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Development of kisspeptin-GnRH neural circuit in utero and mapping of GnRH receptor neurons in mice brain

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Zusammenfassung

'Gonadotropin releasing hormone (GnRH)'-Neurone repräsentieren die gemeinsame ZNS-Endstrecke zur hormonalen Kontrolle der Keimdrüsen. Dabei unterliegt die GnRH-Freisetzung einer Feedback-Regulation durch das gonadale Steroidhormon Östrogen. GnRH-Neurone exprimieren selbst allerdings nicht Östrogen-Rezeptor-alpha (ERα), was nahelegt, dass das Feedback-Signal über einen vorgeschalteten Steroid-sensitiven Signalweg auf GnRH-Neurone übertragen wird. Tatsächlich fungieren 'Kisspeptin'-Neurone als Upstream-Regulator der GnRH-Freisetzung und dienen als Angriffspunkt für Steroidhormone in der Regulation der GnRH-Sekretion. GnRH-Neurone exprimieren den Kisspeptin-Rezeptor GPR54. Kisspeptin-Neurone sind im Gehirn vor allem im Nucleus arcuatus (ARC) und im anterolateralen Nucleus paraventricularis (AVPV) lokalisiert. Kisspeptin-Neurone projizieren in die mediane Eminenz und auf GnRH-Neurone der prä-optischen Region. Kisspeptin bindet an GPR54 und stimuliert GnRH-Neurone der prä-optischen Region zur GnRH-Freisetzung in die Zirkulation. In der vorliegenden Arbeit wurde das genaue raumzeitliche Expressionsmuster der dem GnRH-Neuron vor- und nachgeschaltetem Neuronensysteme, des 'Kisspeptin'-Systems und des GnRH-exprimierenden Systems, bestimmt.

Dazu wurde zunächst das Einsetzen der Expression von Kisspeptin und seinem Rezeptor GPR54 in männlichen KissIC/eR26-τGFP-Embryonen sowie in männlichen GPIC/eR26-τGFP-Embryonen untersucht. Kisspeptin und GPR54 erschienen gleichzeitig am Embryonaltag E13.5. Während der ganzen embryonalen Hirnentwicklung wurde Kisspeptin nur im ARC des Hypothalamus gefunden. Hingegen blieb die Expression von GPR54 auf GnRH-Neurone beschränkt. Die detailierte Analyse zeigt jedoch, dass das GPR54-Expressionsmuster unabhängig von der Lage der GnRH-Neuronen war. Zur Bestimmung des Zeitpunkts, an dem Kisspeptin-Neurone sensitiv gegenüber Sexualsteroiden werden, wurde die Expression von Östrogen-Rezeptor ERα und von Androgen-Rezeptor (AR) in ARC Kisspeptin-Neuronen untersucht. Es stellte sich heraus, dass ERα und AR-positive Neurone die embryonale Hirnregion markieren, in der sich Kisspeptin-Neurone entwickeln. Trans-synaptisches Tracing zeigte schließlich, dass im männlichen embryonalen Mäusegehirn Kisspeptin-Neurone des ARC mit dem GnRH-Neuronensystem kommunizieren. Die Verbindung zwischen ARC-Kisspeptin-Neuronen und GnRH-Neuronen ist unabhängig von der Lage der Neurone im männlichen embryonalen Mäusegehirn. Diese Beobachtung legt den Schluß nahe, dass im Gegensatz zum adulten Gehirn, in dem Kisspeptin-Neurone eine geschlechtsspezifische Verteilung aufweisen, Beginn und Entwicklung des Kisspeptin-GPR54-Systems im

männlichen embryonalen Mäusegehirn eher dem weiblichen Mäusegehirn ähneln und dass der Sexualdimorphismus erst später in der Entwicklung entsteht.

Es folgte die Untersuchung des Downstream-Targets, dem Gonadotropin releasing hormone receptor (GnRHR)-Neurons. In der Hypophyse triggert GnRH-Rezeptorbindung die Synthese und Freisetzung von Luteinisierungshormon (LH) und von follikelstimulierendem Hormon (FSH). Die Rolle von GnRHR in den gonadotropen Zellen der Hypophyse ist bekannt, aber die Funktion der GnRHR-Neurone im Gehirn ist noch nicht vollständig geklärt. Zur Bearbeitung dieser Frage wurde die Verteilung der GnRHR-Neurone im embryonalen Gehirn von weiblichen GnRHR/eR26-τGFP-Mäuseembryonen kartiert. Es stellte sich heraus, dass ihre Anzahl signifikant im Lauf der Entwicklung ansteigt. Die GnRHR-Neuronen reicherten sich in der olfaktorischen Hirnregion und in Sexualzentren wie der medialen Amygdala (MeA), der medialen prä-optischen Region (MPA), dem ventromedialen Hypothalamus (VMH) und der peri-aquaeduktalen grauen Substanz (PAG) an. Die GnRHR-Neuronen der olfaktorischen Regionen und Sexualzentren erwiesen sich als sensitiv gegenüber Sexualsteroiden. Erstmalig konnte die olfaktorische Stimulation von GnRHR-exprierenden Neuronen gezeigt werden. Die Ergebnisse der vorliegende Untersuchung liefern Einblicke in die Entwicklung des Kisspeptin-GPR54-Systems und können zum besseren Verständnis von Störungen der Geschlechtsentwicklung wie dem hypogonadotrophen Hypogonadismus oder der Pubertas praecox beitragen. Die anatomische Kartierung der GnRHR-Neurone im Gehirn beleuchtet die Rolle von GnRH-Signalwegs in der Säugetier-Hypothalamus/Hypophysen/Gonaden-Achse.

Abstract

Gonadotropin releasing hormone (GnRH) neurons are the final common output pathway by which brain controls reproduction. Gonadal steroid hormone, estrogen regulates GnRH release by feedback signaling. Interestingly, GnRH neurons do not express estrogen receptors (ERα), suggesting that a steroid sensitive pathway might mediate these effects on GnRH neuron. Kisspeptin, a key upstream regulator of GnRH secretion serves as a target for steroid hormone in the control of GnRH secretion. GnRH neurons express kisspeptin receptor, GPR54. Kisspeptin neurons are primarily located in two major locations of the hypothalamus, the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV). Kisspeptin neurons project to GnRH neurons located in the preoptic area (and to the median eminence). Kisspeptin binds to GPR54 to stimulate GnRH neuron in the preoptic area to release GnRH into the circulation. The goal of this study is to determine the precise spatio-temporal expression pattern of the upstream and the downstream target of GnRH neuron, Kisspeptin system and GnRHR expressing neurons respectively.

I examined the onset of kisspeptin and its receptor, GPR54 in the KissIC/eR26-τGFP and GPIC/eR26-τGFP male mouse embryo respectively. Interestingly expression of Kisspeptin and its receptor GPR54 initiates at the same time (E13.5). Throughout the embryonic brain development kisspeptin neurons remain restricted to the ARC of the hypothalamus whereas GPR54 expression is restricted to the GnRH neuron. Detailed analysis revealed that the GPR54 expression is independent of the location of the GnRH neuron. Next I analyzed when kisspeptin neurons becomes sensitive to gonadal steroid hormones. I investigated the expression of estrogen receptor alpha (ERα) and androgen receptor (AR) in the ARC kisspeptin neuron. I observed that ERα and AR positive neurons marks the birthplace of kisspeptin neuron in the embryonic brain. Transsynaptic tracing in the embryonic mouse brain revealed that ARC kisspeptin neurons communicate with GnRH neurons in utero. The connectivity between ARC kisspeptin neurons and GnRH neurons is independent of the location of the GnRH neurons in the embryonic male mouse brain. These observations suggests that in contrast to the adult brain where kisspeptin neurons are present in sexually dimorphic manner, onset and the development of Kisspeptin-GPR54 system in embryonic male brain is highly similar to female brain and sexual dimorphism arises later in development.

Next, I investigated the downstream target of GnRH neuron, gonadotropin releasing hormone receptor (GnRHR) expressing neurons. GnRH binds to its receptor in pituitary to trigger the

synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). The role of GnRHR in pituitary gonadotropes is well understood but the function of GnRHR neurons in the brain is not well established. Using GnRHR/eR26-τGFP mouse I mapped the distribution of GnRHR neurons in the female brain. I observed that the GnRHR neurons are concentrated in olfactory processing areas and reproductive centers in the brain such as medial amygdala (MeA), medial preoptic area (MPA), ventromedial hypothalamus (VMH) and periaqueductal grey (PAG). I found that the number of GnRHR neurons significantly increases across developmental stage I also observed that GnRHR neurons in olfactory and reproductive centers are sensitive to steroid hormones. For the first time I also identified that GnRHR expressing neurons are activated upon olfactory stimulation. Taken together, the present study provides insight into the (I) development of kisspeptin-GPR54 system which will help to better understand reproductive disorders such as hypogonadotropic hypogonadism and precocious puberty (II) detailed anatomical mapping of GnRHR neurons in the brain will help in understanding the role of GnRH signaling in the mammalian brain and its effect on reproductive axis.

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List of abbreviations

μm	Microgram
μl	Microliter
μm	Micrometer
^{o}C	Degree Celsius
AON	Anterior cortical nucleus
AVPe	Anteroventral periventricular nucleus
AR	Androgen receptor
ARC	Arcuate nucleus
BNST	Bed nucleus of the stria terminalis
BL	Barley lectin
β-Gal	Beta –galactosidase
Cre	Cre-recombinase
d	Day
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
E	Embryonic day
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
eROSA26	Enhanced Rosa26
et al.	et altera
FSH	Follicle stimulating hormone
g	Gram
GFP	Green fluorescent protein
GnRH	Gonadotropin releasing hormone
GnRHR	Gonadotropin releasing hormone receptor
GRIC	GnRHR-IRES-Cre
HPG	Hypothalamic Pituitary gonadal
IRES	Internal ribosome entry site

Kiss1	Kisspeptin gene
LH	Luteinizing hormone
ME	Median eminence
ml	Milliliter
mM	Millimolar
MPA	Medial preoptic area
mRNA	Messenger ribonucleic acid
n	Sample number
OE	Olfactory epithelium
OVLT	Organum vasculosum of the lamina terminalis
PND	Postnatal day
PAG	Periaquectal gray
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMCO	Posteromedial cortical amygdaloid nucleus
POA	Preoptic area
RNA	Ribonucleic acid
RT	Room temperature
SEM	Standard error of the mean
TH	Tyrosine hydroxylase

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1 Introduction

1.1 GnRH neuronal system

The mammalian reproductive function is maintained by hypothalamic pituitary gonadal (HPG) axis (Figure 1.1), the neuroendocrine system consisting of hypothalamus, pituitary and gonads (Carolsfeld, et al., 2000). At the center of the HPG axis are the hypothalamic gonadotropin releasing hormone (GnRH) producing neurons that are scattered along the medial septum/ preoptic area to the posterior hypothalamus. Adult mouse brain has about 800-1000 GnRH neurons in number (Tobet et al., 2001). GnRH neuron is the master regulator of the HPG axis. The GnRH neuron which are the final common output pathway of the brain controlling reproduction integrate intrinsic and extrinsic cues resulting in a pulsatile secretion of the decapeptide GnRH(also known as luteinizing hormone-releasing hormone) into the portal stream. The GnRH acts on the GnRH receptor (GnRHR) expressed on the surface of the gonadotropes that are scattered in the anterior pituitary triggering the synthesis and release of the gonadotropins (luteinizing hormone (LH) and follicle stimulating hormone (FSH)) into the systemic circulation (Clayton et al., 1981).

In the female, LH acts on theca cells in the ovary and induces ovulation. In the male, LH acts on leydig cells in the testis and stimulates the production of testosterone. FSH stimulates the maturation of germ cells in both testis and ovary (Gharib et al., 1990; Hillier, 2001). The LH and FSH acts on the gonads triggering gametogenesis and stimulates the release of gonadal steroid hormones which have positive and negative feedback effect on the HPG axis by modulating either the gonadotropes in the pituitary or the release of GnRH from the hypothalamus. The circuit controlling reproduction undergoes dramatic functional changes during development. The HPG axis is tightly regulated and its function is important for the development and the maintenance of reproductive physiology (Varykina, 2010).

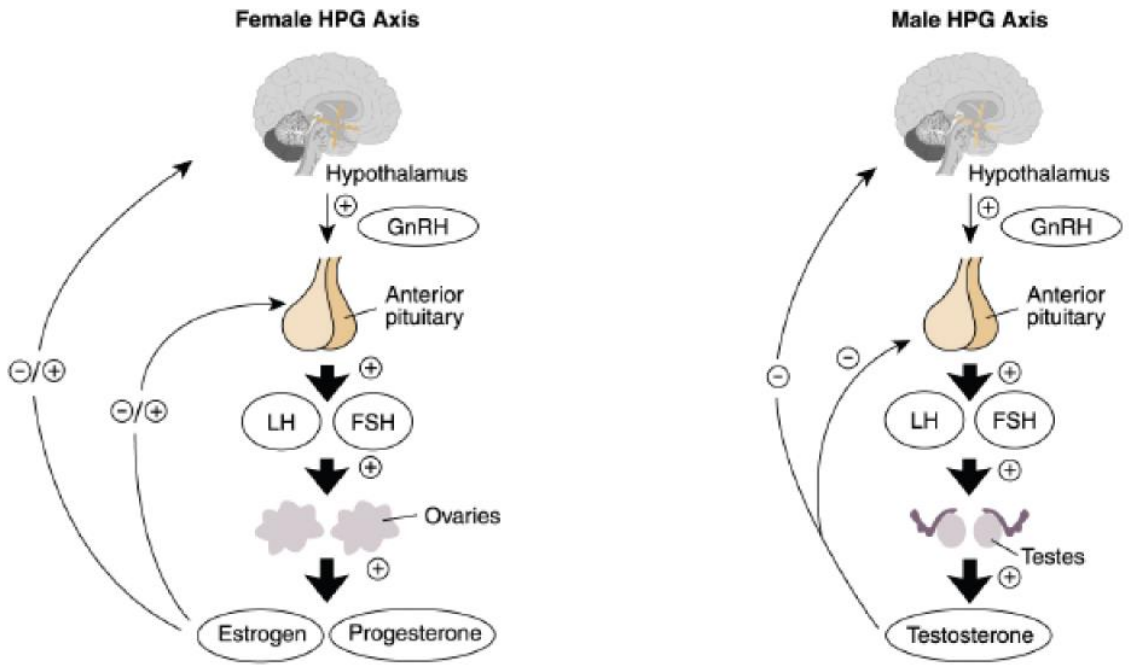


Figure 1.1 The Hypothalamic-Pituitary-Gonadal (HPG) axis. The schematic diagram represents the components of the HPG axis. GnRH neurons in the hypothalamus releases gonadotropin releasing hormone (GnRH) into the median eminence in a pulsatile fashion. GnRH acts on the anterior pituitary to secrete gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the circulation. The gonadotropins act on the receptors located in the gonads to stimulate the production of steroid hormones such as estrogen/ progesterone in female and testosterone in male. In females the gonadal hormones, estrogen and progesterone exert positive and negative feedback at the level of both pituitary and hypothalamus to regulate the secretion of GnRH. In males, testosterone exert negative feedback both at the level of pituitary and hypothalamus to regulate the secretion of GnRH. Abbreviations: HPG axis, hypothalamic-pituitary-gonadal axis; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone. Adapted from Hilmer Sturmhoefel and Bartke, 1998.

1.1.1 Development of GnRH neurons

During embryonic development GnRH neurons originate outside the brain in the olfactory placodes and migrate to their final destination in the forebrain (Figure 1.2). GnRH neurons are born at embryonic day (E)10 in the mouse olfactory placode (Schwanzel et al., 1989; El Amraoui et al., 1993). At E12.5 the number of GnRH neurons is same as that of the number of GnRH neurons in the postnatal mouse but are predominantly located in the nasal regions (Schwanzel et al., 1989; Wray et al., 1989; Wray et al., 1994). The GnRH neurons migrate in clusters along the vomeronasal axons cross the cribriform plate to destinations in the forebrain. The GnRH neurons in mice migrate from olfactory areas to the forebrain between E11 and birth (Schwarting et al., 2007). GnRH neurons complete their migration by birth. At E12.5, GnRH neurons are not detected in the forebrain. At E14.5 GnRH neurons can be located in the forebrain and at E16.5 they are predominantly detected in the forebrain areas such as preoptic area (POA) and medial septum (Livne et al., 1993). From E12.5 to E16.5 as the GnRH neurons migrate in a rostral to caudal direction the number of GnRH neurons in the nasal area decreases with increase in the number of GnRH neurons in the forebrain areas. The number of GnRH neurons changes during embryonic development. Some report suggest that the number of GnRH neurons peaks to around 1500 at E15 and then declines to 800 postnatally (Wu et al., 1997). The adult like spatial distribution of GnRH neurons are established by birth (Wierman et al., 2004). The neurons reach their final destination in the hypothalamus by birth and extend their processes to the median eminence (ME), the organum vasculosm of the lamina terminalis (OVLT) or both to release GnRH into the circulation (Tobet et al., 2001; Schwanzel et al., 1989). At the median eminence GnRH neurons are in contact with the blood vessels and release GnRH into circulation to activate pituitary gonadotropin production and secretion (Herbison et al., 2006; Palkovits et al., 1978).
GnRH neurons are not sexually dimorphic. GnRH modulates reproductive physiology by binding to GnRH receptor expressed by the gonadotropes in the anterior pituitary to release LH and FSH. Gonadotropes constitute 7-15% of the total number of cells in the anterior pituitary gland (Kaiser, 2011). Studies have revealed the presence of heterogeneous gonadotrope population, monohormonal (15%) positive for either LH or FSH and bihormonal (70%) gonadotrope positive for both LH and FSH (Moriarty, 1976; Meeran et al., 2003). GnRH neuronal migration and the secretion of GnRH is critical for the regulation of HPG axis. The precise mechanism that control GnRH neuron migration is not known.

Abnormal migration of GnRH neuron causes Kallmann syndrome and hypogonadotropic hypogonadism characterized by infertility (MacColl, et al., 2002; Clayton et al., 1981). Kallmann

syndrome is a genetic disorder associated with the mutation in the KAL 1 gene. KAL 1 gene encodes the secreted glycoprotein anosmin-1 that play a critical role in axon pathfinding and migration during CNS development (de Castro et al., 2014). In Kallmann syndrome the GnRH neurons were found to be in clusters in the olfactory area of the human fetus brain (Schwanzel-Fukuda et al., 1989). Molecules such as membrane receptors, neurotransmitters, and chemorepellent molecules has influence on the guidance of GnRH neurons. Mutations in several other genes which alter the development of GnRH and olfactory systems has been identified in Kallmann syndrome patients such as NELF, FGFR1, prokineticin and its receptor (Miura et al., 2004; Dodé et al., 2003; Dodé et al., 2006).

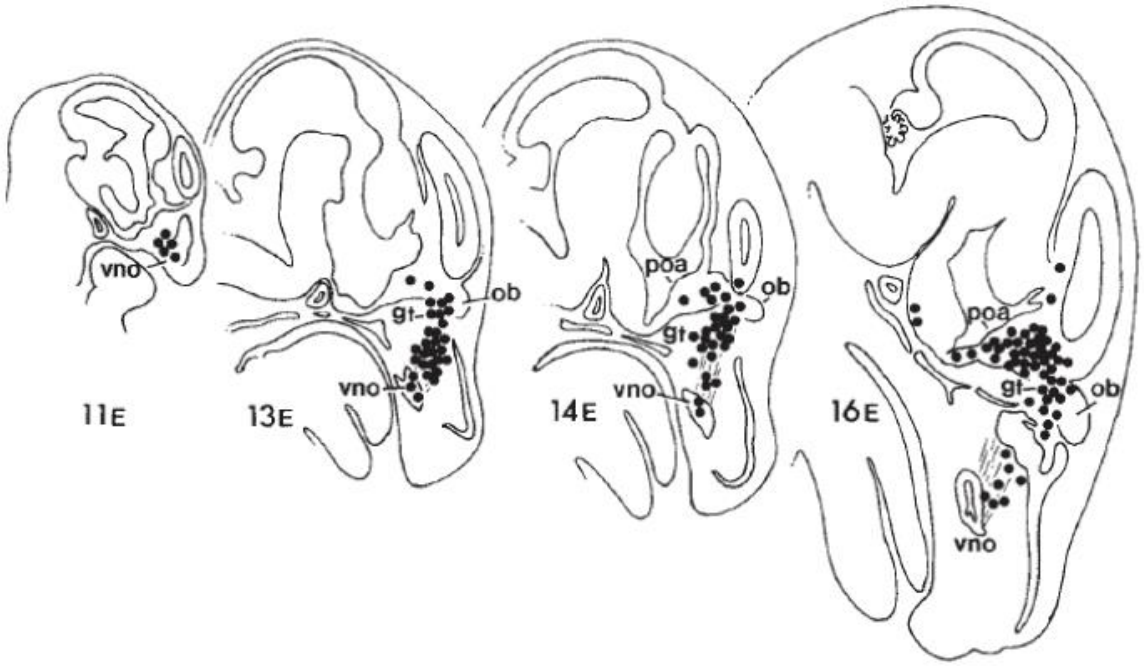


Figure 1.2 Gonadotropin releasing hormone (GnRH) neuronal migration. Photo depicting the migratory route of GnRH neuron during developmental stages on a sagittal section in the mouse brain. The black dots on embryonic day (E) 11, 13, 14 and 16 represents GnRH neuron. The GnRH neurons at E11 were detected in the VNO. As the development progresses, at E13 most of the GnRH migrate along the olfactory nerve towards the base of the telencephalon. The olfactory nerves serve as a migratory routing guide. On day E14, most of the GnRH neurons are located at the ganglion terminale. By day E16, GnRH neurons reach POA in the basal forebrain and adult like distribution is achieved. Abbreviations; VNO, vomeronasal organ; gt, ganglion terminale; ob, olfactory bulb; poa, preoptic area. Adapted from Schwanzel Fukuda et al., 1989.

