ABSTRACT

Title of Document:	THE GONADOTROPIN RELEASING HORMONE-3 SYSTEM IN ZEBRAFISH: EARLY DEVELOPMENT AND REGULATION
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The objective of this study was to expand our understanding of the early development of forebrain Gonadotropin Releasing Hormone (GnRH) neurons in vertebrates in general and in fish in particular. The correct migration during early development of the hypophysiotropic GnRH neurons from the olfactory region to the hypothalamus is crucial for normal gonadal development and reproduction. We developed a Tg(GnRH3:EGFP) zebrafish line in which EGFP is specifically expressed in GnRH3 neurons. Using this line, we have studied in detail the early spatiotemporal development of the GnRH3 system *in vivo*. In addition, we have studied various factors, including GnRH3, Netrins and Hedgehog to better understand some of the mechanisms that mediate this complex axophilic neuron migration event. Lastly, we have conducted targeted GnRH3 neuron ablation experiments in view of

determining the embryonic origin of POA-hypothalamic GnRH3 neurons and the effect of lack of GnRH3 neurons in the CNS.

Our findings show that: 1) GnRH neurons first differentiate and express GnRH3 at 24-26 hours post fertilization (hpf) and immediately thereafter begin to extend fibers. 2) GnRH3 neurons project a complex network of fibers, prior the GnRH3 soma migration, to various CNS regions, and to the pituitary. 3) GnRH3 soma begin migrating towards the hypothalamus at 3 days post fertilization (dpf), passing through the terminal nerve (TN), lateral telencephalon, and reaching the hypothalamus by 12 dpf. 4) expression of GnRH3 itself is necessary for the normal early differentiation and fiber extensions of GnRH3 neurons. 5) Netrin1a is directly involved as a chemoattractant in GnRH3 fiber organization and subsequently, in GnRH3 soma migration to the hypothalamus. 6). Netrin2 is required for normal early ZF embryogenesis. 7). Sonic hedgehog a does not serve as a specific factor in the development of the GnRH3 system. 8). GnRH3 neuron regeneration capacity is temporally limited. 9). Successful ablation of olfactory GnRH3 neurons during development results in lack of GnRH3 neurons in the entire sexually mature brain as well as abnormal gonadal development and inability to reproduce.

This study expands our understanding vis-à-vis the early events that occur during GnRH3 system development and that regulate this complex process. In a broader sense these findings augment current knowledge regarding the regulation of long range tangential neuron migration during development.

THE GONADOTROPIN RELEASING HORMONE-3 SYSTEM IN ZEBRAFISH: EARLY DEVELOPMENT AND REGULATION

By

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Abbreviations

CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
cGnRH-II	Chicken GnRH-II
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CNS	Central nervous system
Dcc	Deleted in colorectal carcinoma
dpf	Days post fertilization
E2	Estradiol
ECM	Extra cellular matrix
EGFP	Enhanced green fluorescent protein
F(x)	Transgenic line generation (x)
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GnRH	Gonadotropin releasing hormone
Hh	Hedgehog
hpf	Hours post fertilization
HPG	Hypothalamic-Pituitary-Gonadal
hpg	Hypogonadal
Hv	Ventral periventricular hypothalamus
ICC	Immunohistochemistry
ISH	*In situ* hybridization
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
MAB	Maleic acid buffer
ME	Median eminence
MO	Morpholino
Mtz	Metronidazole

N-CAM	Neural cell adhesion molecules
nfsB	Nitroreductase B enzyme
NMDA	N-methyl-D-aspartic acid
NTR	Nitroreductase
O/N	Over-Night
OB	Olfactory bulb
OE	Olfactory epithelium
ON	Olfactory nerve
PBST	Phosphate buffered saline
POA	Preoptic area
PPa	Anteroventral parvocellular preoptic nucleus
PPp	Posteroventral parvocellular preoptic nucleus
PRDM	pineal-restrictive downstream module
RT-PCR	Reverse transcriptase polymerase chain reaction
sGnRH	Salmon GnRH
Shha	Sonic hedgehog a
smo	Smoothened
T	Testosterone
Tel	Telencephalon
TeO	Optic tectum
Tg	Transgenic
TN	Terminal nerve
TNg	Terminal nerve ganglion
Vv	Ventral telencephalic area
VNN	Vomeronasal nerve
ZF	Zebrafish

Chapter 1: GENERAL INTRODUCTION

GnRH nomenclature

Multiple forms of Gonadotropin Releasing Hormone (GnRH) have been found in the brain of single species (J. Powell et al., 1994; N. Sherwood et al., 2002). However, the functions of the respective forms have not been determined in all cases. Throughout this thesis the term GnRH3 will be used in reference to the forebrain-hypophysiotropic GnRH in zebrafish and the term GnRH1 will refer to the forebrain-hypophysiotropic form of GnRH in humans and fish species that have two GnRHs. Similar to GnRH1 in mammals, GnRH3 (sGnRH) in mature zebrafish is present in the hypothalamus, and thus is the hypophysiotropic GnRH, but is also present in the TN (Steven et al., 2003). In some cases, when generically referring to hypophysiotropic GnRH in both ZF and mammals, the term forebrain GnRH is used. This section will clarify why this nomenclature was adopted.

The history of GnRH nomenclature is complex; originally this decapeptide was termed luteinizing hormone releasing hormone (LHRH) by Dr. Schally, whose group isolated and discovered the amino-acid sequence of this peptide (Baba et al., 1971). At the same time GnRH was isolated and described also by Dr. Guillemin who termed it Luteinizing Hormone Releasing Factor (LRF) (Burgus et al., 1972). However, with the discovery that LHRH/LRF not only regulates the release of luteinizing hormone (LH) but also that of follicle stimulating hormone (FSH), Dr. Schally coined the more general term 'Gonadotropin Releasing Hormone' (Schally et al., 1971). With the discovery that more than one form of GnRH exists among the

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vertebrates, various nomenclature systems were proposed. One scheme delineates the difference in amino acids in positions 5, 7 and 8 of the various GnRHs (King and Millar, 1985), which are the positions that differ between GnRH forms. This scheme specifies notation of distinct forms as $\{X^5X^7X^8\}$GnRH. A second nomenclature system identifies the GnRH forms based on the species in which the individual forms were first discovered, i.e., cGnRH-II (Chicken GnRH-II) or sGnRH (salmon GnRH) (Chou et al., 1985; Sherwood et al., 1983). The nomenclature was further complicated with the finding that some fish species have three forms of GnRH (King and Millar, 1985; Powell et al., 1986). Given these developments, the proposed methods of nomenclature were either cumbersome or confusing.

The current adopted GnRH nomenclature convention, in accordance with the recommendations of the March 1997 genome database nomenclature committee, is based on the terminology of GnRH 1/2/3 (White and Fernald, 1998). However, this nomenclature is differentially used based on two incompatible rationales. According to one rationale, the GnRH 1/2/3 terminology should be based on the location and function of the GnRH in question. GnRH1 being the hypophysiotropic form found in the hypothalamus and GnRH2 the midbrain form of GnRH. In fish that have three GnRH forms, GnRH3 would be the non-hypophysiotropic form found in the TN. However this method is inadequate when attempting to discuss GnRHs in fish such as the zebrafish, catfish and salmon, which have two forms of GnRH. In these fish, there is only one forebrain form of GnRH that is found both in the TN and in the hypothalamus. Presumably this form has dual functionality. In such cases, it is unclear whether the forebrain GnRH should be termed GnRH1 or GnRH3.

The second rationale, which has been adopted in this thesis, is that GnRH nomenclature be based not on function/location, but rather in accordance with GnRH phylogeny. According to this method, there are three branches in the GnRH phylogenetic tree. GnRH2 includes the midbrain "cGnRH-II" forms, GnRH3 includes the "sGnRH" forms, and GnRH1 includes mammalian GnRH, as well as species-specific fish GnRHs (mGnRH, sbGnRH, hrGnRH, etc.). GnRH1 is the form that, in most species, is the hypophysiotropic form, excluding fish that have two GnRH forms (such as zebrafish) in which GnRH3 is the hypophysiotropic form (Kah et al., 2007; Kuo et al., 2005) (Fig. 1). This nomenclature, although less informative, is straightforward and eliminates the need to predetermine the function/location of each given GnRH form. Therefore, we have followed the phylogenetic-based GnRH nomenclature scheme. Because zebrafish forebrain GnRH (sGnRH) clusters with all other GnRH3 (sGnRH) forms, it is termed GnRH3. It is noteworthy that recently Dr. Tobet has proposed a fourth branch in the GnRH phylogenetic tree. This branch includes the lamprey GnRHs (GnRH4). These GnRHs are significantly divergent in sequence from all other GnRHs (Kavanaugh et al., 2008).
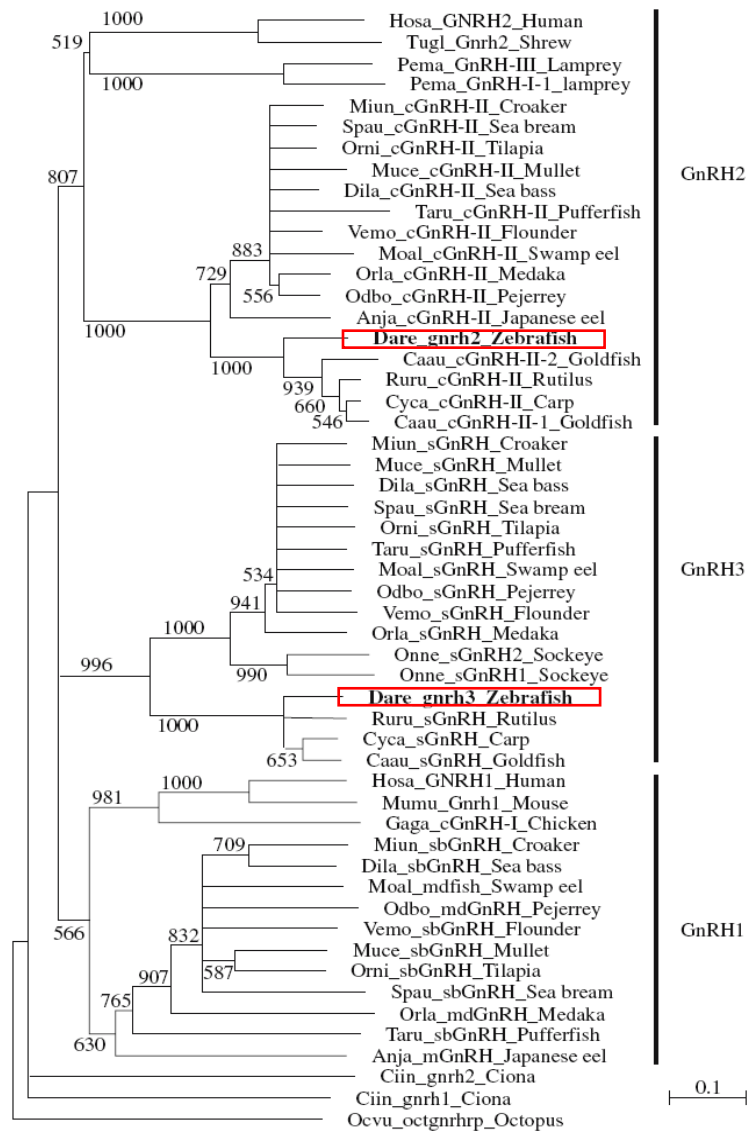
Figure 1.

GnRH phylogenetic tree illustrating the three branches of GnRH in vertebrates.
ZF GnRH2 and GnRH3 are highlighted (Kuo et al., 2005).

Reproduction in fish

Reproduction in all vertebrates is controlled by the CNS via hormones that are released and exert endocrine control over various tissues, primarily the pituitary and gonads. The capacity to reproduce is contingent on two separate events that are mainly controlled by the CNS. The first event is the development of functioning gonads during puberty, and the second is the maturation and release of gametes from the gonads. These two separate events are differentially controlled, however both processes share controlling factors including, among others, GnRH.

Although the reproduction of most fish is oviparous (external fertilization, external embryonic development) and mammalian reproduction is mostly viviparous (internal fertilization, internal embryonic development), the hormonal control over reproduction and the development of reproductive organs is conserved. Specifically, GnRH, gonadotropins and steroids are integral components of the hypothalamo-pituitary-gonad (HPG) axis, which is the reproductive regulatory axis in all vertebrates. Hence, the correct development of the GnRH and gonadotroph systems is crucial for reproduction in all vertebrates.

The pituitary-gonad axis

The HPG axis is the main endocrine-reproductive axis in vertebrates. In all vertebrates, this axis controls the development and maturation of the gametes and their release. The HPG axis cascade is triggered by environmental cues (temperature, photoperiod, social context) and internal signals. These signals are transduced into hormonal cues, which in turn cause the release of the hypophysiotropic form of GnRH from the hypothalamus to the adenohypophysis. In mammals, GnRH is

delivered to the pituitary via a hypophyseal blood portal system. An analogous system does not exist in fish, instead the GnRH neurons directly innervate the pituitary. Binding of GnRH to gonadotroph cell GnRH receptors in the pituitary elicits the biosynthesis and release of gonadotropins from the pituitary to the blood, specifically LH and FSH. These two hormones travel through the blood stream to the gonads where they trigger steroidogenesis, gametogenesis, vitellogenesis, and other processes (Cheng and Leung, 2005; Yoshitaka Nagahama, 1994).

Various gonadal hormones and factors such as E_2, T, activin and inhibin feed back to the pituitary and the hypothalamus, either up-regulating or down-regulating further transcription, translation and release of GnRH and gonadotropins (Nagahama, 1994).

When the HPG axis functions properly, and given sufficient environmental and behavioral cues, this cascade culminates in oocyte development, ovulation and spawning in females and sperm maturation and release in males.

GnRH

GnRH is a decapeptide neurohormone that is necessary for reproduction. To date, 14 variants of GnRH have been described in vertebrates, the sequence of which varies mainly at amino acid positions 5, 7 and 8 (Lethimonier et al., 2004). All vertebrate species studied have two or three paralogous forms of GnRH. The hypophysiotropic GnRH neurons are found primarily in the hypothalamus. These neurons release GnRH, which binds to specific G protein-coupled receptors on gonadotroph cells, subsequently inducing the biosynthesis and release of

gonadotropins from the adenohypophysis. In mice, a deletion of a segment of the GnRH1 gene prevents production of functional GnRH1 peptide, resulting in low levels of sex hormones and hypogonadal and infertile individuals (Mason et al., 1986). Hypophysiotropic GnRH is therefore crucial for normal development of the male and female reproductive systems, onset of puberty, and normal reproductive cycling (Amano et al., 1997a; Attardi et al., 1997; Khakoo et al., 1994; Krieger et al., 1982; Ojeda et al., 2003; Senthilkumaran et al., 1999; Whitlock et al., 2006).

The amino acid sequence of the hypophysiotropic GnRH varies between species in positions 5, 7 and 8. The hypophysiotropic form of GnRH is not limited to the hypothalamus. Forebrain GnRH neurons are found in a continuum from the olfactory region to the hypothalamus (Herbison, 2006). In addition, forebrain GnRH fibers are found in many CNS regions and GnRH transcripts are found in the placenta, ovary, and immune system (Chen et al., 2002a; Cheng and Leung, 2005; Kang et al., 2001; Wong and Zohar, 2004). Given this wide distribution, hypophysiotropic GnRH is increasingly viewed not only as a gonadotropin releasing factor but also as having multiple additional roles. Synthetic analogs of GnRH1 are routinely used clinically as a part of various human-assisted reproductive techniques, to treat cancers, and to induce spawning in livestock and fish (Kiesel et al., 2002; Kuo et al., 2008; Mylonas et al., 1997; Zohar, 2000).

GnRH2 represents the most ancient and conserved form of GnRH. It is found in the midbrain tegmentum of all jawed vertebrates and is believed to play a role in reproductive behavior and/or in control of appetite and metabolism (Amano et al., 1997b; Barnett et al., 2006; Sherwood et al., 1993; Temple et al., 2003). GnRH3 is

present in all fish species. In fish that have two forms of GnRH, GnRH3 is located in the TN and hypothalamus. In fish that have three forms of GnRH, GnRH3 is located only in the TN (Gothilf et al., 1996; Okubo et al., 2000). The function of GnRH3 in fish that have three forms of GnRH is not yet clear, although a role in reproductive behavior has been suggested (Ogawa et al., 2006).

In species that have two forms of GnRH, the forebrain GnRH is located in both the TN and hypothalamus. As in mammalian species, in ZF only two forms of GnRH, GnRH3 and GnRH2, have been isolated and characterized at the nucleotide level (Steven et al., 2003). The lack of a third form of GnRH, which appears to have been lost during evolution, would suggest that any non-redundant functions played by GnRH1 have been assimilated by GnRH3 (Kuo et al., 2005; Okubo and Aida, 2001; Okubo et al., 2002).

Forebrain GnRH neuronal development

Neuronal migration

Progenitor and post-mitotic neuron migration is an integral phase in CNS development. Two main forms of neuron migration exist. In glial migration, newborn neurons migrate along radial glial cell fibers from their birth place in the germinal layers of the neural tube, to their final destination in the cortex and other CNS regions. Once these neurons are in place, they establish synaptic connections with various other neurons. Tangential neuron migration is a form of migration that is perpendicular to the germinal layer. This migration includes movement of neurons from their birthplace to their final destination in a direction that is parallel to the

ventricle (Corbin et al., 2001). Contrary to radial migration, tangentially migrating neurons attach to one another or to preexisting fibers and migrate as chains of neurons (Lois et al., 1996). A classic example of neurons that conduct tangential migration are precursors of inhibitory interneurons, which originate in the germinal layers of the ganglionic eminences, and migrate to the cortex.

Although migration of forebrain GnRH neurons is not 'classical' tangential migration, the basic characteristics of these neurons is in essence tangential, this includes the fact that large portions of this migration occurs parallel to the ventricle, the fact that GnRH3 neurons attach to preexisting fibers (i.e. axophilic migration), and the finding the GnRH3 neurons migrate in chains from the olfactory region. For these reasons forebrain GnRH neuronal migration can be defined as tangential. Forebrain GnRH neurons probably shares regulatory characteristics with other neuronal populations that conduct tangential migration.

