the MePD result in marked hyperphagia and excessive weight gain (King 2006). The effect of MePD lesions on food intake and body weight is highly site specific because lesions more posteriorly, through the amygdalohippocampal area or into the subiculum, do not affect food intake or body weight (King et al. 1996a). Damage to large areas of the amygdala, including the anterior, basolateral, and corticomedial group of nuclei, are also without effect (King et al. 1994). Similarly, in the present study, rats fed a normal diet with lesions to the anterior or ventral MeA did not show differences in body weight gain compared with sham controls. Since it is thought that body weight influences the timing of puberty and a critical body weight is necessary for its onset (Frisch 1983), it is reasonable to assume that the increase in body weight in MePD lesioned rats may underlie the advancement of puberty in the present study. However, by feeding MePD lesioned animals a 25% non-nutritive bulk diet (NND), we could tease apart whether the advancement of pubertal timing was related to this increased calorie intake and body weight gain. On the contrary, the lesioned rats fed the NND had an identical dramatic advancement of puberty without the increased body weight. Moreover, in both dietary groups, puberty occurred in lesioned animals at a significantly lower body
weight compared with controls, because of their younger age, which is inconsistent with the “critical body mass hypothesis” (Frisch 1983). Therefore, our results suggest the MePD plays a critical and novel role in pubertal timing via mechanisms independent of change in body weight or caloric intake.

The amygdala is commonly known for its role in higher-order emotion processing as suggested by Kluver and Bucy in their classical temporal lobectomy studies in rhesus monkeys, which resulted in major changes in their emotional behaviour, cumulatively termed “psychic blindness” (Kluver and Bucy 1937). Essentially the monkeys became tame, fearless and emotionally-flattened. The MePD is considered part of an integrated neural circuit involved in responses to fear and stressful conditions, and in modulating social and aggressive behaviours. As expected, in the present study the MePD lesioned rats showed a decrease in social play behaviour during play fighting tests consistent with previous data in rats that had received amygdala lesions on pnd 7 or 21 (Daenen et al. 2002). Activation in the MePD in rats, following restraint stress, confirms its participation in psychological stress (Rasia-Filho et al. 2012). Exposure to a predator (e.g. a cat) similarly activates the rat MePD (Martinez et al. 2011), indicating its functional relationship to anxiety or fear. The MePD is also robustly activated by social defeat (Fekete et al. 2009) and exposure to aggressive social encounters in the juvenile social subjugation paradigm, which is considered an animal model for child abuse (Weathington et al. 2012). In the present study, the marked impact of intra-MePD administration of D-AP5 on
decreasing social interaction behaviour, and the significant increase in social play
and social interaction behaviour in the rats treated with bicuculline, suggest that the
GABAergic and glutamatergic neuronal systems in the MePD may be key players
in these behaviours. The MePD connects to specific hypothalamic nuclei,
participates in interpretation of olfactory (Meredith and Westberry 2004) and
genitosensorial (Oberlander and Erskine 2008) signalling, and modulates social and
The MePD sends direct efferents to the GnRH-rich mPOA (Canteras et al. 1995,
Hahn et al. 2003) and stimulation of the former induces GnRH release (Beltramino
and Taleisnik 1978). The MePD also contains one of the highest concentrations of E₂,
P₄ and androgen receptors (Simerly et al. 1990). The MePD is, therefore, ideally
positioned to integrate systems required to optimise behavioural adaptation and the
timing of reproductive events in a myriad environment. In fact, social rank is closely
correlated to the age of puberty onset in nonhuman primates, with higher social rank
related to earlier testicular development in males (Bercovitch 1993) and sexual skin
swelling and menarche in females (Bercovitch 1993, Stephens and Wallen 2013).
Neonatal amygdala lesions advance pubertal timing, although they delay first
ovulation, and concomitantly reduce fear and increase exploratory behaviour in
2015).

There is also evidence that psychosocial stress can delay or advance puberty in
animals, including humans (Wise et al. 2009, Wilson et al. 2013, Li et al. 2014a, Li et al. 2014b). These data support a key role for the MePD in stress-induced change in pubertal timing and support the contention that adaptive reproductive behaviour necessitates integrating external signals about the suitability of the surrounding environment for reproduction with the internal control of the reproductive state (Gowaty and Hubbell 2009). This is of interest because the rats in the present study appear to be afflicted by a similar “psychic blindness”, evidenced by their increased playful socialisation and loss of play fighting behaviour. The advancement of puberty in this case might be that MePD lesion prevents the rat experiencing fear, or prevents translation of the fear experience into a suppression of the reproductive axis. This might suggest that, under normal circumstances, the MePD functions to inhibit puberty until such a time as the environment appears safe to allow for reproduction. However, such a causal relationship between these two aspects of MePD function awaits further investigation.