1.1.2 Distribution of GnRH neurons in the brain

In most mammals GnRH neurons are bipolar and fusiform in shape. A small subtype of GnRH neurons are multipolar with triangular cell body (Dudas B et al., 2000). The location of the GnRH neurons are dependent on the species. In rodents, the GnRH neurons are present in clusters within the hypothalamus (Figure 1.3). The clusters are found in the organsum vasculosum laminae terminalis (OVLT) and medial preoptic nucleus (MPA) (Merchenthaler et al., 1984).

GnRH neuron distribution is referred as an inverted Y shaped where the bottom of the Y is in the medial septum. In mice GnRH neurons exhibit remodeling of the dendritic tree to increase direct input during reproductive maturation. During reproductive maturation the morphology of GnRH neuron is altered. Spine like processes increases in GnRH neurons located in the preoptic area (Xue et al., 2014).

Studies have shown that GnRH fibers communicate to approximately 50000 neurons and innervate approximately 53 different areas in the brain (Boehm et al.,2005). However the major targets of the GnRH neurons are the OVLT which is located at the rostral end of the third ventricle and the median eminence which is located along the ventral surface of the hypothalamus (Tobet et al., 2001). In humans three morphological subtypes of GnRH neurons are scattered in medial basal hypothalamus, preoptic area, septal area, bed nucleus of the stria terminalis to the amygdala, magnocellular basal forebrain complex, ventral pallidum and putamen (Rance et al., 1994).

A. **B.**

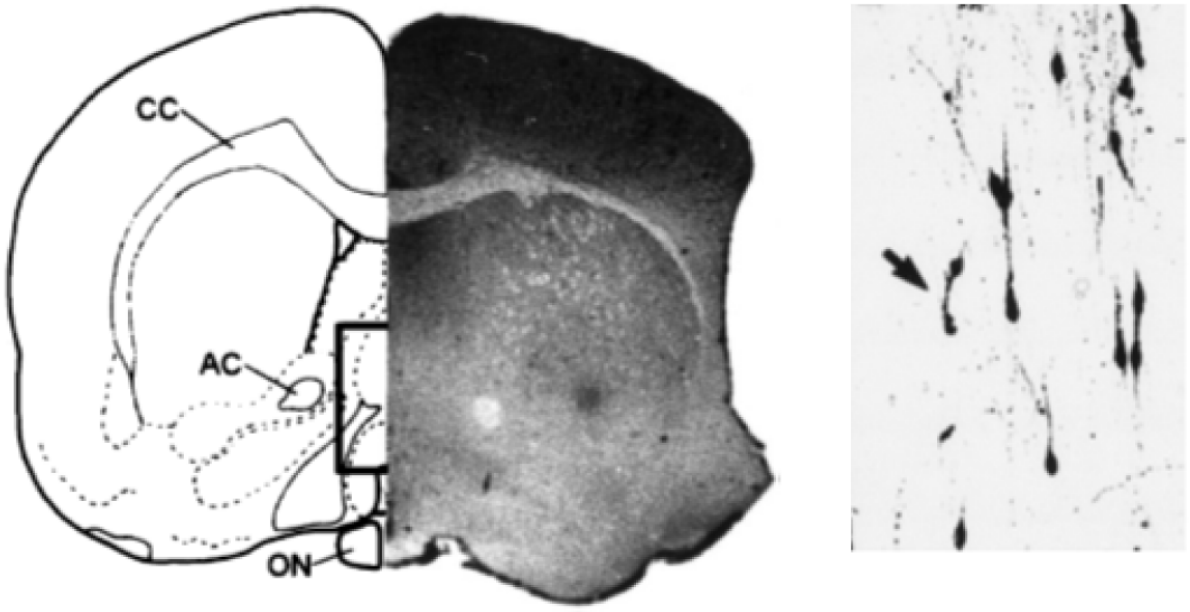


Figure 1.3 Localization of GnRH neurons in the adult mouse brain. Image showing the localization of GnRH neurons in the mouse forebrain. A. Coronal section of the adult mouse brain containing GnRH neurons in forebrain area. (B) A photomicrograph representing the distribution of GnRH neuron in the POA. Abbreviations; CC, corpus callosum; AC, anterior commissure; ON, optic nerve; POA, preoptic area. Adapted from Grober et al., 1998.

1.1.3 GnRH and GnRHR

Gonadotropin releasing hormone is an evolutionary conserved decapeptide synthesized and released in synchronized pulses from the nerve endings of the GnRH neuron into the portal circulation every 30-120 min to stimulate the synthesis and secretion of gonadotropin from pituitary gonadotropes. During ovulatory LH surge the pulse frequency is the highest whereas during luteal phase the pulse frequency is the lowest (Millar et al., 2004). For the release of LH and FSH the pituitary requires pulsatile secretion of GnRH. When the GnRH receptors are stimulated continuously by exogenous GnRH, the GnRH receptors are desensitized and downregulated (Miura et al., 2004). GnRH is critical in maintaining FSH levels. In GnRH null mice FSH levels are reduced approximately 60% when compared to normal mice (Mason et al., 1986). Upon single pulse administration of GnRH led to fourfold increase in the levels of FSH beta gene expression (Burger et al., 2001).

There are 3 isoforms of GnRH- GnRH1, GnRH2, and GnRH3. Most vertebrates have GnRH1 and GnRH2 (King et al., 1990). In vertebrates atleast two major isoforms of GnRH are identified, namely GnRH1 which acts on the hypothalamus, pituitary and GnRH 2 is extra hypothalamic found primarily in the midbrain for which the function is still under investigation (Gorbman et al., 2003). GnRH3 has been identified only in teleosts (Yamamoto et al., 1995).

GnRH exerts its effect by binding to GnRH receptor (GnRHR). The primary structure of GnRHR was first elucidated by cloning and sequencing cDNA from a murine gonadotroph derived cell line (αT3-1) (Reinhart et al., 1992). GnRH receptors belong to the G protein coupled receptor (GPCR) family of proteins, characterized by seven hydrophobic extra and intracellular loops. The extracellular domains and superficial regions of the TM domains are typically responsible for binding events, especially the third extracellular loop (EC3) (Millar et al., 2004; Stojilkovic et al., 1994).

In vertebrates three forms of GnRHR (GnRHR I, GnRHR II, and GnRHR III) have been identified (Millar, 2005). Among the three forms of GnRHR, GnRH type I is the predominantly expressed form of the receptor. In man hypothalamic GnRH binds to GnRH type I receptors in the pituitary to regulate the secretion of LH and FSH (Millar et al., 2004). The mouse type I GnRH receptor amino acid sequence is atleast 80% homologous to pigs, sheep and humans. GnRHR II was first identified in chicken and is expressed by most of the vertebrates (Miyamoto et al., 1984). In this thesis GnRH and GnRHR refers to GnRH1 and type 1 GnRH receptor if not mentioned.

GnRHR consists of 328 amino acids in human, 327 amino acids in mouse and rat (Reinhart et al., 1992). Mammalian GnRHR do not contain intracellular carboxy terminal domain. The lack of C-terminal domain does not allow rapid desensitization and internalization of the receptor. Non- mammalian GnRHR contain C- terminal tail and the internalization is rapid (Hislop et al., 2001). Continuous activation of GnRHR results in desensitization and leads to the suppression of gonadotropin secretion (Limonta et al., 2012). GnRHR is primarily expressed by the gonadotrope in the anterior pituitary, GnRHR is also detected in the brain, breast, endometrium and prostate (Cheung et al., 2008).

When GnRH binds to GnRHR, signaling cascade is activated. GnRHR activates Gq/11 which leads to the activation of phospholipase C (PLC) (Figure 1.4). Activated PLC generates inositol 1, 4, 5- triphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol 4, 5 bisphosphate (PiP2). This leads to the activation of protein kinase (PKC). IP3 binds to its receptor in the endoplasmic reticulum (ER) which mobilizes calcium from the intracellular stores and activates CaMK downstream cascade. This results in the influx of extracellular calcium triggering the release of gonadotropins (Ciccone et al., 2009; Haisenleder et al., 2003).

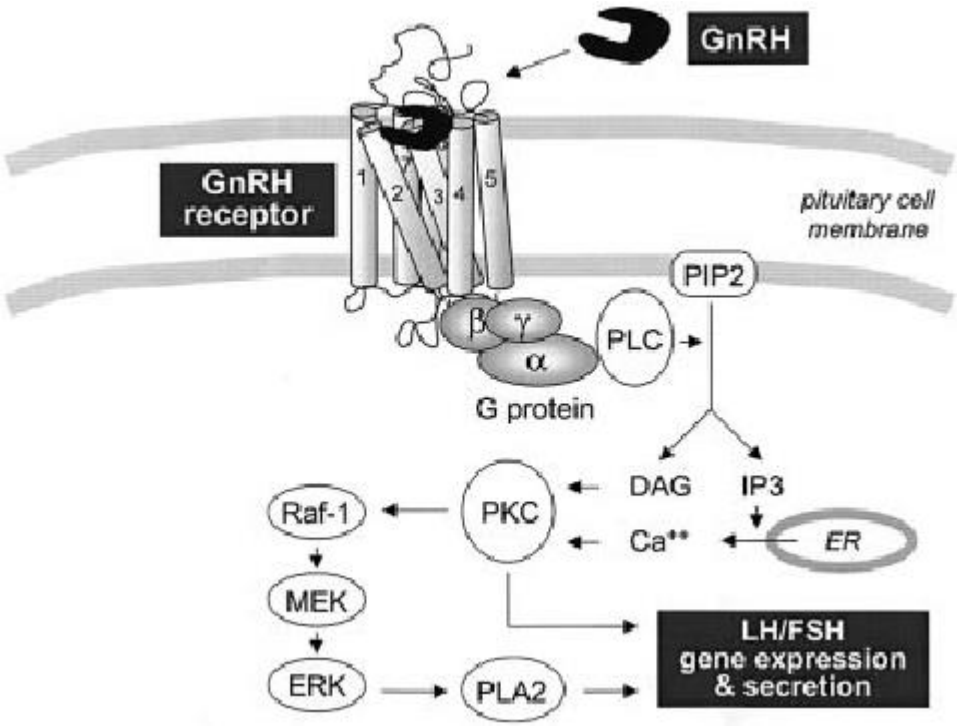


Figure 1.4 Schematic picture of the signal transduction cascade in pituitary gonadotrope in response to GnRH binding to the GnRHR. Activation of GnRHR induces PLC which generates IP3 and DAG from PiP2. The cascade of events lead to the release of calcium from the intracellular stores and activates the downstream pathway. Abbreviations; PLC, Phospholipase C; IP3, inositol phosphate; DAG, diacylglycerol; PIP2 phosphatidyl inositol phosphate; Ca^{++}, calcium ions; ER, endoplasmic reticulum; PKC, protein kinase C; ERK, mitogen activated protein kinase; PLA2, phospholipase A_2; LH, luteinizing hormone; FSH, Follicle-stimulating hormone. Adapted from Karges et al., 2003.

1.2 Kisspeptin/GPR54 neuronal system

1.2.1 Kisspeptin

The *Kiss1* gene and its product kisspeptin play an important role in the neural control of fertility (Kauffman, 2010; Clarkson et al., 2008). In 1996, *Kiss1* gene was identified in the tumor cells for its antimetastatic properties (Lee et al., 1996). In humans the *Kiss1* gene is located on chromosome 1(1q32) (Ohtaki et al., 2001). The protein product of *Kiss1* gene is a 145 amino acid peptide which is proteolytically cleaved to 54 amino acid peptide (Kisspeptin-54). The Kisspeptin-54 can further be cleaved to yield C-terminal fragments of 14, 13 and 10 amino acid peptides (Figure 1.5). The physiological role of the shorter peptides are yet to be studied (Kotani et al., 2001).

Kisspeptin-54 is the most abundant form in humans. Kisspeptin-54, the longer form of kisspeptin could be unstable and cleave into shorter forms kisspeptin-14, kisspeptin-13 and kisspeptin-10 during the purification process. All the forms of kisspeptin with a common RF- amide C terminus have similar binding affinities to its receptor, GPR54 (Kotani et al., 2001). Since all the truncated form of kisspeptin are biologically active this suggests that the C-terminal domain is responsible for binding to GPR54.

Kisspeptin neuron integrates both the central and peripheral signals and acts as an important upstream regulator of GnRH neurons. The distribution of kisspeptin neurons varies between species (Colledge, 2009). In rodents kisspeptin neurons are primarily located between two hypothalamic regions, the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV)/ rostral periventricular (PN) nuclei (Semaan et al., 2010). One of the major circuits involved in the secretion of GnRH and puberty onset is the kisspeptin neural circuit. The ARC kisspeptin neurons co-express two additional peptides, neurokinin B (NKB) and dynorphin whereas the kisspeptin neurons in the anteroventricular nucleus (AVPe) do not express any of these peptides. This population of kisspeptin neurons in the ARC are known as KNDy neurons (Cheng et al., 2010; Foradori et al., 2006).

In the hypothalamus kisspeptin fibers are identified in lateral septum, preoptic area and arcuate nuclei. Outside the hypothalamus kisspeptin fibers are located in medial amygdala (MeA), bed nucleus of stria terminalis (BNST) and periaqueductal grey (PAG). Role of kisspeptin in regions other than hypothalamus are yet unknown (Clarkson et al., 2009; Brailoiu et al., 2005; Kim et al., 2011).

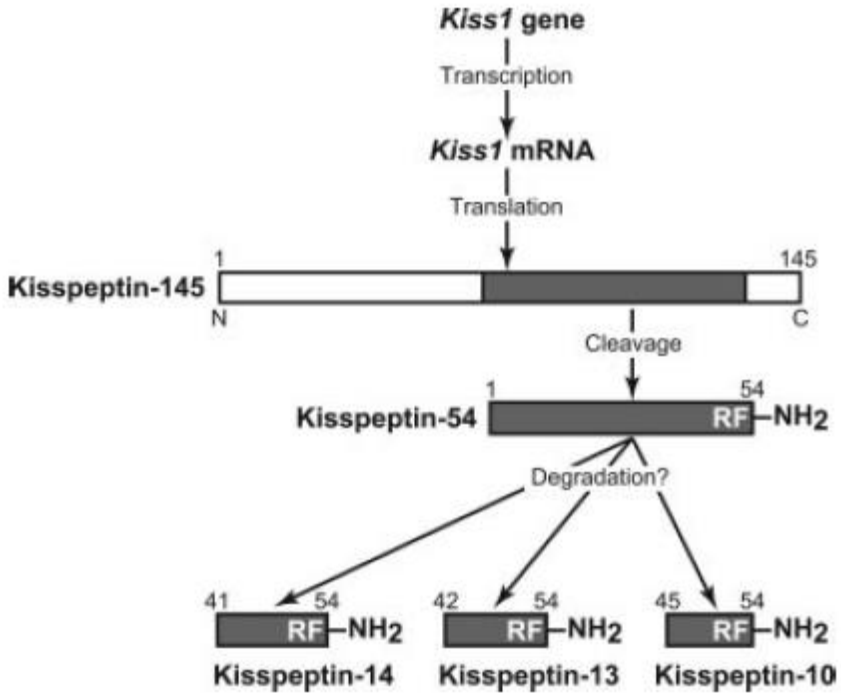


Figure 1.5 Products of Kiss1 *gene*: The primary translated product of *Kiss1* gene is a 145 amino acid peptide called kisspeptin-145. Kisspeptin-145 undergoes proteolytic cleavage to yield RF amidated peptide known as kisspeptin-54. Unstable kisspeptin undergoes degradation to result in shorter peptide with C-terminal region (Kisspeptin-14, Kisspeptin-13 and Kisspeptin- 10). Adapted from Oakley et al., 2009.

1.2.2 Development of kisspeptin

During embryonic development *Kiss1* expression in rodents is restricted to the arcuate nucleus (ARC) (Desroziers et al., 2012). Kisspeptin protein expression in the AVPV occurs during early postnatal life (Semaan et al., 2010). The number of kisspeptin neuron increases during embryonic development and some reports suggest that the number decreases during the late embryonic stage (Desroziers et al., 2012). Kisspeptin exhibit sexual dimorphism during a critical developmental window due to the exposure of steroid hormones such as estradiol and testosterone. The critical developmental window extends from the late embryonic development to the early postnatal development (Poling and Kauffman, 2013).

Several studies have shown that steroid hormones establish kisspeptin dimorphism. New born female mice treated with steroid hormone had fewer kisspeptin neuron in the AVPV whereas the number of kisspeptin neurons increased when a male mice is castrated (Homma, 2009). One mechanism is because developing males are exposed to higher levels of steroid hormone than females resulting in estradiol induced apoptosis and establishment of organizational effect. In developing male brain the circulating testosterone produced by the testis reaches the brain and it is aromatized to estradiol which then leads to the masculinization of the brain (Naftolin et al., 1974). Estradiol is from the aromatization of testosterone (Poling and Kauffman, 2013). The AVPV kisspeptin neurons are sexually dimorphic. Female rodents have atleast 10 fold higher number of AVPV kisspeptin neurons than male rodents. The kisspeptin neurons in the periventricular (Pe) region increases from P25 to adult along with the innervation of its fibers on GnRH neuron (Clarkson and Herbison, 2006).

Administration of kisspeptin activates GnRH neurons and increases the secretion of GnRH while increase in the secretion of GnRH was not observed in kisspeptin receptor knockout mice suggesting that kisspeptin induces GnRH secretion (d'Anglemont de Tassigny et al., 2008). Neuronal activity marker c-Fos is detected in 85% of GnRH neurons upon application of kisspeptin suggesting that kisspeptin activates GnRH neuron (Irwig et al., 2005). Activation of kisspeptin receptor induces the release of luteinising hormone (LH) (Gottsch et al., 2009). Administration of an antagonist to GnRH (Acyline) results in the inhibition of LH reporting that the action of kisspeptin is via GnRH neuron (Irwig et al., 2005). These studies confirm that kisspeptin is a potent activator of GnRH neuron.

Steroid hormones regulate the release of GnRH via feedback. GnRH neurons do not express ERα and AR, therefore the sex steroids regulate GnRH neurons indirectly (Roseweir et al., 2009).

Kisspeptin neuron being a potent regulator of GnRH neuron express steroid hormone receptor (Lehman et al., 2010). The AVPe kisspeptin neuron mediates the positive effect of estrogen which is necessary for the preovulatory surge in females whereas the arcuate nucleus (ARC) mediates the inhibitory effect of estrogen in both male and female (Smith et al., 2005a; Smith et al., 2005b). When estrogen levels are low in diestrus stage ARC *Kiss1* levels are increased and during proestrus stage when estrogen level peaks the levels of ARC *Kiss1* decreased. AVPV *Kiss1* levels are increased in proestrus when estrogen level peaks. Studies have revealed that estrogen exert positive effect on AVPV kisspeptin neurons and exert negative effect on ARC kisspeptin neurons (Keen et al., 2008).

Past studies have shown that ERα plays a critical role in modulating LH response to kisspeptin and not ERβ. Selectively blocking ERα resulted in the elimination of LH rise and blocked ovulation while blocking ERβ did not have any effect on LH rise. This result showed that estrogen acts through ERα in mediating both positive and negative effects on kisspeptin (Roa et al., 2008). Factors that affect kisspeptin neurons will modulate GnRH release. Estrogen and testosterone both can act on kisspeptin neurons and modulate the *kiss1* gene expression (Smith et al., 2005a). Sex steroid hormones regulate the two population of *Kiss1* mRNA differentially via positive and negative feedback in the rodent brain. They increase *Kiss1* mRNA expression in the AVPV whereas they decrease the *Kiss1* mRNA expression in the ARC (Clarkson et al., 2008; Smith et al., 2005b; Adachi et al., 2007). Most of the ARC and AVPV kisspeptin neurons express ERα and approximately 25-40% express ERβ (Smith et al., 2005b).

Upon administering developing female mice with gonadal steroid hormones at 2, 5, 10 and 20 days old mice and when tested for fertility after 100 days. Animals to which the gonadal steroid hormone was administered at day 2 and day 5 revealed that the ovarian and uterine weight significantly reduced and sterile. The animal to which the gonadal steroids were administered at day 20 were fertile (Barraclough., 1961). This study demonstrated that exposure to steroid hormones during the critical perinatal window could severely affect fertility. Alpha fetoprotein which is a plasma protein found in high levels during late gestation and early stages of development binds to circulating estrogen and prevents the developing female brain from masculinization (Bakker et al., 2006).

1.2.3 GPR54

The receptor for kisspeptin, Kiss1r also known as GPR54 is a G protein coupled receptor important for gonadotrophin physiology (Lee et al., 1999). In 2003, it was discovered that GPR54 mutation cause hypogonadotropic hypogonadism and absence of puberty in mice and humans (de Roux et al., 2003).