Regardless of the form of migration, the migratory process is divided into several stages. First, the soma switches from a stationary state to a migratory state. This migratory state lasts as long as migration persists and during this time the soma must respond to guidance cues that guide the cell to its correct destination. Guidance factors can influence movement, directionality and speed. Upon reaching its destination, the soma transforms back to a stationary phase and begins to form connections with other cells. The migration phase itself is characterized by three steps: a) leading-edge extension directed by microfilament polymerization, b) nucleokinesis, and c) retraction of the trailing process. All these stages are mediated

by changes in the microtubule network and cytoskeleton (Lambert de Rouvroit and Goffinet, 2001; Sobeih and Corfas, 2002).

Fiber outgrowth and pathfinding is in many respects similar to soma migration. Fibers follow a growth cone and movement is mediated by the cytoskeleton, specifically by actin filaments and microtubules. The main difference between fiber and soma movement is that during fiber outgrowth the soma remains stationary (Bouquet and Nothias, 2007). Generally speaking, fibers follow two growth patterns, parallel (i.e., away from the midline) or across the midline (commissural fibers).

The factors that mediate soma migration and fiber pathfinding are numerous, as is expected given the complexity of the CNS. These extracellular factors can be separated into four main classes. The first are extracellular matrix molecules (ECM) that are secreted from cells and which are thought to compose a large proportion of the brain volume (Ruoslahti, 1996). These proteins and polysaccharides fill the intercellular space and interact with soma and fibers. ECMs that are involved in neuronal migration include Reelin, Integrins, HSPGs, Laminin and Anosmin-1 (Doetsch et al., 1997; Sobeih and Corfas, 2002; Walsh and Doherty, 1997). The gene *kal1* encodes for Anosmin-1. A mutation in *kal1* is one of the causes for Kallmann syndrome, which results in hypogonadism (Soussi-Yanicostas et al., 1998). Thus ECM proteins, as well as cell surface molecules responsible for interactions with the ECMs, are involved in tangential migration of neurons. The ECM serve not only to directly modulate neuronal migration, but also form a distinct region in which soluble

factors accumulate. Thus, the ECMs play a part in regulating chemoattractant and chemorepellent factor gradients (Sobeih and Corfas, 2002).

The second group of factors involved in neuronal migration is the cell adhesion molecules (CAMs). These are cell surface molecules that mediate cell-cell adhesion and recognition. Similar to ECMs, CAMs also participate both in fiber pathfinding and fasciculation and in neuron migration. Neural cell adhesion molecules (N-CAM) are a subset of CAMs that act via homophilic and heterophilic interaction to mediate adhesiveness to other neurons or to the ECM. Examples of CAMs are neurolin and astrotactin.

 The third group of factors is soluble and membrane-bound factors, such as secreted guidance molecules that have the capacity to bind to receptors. These factors play a crucial role in many processes, ranging from cell survival and morphology to neuron migration and fiber-targeting. By virtue of being soluble, these factors can form gradients that regulate fiber pathfinding and neuron migration at a distance. Secreted guidance molecules can provide both positional and directional signals to soma and fibers, although sometimes it is difficult to differentiate between the two signals. However, in both cases their action is dependant on location, timing and gradient. Examples are Neuregulin, Notch, Neurotrophins, Chemokines, and factors that have been implicated with tangential migration of neurons including Slit, Ephrins, Semaphorin and Netrin1 (Guthrie, 2004; Song and Poo, 2001).

Netrin1 has been implicated in GnRH1 migration (Schwarting et al., 2004), but is better known for its involvement in migration of cells from the rhombic lip to their destination in the hindbrain. Netrin1 has is expressed by the floor plate of the

hindbrain and acts as an attractant to induce and guide the processes involved in cell

migration from the dorsal rhombencephalic neuroepithelium (Alcantara et al., 2000)**.**

A prevalent hypothesis regarding the action of secreted guidance molecules is

as follows. Growth cone exposure to a factor induces activation of receptors. The

activated receptors then cluster in the growth cone area in which exposure to the

factor is highest. This spatial-specific clustering of receptors provides directional

information, which is conferred to the cytoskeleton via downstream effectors. This

process allows for directionality of neuronal movement based on factor gradients.

Movement itself is modulated by actin filaments and may be either away from or

towards the factor, depending on whether it is repulsive or attractive (Song and Poo,

2001).

The last group of factors involved in neuronal migration is neurotransmitters

and ion channels. An example of an ion-channel factor is Ca^{2+}. The rate of neuron

migration is correlated to the fluctuation rate of intercellular Ca^{2+} levels (Komuro and

Rakic, 1992). Neurotransmitters are also involved in neuronal migration. For

example, blockade of the NMDA receptor inhibits migration (Komuro and Rakic,

1993) and blockade of GABA receptors enhances migration of GnRH neurons

(Fueshko et al., 1998)

Migration of GnRH neurons

During early development, forebrain GnRH neurons traverse a unique

tangential migration route from the region of the olfactory placode into the central

nervous system (Marin and Rubenstein, 2003). Forebrain GnRH neurons are born in

the olfactory region and migrate apposed to the vomeronasal and olfactory nerve

tracts to the TN ganglion (TNg). GnRH neurons then cross the nasal/brain junction to enter into the forebrain at the cribriform plate. Once in the forebrain, GnRH neurons turn caudoventrally towards the preoptic area (POA)-hypothalamus, eventually reaching the preoptic region of the hypothalamus (Norgren et al., 1995; Parhar et al., 1995b; Wray, 2001; Wray et al., 1989b). The GnRH neurons in the POA-hypothalamus extend fibers that innervate the pituitary portal blood system in mammals or the adenohypophysis directly in fish.

Evidence exists suggesting that GnRH soma migration is axophilic and involves various cell adhesion molecules. Although GnRH neurons migrate along the tract of the olfactory/vomeronasal fibers, the exact identity of the fibers involved has not been fully elucidated (Schwarting et al., 2004; Wray et al., 1994). In zebrafish, GnRH3 soma also follow their own fiber tracts (Palevitch et al., 2007).

It is important to note that the GnRH migrational path is very similar across vertebrates from fish to mammals. However, there are some slight differences, mainly in the trajectory of migration, which are a consequence of CNS architecture differences between classes. In fish, the elongated shape of the CNS translates into GnRH migration that is mainly anteroposterior and less dorsoventral, while in mammals the CNS architecture translates into GnRH migration that involves more dorsoventral changes. This difference is especially apparent in mammals when GnRH neurons cross the cribriform plate and turn ventrally into the ventral forebrain, while in fish the turn made by the neurons is less prominent. Another difference is the timeframe of migration. In the mouse, migration begins at E10.5 and ends at E18.5, taking approximately 8 days. In ZF, migration begins at 3 dpf with the first soma

reaching the hypothalamus at 13 dpf, taking approximately 10 days. This difference in duration of migration coupled with a difference in distance of migration, which is shorter in ZF, dictates a difference in GnRH soma rate of migration (see chapter 2).

Failure of GnRH neurons to migrate properly and/or of GnRH fibers to target the pituitary correctly will result in a failure to deliver GnRH to the pituitary, ultimately leading to abnormal gonadal development and infertility (MacColl et al., 2002; Sato et al., 2004; Zenteno et al., 1999).

In addition to information regarding GnRH soma migration during early development, several reports have addressed the topic of GnRH fiber tract location. In mammals, the focus has been on GnRH fibers along the GnRH migration tract from the olfactory region into the forebrain and on fiber projections to the median eminence. Fibers have also been described in the amygdala, organum vasculosum of the lamina terminalis, hippocampus and midbrain (Parhar et al., 1995b; Rogers et al., 1997; Schwarting et al., 2001; Spergel et al., 2001; Stopa et al., 1991; Wray, 2001). In fish, GnRH fibers as well as multiple types of GnRH receptors have been localized to various regions of the CNS including the telencephalon, preoptic region, hypothalamus, thalamus, midbrain tegmentum, rhombencephalon, optic chiasm, and retina (Amano et al., 1997b; Chen and Fernald, 2006; Gonzalez-Martinez et al., 2004b; Gothilf et al., 1996; Grens et al., 2005; Hoffman and Gibbs, 1982; Lethimonier et al., 2004; Parhar et al., 1995a; Parhar et al., 1995b; Whitlock et al., 2006; Wong et al., 2004). This widespread location of GnRH fibers and receptors suggests a multifaceted regulatory mechanism (Chen and Fernald, 2006) and indicates that GnRHs play multiple neuroregulatory roles that potentially include

control over sensory, metabolic and motor functions. Although location of GnRH fibers in mature fish has been described in several species, their precise spatiotemporal developmental pattern has not been elucidated to date. It is noteworthy that GnRH mRNA has been detected in several extra-CNS tissues including the pituitary, ovary, immune cells, and seminiferous tubular cells (Cheng and Leung, 2005; Wong and Zohar, 2004).

Factors involved in GnRH differentiation and migration

A plethora of factors influence and guide GnRH neuron migration and fiber-targeting during early development. These factors include secreted molecules such as GABA and Netrin1, cell surface proteins such as various receptors and adhesion molecules, and nuclear factors (Cariboni et al., 2007; Kramer and Wray, 2000b; Schwarting et al., 2004; Tobet and Schwarting, 2006; Wray, 2001). Given the complexity of GnRH neuron migration, and the diversity of regions that these neurons encounter, it is safe to assume that different factors control different stages of migration in different regions of the migratory route.

The first stage of forebrain GnRH neuron development is their differentiation in the olfactory region. It is not clear exactly which factors control GnRH neuron differentiation, but it is assumed that they include factors that are involved in the development of the olfactory placode such as OTX1/2, six-3 and Pax-6. These factors appear prior to GnRH neurons, are necessary for olfactory placode formation, and impact GnRH neuron development. A second set of implicated factors are those

expressed early on by GnRH neurons themselves. These include GATA-4, AP-2α, NSCL1, Olf-1, and GnRH (Cariboni et al., 2007).

GnRH soma and fibers begin migration from their birthplace in the olfactory region. Migration within the olfactory region is tied to several factors, including LIF, a cytokine that is highly expressed in the nasal region in developing embryos and elicits a chemotactic response from GnRH neurons *in vivo* (Magni et al., 2007). Another cytokine that is involved at this stage is HGF and its receptor Met. The Met receptor is expressed by GnRH neurons in the nasal compartment and migration of GnRH neurons in nasal explants was directly correlated to HGF concentrations (Giacobini et al., 2007). A chemokine that has been implicated in nasal GnRH neuron migration is Cxcl12 (previously SDF-1) and its receptor Cxcr4. Both are expressed in the olfactory region during development. Cxcr4-deficient mice and ZF exhibit various GnRH neuron abnormalities (Toba et al., 2008). Other factors that are associated with GnRH neurons or with the vomeronasal nerve in the olfactory region include N-CAM, Dcc and NELF. NELF is expressed in the olfactory area and in GnRH neurons. Reduced NELF expression effects both GnRH fibers and the number of GnRH soma that exit the nasal region (Kramer and Wray, 2000b).

N-CAM is a neural cell adhesion molecule that is expressed along the migration path of the GnRH neurons by the olfactory, vomeronasal, and terminal nerves. The distribution of N-CAM strongly suggests that it is important for GnRH neuron migration (Schwanzel-Fukuda et al., 1992). Another factor that influences the axophilic aspects of GnRH migration in this region is EphA5, a tyrosine kinase receptor that is an ephrin receptor. Ephrin is expressed by the vomeronasal tract and

over-expression of EphA5 causes delay of GnRH migration initiation as well as clustering of neurons on the olfactory fibers (Gamble et al., 2005). A similar approach to studying GnRH neurons was taken when studying surface glycoconjugate lactosamine, a factor that is highly expressed in the olfactory epithelium as well as by GnRH neurons. The peak expression of lactosamine by GnRH neurons occurs during their most rapid migration phase. In mice in which acetylglucosaminyltransferase-1, an enzyme required for lactosamine expression, is knocked-out, there is an increase in the number of GnRH neurons that remain in the olfactory region (Bless et al., 2006). Netrin1 and its receptor Dcc, factors also implicated in migration of GnRH neurons in the nasal compartment, are discussed in more detail below.

The next phase of GnRH neuron migration is the passage into the CNS via the nasal-forebrain junction at the cribriform plate. In mice, GnRH neurons split at this junction. The majority of neurons continue migration towards the hypothalamus via the caudal branch of the vomeronasal nerve, and few neurons continue into the accessory olfactory bulb. At this point, multiple repulsive and attractive factors, as well as cell adhesion molecules, are likely to participate (Cariboni et al., 2007).

In Kallmann syndrome, GnRH neurons appear to stop migration in proximity to the cribriform plate. A factor that is crucial in this juncture is the extracellular matrix protein Anosmin-1, which is a product of the gene *kal1*. *In vitro* studies using the immortalized GnRH GN11 cell line revealed a chemotactic response of GN11 cells to Anosmin-1. In medaka, knock-down of the *kal1* ortholog resulted in disruption of GnRH migration into the forebrain (Cariboni et al., 2004; Okubo et al., 2006). Accordingly, mutation in the *kal1* gene in humans results in abnormal

targeting of GnRH neurons, causing idiopathic hypogonadotropic hypogonadism. This condition can be reversed by exogenous GnRH pulses (MacColl et al., 2002; Sato et al., 2004; Zenteno et al., 1999).

A neurotransmitter that is involved in GnRH migration at the nasal-forebrain junction, and thereafter, is GABA. Antagonists of the GABA-A receptor increase GnRH neuron migration, and GABA-A receptor agonists inhibited the migration of GnRH neurons in nasal explants. Moreover, in transgenic mice in which GABA is over-expressed in GnRH neurons by the GAD67 enzyme, abnormal migration and location of GnRH neurons is observed. These animals are subfertile (Fueshko et al., 1998; Heger et al., 2003). Additionally, Reelin, an extracellular matrix protein found along the GnRH migrational path has an inhibitory role in guiding GnRH neurons across the nasal/brain junction and into the basal forebrain (Cariboni et al., 2005).

The final phase of GnRH neuron migration is the caudoventral turn into the basal forebrain, which also involves the vomeronasal fiber subset that takes the same path. It is not clear which factors regulate this phase of migration, however Fibroblast Growth Factor 1 (FGF1) and GABA have been suggested as participants in this final approach. FGFR mutant mice exhibit a 30% reduction in GnRH neuron number. This reduction was especially pronounced in the region of the organum vasculosum of the lamina terminalis, but occurred throughout the forebrain (Tsai et al., 2005). In addition to effects on soma, FGF has an effect on GnRH fiber outgrowth and final targeting to the median eminence (Gill and Tsai, 2006).

This short overview of some of the factors that are implicated in GnRH neuron migration and fiber-targeting indicates the complexity of the GnRH neuron

migration process. It is clear that a large number of factors are involved in a spatiotemporal-specific manner in GnRH system development. Some interact directly with GnRH neurons, some interact with the olfactory and vomeronasal nerves that the GnRH neurons migrate apposed to, and others seem to act in a more general fashion to modulate relevant CNS regions. What is clear is that GnRH neurons migrate along the path taken by the olfactory and vomeronasal nerves. However, while the correct migration of these nerves is a necessity, it is not wholly sufficient for correct GnRH migration and final location.

Given the current knowledge regarding the regulation of forebrain GnRH neuron development, we chose to focus on three factors, Netrin, Shh and GnRH. One of these factors, Netrin1, has already been directly implicated in GnRH1 neuronal migration in mammals. However, many questions remain regarding its specific role. Although the published data is not conclusive, GnRH and Shh are also suspected to be relevant factors due to association or *in vitro* studies.

The role of Netrins, Shh and GnRH in GnRH migration

As noted above, one chemoattractant factor implicated in GnRH1 migration is Netrin1, which is a diffusible Laminin-related protein. This secreted molecule attracts and repels various fiber types, as well as neuron cells (Colamarino and Tessier-Lavigne, 1995; Kennedy et al., 1994; Serafini et al., 1996). This dual activity depends on the constellation of receptors involved (Dickson, 2002.). Depending on the receptors expressed by the growth cone, Netrin1 fluctuates between a chemoattractant and chemorepellent. It seems that expression of the Netrin1 receptor Dcc promotes

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attraction, while expression of the Unc5 receptor promotes short range repulsion. A combination of Dcc and Unc5 followed by interaction of the cytoplasmatic domains of these two receptors mediates long range repulsion (Barallobre et al., 2005; Hong et al., 1999). Interestingly, Netrin not only plays a role in the attraction of commissural fibers towards the midline, but is also a factor in tangential and radial migration of neurons (Alcantara et al., 2000; Goldowitz et al., 2000; Kawasaki et al., 2006). In addition, Netrin is an important factor in morphogenesis of the vascular system (Lu et al., 2004), as well as in tumorigenesis (Arakawa, 2004). During development in mice, the Dcc receptor is expressed by cells of the olfactory epithelium and the vomeronasal nerve, and in cells migrating along this tract including GnRH1 neurons (Schwarting et al., 2001). Netrin1 is expressed in the ventral forebrain and may mediate the attraction of these cells and fibers. Studies in mice show that in either Netrin1- or Dcc-deficient animals, GnRH1 neurons migrate to inappropriate destinations in the CNS (Deiner and Sretavan, 1999; Schwarting et al., 2001; Schwarting et al., 2004). Specifically, in Dcc- and Netrin1-deficient mice, fewer GnRH1 cells than normal migrate to the hypothalamus; these cells fail to turn into the ventral forebrain and are displaced to the cerebral cortex and dorsal forebrain (Schwarting et al., 2001; Schwarting et al., 2004). The aforementioned results indicate that Netrin1 and its receptor, Dcc, play a role during the later stages of GnRH1 migration, once the neurons cross the cribriform plate and turn ventrally towards the hypothalamus. In ZF, similar to other vertebrates, Netrin1a is expressed in the caudal diencephalon, midbrain, hindbrain and spinal cord, and in the forebrain along various commissures, as well as in the optic cups (Lauderdale et al., 1997).

