Genetic labeling and functional characterization of GnRH target cells in the house mouse (*Mus musculus* (Linnaeus, 1758))

Dissertation

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To Whom It May Concern,

I am a native English speaker, I have read the thesis prepared by Wen Shuping, provided that the corrections that I have suggested are incorporated in the final thesis. I can affirm that the thesis is written in grammatically correct English that would be understandable to any native English speaker.

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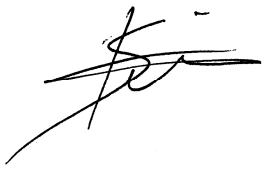

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Abbreviations

αGSU	Glycoprotein hormone α -subunit
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
°C	degree Celsius
ACTH	Adrenocorticotropic hormone
AVP	Vasopressin
BL	Barley lectin
bp	Base pair
BSA	Bovine serum albumin
CNS	Central nervous system
Cre	Cre recombinase
d	Day
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
DTA	Diphtheria toxin A fragment
DTR	Diphtheria toxin receptor
E	Embryonic day
EDTA	Ethylene diamine tetra acetic acid
et al.	*et altera*

Abbreviations

FRT	Flp recombination target
FSH	Follicle stimulating hormone
FSHß	Follicle stimulating hormone ß subunit
g	Grams
G418	Geneticin
gDNA	Genomic DNA
GFP	Green fluorescent protein
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GnRHR	GnRH receptor
GnRHR cell	GnRH receptor expressing cell
GRIC	GnRHR-IRES-Cre
hr(s)	Hour(s)
Hepes	4-(2-Hydroxyethyl)-piperazin-1-ethansulfonic acid
hpg	GnRH-deficient hypogonadal
HPG	Hypothalamic-pituitary-gonadal
hPLAP	human placental alkaline phosphatase reporter
IF	Immunofluorescence
IHH	idiopathic hypogonadotropic hypogonadism
IHC	Immunohistochemistry
IRES	Internal Ribosome Entry Site
Kb	Kilobase
KS	Kallmann syndrome
l	Litter
LH	Luteinizing hormone
LHß	Luteinizing hormone ß subunit

Abbreviations

M	Molar
ME	Median eminence
mg	Milligram
Min(s)	Minute(s)
ml	Milliliter
mM	Millimolar
MPN	Medial preoptic nucleus
mRNA	Messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone
n	Sample number
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Neo	Neomycin resistance gene
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
OE	Olfactory epithelium
OT	Oxytocin
P	Postnatal day
PAG	Periaqueductal gray
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pgk	phosphoglycerate kinase
pH	*potentium hydrogenii*
POA	Preoptic area
PR	Progesterone receptor
PRL	Prolactin
RHPA	Reverse hemolytic plaque assay

Abbreviations

RNA	Ribonucleic acid
RT	Room temperature
SC	Superior colliculus
SDS	Sodium dodecyl sulfate
Sec(s)	Second(s)
SEM	Standard error of the mean
SSC	Sodium chloride sodium citrate
SSPE	Sodium chloride sodium phosphate-EDTA buffer
TAE	Tris-acetate-EDTA
Tris	Tris-(hydroxymethyl)-aminomethane
TSH	Thyroid-stimulating hormone
TSHß	Thyroid-stimulating hormone β subunit
U	Unit (enzymatic activity)
UV	Ultraviolet
V	Volume
VMHvl	Ventromedial hypothalamic nucleus, ventrolateral part
VNO	Vomeronasal organ
W	Weight
YFP	Yellow fluorescent protein
ZMNH	Center for Molecular Neurobiology Hamburg

1. Introduction

1.1 Central control of reproduction: the HPG axis

In vertebrates, sexual maturation and reproductive function are highly regulated by the hypothalamic-pituitary-gonadal (HPG) axis, which is hierarchically organized and consists of three endocrine organs: the hypothalamus, pituitary and gonads (Figure 1.1). Each of them produces specific hormones that regulate the next downstream organ.

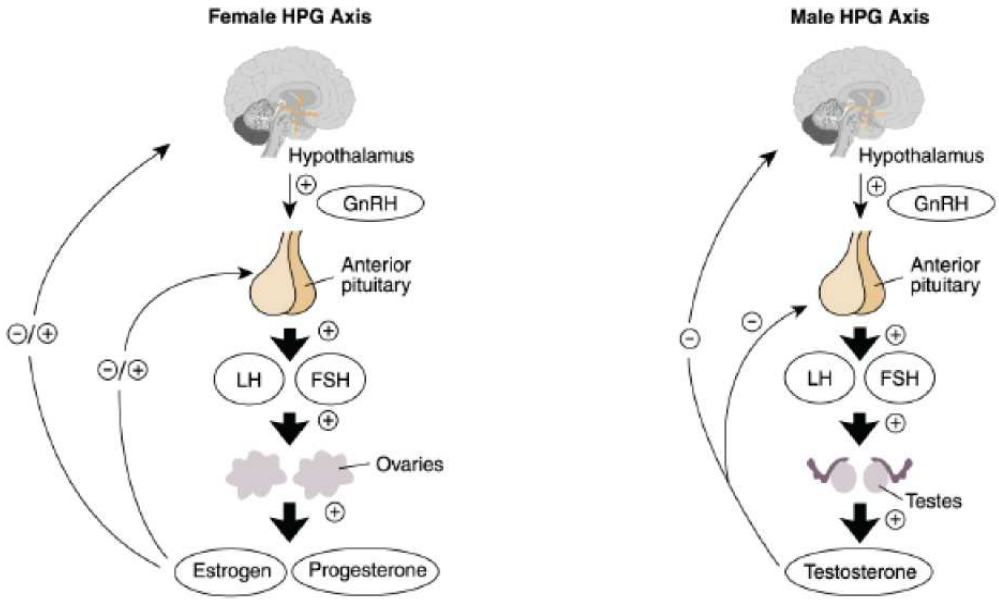

Figure 1.1 The hypothalamic-pituitary-gonadal axis.

GnRH neurons in the hypothalamus synthesize and release GnRH, which acts on the anterior pituitary and triggers gonadotropin (LH and FSH) release from gonadotropes. LH and FSH then stimulate the production of steroid hormones in the gonads, which in turn send either positive or negative feedback to the hypothalamus and the pituitary gland to regulate the synthesis and release of GnRH and gonadotropins. Abbreviations: FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; HPG,

hypothalamic-pituitary-gonadal; LH, luteinizing hormone. Adapted from Hiller-Sturmhofel and Bartke, 1998.

The hypothalamus synthesizes and releases the decapeptide gonadotropin-releasing hormone (GnRH) in discrete pulses. In response to GnRH, gonadotropes in the anterior pituitary gland produce and secrete two gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), which travel along the systemic circulation and bind to their specific receptors on ovarian or testicular cells to regulate spermatogenesis and sex steroid hormone production in the gonads. The sex steroid hormones produced by the gonads in response to gonadotropins are in turn released into the circulatory system and act on different target tissues. Importantly gonadal steroid hormones also provide feedback to the hypothalamus and the pituitary gland to modulate the synthesis and release of GnRH and gonadotropins.

1.2 GnRH

1.2.1 GnRH structure

The decapeptide GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH_2) is the primary regulator of the HPG axis. It was first isolated and sequenced from pig hypothalami during the early 70s (Baba et al., 1971; Matsuo et al., 1971; Schally et al., 1971) and subsequently found in the brain of all vertebrates (Gore, 2002). Its amino acid sequence is highly conserved in all mammals studied so far, except guinea pig, in which there are substitutions of amino acids 2 and 7 (Jimenez-Linan et al.,1997).

The mammalian *GnRH* gene was cloned in the 80s (Seeburg and Adelman, 1984; Adelman et al., 1986). The gene codes for a precursor molecule of 92 amino acids in which the GnRH decapeptide is preceded by a signal peptide of 23 amino acids and followed by a GnRH-associated peptide.

Most vertebrate species possess three GnRH molecules. In addition to hypothalamic GnRH, which is referred to as GnRH-1, GnRH-2 and GnRH-3 molecules are found in the

brain (Table 1.1). Both are also decapeptides, however with different structures, functions and are produced by different cells (Gore, 2002). Importantly, so far GnRH-2 and GnRH-3 have not been identified in the mouse (Morgan and Millar, 2004). Therefore unless indicated otherwise, "GnRH" refers to GnRH-1 in this thesis.

Molecule	Signal peptide	GnRH	C.S.	GnRH-associated peptide
GnRH-1				
human	MKPIQKLLAGLILLTWCVEGCSS	QHWSYGLRPG	GKR	DAENLIDSFQEIVKE…
monkey	MEPIPKLLAGLILLTVCVEGCSS	QHWSYGLRPG	GKR	DAENLMDSFQEIVK…
mouse	M--ILKLMAGILLLTVCLEGCSS	QHWSYGLRPG	GKR	NTEHLVESFQEMGK…
rat	MEIPKLMAAV VLLTVCLEGCSS	QHWSYGLRPG	GKR	NTEHLVDSFQEMGK…
tree shrew	MELVPKLAGLILLTLCVGGCYA	QHWSYGLRPG	GKR	NAENLIDSFQEIAKE…
frog	MKAFPTFALLFLVLLFSAHVSDA	QHWSYGLRPG	GKR	DTESLOMYHETPNE…
GnRH-2				
goldfish	MVHICRLFVVMGMLLCLSAQFASS	QHWSHGWYPG	GKR	EIDVYDPSE-…
cichlid	MCVSRLALLLGLLLCVGAQLSFA	QHWSHGWYPG	GKR	ELDSFGTSE…
seabream	MCVSRLVLLLGLLLCVCAQLSNG	QHWSHGWYPG	GKR	ELDSFGTSE…
tree shrew	MASSMLGFLLLLLLLMAAHPGPSEA	QHWSHGWYPG	GKR	ASNSPQDPQ…
GnRH-3			GKR	
goldfish	MEGKGRVLVQLLMLACVLEVSLC	QHWSYGWLPG	GKR	SVGEVEATFRMMD...
cichlid	MEAGSRVIMQVLLLAVVQVTLS	QHWSYGWLPG	GKR	SVGELEATIRMMGT...
masu salmon	MDLSSKTVVQVVMLALIAQVTFS	QHWSYGWLPG	GKR	SVGELEATIRMMDT...

Table 1.1 Amino acid alignment of GnRH precursors.

C.S. =cleavage site. (Adapted from Gore, 2002)

1.2.2 GnRH release and GnRH signaling in gonadotropes

One important function of GnRH is the regulation of gonadotropin synthesis and release from the anterior pituitary. After being released into the hypophyseal portal vasculature

from axon terminals of GnRH neurons in the median eminence (ME), GnRH binds to GnRH receptor (GnRHR) expressed by gonadotropes in the anterior pituitary to regulate the synthesis and secretion of LH and FSH (Clayton & Catt, 1981).

GnRH is released in discrete pulses which in turn stimulate the pulsatile release of gonadotropins. High GnRH pulse frequencies (one pulse every 30 minutes) are optimal for LHß synthesis and release, whereas low GnRH pulse frequencies (one pulse every 120 minutes) are optimal for FSHß synthesis and release (Kaiser et al., 1997).

To date, attention has been focused on the role of GnRH in regulating gonadotropin release from mature gonadotropes in adult animals. However, earlier studies in fetal mice have shown that GnRH stimulates pituitary to secret LH at embryonic day (E) 16 (Pointis and Mahoudeau, 1979), and that plasma LH is first detected at E16 (Pointis et al., 1980). These experiments raise the possibility that GnRH signaling might also play a role during embryonic development of gonads.

1.2.3 GnRH neurons and their neural circuitry

GnRH is synthesized and released by GnRH neurons, a small population of neurons (ranging from 800 to 2000 neurons depending on the species) localized in the caudal telencephalon and the most rostral regions of the diencephalon (Silverman et al., 1994).

In mice, ~800 GnRH neurons locate in the basal forebrain with most of them concentrated in the preoptic area (POA) of the hypothalamus (Figure 1.2). Recently, the neural circuitry of GnRH neurons was visualized using genetic transneuronal tracing in mice (Boehm et al., 2005). The locations of tracer-labeled cells identified neurons directly presynaptic or postsynaptic to GnRH neurons in the brain's reproductive neural circuitry. GnRH neurons were found to communicate with ~50000 neurons in 53 functionally diverse brain areas, suggesting that GnRH neurons integrate a variety of information and influence numerous brain functions. Several of these presynaptic neurons were found in areas involved in pheromone signal processing and are activated in mice

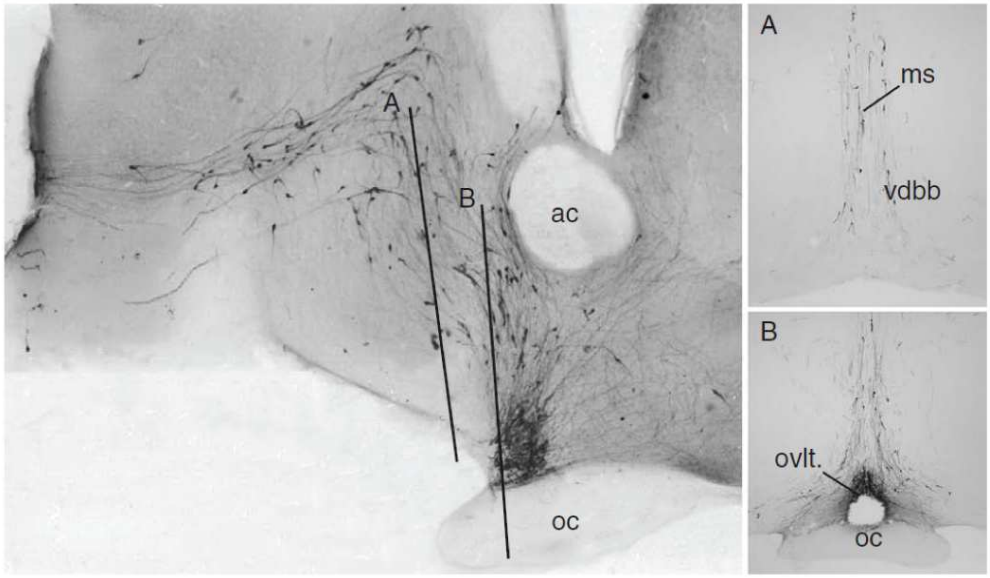

Figure 1.2 Distribution of GnRH neurons in adult mouse brain.

Distribution of GnRH neurons on a parasagittal section of adult mouse brain. Scattered GnRH-immunoreactive neurons were observed from the olfactory bulbs (left) into the hypothalamus (lower right). A and B, Photographs of GnRH-immunoreactive neurons on coronal sections of adult mouse brain. A, GnRH neurons are located in the medial septum (ms) and the vertical limb of the diagonal band of Broca (vdbb). B, GnRH neurons and dense fibers are located in the organum vasculosum of the lamina terminals (ovlt) on both sides of the third ventricle. Abbreviations: ac, anterior commissure; ms, medial septum; oc, optic chiasm; ovlt, the organum vasculosum of the lamina terminals; vdbb, vertical limb of the diagonal band of Broca. Adapted from Knobil and Neill's physiology of reproduction, Volume 1, 2006.

exposed to pheromones, indicating direct synaptic connections between GnRH neurons and neurons relaying vomeronasal signals. In addition, these studies have revealed feedback loops between the neuroendocrine hypothalamus and both the main and accessory olfactory systems, suggesting that the animal's neuroendocrine status might modulate its susceptibility to chemosensory cues. Postsynaptic neurons were also found in a surprisingly large number of brain areas involved in diverse functions, including sexual behavior (Boehm et al., 2005).

1.2.4 Migration of GnRH neurons

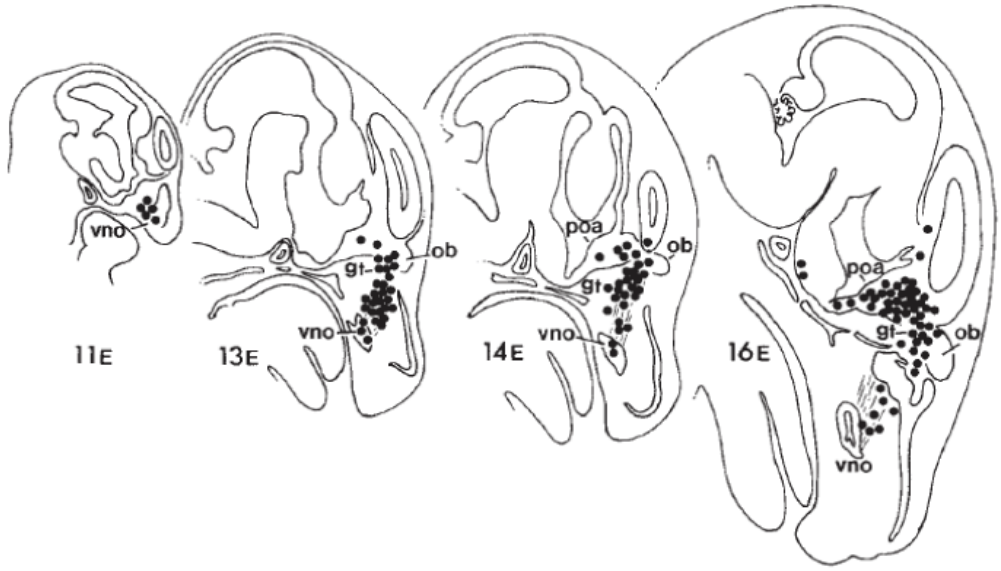

Figure 1.3 Embryonic migration of GnRH neurons.

Embryonic migration of GnRH neurons from the olfactory placode to the forebrain is illustrated on sagittal sections through the heads of fetal mice on embryonic days 11, 13, 14, and 16. The black dots represent GnRH-immunoreactive neurons. On day E11, most GnRH neurons are born in the vomeronasal organ. On day E13, the majority of GnRH neurons migrate along the terminal nerve in the nasal septum. On day E14, many GnRH neurons have arrived at the ganglion terminale and the central roots of the nervus terminalis. And by day E16, GnRH neurons mainly locate in the POA of the hypothalamus and this pattern is similar to that in the adult. Abbreviations: gt, ganglion terminale; ob, olfactory bulb; poa, preoptic area; vno, vomeronasal organ;. Adapted from Schwanzel-Fukuda and Pfaff, 1989.

GnRH neurons are born outside of the CNS and then migrate from their birthplace into the hypothalamus during embryonic development. In mice, GnRH neurons are born in the olfactory placode at E11 to 11.5 （Figure 1.3）, migrate along the terminal nerve, cross the cribriform plate and enter the forebrain (Schwanzel-Fukuda and Pfaff, 1989). At E12.5, GnRH neurons are located outside of the olfactory epithelia. At E14.5, GnRH neurons are already detected throughout the forebrain (Livne et al., 1993). At E16.5, the

distribution of GnRH neurons is similar to that in adult animals, with their cell bodies mainly in the preoptic area (POA) and the anterior hypothalamus (Gore, 2002). GnRH axons project to the presumptive ME as early as E13.75 (Wu et al., 1997). The total number of GnRH-immunoreactive cells changes during embryonic development. It peaks at around 2000 at E12.75 (Wu et al., 1997) and then declines to 800 cells postnatally (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989; Wu et al., 1997). It is still not known how the size of the GnRH neuronal population is established during development (Tobet and Schwarting, 2006).

1.2.5 GnRH target cells

1.2.5.1 GnRHR structure

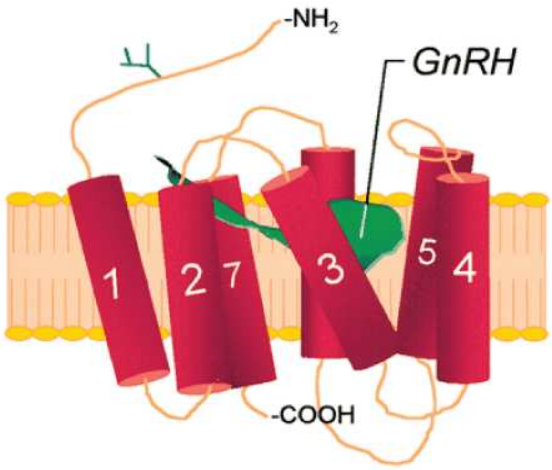

Figure 1.4 Structure of the human GnRH receptor.

Counterclockwise orientation of the human GnRHR, a prototypic G protein-coupled receptor with seven transmembrane domains (TMD). TMD 2, 3, 5 and 6 comprise the core of the receptor. Adapted from Ulloa-Aguirre et al., 2003.

The GnRHR was first cloned from mouse (Reinhart et al., 1992), then sequentially from other species (Jeong and Kaiser, 1994). The GnRHR consists of 328 amino acids and is a member of the seven-transmembrane G-protein-coupled receptor (7TM-GPCR)

superfamily (Stojilkovic et al., 1994). It has a short N-terminal extracellular domain and no C-terminal intracellular tail (Figure 1.4), which is thought to be involved in receptor desensitization and internalization in other GPCRs (Sealfon et al., 1997). The mouse GnRHR is 97% homologous to the rat GnRHR, 89% to the human GnRHR, and 87% to the ovine GnRHR (Jeong and Kaiser, 1994).