Kisspeptin binds to its receptor expressed by GnRH neuron, GPR54 to stimulate the release of GnRH (d'Anglemont de Tassigny et al., 2008). GPR54 share 45% identity with galanin receptors. GPR54 is detected in multiple regions in the brain such as pons, thalamus, hypothalamus, amygdala, arcuate nucleus, cortex, frontal cortex and striatum. Kisspeptin-54, -14 and -13 were found to bind and activate to GPR54 (Lee et al., 1999). GnRH neurons express GPR54 mRNA and kisspeptin depolarize GnRH neuron documenting that kisspeptin neuron regulate GnRH neuron (Irwig et al., 2004; Han et al., 2005).

In human tissues, quantitative PCR with reverse transcription (RT-PCR) method revealed that GPR54 is also highly expressed in regions other than brain such as placenta, testis and moderate levels in pancreas, liver and small intestine (Ohtaki et al., 2001). In the preoptic area more than 60% of GnRH neurons express GPR54 in mice. Dual labelling in mice across development ages P1, P5, P20 and P30 revealed that the percentage of GnRH neurons expressing GPR54 increases from 40% to 70% (Herbison et al., 2010). The GPR54 expression along with *Kiss1* varies during different stages of the estrous cycle.

Kisspeptin signaling also plays a role in the regulation of metabolism. Adult GPR54 knockout female mice have high levels of leptin and fat whereas adult GPR54 knockout male mice have regular levels of leptin. These experiments state that the role of kisspeptin in metabolism is sexually dimorphic (Holmes, 2014).

Kisspeptin on binding to its receptor GPR54 (Kiss1r) activates G protein, Gq/11 and phospholipase C to cleave phosphatidylinositol 4,5-bisphosphate (PiP2) resulting in the generation of intracellular inositol triphosphate (IP3) and diacylglycerol (Figure1.6). IP3 causes Ca2+ release from the endoplasmic reticulum which results in the activation of ERK1/2 and p38mitogen activated MAPK pathways (Kotani et al., 2001; Stafford et al., 2002). Although GPR54 is expressed in GnRH neurons and pituitary, the stimulatory effect of kisspeptin on gonadotropin release is only via the activation of GnRH neuron (Clarkson et al., 2008).

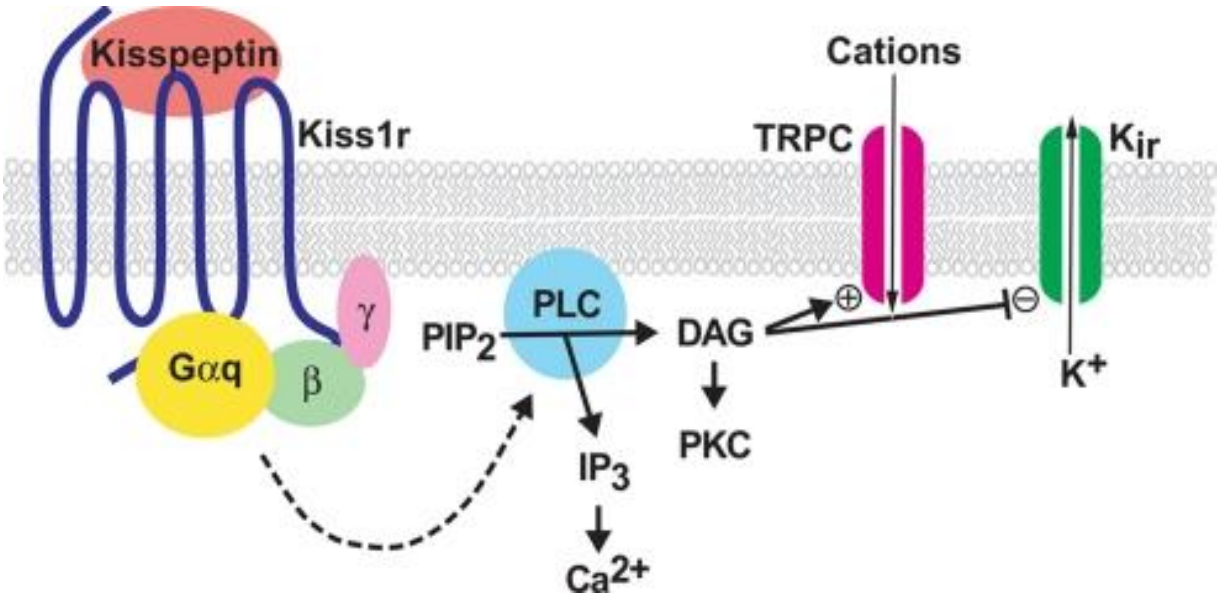


Figure 1.6 Kisspeptin and Kiss1r (GPR54) signaling. Schematic picture of how kisspeptin neuron activates Kiss1r (GPR54) in the GnRH neuron. Kisspeptin binds to GPR54 and activates phospholipase C which results in the generation of IP3 and DAG, which in turn results in the increase of calcium. Abbreviations ; PIP2, phosphatidylinositol 4,5- phosphate PLC, Phospholipase C; IP3, inositol phosphate; DAG, diacylglycerol; Ca^{++}, calcium ions; PKC, protein kinase C; Adapted from Oakley et al., 2009.

1.2.4 Role of kisspeptin and GPR54 in the regulation of puberty

Kisspeptin and GPR54 are established as a key gatekeeper of puberty and preovulatory LH surge (Figure 1.7). Kisspeptin neurons initiating puberty was first identified in humans (Seminara et al., 2003; Mayer et al., 2010).

In mice, kisspeptin neurons in the AVPV are detected at P25 and the number reaches adult level at P31 when the puberty onset occurs (Clarkson et al., 2006). Administration of kisspeptin from P25 induced precocious puberty, increase in uterus weight, increase in the levels of LH and estrogen in mice compared to controls (Navarro et al., 2004). An autosomal dominant mutation in GPR54 where proline is substituted for arginine at 386 codon in which the receptor is continuously active triggering signaling pathways are associated with precocious puberty (Teles et al., 2008). In mice and in primates intravenous administration of kisspeptin resulted in the advancement of puberty (Navarro et al., 2004; Plant et al., 2006). The expression level of *Kiss1* mRNA increases during puberty both in male and female (Shahab et al., 2005). Another study has shown that the administration of human kisspeptin (hkp-10) resulted in increased GnRH release whereas administration of kisspeptin antagonist peptide (Kp232) resulted in reduced GnRH release both in prepubertal and pubertal monkey. In ovariectomized pubertal monkey, kisspeptin failed to stimulate GnRH due to the absence of steroids but when estrogen is administered hkp10 triggered GnRH release partially. In ovariectomized prepubertal monkey kisspeptin administration stimulated GnRH release demonstrating that it is independent of steroid hormones (Guerriero et al., 2011).

Administration of kisspeptin antagonist result in the impairment of puberty onset whereas mutations that activate GPR54 cause precious puberty (Pineda et al., 2010; Teles et al., 2008). GPR54 knockout mice were identified with the absence of puberty, smaller gonads and low levels of gonadotropins and steroids (Holmes et al., 2014). During puberty, kisspeptin fibers that are in vicinity to GnRH neurons has been shown to increase in mice (Clarkson et al., 2006). During pubertal development the levels of both *Kiss1* and GPR54 mRNA increase in rodents and in primates (Han et al., 2005; Keen et al., 2008). Kisspeptin fibers have been found to be in close vicinity to GnRH neurons in the preoptic area in mice and in rats (Clarkson et al., 2006; Kinoshita et al., 2005). The sensitivity of kisspeptin to the GnRH neurons changes during different stages of reproductive development. During juvenile stage 27% of GnRH neurons are depolarized to kisspeptin, 45 % of GnRH neurons are depolarized to kisspeptin during prepubertal stage and 90% in post pubertal stage (Han et al., 2005). The number of GnRH neurons that respond to kisspeptin are not sexually dimorphic (Liu et al., 2008). Steroid hormone is critical for the

pubertal development and the maintenance of reproductive physiology (Takase et al., 2009). Ablation of ERα in kisspeptin neuron result in the decreased inhibition of GnRH neuron and has been shown to cause precocious puberty (Mayer et al., 2010). GPR54 and Kiss1 knockout mice exhibit infertility and impaired reproductive function (Funes et al., 2003; d'Anglemont de Tassigny X et al., 2007; Lapatto et al., 2007).

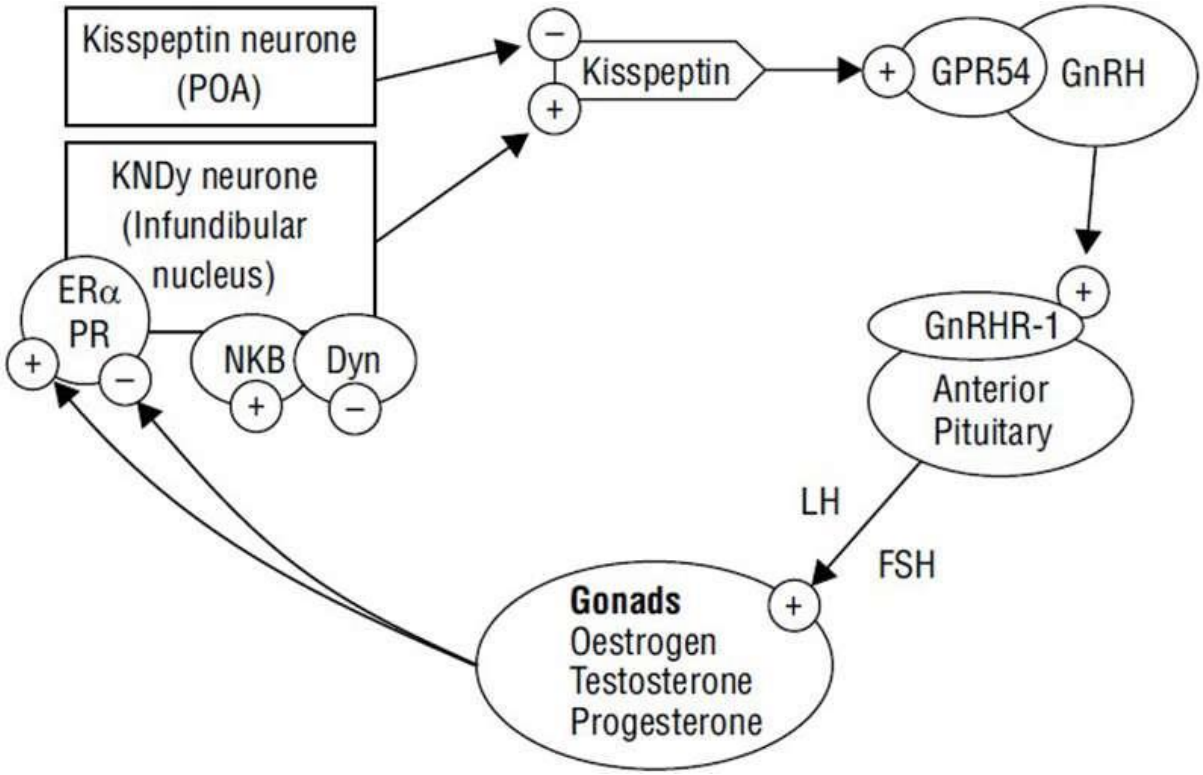


Figure 1.7 Schematic representation of kisspeptin neuron and GnRH neuron interaction. Abbreviations; POA, preoptic area of hypothalamus; KNDy neuron, kisspeptin dynorphin neuron; GPR54, G protein coupled receptor 54(GPR54); GnRH, gonadotrophin releasing hormone; GnRHR-1, gonadotrophin releasing hormone receptor 1; LH, luteinizing hormone; FSH, follicle stimulation hormone; ERα, estrogen receptor alpha; PR, progesterone receptor; NKB, neurokinin B; Dyn, dynorphin. Adapted from Javed et al., 2015.

1.3 Neuronal circuit underlying chemosensory signaling and reproduction

1.3.1 Interaction of GnRH neurons with chemosensory system

Pheromone exerts its effects on GnRH neurons and thereby modulate the neuroendocrine status. One study dissected the neurons communicating with GnRH neuron and revealed that olfactory cue could influence reproductive function. They used a transgenic mice in which barley lectin gene (a tracer that travels both across upstream and downstream synapses) placed next to the gene encoding GnRH neuron in which only the GnRH neuron produces barley lectin. The study concluded that the Bl+ve cells which communicate with GnRH neurons are found in approximately 53 different functional areas. The expression of BL was identified in regions including the vomeronasal amygdala which receives vomeronasal input, olfactory cortex indicating direct synaptic connection with GnRH neuron. Upon pheromone exposure the GnRH downstream and upstream neurons were activated which was detected by the expression of c-Fos. This indicates that the GnRH neurons could influence a variety of functions including the processing of chemosensory signals (Boehm et al., 2005).

Another important study traced GnRH neurons using modified pseudo rabies virus to infect only GnRH expressing neurons to anatomically trace the afferent pathways to GnRH neurons in the hypothalamus. The transneuronal retrograde travelling property of the virus allowed them to identify olfactory receptor neurons in the main olfactory epithelium that are polysynaptic connected to GnRH neurons in mice (Yoon et al., 2005). Several other studies have showed the significance of pheromone mediated behavior either by surgical removal of vomeronasal organ (VNOx) which resulted in the decrease of lordosis behavior to male mount, decrease in urine marking and intermale aggression, reduced copulatory behavior and decrease in pregnancy block (Keller et al., 2006a; Maruniak et al., 1986; Clancy et al., 1984; Lloyd-Thomas et al., 1982; Li et al., 2013). While chemical ablation of the main olfactory epithelium resulted in decreased lordosis behavior and sex discrimination (Keller et al., 2006b).

The olfactory cues can modulate endocrine status and cause certain effects such as (i) Vandenberg eefect (ii) Bruce effect (iii) Lee-Boot effect and (iv) Whitten effect.

(i) Vandenbergh effect

When a female mice is exposed to the chemosignals of the male mice, the pubertal development of the female mice is accelerated .This phenomenon is known as Vandenbergh effect (Castro, 1967; Vandenbergh, 1975). Female mice raised with a castrated male mice do not undergo

accelerated puberty (Bronson et al., 1975). Removal of VNO in the female mice eliminated the role of male urine in accelerating puberty (Kaneko et al., 1980).

(ii) Bruce effect

In Bruce effect, the female mice terminates its pregnancy upon exposure to the odors of unfamiliar male. The implantation failure is due to the decrease in prolactin (Bellringer, J et al., 1980). Pregnancy is unaffected when the female mice is exposed to the mated male. Thus females are able to respond to odor of known and unknown mice. The first vomeronasal stimuli identified that can induce Bruce effect are MHC peptides (Bruce, 1959; Singh et al., 1987).

(iii) Lee-Boot effect

Lee Boot effect is a phenomenon in which when the females are grouped together the estrous cycle of the females are prolonged (Van Der Lee et al., 1955). The stimuli for Lee Boot effect is an estrogen dependent pheromone which does not suppress estrous cycle in females (Lepri et al., 1985).

(iv) The Whitten Effect

The Whitten effect is a tendency in which estrous can be induced in grouped females when exposed to male mouse urine (Whitten, 1956). The stimuli inducing the Whitten effect is unidentified but studies have shown that it is species specific volatile and androgen dependent urinary metabolites (Bronson, 1974).

1.3.2 Brain pathways mediating the detection of chemosensory cues in mice.

In rodents chemosignals can be detected by two olfactory systems, namely the main olfactory system and the accessory olfactory system (Zufall,et al., 2001; Liberles et al., 2006). The main olfactory system is responsible for detecting volatile odor compounds. It is mediated by G protein coupled receptor (GPCR). The vomeronasal olfactory system is responsible for detecting pheromones. It is mediated by pheromone receptors V1 and V2 (Dulac and Wagner, 2001).
Sensory neurons from the main olfactory epithelium (MOE) and vomeronasal organ (VNO) project into the main olfactory bulb and accessory olfactory bulb respectively (Figure 1.8). The MOB receives sensory input from the main olfactory receptors and projects to the lateral olfactory tract (NLOT), the anterior cortical nucleus (ACN), and the posterolateral cortical amygdaloid nucleus (PLCN) (Westberry et al., 2003). The AOB receives sensory input from the VNO which then projects to the anterior amygdala (MeA), posterior amygdala (MeP), posteromedial cortical nucleus (PMCN) of the amygdala and the bed nucleus of the stria

terminalis (BNST) (Meisami et al., 1998; von Campenhausen et al., 2000). Neurons from these regions project to the medial preoptic area (MPOA), ventromedial hypothalamus (VMH), the premammillary nuclei and the supra optic nuclei in the hypothalamus that are associated with reproduction and maternal behavior (Petrovich et al., 2001).

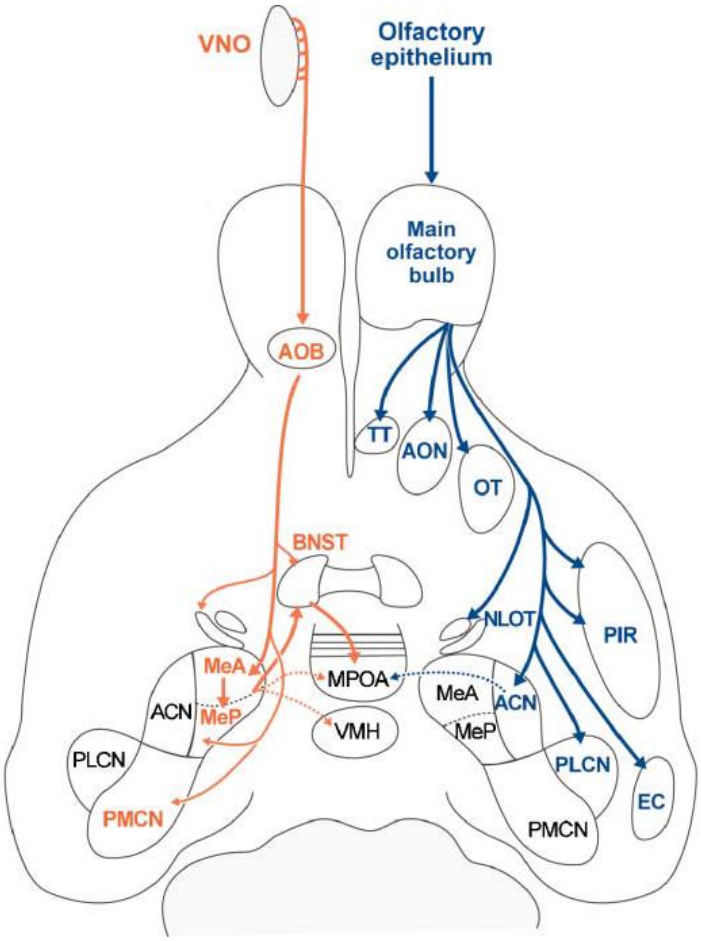


Figure 1.8 Neural pathways underlying the detection of chemosensory cues in mice.
Chemosensory input can be detected by vomeronasal organ (VNO) or main olfactory epithelium. The vomeronasal input is transmitted from the vomeronasal organ to the accessory olfactory bulb (AOB) and projects to the anterior and posterior medial amygdala (MeA, MeP) and posterior medial cortical nucleus (PMCN) and bed nucleus of the stria terminalis (BNST). The input from these areas further goes to the medial preoptic area (MPOA). The chemosensory input detected by the main olfactory epithelium is transmitted to the main olfactory bulb (MOB) and projects to the anterior cortical nucleus (ACN), posterolateral cortical nucleus (PLCN), anterior olfactory nucleus (AON) and olfactory tubercle (OT), tenia tecta (TT), piriform cortex (Pir) and entorhinal cortex (EC). Adapted from Dulac and Wagner, 2006.

1.4 Aim of the thesis

The main goal of this study was to investigate the development of the upstream neuroendocrine circuit of GnRH neurons, Kisspeptin-GPR54 system during embryonic brain development and the downstream target of GnRH, GnRHR expressing neurons. The following questions were addressed in three aims:

Aim I: To understand the development of kisspeptin and GnRH neural circuit in the embryonic male mouse brain I investigated the precise spatio-temporal expression pattern of Kisspeptin and GPR54 utilizing a binary genetic strategy in KissIC/eR26-τGFP and GPIC/eR26-τGFP male embryonic mice respectively. To examine when kisspeptin neurons are sensitive to steroid hormones I decided to study when Kisspeptin neurons starts to express steroid hormone receptors. And finally, combinatorial transsynaptic tracing was used to dissect the kisspeptin neuron and GnRH neuron circuit in KissIC/R26-BIZ male embryonic mice. These results are presented in Chapter 3.1.

Aim II: To identify the role of estrogen signaling during reproductive development in the AVPV and ARC kisspeptin neurons I studied the expression of ERα in AVPV and ARC kisspeptin neurons. In addition I also studied the expression of dopaminergic kisspeptin neurons during reproductive maturation in KissIC/eR26-τGFP female mice. These results are discussed in Chapter 3.2.

Aim III: The objective of this aim is to investigate the role of GnRH receptor (GnRHR) neurons in the brain. I first mapped the distribution the GnRHR expressing neurons in the GRIC/eR26-τGFP adult female mice brain. I assessed GnRHR expressing neurons for steroid hormone sensitivity and investigated their potential role in olfactory process. These results are described in Chapter 3.3.