The Netrin receptors in ZF are Dcc, neogenin (a Dcc-like receptor) and Unc5b. As in other vertebrates, Dcc in ZF seems to be the central receptor for Netrin1-mediated signaling that results in chemoattractive activity (Suli et al., 2006).

Recently, two novel Netrins in addition to Netrin1a and Netrin1b have been discovered in ZF, these Netrin orthologs are named Netrin2 and Netrin4 (Park et al., 2005). Although the role of these new Netrins is not known, Netrin2 is of special interest with respect to forebrain GnRH due to the fact that it has a unique expression pattern within the CNS. In contrast to Netrin1, which is expressed primarily along the midline, Netrin2 is expressed at 48 hpf in bilateral clusters in the telencephalon, tectum and cerebellum (Park et al., 2005). The time frame and expression pattern of Netrin2 suggests that it may play a role as a chemoattractant for forebrain GnRH soma and fibers.

An additional factor that is involved in CNS development in general, but more specifically in forebrain patterning, pituitary development and Netrin activity, is the protein hedgehog (Hh) (Charron et al., 2003; Karlstrom et al., 1999; Sbrogna et al., 2003). Members of the Hh family are signaling molecules that are secreted by the notochord and floor plate and act as differentiation factors in a concentration-dependent manner (Charron and Tessier-Lavigne, 2005). The Hh pathway plays a crucial role in embryo patterning, including that of the neural tube, but also exhibits specific fiber chemoattractant and chemorepellent activities (Charron et al., 2003; Kolpak et al., 2005), leading to the hypothesis that morphogenes such as Hh are reused later in development as soma and fiber guidance cues. As in mammals, ZF have five forms of Hh – Desert hedgehog, Indian hedgehog a/b and Sonic hedgehog a

(Shha) and b. All three activate a downstream signaling cascade that includes the transmembrane receptors Patched1 and Smoothened, as well as the family of Gli transcription factors. There are several ZF mutant lines in which the Hh pathway is disrupted including the sonic-you (syu), in which Shha is mutated, the smu, in which smoothened is mutated, and the you-too (yot), in which gli2 is mutated. These mutants display various abnormalities including defects in the spinal cord and ventral forebrain. The yot mutant displays malformations in the optic chiasm, postoptic commissure and abnormal development of the pituitary, suggesting that the yot mutation leads to defects in fiber guidance, ventral diencephalon development and pituitary formation (Karlstrom et al., 1999). However, the specific effect of these mutations on the GnRH system is unknown.

Interestingly a link was found between activity of Netrin1 and that of Hh. The formation of commissures that meet or traverse the midline is believed to be mediated by Netrin1, which acts as a long range chemoattractant (Charron and Tessier-Lavigne, 2005; Serafini et al., 1996). However, in Netrin1 or Dcc mutant mice some commissural fibers do reach the midline, suggesting that other chemoattractants are involved. Indeed, Shha functions as a commissural fiber chemoattractant that can mimic Netrin1 activity and act independently of Netrin1 (Charron et al., 2003). This Shha activity can be abolished by cyclopamine treatment, which inhibits the Hh mediator Smoothened (Charron and Tessier-Lavigne, 2005). An additional study determined that retinal ganglion cells are guided along a constrained pathway in the ventral midline and prevented from crossing the midline by Shha expression at the optic chiasm border (Trousse et al., 2001). Lastly, in the ZF smu mutant there is a

reduction in Netrin1 expression in areas that are important for fiber pathfinding (Varga et al., 2001). These studies demonstrate that Shha is a chemoattractant that acts with Netrin1 in commissural fiber attraction. Furthermore, as demonstrated in multiple studies including GnRH studies; both Netrin1 and Shha play roles in the migration and targeting of non-commissural fibers. In addition to the involvement of Shha in fiber-targeting in ZF, this pathway is thought to be necessary for development of the pituitary. To assess the involvement of Shha in pituitary development Sbronga et al. (Sbrogna et al., 2003) used cyclopamine treatment by immersion of ZF at different stages of development. Post-immersion, measurements were conducted of the pituitary marker lim3 and the homeobox transcription factor nk2.2, which is expressed in the adenohypophysis and is positively regulated by Hh..Alteration of the time frame of cyclopamine exposure resulted in specific effects on pituitary development. These effects ranged from elimination of the adenohypophysis when ZF embryos were treated pre-somatogenesis to a slightly reduced adenohypophysis with a decrease in hormone-producing cells when treated post-somatogenesis. The defects observed in the cyclopamine-treated embryos are similar to abnormalities observed in the smu mutant.

Lastly, *in vitro* experiment indicate that GnRH itself may be a factor that plays a role in GnRH neuron modulation. GnRH receptors are expressed by GnRH1-producing FNC-B4 cells. The same GnRH cell line, when exposed to exogenous GnRH underwent various modulatory changes that include (i) stimulated cAMP production and calcium mobilization, (ii) cytoskeletal remodeling, growth cone extension abnormalities, and a dose-dependent increase in migration, (iii) down-

regulation of Nestin expression. (Romanelli et al., 2004). These effects of GnRH on the GnRH FNC-B4 cell line suggest that GnRH acts as an autocrine/paracrine regulatory factor in GnRH neuron development.

Zebrafish as a model system

Many models have been used to study early development of vertebrates. An especially challenging area of research is the field of CNS development. Zebrafish are an extremely powerful model for studying vertebrate developmental biology, as well as for research related to human diseases and potential targets for therapeutic intervention. Their value stems from several traits found in zebrafish and the many molecular tools available for use in this species. Specifically, zebrafish possess: 1) large number of embryos in each spawn 2) fast development from fertilization to the phylotipic stage (~24 hours post-fertilization) 3) transparent embryos and larvae during development allowing for visualization of processes *in vivo* 4) external fertilization and development 5) the ability to raise hundreds of zebrafish inexpensively and in a small space, and 6) the relatively short time in which zebrafish reach reproductive maturity. In terms of available molecular tools, a) the zebrafish sequencing project is close to completion, b) forward genetics via mutagenesis results in a plethora of well defined zebrafish mutants, c) reverse genetics methods enable the creation of zebrafish transgenic lines in which markers or other proteins are specifically expressed d) gene knock-down and over-expression (OE) methods are readily available (Bradbury, 2004). Many transgenic zebrafish lines and mutant lines can be obtained from the zebrafish international resource center (ZIRC). The

available methodologies used in zebrafish are continually being expanded and improved. Two examples of recent progress in genetic manipulation of zebrafish are the advent of Tol2 transposon element usage enabling faster and easier creation of transgenic lines (Kawakami, 2007; Kawakami et al., 1998), and the recent development of the synthetic zinc finger nuclease-mediated knock-out (Ekker, 2008).

With regard to developmental events, in zebrafish, it is possible to endogenously express markers in specific tissues and observe of tissue/organ development in the whole animal. When this is coupled to knock-down or OE of specific genes, *in vivo* analysis of downstream morphological, developmental and physiological effects are possible. In contrast to the use of ISH and ICC, in which development is only seen as a snapshot in time, endogenous expression of proteins such as EGFP or RFP is temporally sensitive. In comparison to transgenic mammals, zebrafish afford the advantage of optic transparency, hence the ability to fully take advantage of endogenous marker expression.

One of the main issues in studying GnRH neurons and their development is the fact that only a modest number (700-1200 in mammals) of neurons are scattered along a continuum in an adult CNS (Rubin and King, 1994; Tsai et al., 2005). This has made studying GnRH neuron properties and development a challenge. With the advent of transgenesis and promoter-reporter protein expression, the use of animals in which GnRH neurons express florescent proteins has become possible, vastly improving visualization of GnRH neurons. Indeed, there are several lines of GnRH-GFP transgenic mice and rats in use (Han et al., 2004; Spergel et al., 1999; Suter et al., 2000). Compared with these animals, use of a transgenic GnRH3:EGFP

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zebrafish allows observation of GnRH neurons not only in tissue slices, but also in the intact live animal, resulting in real-time high resolution analysis of developmental events, and of the factors effecting these events in a whole-animal context.

Similar to the case in mammals, and unlike many other fish species, zebrafish have two GnRH forms (GnRH3, GnRH2). In addition, the migratory path of hypophysiotropic GnRH neurons in teleosts is very similar to mammals. Combining the use of a *Tg(GnRH3:EGFP)* zebrafish line with the various traits of zebrafish as well as the molecular tools and mutants available, has greatly aided in a better understanding of GnRH system development, as well as the factors that control this process.

Project relevance

Normal early development of the forebrain GnRH system is a crucial step in gonadal development and the establishment of a functional reproductive system. In addition, the forebrain GnRH neuronal development is a unique model for studying long range tangential neuronal migration and fiber-targeting. For these reasons, studying the events and factors that are involved in the early development of the GnRH system are crucial.

The GnRH system has been studied in various fish species, however much of the current research related to the morphology and regulation of the system in fish has focused on adult stages rather than early development. Previous studies in fish and mammals that addressed the early development of the GnRH system have produced invaluable data regarding the development of the system and the factors that govern it.

However, these studies are limited by available methodologies (e.g., ISH and ICC) or by the inherent limitations posed by mammalian species, primarily the fact that mammalian development is viviparous and embryos are not optically translucent (Chen and Fernald, 2006; Gonzalez-Martinez et al., 2004a; Grens et al., 2005; Lethimonier et al., 2004; Tobet and Schwarting, 2006; Whitlock et al., 2006; Wong and Zohar, 2004; Wray et al., 1989a; Wray et al., 1989b; Wu et al., 2006). These limitations have made it challenging to examine the very early development of the forebrain GnRH neurons and the development and location of the forebrain GnRH fibers. Much remains to be elucidated regarding the spatiotemporal development of the forebrain GnRH system, including questions regarding why and how fibers target non-pituitary regions, as well as which factors mediate GnRH soma migration and fiber pathfinding. This study expands the existing knowledge regarding the spatiotemporal pattern of early forebrain GnRH soma migration, fiber development and the factors that govern this process through the use of transgenic ZF.

The use of the ZF model allows *in vivo* observation of the development of the GnRH system with unprecedented resolution, especially with regard to delineating the development and location of GnRH fibers. Using a whole animal *in vivo* model also allows the study of GnRH neuronal development in the context of the entire milieu of CNS factors that affect the migration process.

The hypophysiotropic GnRH neurons conduct a long tangential migration during early development. This migration traverses various non-CNS and CNS regions and is undoubtedly controlled by multiple chemoattractant, chemorepellent, autocrine and other factors. Understanding the process by which various factors participate and control the

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complex GnRH neuronal migration is important not only on a basic level but also due to the fact that the Kallmann syndrome, in which afflicted humans are sterile and anosmic, is caused by an early GnRH migrational defect.

As discussed above, multiple factors are involved in GnRH neuron migration. However, much remains to be understood about the control of GnRH system during development. This study focuses on several factors that are implicated, either directly or indirectly, as participants in the process of forebrain GnRH neuronal migration. Examining the role of these factors was achieved by knock-down, OE and mutation of these factors. The effect of OE and knock-down of various factors during early development was observed *in vivo* and thus has provided a better understanding regarding the exact location, time frame and function of these factors vis-à-vis GnRH system development.

GnRH peptide and receptor transcripts begin to be expressed very early during development. The function of this early expression is probably not aimed at direct modulation of the gonads since, at this time, GnRH neurons have not reached the adenohypophysis/portal blood system, nor have the gonads developed (Palevitch et al., 2007; Wray et al., 1989a). The GnRH1 decapeptide can modulate the cytoskeletal and fiber development of GnRH1-producing neurons *in vitro*, yet the *in vivo* effects of GnRH1 on the development of the GnRH system are not known. In view of these facts, we investigated the hypothesis that the *in vivo* expression of GnRH modulates the development of the GnRH system. This hypothesis was tested via GnRH knock-down experiments in Tg(GnRH-GFP) ZF.

Studies examining the location of GnRH1 neurons in mice that are deficient in Netrin1a or Dcc have focused on the pattern of GnRH1 cell location after abnormal migration occurred (at E13 and E15). In addition, GnRH1 location focused on GnRH1 soma and not on potential abnormalities in the targeting of the GnRH1 fibers that innervate the portal blood system, or other CNS regions (Schwarting et al., 2001; Schwarting et al., 2004). It is therefore of interest to observe whether there are earlier migrational soma defects, as well as mistargeting of fibers in Netrin1a- or Dcc-deficient animals, and to observe such migrational defects as they develop in real time. One of the unexplained findings of the aforementioned studies was that although Netrin1a is expressed during development in the caudal olfactory epithelium, loss of Netrin1 had no measurable effect on cells in the nose that express Dcc. Our hypothesis is that such an effect may manifest as abnormalities in the early development of GnRH1 fibers, rather than in final cell location. Also, given that Netrin1a is established as a chemoattractant for GnRH neurons/fibers or for the scaffold of fibers that they are associated with, we tested whether tissue-specific over-expression of Netrin1a disrupts development of the GnRH system.

Although the role of Netrin2 is currently unknown, the spatiotemporal expression pattern of the recently discovered ZF Netrin2 suggests that this factor may play a unique and important role as a chemoattractant for GnRH soma and fibers during early development. We examined the specific effect of Netrin2 knock-down on GnRH3 neurons and embryo development.

There is little information regarding the effect of disrupting the Shh pathway on the development of the GnRH system. Disruption of the Shha pathway at various

timepoints by immersion in cyclopamine was expected to result in two separate types of abnormalities: 1) Early disruption resulting in various severities of embryonic and pituitary developmental abnormalities, a result of Shha activity as a morphogen, and 2) later disruption resulting in commissural fiber pathfinding abnormalities and tangential migration abnormalities, a result of Shha activity as a neuromodulatory factor.

From the available data, it seems that both classes of abnormalities caused by disrupting this pathway affect CNS infrastructure in a manner that could alter development of the GnRH system, such as structures and commissures in the ventral forebrain, as well as the development of the pituitary itself. We have examined the effect of blocking the Shha pathway on the development of the GnRH3 system. To allow temporal isolation of Shha effects during development, we used cyclopamine, which blocks the Shha pathway and can be used discriminately by immersion at specific temporal regimes. Such immersions enabled us to target specific developmental stages and levels of effect before and after the major patterning events in the CNS have occurred. These experiments were further corroborated by utilizing a Shh mutant zebrafish line.

The use of a model system that allows expression of EGFP specifically in GnRH3 neurons, coupled with the optical transparency of the larvae, allowed us to physically ablate the GnRH3 neurons with great specificity and at an early developmental time point. These experiments allowed us to examine the origin of GnRH3 neurons as well as to understand the effects of the absence of GnRH3 neurons on the morphology and development of the CNS and the gonads over time. In the past, the origin of GnRH

neurons was studied by ablating the entire olfactory placode (Akutsu et al., 1992). However, targeted ablation of the GnRH neurons during early development has not been accomplished to date. The laser ablation method used allowed us to perform specific bilateral and unilateral ablation of GnRH3 neurons, without harming adjacent cells. The recently developed method of using constructs to specifically deliver promoter-driven toxin to a subset of cells is an alternative ablation method that was attempted. Exploring the phenotypic effect of GnRH3 neuron ablation on the CNS and gonads enhances our understanding of the system in its entirety.

Moreover, one of our long-term goals is to develop methodologies to manipulate the hypothalamo-pituitary-gonad (HPG) axis in fish with the objective of inducing either better fecundity or sterility. In aquaculture, increased fecundity in broodstock fish is obviously a desirable trait. Conversely, sterility of fish raised for consumption is a sought-after attribute. Sterility is desirable for several reasons: a) growth of gonads diverts energy away from muscle growth thus prolonging the amount of time that it takes for food fish to reach market size, b) in some cases puberty and the development of gonads can negatively affect the flesh quality (flavor) of the fish, c) from an ecological perspective, the benefits of sterility include the inability of escaped farm fish to reproduce with wild populations, thus preventing genetic contamination, d) if a non-endemic species is being cultured, sterility will again prevent such a species from propagating in the wild, and e) from an intellectual property standpoint, sterility will ensure the exclusive ability to propagate transgenic fish in the hands of patent holders. Better understanding of forebrain GnRH development will potentially lead to applied methods for manipulating the GnRH system along these lines.

From a human disease perspective, the study of the GnRH system is relevant in two ways; one is to better understand GnRH-specific tangential migration developmental diseases such as Kallmann syndrome. Gaining a better understanding of the forebrain GnRH soma migration and fiber pathfinding processes that lead to Kallmann syndrome associated neuronal defects serves to elucidate how these reproductive abnormalities emerge and their basic etiology and biology. The other is to expand our basic general understanding of tangential neuronal migration regulation. In addition to forebrain GnRH neurons, various other neuronal populations use this form of migration. These include inhibitory interneurons (Tanaka et al., 2003), as well as neuron migration from the subventricular zone to the olfactory bulb. The latter is a process that occurs also in adults and is thus potentially crucial for maintenance and function in the adult CNS (Doetsch et al., 1997). These examples signify the importance of better understanding neuronal tangential migration.

Project hypothesis, objectives and organization

The overall goal of this research was to advance our understanding of forebrain GnRH system development and the factors regulating this development, using the ZF as a model system.

In order to achieve this goal and given available knowledge, we stipulated the following five hypotheses. Each hypothesis was tested via the following detailed objectives and methods:

Hypothesis 1: GnRH3 neurons in zebrafish emerge in the olfactory region and migrate during early development to the POA-hypothalamus, from which they innervate the pituitary as well as additional CNS regions.

To test this hypothesis, our objectives were: (i) develop a transgenic ZF model in which GnRH3 neurons specifically express EGFP and (ii) use this model for following GnRH3 developmental events *in vivo* and in real time, thus enabling high resolution spatiotemporal understanding of the early development of the ZF GnRH3 system with an emphasis on GnRH3 fiber pathfinding and targeting.