The GnRHR density on the cell surface can be regulated by GnRH, and GnRHR mRNA levels can be increased by pulsatile GnRH administration (Clayton et al., 1985; Yasin et al., 1995). GnRHR expression is also modulated by steroid hormones, such as estrogen (Gregg and Nett, 1989; Turgeon et al., 1996), progesterone (Cheng et al., 2001), androgen (Spady et al., 2004), and glucocorticoids (Maya-Nunez and Conn, 2003).

1.2.5.2 GnRH signaling in the central nervous system

In addition to regulating gonadotropin synthesis and release from gonadotropes in the anterior pituitary, GnRH can stimulate specific reproductive behaviors. In female mice and rats reproductive behaviors such as lordosis were facilitated by subcutanous injection of GnRH (Moss and McCann, 1973; Pfaff, 1973; Luttge and Sheets, 1977) or infusion of GnRH into the subarachnoid space (Sirinathsinghji, 1983), the mesencephalic central gray (Riskind and Moss, 1979; Sakuma and Pfaff, 1980), or the ventral hypothalamus (Moss and Foreman, 1976). In male orchiectomized rats, GnRH application decreased the latency to intromission and ejaculation (Moss and McCann, 1973).

Anatomical studies in mice showed that about 30% of GnRH neurons send nerve fibers intracerebrally to areas, such as the amygdala and central gray (Jennes and Stumpf, 1980). GnRH was also found in dense core vesicles in axon terminals of GnRH neurons (Jennes et al., 1985). These data raise the possibility that GnRH itself might be released locally within the CNS and act on downstream neurons expressing the GnRHR. Consistent with this, GnRH neurons were shown to have direct synaptic contacts with neurons in brain regions involved in sexual behavior, such as the MPN and VMHvl (Boehm et al., 2005).

Radioligand receptor binding assays and in situ hybridization studies revealed GnRH binding sites and GnRHR mRNA expression in several brain regions (Badr and Pelletier 1987; Jennes et al. 1988; Jennes et al. 1997). However these studies are partially contradictory to each other in respect to which brain areas contain GnRHR neurons. One transgenic mouse model has been described in which a 3.3 kb fragment of the rat *GnRHR* promoter drives expression of the *hPLAP* gene (Granger et al., 2004). Expression of this reporter gene was found in gonadotropes in the anterior pituitary as well as in several areas in the brain. However in the absence of established antibodies against the GnRHR protein, aberrant and ectopic activation of this rat *GnRHR* promoter fragment cannot be ruled out in this mouse model, leaving the precise localization of GnRHR expression in the brain to be defined.

1.3 Development of the pituitary gland

1.3.1 Structure of the pituitary gland

The pituitary gland is a small endocrine organ located at the base of the brain, just beneath the optic chiasm, and is essential for the development and function of many other organs in the body. It comprises of three different parts, the anterior, posterior pituitary and the intermediate lobe in most mammals (Kelberman et al., 2009).

The anterior pituitary contains five major hormone secreting cell types (Table 1.2). Somatotropes secrete growth hormone (GH), lactotropes secrete prolactin (PRL), corticotropes secrete adrenocorticotropic hormone (ACTH), thyrotropes secrete thyroid-stimulating hormone (TSH), and gonadotropes secrete LH and/or FSH (Daughaday, 1985). Some cells in the anterior pituitary produce more than one hormone. For example, most gonadotropes produce LH as well as FSH in the same cell. Some gonadotropes also produce GH in addition to LH and FSH (Childs et al., 1994).

The posterior pituitary consists of axonal projections extending from the hypothalamus and pituicytes, a type of astrocytic glial cells. Two nonapeptides, vasopressin and oxytocin are secreted from the posterior pituitary (Zhu et al., 2007). Both are synthesized

by peptidergic neurons in the supraoptic (SON) and paraventricular nuclei (PVN) of the hypothalamus (Hadley and Levine, 2007). Vasopressin is involved in the water reabsorption in the kidneys, glycogenolysis in the liver, and vasoconstriction (Ball, 2007). Oxytocin stimulates smooth muscle contraction and is involved in parturition and milk ejection during nursing (Gainer and Wray, 1994).

The intermediate lobe contains melanotropes, which produce melanocyte-stimulating hormone (MSH) (Evans et al., 1994). MSH stimulates the production and release of melanin in skin and hair (Slominski et al., 2004).

	Somatotropes	**Lactotropes**	**Thyrotropes**	**Corticotropes**	**Gonadotropes**
Hormone product	GH	PRL	TSH	ACTH, MSH, lipotropins, endorphins, enkephalin.	LH, and/or FSH
Site of action	Liver, kidney, most other tissues	Mammary	Thyroid	Adrenal gland	Ovary, testis
Positive regulator	Growth-hormone-releasing hormone (GRH)	Estrogen, thyrotropin-releasing hormone (TRH)	TRH	Corticotropin-releasing hormone (CRH)	GnRH
Negative regulator	Somatostatin, insulin-like growth factor	Dopamine	Thyroid hormone	Corticosteroids	Gonadal steroids, inhibins
Hypopituitarism phenotype	Dwarfism	Failure to lactate	Thyroid hypoplasia, dwarfism, cretinism, hypothyroidism	Adrenal hypoplasia	Sexual immaturity
Hyperpituitarism phenotype	Gigantism, acromegaly	Galactorrhea, infertility	Thyroid hyperplasia, hyperthyroidism	Cushing disease	Precocious puberty

Table 1.2 Cell types in the anterior pituitary and their functions.

Adapted from Watkins-Chow and Camper, 1998.

1.3.2 Development of the anterior pituitary

The pituitary appears at the third week of development in human and at day 8 in mouse embryos. The anterior, intermediate and posterior lobes appear at proximately the same time (Sheng et al., 1997).

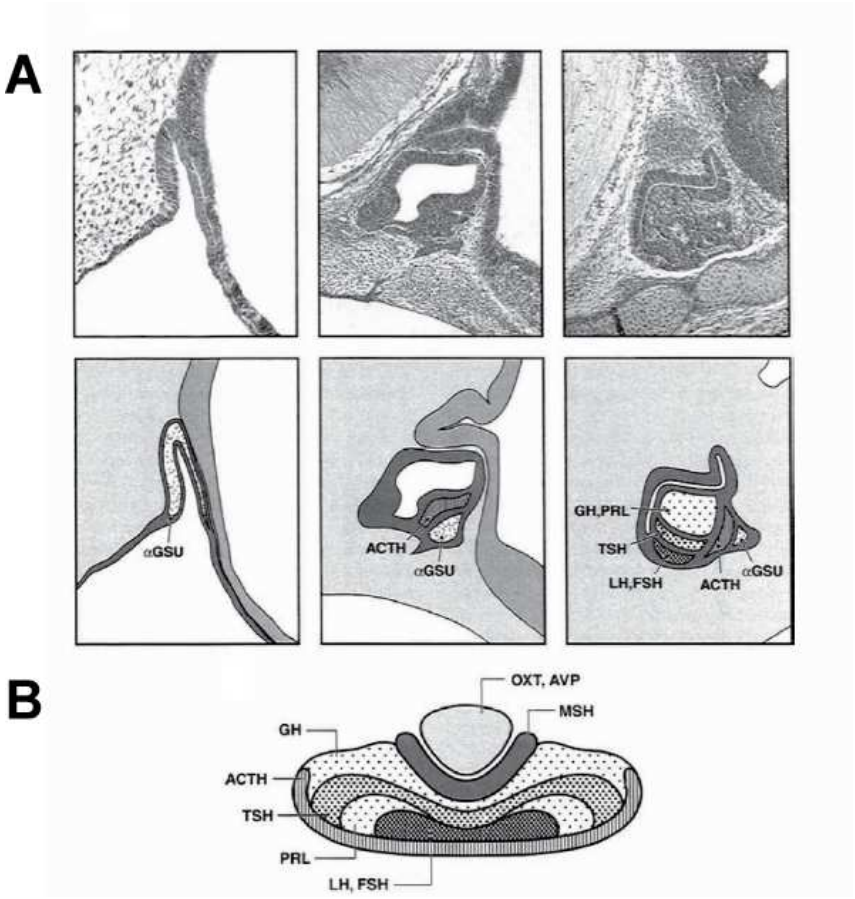

Figure 1.5 Development of the mouse anterior pituitary.

A, Pictures of pituitary sections from mouse embryos at E10.5, E12.5, and E16.5 in the brightfield (top) and in the diagrammatic form (bottom). At E10.5 Rathke's pouch (dark gray) expresses αGSU. By E12.5 ACTH expression appears in the anterior pituitary. By E16.5 all types of hormone transcripts are detectable in a spatially restricted pattern in the anterior pituitary. B, Coronal pictures of mouse pituitary sections of E16.5 embryos. From top to bottom lays the posterior pituitary (light gray), the intermediate (dark gay) and the anterior pituitary (patterned). The anterior pituitary expresses hormone transcripts for GH, PRL, TSH,

ACTH, LH and FSH in a spatially restricted pattern, which is lost as the animal matures. Adapted from Camper et al., 2002.

The anterior pituitary originates from Rathke's pouch from the stomodeum in most species. The posterior pituitary is derived from the floor of the third ventricle and is connected to the ventricular floor through the infundibulum (Couly and Le Douarin, 1985; 1987). The intermediate lobe is a subdivision of the anterior pituitary (Kelberman et al., 2009).

The development of the pituitary gland in mouse can be divided into several steps (Figure 1.6). First a rudimentary pouch forms via thickening and invagination of the oral ectoderm at E8, followed by the formation of a definitive pouch as it extends and maintains contacts with the evaginating neural ectoderm. Finally the pouch separates from the mouth at E12.5 and expands to generate the anterior and intermediate lobes (Sheng et al., 1997). It is thought that by E16.5 all five hormone producing cells of the anterior pituitary have finished their differentiation and produce their specific hormone products (Figure 1.5; Camper et al., 2002).

During the development expression of different transcription factors is induced in spatially distinct areas in the developing pituitary by extrinsic factors secreted from surrounding tissues (Zhu et al., 2007). Specific transcription factor expression leads to particular hormone transcription in respective pituitary cell types (Treier and Rosenfeld, 1996; Watkins-Chow and Camper, 1998). As the pituitary gland develops, this pattern finally disappears and the target organs of the hypothalamus and the pituitary hormones start to regulate pituitary development by different inhibitory or stimulatory factors.

1.3.3 Development of gonadotropes

Gonadotropes form a heterogeneous cell population in the anterior pituitary, which consists of monohormonal (18% LH and 22% FSH containing cells) as well as multihormonal gonadotropes containing both LH and FSH (about 60% of the gonadotropes) (Childs, 2006). LH and FSH share a common α subunit (α GSU) with

TSH, but have distinct ß subunits, LHß and FSHß, which confer their specificities (Gharib *et al.,* 1990). FSH and LH have distinct functions in both males and females (Table 1.3).

The gonadotrope is the last anterior pituitary cell type that reaches maturation and initiates expression of its specific hormones. So far there is no single transcription factor found to be crucial and sufficient for gonadotrope development. The specification of gonadotrope cell fate occurs with the onset of nuclear receptor steroidogenic factor 1 (SF1) expression at E13.5 (Ingraham et al., 1994). Targeted disruption of the *SF1* gene leads to impaired LHß, FSHß and GnRHR expression in the pituitary (Zhao et al., 2001; Parker et al., 2002). However this defective gonadotropin expression can be reversed by GnRH treatment (Ikeda et al., 1995). Other transcription factors, including Gata2, Pitx1, Pitx2, Prop1, and Otx1, also contribute to the regulation of gonadotrope differentiation (Zhu et al., 2007).

	FSH	LH
Female	Stimulates ovary to produce steroids.	Stimulates ovary to produce steroids.
	Stimulates ovary to produce estradiol during follicular phase and progesterone during luteal phase.	LH surge at midcycle triggers ovulation.
	FSH surge at midcycle, together with LH, triggers ovulation.	Triggers ovulation and turns follicles into corpora lutea.
Male	Stimulates Sertoli cells to produce androgen-binding protein (ABP), thereby stimulates spermatogenesis.	Stimulates Leydig cells to produce testosterone, which provides negative feedback to the anterior pituitary and hypothalamus.
	Stimulates Sertoli cells to produce inhibin which provides negative feedback to the anterior pituitary.	

Table 1.3 Functions of gonadotropins in both females and males.

Basal FSHß expression is regulated by LIM homeobox protein 3 (Lhx3) (West et al., 2004), heterotrimeric nuclear factor-Y (NFY), and SF1. Lhx3 carries the LIM domain, a unique cysteine-rich zinc-binding domain, and is also expressed in other cell lineages in

the pituitary. However SF1 interacts with NFY to specifically regulate basal FSHß expression (Jacobs et al., 2003). FSHß expression can also be regulated by GnRH and members of the TGF-ß superfamily including activins (Burger et al., 2004). Mutations in either the *GnRH* or *GnRHR* gene dramatically decreased LHß and FSHß expression in the pituitary (Mason et al., 1986; Pask et al., 2005). Activin secreted by gonads and the pituitary also induces *FSHß* transcription. Mice deficient for activin or its receptor exhibit diminished levels of FSHß in the pituitary (Matzuk et al., 1995). LHß expression can be stimulated by SF1 and Egr1, a GnRH-inducible zinc finger transcription factor, which function alone or in synergy with each other (Halvorson et al., 1998; Tremblay and Drouin, 1999). Mice deficient for Egr1 exhibit diminished LHß but intact FSHß expression (Lee et al., 1996). Expression of LHß can also be stimulated by GnRH. Mutations in the *GnRH* or *GnRHR* gene decreased LHß expression in the pituitary (Mason et al., 1986; Pask et al., 2005).

1.4 Defects in the HPG axis development

Several mutations in either the *GnRH* or *GnRHR* gene have been identified and compromise GnRH signaling in the HPG axis (Cheng and Leung, 2005). Affected individuals suffer from hypogonadotropic hypogonadism (Layman, 2002).

In humans, mutations in the *GnRH* or *GnRHR* gene cause normosmic idiopathic hypogonadotropic hypogonadism (IHH) with normal olfaction (Bouligand et al., 2009; Chan et al., 2009), or Kallmann syndrome (KS) associated with hypo/anosmia (Pedersen-White et al., 2008). Patients with IHH or KS display either partial or complete lack of pubertal development (Seminara et al., 1998). In many cases the mutant peptide and receptor still retain some activities which might explain the broad spectrum of phenotypes, ranging from delayed puberty or reduced fertility, to sexual infantilism, primary amenorrhea or cryptorchidism (Seminara et al., 1998).

A naturally occurring mouse strain was identified which bears a deletion in the coding region of the *GnRH* gene (Cattanach et al., 1977; Mason et al., 1986). These mice lack

the synthesis of the GnRH decapeptide in the POA and the anterior hypothalamus, and were called GnRH-deficient hypogonadal (*hpg*) mice. *hpg* mice display a severe disruption of gonadotropin synthesis and release, and little or no postnatal gonadal development (Mason et al., 1986). These mice do not undergo puberty and stay sexually immature for their entire lives. Transplantations of fetal or early neonatal brain tissues containing GnRH neurons into the 3rd ventricle of *hpg* mice reverse the hypogonadotropic hypogonadism phenotype (Charlton, 2004).

In addition a mutation in the *GnRHR* gene was induced by N-ethyl-N-nitrosourea (ENU) mutagenesis in mice (Pask et al., 2005). A single amino acid was changed in the third transmembrane domain leading to inactivation of the receptor. These mice display hypogonadotropic hypogonadism with dramatically reduced numbers of gonadotropes and disrupted development of the gonads (Pask et al., 2005).

Interestingly, the GnRH neuronal population remains normal in both hypogonadal mouse lines. Despite deficiencies in GnRH signaling, the size of the GnRH neuronal population, their distribution throughout the anterior hypothalamus and their axonal projections were unimpaired in these mice (Gill et al., 2008), which suggests that GnRH neuronal development is independent of GnRH signaling. However it remains possible that the mutant genes or gene products retain some activity and enable normal GnRH neuronal migration.

1.5 Scientific aims and strategy

This thesis aims to address three questions:

1). What is the role of GnRH signaling during embryonic development of the HPG axis?

2). Are there GnRHR neurons in the mouse brain? If so, where are these cells? What are their physiological functions?

3). What are the physiological properties of primary mouse gonadotropes?

To be able to address these questions, a binary genetic strategy was adopted, in which different transgenes were expressed in GnRHR cells allowing *in vivo* visualization or ablation of these cells in the entire mouse.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals

Chemicals were mainly bought from Sigma, Merck, Gibco/BRL, or Roth. Molecular weight standards and restriction enzymes were obtained from Fermentas.

2.1.2 Solutions and buffers

All buffers and media were made with deionized water (ddH_2O, membranePure GmbH), and autoclaved (20 mins, 121°C) or filtered with 0.22 µm Millex–GP sterilizing filters (Millipore).

Table 2.1 List of buffers.

Antibody buffer (for IF and IHC)	0.5% lambda-carrageenan (Sigma), 0.02% sodium azide, in PBS, pH 7.4.
Betaine (5 M) (for PCR)	6.76 g betaine in 10 ml ddH_2O, autoclave.
Blocking buffer A (for IF and IHC)	5% normal donkey serum, 0.2% Triton X-100, 0.02% sodium azide, in PBS, pH 7.4.
Blocking buffer B (for IF and IHC)	1% BSA, 0.25% TX-100, in PBS, pH 7.4.
Denaturing solution (for Southern blot analysis)	1.5 M NaCl, 0.5 M KOH.

Dispersion medium (for pituitary primary cell culture)	10 mM D-glucose, 10 mM HEPES, 0.5 mg/ml BSA, in Hank´s F10 medium.
DNA loading buffer (5x) (for PCR)	50% glycerol, 60 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol FF.
Ethidiumbromide-staining solution (for electrophoresis)	10 µ g/ml ethidiumbromide, in TAE buffer.
Growth medium (for pituitary primary cell culture)	10% fetal bovine serum, 1.78 mM L-glutamine, penicillin (100 U/ml), streptomycin (1 mg/ml), in low-glucose DMEM.
Hoechst solution (for nuclear staining)	5 µ g/ml Hoechst 33258 dye, in PBS, pH 7.4.
Lysis buffer (for ear punches or embryo biopsies)	50 mM Tris-HCl, pH8, 100 mM NaCl, 0.2% NP40, 0.2% Tween, 1 mM EDTA, mg/ml proteinase K.
Low TE buffer (for dissolving gDNA)	10 mM Tris-HCl, 1 mM EDTA.
Neutralization solution (for Southern blot analysis)	0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl.
PBS (20x)	3 M NaCl, 161 mM Na_2HPO_4, 39 mM KH_2PO_4, pH 7.4.

SSC (20x) (for Southern blot analysis)	3.0 M NaCl, 0.3 M Na_3citrate, pH 7.0.
SSPE (20x) (for Southern blot analysis)	3.0 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA, pH 7.4, treated with DEPC overnight.
Speed Hyb II buffer (for Southern blot analysis)	1% (w/v) SDS, 10% (w/v) PEG, 1.5x SSPE.
TAE buffer (50x) (for gel electophoresis)	2.5 M Tris-acetate, 50 mM EDTA, pH 8.0.
TE Buffer (pH 7.4, 7.5, or 8.0)	10 mM Tris-Cl, 1 mM EDTA, adjust pH with concentrated HCl.
Tris-HCl (1 M, pH 6.8)	12.1 g Tris base in 100 ml H_2O, adjust pH with concentrated HCl.
Wash buffer A (for Southern blot analysis)	2x SSC, 0.5% SDS.
Wash buffer B (for Southern blot analysis)	0.2x SSC, 0.1% SDS.

2.1.3 Antibodies

2.1.3.1 Primary antibodies

Table 2.2 List of primary antibodies

Antibody	Host	Source	Cat.Nr./Clone	Dilution
Anti-ACTH	rabbit	NIDDK	AFP39013083	1:10000

anti-FSHß	guinea pig	NIDDK	AFP-28122491	1:1000
anti-FSHß	rabbit	NIDDK	AFP-77981289	1:1000
anti-GFP	rabbit	Invitrogen	A11122	1:5000
anti-GFP Alexa Fluor® 488 conjugate	rabbit	Invitrogen	A21311	1:800
anti-GH	guinea pig	NIDDK	AFP-222387790	1:10000
anti-GnRH	mouse	Covance	SMI-41R	1:500
anti-GnRH	rabbit	ABR	PA1-121	1:800
anti-LHß	guinea pig	NIDDK	AFP-22238790	1:500
anti-LHß	rabbit	NIDDK	AFP-C697071P	1:1000
anti-PR	rabbit	Thermo	RM-9102-S0	1:200
anti-PRL	rabbit	NIDDK	AFP-131581570	1:10000
anti-TSHß	guinea pig	NIDDK	AFP-98991	1:50000

2.1.3.2 Secondary antibodies

All Alexa Fluor 488 or 546, Cy3™ or Cy5™ conjugated secondary antibodies against goat, guinea pig, mouse and rabbit were purchased from Invitrogen or Jackson ImmunoResearch via Dianova. The dilution used was 1:500.