2. Materials and Methods

2.1 Animals

All experimental procedures were conducted with the guidelines established by the animal welfare committee of the University of Saarland. Mice were kept under standard light/dark cycle with food and water ad libitum. Kisspeptin-IRES-Cre (KissIC) mice (Mayer et al., 2010), GPR54-IRES-Cre (GPIC) mice (Mayer and Boehm, 2011), GnRHR-IRES-cre (GRIC) mice (Wen et al., 2008), ROSA26-CAGS-τGFP (eR26-τGFP) mice (Wen et al., 2011), ROSA26-BL-IRES-τlacZ (R26-BIZ) (Kumar et al., 2014) mice were kept in a mixed (129/SvJ and C57BL/6J) background.

2.2 Extraction of genomic DNA from tail biopsies

Genomic DNA isolation was carried out by incubating tail biopsies (approx. 1 cm) in 150 µl of tail lysis buffer containing proteinase K (1mg/ml) at 55^0C overnight in a shaking incubator. To the viscous sample equal volumes of isopropanol was added and then centrifuged at 1300 rpm for 10 minutes at room temperature. The supernatant was removed to a new tube containing isopropanol and then centrifuged at maximum speed. The pellet was washed twice with 70% ethanol, decanted and dried at room temperature. The genomic DNA was resuspended in distilled H_2O. The genotyping for all embryos were performed using genomic DNA isolated from tail biopsies.

Lysis Buffer

50 mM Tris HCl
100 mM NaCl
0.2% Tween
0.2% NP40
1 mM EDTA 1
1 mg/ml Proteinase K

2.3 Extraction of genomic DNA from ear biopsies

The genotyping for adult animals were done using genomic DNA isolated from ear biopsies. Ear biopsies were lysed by incubating overnight with lysis buffer containing proteinase K at 55ºC. The extracted genomic DNA were stored at 4ºC until use for PCR.

2.4 Gender Determination by PCR

To identify the gender of the embryo, genomic DNA were isolated from the tail biopsies and PCR amplification was performed. The primers were purchased from Eurofins MWG operon

The reaction mixture for the PCR to identify the gender is described below

gDNA template	1.0 µl
PCR buffer (10X)	5.0 µl
dNTPs (25 mM)	1.0 µl
$MgCl_2$ (25 mM)	5.0 µl
Betaine (5 M)	10 µl
DMSO	2.5 µl
Primers (25 µM)	0.5 µl
Taq Polymerase 1U	
ddH_2O make final volume upto 50 µl	

2.5 Genotyping by PCR

Program for genotyping

Temperature	Time
94^0C	5 mins
94^0C	30 secs
55^{o}C	1 min
72^{o}C	2 mins
72^{o}C	10 mins
4^{o}C	hold

2.6 Primers

Cre

Fwd: GTCGATGCAACGAGTGATGAGGTTCG

Rev: CCAGGCTAAGTGCCTTCTCTACACCTGC

KissIC

Fwd: CAAAGCTATCAGAGGGAGAAGCAAACAGCC

Rev1: CGGAATTCATCGATGATATCAGATCCGG

Rev2: CGACTTTGGCACCGAGGACATCTTG

GPIC (GPR54)

Fwd: GGCTATTATTGTGCTTGTGTGGAGGTACACAG

Rev1: CATAAACACAAACTCCTGGTTCGGTTCTCAG

Rev2: CGGAATTCATCGATGATATCAGATCCGG

Primer used in gender PCR

XY Fwd: TGAAGCTTTTGGCTTTGA

XY Rev: CCGCTGCCAAATTCTTTG

2.7 Adult mouse tissue preparation

All animals were deeply anaesthetized using intraperitoneal injection of ketamine/xylazine. Transcardial perfusions were performed using 100 ml PBS to remove the circulatory blood completely. Animals were then perfused with 100 ml 4% PFA. Brains were then dissected out and post fixed in 4% PFA for 2 hours at 4°C. Then the brains were transferred to 30% sucrose solution until the brain completely sinks. The brains are then stored in cryoprotectant. The brains were sectioned into 5 series of 14 µm on a cryostat. The sections were collected on super frost glass slide (Roth, Karlsruhe, Germany) with adjacent sections placed across different slides to produce a series of slides.

2.8 Preparation of embryonic mouse brain

In order to obtain the embryonic brains of KissIC/eR26- τGFP, GPIC/ eR26- τGFP, pregnant mice were anaesthetized with isofluorane and sacrificed by decapitation. The embryos were isolated with the amniotic sac in ice cold PBS. The embryos were carefully dissected out from the amniotic sac. To identify the gender tails of the embryo were cut and used for gender PCR. Isolated embryos were rinsed thoroughly in ice cold PBS and were then fixed with 4% PFA for 1.5-4 hrs. After fixation the embryos were washed in ice cold 1XPBS. The embryos were then soaked in 30% sucrose until the embryo completely sinks in sucrose and frozen using Leica cryoprotectant. Series of 14 µm sagittal sections were taken on superfrost plus glass slide for immunohistochemistry analysis.

2.9 Immunofluorescence analysis

The sections were washed with 1X PBS and incubated with ice cold MeOH for 20 minutes at room temperature (RT). After washing with PBS the sections were incubated with blocking solution for 2 hours at RT. Then the sections were incubated with primary antisera diluted in blocking solution overnight at 4 C. The next day sections were washed thoroughly with PBS and incubated with secondary antibody diluted in blocking solution for 2 hrs at RT followed by washing with PBS. Then nuclear staining was done using Hoechst for 10 minutes and the sections were mounted with Fluromount G. For dual immunostaining after incubating with the first primary antibody the sections were washed with 1XPBS and then incubated overnight with the second primary antibody at 4 ^{o}C. The sections were washed with PBS and the secondary antibody for both the antibody diluted with blocking solution were incubated together.

Blocking solution	5% donkey serum
	0.02% sodium azide
	0.2% Triton X-100 in PBs, pH 7.4
20XPBS	3 M NaCl
	161 mM Na2HPO4
	39 mM KH2PO4
	pH 7.4

2.10 LacZ staining

The embryonic sections were fixed in 0.2% glutaraldehyde, 2mM $MgCl_2$, 5mM EGTA, and 1XPBS (pH 7.3) for 2 minutes at 20°C–25°C. Then the sections were washed for 30 minutes using LacZ wash buffer A and then washed for 10 minutes using LacZ wash buffer B. LacZ staining solution was added to completely cover the sections. Sections were incubated in a humidified chamber at 30°C overnight. The sections were washed with LacZ wash buffer A and then mounted with Mayer's glycine gelatin. The brain sections were analyzed using Zeiss Axiophot light microscope.

LacZ wash buffer A

2Mm $MgCl_2$

5mM EGTA

0.1M Phosphate buffer pH 7.4

LacZ wash buffer B

0.1% Sodium deoxycholate

0.02% Nonidet P40, 2mM $MgCl_2$

5 mM EGTA, 0.1 M Phosphate buffer pH 7.4

LacZ staining buffer

0.02% Nonidet P40, 2Mm $MgCl_2$

0.01% Sodium deoxycholate

5mM EGTA, 20 mM K_3FeCN_6, 20mM K_4FeCN_6

2mg/ml X-gal, 0.1 M Phosphate buffer pH 7.4

LacZ staining buffer

0.01 % sodium deoxycholate

0.02% Nonidet P40, 2mM $MgCl_2$

5mM EGTA, 0.5 mg/ml of NBT

0.5 mg/ml of X-Gal

0.1M Phosphate buffer pH 7.4

2.11 Immunostaining for Barley Lectin (BL) using TSA amplification

To detect BL from KissIC/R26BIZ mice, sections were washed with 1X PBS for 10 minutes. Then the sections were incubated with ice cold MeOH:H_2O_2 for 30 minutes at room temperature. Following a 10 minutes incubation in 0.5% Triton X 100 the sections were washed with TNT (TRIS NaCl Tween) buffer and then blocked with TNB blocking solution for 30 minutes at room temperature. The sections were then incubated with Goat antiBL antibody (WGA) 1:1000 in TNB overnight at 4^0C. The sections were washed with TNT solution, incubated with 1:1000 HRP in TNB for 30 minutes, followed with a wash the sections were incubated briefly for 5-10 minutes with tyramide signal amplification (TSA) solution. After 3X wash with TNT the sections were incubated with 1:500 streptavidin Alexa 546 in TNB buffer for 1 hour. Then nuclear staining was done using Hoechst for 10 minutes and the sections were mounted with Fluromount G.

For DAB staining, following incubation with TSA amplification solution wash thoroughly with TNT solution and incubate with ABC for 30 minutes at RT. Then wash the sections and add DAB and monitor the colour development, wash with PBS then with distilled water before air drying. Dehydrate with increasing grades of alcohol 50%, 70%, 80%, 90%, 95%, 2X 100%, 2 X Xylene. Mount with DPX.

TNT wash buffer 1.5 M NaCl

	0.1 M Tris HCl pH7.4
	0.02% Tween 20
TNB blocking solution	1.5M NaCl
	2.5g blocking powder (Perkin Elmer)
	0.1 M Tris HCl pH7.4

ABC Vector laboratories 2 drops of A + 2 drops of B in 5 ml of PBS prepare 30 minutes before use.

2.12 Exposure to Odorants

The experiments were conducted with 13-18 week old sexually naive GRIC/eR26-τGFP male mice. The mice were housed individually before the experiments. Prior to the experiment the animals were habituated to testing environment (clean bedding) for atleast 10 days. The chemosensory stimuli (bedding) were collected from a group of atleast 5 housed females. The female bedding were collected mixed and used fresh as a stimuli. After habituation the test animals were exposed to soiled bedding simultaneously the control animals were exposed to clean bedding for 90 minutes. The animals were sacrificed were perfused intracardially with ice cols 4% PFA.

2.13 Antibody

Name of the antibody	Host	Manufacturer	Dilution
GnRH	Rabbit (Polyclonal)	Affinity Bio reagents PA1-121	1:1000
ER α	Rabbit (Polyclonal)	Millipore 06-935	1:1000
Kisspeptin	Rabbit (Polyclonal)	Millipore AB9754	1:500
AR	Rabbit (Polyclonal)	Santa Cruz SC816	1:200
GFP	Chicken (Polyclonal)	Molecular Probes A10262	1:1000
WGA	Goat (Polyclonal)	Vector laboratories AS 2124	1:1000
TH	Rabbit (Polyclonal)	Millipore AB 152	1:1000
c-Fos	Goat(Polyclonal)	Santa Cruz SC52	1:300
Rabbit IgG	Cy3 conjugated IgG	Molecular Probes A10520	1:500
Chicken IgG	Alexa Fluor 488	Molecular Probes A11039	1:500
Goat IgG	Biotinylated	Vector Laboratories BA 9500	1:500

2.14 Image acquisition and Processing

The images were captured using Axiovision software with Zeiss Axioskop microscope. The captured images were processed using adobe Photoshop CC 2015.

2.15 Quantification of neuron

Kisspeptin, τGFP, GnRH, AR and ERα immunoreactive neurons were manually counted in every fifth section both in 14 μm thick sagittal sections for embryonic brain study and 14 μm thick coronal sections for adult brain study. The neurons were counted using Image J cell counter plugin. The nuclei was visualized by staining with Hoecht 33258. The total number of immunopositive neurons in the embryonic brain and the adult brain were multiplied by 2.5 to estimate the total number of neurons per brain.

2.16 Statistics

All experiments were performed in triplicate. All data quantification were expressed as mean ± standard error of the mean (SEM). Statistical significance was assessed using two-tailed unpaired Student's tests.

3. Results

3.1 Development of Kisspeptin-GPR54 system in male mice

3.1.1 Temporal and spatial expression of kisspeptin neurons in male mice

To detect kisspeptin neurons I bred Kiss-IRES-Cre with eR26-τGFP mice. In Kiss-IRES-Cre mice, Cre recombinase is coexpressed along with Kiss1 gene. Transcription of *Kiss1* allele yields a biscistronic mRNA from which Kiss1 and Cre are independently translated. The reporter strain eRosa 26-τGFP carries a fusion protein in the rosa locus. Due to the presence of loxp flanked stop signal the transcription normally terminates prematurely, but when crossed with Kiss-IRES-Cre mice, the Cre mediated excision of the transcription stop signal leads to τGFP expression. Therefore kisspeptin neurons in double heterozygous KissIC/eR26-τGFP mouse brain express τGFP and can be visualized by fluorescence signals (Figure 3.1 A).

To anatomically map kisspeptin neuron in the male embryonic brain I examined KissIC/eR26-GFP embryonic mouse brain at different developmental stages. GFP immunostaining on sagittal sections of mouse brain across different ages shows the distribution of kisspeptin neurons. I did not detect τGFP cells at E12.5. I detected the first expression of τGFP cells (94.3 ± 8.3, n=3) at E13.5 in ARC (Figure 3.1 B). At E16.5, I observed an increase the number of τGFP expressing kisspeptin neurons (962.7 ± 68, n=3) (Figure 3.1 E). The kisspeptin neurons remained restricted to the ARC (Figure 3.1 C). I performed immunohistochemistry with polyclonal antibody directed against kisspeptin and detected the first expression of kisspeptin neuron at E16.5. On performing double immunohistochemistry I found that that 92.1 ± 1% of τGFP cells express kisspeptin showing faithful expression of τGFP in kisspeptin neurons demonstrating the sensitivity of binary genetic system over immunohistochemistry (Figure 3.1 C-F).

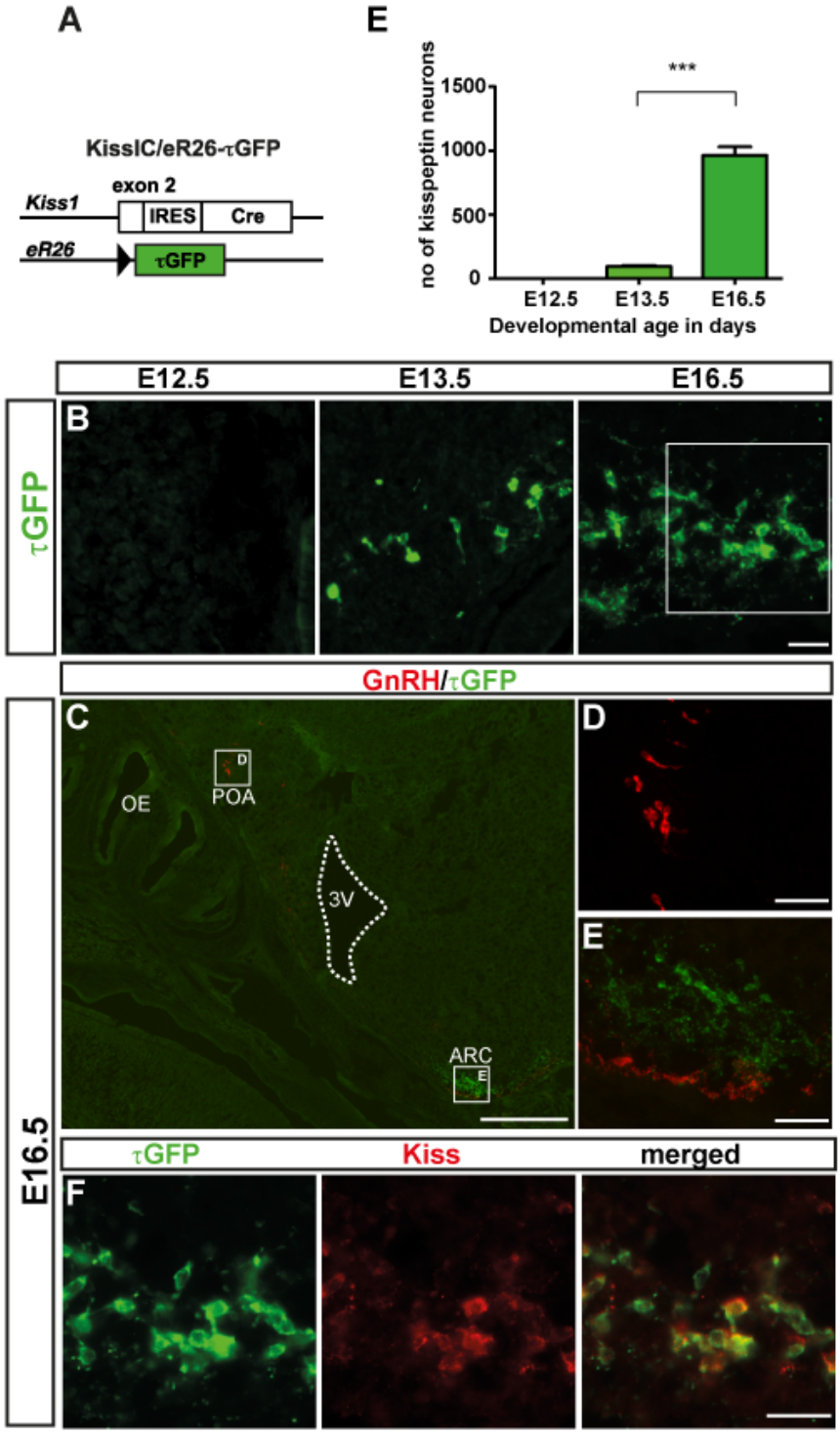


Figure 3.1 Temporal and spatial expression of Kisspeptin neurons in male mouse embryos. **A,** Genetic strategy to analyze the temporal and spatial expression pattern of kisspeptin neuron. In double heterozygous KissIC/eR26-τGFP male embryos, Kisspeptin neurons express τGFP. **B,** Representative Immunolabelling images for τGFP on a sagittal section in KissIC/eR26-τGFP male mouse embryo during early stages of embryonic development. Note, τGFP was not detected E12.5 (left). The first expression of τGFP was detected at E13.5 (middle) in the arcuate nucleus (ARC) of the hypothalamus. At E16.5 (right) the expression of τGFP increased

significantly, the expression of τGFP remained restricted to the ARC throughout the development. **C,** Expression of τGFP in kisspeptin neurons is confirmed by the double immunolabelling for τGFP (green) and kisspeptin (red) against antisera in KissIC/eR26-τGFP male embryo at E16.5 male embryo. **D-F,** Representative immunolabelling images for τGFP (green) and GnRH (red) on a sagittal section in in KissIC/eR26-τGFP male mouse embryo at E16.5. 3V, third ventricle; POA, preoptic area; OE, olfactory epithelium. **E,** Quantification of kisspeptin neurons at E12.5, E13.5 and E16.5 in KissIC/eR26-τGFP male mouse embryo. ***, P <0.001. Scale bars: 25 μm (**B and C**), 500 μm (**D**) and 50 μm (**E and F**).

3.1.2 Temporal and spatial expression of GPR54 receptor expressing neurons in male mice

Kisspeptin controls the secretion of GnRH via acting on GPR54 expressed by GnRH neurons. To map the temporal and spatial expression of GPR54 receptor expressing neurons in male embryonic mice I used GPIC/eR26-τGFP double heterozygous mice.

To detect GPR54 expressing neurons I bred GPR54-IRES-Cre (GPIC) with eR26-τGFP mice. In GPR54-IRES-Cre mice, Cre recombinase is coexpressed along with GPR54 gene. Transcription of Gpr54 allele results in a biscistronic mRNA from which GPR54 and Cre are independently translated. The reporter strain eRosa26-τGFP carries a fusion protein in the Rosa locus. Due to the presence of loxp flanked stop signal the transcription normally terminates prematurely. But when crossed with GPIC-IRES-Cre mice the Cre mediated excision of the transcription stop signal leads to τGFP expression. Therefore GPR54 expressing neurons in double heterozygous GPIC/eR26-τGFP mouse brain express τGFP and can be visualized by fluorescence signals (Figure 3.2 A). I performed dual immunolabelling using antisera against GFP and GnRH. During embryonic development GnRH neurons are born outside the brain in the olfactory placode cross the cribriform plate and migrate towards basal forebrain.

I did not detect the τGFP expressing cells at E12.5 (Figure 3.2 B). I detected first τGFP expressing cells at E13.5. At E13.5 approximately 3% of GnRH neurons expressed GPR54 (5.3 ± 1.3 τGFP+ of 183 ± 6.8 GnRH+, n=3). At E13.5 GnRH neurons are primarily located in the nasal septum and rostral forebrain, and all the τGFP+ cells were GnRH neurons (Figure 3.2 C). At E16.5 GnRH are distributed in nasal septum, preoptic area and OVLT. At E16.5, 43% of GnRH neurons express GPR54 (100.3 ± 2.8 τGFP+/ GnRH+ of 262.7 ± 8 GnRH+, n=3 mice).

At E16.5 GnRH neurons are primarily located in the anterior forebrain and few neurons are distributed along their migratory path (Figure 3.2 D). At PND2, 72% GnRH neuron expressing GPR54 (102.3 ± 12 τGFP+/GnRH+ of 141 ± 11 GnRH+, n=3 mice) (Figure 3.2 E). The number of GnRH neurons expressing GPR54 had greatly increased across ages. During embryonic

development all the τGFP expressing neurons are GnRH neurons independent of their location within the brain (Figure 3.2 F).