To achieve these objectives, we have: 1) produced a construct that expresses EGFP under the control of the GnRH3 promoter, 2) produced a stable ZF transgenic line using this construct that expresses EGFP specifically in GnRH3 neurons, 3) validated the specificity of EGFP expression, and 4) used light and confocal microscopy to track and describe the precise spatiotemporal development of the GnRH3 system *in vivo*.

Hypothesis 2: GnRH3 expression plays a role in early development of the GnRH3 system.

To test this hypothesis, our objectives were: (i) achieve GnRH3 knock-down in the Tg(GnRH3:EGFP) larvae during early development and (ii) examine GnRH3 neuronal development in Tg(GnRH3:EGFP) larvae in which GnRH3 has been knock-down to elucidate the *in vivo* role that GnRH3 plays in early establishment of the GnRH3 system.

To achieve these objectives, we have: 1) used anti-GnRH3 MOs targeting the AUG or anti-splice site to knock-down GnRH3 in Tg(GnRH3:EGFP) larvae, 2) followed the development of the GnRH3 system and compared it to controls using light and confocal microscopy and 3) validated GnRH3 MO efficacy and results by using anti-splicing MO-treated larvae to conduct RT-PCR.

Hypothesis 3: Netrin1a and its receptor Dcc, as well as Netrin2, are important factors in GnRH3 neuron migration and fiber-targeting.

To test this hypothesis, our objectives were: (i) achieve knock-down of Netrin1a and its receptors Dcc and Unc5b in Tg(GnRH3:EGFP) larvae during early development,
(ii) over-express Netrin1a in the pineal gland of Tg(GnRH3:EGFP) larvae during early development, (iii) achieve knock-down of Netrin2 in Tg(GnRH3:EGFP) larvae during early development, and (iv) examine GnRH3 neuronal development in

Tg(GnRH3:EGFP) larvae in which Netrin1a, Dcc, Unc5b and Netrin2 have been knock-down to elucidate the *in vivo* role that these factors play in early establishment of the GnRH3 system.

To achieve these objectives, we have: 1) knock-down Netrin1a, Dcc, Unc5b, and Netrin2 in Tg(GnRH3:EGFP) larvae via MO injections, 2) specifically over-expressed Netrin1a in the pineal gland of Tg(GnRH3:EGFP) embryos by injecting plasmids expressing Netrin1a under the control of the pineal-specific aanat2 promoter, with or without simultaneous knock-down of naturally occurring Netrin1a, 3) followed the development of the GnRH3 system in these knock-down larvae and compare it to controls using light and confocal microscopy, and 4) validated Netrin1a and Unc5b MO efficacy and results by using anti-splicing MO-treated larvae to conduct RT-PCR.

Hypothesis 4: Shha is involved, in a neuroregulatory-specific manner, in modulating GnRH3 system development.

To test this hypothesis, our objectives were: (i) downregulate Shha activity at various developmental timpoints, thus differentially effecting Shha activity either as a morphogen or as a factor that is specifically involved in neuronal development, (ii) examine GnRH3 neuronal development in Tg(GnRH3:EGFP) larvae in which the Shha pathway has been disrupted at various timepoints and compare these larvae to controls using light and confocal microscopy.

To achieve these objectives, we have: 1) exposed Tg(GnRH3:EGFP) embryos and larvae to cyclopamine by immersion, thus disrupting the Shha pathway at specific timeframes, 2) developed and utilized an smo-GnRH3:EGFP ZF line, in which the smo protein is mutated and thus the Shha pathway is continuously disrupted, and 3) followed the development of the GnRH3 system in both cyclopamine-treated and smo-GnRH3:EGFP larvae and compared it to controls using light and confocal microscopy.

Hypothesis 5: Ablation of GnRH3 neurons in the olfactory region during early development adversely effects gonadal development and, consequently, fecundity.

To test this hypothesis our objectives were: (i) use the Tg(GnRH3:EGFP) line to specifically ablate GnRH3 neurons *in vivo* during larval stages, both bilaterally and unilaterally, (ii) apply ZF molecular techniques to achieve conditional ablation of GnRH3 neurons at larval and post-larval stages, (iii) use ablated animals to examine the specific role, with respect to fecundity and gonad development, of GnRH3 neurons that immerge in and migrate from the olfactory region, and (iv) examine the effect ablating olfactory region GnRH3 neuron during early development on location of GnRH3 neurons in the adult CNS.

To achieve these objectives, we have: 1) mechanically laser-ablated GnRH3 neurons in Tg(GnRH3:EGFP) larvae, both unilaterally and bilaterally, 2) developed a Tg(GnRH3:EGFP) line in which a construct enables conditional GnRH3-specific toxin delivery and activation, 3) evaluated ablation success and GnRH3 neuron

regeneration capacity, and 4) raised fish to maturity in which GnRH3 neurons were successfully bilaterally and unilaterally ablated, as well as control fish, at which time their fecundity, gonad morphology, and GnRH3 neuron location were assessed.

Current scientific knowledge regarding the above topics is either non-existent or incomplete due to the limitations presented by the models and techniques used to date to study the forebrain GnRH system.

Chapter 2: EARLY DEVELOPMENT OF THE GnRH3 SYSTEM

Abstract

Normal migration of the gonadotropin-releasing hormone (GnRH) neurones during early development, from the olfactory region to the hypothalamus, is crucial for reproductive development in all vertebrates. The establishment of the GnRH system includes tangential migration of GnRH soma, as well as extension of GnRH fibers to various areas of the central nervous system (CNS). The exact spatio-temporal nature of this process is not fully understood, nor are the factors governing it. We studied the development of the GnRH system using a newly developed Tg(GnRH3:EGFP) zebrafish line. We found that EGFP is specifically and robustly expressed in GnRH3 neurones and fibers. GnRH3 fibers in zebrafish began to extend as early as 26 hpf and by 4–5 dpf had developed into an extensive network reaching the optic tract, telencephalon, hypothalamus, midbrain tegmentum and hindbrain. GnRH3 fibers also innervated the retina and projected into the trunk via the spinal cord.

GnRH3 soma were observed migrating along their own fibers from the olfactory region to POA-hypothalamus via the terminal nerve ganglion and the ventral telencephalon. GnRH3 cells were also observed in the trigeminal ganglion and in the pronephric ducts. Novel data regarding the early development of the GnRH3 fiber network in the CNS and beyond is described.

Introduction

GnRH is a decapeptide neurohormone that is necessary for reproduction. However, it is increasingly viewed as also having additional neuroregulatory and neuromodulatory functions. All vertebrate species studied have two or three paralogous forms of GnRH. In most species GnRH1 is found primarily in the preoptic area (POA) and the caudal hypothalamus, it is the hypophysiotropic form that induces the release of gonadotropins from the adenohypophysis, which in turn promotes gonad development. GnRH2 is found in all jawed vertebrates in the midbrain and is implicated in reproductive behavior and food intake (Amano et al., 1997b; Barnett et al., 2006; Sherwood et al., 1993; Temple et al., 2003). In fish species that have three forms of GnRH, GnRH3 is located in the TN. The role of TN GnRH is not clear, however, a role in reproductive behavior has been suggested (Ogawa et al., 2006). In fish species that have only two forms of GnRH, including ZF, GnRH3 is located in both the TN and POA-hypothalamus and is, therefore, the hypophysiotropic form. As in mammalian species, in zebrafish (*Danio rerio*), only two forms of GnRH have been isolated and characterized at the nucleotide level, GnRH2 and GnRH3 (Steven et al., 2003). The lack of a third form of GnRH, which appears to have been lost during evolution, would suggest that nonredundant functions played by GnRH1 have been assimilated by GnRH3 (Kuo et al., 2005; Okubo and Aida, 2001; Okubo et al., 2002).

During early development, forebrain GnRH neurones traverse a unique tangential migration route from the olfactory region into the central nervous system (CNS) (Marin and Rubenstein, 2003; Steven et al., 2003). These neurones migrate

along the terminal/olfactory nerve tracts to the terminal nerve ganglion (TNg) and continue to the ventral telencephalon, eventually reaching the POA and caudal hypothalamus. Evidence suggests that the forebrain GnRH soma migration is axophilic and involves various cell adhesion molecules. Although the forebrain GnRH neurones migrate along the vomeronasal nerve, the exact identity of the fiber fibers involved has not been elucidated (Schwarting et al., 2004; Wray et al., 1994). In zebrafish, the GnRH3 soma may associate with their own fibers during migration (Palevitch et al., 2007).

In addition to information regarding forebrain GnRH soma migration during early development, several reports have addressed the topic of GnRH fiber tract location. In mammals, the focus has been on GnRH1 fibers in the olfactory region, basal hypothalamus, along the GnRH1 migration tract and fiber projections to the POA, and the median eminence (Rogers et al., 1997; Stopa et al., 1991). During mammalian development, a subset of GnRH1 associated fibers turn caudally after crossing the cribriform plate and continue into the ventral forebrain, whereas another subset continue dorsally to the accessory olfactory bulb (Schwarting et al., 2001; Wray, 2001). A similar fiber development pattern has been described in teleosts. In sexually mature fish, GnRH fibers, as well as multiple types of GnRH receptors, have been localized to various regions of the CNS, including the telencephalon, POA, hypothalamus, thalamus, midbrain tegmentum, rhombencephalon, and retina (Chen and Fernald, 2006; Gonzalez-Martinez et al., 2004b; Grens et al., 2005; Lethimonier et al., 2004; Whitlock et al., 2006). This widespread location of GnRH fibers and receptors suggests a multifaceted regulatory mechanism and indicates that GnRHs

play multiple neuroregulatory roles that potentially include control over sensory, metabolic and motor functions. It is noteworthy that GnRH mRNA is identified in several extra-CNS tissues, including the ovary, immune cells, and seminiferous tubular cells (Cheng and Leung, 2005).

The characterization of both the GnRH soma and GnRH fiber migratory route and their final location has been conducted primarily by means of immunohistochemistry (ICC) and *in situ* hybridization (ISH) and are thus limited by the sensitivity of these methods. In the present study, we developed a novel zebrafish line in which a GnRH3 promoter/EGFP reporter construct is used to induce stable transgenic EGFP expression in GnRH3 neurones. This zebrafish line exhibits robust and lasting expression of EGFP in GnRH3 neurones, providing extremely high resolution *in vivo* data regarding the early development of the GnRH3 system. We have used this construct previously in a transient fashion to observe GnRH3 soma migration; however, in these fish, EGFP expression was short-lived and at times ectopic (Palevitch et al., 2007). In the present study our stable transgenic line, Tg(GnRH3:EGFP), was used to provide a detailed delineation of the spatiotemporal development of the extensive GnRH3 fiber network in the CNS and beyond, as well as GnRH3 soma migration and location.

Materials and methods

Experimental Animals

Adult zebrafish were spawned and larvae raised in filtered fresh water under a 14:10 h light/dark cycle at 28–29° C. 1-phenyl-2-thiourea was added to the water every second day during 2–10 dpf at a concentration of 0.2 mM to prevent pigmentation. Starting at 4 dpf, larvae were given *Paramecia* once daily. Larvae used for experiments were anaesthetized with MS-222 and either observed under a light/confocal microscope or fixed in 4% paraformaldehyde for further treatment. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland Biotechnology Institute.

Generation of promoter–reporter construct

A 2.4-kb sequence upstream of the coding region for the GnRH3 precursor sequence was cloned and isolated from zebrafish genomic DNA. This fragment [corresponding to nt 365–2797 (GenBank: AF490354)] contains 1.3 kb of 5' upstream flanking sequence, exon-1, intron-1 and part of exon-2 of the GnRH3 gene. The fragment was cloned into pBlueScript vector and the clone was digested with HindIII and BamHI. The insert was then ligated into a HindIII/BamHI cut green fluorescent protein-expressing vector, p-EGFP-1 (Clontech, Palo Alto, CA, USA); (Fig. 2).

Microinjections

To produce the Tg(GnRH3:EGFP) line, the construct (GnRH3:EGFP) was linearized using SalI and diluted in distilled water to a final concentration of 50 μg/ml. Phenol red was added to the injection solution to a final concentration of 0.1% for visualization during microinjection. Approximately 2 nl of DNA solution was microinjected into the cytoplasm of zebrafish embryos at the one- or two-cell stage. Microinjections were carried out under a dissection microscope (Model MZ8; Leica, Deerfield, IL, USA) using a PLI-100 pico-injector (Medical System Corp., New York, NY, USA).

Production of the Tg(GnRH-EGFP) line

P0 fish injected with the linearized GnRH3:EGFP construct were grown to maturity and crossed. F1 offspring were screened at 3 dpf for expression of the EGFP reporter gene and grown to maturity. Mature F1 fish were screened for incorporation of the reporter construct into the genome by a finclip polymerase chain reaction (PCR) screening method (Du and Dienhart, 2001) using EGFP transgene-specific primers. For experiments detailed herein, F2 and F3 larvae were used.
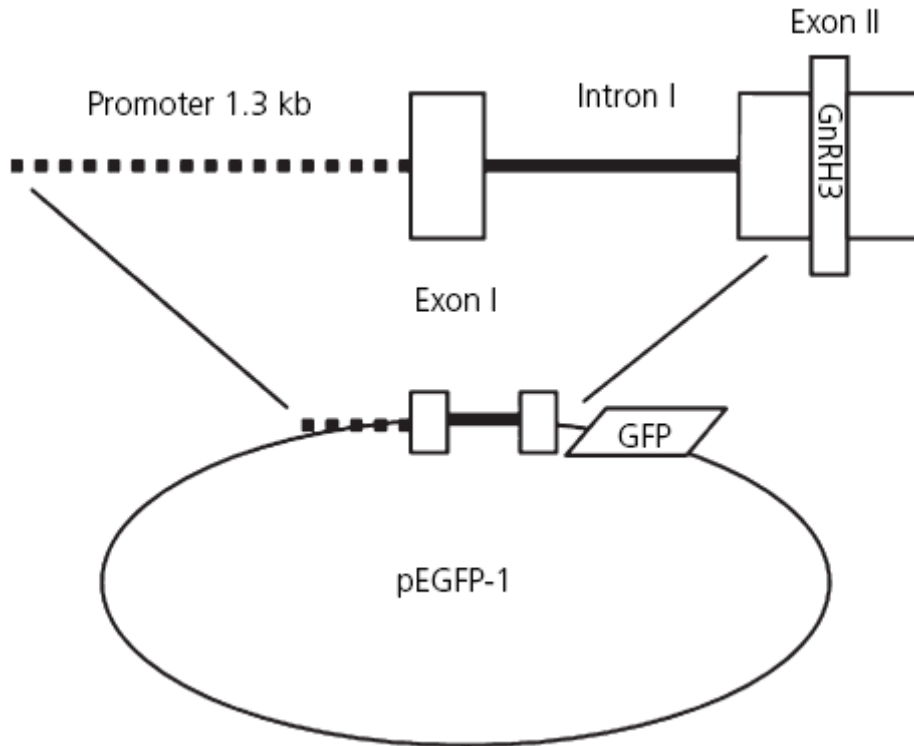
Figure 2.

GnRH3:EGFP expression vector. A 2.4 kb sequence upstream of the gonadotropin-releasing hormone (GnRH3) decapeptide coding region including 1.3 kb of 5' flanking sequence, exon I, intron I and part of exon II was cloned and inserted into an expression vector p-EGFP1 that includes an EGFP coding sequence.

ISH and ICC

Whole-mount double-labeling of GnRH3 cells and fibers in transgenic zebrafish was based on published procedures (Novak and Ribera, 2003). A fragment of zebrafish GnRH3 cDNA 27-378 (GenBank: AJ304429) was cloned and introduced into pGEM-T Easy Vector (Clontech, Madison, WI). The construct was linearized with either NcoI or SalI restriction enzymes and used as templates to synthesize digoxigenin (Dig)-labeled sense or anti-sense probes (Dig RNA labeling kit; Roche Diagnostics, Penzberg, Germany). Transgenic larvae were anaesthetized, fixed

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O/N and rinsed 2 - 5 min in phosphate-buffered saline (PBS). Larvae were then incubated for 2 h at 37° C with 1 mg/ml of collagenase and washed 2-5 min in PBS-Tween 20 (PBST) following pre-hybridization in hybridization buffer (50% formamide, 5X SSC, 5 mM ethylenediaminetetraacetic acid, 0.1% Tween 20, 0.1% CHAPS, 50 mg/ml heparin, and 1 mg/ml yeast RNA) for 2 h at 50° C. Antisense/sense RNA riboprobe was added to the hybridization buffer at a concentration of 1 μg/ml and heated to 85° C for 5 min. The larvae were then added to this solution and hybridized with the Dig-labeled GnRH3 anti-sense RNA at 50° C for 3 h. Following hybridization, the samples were rinsed at 50° C twice for 30 min in washing buffer (50% formamide; 2X SSC, 0.15% CHAPS), once for 15 min in washing buffer (2X SSC, 0.3% CHAPS), twice for 30 min in washing buffer (0.2% SSC, 0.3% CHAPS) and finally in MAB buffer (0.1 M maleic acid, 150 mM NaCl, pH 7.4) at room temperature. Following a 3 h pre-incubation in blocking solution (2% blocking reagent, 5% calf serum in MAB buffer), samples were incubated for 3 h in anti-Dig-AP (1:5000 in blocking solution). Next, samples were washed in PBST for 15 min three times and then O/N in PBST at 4° C. Larvae were transferred to 100 mM Tris, pH 8.4, for 5 min and then placed either in FastRed solution for 20-40 min (FastRed tablets; Sigma-Aldrich, St Louis, MO, USA), or in purple AP substrate. After sufficient color development, larvae were washed 4-15 min in PBST, blocked in 10% normal goat serum/PBST for 2 h, and rinsed 2-5 min in PBST.