2.2 Methods

2.2.1 Mice

Mice were kept under a standard light/dark cycle with food and water *ad libitum*. All experiments were conducted in accordance with the guidelines established by the Animal Welfare Committee of the University of Hamburg.

2.2.2 Genomic DNA preparation

Genomic DNA (gDNA) was extracted with the DNA Isolation Reagent (AppliChem GmbH, Darmstadt, Germany) from mouse tail biopsies after overnight incubation at 55°C in tail lysis buffer supplemented with proteinase K (Roche Diagnostics, Mannheim, Germany). Ear punch tissue samples were digested in 100 µl ear lysis buffer supplemented with proteinase K.

2.2.3 Mouse genotyping

Mice were genotyped by Southern blot analysis or polymerase chain reaction (PCR).

2.2.3.1 Southern blot analysis

The gDNA samples (5 - 10 µg) were digested overnight with 20 units of restriction enzyme *Eco*RV or *Bsp*TI. The digested DNA was applied to a 0.7 % agarose gel and separated by electrophoresis. Then the gel was incubated for 45 mins in denaturing solution followed by 45 mins in neutralization solution before being blotted onto a nylon membrane 35 (Hybond N+, Amersham - Pharmacia). The DNA was transferred onto the nylon membrane by the capillary method using a reservoir of 20X SSC. After transfer the membrane was dried and the DNA was cross-linked to the membrane using UV-light.

DNA probes (20-50 ng) specific for exon 2 of the *GnRHR* gene were radioactively labeled with [α-32P]dCTP using Megaprime DNA labeling system (GE Healthcare Life Sciences) and purified over Sephadex- G50 spin columns (Probe Quant G50, Amersham Pharmacia). Before hybridization probes were denatured by boiling for 5 mins.

Prehybridization was carried out by saturating the membrane in Speed Hyb II buffer containing 1mg/1ml sonicated herring sperm DNA (denatured at 95ºC for 5 mins) at 65ºC for 30 mins in the hybridization oven. Hybridization was carried out at 65ºC for 16-24 hrs in incubation buffer containing denatured probes. To remove non-specifically bound

probes, membranes were washed with Wash buffer A and Wash buffer B at 60°C for 30 mins separately. The membrane was then sealed in plastic foil for final readout.

2.2.3.2 PCR genotyping

PCR was performed in 50 µl reaction mixture in 0.2 ml eppendorf tubes using a thermal cycler (Biometra or DNA Engine).

The reaction mixture is described as following:

gDNA template	1.0 µl
PCR buffer (10x)	5.0 µl
dNTPs (20 mM)	1.0 µl
$MgCl_2$ (25 mM)	3.0 µl
Betaine (5 M)	10 µl
DMSO	4.0 µl
Primers (25 µM)	0.5 µl for each primer
Taq-Polymerase	1 U
ddH_2O	Add ddH_2O to make a final volume of 50 µl.

2.2.3.2.1 Primers

All primers for PCR were from Eurofins MWG Operon.

Table 2.3 List of primers

Mouse	Mutant allele	Primers (from 5' end to 3' end)
GRIC	Cre	Cre 1: TAACATTCTCCCACCGTCAGTACG Cre 2: AAACGTTGATGCCGGTGAACGTGC Cre 3: TAAGAACTAGACCCAGGGTACAAT Cre 4: AACAGCAGCGAGCCCGAGTAGTG

R26-DTA	DTA	RosaFA: AAAGTCGCTCTGAGTTGTTAT RosaRA: GGAGCGGGAGAAATGGATATG SpliAcB: CATCAAGGAAACCCTGGACTACTG
R26-YFP	YFP	YFP1: AAAGTCGCTCTGAGTTGTTAT YFP2: GCGAAGAGTTTGTCCTCAACC YFP3: GGAGCGGGAGAAATGGATATG
FLIP	FLIP	Flp1: CACTGATATTGTAAGTAGTTTGC Flp2: CTAGTGCGAAGTAGTGATCAGG
Embryo (for gender identification)	X/Y	XY-F: TGAAGCTTTTGGCTTTGA XY-R: CCGCTGCCAAATTCTTTG

2.2.3.2.2 PCR conditions

Mouse	PCR condition	Pattern of Bands
GRIC	95°C : 5 mins 94°C : 30 secs 55°C : 30 secs 72°C : 45 secs } 35 cycles 72°C : 10 mins 4°C : hold	2 bands: wild-type band: 400 bp knock-in band: 200 bp
FLIP	94°C : 1 mins 94°C : 30 secs 60°C : 30 secs 72°C : 1 min } 35 cycles 72°C : 10 mins 4°C : hold	0 band: wild-type 1 band: knock-in
R26-DTA	94°C : 5 mins 94°C : 30 secs 55°C : 1 min 72°C : 2 mins } 35 cycles 72°C : 10 mins 4°C : hold	2 bands: wild-type band: 580 bp knock-in band: 320 bp
R26-YFP	95°C : 2 mins 94°C : 20 secs 55°C : 35 secs 72°C : 35 secs } 35 cycles 72°C : 10 mins	2 bands: wild-type band: 600 bp knock-in band: 250 bp

	4°C : hold		
Embryo (for gender identification)	94°C : 1 min 94°C : 30 secs 60°C : 30 secs 72°C : 1 min 72°C : 10 mins 4°C : hold	} 35 cycles	1 band: female 2 bands: male

2.2.4 Mouse tissue preparations

2.2.4.1 Mouse perfusion

Mice were anesthetized with ketamine/xylazine (Bayer, Germany) anesthesia and perfused transcardially with 10 ml 37°C PBS, then 100 ml ice cold fixative, Roti ®-Histofix 4% (Roth, Karlsruhe, Germany). Organs were removed and soaked in fixative at 4°C for 2-4 hrs. For cryostat sectioning, organs were transferred to 30% sucrose in PBS until the organs sank to the bottom, and then frozen in tissue-freezing compound, O.C.T. compound (Leica Microsystems GmbH, Wetzlar, Germany). Serial 14 or 50 µm thick sections were generated with a cryostat (CM3050; Leica, Nussloch, Germany) and collected either on SuperFrost® Plus glass slides (Roth, Karlsruhe, Germany) or PBS for further immunofluorescence (IF) analysis.

2.2.4.2 Preparation of mouse embryos

The day when the plug was observed was determined as E0.5. At 1 pm on the day when the embryos were to be collected, the pregnant female was anesthetized with Halothane (Willy Rüsch Hospital Vertriebs GmBH, Germany) and killed by decapitation. Embryos were immediately removed from the uterus and washed with ice cold PBS. Tails were cut for gender and knock-in allele genotyping. The whole embryos were soaked in ice cold Roti®-Histofix 4% on ice on a shaker for 1.5 to 6 hrs depending on the age of the embryos. For E16.75 or older embryos, the skin was removed from the backside of the embryos for better exposure to fixative. Afterwards embryos were transferred to 30% sucrose in PBS and kept in 4°C until the embryos sank to the bottom. Embryos were then

frozen in O.C.T. compound, and 14 µm sagittal sections were prepared for further IF analysis.

2.2.5 Application of LH for pregnant female mice

1 µg LH (ovarian LH, NIDDK) dissolved in 100 µl physiological saline was injected subcutaneously into pregnant female carrying E16.75 embryos. The injection was repeated twice 2 and 4 hrs after the 1st injection. 2 hrs before the preparation of E17.75 embryos, a 4th injection was applied.

2.2.6 Immunofluorescence analysis of tissue sections

Sections were blocked in blocking buffer A for 1 hr at RT and then treated at 4 °C overnight (14 µm thick sections), or 3 nights (50 µm thick sections) with primary antibodies in antibody buffer, followed by secondary antibodies for 1 hr at RT. Sections were then incubated with Hoechst solution for nuclear staining for 10 mins and coverslipped with Fluoromount-G (SouthernBiotech). Appropriate controls omitting primary antibodies were performed and did not yield any staining.

2.2.7 Hematoxylin and eosin (H&E) stain

For gonad histology, 14 µm thick sections of testis, ovaries and uterus were air-dried overnight and hydrated in 50% ethanol. They were then stained with hematoxylin (Fisher Scientific) for 1 min, rinsed in tap water for 10 mins, and counterstained with eosinY (Sigma) for 1 min. Sections were dehydrated in ethanol and xylene baths and coverslipped with mounting medium DPX (Sigma).

2.2.8 Pituitary primary cell culture

Mice were anesthetized with Halothane and killed by decapitation. Pituitaries were quickly removed and transferred into Dispersion medium. Pituitaries were cut into small pieces and digested with collagenase type CLS-II (678 U/ml in dispersion medium; Biochrom KG, Berlin, Germany) for 30 mins at 37°C. Cells were gently triturated with

glass pipettes, centrifuged for 15 mins at 4°C and resuspended in Growth medium. The cell suspension was then plated on 8-chamber slides (Nunc International, New York, USA) precoated with poly--ornithine (Sigma), and kept overnight at 37°C in a humidified incubator with 5% CO_2. This experiment was done by Ai Wei.

2.2.9 Quantitation of pituitary hormone producing cells

Cells were fixed on 8-chamber slides with 4% paraformaldehyde (PFA) containing 4% sucrose for 10 mins at RT and blocked in blocking buffer B. Individual chambers were then treated with different primary antibodies overnight at 4°C in blocking buffer B, followed by secondary antibodies for 1 hr at RT. After removal of the medium chambers, slides were treated with Hoechst solution for nuclear staining and mounted with Fluoromount-G. Appropriate controls omitting primary antibodies were performed and did not yield any staining.

To determine the relative abundance of hormone producing cells, four pictures representing non-overlapping areas (each 1900 X 2600 μm^2) were taken for each chamber (staining). Individual cells identified by the antibody staining were counted using ImageJ software. This was done by the Ai Wei.

2.2.10 Quantitation of GnRH neurons

14 µm thick coronal brain sections of adult mouse brains were stained with rabbit anti-GnRH antibody followed by Alexa Fluor 488 conjugated secondary antibody. Numbers of GnRH neurons in sections corresponding to figure 19-50 (Bregma 1.42 mm to -2.30 mm) in the Mouse Brain in Stereotaxic Coordinates (Paxinos and Franklin, 2001) were counted, and summed to calculate the total number of GnRH neurons in the hypothalamus. Numbers of GnRH neurons in sections corresponding to figure 19-30 (Bregma 1.42 mm to 0.14 mm) were counted for the total number in the anterior hypothalamus.

2.2.11 Measurement of immunofluorescence intensity

Photographs used for IF intensity measurement were taken with the same exposure time in each control and experimental group. ImagJ software was used for the measurements of IF intensity. Mean values of 20 cells chosen randomly from each photograph were used for analysis and comparison.

2.2.12 Measurement of circulatory levels of gonadotropins

Mice were killed by CO_2 exposure between 13 and 15 pm. Blood was collected from the heart using heparinized syringes and then centrifuged at 1000 g for 10 mins at 4°C. The plasma supernatant samples were collected and stored at -80°C until assayed. The hormone assays for FSH and LH were done by Brigitte Mann (Northwestern University, USA).

2.2.13 Photographic documentation

Tissue sections were analyzed using a confocal laser-scanning microscope (Fluroview 1000, Olympus) or an Axionskop 2 microscope equipped with AxioVision software (Zeiss). Photographic images were processed using Adobe Photoshop CS3 and Adobe Illustrator CS3 software (Adobe Systems, San Jose, CA).

2.2.14 Statistical analysis

All numerical data is presented as mean values ± SEM. Statistical significance was analyzed using the student's t-test. The threshold value for significant difference was $P < 0.05$.

3. Results

3.1 Genetic labeling of GnRH target cells in mice

3.1.1 Generation of GnRHR-IRES-Cre (GRIC) mice

So far different approaches have been employed to indentify GnRH target cells (GnRHR cells) in the brain. Receptor binding studies using radiolabeled GnRH peptide (Badr and Pelletier 1987; Jennes et al. 1988) and *in situ* hybridization studies (Jennes et al. 1997) with GnRHR probes have been described. In addition a transgenic mouse model in which a 3.3 kb fragment of the rat *GnRHR* promoter driving expression of *hPLAP* gene in GnRHR cells has also been generated (Granger et al., 2004). None of these studies provide means to manipulate live GnRHR cells *in vivo*, to visualize, stimulate or even ablate them in live animals. Therefore in this study a binary genetic strategy was adopted to express different transgenes in GnRHR cells, which would allow *in vivo* visualization and manipulation of these cells in mouse. This binary strategy was achieved by firstly generating a GnRHR specific Cre knock-in mouse, in which a *Cre* recombinase gene was placed downstream of the endogenous *GnRHR* promoter to avoid potentially ectopic Cre recombinase expression in cell types or regions not associated with the original specificity of the *GnRHR* promoter, then breeding this knock-in mouse with different Cre reporter mouse strains carrying various reporter genes to facilitate Cre- mediated expression of these genes in GnRHR cells.

The generation of the Cre knock-in mouse was accomplished by homologous recombination in ES cells (Nagy et al., 2003). A clone containing exon 3 of the *GnRHR* gene (Figure 3.1) was isolated from a 129/SvJ mouse genomic library (Stratagene) and subcloned into the pKO-V901 plasmid (Lexicon Genetics) with a *phosphoglycerate kinase (pgk)* promoter-driven diphtheria toxin A cassette. An *Asc*I restriction enzyme site was created 3' to the stop codon of the *GnRHR* gene using PCR mutagenesis. An IRES-Cre-FRT-PGKneo-FRT cassette was obtained from J. Gogos and R. Axel (Columbia

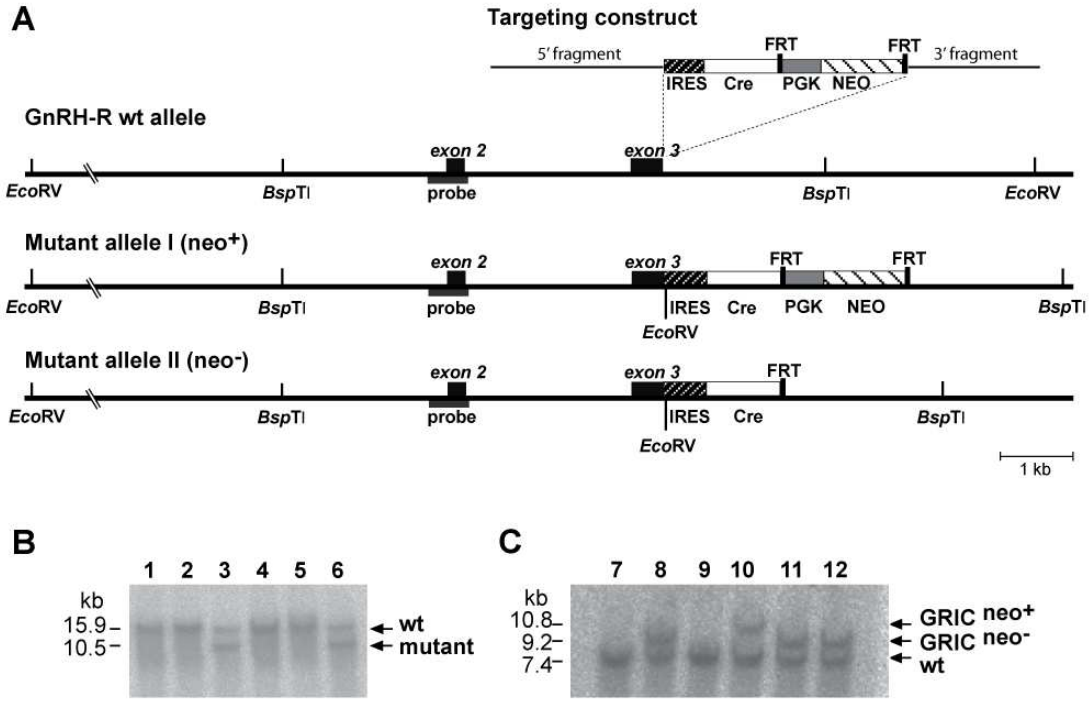

Figure 3.1 Targeted integration of the IRES-Cre cassette into the *GnRHR* locus.

A, Schematic representation of the targeting strategy used to express Cre recombinase under control of the *GnRHR* promoter. From top to bottom, the targeting vector, the *GnRHR* wild-type (wt) allele, and the targeted *GnRHR* allele before (neo^{+}) and after (neo^{-}) removal of the neo cassette are shown. Restriction sites for *Eco*RV and *Bsp*TI, as well as the location of the probe are indicated. Black boxes represent exons 2 and 3. The inserted cassette is composed of an internal ribosomal entry site (IRES) followed by the coding sequence for Cre recombinase (Cre), and a *pgk* promoter driven neo selection cassette flanked by Flp recombinase recognition sites (FRT). B, Southern blot analysis of DNA from wild-type and heterozygous mutant mice after digestion with *Eco*RV. The expected fragment sizes detected by the probe used for hybridization (shown in A) are indicated (wild-type, 15.9 kb; mutant, 10.5 kb). Mice Nos. 3 and 6 carry the mutant *GnRHR* allele (GRIC^{neo+}). C, Southern blot analysis of DNA digestion with *Bsp*TI from wild type, and heterozygous mutant mice before and after removal of the neomycin selection cassette. The expected fragment sizes detected by the probe shown in A are indicated (wild-type, 7.4 kb; mutant allele I GRIC^{neo+}, 10.8 kb; mutant allele II GRIC^{neo-}, 9.2 kb). Mouse No.10 carries mutant allele I GRIC^{neo+}, and mice Nos. 8, 11, and 12 carry mutant allele II after Flp recombinase-mediated excision of the neomycin cassette (GRIC^{neo-}).

University) (Eggan et al., 2004). This cassette contains, in order, an IRES (internal ribosome entry site) sequence, the coding region of Cre recombinase, an FRT site, a neomycin selection cassette under the control of the promoter of the *pgk* gene, and another FRT site (IRES-Cre-FRT-PGKneo-FRT). The cassette is flanked by *Asc*I sites and was then inserted into the *Asc*I site of pKO-GnRHR, resulting in the final targeting vector, 5' GnRHR sequence-IRES-Cre-FRT-PGKneo-FRT- GnRHR 3' flanking sequence (Figure 3.1. A). The integrity of the construct was confirmed by restriction mapping and sequencing. The targeting construct was next used to prepare ES cells in which one wild-type *GnRHR* allele was replaced by the altered *GnRHR* allele (Figure 3.1. A). After transfection of ES cells, G418-resistant clones were screened by Southern blot analysis for homologous recombination with external probes (Figure 3.1. A). 2 recombinant ES cell clones were used to prepare chimeric mice (by blastocyst injection (Nagy et al., 2003) into C57BL/6J embryos at the ZMNH transgenic animal facility). Cloning of the targeting construct and evaluation of targeted embryonic stem cells were done by Dr. Ulrich Boehm.

The chimeric mice were then bred with C57BL/6J mice to obtain mice heterozygous for the altered *GnRHR* allele ($GRIC^{neo+}$ mice) (Figure 3.1. B). These mice still containing the neo resistance cassette were then bred with Flp deleter mice to delete the neo selection cassette and obtain mice carrying the mutated *GnRHR* allele but not the neo resistance cassette ($GRIC^{neo-}$ mice) (Figure 3.1.C) (Rodriguez et al., 2000). Heterozygous $GRIC^{neo-}$ (GRIC) mice were interbred to obtain mice homozygous for the altered allele. GRIC mice are viable, fertile, and produce litters with frequency and size indistinguishable from those of wild-type animals breeding in the same animal house (data not shown).