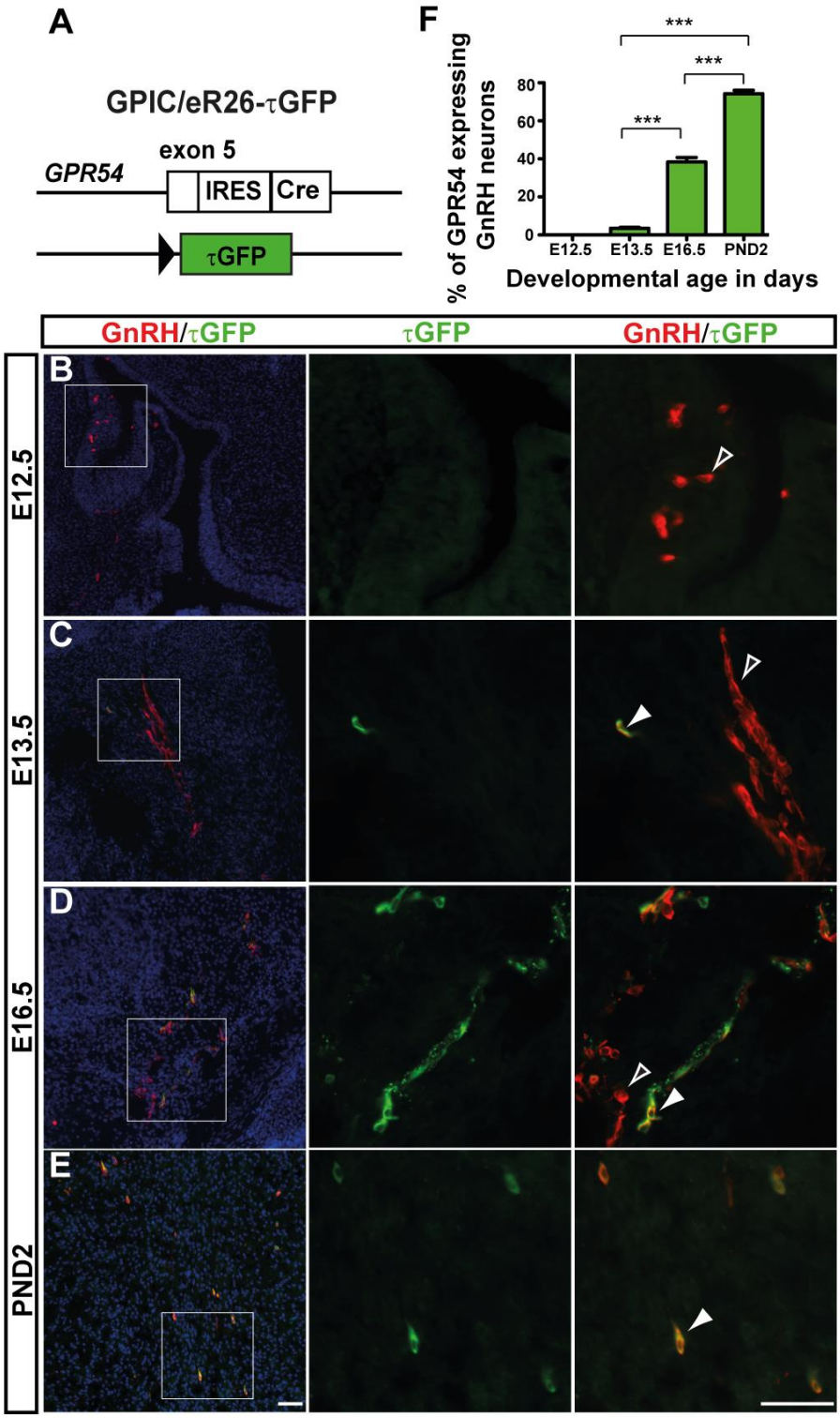


Figure 3.2 Temporal and spatial expression of GPR54 expressing neurons in male mouse embryos. **A,** Genetic strategy to analyze the temporal and spatial expression pattern of GPR54 neuron in heterozygous GPIC/ eR26-τGFP male embryos. **B-E,** Double immunolabelling with τGFP (green) and GnRH (red) on a sagittal section of the head at E 12.5, E13.5, E16.5 and coronal section of the head at PND2 in a heterozygous GPIC/ eR26-τGFP PND2 male mouse. Note, GPR54 expression starts at E13.5

in the olfactory area. Open arrows points GnRH neurons that do not express GPR54, whereas close arrow shows GnRH neuron that express GPR54. NS, nasal septum; OE, olfactory epithelium; FB, forebrain; POA, preoptic area. Scale bars, 50 μm. F, Quantification of GPR54 expressing GnRH neurons during the development. ***, P<0.001.

3.1.3 ERα and AR immunoreactivity within the male embryonic brain marks the birthplace of kisspeptin neurons

It is known that steroid hormones regulate HPG axis through both positive and negative feedback loops between the gonads and brain. Previous studies have shown that estrogen regulate Kiss1 expression in AVPV and ARC within the brain. Sex steroids acts on kisspeptin neuron which in turn modulate GnRH neurons. To address the question when during embryonic development ARC kisspeptin neurons becomes sensitive to steroid hormone I used double heterozygous KissIC/eR26- τGFP mouse brain in which kisspeptin neurons express τGFP. I performed immunohistochemistry using antisera against estrogen receptor alpha (ERα**)** and androgen receptor (AR).

While I did not detect AR signal at E12.5 but I first observed AR signal first at E13.5 (Figure 3.3 A-B). Results from my previous experiments showed that the onset of kisspeptin neuron expression is also at E13.5. At E13.5, 63.4 ± 6.9% of kisspeptin neurons expressed AR (45.8 ± 15.3 AR+/τGFP+ of 69.3 ± 20.2 τGFP+, n=3 mice). The number of kisspeptin neurons expressing AR increased to 96.4 ± 1 % (376 ± 21.2 AR +/τGFP+ of 390.7 ± 26.3 τGFP+, n=3 mice) at E16.5 (Figure 3.3 C-D).

To address the question when ARC kisspeptin neuron starts to express ERα. I used double heterozygous KissIC/eR26-τGFP mouse brain and performed immunolabelling against ERα and GFP. I detected ERα expression in the ARC at E13.5 (Figure 3.4 A). At E13.5, 61.6 ± 6.7% of kisspeptin neurons expressed ERα (45.6 ±16.7 ERα +/τGFP+ of 70 ± 18.9 τGFP+, n=3 mice). The number of kisspeptin neurons expressing ERα increased to 93.1 ± 1.7% (317 ± 7.6 ERα+/τGFP+ of 340.7 ± 10.6 τGFP+, n=3) at E16.5 (Figure 3.4 C-D).The expression of AR and ERα marks the birthplace of kisspeptin neuron in the ARC (Figure 3.3 B and Figure 3.4 B).

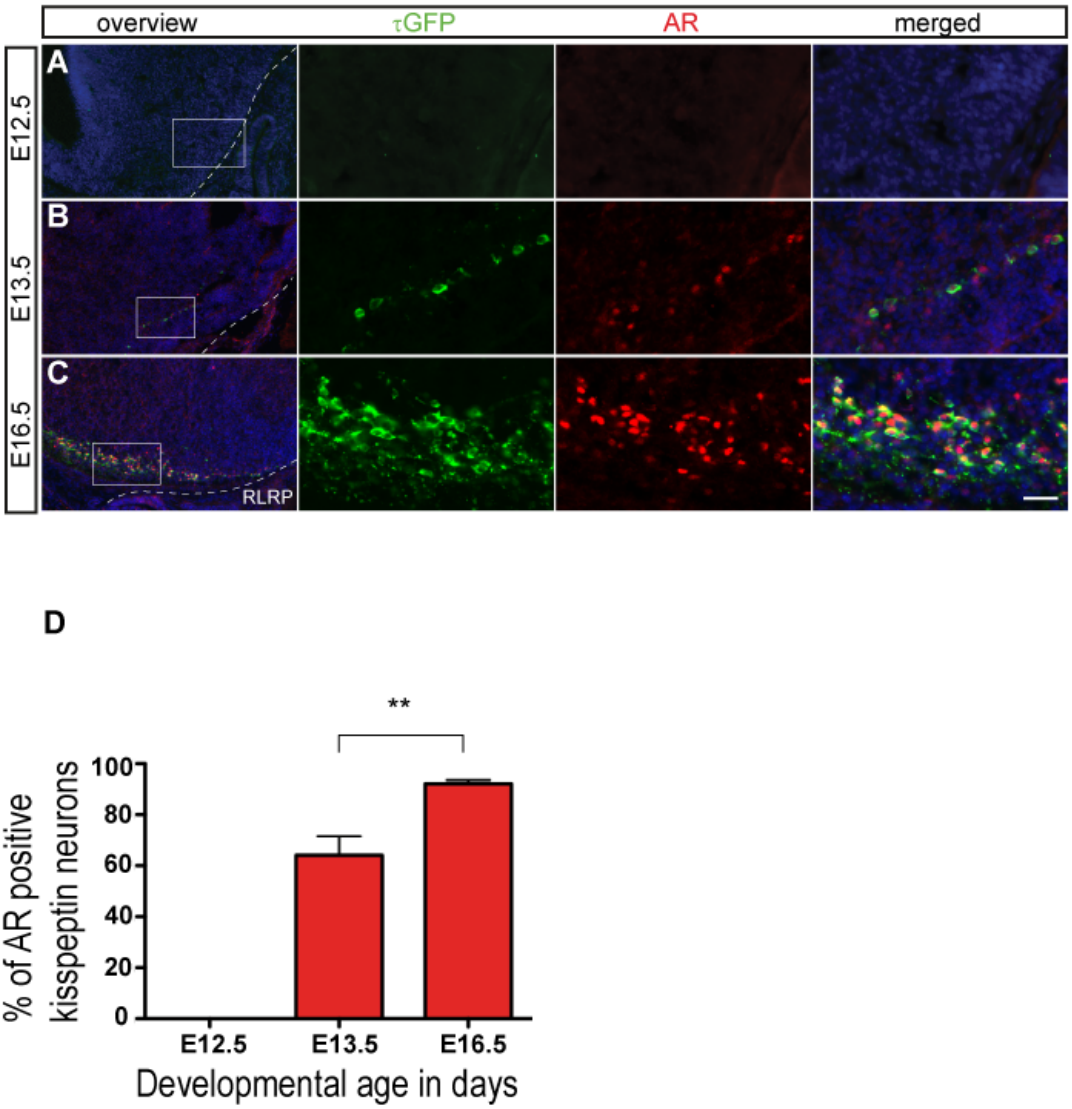


Figure 3.3 ARC kisspeptin neurons express AR in the embryonic male brain. A-C, Double immunolabelling with antibody against AR (red) and τGFP (green) on whole head sagittal section in KissIC/eR26-τGFP male embryos at E12.5, E13.5 and E16.5. Note, the AR expression was not detected at E12.5. The first expression of AR was observed in ARC at E13.5. The kisspeptin neuron born at ARC express AR demonstrating steroid sensitivity. The number of AR expressing kisspeptin neurons increase in population at E16.5. **D,** Graph showing the ratio of kisspeptin neurons co-expressing AR in the developing embryonic brain. **, $P<0.01$; Scale bar, 50 μm (overview) and 25 μm (insert)

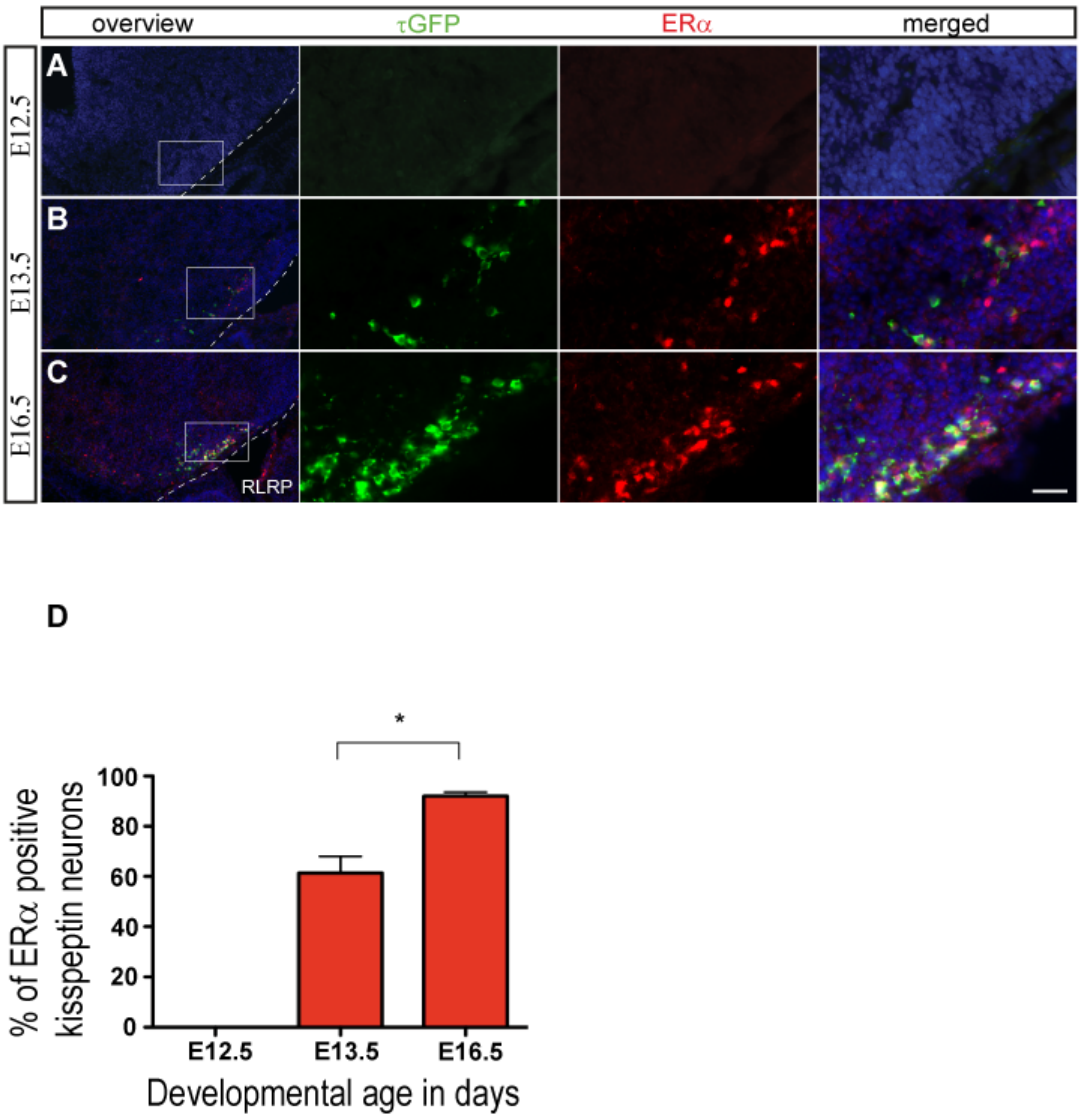


Figure 3.4 ARC kisspeptin neurons express ERα in the embryonic male brain. A-C, Double immunolabelling with antibody against ERα (red) and τGFP (green) on whole head sagittal section in KissIC/eR26-τGFP embryos at E12.5, E13.5 and E16.5. Note, ERα expression was not detected at E12.5. The first expression of ERα was observed in ARC at E13.5. The kisspeptin neurons born at ARC express ERα demonstrating steroid sensitivity. **D,** Graph showing the ratio of kisspeptin neurons co-expressing ERα in the developing embryonic brain. **, $P<0.05$; Scale bar, 50 μm (overview) and 25 μm (insert).

3.1.4 Establishment of kisspeptin and GnRH neuronal connectivity in utero.

The establishment of kisspeptin-GnRH neuron circuit in utero was analyzed using KissIC/R26-BIZ male embryonic mice in which barley lectin (BL) is produced by kisspeptin neurons (Fig 3.5 A). BL is a bidirectional transneuronal tracer marking both the upstream and downstream neuron while LacZ acts as a stationary marker labeling the primary neurons. Immunostaining for βGalactosidase (βgal) activity which is a gene product of LacZ to identify BL producing neurons revealed that BL producing neurons were found primarily in the ARC (Fig 3.5 B-D). In addition to the ARC, BL was detected in the anterior brain regions such as POA and OVLT where GnRH are present. The presence of transynaptically transferred BL in the anterior brain shows that BL producing (kisspeptin) neurons are synaptically connected to the neurons in the anterior brain. Tracing of kisspeptin neurons using transneural tracer BL revealed that kisspeptin neurons in the ARC communicate with the GnRH neurons (Fig 3.5 E-G). At E16.5 approximately 40% (130.7 ± 14.3 BL+/GnRH+ of 333 ± 33 GnRH+, n=3) of the GnRH neurons are connected to kisspeptin neurons. The number of connected neurons increased to 60.4% (147.7 ± 32 BL+/ GnRH+ of 243 ± 50 GnRH+, n=3) at E18.5. At PND2 nearly 57% (127.3 ± 19 BL+/ GnRH+ of 222 ± 17 GnRH+, n =3) of the GnRH neurons are connected to ARC kisspeptin neurons (FIG 3.5 H). These experiments demonstrate that embryonic GnRH neurons are synaptically connected to ARC kisspeptin neurons.

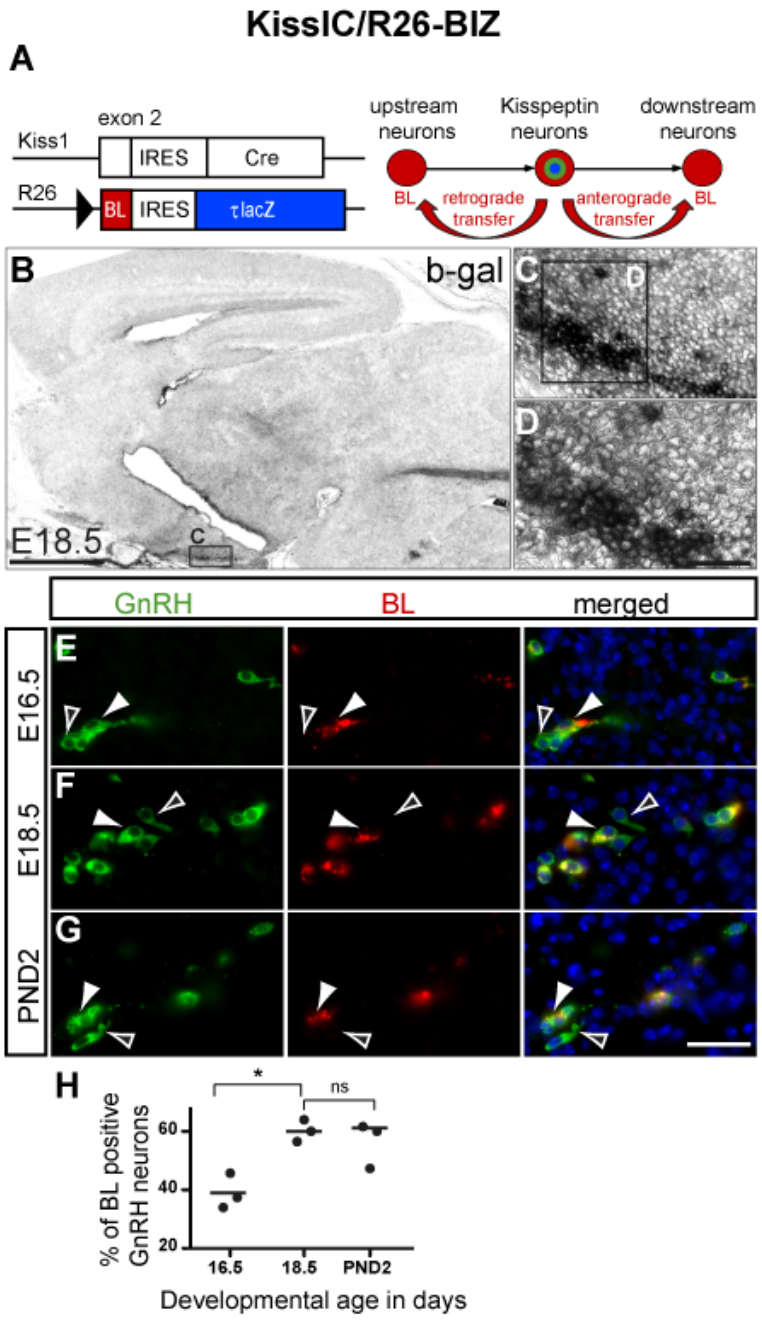


Figure 3.5 ARC kisspeptin and GnRH connectivity is established in utero. A, Genetic strategy for KissIC/R26-BIZ mice in which τlacZ acts as a stationary marker and BL is transferred to synaptically connected neurons. **B-D,** At E18.5, b-gal producing primary neurons are restricted to the ARC. **E-G,** Immunostaining against GnRH (green) and BL (red) at E16.5, E18.5 and PND2. Closed arrowhead indicates that GnRH neuron contain BL whereas open arrowhead points to GnRH neuron that do not contain BL (right). **H,** Quantification of the percentage of BL positive GnRH neurons across different developmental stages. Scale bars: 200 μm **(B)**, and 50 mm **(E-G)**. **Declaration: Contributed by Kumar et al., 2015b.**

3.2 Differential expression of ERα and dopaminergic AVPV kisspeptin neurons during puberty

3.2.1 Expression of ERα in AVPV and ARC kisspeptin neurons during reproductive maturation

Kisspeptin neuron signaling is a crucial component of the reproductive circuit maturation. Previous studies have shown that AVPV and ARC kisspeptin neurons are sensitive to estrogen and control GnRH neuron. The question that I asked was whether the sensitivity (responsiveness) of AVPV and ARC Kisspeptin towards estrogen change before puberty (P21) and after puberty (P84).