For the ISH-ICC results, the same larvae were then incubated O/N at 4° C in 0.5 ml 10% goat serum/PBST containing a monoclonal anti-EGFP immunoglobulin G at 1:500 dilution (Molecular Probes, Eugene, OR, USA) and washed in PBST for

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4-30 min at room temperature. Next, the secondary Alexa 488 goat-anti-mouse Ab

(1:500) was added (Alexa Fluor 488; Molecular Probes, Inc.) and incubated O/N at 4°

C. ICC of transgenic zebrafish cryosections was also performed following the above

ICC protocol. Transgenic zebrafish larvae were fixed for 2-4 h in 4%

paraformaldehyde and then O/N in 30% sucrose. Fixed animals were then washed in

PBS for 2-5 min, embedded in Tissue-Tek compound (Sakura Finetechnical, Tokyo,

Japan) and frozen on dry ice. 10 µM sections were cut using a cryostat (CM3000;

Leica, Nussloch, Germany). Sections were placed on Superfrost Plus slides (Erie

Scientific Company, Portsmouth, NH, USA), and ICC was conducted as described

above using a primary anti-EGFP 1:500 Ab (Molecular Probes, Inc.) and a secondary

anti-mouse immunoglobulin G TRITC conjugate (Sigma-Aldrich).

Microscopy

Bright field fluorescence microscopy was conducted using a Zeiss

Axioplan 2 microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) with

appropriate filters, an Attoarc HBO100 W power source and an Olympus DP70

digital camera (Olympus, Tokyo, Japan). Confocal microscopy was conducted using

a Zeiss Radiance 2100 laser scanning system together with Laser-Sharp and LSM

imaging software (Carl Zeiss MicroImaging, Inc.). All confocal pictures are of

extended focus views. Determining the location of GnRH3 soma and fibers to

specific regions was performed in accordance with previous studies (Kimmel et al.,

1995; Liu et al., 2003; Thomas Mueller, 2005; Wullimann, 1996). The average

distance of GnRH3 soma migration was determined by measuring the distance

between the two furthest GnRH3 soma in no less than eight larvae on a daily basis.

Results

Specificity of EGFP expression in GnRH3 soma

To verify that the EGFP expression is specific to GnRH3 cells, we conducted a whole-mount double-labeling assay in Tg(GnRH3:EGFP) zebrafish. ICC was used to detect EGFP protein (Fig. 3A). ISH was used to label GnRH3 neurones (Fig. 3B). These two signals were photographed in the same larvae using appropriate filters and the photographs were superimposed (Fig. 3D). We conducted this labeling in fifteen 3 dpf larvae. Specific co-location of the GnRH3 red signal and the EGFP signal (Fig. 3) was repeatedly observed, demonstrating that the EGFP reporter is driven by the GnRH3 promoter and is expressed exclusively in GnRH3 neurones. The pattern on EGFP expression at later time points is in agreement with previous GnRH3 location studies (Palevitch et al., 2007; Steven et al., 2003).

Localization of EGFP to GnRH3 fibers

As described below in detail, during the first days of larval development and prior to GnRH3 soma migration, extensive fiber tracts were visualized extending from the GnRH3 cells in the olfactory region. These tracts extended to various regions of the CNS, some of which have not been described previously as containing GnRH3 fibers. In order to ascertain that the signal is not auto-fluorescence, the presence of EGFP in the fibers was verified by ICC on horizontal cryosections of 5 dpf larvae. When superimposing images of the endogenous fluorescent signal (green) in the fiber tracts (Fig. 4A) and those of fluorescence (red) emitted by anti-EGFP ICC (Fig. 4B), we observed a precise overlap (Fig. 4C). This demonstrates that

these fiber tracts contain EGFP protein originating from the GnRH3 soma in the region. No green signal was detected in relevant regions in nontransgenic animals. Taken together, the whole-mount double-labeling results and the cryosection ICC results validate the supposition that EGFP is expressed exclusively in GnRH3 cells and that the observed fiber tracts extend from GnRH3 soma.
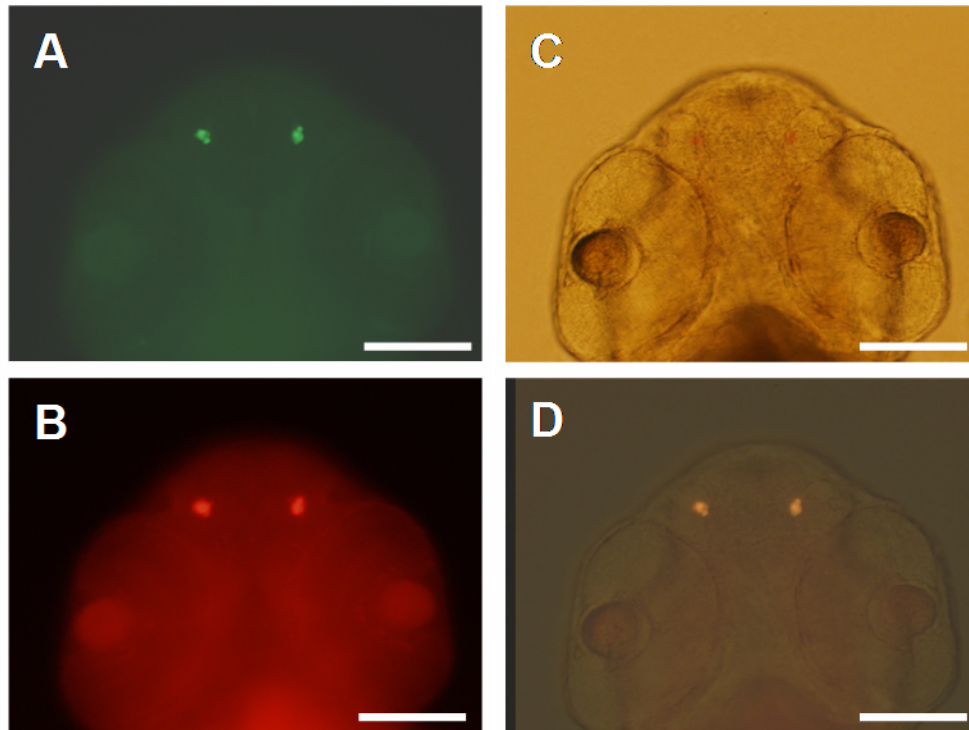
Figure 3.

Whole-mount EGFP-GnRH ISH and ICC double-labeling. Verification of specific and exclusive expression of EGFP in GnRH3 neurones of transgenic zebrafish line. At 3 dpf, EGFP was specifically co-expressed in GnRH3 expressing neurones in the olfactory region of transgenic zebrafish. (A–D) Single 3 dpf larvae, ventral view with anterior towards the top. (A) ICC using primary anti-GFP Ab and secondary Alexa 488 Ab. (B) ISH using a GnRH3-specific Dig probe and FastRed. (C) White light picture of larvae, FastRed coloring can be seen in GnRH3 neurones. (D) Overlay of Figures (A) and (B). Scale bars: 150 µm.
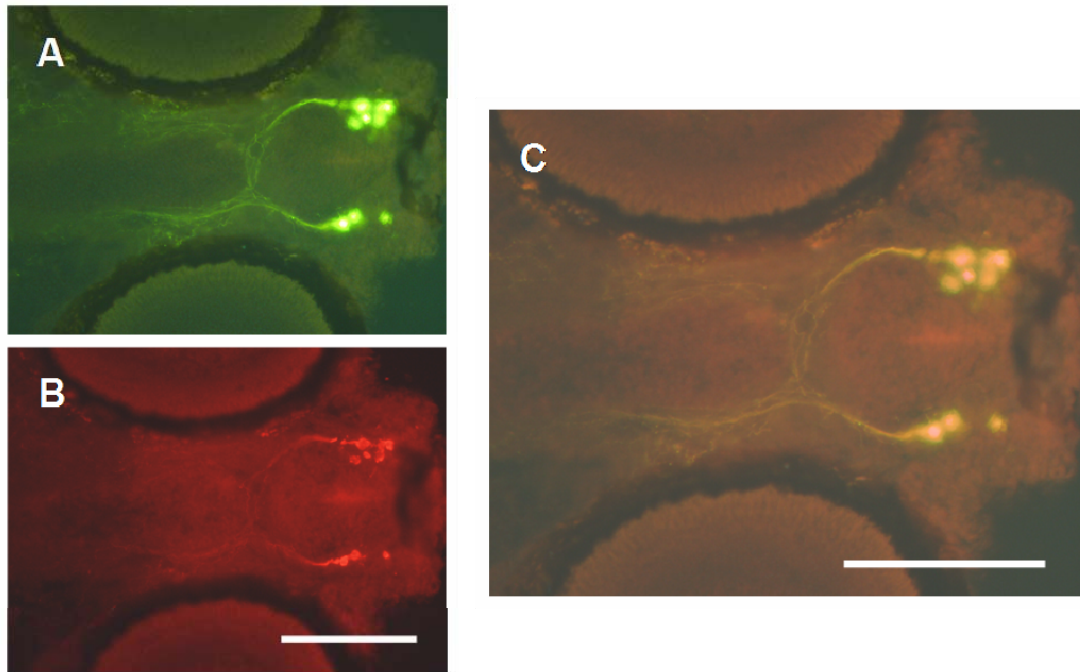
Figure 4.

Verification of EGFP expression in GnRH3 fiber projections of GnRH3: EGFP transgenic zebrafish line. (A–C) Cryosections of single 5 dpf larvae head, dorsal view with anterior towards the right. (A) Green fluorescence in GnRH3 neuron soma in the olfactory region and in fiber projections along the optic commissure. (B) Immunohistochemistry using primary anti-GFP Ab and secondary TRITC Ab reveals location of EGFP protein to GnRH3 neuron soma and fiber tracts. (C) Overlay of Figures (A) and (B). Scale bars: 150 µm.

Early development of GnRH3 fiber tracts

The GnRH3 fiber developmental pattern was observed via confocal microscopy in live embryos. Confocal microscopy was found to be the best method of visualizing fibers during early development. The fibers consisted of projections extending bilaterally from both GnRH3 soma clusters located in the olfactory region.

The majority of this fiber network was established by the time that the GnRH3 soma began migrating caudally at 3–4 dpf. At approximately 26 hpf, 2 h following the first expression of EGFP in the GnRH3 soma, the GnRH3 neurones begin to extend fibers. These first extensions project dorsocaudally towards the pallium, developing several fiber branches, some of which also project caudoventrally (Fig. 5A,B).

Between 32–36 hpf, the neurones begin to develop multiple additional fiber extensions, projecting caudoventrally and eventually meeting at the midline to form a commissure in the subpallium between the two GnRH3 neuron clusters (Fig. 5C). Two to three hours later, new fibers begin extending from the soma in two additional directions, caudoventrally along the anterior commissure and the optic commissure, eventually innervating the eye (Fig. 5D,E). At the same time, a fourth subset of fibers begins to extend caudally towards the hypothalamus. By 48 hpf, the fibers extending dorsocaudally meet at the midline, forming a second commissure in the pallium (Fig. 5G,H). Thus, within 48 h of development, the GnRH3 soma in the olfactory region that have not yet begun migration extend four distinct subsets of fiber tracts: two commissures between the bilateral GnRH3 soma clusters, including one in the pallium and one in the subpallium, along the optic tract to the eyes, and caudally towards the hypothalamus (Fig. 5G,H). An additional subset of fibers targets the pineal gland region in the dorsal midbrain (not shown).

Throughout the next 2 days, fiber tracts extending caudally develop rapidly and branch into multiple CNS regions. By 4 dpf, an extensive network of fibers is in place projecting to numerous brain regions, including the olfactory bulbs, ventral and

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dorsal telencephalon, thalamus, POA and caudal hypothalamus, optic tectum, midbrain tegmentum and hindbrain (Fig. 6A–D). At 4–5 dpf, a subset of GnRH3 fibers coursing caudally reach the POA and turn caudoventrally towards the pituitary, while some of the fibers in this tract continue to project caudally towards the hindbrain and the trunk (Fig. 6C,D).

The GnRH3 fiber network develops in a 3-dimensional pattern that can be visualized in the depth-coded figures (Fig. 6B,D). This extensive GnRH3 fiber network is established by 4–5 dpf and remained mostly unchanged throughout our observation period until day 15 post-fertilization.
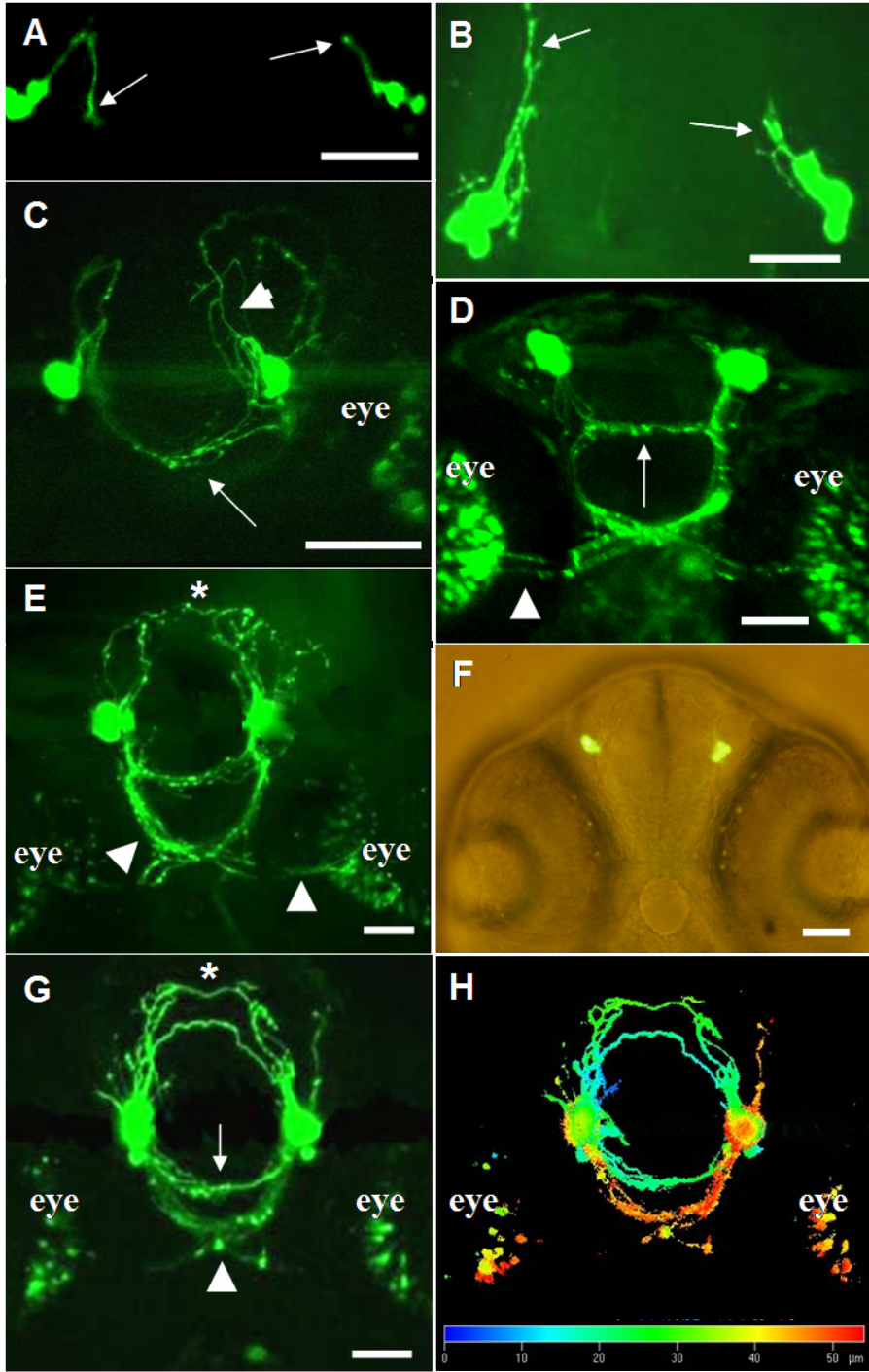
Figure 5. (previous page)

Images of fiber projections from GnRH3 neurones at 26–48 hpf (confocal microscopy). (A–C,E,G,H,F) Frontal view. (D) Ventral view, eyes are to the right and left of the two GnRH3 neurones clusters. (A) At 26 hpf, the beginning of fiber projections from the GnRH3 soma can be observed extending dorsocaudally towards the pallium (arrows). (B) Over the next few hours, these projections multiply, and extend further dorsocaudally (arrows). (C) By 36 hpf, fibers extending caudoventrally from a commissure (arrow) and fibers extending dorsocaudally (arrowhead) continue to develop bilaterally. (D,E) Fiber projections at 36–48 hpf. Three bilateral projection tracts can clearly be seen, including one projecting dorsocaudally towards the pallium (E; asterisk), another towards the subpallium commissure (D; arrow) and, finally, fibers extending along the optic tract into the eyes (D,E; arrowheads).
(F) Bright field fluorescence microscopy of transgenic larvae at 45 hpf. (G, H) At 48 hpf, projections extend toward the pallium connect (G; asterisk), subpallium commissure (G; arrow), optic chiasm (G; arrowhead). (H) Depth color-coded image of (G). Scale bars (A,B) 25 µm; (C–H) 50 µm. (the color signal in the eyes is due to autofluorescence).
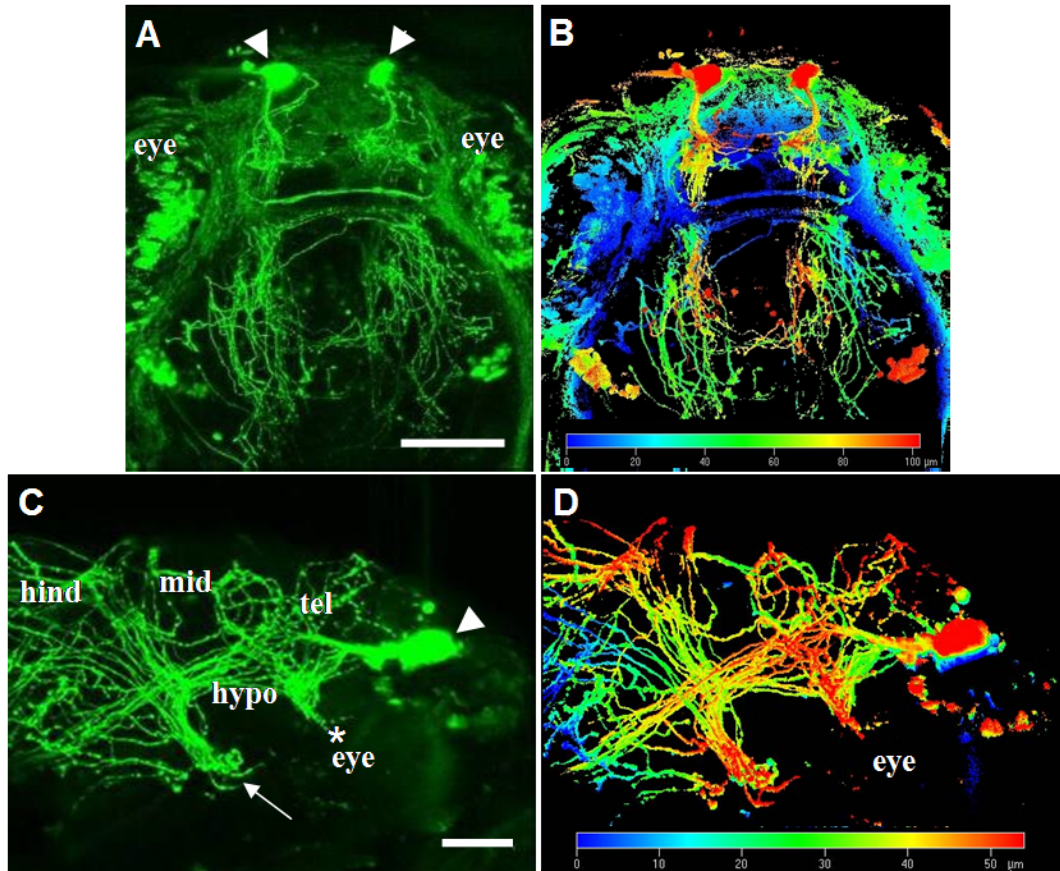
Figure 6.