The mechanism whereby MePD lesions result in early onset of puberty is unknown. However, we have shown in present studies that the dramatic advancement of puberty in the MePD lesioned animals was associated with an earlier onset of higher LH pulse frequency. This suggests an earlier acceleration of GnRH pulse generator frequency in the prepubertal period. It is well established that a decrease in GABAergic tone and increase in glutamatergic tone in the hypothalamus are critical for pubertal timing in rodents and primates (Urbanski and Ojeda 1987, Plant et al.
Prior to puberty, there is a decline in GABA release concomitant with increased GnRH release (Mitsushima et al. 1994). Chronic microinfusion of the GABA$_A$ receptor antagonist, bicuculline, into the hypothalamus significantly increased GnRH release and accelerated the timing of puberty in female monkeys (Keen et al. 1999). More recently, it was demonstrated that GABA$_A$ receptor antagonism dramatically stimulated kisspeptin release in the mediobasal hypothalamus of prepubertal monkeys, and that bicuculline-induced GnRH release is blocked by kisspeptin receptor antagonism. This suggests that kisspeptin neurones may relay inhibitory GABAergic signals to GnRH neurones (Kurian et al. 2012). Similarly, glutamatergic signalling plays an important role in pubertal timing. NMDA induces GnRH release, resulting in precocious puberty in rats (Urbanski and Ojeda 1987) and monkeys (Plant et al. 1989), whereas NMDA receptor antagonism delays puberty (Urbanski and Ojeda 1990). However, what regulates hypothalamic GABAergic or glutamatergic tone remains to be established. The MePD undergoes dramatic change during puberty in rodents, including volumetric growth due in part to increased neuronal soma size and dendritic spine density, and increased glutamatergic synaptic activity, but without an apparent corresponding GABAergic change, indicating an overall increased excitability and activation of this area at the onset of puberty (Zehr et al. 2006, Cooke and Woolley 2009, Cooke 2011). The volume of grey matter within the human amygdala also increases during puberty and these morphometrics are associated with
changing levels of circulating sex steroids (Neufang et al. 2009). The delay of puberty following antagonism of NMDA receptors in the MePD supports a role for amygdala glutamatergic signalling in stimulating puberty onset. However, these data are seemingly inconsistent with the advancement of puberty following discrete lesions of the MePD. One explanation for this conundrum is that, the lesion causes loss of inhibitory GABAergic outputs, which constitute the majority (50–70%) of MePD projection neurones (Keshavarzi et al. 2014). This may result in release of a tonic inhibitory break exerted by these amygdala projections on key hypothalamic reproductive nuclei, including the GnRH-rich mPOA (Canteras et al. 1995, Hahn et al. 2003), and kisspeptin-rich AVPV and ARC (Choi et al. 2005, Pardo-Bellver et al. 2012, Hu et al. 2015). However, the neuronal phenotypes of these direct projection fibres and whether they contact the essential reproductive neurones remains to be established. We have previously shown the MeA suppresses pulsatile LH secretion through activation of GABAergic neurones intrinsic to the mPOA (Lin et al. 2011), adding to the complexity of amygdaloid control of gonadotropic hormone secretion. Since the glutamate component of the MePD is considerably smaller than GABA, which constitutes 70% of the total nuclear cell count (Choi et al. 2005, Keshavarzi et al. 2014), it may suggest a differential dominance of inhibition in this nucleus. Our data suggest that the balance between GABA and glutamate function intrinsic to the MePD is crucial in triggering puberty onset, since their antagonism advances and delays puberty, respectively. Nevertheless, the hierarchical and developmental
relationship between MePD GABA and glutamate systems, both the intrinsic and extrinsic projecting components, in modulating pubertal timing remains to be established.

In the present study, lesioning the MePD disrupted oestrous cyclicity including a lengthening of the cycle in the immediate post-puberty period. The time spent in metaoestrus was profoundly increased in addition to a reduction in dioestrus in MePD lesioned rats. This may indicate abnormal levels of oestrogen or progesterone caused by abnormal follicle development (vom Saal et al. 1994). Although it is reported that bilateral electrolytic lesioning of the MePD in adult rats did not affect oestrous cyclicity, only the time taken to display oestrus after surgery was recorded without evaluation of cyclicity per se (King et al. 1999). However, whole amygdala lesion caused increased cycle length (Meyer et al. 1983). The reason for the prolonged oestrous cycles in the prepubertal MePD lesioned rats may be due to disruption of ovulation, because stimulation of the amygdala causes advancement of ovulation or resumption of oestrous cycles after periods of prolonged dioestrus (Sherwood 1977). Additionally, lesion of the cortico-medial amygdala blocks ovulation (Velasco and Taleisnik 1971). Further studies are necessary to establish whether prepubertal MePD lesions affect the preovulatory LH surge and whether the disruption to oestrous cyclicity persists long-term.