To address this question I used KissIC/eR26-τGFP brain (P21) and stained them with ERα antisera. The number of ARC kisspeptin neurons expressing ERα at P21 was approximately 90 % whereas at P21, the number of AVPV kisspeptin neurons expressing ERα was approximately 30%. Surprisingly at P84 the number of AVPV kisspeptin neurons expressing ERα increased to approximately 73% whereas the sensitivity of ARC kisspeptin neurons remained the same. These experiment showed that the sensitivity of AVPV kisspeptin neuron towards estrogen significantly increase during reproductive maturation.

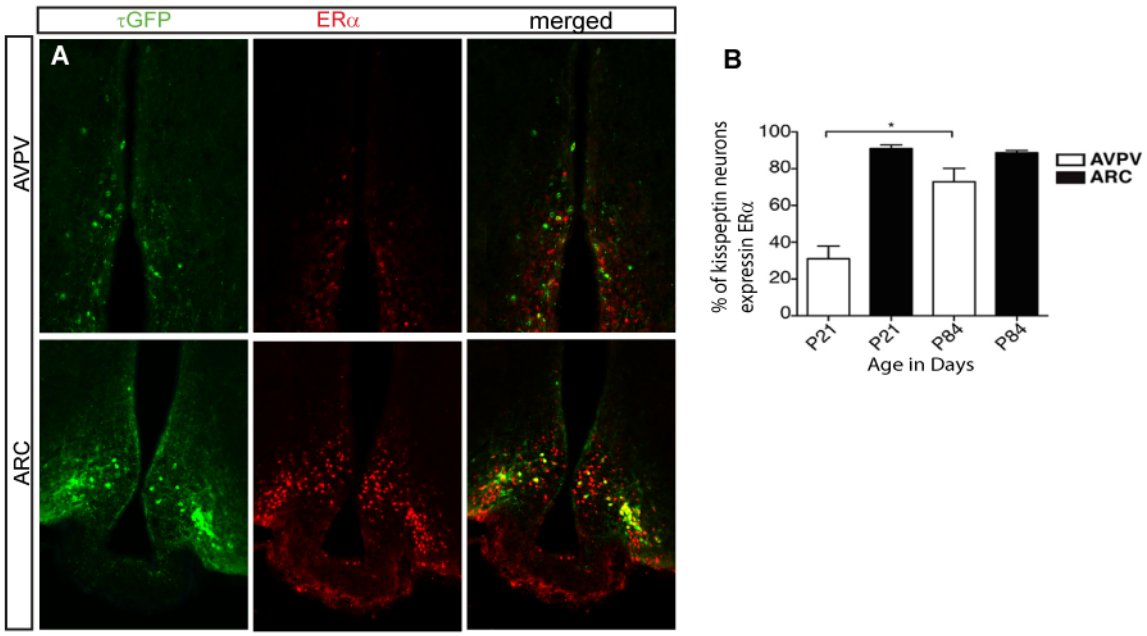


Figure 3.6 AVPV and ARC kisspeptin neurons express ERα. A, Immunohistochemistry analysis of AVPV (top) and ARC (bottom) in kissIC/R26-GFP using antisera against GFP (green) left , ERα (red) middle, kisspeptin neuons expressing ERα (yellow) left. **B,** Quantification of the percentage of kisspeptin

neurons expressing ERα in P21 and P84 female kissIC/R26-GFP mice. *, P < 0.05. **Declaration: P84 quantification and Figure B contributed by Kumar et al., 2015a.**

3.2.1 Expression of dopaminergic AVPV and ARC kisspeptin neurons during reproductive maturation

Kisspeptin neuron has been shown to produce dopamine and dopamine has an inhibitory effect on GnRH neuron. To understand if dopaminergic kisspeptin neurons increase during maturation. I performed dual immunohistochemistry using antisera against GFP and tyrosine hydroxylase (TH) which is a catecholaminergic neuronal marker in double heterozygous KissIC/eR26-τGFP female mice. At P21 (before puberty), I found that the number of AVPV kisspeptin neurons positive for TH was 10.4% ± 1.3% (n = 3). At P84 (after puberty), the number of AVPV kisspeptin neurons positive for TH had greatly increased to 47.3% ± 2% (n = 3). This suggest that dopamine being an inhibitor on GnRH neuron might play a crucial rule during the onset of puberty and modulate GnRH neuron firing during estrous cycle.

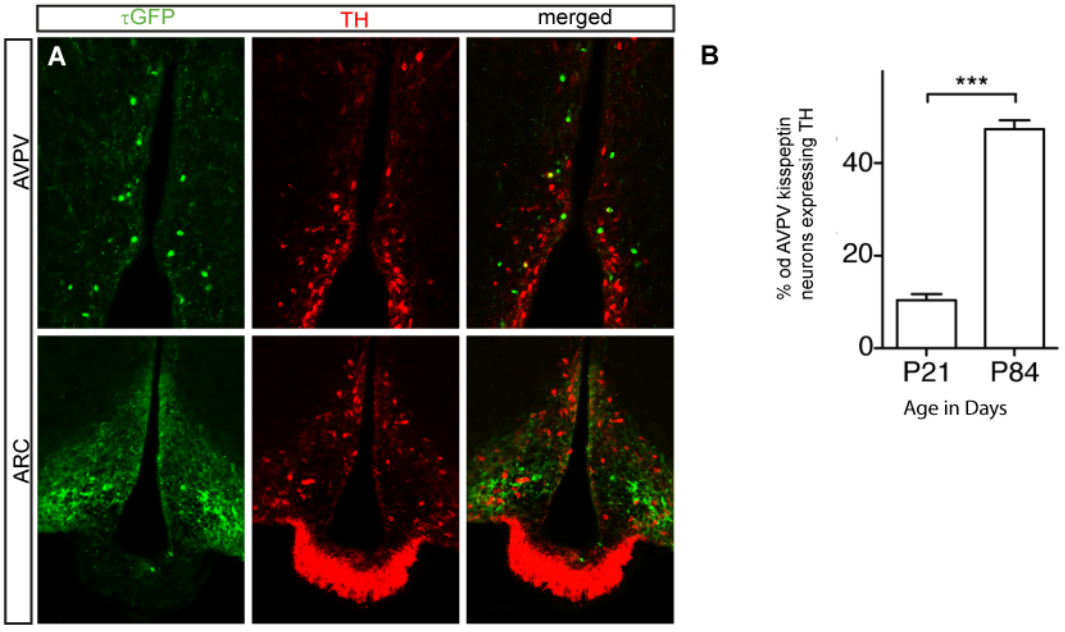


Figure 3.7 AVPV and ARC kisspeptin neurons express TH. A, Immunohistochemistry analysis of AVPV (top) and ARC (bottom) in kissIC/R26-GFP using antisera against GFP (green) left , TH (red) middle, kisspeptin neurons expressing TH (yellow) left. **B,** Quantification of the percentage of kisspeptin neurons expressing TH in P21 and P84 female kissIC/R26-GFP mice. ***, P < 0.001. **Declaration: P84 quantification and Figure B contributed by Kumar et al., 2015a.**

3.3 Distribution and characterization of GnRH receptor (GnRHR) neurons in the female mouse brain

3.3.1 Distribution of GnRH receptor neurons in the female mouse brain

I examined GnRH receptor (GnRHR) expressing neurons which is the target of the GnRH neurons using binary genetic strategy. I mapped the neuroanatomical distribution of GnRHR neurons across different developmental ages using immunofluorescence. Previous studies have shown that GnRH neurons communicate with neurons in approximately 50 functionally different brain areas indicating that GnRH neurons integrate a variety of information and influence numerous brain functions.

To visualize GnRHR expressing neurons in female mice I bred GnRHR-IRES-cre (GRIC) mice with eROSA26-CAGS-τGFP female mice.

In GRIC/eRosa26-τGFP mice, τGFP serves as a readout for GnRH receptor (GnRHR) neurons.

Binary genetic strategy to genetically label GnRH receptor (GnRHR) neurons

GnRHR-IRES-Cre (GRIC) x eROSA-τGFP

The anatomical locations of GnRH receptor neurons were identified in the coronal sections of 13 weeks and 10 weeks old adult GRIC/eR26-τGFP female mice. The brains were cryosectioned into five serial sets of 14 μm coronal sections on superfrost slides. First set of sections from each animal was used for immunohistochemistry studies to examine GnRH receptor neuron expression. GnRH receptor neurons were identified between bregma 1.34 mm and bregma -6.12 mm in the mouse brain atlas (Paxinos and Franklin, 2001).

Mouse brain regions expressing GFP

TELENCEPHALON

ACo	Anterior cortical amygdaloidal nucleus (ACo)
BMA	Basomedial amygdaloid nucleus
MeA	Medial amygdala
MePD	Posterodorsal medial amygdala
Pir	Piriform cortex
PLCO	Posterolateral cortical amygdale nucleus
PMCO	Posteromedial cortical amygdaloid nucleus
BMA	Basomedial amygdale nucleus

DIENCEPHALON

MPA	Medial preoptic area
BSTMPM	Bed nucleus of the stria terminalis, posteromedial
BSTMPL	Bed nucleus of the stria terminalis, posterolateral
AV	Anteroventral thalamic nucleus
LD	Laterodorsal thalamic nucleus
LHb	Lateral habenular nucleus
LHA	Lateral hypothalamic areas
AD	Anterodorsal thalamic nucleus
Pe	Periventricular hypothalamic nucleus
AM	Anteromedial thalamic nucleus
VMH	Ventromedial hypothalamic nucleus
MD	Mediodorsal thalamic nucleus
AH	Anterior hypothalamic area
DM	Dorsomedial hypothalamus nucleus
LA	Lateroanterior hypothalamic nucleus
PH	Posterior hypothalamic area

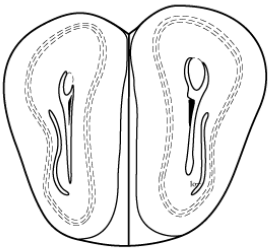
MESENPHALON

DL PAG	Dorsolateral periaqueductal gray
DM PAG	Dorsomedial periaqueductal gray
SC	Superior colliculus
VLPAG	Ventrolateral periaqueductal gray

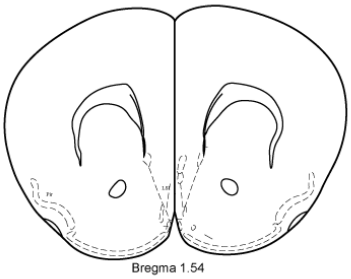
METENCEPHALON AND MYELENCEPHALON

Cerebellum

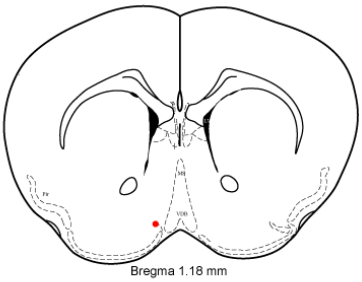
Raphe nucleus



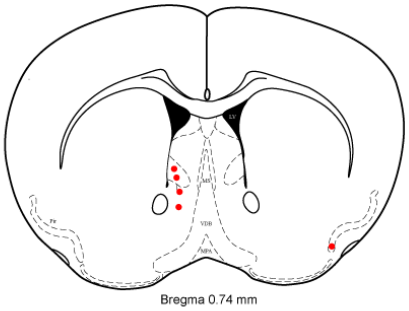
Bregma 3.92 mm



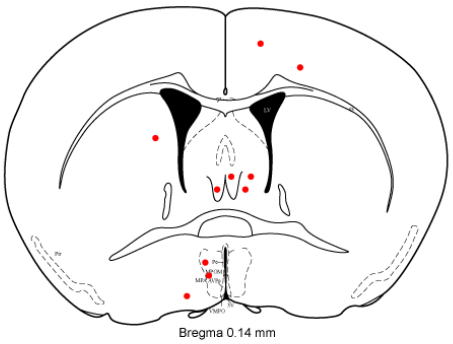
Bregma 1.54



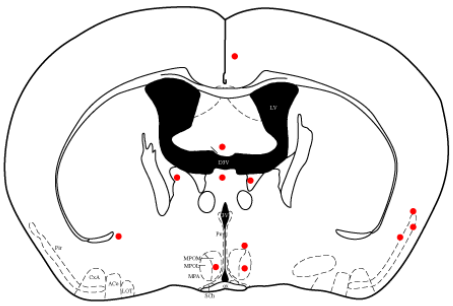
Bregma 1.18 mm



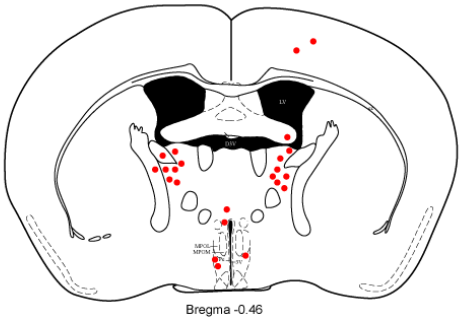
Bregma 0.74 mm



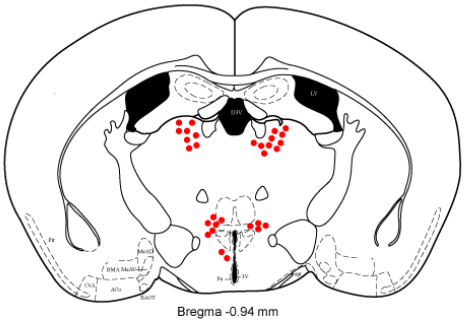
Bregma 0.14 mm



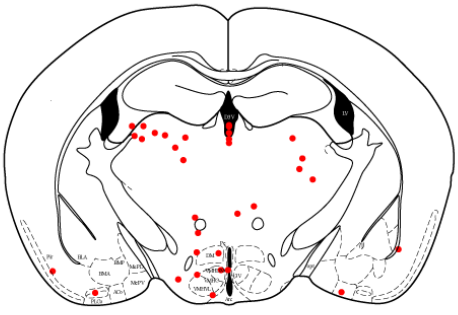
Bregma -0.22 mm



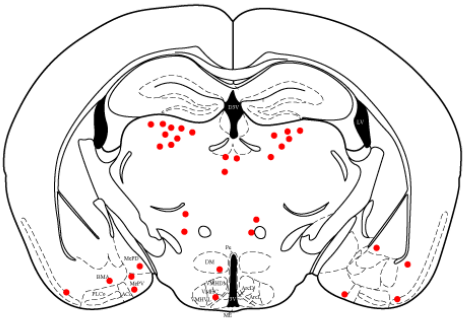
Bregma -0.46



Bregma -0.94 mm



Bregma -1.46 mm



Bregma -1.70 mm

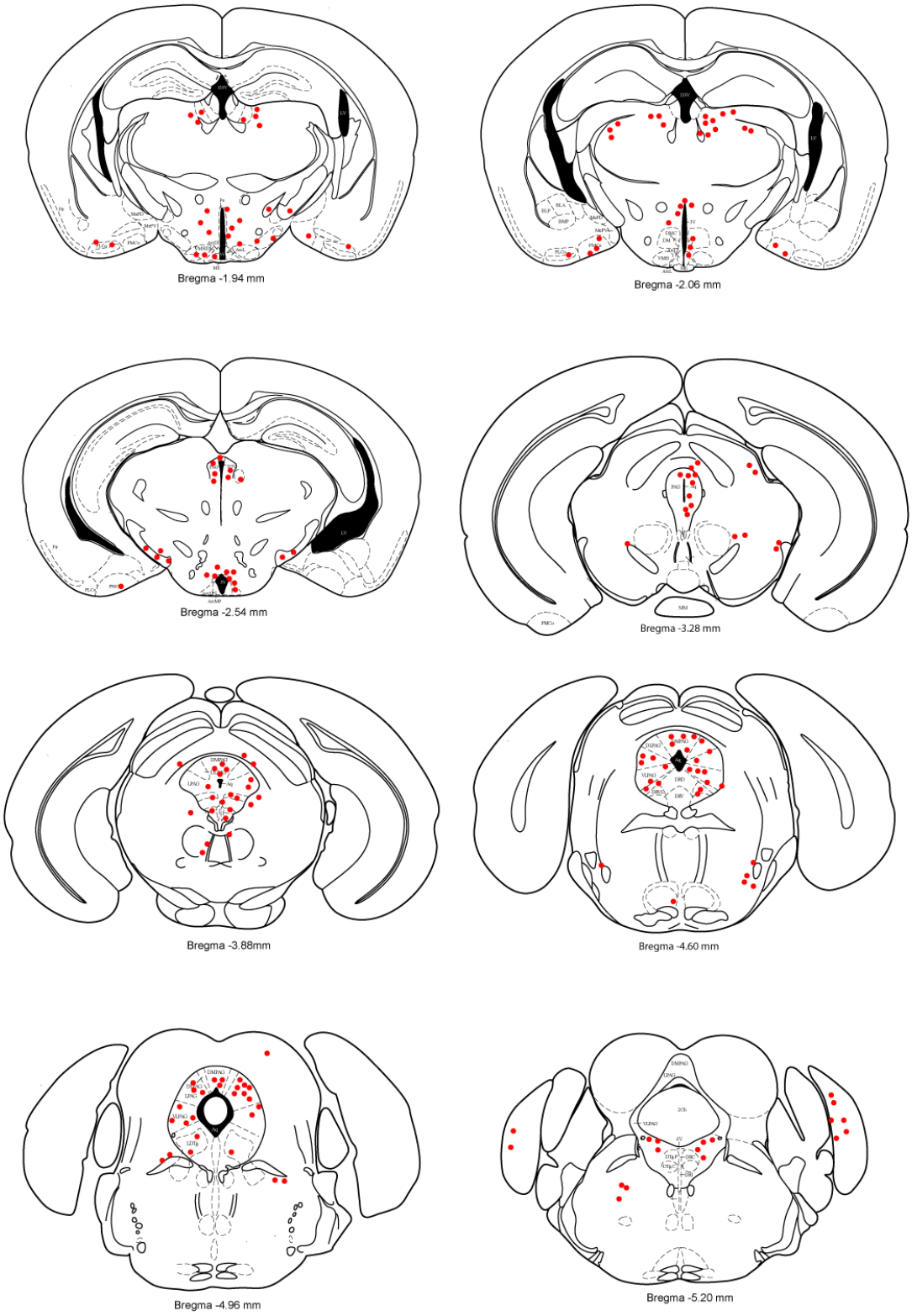


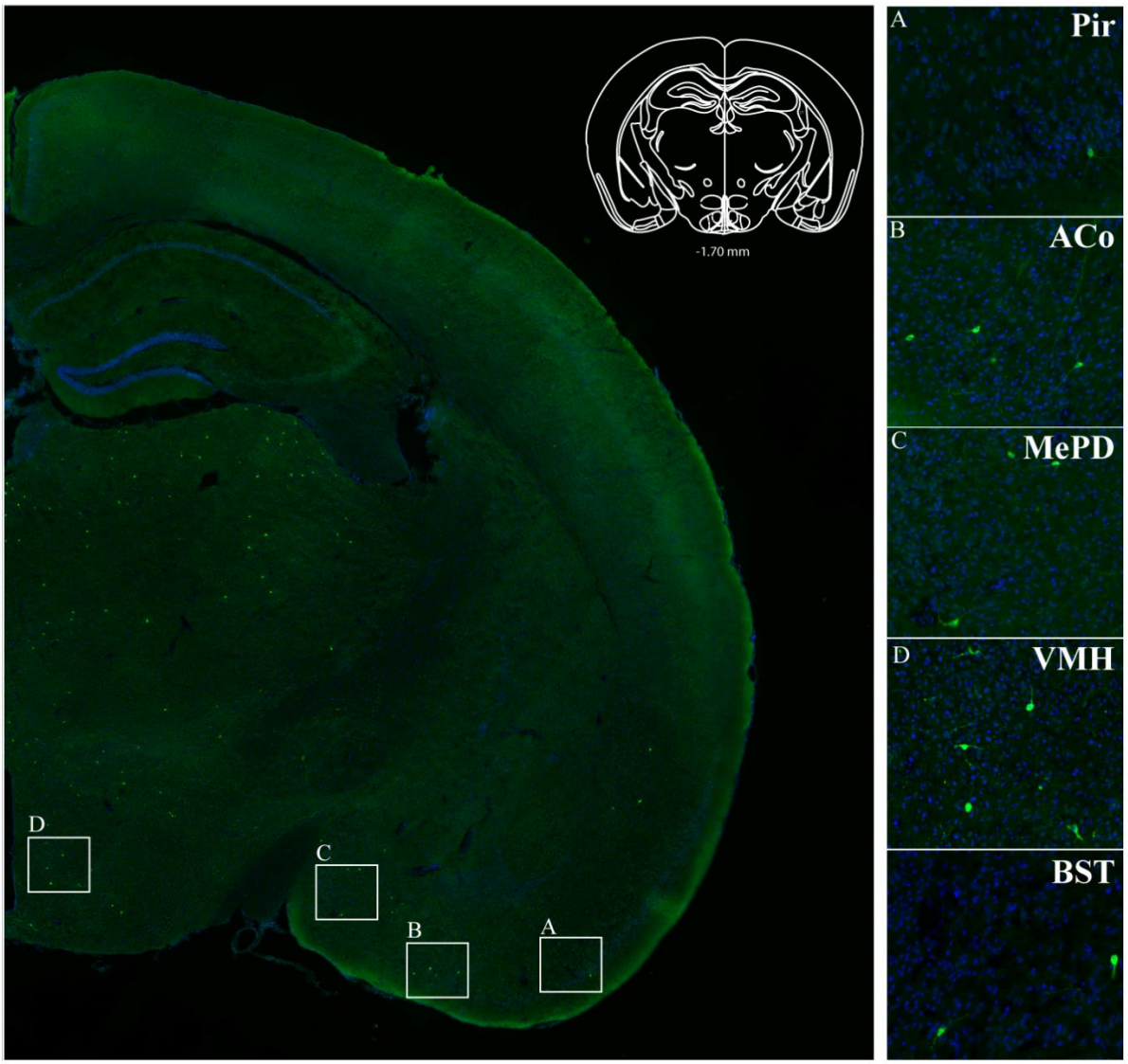
Figure 3.8 Schematic diagrams showing the distribution of GnRH receptor neurons in the adult female mouse brain. Numbers below each diagram points to the distance from bregma in mm. Red

dots, location of GnRHR neurons. Arc, arcuate nucleus; Diagrams are adapted and modified from the Mouse Brain Atlas, Paxinos and Franklin, 2001.