3.1.2 Fluorescent visualization of gonadotropes

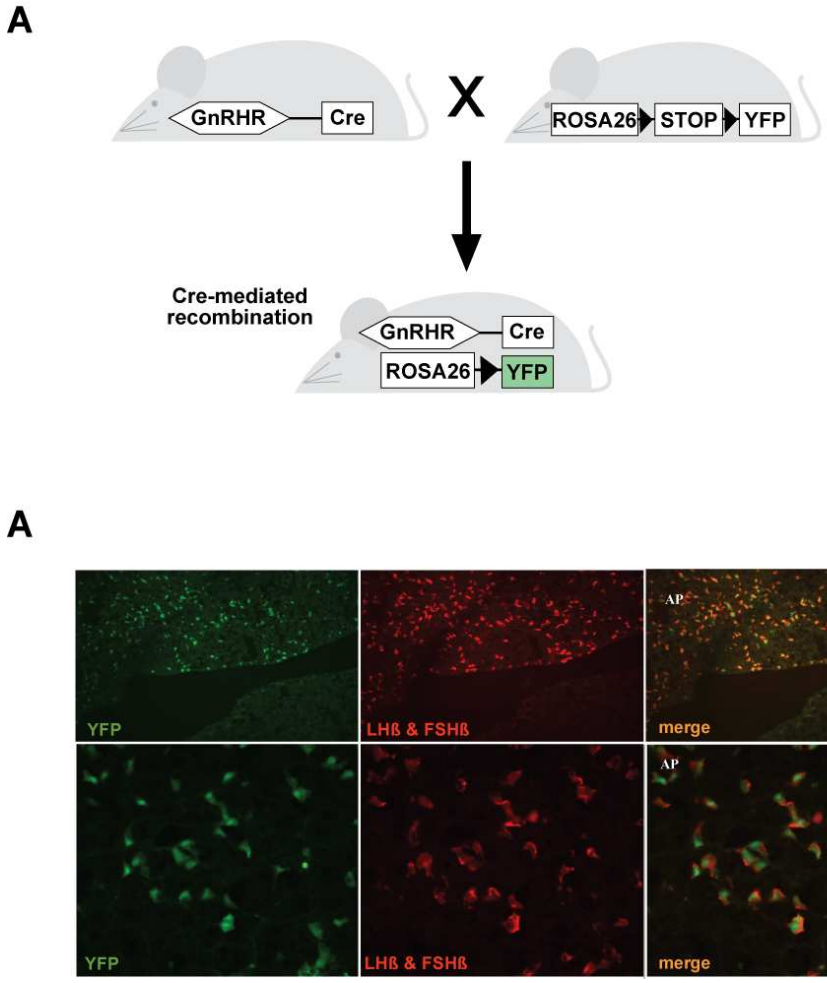

Figure 3.2 Cre recombinase mediated YFP expression in gonadotropes.

A, Breeding strategy to activate YFP expression in GnRHR cells. Coexpression of Cre recombinase with GnRHR leads to excision of the floxed stop cassette, which in turn activates ROSA26 driven transcription of *YFP* in GRIC/R26-YFP double knock-in mice. B, IF analysis of pituitary sections prepared from GRIC/R26-YFP mice using antibodies against LHß and FSHß. Nearly all gonadotropin-containing cells (*red*) display YFP fluorescence (*green*), demonstrating faithful activation of the *ROSA26-YFP* reporter gene in gonadotropes. Abbreviations: AP, anterior pituitary; PP, posterior pituitary.

To monitor Cre recombinase activity in GRIC mice, these mice were bred to ROSA26-YFP (R26-YFP) mice, which carry a targeted insertion of a *YFP* gene in the ubiquitously expressed *ROSA26* locus (Soriano, 1999; Srinivas et al., 2001). Due to a *loxP* flanked (floxed) strong a transcriptional terminator, the *R26-YFP* allele terminates transcription prematurely. However when these mice are bred with Cre-expressing mice, the Cre-mediated excision of the floxed terminator leads to constitutive YFP expression (Figure 3.2.A). Therefore gonadotropes in GRIC/R26-YFP double-heterozygous mice should express YFP from the recombined *R26-YFP* allele and be identifiable by their endogenous fluorescence signal (Figure 3.2.A).

Pituitary slices prepared from GRIC/R26-YFP mice contained brightly fluorescent cells in the anterior pituitary (Figure 3.2. B), but not in the posterior pituitary, consistent with the distribution of gonadotropes in the mouse pituitary (Baker and Gross, 1978). IF analysis on pituitary sections prepared from GRIC/R26-YFP mice using antibodies against LHß and FSHß showed that 99.9% of gonadotropin-containing cells are labeled by YFP fluorescence (Figure 3.2. B), demonstrating faithful activation of the *ROSA26-YFP* reporter gene in gonadotropes. YFP fluorescent cells without immunofluorescent LHß/FSHß signals were observed with a frequency of 1.8%. These cells may produce gonadotropins at a level below detection threshold.

3.1.3 Initial characterization of YFP-tagged gonadotropes

Gonadotropes form a small cell population, which is scattered throughout the anterior pituitary and thus difficult to isolate and characterize (Lloyd and Childs, 1988). Earlier functional characterizations of gonadotropes were performed mainly in gonadotrope-derived cell lines (Windle et al., 1990; Thomas et al., 1996). Fluorescent tagging of gonadotropes in GRIC/R26-YFP mice provides a simple means to identify and isolate primary gonadotropes for further characterization of GnRH signaling in these cells. In collaboration with Prof. Schwarz and his colleagues (ZMNH, Hamburg), the physiological properties of primary fluorescent gonadotropes prepared from male adult

GRIC/R26-YFP mice were studied using electrophysiology, calcium imaging, and the reverse hemolytic plaque assay (RHPA) (Wen et al., 2008).

Pituitary primary cell cultures were prepared from male adult GRIC/R26-YFP mice. YFP-positive (YFP+) cells were detected by their endogenous fluorescence using a YFP filter set (Figure 3.3. A). Fluorescent cells were observed with a mean frequency of 15.4%. For electrophysiological experiments only those cells that were not in contact with other cells were chosen. After selection of a particular cell, the experiment was continued in the bright-field mode of the microscope.

It was found that the resting potential of male mouse gonadotropes varies between -13 and -75 mV with a mean of -52 mV and that about 25% of gonadotropes were spontaneously active. After GnRH application, an initial hyperpolarization followed by various types of membrane potential changes was observed in all responding cells. Some cells initiated oscillations of the membrane potential of low frequency (~ 0.2 Hz), whereas cells that were initially silent responded to GnRH with ongoing firing of action potentials after initial transient hyperpolarizations. Gonadotropes with more depolarized resting membrane potentials responded to GnRH either with a large sustained hyperpolarization or with the development of slow membrane potential oscillations consisting of hyperpolarizing and depolarizing waves.

Calcium imaging experiment was also performed in YFP-tagged gonadotropes prepared from GRIC/R26-YFP male mice. Fura-2 fluorescence was measured as an indicator for a change in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). After application of GnRH, a broad range of response patterns were observed. Of 25 cells (from four mice), 23 responded to GnRH (10 nM) with an increase in $[Ca^{2+}]_i$. Most cells (n = 15) exhibited oscillations in $[Ca^{2+}]_i$ ranging between 0.08 and 0.25 Hz (0.17 ± 0.02 Hz; mean ± SEM); eight cells responded with a plateau-like increase. Figure 3.3 shows two examples. One cell responded to application of 10 nM GnRH with a biphasic response, consisting of an initial transient and a subsequent plateau-like increase in $[Ca^{2+}]_i$ (Figure 3.3.B). The other

cell responded with slow oscillations in the $[Ca^{2+}]_i$ of 0.12 Hz (Figure 3.3. C). No GnRH-induced increase in $[Ca^{2+}]_i$ was observed in non-fluorescent cells (Wen et al., 2008).

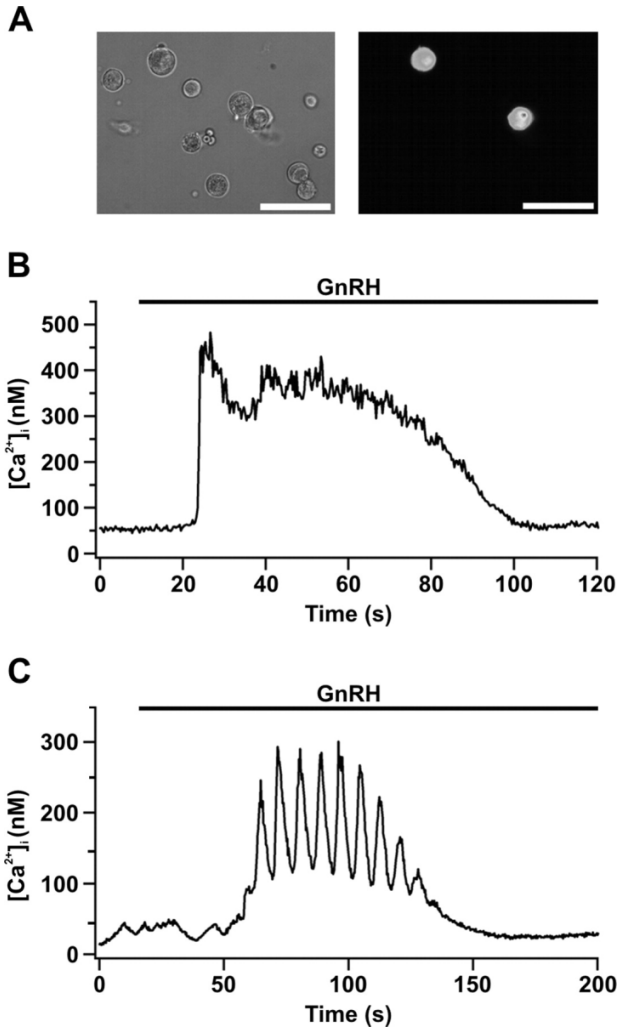

Figure 3.3 Calcium imaging reveals a large heterogeneity of GnRH responses in gonadotropes.

Primary pituitary cells from GRIC/R26-YFP mice held in culture were loaded with fura-2/AM, and the changes in $[Ca^{2+}]_i$ were recorded. A, The transmission *(left)* and the YFP fluorescence (*right*) images of YFP-tagged gonadotropes. *Scale bar*, 50 µm. B and C, Examples of GnRH responses recorded in two different cells. *Bars above the traces* indicate the presence of GnRH. (Adapted from Wen et al., 2008).

To determine whether all gonadotropes can be identified by their secretion of LH/FSH, the reverse hemolytic plaque assay (RHPA) was performed on pituitary cells obtained from GRIC/R26-YFP mice (Figure 3.4). GnRH (200 nM) was present during the incubation period in some of the Cunningham chambers, whereas in other chambers, no GnRH was applied. It was shown that only about 50% of the fluorescent cells exhibited hemolytic plaques (without GnRH: 46.6%, with GnRH: 51.4%; 482 fluorescent cells evaluated) (Figure 3.4. B). Figure 3.4. A shows examples of fluorescent cells with and without plaque formation. This result shows that by using the RHPA, even in the presence of GnRH, only about 50% of gonadotropes can be detected.

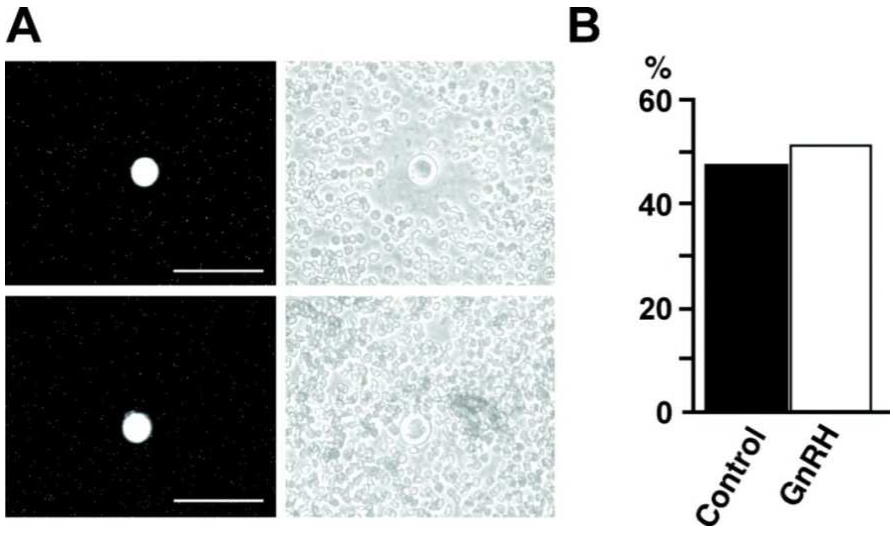

Figure 3.4 LH/FSH secretion of single gonadotropes detected with the RHPA.

The RHPA was performed on pituitary cells prepared from GRIC/R26-YFP mice. A, Example of a fluorescent cell surrounded by a hemolytic plaque (*top*) and another fluorescent cell without plaque (*bottom*); *scale bar*, 50 µm. B, Percentage of plaque-forming cells in the absence (*control*) or presence of 200 nM GnRH during the incubation period. (Adapted from Wen et al., 2008).

In summary, in primary gonadotropes prepared from GRIC/R26-YFP male mice, heterogeneity was demonstrated in terms of their electrophysiological properties, effects

of GnRH on intracellular calcium concentrations, and resting and GnRH-stimulated LH and FSH secretion.

3.2 Ablation of GnRHR cells in mice

3.2.1 Genetic strategy to ablate GnRHR cells

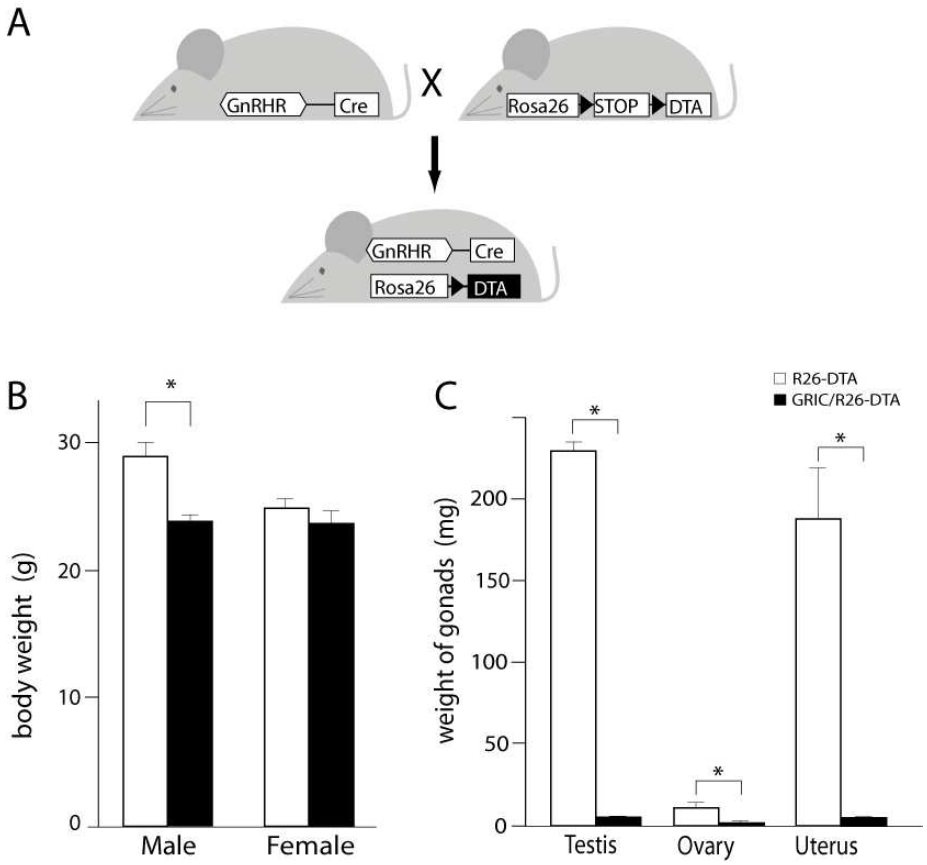

Figure 3.5 Genetic ablation of GnRHR cells leads to hypogonadism.

A, Genetic strategy to ablate GnRHR cells. Coexpression of Cre recombinase with GnRHR leads to excision of the floxed transcription terminator, which in turn activates ROSA26 driven transcription of *DTA* in GRIC/R26-DTA double knock-in mice, resulting in death of GnRHR cells. B, Compared to R26-DTA control mice, the body weight was significantly decreased in GRIC/R26-DTA male mice, but not in

female mice. C, The weight of gonads was dramatically decreased both in GRIC/R26-DTA male and female mice.

GnRHR expression has been described at all levels of the HPG axis. It was shown to be present in the pituitary (Clayton & Catt, 1981), in the CNS (Jennes et al. 1997), and in the gonads (Olofsson et al. 1995; Bull et al., 2000) which are further supported by our finding in GRIC/R26-YFP mice that YFP-tagged cells are present in the anterior pituitary (Wen et al., 2008), brain (Figure 3.17) and gonads (data not shown), suggesting various functions of GnRHR cells in the mouse. In order to study the functions of GnRHR cells in the HPG axis, cell death was induced in these cells by expressing diphtheria toxin A fragment (*DTA*) gene specifically in GnRHR cells, to check what kind of defects are caused in gonadal maturation, gonadotrope development and GnRH neuronal migration in mice.

	Female		Male	
	R26-DTA (n = 5)	GRIC/R26-DTA (n = 10)	R26-DTA (n = 8)	GRIC/R26-DTA (n=4)
Kidneys	299.6±9.3	244.8±5.5*	459.6±15.2	263.5±9.9#
Liver	1,123.2±50.8	943.0±25.9**	1,504.4±51.6	987.0±50.3#
Adrenals	8.31±1.35	6.65±0.68	8.06± 1.01	7.54±1.59
Brain	415.8±73.5	465.7±9.0	493.6±6.4	499.5±7.3
Spleen	75.0±1.7	76.5±3.2	79.9±5.7	96.3±8.6
Heart	116.2±2.5	100.1±3.8*	160.0±4.5	98.8±5.6#

Table 3.1 Organ weights of GRIC/R26-DTA and R26-DTA mice.

Organ weights (in mg) of GRIC/R26-DTA and R26-DTA mice. *, $P < 0.05$; **, $P < 0.005$; #, $P < 0.001$. All P values are based on the comparison between GRIC/R26-DTA and R26-DTA mice of the same gender.

GRIC mice were bred to ROSA26-DT-A (R26-DTA) mice (Figure 3.1), which carry a targeted insertion of the *DTA* gene in the *ROSA26* locus (Soriano, 1999; Brockschnieder et al., 2006), to produce GRIC/R26-DTA double knock-in offspring (Figure 3.5.A). The

A chain of diphtheria toxin inhibits translation by catalyzing the ADP ribosylation of the eukaryotic elongation factor 2 and leads to cell death (Collier, 2001). In GRIC/R26-DTA mice, Cre-mediated recombination triggers DTA expression (Brockschnieder et al., 2006) and leads to ablation of Cre expressing cells, the GnRHR cells in these mice (Figure 3.5.A). GRIC/R26-DTA mice were born at Mendelian frequencies and viable. The body weight of male GRIC/R26-DTA mice was decreased significantly compared to R26-DTA littermates, whereas no significant reduction was observed in female GRIC/R26-DTA mice (Figure 3.5.B and Table 3.1). The weight of different organs in GRIC/R26-DTA mice was also analyzed. The weight of the heart, kidneys and liver was significantly reduced in both male and female GRIC/R26-DTA mice, while the weight of adrenals was only decreased in GRIC/R26-DTA males (Table 3.1).

3.2.2 GRIC/R26-DTA mice display hypogonadotropic hypogonadism

Ablation of GnRHR cells in GRIC/R26-DTA mice led to underdeveloped gonads in these mice. Although the length of the thread-like uterus and the genital-anal distance were not significantly changed (Figure 3.6 and data not shown), both size and weight of the testis, ovary and uterus were dramatically reduced in GRIC/R26-DTA mice compared to R26-DTA littermates (Figure 3.5.C and Figure 3.6). Histological abnormalities were also found in the gonads of both male and female GRIC/R26-DTA mice (Figure 3.6). Testis histology revealed drastically smaller seminiferous tubules and the absence of spermatids and spermatozoa in mutant males, representing incomplete spermatogenesis in these mice (Figure 3.6). The ovaries of GRIC/R26-DTA mice contained a large number of small atretic follicles, and only few of these follicles contained immature oocytes. However in R26-DTA control animals many more follicles were observed containing oocytes at different developmental stages (Figure 3.6). In addition, far less uterine glands were found in the endometrial layer of the uterus in GRIC/R26-DTA mice than in littermate controls (Figure 3.6). Circulatory levels of FSH and LH were also measured in GRIC/R26-DTA mice, which show significant reductions in both male and female mutants compared to R26-DTA control animals (Figure 3.7). All these data reveal severe hypogonadotropic hypogonadism phenotype in mice with ablation of GnRHR cells.

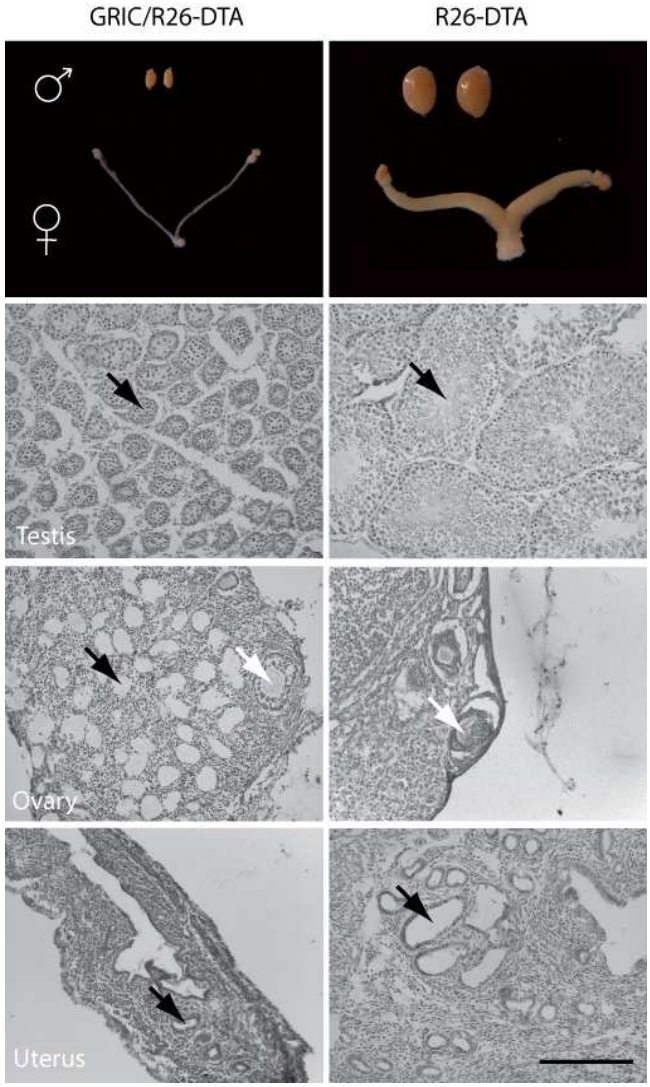

Figure 3.6 Defects in gonadal development in GRIC/R26-DTA mice.