In conclusion, our results suggest that the MePD alter the timing of puberty onset via
a mechanism independent of change in body weight and food intake. MePD glutamatergic systems advance the timing of puberty, whereas local GABAergic activation results in a delay.
Puberty is the process of physical changes through which a child’s body matures into an adult body capable of sexual reproduction. The timing of normal pubertal maturation has received increased attention recently. Age of menarche showed a dramatic decrease in the first half of the 20th century in Western nations. This trend was seen again more recently since 2000, especially in girls (Biro et al. 2006). Abnormal maturation of puberty in girls is a risk factor for anti-social behaviour, anxiety and other mental health disorders, in additions to gynaecological disorders and cancers consequent to earlier sexual activity and first pregnancy (Apter and Vihko 1983, Michaud et al. 2006, De Leonibus et al. 2012). It is now increasingly important to understand the mechanism of puberty onset. It is well established that puberty onset mainly depends on the maturation of HPG axis (Holder and Blaustein 2014). The genetic evidence for the view that Kiss1 neurones are critical for the onset of puberty is overwhelming (Han et al. 2005, Kurian et al. 2012, Pinilla et al. 2012). It has been shown that humans with inactivating mutations in genes encoding Kiss1 or Kiss1R lack pubertal development (Topaloglu et al. 2012). Understanding the role and interactions of Kiss1 in the reproductive system allows us to identify a number of potential targets in the treatment of subfertility and other associated disorders of reproduction. Furthermore, the timing of puberty may also be altered by conditions prevailing in late juvenile development and continuing thereafter, including under nutrition and the presence of social factors such as stress.
(Plant 2015). The mechanism under this complex interaction between genetic and environmental factors remains unknown. The experimental work detailed in this thesis has addressed the question of distinct neural circuits in regulation of puberty timing.

The first aim of this thesis was to elucidate the distinct roles of the AVPV and ARC Kiss1 in regulating pubertal timing using the female rat as a model. The present studies have shown that Kiss1 knockdown in the AVPV resulted in a significant delay in the timing of puberty onset and suppressed the incidence of LH surges, a key component of the complete pubertal developmental process. In contrast, Kiss1 knockdown in the ARC failed to alter pubertal timing, but did decrease the amplitude, although not the incidence, of LH surges. Both ARC and AVPV Kiss1 knockdown resulted in abnormal oestrous cyclicity (see Chapter 3). These data indicate that AVPV Kiss1 is more crucial for initiating the onset of puberty compared with ARC Kiss1. Despite the classical association of ARC Kiss1 with LH pulse generation, and AVPV Kiss1 with LH surge generation, it is worth noting that both AVPV and ARC Kiss1 are essential for normal LH surges. Therefore, ARC Kiss1 may have a novel amplificatory role in the mechanism underlying LH surge production. Both ARC and AVPV Kiss1 appear to be important for maintaining normal oestrous cyclicity. As has been concluded previously (Beale et al. 2013), it seems that Kiss1 expression must be kept within a fairly narrow range to maintain normal fertility, as well as to achieve timely puberty. The present studies showed
that the AVPV Kiss1 plays a more important role than the ARC Kiss1 in controlling timing of puberty, which is consistence with previous work. It has been shown that prepubertal female rats exposed to immunological stress, showed delayed puberty onset and down-regulated Kiss1 expression in the mPOA (including the AVPV), but with no significant change in ARC Kiss1 expression (Knox et al. 2009). Furthermore, Kiss1 expression in the AVPV rises markedly between pnd 32 and the day of vaginal opening, whereas the Kiss1 neurones in the ARC do not appear to undergo such changes during this prepubertal period (Knox et al. 2009). Therefore, disturbing ARC Kiss1 signalling may not be expected to affect the pubertal timing.

Both ARC and AVPV Kiss1 have been implicated in the central regulation of stress induced suppression of the HPG axis (Kinsey-Jones et al. 2009, Knox et al. 2009). CRF, an important central stress modulator, can suppress Kiss1 and Kiss1r mRNA expression in both the mPOA and the ARC (Kinsey-Jones et al. 2009). Moreover, early life stress delays puberty and downregulates Kiss1 in the AVPV (Kinsey-Jones et al. 2010). Thus, CRF appears to be a vital regulator of pubertal timing. Indeed it has been shown, in Chapter 4 of this thesis, that up-regulation of CRF expression in the CeA can advance the timing of puberty onset in female rats, although the underlying neural mechanisms remain to be established. Previous studies have shown that lesioning of the CeA does not affect the pulsatile release of LH in adult female rats (Lin et al. 2011). Therefore, the up-regulation of CeA CRF per se may not affect
the GnRH pulse generator activity in basal stress-free conditions. It is worthy to note that the CeA may play an excitatory role in controlling puberty, since over-expression of CRF in CeA accelerated puberty timing. It was also shown in the present study that over-expression of CRF in the CeA can reduce the immunological stress-induced suppression of pulsatile LH release. This indicates that the advanced puberty onset by up-regulation of CRF signalling in the CeA may be due to an habituation mechanism that reduces the inhibitory effect of daily life stress on the GnRH pulse generator. This is in keeping with the evolutionary theory of reproductive development (Ellis et al. 2011). In particular, early onset puberty is reported in children who have migrated from developing to developed countries (Gluckman and Hanson 2006). Evolutionary perspectives suggest that these effects can be explained by adaptive mechanisms (Gluckman and Hanson 2006).