3.3.2 Expression of GnRH receptor neurons in olfactory and reproductive centers in the female mouse brain

I prepared coronal sections from GRIC/eR26-τGFP adult female mice and performed immunostaining using antisera against GFP. I found that the GnRH receptor expressing neurons to be highly concentrated in olfactory and reproductive centers such as piriform cortex (Pir), anterior cortical nucleus of amygdala (ACo), posterodorsal medial amygdala (MePD), ventromedial hypothalamus (VMH) and bed stria terminalis and periaqueductal grey (PAG).

A



B

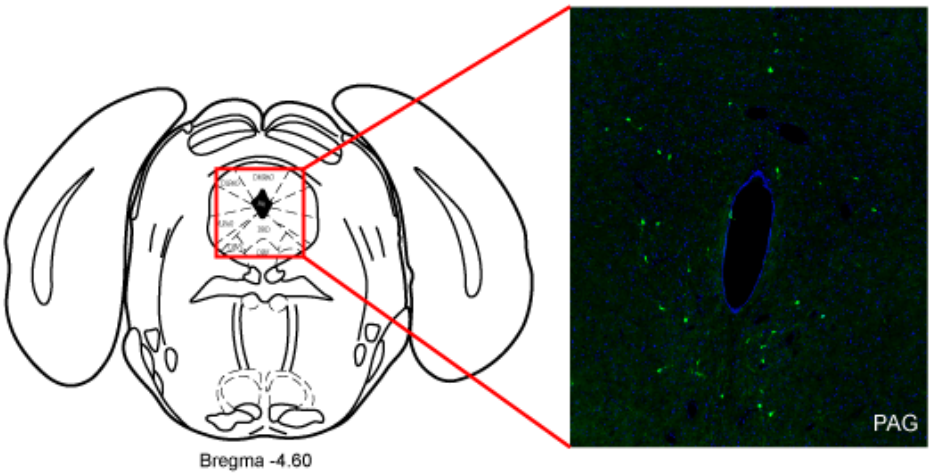


Figure 3.9 Expression of GnRH receptor neurons in olfactory and reproductive centers: A-B, GnRHR neurons expression in olfactory processing area and reproductive centers in 13 week old adult female mouse brain. A, Bregma -1.70mm; B, Bregma -4.60mm.

3.3.3 GnRH receptor neurons are upregulated during reproductive maturation

To identify if GnRH receptor neurons are upregulated during reproductive maturation I analyzed GnRH receptor expression at different developmental stages 6 weeks, 10 weeks and 13 weeks GRIC/eR26-τGFP female mice. I identified that the GnRH receptor neurons are upregulated across developmental stages, especially in olfactory and reproductive centers between 10 weeks and 13 weeks.

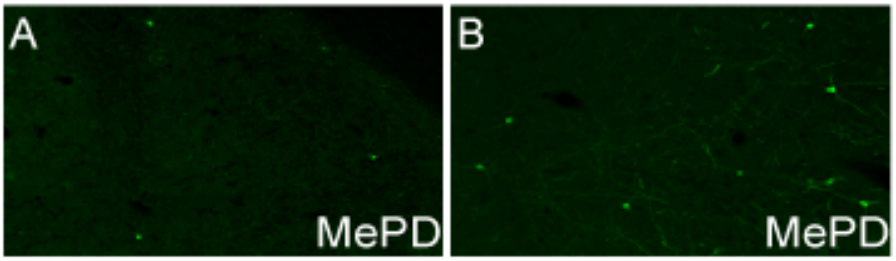


Figure 3.10 GnRH receptor neurons are upregulated in mouse brain. Representative images depicting the upregulation of GnRH receptor neurons in the posterodorsal medial amygdala (MePD). **A,** Expression of GnRH receptor neurons in 10 weeks old GRIC/eR26-τGFP female mice. **B,** Expression of GnRH receptor neurons in 13 weeks old GRIC/eR26-τGFP female mice.

3.3.4 GnRH receptor neurons express steroid hormone receptor

To determine if GnRH receptor neurons are sensitive to steroid hormones, I used 13 weeks old GRIC/eR26-τGFP female mice to analyze whether GnRH receptor neurons express ERα.

I performed double immunostaining using antisera against ERα and GFP. I identified that a subset of GnRH neurons in medial preoptic area (MPO), posterodorsal medial amygdala (MePD) and all the GnRH receptor neurons in ARC region express ERα.

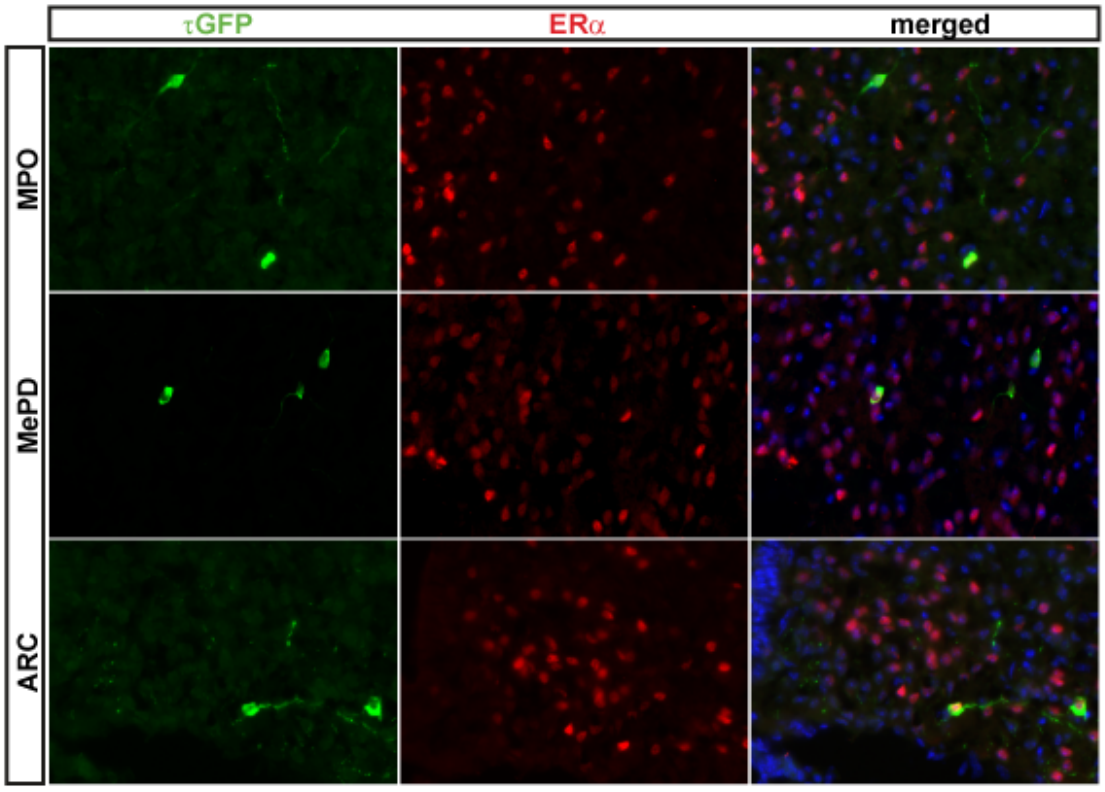


Figure 3.11 GnRH receptor neurons express ERα in GRIC/eR26-τGFP female mice. Dual immunostained photomicrograph of τGFP (green) and estrogen receptor alpha (ERα) (red) showing high degree of colocalization in the MPO (top), MePD (middle) and ARC (bottom) in a 13 week old female GRIC/eR26-τGFP mice.

3.3.5 GnRH receptor neurons and kisspeptin expression

To detect whether kisspeptin neuron which is a key upstream regulator of GnRH neuron also express GnRH receptor. I performed dual immunolabelling on GRIC/eR26-τGFP female mice using antisera against GFP and kisspeptin. Dual label immunohistochemistry showed that kisspeptin neuron does not express GnRH receptors.

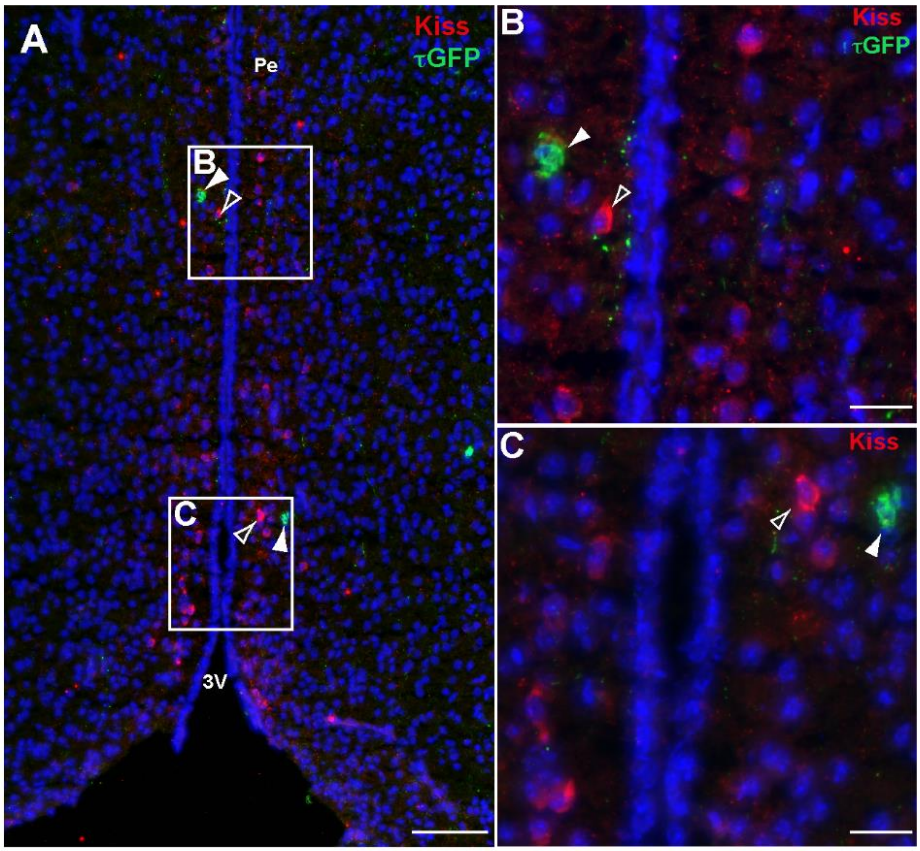


Figure 3.12 GnRH receptor and kisspeptin expression. A-C, Immunohistochemistry analysis on 14 µm coronal section of the medial periventricular nuclei (Pe) from 13 week old female GRIC/eR26-τGFP mice (n=3) with anti GFP and anti Kisspeptin antibody reveals that GFP signal (closed arrow head)does not overlap with kisspeptin signal (open arrowhead). 3V, Third ventricle, A, 100 µm, B, C, 25 µm, Bregma 0.02mm.

3.3.6 GnRH receptor neurons are activated after olfactory stimuli

In order to examine whether the chemosensory cues activate GnRH receptor neurons, I exposed GRIC/eR26-τGFP male mice to female mice soiled bedding as a source of olfactory input for 2 hr. Soiled bedding from adult female mice were collected, mixed and were used to stimulate GRIC/eR26-τGFP male mice. I used c-Fos as a neuronal activity marker to determine the neurons activated by olfactory input. I used immunohistochemistry to detect the expression of c-Fos in GnRH receptor neurons after chemosensory stimulation. Exposure to clean bedding did not induce c-Fos expression in the neurons whereas stimulation with female soiled bedding significantly induces c-Fos expression in GnRH receptor neurons in the male mice. The increased density of c-Fos were found in main olfactory bulb (MOB), accessory olfactory bulb (AOB) and posterodorsal medial amygdala (MePD). The medial amygdala integrates input, both the main olfactory input, accessory olfactory input and the hormonal state to trigger behavioral response. This data suggest that GnRH receptor neurons could be a downstream target of olfactory stimuli.

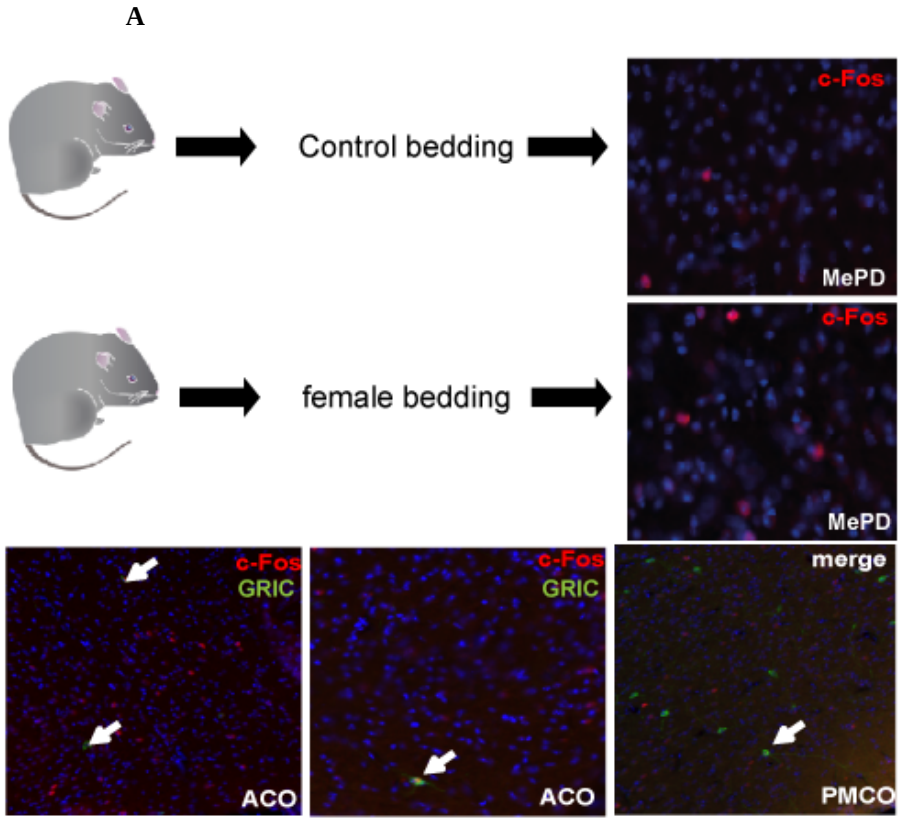


Figure 3.13 GnRH receptor neurons are activated after olfactory stimuli. A, Image illustrating of c-Fos immunoreactivity in GnRH receptor neurons in MePD, ACO and PMCO of GRIC/eR26-τGFP male mice when exposed to female mice bedding for 2 hours.

4. Discussion

4.1 Establishment of Kisspeptin-GnRH neural circuit in utero

Reproductive capacity is essential for the survival of the species. In all vertebrates, gonadotropin releasing hormone (GnRH) neuron is the master regulator of reproduction. Fertility depends on the pulsatile release of gonadotropin releasing hormone (GnRH) by GnRH neurons located in the hypothalamus. GnRH neurons are modulated by endocrine, metabolic and environmental inputs (Skorupskaite et al., 2014; Jeong et al., 2006). As GnRH neurons do not express sex steroid receptors, kisspeptin neurons mediate in relaying gonadal steroid feedback signals to GnRH neurons (Javed et al., 2015). Kisspeptin signaling is essential both for the initiation of puberty as well as for the maintenance of reproductive physiology. Loss of function mutation of kisspeptin signaling results in infertility (Topaloglu et al., 2012; Seminara et al., 2003). Although the function of kisspeptin/GPR54 system in the reproductive axis has been well studied during postnatal life, very few studies have been conducted on the development of kisspeptin/GPR54 system during fetal life.

The first aim of this thesis is to understand the development of the kisspeptin/GPR54 system in the male embryonic brain. I studied the development of kisspeptin/GPR54 expressing neurons, and when kisspeptin neurons becomes sensitive to steroid hormone receptors. In addition I studied when communication between kisspeptin neurons and GnRH neurons are established in the male mouse brain to better understand how kisspeptin in the hypothalamus regulate GnRH secretion from GnRH neurons. In the present study, I used genetic strategies and investigated the birth and the neuroanatomical distribution of kisspeptin neurons in KissIC/eR26-τGFP male embryos (Figure 3.1). Kisspeptin expression was not detected at E12.5. I observed the first expression of kisspeptin at E13.5 in the arcuate nucleus (ARC) of the hypothalamus, while I did not detect kisspeptin expression in the AVPV region (Figure 3.1 B). Previous studies have shown that upon administration of bromodeoxyuridine (BrdU) at different gestational period to female rats and upon performing double immunolabelling for kisspeptin and BrdU revealed that kisspeptin expression begun between E12.5 and E13.5 in the ARC. Kisspeptin immunoreactive cells in the ARC increased in their number until E18.5 (Desroziers et al., 2012). Studies using in situ hybridization (ISH), quantitative reverse transcription real time PCR (QPCR) and immunohistochemistry have showed that the *kiss1* mRNA is detected at E13 within the ARC. At E17, QPCR revealed that female have higher number of cells than male but ISH showed that there is no difference in the number of cells between male and female (Knoll et al., 2013). My

results are consistent with both the studies. My study revealed that the number of kisspeptin neuron increase significantly from E13.5 to E16.5 (Figure 3.1 E). At E 16.5, approximately 92% of the τGFP cells are positive for kisspeptin immunolabelling confirming the faithfulness of the binary genetic system (Figure 3.1 F).

It is known that kisspeptin, a potent activator of GnRH neurons acts via GPR54. GnRH neurons express GPR54. Injection of kisspeptin in GPR54 knockout mice did not result in the increase of LH and FH suggesting that kisspeptin acts directly on GnRH neuron via GPR54 (Messager et al., 2005). Mutation in GPR54 result in autosomal recessive idiopathic hypogonadotropic hypogonadism where the gonadotropin level is low in both humans and mice pointing that GPR54 signaling is critical for reproductive regulation and puberty (Seminara et al., 2003; Kauffman et al., 2007). GPR54 activating mutation are associated with precocious puberty (Teles et al., 2008). Though the role and distribution of GPR54 expressing neurons has been studied the development of GPR54 expressing neurons has not been well characterized during embryonic male brain development. In the next experiment, I used GPIC/eR26-τGFP male embryos and analyzed the expression of GPR54 (Figure 3.2). I did not observe GPR54 expression at E12.5. I found that GPR54 expression starts at E13.5. At E13.5, I detected GnRH neurons mostly in the nasal septum and 3% of GnRH neuron expressed GPR54 (Figure 3.2 B-C). At E16.5 most of the GnRH neurons has reached their final location in the forebrain. At E16.5, 43% of the GnRH neuron expressed GPR54 respectively (Figure 3.2 F). Previous studies using ISH demonstrated that the expression of kiss1 mRNA and GPR54 mRNA at E13 (Knoll et al., 2013). My studies show that the expression of both kisspeptin and GPR54 start at E13.5 and their coordinated increase at E16.5 suggest the possibility of signaling between kisspeptin neurons and GnRH neurons during the embryonic development.

Since kisspeptin/GPR54 signaling has been associated with anti-metastatic effects it has been speculated that kisspeptin/GPR54 signaling might act as a pausing signal for migrating GnRH neurons (Schwarting et al., 2007). Studies in GPR54 knockout mice have shown that the GnRH neuron exhibited normal migration and projection indicating that hypogonadism is only due to the lack of release of GnRH (Messager et al., 2005). My study revealed that all GPR54 expressing neurons are GnRH neurons but not all GnRH neurons express GPR54 during embryonic brain development. In addition, my data indicate that the expression of GPR54 in GnRH neuron was independent of the migratory path of the GnRH neuron (Figure 3.2 D). Hence kisspeptin/GPR54 signaling might not act as a cessation signal for migrating embryonic GnRH neurons.

At postnatal day 2, I detected 72% of the GnRH neurons express GPR54 (Figure 3.2 F). Similar observations has been made in studies using knockin LacZ mice in which 40% of the GnRH neurons express GPR54 at birth and the number increases to 70% by PND20 (Herbison et al., 2010). GPR54 was detected in 14 regions in the brain including dentate gyrus, hypothalamus, PAG. Northern blot and in situ hybridization analyses revealed that GPR54 is expressed in brain regions such as pons, midbrain, thalamus, hypothalamus, amygdala, cortex, frontal cortex, and striatum as well as peripheral regions such as liver and intestine (Herbison et al., 2010; Lee et al., 1999). GPR54 is also located in regions that do not contain GnRH neurons such as in pituitary and gonads raising the possibility to have additional functions (Irwig et al., 2005). The presence of GPR54 in the PAG suggests that GPR54 might play a role in sexual and motor behaviour.