The sizes of testis, ovary and uterus were profoundly reduced in GRIC/R26-DTA mice compared to R26-DTA littermates. Immature seminiferous tubules (*arrows*) without spermatids and spermatozoa in the testis were oberserved in GRIC/R26-DTA mice, indicating incomplete spermatogenesis. In contrast, mature seminiferous tubules containing spermatogonia, spermatocytes and spermatids were found in control littermates. The ovaries of GRIC/R26-DTA mice contained small atretic follicles (*black arrow*) and few oocytes (*white arrows*) and the uterus contained a reduced number of uterine glands (*arrows*) in the endometrial layer in these mice.. Sections were stained with hematoxylin & eosin. *Scale bar*, 200 µ m.

3.2.3 FSHß+/TSHß+ bihormonal cells are not ablated in GRIC/R26-DTA mice

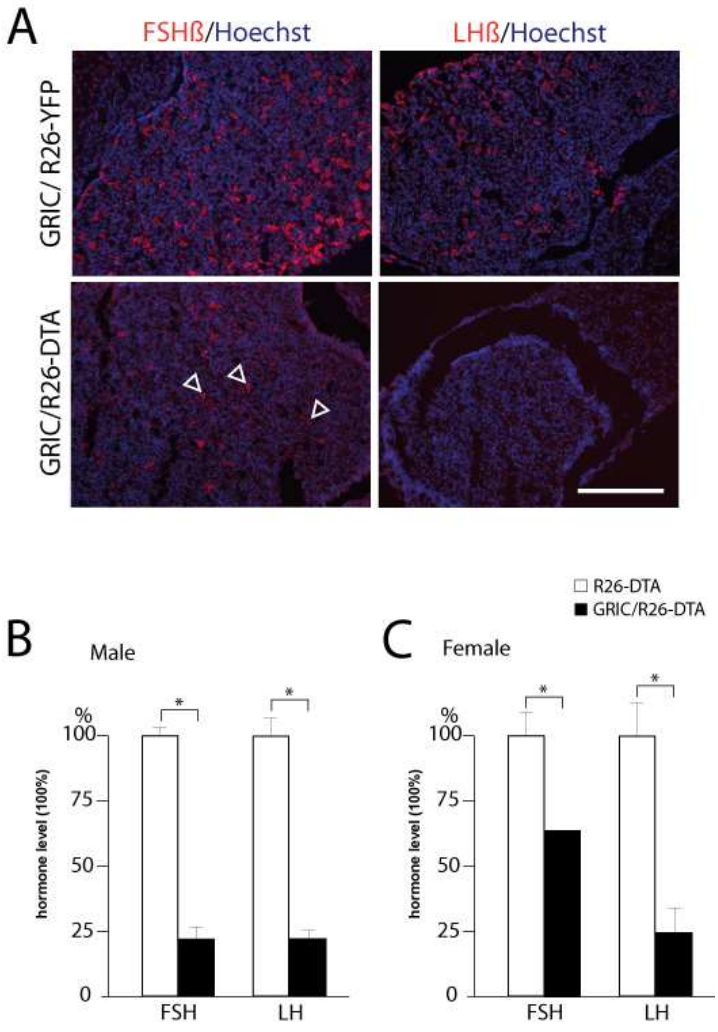

Figure 3.7 Gonadotropin expression and secretion in GRIC/R26-DTA mice.

A, IF staining for LH β or FSH β in pituitary sections prepared from GRIC/R26-DTA and R26-DTA male mice. Whereas LHß+ gonadotropes were efficiently ablated, a significant number of FSHß+ gonadotropes (*white arrows*) were detected in GRIC/R26-DTA mice. Nuclei were stained with Hoechst (*blue*). B and C, Circulatory levels of FSH and LH were significantly decreased in GRIC/R26-DTA mice compared to R26-DTA control animals in both genders. *Scale bar*: 200 µm.

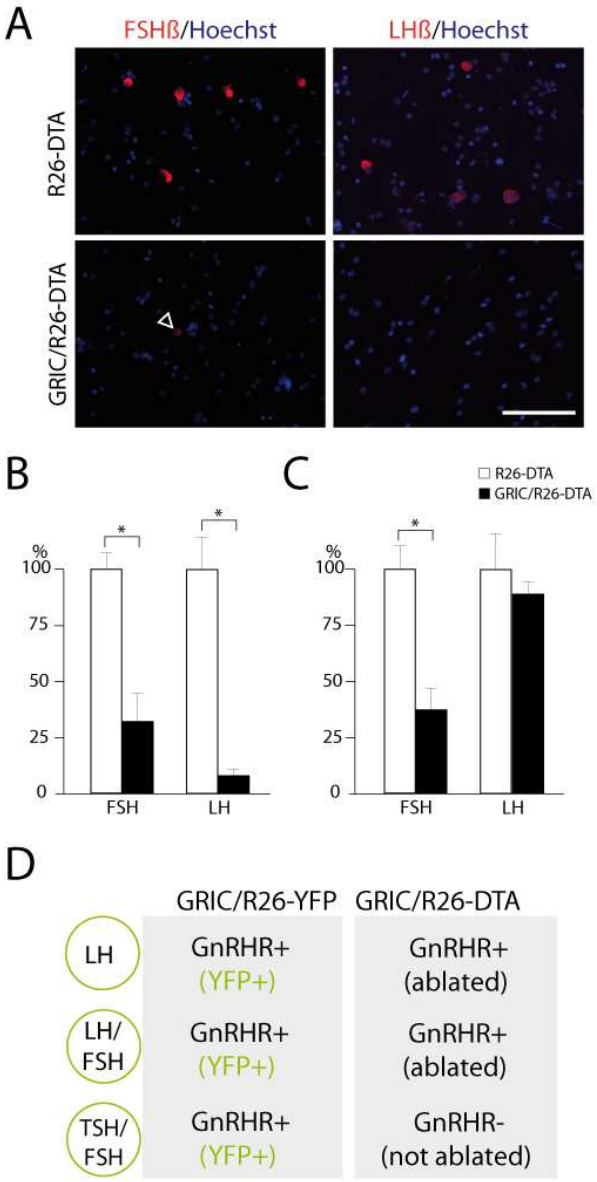

Figure 3.8 Efficient ablation of LHß+ but not FSHß+ gonadotropes in GRIC/R26-DTA mice.

A. IF for LHß or FSHß in pituitary primary cell cultures prepared from GRIC/R26-DTA and R26-DTA mice. *Scale bar*, 100 µm. B. Comparison of ablation efficiency between LHß+ and FSHß+ gonadotropes. 94.4% of LHß+ gonadotropes were ablated in GRIC/R26-DTA mice, whereas only 68.3% FSHß+ gonadotropes were reduced in these mice compared to R26-DTA littermates. C. IF

intensity of FSHß but not LHß was significantly decreased in GRIC/R26-DTA mice compared to R26-DTA mice.

Pituitary sections prepared from GRIC/R26-DTA mice were analyzed by IF using antibodies against LHβ or FSHβ (Figure 3.7). As shown in chapter 3.1.2, more than 99% of gonadotropes express Cre recombinase and display YFP fluorescence in GRIC/R26-YFP mice. Consistent with this, pituitary sections of GRIC/R26-DTA mice showed a dramatic reduction of cells expressing LHβ (LHß+) (Figure 3.7. A). Unexpectedly however, a clearly reduced but significant number of FSHβ producing cells (FSHß+) were present in the anterior pituitary of GRIC/R26-DTA mice (Figure 3.7. A; Figure 3.8. A, B). The IF intensity of these FSHß+ gonadotropes in GRIC/R26-DTA mice was significantly reduced compared to R26-DTA mice (Figure 3.8. C). Since gonadotropes are heterogeneously distributed in the anterior pituitary (Baker and Gross, 1978), LHß+ and FSHß+ gonadotropes were quantified in pituitary primary cell cultures to accurately determine the efficiency of gonadotrope ablation in GRIC/R26-DTA mice. LHß+ gonadotropes were found with a mean frequency of 0.2% in mutant mice compared to 3% observed in control animals, which corresponds to a reduction by 94.4% (Figure 3.8. B). In contrast, FSHß+ gonadotropes were only reduced by 68.3% in the mutant mice (Figure 3.8. B). These data suggest that the remaining FSHß+/LHß- gonadotropes in GRIC/R26-DTA mice do not express GnRHR and thus escape ablation (Figure 3.8. D). Circulatory levels of gonadotropins were significantly decreased in both male and female GRIC/R26-DTA mice.

Next it was examined whether the FSHß+/LHß-/GnRHR- gonadotropes in GRIC/R26-DTA mice express other anterior pituitary hormones (Figure 3.9.A). Double IF analyses show that 91.6% of the FSHß+ gonadotropes in GRIC/R26-DTA mice expressed TSHß compared to 4.2% in littermate controls. The percentage of bihormonal FSHß+/TSHß+ gonadotropes in FSHß+ gonadotropes was significantly increased in GRIC/R26-DTA mice, however not in littermate controls (Figure 3.9. B). A small percentage of FSHß+ gonadotropes in mutant mice expressed ACTH, however in control mice the percentage was significantly increased and 5.1% of FSHß+ gonadotropes expressed ACTH (Figure

3.9.C). Coexpression of FSHß with neither GH nor PRL was found in both mutant and control animals (Figure 3.9. A).

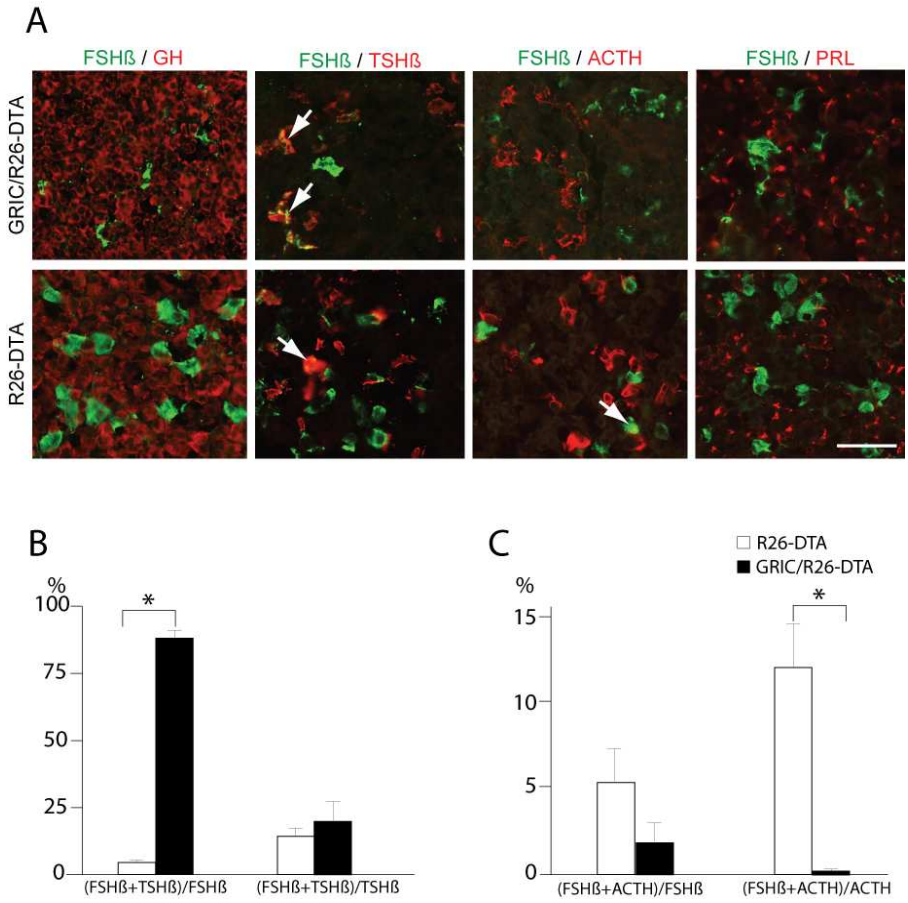

Figure 3.9 Non-ablated FSHß+ gonadotropes in GRIC/R26-DTA mice coexpress TSH β .

A, Double IF analyses for FSH β (*green*) and TSHß, ACTH, GH or PRL (*red*), respectively in pituitary sections prepared from GRIC/R26-DTA and R26-DTA mice. Coexpression of FSHß with either TSHß or ACTH within single gonadotropes was observed in both mutant and control animals. *Scale bar*: 50 µm. B, The percentage of bihormonal gonadotropes coexpressing FSHß and TSHß was significantly increased in FSHß+ gonadotropes, but not in TSHß+ cells in GRIC/R26-DTA mice. C, The percentage of bihormonal gonadotropes expressing both FSHß and ACTH was drastically decreased in ACTH+ cells, but not in FSHß+ gonadotropes in GRIC/R26-DTA mice compared to R26-DTA littermates.

3.2.4 Temporal orchestration of GnRH signaling in the anterior pituitary during embryonic development

Heterogeneity in YFP-tagged gonadotropes prepared from GRIC/R26-YFP adult male mice was well characterized (Chapter 4.1.3; Wen et al., 2008). Of particular interest is whether this heterogeneity also exists in embryonic gonadotropes. Do gonadotropes respond to GnRH heterogeneously during embryonic development? Does GnRH signaling play a role in embryonic development of gonadotropes? The GRIC/R26-YFP mouse provides a feasible tool to answer these questions, as the activity of *GnRHR* promoter is coupled to YFP expression in these mice, presenting a sensitive fluorescent readout for *GnRHR* promoter activity and thus GnRH signaling in the pituitary during mouse embryonic development.

To monitor the initiation of GnRHR expression in mouse embryonic pituitary, YFP expression was examined on pituitary sections prepared from GRIC/R26-YFP mouse embryos at different embryonic days. YFP expression was detected at E12.75 (Figure 3.10), much earlier than E16.75, when LHß and FSHß start to be expressed (Figure 3.11). However none of these YFP+ cells coexpressed α GSU at this age, in contrast to the observations made in embryos of E16.75 and E18.75, in which a significant number of YFP+ cells contained α GSU (Figure 3.10). α GSU is the common subunit for gonadotropins and TSH. It was suggested that α GSU expression is regulated by different cis-acting elements in gonadotropes and thyrotropes (Kendall et al., 1991). These data indicate that α GSU might be the pioneer molecule for TSH expression in thyrotropes, while GnRH signaling navigates the development of gonadotropes in the early stages and initiates expression of α GSU, LHß and FSHß in these cells.

In addition LHß+ and FSHß+ gonadotropes become GnRH responsive in a temporal orchestration during mouse embryonic development. In E16.75 GRIC/R26-YFP mouse embryos, virtually nearly all LHß+ cells were YFP, while none of the FSHß+ cells was YFP+ (Figure 3.11), indicating FSHß+ gonadotropes are not GnRH responsive and FSHß

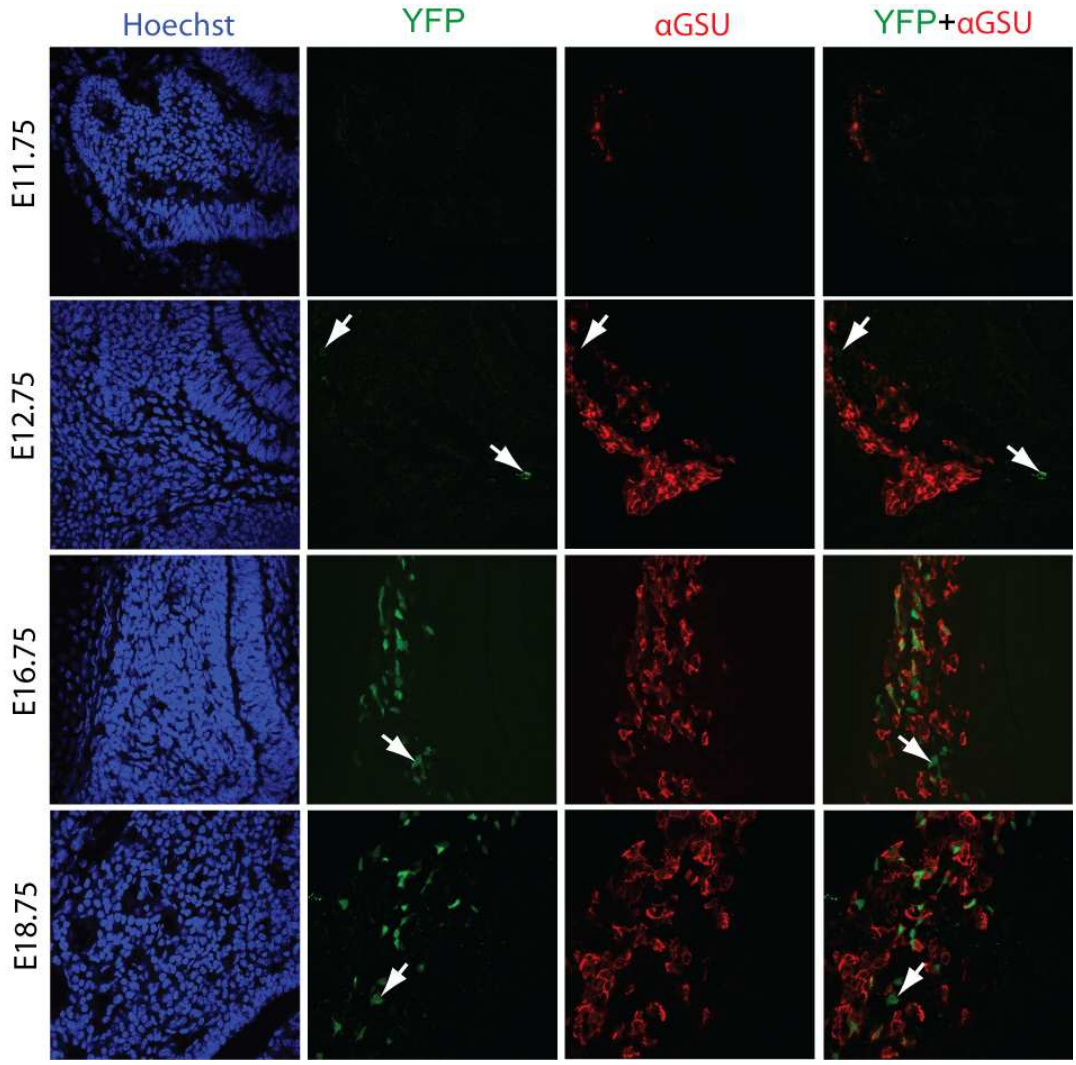

Figure 3.10 GnRH regulation of α GSU expression in the moue embryonic pituitary.

α GSU expression was detected in E11.75 embryos. And YFP expression was observed in E12.5 embryos, which was not co-localized with α GSU (*white arrows*). However in embryos of E16.75 and E18.75, a significant number of YFP+ cells coexpressed α GSU.

expression is independent of GnRH signaling at this age. However in E18.75 mouse embryos or P0 newborn pups, some of FSHß+ gonadotropes were YFP+ (Figure 3.11). All these indicate that initiation of FSHß expression needs signals mediated not by GnRH

but possibly by LH, and later on, GnRH signaling is turned on sequentially in FSHß+ gonadotropes during mouse embryonic development.