An additional aim of this thesis was to investigate the effect of lesioning the MePD on pubertal timing in female rats. This study provides the first evidence for a role of the MePD in regulating the timing of puberty onset. In MePD lesioned animals the decrease in anxiety-like behaviour during the juvenile developmental period may contribute to the precocity of pubertal timing. The results from this study demonstrate that bilateral neurotoxic lesions of the MePD accelerate body weight gain compared with sham rats in normal diet fed rats. These data are consistent with previous reports of hyperphagia and moderate obesity in adult female rats with lesions of the most posterodorsal aspects of the medial amygdala nucleus (King et al.
1996b, Rollins et al. 2001, King 2006). However, since the MePD lesions induce accelerated growth (in terms of rate of body weight gain), factors associated with body weight cannot be excluded. To address these limitations, an alternative strategy, involving the use of a diet with a 25% non-nutritional component was introduced so as to differentiate the body weight gain and pubertal timing. Under these conditions there was no significant difference of the body weight gain between lesioned and sham rats; the rats had the same body weight gain from weaning until puberty. In these animals, the MePD lesions still advanced puberty, which was associated with a significantly lower body weight on the day of vaginal open. Thus, it can be concluded that MePD lesions results in precocious puberty independent of body weight. These data suggest a novel role for the MePD in regulation of pubertal development, the mechanism of which remains to be determined.

In the present study, the reduction of pinning behaviour in MePD lesioned rats is in agreement with the loss of reward system activity and less anxiety, which might suggest that lesioned rats have lower psychological stress reactivity. The amygdala is well known for its role in higher-order processing as one of the key emotion-controlling brain areas (LeDoux 2000). It has been established that the MeA is involved in psychological stress-induced suppression of the HPG axis (Lin et al. 2011). The behavioural observations in the present study support the role of the MeA in integrating emotional and neuroendocrine responses to differing social and physiological environments, and localise this to the MePD region. In summary, the
MePD lesioned animals may not have an intact reward system, which might lead to decreased pinning behaviour and reduced susceptibility to environmental stress, which could contribute to the advancement of puberty.

From a neuroanatomical perspective, the MePD also projects to the AVPV and ARC, which contain key Kiss1 neuronal populations essential for gonadotrophic hormone secretion and puberty (Li et al. 2009b, Li et al. 2012). In addition, it was recently discovered that extra-hypothalamic Kiss1 neurones in the MePD are developmentally upregulated between the juvenile and adult state (Cao and Patisaul 2013). We have recently shown that intra-MePD administration of Kiss1 resulted in an increased LH secretion while administration of a Kiss1 antagonist directly into the MePD profoundly suppresses LH pulse frequency in the female rats (Comninos et al. 2015). Thus, Kiss1 signalling within the amygdala per se appears to contribute to the modulation of gonadotrophin release and pulsatility, in addition to hypothalamic Kiss1. This raising the possibility that the MePD Kiss1 population may be involved in the timing of puberty.

In light of the advancement of puberty induced by lesioning the MePD, it suggests that the MePD may have an inhibitory effect on the control of gonadotrophin secretion, which is essential to induce puberty onset. This is in line with previous studies which showed that lesioning the whole MeA induces precocious puberty (Docke 1974) in rats, while stimulation of the MeA delays puberty (Bar-Sela and
Critchlow 1966). Furthermore, administration of a GABA<sub>A</sub> receptor antagonist into the MePD advanced puberty whilst glutamate antagonism delayed puberty (see Chapter 5). These data indicate that the endogenous glutamatergic systems in the MePD may play a role in triggering the onset of puberty, whereas local GABAergic activity may form a brake on puberty. Thus, a balance between GABA and glutamate function intrinsic to the MePD may be crucial in triggering the timing of puberty onset.