The gonadal steroid hormones regulate the secretion of GnRH and gonadotropin hormones through positive and negative feedback loop by acting on both hypothalamus and pituitary and enable reproductive function (Thackray et al., 2010). Circulating steroid hormones such as estrogen and testosterone exert profound influence on the reproductive function. In rodents GnRH neurons do not express ERα (Roseweir et al., 2009). Steroid hormones exert their effect on GnRH neurons via the kisspeptin neurons. Gonadal steroids act as a key regulator of kisspeptin neuron at different stages of the reproductive physiology (Irwig et al., 2005). Kisspeptin neurons in the ARC and AVPV have different functions in the HPG axis. In the adult female mice almost all the kisspeptin neuron in the AVPV and ARC express ERα suggesting that kisspeptin neurons in the AVPV and ARC are sensitive to estrogen. Sex steroids also regulate hypothalamic expression of Kiss1 and GPR54. One study revealed that the expression of Kiss1 and GPR54 increased after gonadectomy but this effect was reversed by sex steroid replacement (Navarro et al., 2004). AVPV kisspeptin neurons expressing ERα cause positive feedback of estrogen on GnRH neurons during LH surge and ovulation. ARC kisspeptin neurons that express ERα cause negative feedback of estrogen on GnRH neurons (Smith et al., 2005).

Mayer and colleagues using a conditional knockout of ERα in kisspeptin neuron studied pubertal maturation and found that puberty occurred in WT mice approximately at 29 days whereas in kisspeptin specific ERα knockout mice the puberty onset was approximately at 13 days. These data demonstrate that loss ERα in kisspeptin neurons leads to precocious puberty (Mayer et al., 2010). It is unclear when during development kisspeptin neurons become sensitive to steroid hormones.

I used Kiss/eR26-τGFP female mouse in which kisspeptin neurons express τGFP to shed light on when kisspeptin neurons become sensitive to steroid hormones during development (Figure

3.3). I detected that kisspeptin neurons are born in the ARC of the embryonic male brain at E13.5. The number of kisspeptin neurons increase while being restricted to the ARC (Figure 3.3 B). Interestingly ERα and AR were also first detected at E13.5. At E13.5, approximately 63% of kisspeptin neurons express AR and at E16.5, 96% of kisspeptin neurons express AR (Figure 3.3 D). At E13.5, 61% of kisspeptin neurons express ERα and the number increases to 93 % at E16.5 (Figure 3.4 D). The birthplace of the steroid hormone receptors also marks the birthplace of kisspeptin neurons in the embryonic brain.

In ovariectomized mice, upon gonadal steroid administration that generates LH surge, kisspeptin neurons express neuronal activity marker, c-Fos. In wild type mice that generate LH surge, 50% of the GnRH neurons express c-Fos whereas Kiss1 and GPR54 knockout mice did not generate LH surge nor did the GnRH neurons express c-Fos. This demonstrates that both Kiss1 and GPR54 are important for the action of GnRH and LH surge (Clarkson et al., 2008). In rat, 77% of GnRH neurons express GPR54 and when kisspeptin is administered intracerebroventricularly more than 85% of the GnRH neurons expressed c-Fos demonstrating that kisspeptin can directly act on GnRH neuron. Another study showed that more that 90% of GnRH neurons express GPR54 mRNA both in adult and juvenile mice whereas AVPV *kiss-1* mRNA increased through the reproductive maturation from juvenile to adult (Hans et al., 2005). Administration of kisspeptin peptide induce LH levels in both males and females (Navarro et al., 2004). Continuous administration of kisspeptin leads to the desensitization of GPR54 suggesting that invivo pulsatile release of kisspeptin could trigger GnRH release during puberty (Seminara et al., 2006).

Although studies have shown the ontogeny of kisspeptin and GPR54 in the embryonic mice brain, the development of kisspeptin-GnRH neuronal circuitry has not been well established in the male brain. Studies using calcium imaging demonstrated that GnRH neurons from the embryonic nasal explant respond to kisspeptin application (Constantin et al., 2009). Kisspeptin circuitry has not been well studied due to the insensitivity of existing kisspeptin antibodies. One study using dual immunofluorescence experiments detected the interaction between kisspeptin fibers and GnRH neurons at P25 (Clarkson and Herbison, 2006). My study made use of transynaptic tracing method to dissect when kisspeptin neurons communicate with GnRH neurons during embryonic development. In the present study transynaptic tracing of kisspeptin neurons using transneural tracer BL in KissIC/R26-BIZ embryonic male mice revealed that the connectivity of ARC kisspeptin neurons with the GnRH neurons is established in utero (Fig 3.5 A-G). The connectivity was first established at E16.5. At E16.5 approximately 40% of GnRH neurons are connected to ARC kisspeptin neurons. The number of GnRH neurons connected to

kisspeptin neurons increased to 60% at PND2 (Fig 3.5H). GnRH neurons containing BL was observed in all hypothalamic nuclei containing GnRH neurons indicating that the connectivity is independent of the location of GnRH neurons. A selective population of GnRH neurons are connected to kisspeptin neurons and the connected GnRH neurons are in hypothalamic nuclei. The synaptic connection between kisspeptin neuron and GnRH neuron suggest that kisspeptin signal is operative via GPR54 expressed by GnRH neuron. These findings suggests that the connectivity between kisspeptin neurons and GnRH neurons is established during embryonic development.

4.2 Expression of ERα and dopaminergic kisspeptin neurons during puberty

It is known that the increased frequency of GnRH secretion is critical for puberty. The precise mechanism of how estrogen regulates GnRH secretion is not completely known. GnRH neuron do not express ERα. Estrogen regulates GnRH neurons via AVPV kisspeptin neuron and ARC kisspeptin neuron in adult mice (Radovick et al., 2012). However, different roles have been proposed for AVPV kisspeptin neurons and ARC kisspeptin neurons in mediating estrogen feeback on GnRH neurons (Lehman et al., 2010). Kisspeptin neurons expressing ERα is responsible for generating LH surge which is important for ovulation (Clarkson et al., 2008). In mice 40-60% of the AVPV kisspeptin neurons express gonadal steroid hormone receptors. In ovariectomized mice, upon gonadal steroid administration that generates LH surge, kisspeptin neurons express c-FOS whereas the kisspeptin neurons in the non LH surging mice do not express c-FOS. In wild type mice that generate LH surge, 50% of the GnRH neurons express c-FOS whereas Kiss1 and GPR54 knockout mice did not generate LH surge nor did the GnRH neurons express c-FOS. This demonstrates that both *Kiss1* and GPR54 are important for the action of GnRH and LH surge (Clarkson et al., 2008). Upon injection of monoclonal antibody in the POA that inhibits kisspeptin abolishes estrous cyclicity in rats (Kinoshita et al., 2005). Estrogen plays an important role in the regulation of kisspeptin neuron during puberty. Estrogen exerts its effect via ERα (Clarkson, 2013). The above mentioned studies suggests that AVPV kisspeptin neurons and ARC kisspeptin neurons have distinct roles in mediating estrogen feedback but how their sensitivity to estrogen changes during reproductive maturation has not been established.

To identify estrogen sensitive kisspeptin neurons during reproductive maturation I stained sections obtained from KissIC/R26-τGFP female mice against GFP and ERα (Figure 3.6). My study showed that at P21 (before puberty), 31% of AVPV kisspeptin neurons express ERα and

88% of ARC kisspeptin neurons express ERα (Figure 3.6 B). At P84 (after puberty), 73% of AVPV kisspeptin neurons express ERα and 90% of ARC kisspeptin neurons express ERα (Kumar et al., 2015a). The number of estrogen sensitive ARC kisspeptin neurons remains same pre-puberty and post-puberty. Therefore my results demonstrate that the estrogen sensitive AVPV kisspeptin neurons increased in number during reproductive maturation. Previous studies in mice established that the AVPV kiss1 mRNA peak during proestrous and ARC Kiss1 mRNA remains low. During LH surge, AVPV kiss1 mRNA levels are increased. AVPV kisspeptin neurons were positive for c-Fos only during the LH surge and not during diestrus whereas ARC kisspeptin neuron did not express c-Fos (Smith et al., 2006).

My study extends the understanding of AVPV kisspeptin neurons and ARC kisspeptin neurons in mediating estrogen feedback action on GnRH release. AVPV kisspeptin neurons might be the target to relay estrogen –positive feedback on GnRH release. Studies using genetic tracing strategy demonstrate that only a subpoulation of AVPV and ARC kisspeptin neurons are synaptically connected with GnRH neurons. All the AVPV kisspeptin neurons that are connected to GnRH neurons are estrogen sensitive (Kumar et al., 2015a)

Dopamine exhibits inhibitory action to affect LH secretion (Drouva et al., 1977). Immunohistochemistry studies revealed that in the AVPV most of the kisspeptin neurons express TH but very few TH neurons coexpress kisspeptin (Semaan et al., 2010). 20% of GnRH neurons receive dopaminergic input from AVPV (Liu et al., 2012). The function of AVPV kisspeptin neurons coexpressing TH is not completely understood. To identify if dopaminergic kisspeptin neurons increase during puberty. I stained sections prepared from KissIC/R26-τGFP females against GFP and tyrosine hydroxylase (TH) (Figure 3.7). I identified that at P21 (before puberty), 10% of AVPV kisspeptin neurons express TH (Figure 3.7 B). At P84 (after puberty), 47% of AVPV kisspeptin neurons express TH. Therefore the number of dopaminergic AVPV kisspeptin neuron increased in number during pubertal maturation whereas the number dopaminergic ARC kisspeptin neurons remained the same.

The increase in estrogen sensitive and catecholaminergic AVPV kisspeptin neurons across reproductive developmental stages suggest that estrogen signaling and dopamine might play an important role in reproductive maturation. This was in consistent with the previous studies which showed that Kiss1 expression increases during puberty and the level fluctuates during estrous cycle (Smith et al., 2006). Gonadal steroids influence the level of TH in the AVPV. In rats gonadectomy resulted in the increase of the number of TH mRNA containing cells and estradiol

administration resulted in the decrease of the AVPV TH mRNA neurons in female rats (Simerly, 1989).

Estrogen sensitivity and TH expression significantly increased during puberty in AVPV kisspeptin neurons. Thus AVPV kisspeptin neurons express both stimulator, kisspeptin and inhibitor, dopamine and regulate GnRH release. Estrogen sensitivity and TH expression remained the same pre-puberty and post-puberty in ARC kisspeptin neurons. There are several unanswered questions left to be explored in kisspeptin and GnRH biology. The precise molecular mechanism by which estrogen exert differential effect on ARC and AVPV kisspeptin neurons is still unknown. Previous studies using anterograde and retrograde tracing technique showed that ARC kisspeptin neuronal population innervate to wide number of hypothalamic and limbic region nuclei, whereas AVPV kisspeptin neuronal population innervate to medially located hypothalamic nuclei (Yeo et al., 2011). Kumar and colleagues dissected the connectivity between AVPV and ARC kisspeptin neurons with GnRH neuron using a combinatorial genetic strategy demonstrating that a subpopulation of AVPV and ARC kisspeptin neurons are synaptically connected to GnRH neuron. All the kisspeptin neurons that are connected to GnRH neurons express ERα and some are TH positive neurons (Kumar et al. 2015a).

4.3 Expression and characterization of GnRH receptor neurons in the mouse brain

GnRH acts on the pituitary gland to stimulate the synthesis and release of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). In pituitary gonadotropes, 10% of the cells express GnRH(R) receptor (Naor et al., 1982). The role of GnRHR in pituitary is well established but the role of GnRH receptor in the brain in not known. Several studies have reported that GnRH neurons project to different hypothalamic nuclei. Injecting a retrograde neural tracer, wheat germ agglutinin (WGA) in the median eminence revealed that approximately 70% of GnRH neurons in septal and anterior hypothalamic region project to the median eminence. The rest of the GnRH neurons projects to different hypothalamic and extra hypothalamic regions of the brain (Merchenthaler et al., 1989). In 2005, Yoon and colleagues identified that a subset of olfactory sensory neurons targets the hypothalamus and regulate GnRH synthesis and secretion (Yoon et al., 2005). Another study identified the GnRH neural circuitry using Barley lectin (BL), genetic neural tracer in mice. The study demonstrated that GnRH neurons which are approximately 800 in number located in the hypothalamus have synaptic connection with approximately 50,000 neurons in 53 different brain areas. When male mice were

exposed to female soiled bedding several of the BL labelled neurons in medial amygdala (MeA), Posteromedial cortical amygdaloid nucleus (PMCO) expressed c-Fos (Merchenthaler et al., 1989; Boehm et al., 2005). This suggest that olfactory system transmit information to GnRH neurons and GnRH neurons in addition to projecting to the median eminence also project to different brain areas especially olfactory processing areas. These observations led me to speculate that GnRH neuropeptide could also be released in the brain and act on GnRHR expressing neurons in the brain to modulate reproductive behavior.

Many animals transmit information about their social status, territorial ownership and reproductive status via chemosignals (Nakamura et al., 2007). Chemosensory cues are known to regulate neuroendocrine functions such as estrous induction, pubertal development and elicit behavioral response essential for reproduction (Halpern et al., 2003; Meredith, 1998). In the main olfactory system, main olfactory epithelium receives information and is relayed to anterior cortical nucleus (AON), olfactory tubercle (OT), tenia tecta (TT), and piriform cortex (Pir). The vomeronasal system receive sensory input which is relayed to medial amygdala (MeA), Posteromedial cortical amygdaloid nucleus (PMCO) and posterior bed nucleus of the stria terminalis (BNSTp) (Dulac et al., 2006).

In the present study to understand the potential role of GnRHR neurons in the brain I precisely mapped the distribution of GnRHR neurons at different developmental stages in GRIC/eR26-τGFP adult female mouse. I identified that GnRHR neurons in pheromone information processing areas. The GnRHR neurons are concentrated in posteromedial cortical amygdaloid nucleus (PMCO), anterior cortical amygdaloid nucleus (ACO), piriform cortex (PC) and medial amygdala (MeA) (Figure 3.9). The distribution of GnRH receptors is in consistent with the previous study (Wen et al., 2011).

In the GRIC/eR26-τGFP adult female mice I detected GnRHR neurons in bed nucleus of the stria terminalis (BNST), ventromedial nucleus of the hypothalamus (VMH) which plays a role in mating behavior. I also detected GnRHR neurons in the periaqueductal gray (PAG). PAG is involved in multiple functions such as vocalization, fear and lordosis. Upon injection of retrograde transneuronal tracer pseudorabies virus in the lumbar epaxial muscle, the pseudorabies virus labelled cells that control lordosis behavior such as the PAG (periaqueductal gray, medullary reticular formation (MRF) and ventromedial nucleus of the hypothalamus (VMH) (Daniels et al., 1999). Electrical stimulation of PAG triggered lordosis behavior in female rats and bilateral lesion of PAG resulted in reduced lordosis behavior in response to male mount

(Sakuma et al., 1979a; Sakuma et al., 1979b). I also detected GnRHR expression in medial amygdala which could provide emotional tag to information and, play a role in mate recognition and mating. Lesion in the medial amygdala resulted in significant reduction of the lordosis behavior (DiBenedictis et al., 2012).

Interestingly, I identified that the number of GnRHR neurons are upregulated after 6 weeks. Studies have shown that GnRH neurons reach their final number and location during late embryonic stage but the development of GnRHR neuorns has not been studied (Jasoni et al., 2009). I compared 10 weeks and 13 weeks old GRIC/eR26-τGFP female mice. I found that the expression of GnRHR neurons significantly increased at 13 weeks (Figure 3.10). For the first time my study revealed that the GnRHR neurons are significantly upregulated until 13 weeks. GnRHR expression in the brain could be upregulated by GnRH, as well as by steroid hormones like estrogen during reproductive maturation. Previous studies in pituitary have shown that GnRHR expression is upregulated by estrogen (Choi et al., 2005). To understand if the GnRHR expressing neurons are sensitive to estrogen in the brain. I performed dual immunolabelling against ERα and GFP on sections from GRIC/eR26-τGFP female mice. My experiments revealed that a subpopulation GnRHR neurons neurons in medial preoptic area (MPOA), posterodorsal medial amygdala (MePD) and all the GnRHR neurons in arcuate nucleus (ARC) are sensitive to estrogen (Figure 3.12).

Kisspeptin is a potent activator of GnRH neurons. To study if kisspeptin neurons express GnRH receptor and GnRH regulate kisspeptin via feedback regulation. I performed immunolabelling for kisspeptin in GRIC/eR26-τGFP mouse which revealed that kisspeptin neurons do not express GnRH receptors in the periventricular nucleus (Pe) (Figure 3.13).

My mapping results revealed that GnRHR are highly distributed in reproductive behavior and olfactory processing areas. Previous studies suggest that the brain areas that receive olfactory input also contain GnRH neurons or projections from GnRH neurons raising the possibility that olfactory input could activate GnRH neurons and modulate reproductive behavior (Westberry et al., 2003). Plasma LH levels increases in male mice when exposed to female urine. (Coquelin et al., 1984). Exposure to female vaginal secretions increase androgen surge in male hamster and upon removal of VNO this effect was abolished. This demonstrates that VNO mediates the effect induced by pheromones resulting in the increase of GnRH release which subsequently trigger LH and testosterone secretion in animals (Pfeiffer et al., 1984). My mapping result suggest that GnRH might be released locally and speculate that GnRHR neurons might play a role in olfactory triggered reproductive behavior.

Previous studies have shown that olfactory stimuli could activate kisspeptin neurons and GnRH neurons such as the exposure of male chemosensory cue induced c-Fos expression in AVPV kisspeptin neurons of the mice and stimulate GnRH release (Bakker et al., 2010; Murata et al., 2011). Mating or exposure to chemosignals activated neurons in the same regions such as amygdala, bed nucleus of the stria terminalis, medial preoptic area (Westberry et al., 2003). Main olfactory and vomeronasal pathway converge to the medial amygdala in the brain and plays an important role in integrating chemosignals and endocrine status to trigger behavioral response (Meredith, 1998; Sokolowski and Corbin, 2015). In addition to medial amygdala the response to olfactory input is detected in several regions in the brain containing steroid receptors (Blake et al., 2011).

Exposure of male mice to female chemosensory signals result in LH secretion which can be abolished by VNX demonstrating that vomeronasal input activates GnRH neurons (Meredith, 1989). Brain areas that contain GnRH cell bodies/fibers also expressed increased c-Fos expression when the mice is exposed to chemosensory cues. (Westberry et al., 2003).

Several studies demonstrated that olfactory system activates GnRH neurons but none of the studies shed light on the role of GnRHR neurons in the brain. In my study I observed that exposure of female chemosensory cue to male mice increased neuronal activation marker, c-Fos expression in the amygdala, bed nucleus of the stria terminalis and medial preoptic area (MPOA). I detected that GnRHR neurons in pheromone processing areas PMCO, ACO and MeA are activated in male mice upon stimulating with female bedding for 2 hours (Figure 3.11). This strongly suggests that GnRH is released locally within the brain in response to chemosignals to modulate reproductive behavior. Another study reported that when mice is exposed to chemosensory cues some of the brain areas such as amygdala, bed nucleus of the stria terminals(BNST), medial preoptic area that contain GnRH cell bodies/fibers also expressed increased c-Fos expression , suggesting potential role of GnRH in the olfactory cue triggered behavior (Westberry et al., 2003). Consistent with these studies the activation of GnRHR neurons in MePD, PMCO and ACO in response to olfactory stimuli suggests the potential role of GnRHR neurons in the olfactory cue triggered reproductive behavior.

5. Summary

The experiments presented in this thesis were designed to understand the development of presynaptic and postsynaptic target of GnRH neurons. First, the presynaptic target, kisspeptin neuron development were thoroughly studied. My results demonstrate that kisspeptin neuron and its receptor GPR54 are expressed at E13.5, kisspeptin expression is restricted to ARC and GPR54 expression is restricted to GnRH neurons during embryonic development, androgen receptor (AR) and estrogen receptor alpha (ERα) marks the birthplace of kisspeptin neurons in the male brain, and kisspeptin-GnRH neural circuit is established in utero. This study dissects the development of kisspeptin-GPR54 system which will help to better understand reproductive disorders such as hypogonadotropic hypogonadism and precocious puberty. Secondly, during reproduction maturation AVPV and ARC kisspeptin neurons are sensitive to ERα and express tyrosine hydroxylase (TH). The expression of TH and estrogen sensitivity increased in AVPV kisspeptin neurons during pubertal development whereas in the ARC kisspeptin neurons it remained the same. Thirdly, this study represent the first line of evidence in which the post synaptic target of GnRH neuron, GnRH receptor (GnRHR) expressing neuron was mapped extensively and characterized in female mice. This study provide detailed neuroanatomical framework of GnRHR neurons in a female mice, GnRHR expression is upregulated during reproductive development and olfactory stimuli activate GnRHR neurons. Taken together this will help in further understanding the role of GnRHR in the mammalian reproductive axis.

6. References

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Dedicated

To

My Beloved Parents

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8. List of publications

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