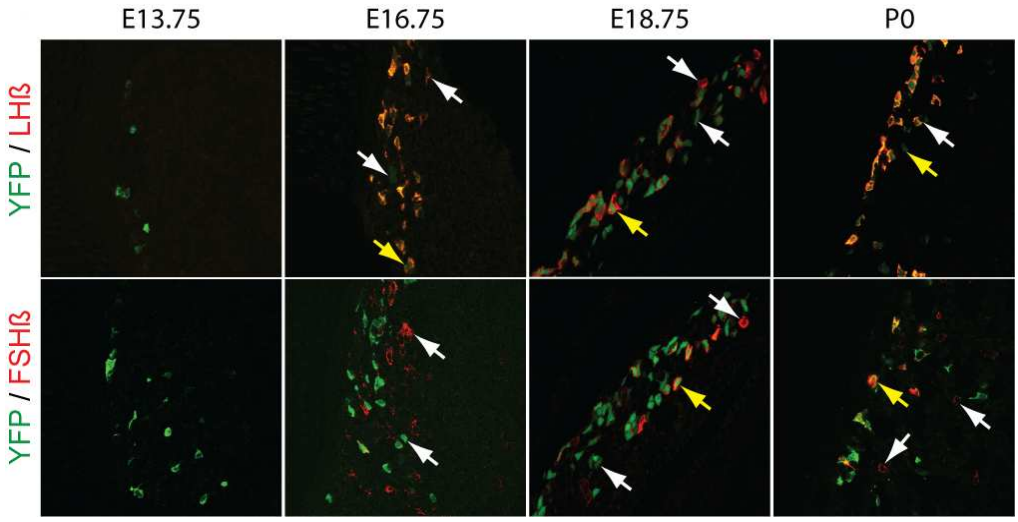

Figure 3.11 GnRH regulation of gonadotropin expression in moue embryonic pituitary.

In pituitary sections prepared from E13.75 mouse embryos, there were no LHß+ or FSHß+ gonadotropes detected. However in embryos of E16.75 and E18.75, and P0 mice, LHß+ gonadotropes were observed in the pituitary, and nearly all of them were colocalized with YFP (*yellow arrows*). In E16.75 embryos, FSHß+ gonadotropes were present, but none of them co-expressed YFP (*white arrows*). In E18.75 embryos or P0 mice, some FSHß+ gonadotropes coexpressed YFP (*yellow arrows*). White arrows indicate cells expressing only LHß or FSHß, or YFP+ cells containing no gonadotropins.

3.2.5 LH injection rescues compromised FSHß+ gonadotrope development in GRIC/R26-DTA mice

It was shown that fetal mouse pituitary becomes GnRH responsive around E16 (Pointis and Mahoudeau, 1979) and can release LH to trigger testosterone production from age-matched fetal mouse testis (Pointis and Mahoudeau, 1976; Pointis et al., 1980). In

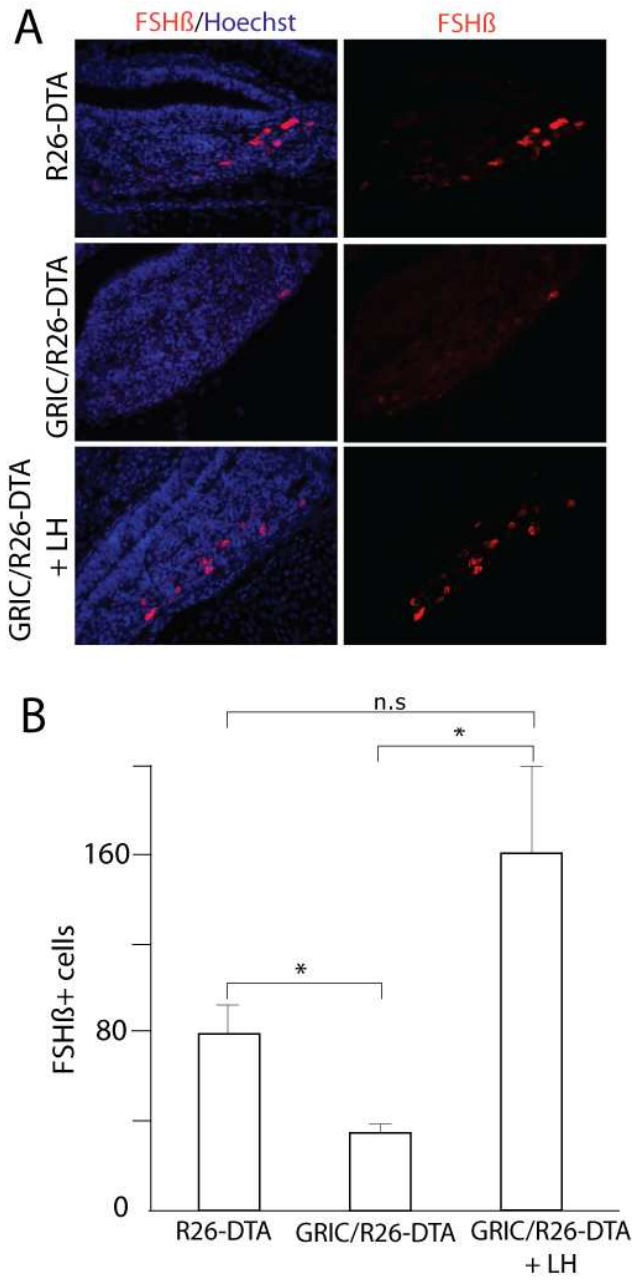

Figure 3.12 FSHß expression induced by LH injection in E17.75 mouse embryos.

A, IF of FSHß in pituitary sections of E17.75 R26-DTA embryos (R26-DTA), GRIC/R26-DTA embryos (GRIC/R26-DTA), and GRIC/R26-DTA embryos prepared from pregnant females injected with LH (GRIC/R26-DTA + LH). B, Statistical analysis of FSHß+ gonadotrope numbers in the embryos. FSHß+

gonadotropes in every 5th of the pituitary sections were quantified to obtain the total number of FSHß+ gonadotropes in one embryo. The number of FSHß+ gonadotropes in GRIC/R26-DTA embryos was significantly smaller than that in R26-DTA embryos. While in GRIC/R26-DTA embryos rescued by LH injection, the number of FSHß+ gonadotropes was significantly increased and became comparable to that in the R26-DTA control embryos.

GRIC/R26-DTA embryos, cell death was induced in GnRHR cells and thus in LHß+ gonadotropes. Surprisingly both the number and the fluorescence intensity of FSHß+ gonadotropes in E17.75 GRIC/R26-DTA embryos were significantly reduced in comparison to R26-DTA littermates, suggesting a compromised FSHß+ gonadotrope development in these embryos (Figure 3.12). The data raise the possibility that an embryonic LH surge around E16 is necessary to initiate FSHß expression. To test this hypothesis, embryonic LH surge was restored via the injection of purified LH into the pregnant female carrying E16.75 GRIC/R26-DTA embryos, and ask whether this would rescue the compromised development of FSHß+ gonadotropes in these embryos. LH could reach embryos via placental circulation and function in the embryonic pituitary gland during development. IF analysis for FSHß expression in pituitary sections of these embryos shows that LH injection indeed reversed the compromised FSHß expression in the pituitary. Both the quantity and fluorescence intensity of FSHß+ gonadotropes were significantly increased and comparable to those in R26-DTA embryos (Figure 3.12). These data show that normal FSHß+ gonadotropes development is compromised in GRIC/R26-DTA mice because nearly all LHß+ gonadotroes express the GnRHR and are thus ablated, therefore there is no LH produced in these GRIC/R26-DTA mouse embryos. However when the LH production is replaced by LH injection into the mother, FSHß expression in the embryonic pituitary is restored. This phenomenon indicates that the initiation of FSHß expression in gonadotropes depends on the existence of LH signaling around E16. Is this dependence mediated by LH receptor (LHR) expressed in FSHß+ gonadotropes? IF analysis of LHR in pituitary sections did not detect any LHR+ signals in FSHß+ gonadotropes at E16.75 (data not shown) and therefore disproved this possibility.

3.2.6 Cellular composition of the anterior pituitary in GRIC/R26-DTA mice

As gonadotrope is not the only cell type existing in the anterior pituitary, ablation of gonadotropes may have a significant impact on other cell types. It was shown that normal

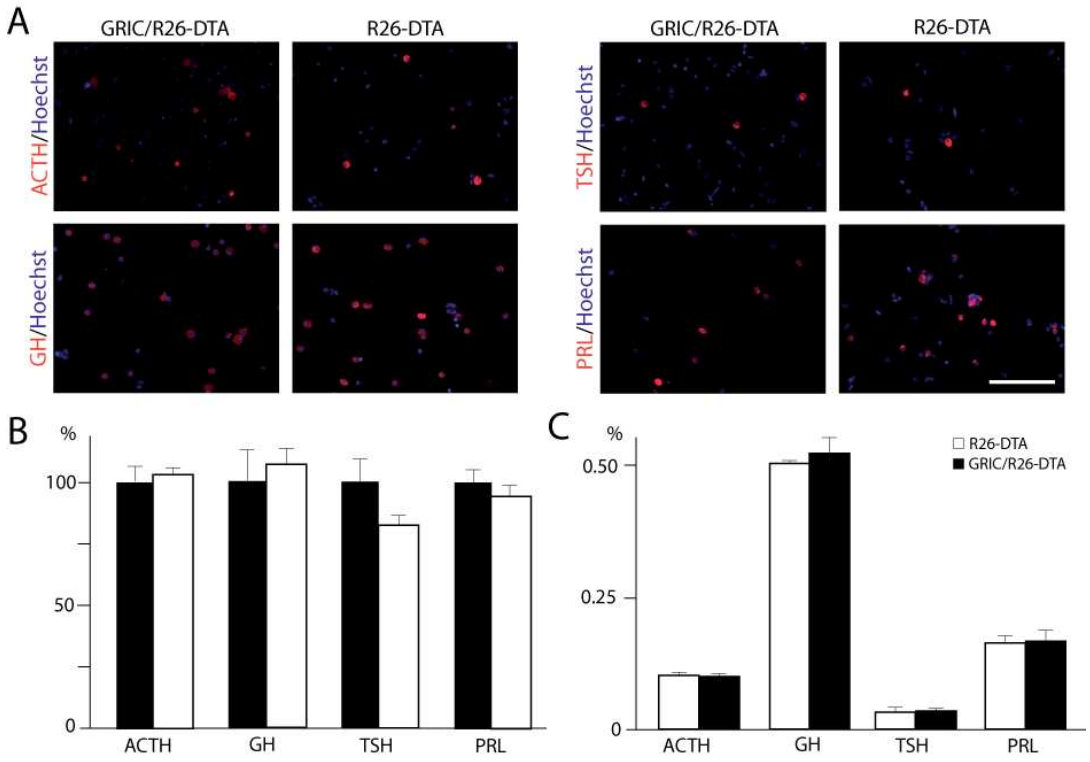

Figure 3.13 Stereotyped ratios of hormone producing cells in the anterior pituitary.

A, IF for TSHß, PRL, ACTH and GH in pituitary primary cell cultures prepared from adult male GRIC/R26-DTA and R26-DTA mice. B, IF intensity analysis shows that there was no significant difference in all of the four other cell populations between GRIC/R26-DTA and R26-DTA mice. C, Statistical analysis revealed stereotyped ratios of the other four hormone producing cells in the anterior pituitary. *Scale bar*, 100 µ m.

development of PRL cells requires existence of gonadotropes (Kendall et al., 1991). Ablation of gonadotropes by expression of DTA directed by 313 base pairs of the bovine-*αGSU* promoter impaired PRL synthesis and storage. In order to see whether the relative

abundance of the other four major hormone secreting cell types in the anterior pituitary was affected in GRIC/R26-DTA mice, IF analysis for TSHß, PRL, ACTH and GH was performed in pituitary primary cell cultures prepared from adult male GRIC/R26-DTA and R26-DTA mice (Figure 3.13. A). IF intensity analysis did not reveal any significant differences (Figure 3.13. B). Quantitation of these cell types also did not show any significant differences between GRIC/R26-DTA and R26-DTA male mice in terms of the ratios of these hormone producing cells in the anterior pituitary (Figure 3.13. C), suggesting stereotyped ratios of these cell populations in the anterior pituitary independent of gonadotrope ablation.

3.2.7 Increased number of GnRH neurons in the anterior hypothalamus of GRIC/R26-DTA mice

It was shown that mutations of *GnRH* or *GnRHR* gene do not affect the size and distribution of GnRH neuronal population (Gill et al., 2008), however it remains possible that one part of the mutant genes or gene products enable normal GnRH neuron development. Therefore it is interesting to check whether ablation of GnRHR cells and thus GnRH signaling in mouse would affect the GnRH neuronal population. The size and distribution of the hypothalamic GnRH neuronal population were analyzed in male GRIC/R26-DTA mice and R26-DTA mice. Strikingly, the total number of the hypothalamic GnRH neurons was significantly increased in GRIC/R26-DTA mice (Figure 3.14) compared to R26-DTA mice. A mean number of 1001 neurons were found in the hypothalamus of GRIC/R26-DTA mice compared to a mean of 739 neurons in R26-DTA mice, which corresponds to an increase of 30% (Figure 3.14.B). Furthermore there was a significant difference in the total number of GnRH neurons in the anterior hypothalamus, but not in the posterior hypothalamus. Next the relative distribution of GnRH neurons along the rostral to caudal axis was studied. There were no significant differences in the relative distribution of GnRH neurons between GRIC/R26-DTA mice and R26-DTA mice, suggesting that the migratory route of GnRH neurons does not depend on GnRHR+ cells (Figure 3.16). To rule out unspecific toxic side effects due to

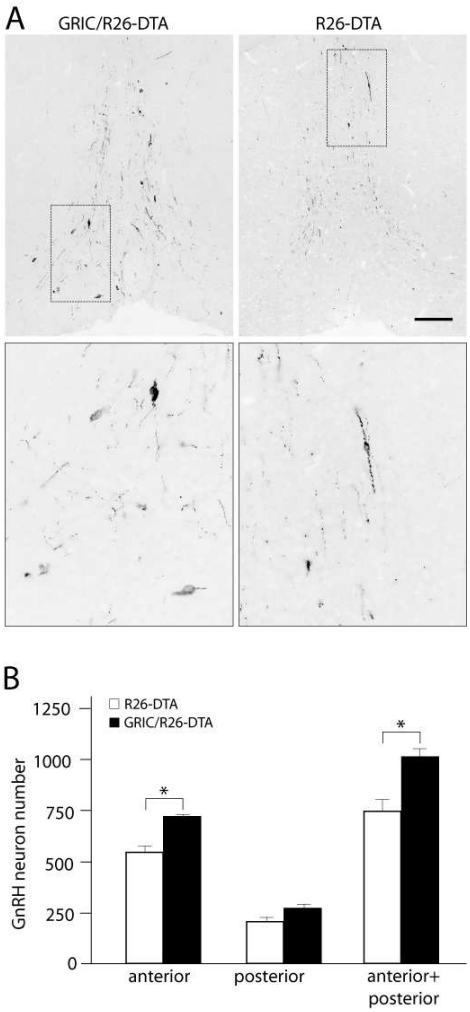

Figure 3.14. Increased GnRH neuron numbers in the hypothalamus of GRIC/R26-DTA mice.

A, Antibodies against GnRH label more neurons in the POA in GRIC/R26-DTA mice than in R26-DTA mice. *Scale bar*, 200 μm. B. Significant increase of GnRH neuron numbers in the anterior but not posterior hypothalamus of GRIC/R26-DTA mice compared to R26-DTA animals.

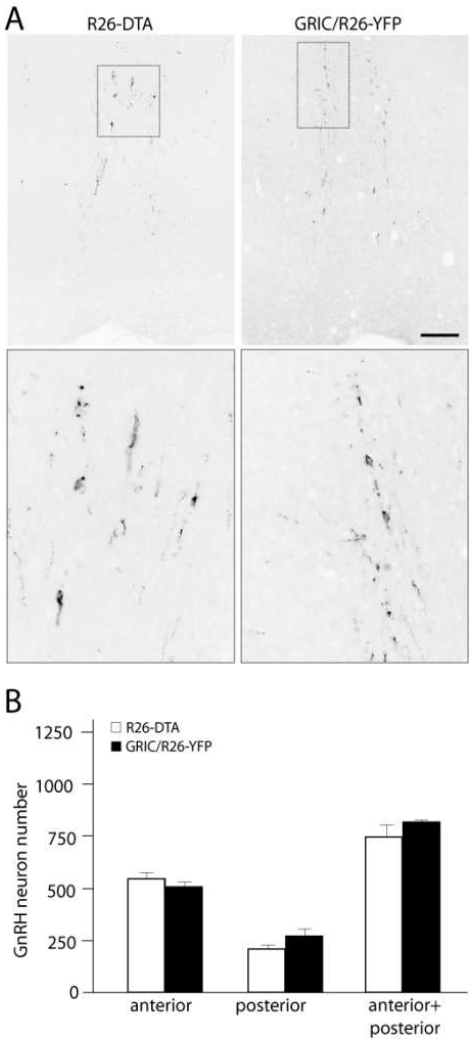

Figure 3.15 GnRH neuron numbers in the hypothalamus of GRIC/R26-YFP and R26-DTA mice.

A, Antibodies against GnRH label similar amount of neurons in the POA in GRIC/R26-YFP mice and R26-DTA mice. *Scale bar*, 200 µm. B. Comparison of GnRH neuron numbers in the anterior and posterior hypothalamus of GRIC/R26-YFP and R26-DTA mice did not reveal any significant differences.

leaky DTA expression from the ubiquitously active *ROSA26* locus, the size and distribution of hypothalamic GnRH neurons were also analyzed in GRIC/R26-YFP mice but no significant difference was detected when compared to R26-DTA mice (Figure

3.15). The gross distribution of GnRH fibers was also analyzed in the mutant mice. Similarly to what was found in R26-DTA mice, dense GnRH fibers were detected in the ME and other areas of the hypothalamus in GRIC/R26-DTA mice (data not shown).

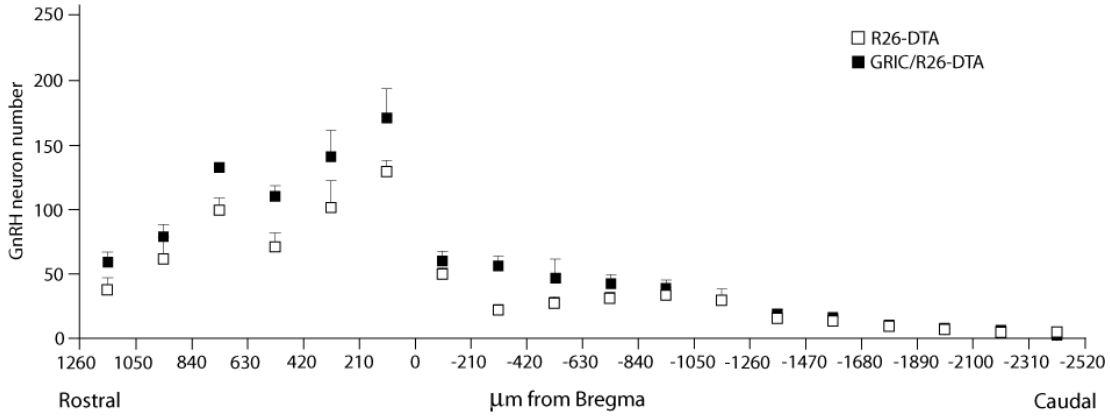

Figure 3.16 Distribution of GnRH neurons in GRIC/R26-DTA mice.

Rostral to caudal distribution of GnRH neurons in GRIC/R26-DTA (*solid square*) and R26-DTA mouse brains (*open squares*; n=3 for each genotype). Numbers on the x-axis indicate the distance from Bregma in µm (Paxinos and Franklin, 2001).

As the size of the hypothalamic GnRH neuronal population has increased in GRIC/R26-DTA mice, it is interesting to check whether the function of this neuronal population, the pulsatile release of GnRH in the ME, has also been changed. In cooperation with Prof. Jon E. Levine (Northwestern University, USA), GnRH release rates were measured *in vivo* in the ME of GRIC/R26-DTA mice by microdialysis. Unexpectedly, GnRH pulse characteristics, including the GnRH pulse frequency, GnRH pulse amplitude, and mean GnRH concentration, in these mice are not significantly different from those in the control animals (data not shown). It implies a stereotyped pulsatile release pattern of GnRH in GRIC/R26-DTA mice, independent of the increased GnRH neuronal population and disrupted HPG axis.

3.3 GnRH receptor is expressed in the mouse brain

3.3.1 Fluorescent visualization of GnRH target cells in the mouse brain

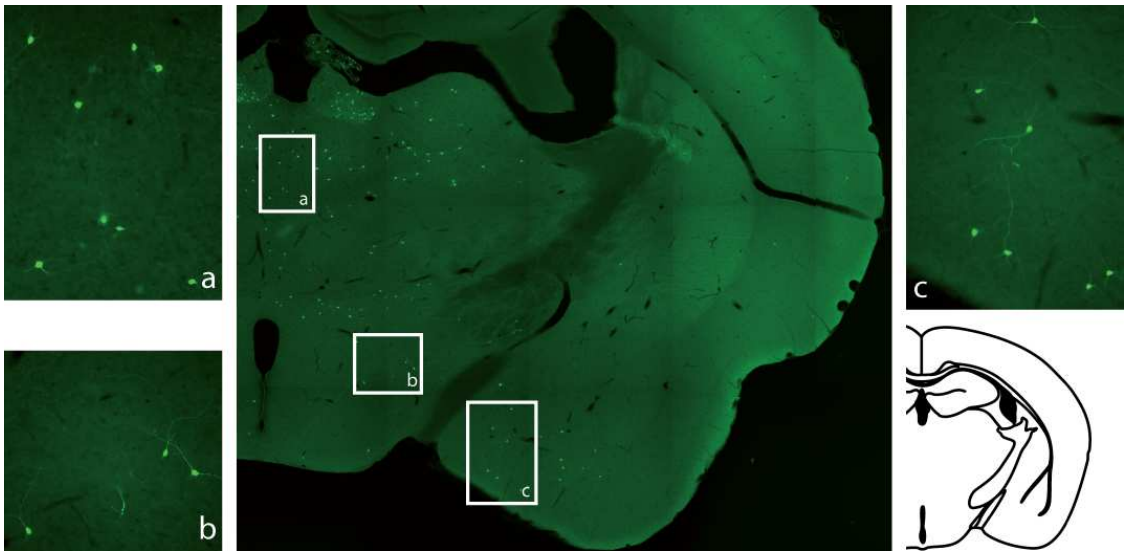

Figure 3.17 Genetic labeling of GnRHR cells in the brain of GRIC/R26-YFP mouse.