The higher-order emotion processing of the amygdala was initially suggested by Kluver and Bucy in their classical temporal lobectomy studies in rhesus monkeys (Kluver and Bucy 1937). Of the many subdivisions of the amygdala, the MeA, which projects to prefrontal cortex, olfactory bulb, hypothalamus, and brainstem structures, demonstrates most neuronal activation in response to psychogenic stressors such as restraint, forced swim, noise, or predator exposure (Canteras <i>et al.</i> 1995, Cullinan <i>et al.</i> 1995, Dayas <i>et al.</i> 2001, Figueiredo <i>et al.</i> 2003b) rather than systemic stressors (Sawchenko <i>et al.</i> 1996, Thrivikraman <i>et al.</i> 1997, Figueiredo <i>et al.</i> 2003b), as measured by c-fos expression. In contrast, the CeA has been shown to be highly responsive to systemic stressors like inflammation or hemorrhage (Sawchenko <i>et al.</i> 1996, Thrivikraman <i>et al.</i> 1997), with specific lesions of this amygdaloid subnucleus resulting in an attenuation of the HPA axis response to interleukin-1β injection (Xu <i>et al.</i> 1999), but not to restraint stress (Dayas <i>et al.</i> 1999). Thus, activation of the MeA and CeA is stressor-specific.
There is evidence that psychosocial stress can also delay or advance puberty in animals, including humans (Wise et al. 2009, Wilson et al. 2013, Li et al. 2014a, Li et al. 2014b). In the present study, it was demonstrated that lesioning the MePD reduced anxiety-like behaviour and advanced puberty. Additionally, we have shown that over-expression CRF in the CeA, which is thought to recapitulate many of the effects of chronic stress (Holmes et al. 2003), resulted in accelerating puberty timing with concomitant enhanced anxiety, providing a novel mechanism for early puberty associated with early life adversity. Thus, the amygdala exerts a dual effect on puberty timing with the CeA being stimulatory and the MePD inhibitory.

Both the MePD and CeA can affect LH secretion, and play an important role in the timing of puberty. This indicates that the amygdala might be high in the hierarchical neural network that controls the GnRH neuronal system critical for puberty onset. Indeed, Comninos and colleagues have recently provided evidence that Kiss1 signalling within the amygdala contributed to the modulation of GnRH pulse generator frequency (Comninos et al. 2015). These data suggest that this limbic circuit might play a critical role in controlling the hypothalamic GnRH pulse generator, which is critical for puberty timing - an hypothesis that needs testing. Furthermore, the hypothalamic Kiss1 may not as yet have a determined role in puberty timing. GnRH neurones represent the final conduit for a variety of neuroendocrine signals that regulate GnRH secretion and mammalian fertility. It is now clear that the Kiss1 neuropeptide is a key regulator of fertility, acting both
directly and indirectly on GnRH neurones to modulate GnRH secretion (Colledge 2009). However, Terasawa et al. (2013) proposed that, although Kiss1 expressing neurones in the ARC are critical for puberty, this is simply because these cells comprise an integral component of the hypothalamic GnRH pulse generating mechanism that generates intermittent release of the decapeptide, an increase of which is obligatory for the onset of puberty. According to this model, Kiss1 neurones in the ARC do not determine the timing of puberty. Rather, as a component of hypothalamic GnRH pulse generation, they subserve an upstream regulatory mechanisms determining the timing of puberty onset (Terasawa et al. 2013). Thus, our data, which are in line with this model, established that the MePD may play an inhibitory role, while the CeA may exert an excitatory role. Both are potentially upstream regulators of hypothalamic Kiss1 neurones to govern the timing of puberty through increased activation of a robust pulsatile mode of GnRH released at the end of juvenile phase of development (Fig.6.1.).
Fig. 6.1. Schematic diagram demonstrating the neural signalling systems regulating the timing of puberty. The MePD and CeA are potentially act as upstream regulators of hypothalamic Kiss1 neurones to govern the timing of puberty through increased activation of a robust pulsatile mode of GnRH released at the end of juvenile phase of development. The MePD modulates pulsatile release of GnRH possibly via direct neural projections to the mPOA and the ARC, and it plays an inhibitory role. GABA is the major inhibitory neurotransmitter in the MePD. The CeA may exert an excitatory role through a indirect functional route via BNST to Kiss1 and GnRH secretion. CRF is the major signalling neuropeptide in the CeA.
FUTURE STUDIES

The preceding experimental work has answered a number of questions regarding the role of the AVPV, ARC and amygdala, in regulating the timing of puberty onset and LH release. The future directions of further work which follows on from the results of these studies include:

1. Elucidating the role of the CeA in regulating sexual maturation.

*Is the CeA important for puberty timing in female rats?*

We have shown that over-expression CRF in the CeA results in accelerating puberty timing. If lesioned CeA could induce suppression of puberty timing, it can support CeA having an excitatory role in the timing of puberty. To address this question, bilateral lesioning of the CeA in pnd10 female rats should be conducted and the VO and first oestrus day and proestrus LH surge examined.

2. Elucidating the role of the MePD Kiss1 in regulating puberty timing.

*Does the MePD Kiss1 affect the timing of puberty onset and the pulsatile LH release?*

It is well-established, from lesion studies, that the MePD affects the timing of puberty in female rats. The MeA Kiss1 may also be involved in this process. To
address this question, administration of Kiss1 or Kiss1 antagonist into the MePD from pnd20 should be carried out, and the puberty onset day, following oestrous cyclicity and LH pulse frequency monitored.

3. Elucidating the innervate patterns of the different Kiss1 neurone populations during the pubertal development period.

   a) Do the AVPV and ARC populations of Kiss1 project to GABAergic or glutamatergic neurones in the amygdala? How do projections from Kiss1 neurones to the amygdala change during the pubertal development period?