Images of fiber projections from GnRH3 neurones at 4–5 dpf (confocal microscopy). (A,B) Dorsal view of 4 dpf larvae head region, anterior towards the top. (A) View of the GnRH3 fiber scaffold including the pallium commissure and bilateral tract formations ventrally into the telencephalon, midbrain and hypothalamus. GnRH3 soma can be seen in the olfactory region (arrowheads). (B) Depth color-coded picture of (A). (C,D) Lateral view of 5 dpf larvae head region, anterior towards the right. (C) Extensive GnRH3 fiber scaffold can be observed including the GnRH3 soma in the olfactory ∕ terminal nerve region (arrowhead), fiber tracts into optic nerve (asterisk), telencephalon, midbrain, hindbrain and hypothalamus. The ventral turn of a main tract innervating the pituitary can be clearly seen (arrow). (D) Depth color-coded image of (C). Scale bars: (A,B) 75 µm; (B, C) 150 µm. (the color signal in the eyes is due to autofluorescence).

Tracking GnRH3 soma migration

The GnRH3 soma migration progress has been documented in various vertebrates using ISH and ICC methods. We have previously described the migration of GnRH3 neurones in zebrafish using both ISH and the transient expression of EGFP in GnRH3:EGFP construct- injected zebrafish (Palevitch et al., 2007). However, as discussed, these methods are limited by various factors. In the present study, we expand on this description using *in vivo* observations of a stable Tg(GnRH3:EGFP) line. EGFP expression first appears at 24 hpf in 2–3 GnRH3 neurones in the region of the olfactory placode (Fig. 7A). In subsequent days, the process of fiber extension from these soma occurs as described above. An increase in the number of GnRH3 neurones and repositioning of the soma also occurs, which appears to be mainly due to developing morphology of the olfactory region and CNS (Fig. 7B). The formation of a continuum between the olfactory region and the TNg (Fig. 7D) can be clearly observed at this point and, by 6 dpf, it reaches the ventral telencephalon (Fig. 7E). During days 7–12 pf, there is a continuous axophilic migration of GnRH3 soma caudally into the ventral telencephalon along existing GnRH3 fiber tracts that innervate the hypothalamus (Fig. 7 F,G). During this migration, clusters of GnRH3 neurones are found along the migration tract and, by day 12, soma that were observed migrating from the TNg reach the presumptive POA (Fig. 7G). In later stages of development, consecutively until our latest observation point of 30 dpf, GnRH3 neurones can be seen along this migrational tract including the olfactory region, TNg, telencephalon and POA (Fig. 7H). Migration of the soma started at day 3 and was

most significant between days 4 and 5 pf (Fig. 8), with the highest rate of migration
being approximately 1 µm/h.
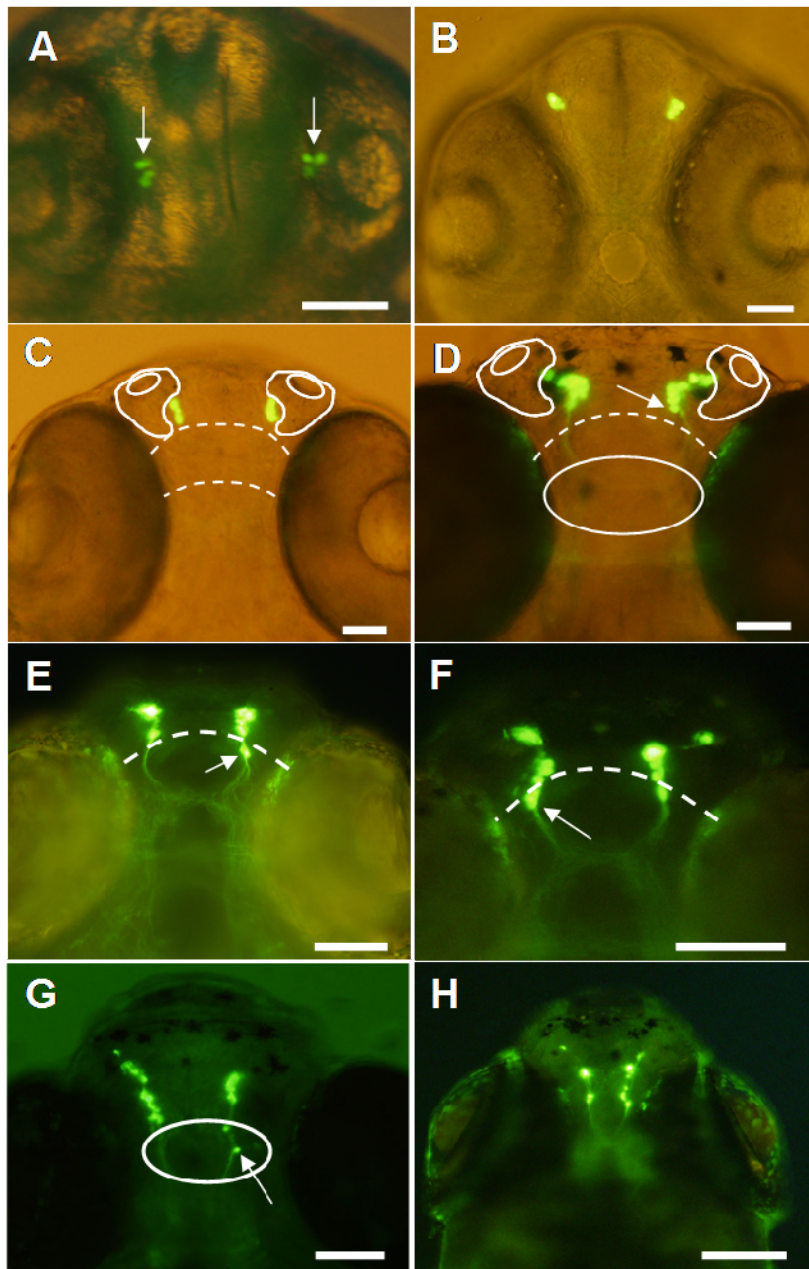
Figure 7.

Time course of GnRH3 soma migration (bright field fluorescence microscopy).
Frontal view (A) of transgenic fish at 26 hpf. Neurones are located in the olfactory
placode region, each cluster containing two to three neurones (arrows). (B) Ventral

view at 45 hpf. Neurones in olfactory region; however, the number of neurones has increased and enhanced green fluorescent protein (EGFP) expression is intensified. (C–H) Dorsal view. (C) At 3 dpf, some neurones migrate posteriorly; areas framed by an unbroken line represent the olfactory organs, the area framed by dashed line represents the ventral diencephalon. (D) At 5 dpf, neurones are migrating and form a continuum from the olfactory region to the terminal nerve ganglion (arrow). Areas framed by unbroken lines represent the deduced olfactory organs; the dashed line represents the telencephalon border and the area framed by an oval represents the deduced diencephalic hypothalamus region. (E, F) At 6 and 8 dpf, migration continues along the ventral aspects of the telencephalon (arrows). The dashed line represents the telencephalon border. (G) At 12 dpf, the first soma reach the hypothalamus (arrow); the oval represents the deduced diencephalic hypothalamus region. (H) At 30 dpf, GnRH3 soma remain clearly visible along the entire migration tract, from the olfactory region to the hypothalamus.

Scale bars: (A–D) 50 µm; (E–G) 100 µm; (H) 500 µm.
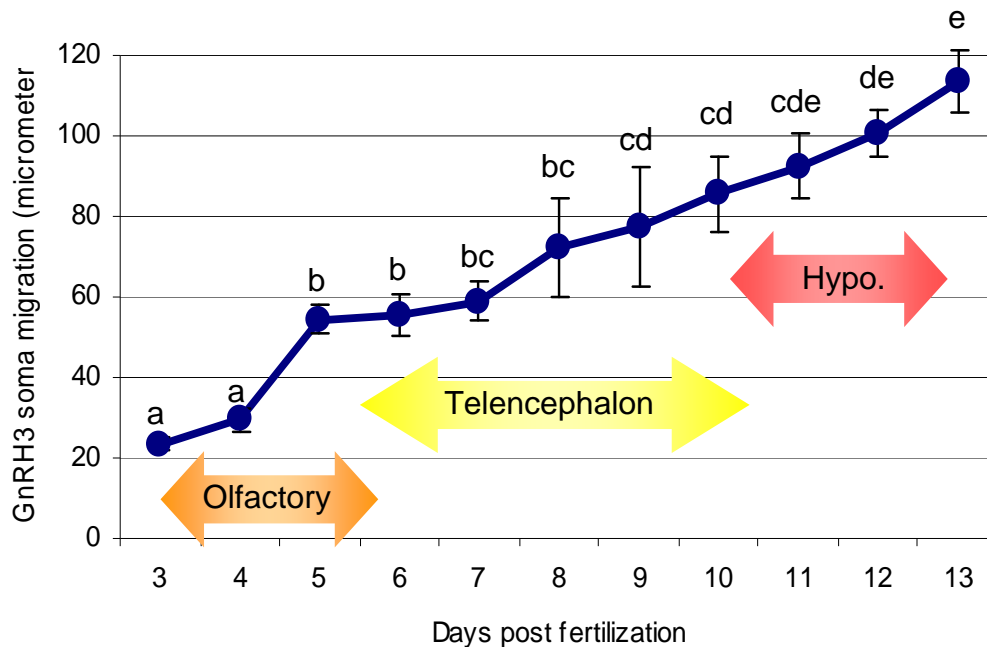
Figure 8 (previous page).

Average migration distance of GnRH3 soma between 3 and 13 dpf. Measurement of migration refers to distance between the olfactory area soma and the farthest soma from that point. Each point represents n>15 larvae. Different letters represent significant differences in means ($P < 0.05$, ANOVA, Tukey HSD test). Arrows represent approximate time frame in which most advanced soma are in various regions.

GnRH3 location in extra-CNS tissues

In the Tg(GnRH3:EGFP) line, EGFP expression was detected also in cells and fibers outside of the brain. Beginning at 4 dpf, clear EGFP expression was localized to soma of the trigeminal ganglia, as well as in some local fiber projections from these neurones (Fig. 9A). Dig-labeling using a GnRH3 RNA probe confirmed expression of GnRH3 in trigeminal nerve cells (Fig. 9B).

Beginning at 6 dpf, expression of EGFP in cells was also clearly visible bilaterally in the pronephric ducts (Figure 8 G,H). However, we were unable to co-localize GnRH3 transcripts to these cells using DIG-labeled probes, presumably due to probe penetration issues.

Fiber tracts were visible along the spinal cord into the trunk. These fibers are an extension of GnRH3:EGFP fibers that extend caudally from the CNS and are found bilaterally along the spinal cord (Fig. 9 C,D). This subset of fibers first projects out of the brain as early as 4 dpf and over the next 6–7 days extends along the trunk, ultimately reaching the proximity of the tail region.

Finally, fiber projections were visible in cryosections of the eye. This expression was localized to a layer of cells in the retina and appears to be the final

location for the GnRH3 fibers that innervate the eye through the optic tract as described above (Fig. 9 E,F).
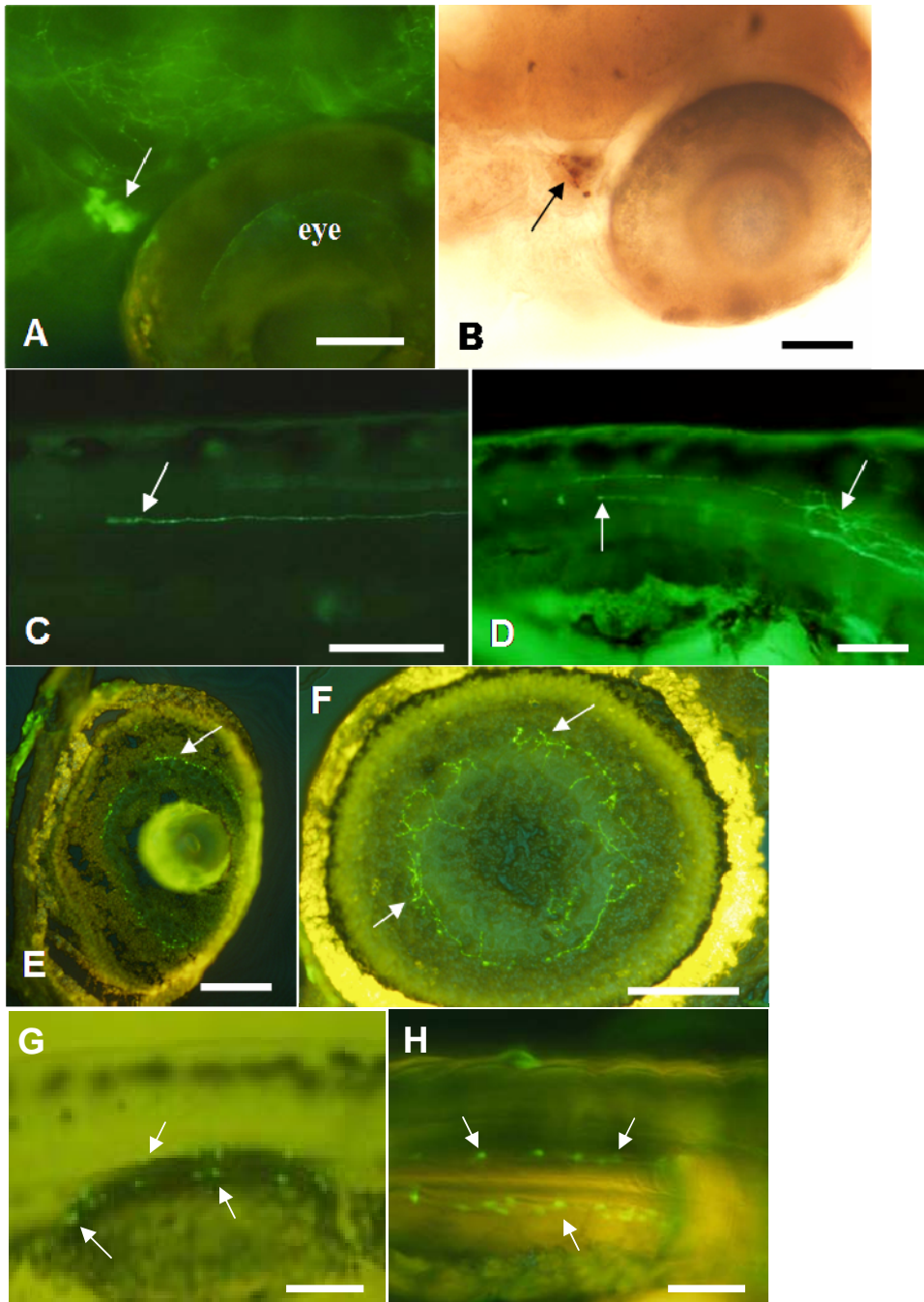
Figure 9.

GnRH3 cells in non-central nervous system tissues (bright field fluorescence microscopy). (A–D) Lateral view with anterior towards the right. (A) At 4 dpf,

GnRH3 cells reach the area of the trigeminal ganglia (arrow). (B) *In situ* hybridization of GnRH3 expression in the trigeminal ganglia (arrow). (C–F) At 4 dpf. (C, D) GnRH3 fibers extend bilaterally along the spinal cord into the trunk (arrows) and (E, F) eye cryosections reveal GnRH3 fibers innervating the retina (arrows). (G,H) GnRH3 cells located at 6 dpf bilaterally in the pronephric ducts. Scale bars: 75 µm.

Discussion

Importance of the Tg(GnRH-EGFP) line

As discussed in the methods section, the Tg(GnRH3:EGFP) line was produced using the GnRH3 ZF promoter. The promoter region used includes a region 2.4 kb upstream of the GnRH3 ATG site. The GnRH3:EGFP construct was first injected into embryos and transient expression was assessed, this transient expression has been described previously (Palevitch et al., 2007). Other than some ectopic expression that is to be expected in the transient model, the expression pattern agreed well with previous work examining the location of GnRH neurons during development. This result indicated that the promoter region chosen encompassed all the necessary regulatory elements for correct GFP expression in GnRH3 neurons. Later generations were screened and the final line is highly stable (currently at F8) and the expression pattern is robust and specific.