A, GnRHR expression was activated in a subset of neurons in the mouse brain. IF analysis of YFP (*green*) expression in coronal sections (50 µm) prepared from the brain of a GRIC/R26-YFP mouse. Magnified images (*white squares*) present YFP+ neurons in representative areas (a, thalamus; b, hypothalamus; c, amygdala) of the brain. *Scale bar:* 200 µm.

In GRIC/R26-YFP mice, *GnRHR* promoter activity in the brain can also be visualized by YFP expression. IF analysis of YFP expression in brain sections prepared from GRIC/R26-YFP mice revealed clearly visualized YFP+ cells (Figure 3.17), demonstrating that cells expressing GnRHR are indeed present in the mouse brain. Photography of a 50 µm-coronal brain section prepared from GRIC/R26-YFP mice represented YFP+ cell bodies as well as fibers in the thalamus, hypothalamus and amygdala (Figure 3.17). All YFP+ cells co-expressed NeuN (data not shown), a neuronal

marker, indicating that all YFP+ cells detected in the brain sections of GRIC/R26-YFP mice were neurons.

However YFP+ neurons in GRIC/R26-YFP mice could not be detected in live brain sections without antibody labeling. The YFP signals in these neurons are not strong enough for *in vivo* visualization. Therefore an eR26-tauGFP reporter mouse line was generated by Oliver Mai in the lab, in which a targeting cassette consisting of a *tau-GFP* gene directed by the CAGGS promoter (Okabe et al., 1997) and followed by a *loxP* floxed transcriptional termination sequence was inserted into the *ROSA26* locus. It was shown that CAGGS promoter increased transgene expression levels up to ~100-fold when compared to the endogenous *ROSA26* promoter (Seibler et al., 2005). Tau-GFP is traditional GFP tagged by tau, the microtubule-associated protein (Weingarten et al., 1975). Compared to untagged YFP, a genetic mutant of GFP (Heim and Tsien, 1996), tau-tagged GFP labels microtubules containing structures and improves the visualization of cell morphology, including axons and fibers. Additionally tau-GFP is anchored to the cytoskeleton and therefore less likely to leak out of cells during live tissue preparations, avoid losing signals and increasing background. In heterozygous offspring GRIC/eR26-tauGFP mice, Cre-mediated recombination removes the floxed termination sequence and thus facilitates tau-GFP expression in GnRHR neurons. Tau-GFP expressing neurons can easily be visualized *in vivo* in live brain sections prepared from GRIC/eR26-tauGFP mice. In cooperation with Prof. Dr. Trese Leinders-Zufall (Homburg), responses of live tau-GFP expressing neurons in acute brain slice preparations from GRIC/eR26-tauGFP mice will analyzed using electrophysiological and Ca-imaging techniques.

3.3.2 GnRHR is expressed in multiple structures through the mouse brain

Distribution of YFP-labeled neurons was analyzed on 50 µm-coronal brain sections through GRIC/R26-YFP male mouse (9-12 weeks) brain. In order to schematically illustrate the distribution of GnRHR neurons in the mouse brain, YFP expression pattern in brain areas between bregma 2.34 mm and bregma -6.12 mm (Paxinos and Franklin, 2001) was drawn from actual 50 µm GRIC/R26-YFP mouse brain sections (Figure 3.18).

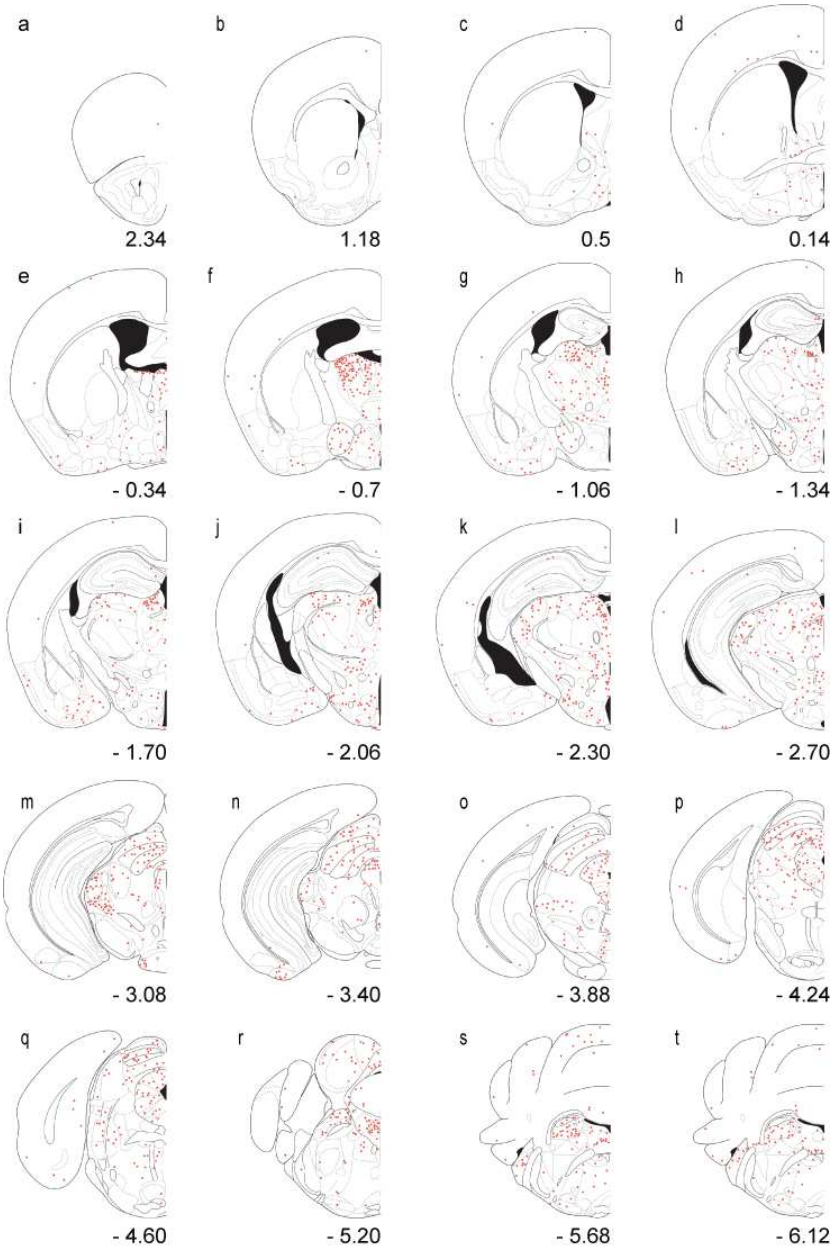

Figure 3.18 Distribution of YFP positive cells in the brain of an adult GRIC/R26-YFP male mouse

Each red dot represents one YFP+ cell. Maps were drawn from actual 50 µm sections. Numbers below each map indicate the distance (*mm*) from bregma. Maps were modified from the Mouse Brain Atlas (Paxinos and Franklin, 2001).

Each red dot represents one actual YFP+ neuron. Numerous areas of the mouse brain were found to contain YFP+ neurons. Except that quite few YFP+ neurons were present in the cortex (including hippocampus), nearly all YFP+ neurons were distributed in the telencephalon (the amygdala and septum), diencephalons (the hypothalamus and thalamus), mesencephalon, metencephalon and myelencephalon.

3.3.2.1 GnRHR expression in the telencephalon

In the telencephalon, YFP expression was observed in olfaction related regions in the amygdala in GRIC/R26-YFP mice. Scattered YFP+ neurons were present mostly in the anterior cortical amygdaloid nucleus (Aco), basomedial amygdaloid nucleus (anterior part, BMA), medial amygdaloid nucleus (posterior dorsal part, MePD), piriform cortex (Pir), and posteromedial cortical amygdaloid nucleus (PMCo) (Figure 3.18 and Figure 3.19). Some YFP+ neurons were also present in the septum, i.e. medial septum (MS) (Figure 3.18 and Figure 3.19).

3.3.2.2 GnRHR expression in the diencephalon

In the diencephalon, most YFP+ neurons were present in the thalamus and hypothalamus. Thalamus connects the sensory pathway with the cerebral cortex, and the hypothalamus coordinates the neuroendocrine system with the limbic and the autonomous nervous system. Numerous YFP+ neurons were observed in the anteroventral thalamic nucleus (AV), lateral geniculate nucleus (LGN), laterodorsal thalamic nucleus (LD), lateral habenular nucleus (LHb) and the lateral hypothalamic areas (LHA) (Figure 3.18 and Figure 3.19). YFP expression was also observed in the anterodorsal thalamic nucleus (AD) and anteromedial thalamic nucleus (AM) (Figure 3.18 and Figure 3.19).

Some YFP+ neurons were scattered in the mediodorsal thalamic nucleus (MD), anterior hypothalamic area (AH), dorsomedial hypothalamus nucleus (DM), lateroanterior hypothalamic nucleus (LA), posterior hypothalamic area (PH) and the medial preoptic area (MPA) (Figure 3.18 and Figure 3.19).

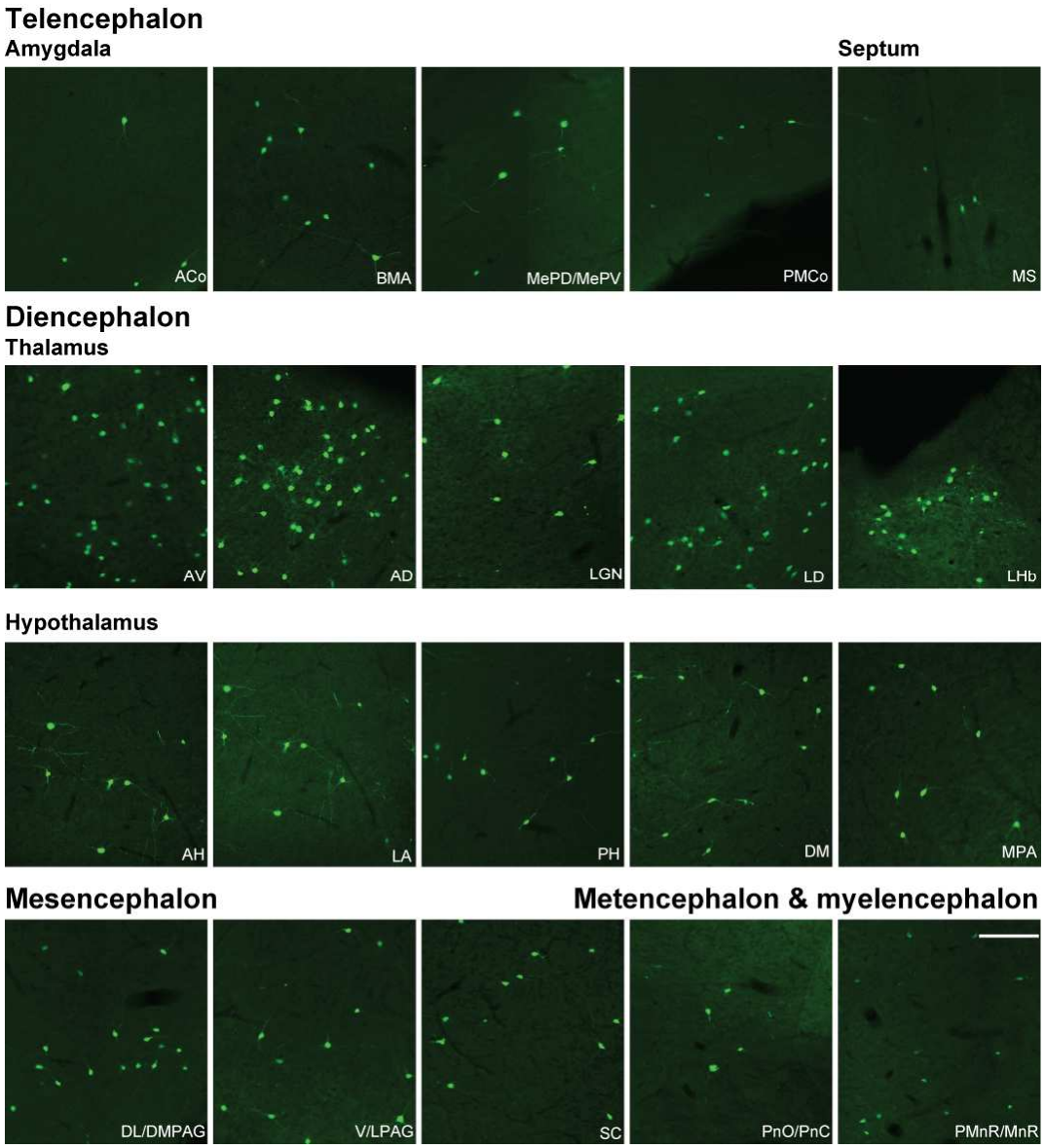

Figure 3.19 Representative photographs of YFP+ neurons in multiple structures of the GRIC/R26-YFP mouse brain.

YFP+ neurons were nicely visualized in multiple structures in 50 µm-coronal brain sections prepared from GRIC/R26-YFP mouse brain. Scale bar, 100 µm. Abbreviations: ACo, anterior cortical amygdaloid nucleus; AD, anterodorsal thalamic nucleus; AH, anterior hypothalamic area; AV, anteroventral thalamic nucleus; BMA, basomedial amygdaloid nucleus, anterior part; LGN, lateral geniculate nucleus; DLPAG,

dorsolateral periaqueductal gray; DM, dorsomedial hypothalamus nucleus,; DMPAG, dorsomedial periaqueductal gray; LA, lateroanterior hypothalamic nucleus; LD, laterodorsal thalamic nucleus; LGN, lateral geniculate nucleus; LHb, lateral habenular nucleus, lateral part, medial part; MePD, medial amygdaloid nucleus, posterior dorsal part; MePV, medial amygdaloid nucleus, posterior ventral part; MnR, median raphe nucleus; MPA, medial preoptic area; MS, medial septal nucleus; PH, posterior hypothalamic area; PLCo, posterolateral cortical amygdaloid nucleus; PnO, pontine reticular nucleus, oral part; PnC, pontine reticular nucleus, caudal part; PMCo, posteromedial cortical amygdaloid nucleus; PMnR, ,paramedian raphe nucleus; SC, superior colliculus; VLG, ventral lateral geniculate nucleus.

3.3.2.3 GnRHR expression in the mesencephalon

In the mesencephalon, numerous YFP+ neurons were present in the dorsolateral and dorsomedial periaqueductal gray (DL/DMPAG) and the superior colliculus (SC) (Figure 3.18 and Figure 3.19). A few YFP+ neurons were scattered in the ventrolateral and lateral periaqueductal gray (V/LPAG) (Figure 3.18 and Figure 3.19).

3.3.2.4 GnRHR expression in the metencephalon and myelencephalon

In the metencephalon and myelencephalon, numerous YFP+ neurons were found in the cerebellum, some YFP+ neurons were observed in the pontine reticular nucleus (caudal part, oral part; PnC/PnO), and few were present in the raphe nucleus (median, paramedian, MnR/PMnR) (Figure 3.18 and Figure 3.19).

3.3.3 GnRHR neurons are connected to GnRH neuronal network

GnRH neurons were found to have synaptic contacts with neurons in multiple brain regions (Boehm et al., 2005). Coexpression analysis of YFP and GnRH was perfomed on 50 µm-coronal brain sections prepared from GRIC/R26-YFP mice to address the question whether YFP+ neurons make synaptic contacts with GnRH neurons. Data from confocal microscope analysis suggest that the axons of YFP+ neurons in the POA might make synapses with GnRH neurons (Figure 3.20, A-C). These connections can be visualized by colocalization analysis of YFP with barley lectin (BL), a transneuronal tracer, in heterozygous offspring from the breeding between GRIC/R26-YFP mice and BIG (BL-

IRES-GFP) mice, in which BL is specifically expressed in GnRH neurons (Boehm, et al., 2005).

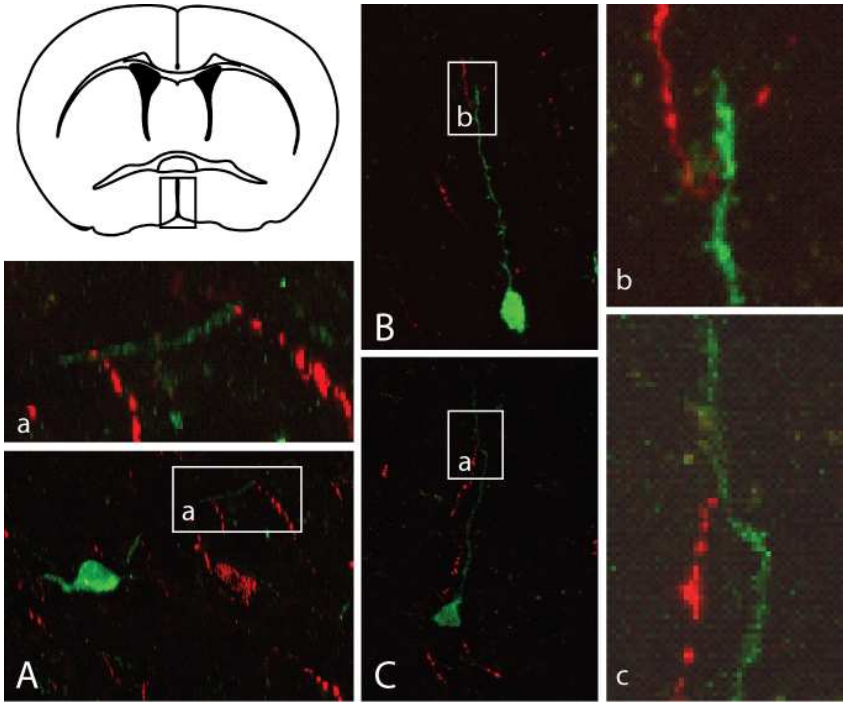

Figure 3.20 GnRH fibers contact GnRHR neurons in the brain of GRIC/R26-YFP mouse.

A-C, Confocal micrographs of 50 µm-coronal brain sections show that axons of YFP+ neurons in the POA making contacts with GnRH fibers in GRIC/R26-YFP mouse brain.

4. Discussion

4.1 Temporal orchestration of GnRH signaling in the anterior pituitary during embryonic development

This study demonstrates a functional role of GnRH signaling during embryonic gonadotrope development. Data from adult GRIC/R26-DTA mice surprisingly show that a significant number of FSHß+ but not LHß+ gonadotropes did not express the GnRHR and thus escaped from ablation, suggesting that LHβ expression is inevitably linked to GnRHR expression (thus leading to cell death) whereas FSHβ expression is not. Consistent with this, GnRH induces LHβ but not FSHβ expression at E16.5 in immature gonadotropes (Japon et al., 1994), while FSH can be expressed independent of GnRH signaling, for example by activin-dependent pathways (Weiss et al., 1995; Gregory and Kaiser, 2004). Therefore immature FSHß+/LHß- gonadotropes need a signal other than GnRH to initiate FSHß expression, which may be provided by GnRH-responsive LHß+ gonadotropes at E16.5. This hypothesis was confirmed by further experiments on mouse embryos.

Data from GRIC/R26-DTA and GRIC/R26-YFP embryos indicated that LH secretion around E16.5 is necessary for FSHß+ gonadotropes to become GnRH responsive and undergo normal development. In order to produce this LH release, firstly GnRH neurons should have reached their final destinations in the basal forebrain and project their axons to the ME to secret GnRH. This stage is reached at E16 in the mouse (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). Secondly, the LHß+ gonadotropes in the anterior pituitary should express the GnRHR and thus be GnRH responsive at this time point, which allows LH synthesis and release triggered by GnRH. GnRHR promoter activity analysis in E16.75 GRIC/R26-YFP mouse embryos revealed that indeed LHß+ gonadotropes expressed GnRHR and were GnRH signaling targets at this age. Interestingly however none of the FSHß+ gonadotropes express GnRHR, suggesting that

production of LH but not FSH is triggered by GnRH at this age. Thirdly, the LH target cells should express the LH receptor (LHR) and thus be LH responsive and stimulated by the LH signal. As LHR expression was not detected in FSHß+ gonadotropes, the LH target cells might be in the gonads, which would send signals, in response to LH signal produced by LHß+ gonadotropes, to stimulate FSHß+ gonadotropes express GnRHR and become GnRH responsive. These data raise the possibility that LH may induce FSHß expression via a gonadal feedback loop. Consistent with this, fetal gonads can secrete steroid hormones in response to LH at E16 (Pointis and Mahoudeau, 1976, 1979; Gross and Baker, 1979; Pointis et al., 1980). Progesterone might be a good candidate to promote FSH β + gonadotrope maturation as it was shown in a gonadotrope-like cell line that progesterone could induce FSHß expression (Thackray et al., 2009).