   Precisely how the different Kiss1 populations control the activity of GnRH neurone during puberty is unknown. It remains that the projections of ARC and AVPV Kiss1 neurones are not known. To address this question, anterograde tract-tracing should be carried out in pnd10, 20, 30 and adult female rats to provide detailed information on the projections of the AVPV and ARC Kiss1 neurone populations. In addition, the distribution of GABAergic and glutamtergic neurones in the amygdala, following the anterograde tract-tracing, should be assessed using dual-immunostaining.

   b) Do the Kiss1 neurones in the ARC activate during LH surge?

   In present study, we have shown that the ARC Kiss1 plays an amplificatory role in
regulating LH surge. To further confirm its role, the FOS expression of ARC Kiss1 neurones during the LH surge, and utilising intra-ARC Kiss1 antagonists to see if the LH surge findings are duplicated, should be carried out.
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APPENDIX

7.1 Radioimmunoassay for determination of luteinising hormone levels

A double-antibody radioimmunoassay supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA) was used to determine LH concentrations in the whole blood samples. The sensitivity of the assay was 0.093 ng/ml.

7.1.1 Reagents used in the radioimmunoassay

General Diluent: 1400 ml Deionised water

8.09 g Na$_2$HPO$_4$ (anhydrous) (Sigma-Aldrich)

2.03 g NaH$_2$PO$_4$·2H$_2$O (Sigma-Aldrich)

0.14 g Thimerosal (Sigma-Aldrich)

7.00 g Bovine Serum Albumin (BSA; Sigma-Aldrich)

The above components were dissolved in 1000 ml water in order, with each component only added once the previous had fully dissolved. A further 400 ml of water was then added to bring the total volume to 1400 ml and give a 0.05 M phosphate buffer (pH 7.4) with 0.5% BSA. This buffer was then used for iodination, preparation of standards, primary antibody diluent, label and precipitating antibody.
buffer.

Primary antibody diluent: 45 ml General diluent

0.84 g EDTA

149.5 μl Normal Rabbit Serum

Primary antibody:

The lyophilised antibody NIDDK-anti-ratLH-S-11 was used as a primary antibody for the LH radioimmunoassay. The lyophilised protein was reconstituted in 1 ml of deionised water to give a buffered solution with an antibody dilution of 1:40. The antibody was then split into 25 μl aliquots and stored at –20 °C. For use in the assay aliquots were thawed and diluted with 1 aliquot per 45 ml of primary antibody diluent.

Precipitating antibody:

Anti-rabbit gamma globulin raised in the donkey was used (IDS, Boldon, UK). For use in the assay this was diluted 1:100 in general diluent.

Iodine\textsuperscript{125}-labelled LH:

Rat LH was labelled with radioactive iodine-125 (\textsuperscript{125}I) by means of an iodination procedure similar to that described previously (Markwell & Fox, 1978) (See sections 7.1.2 & 7.1.3).
Rat LH standards:

The referenced preparation used for the standard curve was NIDDK-rLH-RP-3 (5 μg ampoule) supplied in lyophilised form. The protein was dissolved in 1 ml of deionised water. The protein was then stored in 40 μl aliquots (containing 200 ng LH) at -20 °C until required. The highest concentration standard (16 ng/ml) was produced by diluting one aliquot of rat LH in 12.46 ml general diluent. The resulting 16 ng/ml standard was then split into 400 μl aliquots and stored at -20°C until required. On the first day of an assay one aliquot of the 16ng/ml standard was removed from the freezer. 200 μl of the standard was then used to in a 1/2 dilution series to produce the remaining standards containing 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.01562 and 0 ng/ml rat LH. Three LP2 tubes were then labelled S1 to S12, for each of the 12 standard curve concentrations. 50 μl each of the 12 dilutions of rat LH standard was dispensed in triplicate into pre-labelled LP-2 tubes. A further 50 μl of saline was then added to all standard curve tubes before being used immediately in the assay.

Positive controls:

In order to calculate the coefficient of variation across multiple repetitions of the LH RIA, samples of pooled plasma, obtained by centrifugation of whole blood from OVX rats, were aliquoted into 25 μl, 12.5 μl, 6.25 μl volumes and made up to 100 μl with general diluent. These control samples were run in triplicate in each LH RIA, with one set of plasma pools added to the assay approximately every 500 tubes to
account for potential drift.

Washing solution:  

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Deionised water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>40 g</td>
</tr>
<tr>
<td>(PEG; Sigma-Aldrich)</td>
<td></td>
</tr>
<tr>
<td>NaCl (Sigma-Aldrich)</td>
<td>9 g</td>
</tr>
<tr>
<td>Triton® X-100 (Sigma-Aldrich)</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

The above components were dissolved in 800 ml water in the specified order, with each component only added once the previous had fully dissolved. A further 198 ml water was then added to bring the total volume to 1000 ml. The resulting solution is 0.9% saline with 4% PEG and 0.2% Triton® X-100 detergent.