The production of the Tg(GnRH3:EGFP) line was a crucial step in this research for three main reasons. First, the EGFP expression level coupled with the fact that ZF larvae are optically translucent allows high resolution and visualization of even the smallest soma and fibers. This level of resolution difficult to achieve when

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using ISH or ICC methods or when using mammalian models that are not translucent.

Second, the fact that visualization of GnRH3 system development can be done *in vivo*

and in the intact animal allows the maintenance of a unperturbed environment that

includes all the brain regions, chemoattractants, chemorepellents, cellular

environment and circulating factors that may influence GnRH3 system development.

Lastly, the ease by which visualization is achievable allows us to follow the

progression in real-time and any time frame.

Early development of the GnRH3 fiber network

The transgenic line used in the present study, although not a tool for

determining GnRH3 expression levels, provided robust and specific GnRH3:EGFP

expression that, coupled with the transparency of the larvae, allowed a level of

resolution not achievable previously. This enabled a detailed account of early

development of the GnRH3 fiber network as a continuum of events.

The early establishment of the two commissures between the GnRH3 soma

clusters is interesting and establishes that there is a morphological connection

between the GnRH3 contralateral neurones. The early innervation of the retina further

solidifies the importance of GnRH3 as a neuromodulatory factor that potentially

facilitates lateral processing of visual information (Kinoshita et al., 2007; Maruska

and Tricas, 2007). This could possibly include, but is not limited to, information

pertinent to reproduction.

Two findings directly related to the hypophysiotropic role of GnRH3 in

zebrafish are of particular interest. First, GnRH3 fibers that originate in the olfactory

region reach the hypothalamus and innervate the pituitary prior to migration of

GnRH3 soma. This observation is based on the location of the pituitary in zebrafish as described previously (Chapman et al., 2005). This could facilitate the influence of GnRH3 on the HPG axis well prior to GnRH3 soma reaching the POA, thus GnRH could be involved in exerting an effect on the HPG axis prior to GnRH soma migration. The role of GnRH in this capacity could be in early modulation of gonad organization and sexual differentiation. Second, as we have suggested previously (Palevitch et al., 2007), GnRH3 soma conduct axophilic migration along their own pre-existing fiber tracts, which are located along the existing terminal/olfactory nerve tract. This suggests that early GnRH3 fiber pathfinding is important for normal GnRH3 soma migration to the POA and caudal hypothalamus.

With regard to the overall roles of GnRH3, we observed that GnRH3 fibers are present in wide regions of the CNS, including the optic tract, telencephalon, POA, hypothalamus, midbrain tegmentum and hindbrain. This fact, coupled with the information regarding the wide distribution of GnRH fibers and receptors in the CNS of mature animals, strongly suggests that GnRH3 has multiple neuroregulatory and neuromodulatory roles (Chen and Fernald, 2006; Gonzalez-Martinez et al., 2004b; Pandolfi et al., 2005). Gonzalez-Martinez et al. have shown in European sea bass (*Dicentrarchus labrax*) that while GnRH1 immunoreactive fibers are abundant in the pituitary, GnRH3 fibers are present in other brain regions (Gonzalez-Martinez et al., 2004b). This suggests that, in species that have three GnRH forms, GnRH1 primarily plays a hypophysiotropic role, whereas GnRH3 mediates other functions such as reproductive behavior. However, in zebrafish, GnRH3 fibers are found in CNS areas associated with both GnRH1 and GnRH3 in 'three form' fish (Gonzalez-Martinez et

al., 2004b; Pandolfi et al., 2005), suggesting that, in zebrafish, GnRH3 plays both the hypophysiotropic role as well as additional neuroregulatory roles. How GnRH3 plays multiple roles and differentially affects various GnRH receptor types remains unanswered.

It is also noteworthy that GnRH3 fibers were seen in the midbrain tegmentum, which is the location of GnRH2 cells in all jawed vertebrates. This indicates that GnRH3 fibers may have direct contact with GnRH2 neurones, thus enabling cross-talk between GnRH2, thus affecting reproductive behavior, and the hypophysiotropic GnRH3.

Finally, the presence of GnRH transcript and receptors has been previously reported in the spinal cord of vertebrates (Dolan et al., 2003; Kah et al., 1986). The exact cellular origin of these transcripts has not been clearly determined, although GnRH-producing cells/neurons may be present in the spinal cord (Dolan et al., 2003). The finding that GnRH3 fibers migrate along the spinal cord to the trunk region supports these results. However, it is clear that, in zebrafish, these GnRH3 fibers originate in the brain. The possibility that GnRH3 fibers in the spinal cord region have a role in locomotor functions warrants further investigation. Also, the finding that GnRH3 neurones extend long and complex fiber tracts to various regions is in agreement with the findings of Campbell (Campbell, 2007).

Migration of GnRH3 soma

The findings of the current study regarding GnRH3 soma migration during development are in agreement with the theory that forebrain hypothalamic GnRH

neurones originate in the olfactory region and migrate through the TNg and ventral telencephalon to the POA and caudal hypothalamus (Okubo et al., 2006). Although, based on observations in the Tg(GnRH3:EGFP) ZF larvae, we cannot unequivocally eliminate the alternative hypothesis that hypothalamic GnRH3 neurones appear independently in the hypothalamus (Gopinath et al., 2004; Whitlock, 2004, 2005; Wu et al., 2006) (see Chapter 5). GnRH3 soma migrated caudally from the olfactory region starting at 3 dpf and, by 12-13 dpf, formed a continuum from the olfactory region to the POA. In later stages of development, GnRH3 neurones were still present throughout the entire migrational tract including the olfactory region and TN. This is in agreement with GnRH3 location studies in adult zebrafish (Steven et al., 2003), suggesting that the GnRH3 neurones in non-hypothalamic regions of the CNS have a permanent and specific function that may not be directly affiliated with gonadotropin regulation, but certainly may participate in conveying external information pertinent to reproduction.

It is also noteworthy that GnRH3 soma migration in zebrafish at its highest rate (approximately 1-2 µm/h) is slow compared to GnRH3 migration in mammalian models (approximately 11–24 µm/h) (Bless et al., 2005; Tobet and Schwarting, 2006). This difference in the speed of migration is most likely due to the longer distance and shorter timeframe in which GnRH3 neurones migrate in mammals. The rate of tangential migration of neurons in mammals varies, not only between neuronal populations, but also within the same population depending on spatiotemporal variables. For example, tangential migration of cerebellar granule cells varies between ~14.8 µm/hr to ~4.1 µm/hr depending on the stage of migration

(Komuro et al., 2001). Similarly, the GnRH3 neuron rate of migration in ZF also varies, however, the highest rate of migration is still slow compared with mammalian tangential neuronal migration. The question remains whether this is a ZF-GnRH3 specific characteristic or a more general dynamic of tangential neuron migration in ZF.

Extra-CNS GnRH3 expression

In addition to the finding that GnRH3 fibers are present in the spinal cord, GnRH3 cells and neurons were observed in additional extra-CNS regions. GnRH3 neurons were observed in the trigeminal ganglion, beginning at 4 dpf. Whitlock et al. (Whitlock et al., 2006; Whitlock et al., 2003) have reported the presence of GnRH receptors in the trigeminal ganglion in zebrafish and Okubo et al. have reported the expression of GnRH3 in the trigeminal ganglion of medaka (Okubo et al., 2006). These observations strengthen the evolutionary hypothesis that various locations and functions of GnRH1, as found in species containing three GnRHs (e.g., medaka), have been taken over by GnRH3 in species with two forms of GnRH (e.g., zebrafish) (Kuo et al., 2005). The function of GnRH in the trigeminal nerve is unclear; it may play a role as a neurotransmitter in the sensory or motor functions involving the cranial region. In this context, it is interesting to note that two of the main nerves extending from the trigeminal ganglia are the ophthalmic nerve that participates in control over eye movement, and the maxillary nerve that is involved in transmission of sensory fibers from the nasal cavity. This is of interest due to the fact that some

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individuals with Kallmann's syndrome display eye movement abnormalities, as well as anosmia (Massin et al., 2003).

Lastly, the expression of GnRH3 in the pronephric ducts suggests that GnRH3 may be involved in kidney development. In addition, GnRH2 is expressed in the kidney (White et al., 1998). These findings suggest that GnRH3-producing cells are present in renal tissue during development and may be important for normal kidney development. Interestingly, another phenotype displayed in some Kallmann syndrome patients is renal agenesia (Zenteno et al., 1999). This suggests that the cause for abnormal migration of CNS GnRH neurons may also be the cause for GnRH related renal abnormalities.

Summary

Due to the small number of GnRH cells and low expression of GnRH, the detailed study of GnRH neuron early development has been challenging and limited in the past. The development and use of the Tg(GnRH3:EGFP) zebrafish line has facilitated an easier, more detailed and more direct method of studying GnRH system development, from early fiber development and soma migration to extra CNS GnRH3 expression. Use of this line has provided a wealth of data including novel findings regarding GnRH3 fiber development and soma migration. The transgenic Tg(GnRH3:EGFP) line, as is demonstrated in the following chapters, not limited to tracking normal developmental events.

The timeframe and extent of early GnRH3 fiber development is astonishing. The innervations of the pituitary suggests that GnRH3 may play an endocrine role in

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modulating the early development of the reproductive system, while the extent of CNS innervation suggests a neuromodulatory role of GnRH3. The early dual reproductive/neuromodulatory role that is suggested by GnRH3 fiber and soma location warrants further investigation. What does seem to be clear based on location of GnRH3 fibers and soma is that GnRH3 in ZF has taken over the role of both GnRH1 and GnRH3 in 'three form' fish. In this regard, ZF is a model that is close to mammals, as both have only one form of forebrain GnRH.

The migration of GnRH3 soma as described herein supports the classic view that the hypophysiotropic GnRH neuron population immerges in the olfactory region and migrates to the hypothalamus. GnRH3 soma are axophilic, utilizing preexisting GnRH3 fiber tracts to migrate into the CNS and to the hypothalamus. This mode of axophilic migration along fibers as a stream of migrating neurons from a proliferative zone to a specific CNS destination is classical tangential migration (Lois et al., 1996).

The Tg(GnRH3:EGFP) line has also enabled us to uncover GnRH3 expression in extra-CNS tissues including the trigeminal ganglion and pronephric ducts as well as fiber projections into the trunk. These finding expand even further the hypothesis that GnRH3 plays multiple roles within and outside of the CNS during development.

Chapter 3: THE ROLE OF GnRH3 AS AN AUTOCRINE FACTOR INVOLVED IN DEVELOPMENT OF THE GnRH3 SYSTEM

Abstract

During the first 48 hours of the GnRH3 system development an extensive GnRH3 fiber network is formed. These fibers develop prior to GnRH3 soma migration, extending from the GnRH3 soma in the olfactory region to innervate the optic tract, telencephalon, hypothalamus, pituitary, midbrain tegmentum and hindbrain as well as forming commissures between the two GnRH3 soma clusters. Thereafter, starting at 3 dpf, GnRH3 soma migrate along these fiber tracts to their final destination in the POA-hypothalamus and terminal nerve. Using the TG(GnRH3:EGFP) ZF line, we show *in vivo* that early establishment of the GnRH3 fiber network is disrupted when GnRH3 is knock-down using GnRH3-targeting morpholino-modified antisense oligonucleotides. GnRH3 knock-down causes abnormal fiber development and pathfinding, disrupting early fiber-targeting and commissure formation. In addition, GnRH3 knock-down results in anomalous GnRH3 soma proliferation and location. This suggests that early expression of GnRH3 by GnRH3 neurons, shortly after their birth, is necessary for normal development of the GnRH3 system. These findings are in agreement with *in vitro* experiments, using GnRH cell lines that have shown that GnRH is involved in cytoskeleton modeling and growth-cone targeting. Thus GnRH3 plays an important autocrine or paracrine function in early GnRH3 neuron development.

Introduction

During early development GnRH3 soma traverse a long tangential migration. The GnRH3 soma and fibers originate in the olfactory region, and proceed to migrate through various CNS regions to their final destination. GnRH soma reach the TN and POA-hypothalamus, and GnRH fibers innervate multiple CNS regions including the optic tract, telencephalon, hypothalamus, midbrain tegmentum and hindbrain, as well as various non-CNS regions.

This long range migration and targeting process is regulated by a plethora of factors (Cariboni et al., 2007). The influence of these factors is spatiotemporally differential, and it is safe to hypothesize that at any given time multiple chemoattractants, chemorepellents, and adhesion factors as well as various receptors, are actively influencing GnRH migration. The migration of GnRH neurons is classically divided into three phases: (i) migration in the nasal compartment apposed to the olfactory/vomeronasal fibers, (ii) entrance to the CNS and migration into the telencephalon and (iii) migration into the basal forebrain/hypothalamus (Wray, 2001).

Various factors have been implicated as participating in forebrain GnRH migration. These factors include secreted molecules, cell surface proteins, adhesion molecules and nuclear factors (Kramer and Wray, 2000b; Schwarting et al., 2004) (see Chapter 1). Of the three GnRH migration stages, the one that is arguably most poorly understood is the first, the immergence of GnRH neurons in the olfactory region, their early differentiation, and the projection of fibers. There are two classes of factors that are hypothesized to participate in early GnRH3 neuron development and migration in the olfactory region. The first class is the factors that participate in

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olfactory placode development and formation such as OTX-1, PAX-6 and six-3. The second class is the factors that are expressed at early stages by GnRH neurons such as Olf-1, GATA-4, AP-2α, and slightly later Ebf2, Cxcl12 and NELF (Cariboni et al., 2007; Corradi et al., 2003; Kramer and Wray, 2000b; Schwarting et al., 2006; Tobet and Schwarting, 2006).

In this context, GnRH itself is of interest as a factor that is expressed very early on by GnRH neurons, and thus may be necessary for the early differentiation, development and fiber-targeting of forebrain GnRH neurons. Temporal quantitative measurements of GnRH mRNA or decapeptide levels during early development in fish have not been conducted done to date in ZF. However, GnRH3 mRNA was expressed as early 24-26 hpf (Gopinath et al., 2004; Palevitch et al., 2007) and GnRH receptor transcripts were detected as early as 36 hpf (Whitlock et al., 2006). These time points are prior to the time in which GnRH3 is thought to have a modulatory effect on the pituitary-gonad axis. Similar to ZF, in mice, GnRH1 expression begins in temporal proximity to GnRH1 neuron migration at E11.5, (Wray et al., 1989a), and continues into the GnRH1 soma migration phase (Martinez-Fuentes et al., 2004). In addition, *in vitro* experiments show that GnRH1 causes cytoskeletal remodeling and growth cone extension abnormalities in GnRH1-producing FNC-B4 cells (Romanelli et al., 2005).

Based on these findings, the first expression of GnRH transcripts in both fish and mammals occurs prior to GnRH soma migration events, prior to HPG axis formation and prior to GnRH peptide reaching the pituitary. These facts coupled to the aforementioned GnRH modulatory *in vitro* findings suggest an

autocrine/paracrine role for GnRH in early GnRH neuron development. The role of GnRH in this capacity could be in early differentiation, development or migration of GnRH3 soma and fibers.

To study the role of GnRH3 during early development, we utilized the Tg(GnRH3:GFP) line. This line enabled *in vivo* observation of GnRH3 neuron development in high resolution, both under natural conditions and also when GnRH3 is knock-down via GnRH3 antisense MO.

Materials and methods

Experimental animals

Experimental animals were maintained and treated as specified in Chapter 2. F1 and F2 Tg(GnRH3:EGFP) were crossed and 1-2 cell stage embryos were used for MO injections. Larvae that were positive for EGFP were used for further observations.

Microinjections

To achieve GnRH3 knock-down, two morpholino-modified antisense oligonucleotides (MO; GeneTools, Eugene, OR, USA) were used: (i) one to prevent splicing, (CCACTCCATGCTAAAACTAAAACAC) designed to bind to the last 8 bp of exon 1 and the first 16 bp of intron 2 (ii) another, GnRH3 AUG targeting morpholino (AACAACCTTCCTTTCCACTCCATGC), directed to the AUG translation start codon. 1-2 nl of either MO, at a concentration of 0.5 mM, was

injected into GnRH3 transgenic zebrafish at the 1–4 cell stage. Phenol red was added

to the injection solution to a final concentration of 0.1% for visualization during

microinjection. GeneTools standard control MO

(CCTCTTACCTCAGTTACAATTTATA) was used as a control at 1 mM

concentration. An additional control, an unrelated smyD1a ATG MO,

(ACTTCCACAAACTCCATTCTGGATC) (Tan et al., 2006), was also used.

Microinjections were carried out under a dissection microscope (MZ8; Leica,

Deerfield, IL, USA) using a PLI-100 pico-injector (Medical System Corp., New

York, NY, USA). A total of 400 embryos were injected for the MO experiments.

MO-injected embryos were assessed for gross morphological abnormalities; only

embryos that did not exhibit apparent abnormalities under a dissecting microscope

were selected for further confocal microscopy. The percentage of abnormal embryos

was assessed in three separate groups of 50 embryos injected with GnRH3 MO.

Semi-quantitative reverse transcriptase RT-PCR

1-2 cell stage embryos were injected with GnRH3 anti-splicing MO (1–2 nl,

0.5 mM). In two experiments, 20 injected and 20 control embryos were collected at

24 and 48 hpf, RNA was purified using the NucleoSpin RNA II kit (Macherey-Nagel,

Duren, Germany). First-strand cDNA was synthesized using the transcriptor high

fidelity cDNA synthesis kit (Roche, Mannheim, Germany) and used for PCR

reaction.