The necessity of early LH signal for proper development of FSH β + gonadotropes is further demonstrated in E16.75 GRIC/R26-DTA embryos, in which only rare and faint FSH β IF signals were detected. However, injection of purified LH into the mother is sufficient to promote FSHß+ gonadotrope development in these embryos. This experiment also ruled out the possibility that other deficits in these embryos caused the compromised development of FSHß+ gonadotropes, for example compromised GnRH signaling in the gonads.

However no ablation efficiency differences between FSHß+ and LHß+ gonadotropes were reported in transgenic mice using *α GSU* promoter fragment to express DTA in gonadotropes (Kendall et al., 1991; Seuntjens et al., 1999; Vankelecom et al., 2003), perhaps reflecting earlier ablation of gonadotropes as α GSU is the first gonadotropin transcript expressed during gonadotrope development (Zhu et al., 2007).

4.2 Increased number of GnRH neurons in the anterior hypothalamus of GRIC/R26-DTA mice

This study also showed that GnRHR cells are neither a prerequisite for the proper developmental migration of GnRH neurons into the brain nor for their projections to the ME but do affect the size of GnRH neuronal population. The significantly increased size of the GnRH neuronal population in the anterior hypothalamus of GRIC/R26-DTA mice is a major surprise in comparison to other hypogonadal mice, which had a reduced or unaltered size of the GnRH neuronal population (Mason et al., 1986; Muscatelli et al., 2000; Kruger et al., 2004; d'Anglemont de Tassigny et al., 2007; Gill et al., 2008).

These data suggest that establishing the proper number of GnRH neurons during development is dependent on GnRHR cells. However it is at present unclear how GnRHR cells determine the proper size of the GnRH neuronal population. GnRH neuronal development relies upon cooperative signaling from surrounding tissues first affecting fate specification within the nasal placode and then affecting migration and axon targeting (Tobet and Schwarting, 2006). Both migration and projections of GnRH neurons appeared undisturbed in GRIC/R26-DTA mice, indicating that the latter aspect of GnRH neuron development is intact but the fate specification phase may be affected in GRIC/R26-DTA mice. It remains possible that either more GnRH neurons are generated or less neurons die during development. Inducible ablation of GnRHR+ cells in GRIC/R26-iDTR mice carrying a Cre-activated *diphtheria toxin receptor* gene (DTR) (Buch et al., 2005) will enable us to determine the time window in which the size of the GnRH neuronal population is established.

Although preliminary inspection of GnRH projections did not reveal gross abnormalities, a detailed quantitative analysis of the connectivity of the GnRH neuronal network in the GRIC/R26-DTA mice can be carried out in transgenic mice bearing BL, a transneuronal tracer, specifically in GnRH neurons (Boehm et al., 2005). Given the intimate connection of GnRH neurons to the olfactory system (Boehm et al., 2005) it will be interesting to analyze pheromonal effects on reproduction in those mice.

4.3 GnRHR is expressed in the mouse brain

This study has for the first time mapped the distribution of GnRHR cells in the mouse brain at a single cell resolution. The topographic distribution of YFP+ neurons mapped in GRIC/R26-YFP mouse brain shows that GnRHR neurons exist in multiple brain areas which are involved in different brain functions.

4.3.1 GnRHR neurons and olfaction

In the telencephalon, YFP expression was observed in several substructures of the amygdala that are involved in the processing of pheromone or odor information. Pheromones signals detected in the vomeronasal organ (VNO) are relayed through the accessory olfactory bulb (AOB) to the MEA (medial amygdala) and PMCO in the amygdala, from where signals are relayed further to the hypothalamus (Dong et al., 2001). Whereas odor signals derived from the main olfactory epithelium (MOE) are relayed through the main olfactory bulb (MOB) to the olfactory cortex and cortical amygdala, including the Pir and ACo, from where the information is relayed further to other nuclei. For example information from the ACo is transferred further to the BMA of the amgydala (Shipley et al., 1995).

Scattered YFP+ neurons were observed in several olfaction related amygdala structures, the ACo, BMA, MEA, Pir and PMCo (Figure 3.18 and Figure 3.19). This is consistent with previous study showing that transgene expression was observed in the amygdala of a transgenic mouse model in which a 3.3-kb fragment of the rat GnRH-R promoter is linked to *hPLAP* gene (Granger et al., 2004). Subsets of neurons in the MEA and PMCo were shown to relay signaling of female urinary pheromones and stimulate GnRH neurons to release GnRH (Boehm et al., 2005), which in turn triggers LH surge in the animals (Maruniak and Bronson, 1976). In addition GnRH has also been proposed to modulate detections for reproduction relevant odors (Wirsig-Wiechmann, 2001), and this may lead to the variations in the olfactory performance during menstrual cycles (Hummel et al., 1991). All these data suggest that GnRH may regulate reproduction by modulating

pheromone- and odor-signal processing. via GnRHR activation in the amygdala. In cooperation with Prof. Dr. Trese Leinders-Zufall (Homburg), pheromone or odor-induced activation of GnRHR neurons in these regions will be analyzed.

4.3.2 GnRHR neurons and autonomic activities

YFP+ neurons were also observed in the septum of the telencephalon (Figure 3.18, Figure 3.19), indicating that GnRHR neurons are present in this region. It was shown that injection of GnRH into the septal area can elicit thermoregulatory skin vasomotion in rats (Hosono et al., 1997). Combined with our data, this thermoregulatory regulation is possibly modulated by the activation of GnRHR neurons in the septum. Additionally numerous YFP+ neurons were scattered in the cerebellum where immunoreactive GnRHRs were also detected (Albertson, et al., 2008). The cerebellum plays an important role in movement coordination, in which GnRHR neurons may be involved. Some YFP+ neurons were present in the pontine reticular nucleus which has direct synaptic contacts with GnRH neurons (Boehm et al., 2005) and is involved in head movement. Some YFP+ neurons were also observed in the raphe nucleus which is known for releasing serotonin to activate serotonergic neurons and regulate autonomic activities.. All these data suggest that GnRHR neurons are possibly involved in modulating autonomic activities.

4.3.3 GnRHR neurons and coping and reproductive behavior

The PAG, also called the midbrain central gray, is a cell-dense region surrounding the midbrain with radial and columnar organization. In the brain of GRIC/R26-YFP mice, numerous YFP+ neurons were present in the PAG region (Figure 3.18 and Figure 3.19).

The PAG mediates active and passive coping reactions, such as confrontational defense reactions, escape or flight responses (Bandler et al., 1985; Depaulis and Vergnes, 1986), or quiescence and immobility, decreased vigilance and hyporeactivity (Bandler et al., 1985; Krieger and Graeff, 1985). Earlier studies have showed that administration of GnRH modifies passive and active avoidance responses in rats (Mora and Diaz-Veliz,

1985), supporting our finding that GnRH may modulate coping behavior in the brain via the GnRHR neurons in the PAG region.

Furthermore infusions of GnRH into the dorsal PAG or the ventrolateral PAG improved lordosis reflex performance in female rats (Riskind and Moss., 1979; Sakuma and Pfaff, 1980). The locations of the effective infusions are consistent with the distribution of YFP+ neurons in the PAG in GRIC/R26-YFP mouse brain (Figure 3.18 and Figure 3.19), suggesting that GnRH target cells exist in this area and are involved in particular reproductive behaviors. GnRH was also shown to be released in large quantities into the cerebrospinal fluid (CSF) (Yoshioka et al., 2001), and intracerebroventricular injection of GnRH could compensate the deficit in sexual behaviors following VNO removal in rats (Meredith et al., 1992). Because the PAG region surrounds the third ventricle, GnRH can be transported by CSF to the third ventricle and bind to GnRHR neurons in the PAG to induce sexual behaviors. In addition YFP+ neurons were also observed in the MPA which is also involved in sexual behaviors (Simerly, 2002).

4.3.4 GnRHR neurons as integrator and modulator

Adjacent to the PAG is the superior colliculus (SC). The SC is one part of the tectum, a layered structure containing a high proportion of multisensory neurons. The SC plays a role in the motor control of orientation behaviors of the eyes, ears and head**.** Numerous GnRHR neurons are present in the SC as indicated by the YFP+ neuron distribution in this structure of the GRIC/R26-YFP mouse brain, and this is consistent with previous IHC study (Albertson et al., 2008) as well as ligand binding studies (Jennes et al., 1997). All these data raise the possibility that GnRH acts on GnRHR neurons in the SC to modulate the integration of somatosensory, visual and auditory information and therefore regulate orientation behaviors in mice.

In addition many YFP+ neurons were distributed in the thalamus and hypothalamus. Thalamus is the largest part of the diencephalon in the mouse brain. It forms strong reciprocal connections with the cerebral cortex, and is involved in many different

functions, i.e. learning and memory, visual and motor activities. Numerous GnRHR neurons are present in the anterior thalamic nuclei AV (Figure 3.18 and Figure 3.19), some were observed in the AD and AM (Figure 3.18 and Figure 3.19) of the anterior thalamic nuclei, which are part of an 'extended hippocampal system' (Aggleton et al., 1996). Numerous GnRHR neurons are present in the anterior thalamic nuclei, indicating GnRH regulation may be involved in attentional processes and learning and memory.

GnRH and GnRHR are concentrated in several diencephalic visual processing centers of fishes (Maruska and Tricas, 2007). Interestingly in GRIC/R26-YFP mice, numerous YFP+ neurons were also present in the LGN (Figure 3.18 and Figure 3.19) of the thalamus. LGN is a diencephalic visual processing center in the mouse brain. It is the main relay of visual information received from the retina to the primary visual cortex, and it also receives strong feedback from the primary visual cortex. The presence of GnRHR expression in the LGN provides the possibility that GnRH modulate the processing of visual information in the mouse brain. Lots of YFP+ neurons were also present in the LHb (Figure 3.18 and Figure 3.19) of the thalamus. The existence of GnRHR neurons in this region indicates that GnRH regulates the releasing of dopamine, serotonin and norepinephrine and modulates reward and pain processes.

The hypothalamus acts as an interface between sensory and motor pathways and coordinates stimulus-specific behaviors with appropriate autonomic and endocrine responses. In the hypothalamus, numerous YFP+ neurons were present in the LHA, some were scattered in the AH, DM, LA and PH (Figure 3.18 and Figure 3.19). It was shown that GnRH can inhibit or stimulate neurons in the hypothalamus (Pan et al., 1988) which may be mediated by GnRHR neurons in these regions. Furthermore LHA recieves information from GnRH neurons directly (Boehm et al., 2005). All these indicate GnRH interacts with GnRHR neurons in the hypothalamus and modulate multiple brain functions.

4.3.5 Future experiments

The present study extends previous studies for GnRHR mRNA expression and GnRH binding sites, as well as one study using a transgenic mouse model expressing *hALPA* under the control of a rat GnRHR promoter fragment (Badr and Pelletier, 1987; Jennes and Woolums, 1994; Choi et al., 1994; Granger et al., 2004), and provides evidence that GnRHR neurons are present in multiple brain areas and function as neurotransmitter and/or neuromodulator in addition to its well-known role as being a neurohormone regulating the HPG axis. Except the potential functions described in this thesis, many of the regions identified require further characterization for their physiological characters. In GRIC/R26-iDTR mice carrying a Cre-activated *DTR* (diphtheria toxin receptor) gene (Buch et al., 2005), inducible ablation of GnRHR neurons in specific brain areas following stereotaxic injection of DTA makes it possible to determine physiological functions of GnRHR neurons in specific brain areas. Coimmunohistochemistry of YFP and c-fos expression, a marker for neuronal activity (West et al., 2001), could also be used to determine the physiological functions of GnRHR neurons in distinct brain areas.

Furthermore it remains possible that some other brain areas may also express GnRHR which were undetectable in GRIC/R26-YFP mice at the age being examined or beyond the brain regions being studied in this thesis. For example GnRH immunoreactive fibers were observed in caudal olfactory bulb, and GnRH is indicated to contact with GnRHR cells in the nasal epithelium (Jennes, 1986). GnRHR expression is detected in bovine nasal mucosa although with regional differences (Sundaram et al., 2008). In GRIC/R26-YFP mice, YFP+ neurons were found in the olfactory bulb (data not shown). However because of the high fluorescence background, GnRHR expression in nasal epithelium is not detectable in these mice. GRIC/eR26-tauGFP mice, in which GnRHR cells are tagged by insoluble tau-GFP and thus escape from increased fluorescence background caused by diffusion of soluble fluorescent protein during tissue processing, provide a possible means to identify GnRHR cells in the nose. In addition GnRHR has been suggested to be expressed in olfactory neuroblasts which secret GnRH during embryonic development, and promotes these cells migrate from the olfactory sensory lineage and differentiate into

GnRH neurons (Romanelli et al., 2004). Similarly GnRHR expression in the nose of mouse embryos can also be studied in GRIC/eR26-tauGFP mice.

In addition in GRIC/eR26-tauGFP mice, tau-GFP expressing GnRHR neurons can be clearly visualized without antibody labeling, facilitating *in vivo* characterization of these neurons with electrophysiology approaches or calcium imaging. Tau-GFP tagged GnRHR neurons can also be isolated and pooled by flow cytometry for further characterization of their molecular properties.

4.4 YFP tagging of primary gonadotropes

Gonadotropes represent only about 5–15% of the heterogeneous cell populations and are scattered throughout the anterior pituitary, which makes molecular and cellular characterization of primary gonadotropes a challenge (Lloyd and Childs, 1988). Previous studies about gonadotropes are carried out mainly in gonadotrope-derived cell lines (Windle et al., 1990; Thomas et al., 1996), which may not represent the exact physiological properties of primary gonadotropes. This study has for the first time tagged primary gonadotropes with a fluorescent protein, which facilitates their identification and isolation for further functional characterization.

Gonadotropes in pituitary primary cell cultures prepared from GRIC/R26-YFP mice were easily identified by their endogenous YFP fluorescence and this made it possible to characterize their physiological properties by electrophysiology, calcium imaging, and study of gonadotropin secretion from single isolated gonadotropes. About 50% of primary gonadotropes do not exhibit secretion of LH or FSH. Application of GnRH induced a broad range of both electrophysiological responses and increases in the intracellular calcium concentration. Our collaborators Prof. Dr. J.R. Schwarz and his colleagues have done further studies on these YFP-tagged gonadotropes and made interesting discoveries which have not been revealed by previous studies in cell lines (Hirdes et al., 2010).

Furthermore YFP tagging of primary gonadotropes facilitates the study of correlation of gonadotrope heterogeneity within the estrous cycle in female rodents (Jeong and Kaiser, 1994). It was shown that only 50% of LH containing cells secret LH in diestrus female rats, whereas almost all LH containing cells show secretion in proestrus rats (Smith et al., 1984). Moreover the percentage of GnRH-bound (GnRHR) cells in the pituitary is also changing during estrous cycle in rats and peaks at proestrus and thus supports the preovulatory gonadotropin surge (Lloyd and Childs, 1988). Moreover it was also shown that small and medium-sized gonadotropes enlarge and produce LHß at proestrous and estrous, however shrink to smaller size during metestrus and diestrus (Childs et al., 1992). GRIC/R26-YFP mice provide a mean to study the molecular properties of gonadotropes at different phases of the estrous cycle, in which YFP-tagged gonadotropes can be isolated and pooled by fluorescence-activated cell sorting and then used for further analyses for their gene transcription profiles.

Gondaotropes can perform cellular movements *in vitro* and spatial repositioning in pituitary slices (Navratil et al., 2007). Confocal microscopy analysis of live pituitary slices prepared from GRIC/R26-YFP mice shows that YFP-tagged gonadotropes grew cell processes in response to GnRH stimulation (Iain Stitt and Ulrich Boehm, data not shown). Therefore cell processes formation and repositioning of YFP-tagged gonadotropes can be visualized in live pituitary slices prepared from GRIC/R26-YFP female mice at different phases of the estrous cycle, which would provide detailed information of how gonadotropes contribute to proestrus LH surge by extending cell processes and repositioning themselves in pituitary.

The binary genetic strategy used in this study also allows expression of other reporter genes in gonadotropes. For example, expression of barly lectin in gonadotropes, an axonal marker used for trans-synaptic labeling would facilitate visualization of synaptic contacts of gonadotropes with other cell types in the pituitary.

4.5 A binary genetic strategy to visualize and manipulate GnRH target cells

In order to visualize and manipulate GnRH target cells in the mouse, a knock-in strategy rather than a transgenic approach was used in this study to generate a mouse model, in which expression of Cre recombinase was introduced under the control of endogenous *GnRHR* promoter. By inserting an *IRES-Cre* cassette just downstream of the *GnRHR* coding region, a bicistronic message was produced under the control of *GnRHR* promoter, from which the GnRHR and Cre recombinase were independently translated. In contrast to traditional transgenic approaches, the knock-in approach has the advantage that the transgene is translated under the control of the endogenous *GnRHR* promoter, escaping from nonspecific expression of the transgene caused by different transgene copy number or the site of transgene insertion in the genome. Similar strategies using IRES elements in gene targeting have faithfully directed expression of reporter genes to different cell populations of various tissues with high specificities (Mombaerts et al., 1996; Belluscio et al., 1999; Rodriguez et al., 1999; Eggan et al., 2004).

In this study *GnRHR* promoter activity was visualized in GRIC/R26-YFP mice with a single cell resolution and high sensitivity (Wen et al., 2008). And the coexpression of YFP with GnRHR is independent of the strength of the endogenous *GnRHR* promoter, therefore facilitating detections of weak GnRHR expression in various tissues of the mouse.

Additionally ablation of GnRHR cells was achieved in GRIC/R26-DTA mice, in which DTA expression from the *ROSA26* locus was activated by endogenous *GnRHR* promoter and led to cell death in GnRHR cells. GRIC/R26-DTA mice are produced from breedings between GRIC and R26-DTA mice. Both mouse strains show normal reproductive capacities. This binary genetic approach made it possible to produce genetically identical GRIC/R26-DTA mice for analysis irrespective of the infertility in these mice, which is a major advantage over a conventional transgenic approach.

Furthermore breeding the GnRHR specific Cre knock-in mice with other Cre reporter mice provides versatile tools for functional characterization of GnRHR cells in the mouse. For example expressing light-activated ion channels facilitate activation of GnRHR cells *in vivo*, while expressing a transneuronal tracer in GnRHR neurons allows visualization of GnRHR neuronal circuits

5. Summary

In summary, the experiments presented in this thesis demonstrate a functional role of GnRH signaling in the embryonic development of gonadotropes as well as in the establishment of the GnRH neuronal population. They also map the distribution of GnRH target cells at single cell resolution in the mouse brain for the first time and set the stage for further functional characterization of GnRHR neurons in the brain. Finally, fluorescent labeling of mouse gonadotropes with YFP led to the characterization of GnRH signaling in primary cells.

Firstly, a temporal orchestration of GnRH signaling was discovered during embryonic development of gonadotropes. LHß+ but not FSHß+ gonadotropes express the GnRHR at E16.75 and are thus responsive to GnRH. LH production triggered by GnRH in these LHß+ gonadotropes at this stage then promotes the expression of FSHß in FSHß+ gonadotrope. Moreover, increased numbers of GnRH neurons were found in the anterior hypothalamus of mutant adult mice with ablation of GnRHR cells, suggesting an unexpected role of these cells in establishing the size of the GnRH neuronal population.

Secondly, GnRHR neurons were identified in multiple brain areas, including olfaction-related structures of the amygdala, areas in the thalamus involved in learning and memory and visual processing, and sexual behavior related regions of the PAG. These data suggest that complex interactions are present between the reproductive center of the brain and other brain functions via activation of GnRH target cells by GnRH signaling.

Thirdly, primary gonadotropes were tagged with YFP and could be easily identified *in vivo*, facilitating the characterization of their molecular and physiological properties. Initial characterization of these YFP-tagged gonadotropes revealed a significant heterogeneity in terms of their resting properties and their responses to GnRH. About 50% of gonadotropes do not exhibit secretion of LH or FSH. Application of GnRH induced a broad range of both electrophysiological responses and increases in the intracellular calcium concentration.

Lastly, the GnRHR specific Cre knock-in mouse generated in this study should facilitate the expression of different transgenes in GnRHR cells for *in vivo* manipulation and therefore functional characterization of these cells in the mouse.

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