7.1.2 Reagents used in the iodination of rat luteinising hormone

Purified rat LH:

Iodination grade protein NIDDK-rLH-1-9 was supplied by the NIDDK in lyophilised form. It was reconstituted with 200 µl of 0.5M phosphate buffer. Aliquots of 10 µl (containing 5 µg rat LH) were then stored at -80 °C until required for iodination.

Idoine-125:

37 MBq iodine-125 (\(^{125}\text{I}\)) was supplied by Perkin-Elmer (Cambridge, UK) in the form of 10 µl Na\(^{125}\text{I}\).
Phosphate Buffer (PB) 0.5 M (pH7.4): 140 ml Deionised water

7.1 g Na$_2$HPO$_4$ (anhydrous)

3.12 g Na$_2$HPO$_4$·2H$_2$O

Phosphate Buffer (PB) 0.05 M (pH7.4): 1400 ml Deionised water

7.1 g Na$_2$HPO$_4$ (anhydrous)

3.12 g Na$_2$HPO$_4$·2H$_2$O

Iodination vials:

The catalytic matrix formed within the iodination vials was used as a reducing substrate for the $^{125}$I. 1 mg Iodo-Gen® (1,3,4,6-Tetrachloro-3a,6a-diphenylglyvouril: Sigma-Aldrich) was dissolved in 10 ml chloroform (Sigma-Aldrich). This solution (50 µl) was then placed into the bottom of small glass test-tubes (Thermo Life Sciences). The tubes were left at room temperature until the chloroform had evaporated away leaving the matrix of iodogen at the bottom of the tube. The iodogen coated vials were then sealed with a small rubber stopper and stored at -20 °C until required.
7.1.3 Procedure for iodination of rat luteinising hormone

This procedure was carried out behind a Perspex shield positioned within a ventilated cupboard certificated for work involving radioactivity. 5 g Sephadex G-75 (Sigma-Aldrich) was soaked overnight in 150 ml deionised water at 4 °C. On the morning of the iodination, the Sephadex was allowed to warm to room temperature. The top of a 10 ml polystyrene serological pipette (BD Falcon, Oxford, UK) was sawn off. The tapered end of the pipette was then plugged with approximately 2 cm of glass wool (Surechem Products, Needham Market UK). One aliquot (5/10 µl) of rat LH was diluted with 20 µl of 0.5M PB and mixed. The entire 30 µl was then added to the bottom of the iodination vial. 5 µl \(^{125}\text{I}\) (equivalent to 17.5 MBq) was then immediately added to the iodination vial. The mixture was then left for 30-40 min to allow the reduction of molecular Na\(^{125}\text{I}\) to \(^{125}\text{I}^-\), and the subsequent binding of the ionic iodine-125 to tyrosine residues of the rat LH protein. During the 30-40 min reaction period the elution column was made.

The prepared 10 ml plastic pipette was fixed vertically in a clamp stand with the tapered end plugged with glass wool at the bottom. Soaked Sephadex G-75 was slowly added to the pipette with deionised water in order to prevent the Sephadex from drying. Soaked Sephadex was added to the column and allowed to settle, while the water fraction was allowed to flow out of the column. More water was constantly added to the column to prevent the Sephadex from drying. Once the Sephadex had
settled and formed a distinct meniscus at least 10 ml of general diluent was run through the column, still ensuring the Sephadex was not exposed to air. After 30-40 min the reaction between $^{125}$I and rat LH was quenched by the addition of 150 µl 0.05 M PB. General diluent was allowed to run through the elution column until the meniscus of the buffer had reached the top of the Sephadex layer. At this point, whilst being careful not to allow the Sephadex to come into contact with air, the reaction product was transferred from the iodination vial to the elution column. The position of the radioactivity within the column was judged using a hand-held Geiger counter (Mini 900; Perspective Instruments, Shaw, UK). Once the radioactivity reached approximately half-way through the column a series of forty 500 µl fractions of eluent were collected into pre-labelled 1.5 ml tubes (Eppendorf, Stevenage, UK). During this collection period the column was constantly topped up with general diluent. 10 µl from each collected fraction was transferred into clean pre-labelled LP-3 tubes, which were counted for 1 minute using an automatic gamma counter (WIZARD$^2$; Perkin-Elmer) to determine the fractions that contained iodinated rat LH ($^{125}$I-rLH).

Figure 7.1 illustrates a typical iodination profile. The fractions represented by the first peak contain $^{125}$I-rLH. Any tubes from the first peak with counts per minute over approximately 1,000,000/10 µl were mixed and diluted with general diluent to give approximately 85,000 cpm/µl. Aliquots of 100 µl were then placed into 0.5 ml Eppendorf tubes and stored at -20°C until required.
**Fig 7.1** Representative examples of an elution profile from a Sephadex G-75 column following the iodination reaction of 5 µg rat LH with 18.5 MBq 125I.

### 7.1.4 Procedure for the radioimmunoassay

The assay was carried out over 4 consecutive days. Reagents were added at room temperature, but otherwise samples were stored at 4°C. The sequence of samples assayed was as follows:

Tubes 1-3: Total counts tubes

Tubes 4-6: Non-specific binding tubes

Tubes 7-42: Standard curve; 12 standard concentrations, run in triplicate,
starting with the standard of lowest concentration

Tubes 43-51: Plasma pools

Tubes 52-end: Blood samples of unknown LH concentration. Additional sets of plasma pools were added approximately every 500 samples.

Day 1:

1. 50 µl primary antibody diluent was added to each of the three non-specific binding tubes in the standard curve.

2. All other tubes, with the exception of the total counts tubes, received 50 µl of primary antibody diluted in primary antibody as described above.

3. All tubes were vortexed and stored overnight at 4 °C.

Day 2:

4. An aliquot of $^{125}$I-rLH was diluted with general diluent to 150-200 cpm/µl. 50 µl of the resulting solution was dispensed into the total counts tubes, which were then counted using an automatic gamma counter to confirm adequate radioactivity levels (7,500-10,000 cpm/tube).

5. Each tube received 50 µl $^{125}$I-rLH diluted to 150-200 cpm/µl.

6. All tubes were vortexed and stored overnight at 4 °C.
Day 3:

7. All tubes, with the exception of total counts tubes, received 50 µl precipitating antibody diluted with general diluent as described above.

8. All tubes were vortexed and stored overnight at 4 °C.

Day 4:

9. All tubes were centrifuged at 3,500 rpm for 60 min at 4 °C.

10. Excluding the three total counts tubes, the supernatant in each tube was aspirated away using a water suction aspirator, without disturbing the pellet, and discarded.

11. Excluding the tree total counts tubes, each tube received 200 µl washing solution and was again centrifuged at 3,500 rpm for 60 min at 4 °C.

12. Excluding the three total counts tubes, the supernatant in each tube was again aspirated using a water suction aspirator, without disturbing the pellet, and discarded.

13. The radioactivity in the remaining pellets at the bottom of each tube was then counted for 1 min per tube using an automatic gamma counter. Tubes were counted in the order previously specified. The standard curve was automatically plotted by the computer software, and the unknown concentrations of LH were calculated from this curve.

The detection limit of the LH RIA was 0.093 ng/ml and the three plasma pool
concentrations were 8.41 ± 0.07, 4.64 ± 0.18 and 2.05 ± 0.20 ng/ml (mean ± SEM).

The intra-assay variation was 5.8%, and the inter-assay variation was 7.2%.

7.2 Solutions used for brain perfusion and immunocytochemistry

7.2.1 Solution for brain perfusion

5 IU/ml heparinized 0.9% saline:

9 g NaCl

1000 ml deionised water

0.2 ml heparin sodium (25000 IU/ml)

4% Paraformaldehyde (PFA) in 0.1 M PBS:

500 ml deionised water

40 g PFA

500 ml 0.2 M PBS

Drops of 1M NaOH

Drops of 1M HCL

PFA (40 g) was added to 400 ml deionized water and heated whilst stirring. The mixture was heated to approximately 65 °C at which point the majority of the PFA entered solution. Drops of concentrated sodium NaOH were added to make the remaining PFA go into solution. The solution was then made up to 500 ml and cooled on ice to room temperature (20-25 °C). The pH of the solution was checked to ensure
it was approximately pH 7.4 by adding drops of concentrated HCl. Then the solution was filtered, and the same volume of 0.2 M PBS was added to give a final solution of 4% PFA in 0.1 M PBS. The final PFA solution was kept on ice.

7.2.2 Solution for immunocytochemistry

**Antifreeze:**

1000 ml deionised water

1.77 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

5.47 g $\text{Na}_2\text{HPO}_4$ (anhydrous)

9.0 g NaCl

300 g Sucrose (30% w/v)

300 ml Ethylene Glycol (30% w/v)

10 g Polyvinyl pyrrolidine (1% w/v)

The above were dissolved in 500 ml of deionised water. This solution was then made up to 1 litre and stored at 4°C.

**0.04% bovine serum albumin (BSA) in 0.1 M PBS (PBS-BSA):**

100 ml 0.5 M PBS

400 ml deionised water

0.2 g BSA (Sigma-Aldrich)
**0.3% triton and 0.04% BSA in 0.1 M PBS (PBST-BSA + NaN₃):**

100 ml 0.5 M PBS

400 ml deionised water

15 ml 10% triton (Sigma-Aldrich)

0.2 g BSA

0.1 g NaN₃ (Sigma-Aldrich)

**0.1M Tris buffer (pH7.6):**

1000 ml deionised water

7.45 g Trizma preset crystals pH7.6

**Standard antibody diluent:**

400 ml Milli-Q water

0.1 g MgCl₂·2H₂O

1.017 g Na₂HPO₄·2H₂O

6.1 g Na₂HPO₄ (anhydrous)

2.0 g NaCl

0.5 g sodium azide