To amplify GnRH3, primers targeting GnRH3 exon 1 and exon 3, which

normally produce a 191 bp product were used:

forward: ATCTTGAACAAACACAGCAG

reverse: CAGCACTGTGTCACCTGGAT

As a control, primers for EF1-alpha mRNA were used, which normally produce

an 810 bp product:

forward: GCATACATCAAGAAGATCGGC

reverse: GCAGCCTTCTGTGCAGACTTTG

Microscopy

Microscopy was conducted as described in Chapter 2.

Results

GnRH3 knock-down effects on soma location and differentiation

      The specific effects of GnRH3 knock-down on GnRH3 neurons were initially

manifested at 26 hpf and included soma and fiber abnormalities. Approximately 70%

of MO-injected embryos exhibited some type of GnRH3-specific abnormality.

      Abnormal location of GnRH3 soma was seen in MO-injected animals starting

at 26 hpf. The number of GnRH3 soma was increased compared to controls, in the

olfactory region, the dorsal midbrain, and the subpallium (Fig. 10). This increase in

cell number was observed in two respects, (i) proliferation of the cells in normal

location of GnRH3 neurons, the bilateral clusters in the olfactory region (Fig. 10 A,C)

and (ii) emergence of GnRH3 cells in abnormal locations within the CNS,

specifically in the midbrain and subpallium (Fig. 10 A,C). Interestingly, GnRH3 cells

found in abnormal locations did not project fibers.

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In addition we observed highly abnormal proliferation of GnRH3 cells in the two extra-CNS regions. A significant proliferation of cells was observed in the trigeminal ganglia (Fig. 10 A,C). These cell clusters extended beyond the normal area of the trigeminal ganglia to encompass the lateral aspects of the hindbrain. The GnRH3 neurons in this region extended fibers to various areas, a phenomenon not observed in control animals.

Lastly, in control larvae not treated with GnRH3 MO, GnRH3 fibers project from the CNS along the spinal cord (see Chapter 2). However no soma are present in this region. Contrary to this normal phenotype, when GnRH3 is knock-down EGFP expressing soma and fibers were present in along the spinal cord in areas, which normally only contain GnRH3 fibers (Fig. 10C).
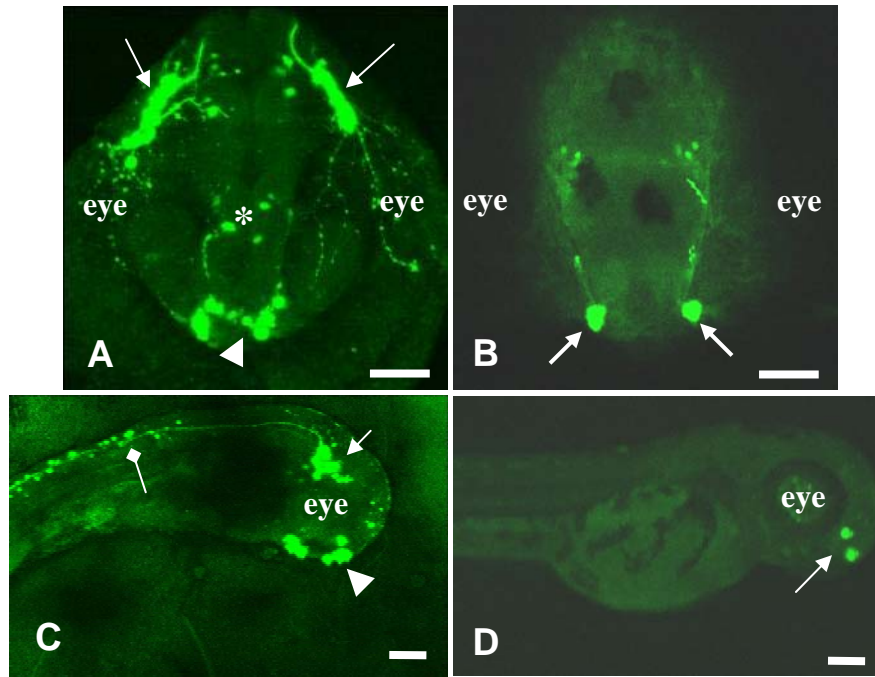
Figure 10.

GnRH3 knock-down by GnRH3 antisense MO (confocal microscopy). (A,B) Frontal and (C,D) lateral view. (A, C) At 30 hpf, there is an abnormal presence of GnRH3

neurones in the dorsal midbrain (asterisk) and abnormal proliferation in the olfactory placode region (arrowhead). Abnormal proliferation was also observed in the trigeminal ganglia (arrows) and abnormal locations in the spinal cord (diamond arrow). (B, D) Control larvae at 30 hpf. Scale bar (A,B,C,D) 50 µm.

GnRH3 knock-down effects on fiber-targeting

In larvae in which GnRH3 was knock-down, early fiber projections from GnRH3 neurones in the olfactory region were affected. When comparing the early fiber projections (28 hpf) in GnRH3 knock-down vs. control animals, the number of fiber projections was increased. Normally, there are 1-2 main fiber tracts that project towards the pallium. In knock-down animals, there were numerous fiber projections that extended in random and sporadic directions (Fig. 11 A,B). In addition to quantity and directionality abnormalities of the fibers, these early fibers in knock-down animals exhibited increased fiber branching points compared to controls.

At 48 hpf, a subset of GnRH3 fibers normally project bilaterally towards the pallium and subpallium to form commissures. In GnRH3 knock-down animals, these fibers did not meet at the midline, rather they developed incompletely or abnormally, in some cases missing their target (Fig. 11C) and in others extending in the correct direction only to loop back to their soma of origin (Fig. 11E).

Finally, GnRH3-expressing cells in the trigeminal ganglia projected fibers into the trunk along the spinal cord. This phenotype was not observed in controls in which the trigeminal population was not observed to project fibers (Fig. 11A).
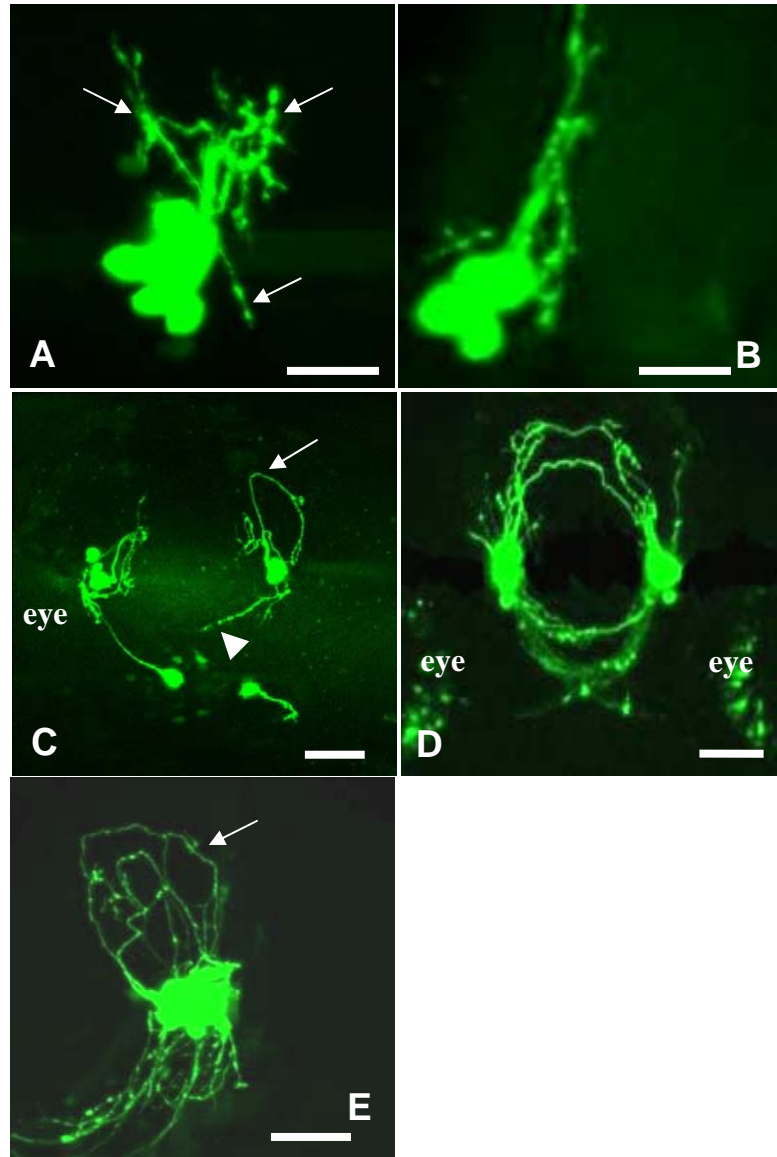
Figure 11.

Abnormal fiber development from GnRH3 olfactory region neuron clusters. (A,C,E) Tg(GnRH3:EGFP) larvae treated with GnRH3 MO knock-down. (B,D) Control Tg larvae (A) 28 hpf: Increased number of fibers extending in various directions (arrows) and increased number of fiber branching points (B) Control. (C–E) Head-on view at 48 hpf. (C,E) Fibers extending from neurones to the pallium loop back to the soma

cluster instead of forming a commissure (arrows). The commissure in the subpallium (arrowhead) is not completed. (D) Control. Scale bars: (A,B,E) 25 µm; (C,D) 50 µm.

Validation of GnRH3 knock-down

The efficacy and specificity of the anti-splicing GnRH3 MO was validated at 24 and 48 hpf by conducting semi-quantitative RT-PCR (Fig. 12). At both time points, levels of normal length GnRH3 mRNA in MO-injected larvae were dramatically decreased. The specificity of the anti-GnRH3 MOs (anti-splicing and anti-translation) was equivalent based on similarity of phenotype and on the fact that injection of control MO and of the unrelated smyd1a MO had no effect at any stage on the development of the GnRH3 neurones or fibers. In addition, gross morphology of 80% of anti-GnRH3 MO-injected larvae was normal, indicating that the GnRH3 knock-down effect is GnRH3 neuron-specific. Only larvae that exhibited normal CNS and trunk morphology post-injection were used for further evaluation of GnRH3 development.
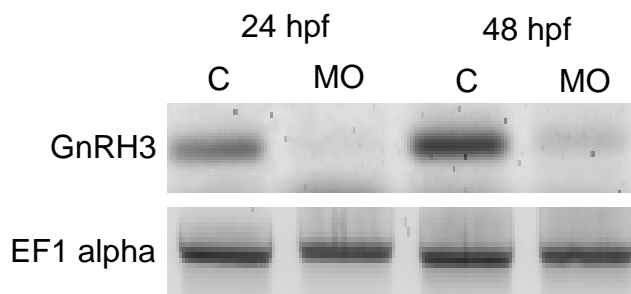
Figure 12.
Semi-quantitative reverse-transcriptase polymerase chain reaction at 24 and 48 hpf. Normal length GnRH3 mRNA transcript is absent or drastically decreased in larvae injected with GnRH3 anti-splicing MO. (C-control, MO-injected).

Discussion

The goal of this study was to examine whether GnRH3 is a factor that is involved in early GnRH3 neuron differentiation, development and fiber-targeting. Expression of GnRH transcripts and GnRH receptors in fish, as well as in mammals, begins during the early stages of development, prior to development of the HPG axis (Palevitch et al., 2007; Romanelli et al., 2004; Whitlock et al., 2006). This expression of both GnRH and GnRH receptors coincides with the onset of GnRH neuronal differentiation and fiber development. In addition, recent information obtained from GnRH cell lines indicates that altering exposure of GnRH neurons to GnRH peptide can induce a switch in coupling of GnRH-R to specific G-protein receptors, thereby differentially affecting biological processes. The downstream mechanism appears to involve stimulation of cAMP production and mobilization of intracellular calcium stores. Thus GnRH was suggested as an autocrine/paracrine factor that can influence differentiation and elicit structural changes in GnRH neurons (Krsmanovic et al., 2003; Romanelli et al., 2004).

Based on these findings, we hypothesized that GnRH3 itself is a factor that is necessary for GnRH3 neuron development and fiber-targeting. *In vivo* observations of GnRH3 neuron development in the Tg(GnRH3:EGFP) line after GnRH3 knock-down confirm that, indeed, GnRH itself or its precursor is a factor involved both in establishment of the early GnRH3 fiber network and in proper location and differentiation of GnRH3 soma.

GnRH3 is necessary for normal GnRH3 development

In GnRH3 MO-treated larvae, there is abnormal location of GnRH3 soma to the subpallium and midbrain, as well as an increased number of neurones in the olfactory region and the trigeminal ganglia. Based on these findings, GnRH3 seems to play a role in GnRH3 cell location and differentiation.

It is not clear which factors are responsible for differentiation of GnRH neurones, although several transcription factors expressed in developing GnRH cells have been suggested as candidates (Wray, 2001). One factor of interest is Nestin, a marker for stem cells and precursor cells in the developing embryonic nervous system. In addition, Nestin is localized to active neurogenesis areas in adult ZF. Interestingly, Nestin is expressed in early GnRH neurons and is suspected to play a role in neurogenesis of olfactory epithelium cells (Dahlstrand et al., 1995; Kramer and Wray, 2000a; Mahler and Driever, 2007). *In vitro* exposure of the FNC-B4 GnRH-producing cell line to GnRH induces a decrease in Nestin(Romanelli et al., 2004), suggesting that under normal conditions a rise in expression of GnRH triggers a decrease in Nestin, thereby signaling a commitment of the cell to differentiate into a GnRH neuron. By the same token, knock-down of GnRH may in the short term prevent a decrease in Nestin levels, thus delaying GnRH neuron differentiation. The abnormal increase in proliferation of GnRH neurons in GnRH knock-down larvae suggests that GnRH may also be a factor that directly or indirectly acts to inhibit surrounding cells from differentiating into GnRH neurons. Thus, on the one hand GnRH may act in an autocrine/paracrine fashion to induce GnRH neuronal differentiation. On the other hand, it may also act in a paracrine fashion to inhibit

surrounding cells from differentiating into GnRH neurons. This dichotomy of actions

would be in line with our finding that GnRH knock-down induces an abnormal

increase in proliferation of GnRH neurons in the olfactory region and trigeminal

ganglia. Further studies are required to elucidate the regulatory relationship between

GnRH and Nestin and the role that Nestin plays in GnRH3 neuronal development.

GnRH3 knock-down causes abnormal GnRH3 neuron fiber developmental

Knock-down of GnRH3 in Tg(GnRH3:EGFP) ZF resulted in distinct early

fiber developmental abnormalities. These abnormalities included several phenotypes,

(i) an increased number of fibers projecting from GnRH3 soma, (ii) a lack of correct

directional targeting by extending fibers, and (iii) inability to form commissures

between the two GnRH3 clusters in the olfactory region. The finding that *in vitro*

exposure of FNC-B4 cells to GnRH causes cytoskeletal remodeling and growth cone

extension abnormalities is in agreement with these results (Romanelli et al., 2004).

Based on previous studies, the structural changes observed that are caused by

abolishing GnRH are possibly mediated by cytoskeleton remodeling, changes in actin

protein levels, and GnRH-R downstream effects on cAMP and Ca^{2+} (Romanelli et al.,

2004). Our *in vivo* study demonstrates that a GnRH3 autocrine/paracrine loop

mechanism is involved in GnRH3 fiber pathfinding and targeting during early

development.

Summary

Our findings show that GnRH3 acts in an autocrine/paracrine fashion in ZF to

mediate correct GnRH3 fiber development and is involved in GnRH3 cell

immergence and proliferation. Thus, GnRH3 plays an important autocrine/paracrine role during early development as a migration/pathfinding and neuron differentiation modulating factor. These findings are in agreement with GnRH *in vitro* studies (Romanelli et al., 2004). It is noteworthy, however, that our findings seemingly differ from findings in the hpg mouse and the GnRH receptor mutant mouse (Gill et al., 2008; Livne et al., 1993). According to these studies, in GnRH mutant mice the size and distribution of GnRH1 neurons in the basal forebrain is normal and GnRH1 fibers project correctly to the median eminence (ME). There are several possible reasons for the difference in findings between the mutant mouse model and both the *in vitro* cell line and zebrafish models. While GnRH1 exon 2 deletion in the hpg mouse results in loss of GnRH1 hypophysiotropic action, it does not appear sufficient to affect the GnRH autocrine/paracrine function that is necessary for normal GnRH neuron development. Regarding the GnRH-R mutation, more than one type of GnRH-R is found in most species, thus a mutation in one receptor does not necessarily imply inactivation of autocrine/paracrine GnRH effects. Second, the findings in mutant mice studies focus on location of the hypothalamic GnRH1 population and innervations of the ME. Herein, we describe abnormalities in GnRH neuron development and fiber pathfinding while GnRH neurons are still in the olfactory region. Early abnormalities at this stage do not necessarily imply that later targeting of GnRH fibers to the ME/pituitary or GnRH neuron migration is abnormal. In fact, a significant number of ZF seemed to overcome the initial defects in GnRH neuron development and proceed to develop a normal looking fiber network and GnRH soma migration. We speculate that this is mainly due to the short half-life (4-5 days) of the anti-GnRH MO, but it

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may also indicate that early developmental defects due to GnRH knock-down are not permanent or do not eventually affect GnRH hypothalamic presence.

It is important to note that other than GnRH3 neuron-specific abnormalities, which were observed in 70% of MO-injected embryos, non-GnRH3-specific morphological abnormalities were observed in 9% of MO-treated larvae (11% of embryos did not survive to 24 hpf). These non-GnRH3-specific abnormalities were similar to CNS deformities observed following GnRH3 knock-down in another study (Wu et al., 2006). However, in the latter study, CNS and eye deformities were observed in more than 50% of MO-injected embryos compared to 9% in the present study. This difference could be due to variations in the amount of MO injected, MO sequence or the ability to detect abnormalities in the GnRH3 system. To exclude the possibility that general CNS abnormalities are causing the observed GnRH3-specific abnormalities, embryos that exhibited general CNS abnormalities (9% of total) were not chosen for further confocal observation.

The findings regarding the extent of GnRH3 fiber distribution laid out in Chapter 2 and the findings herein regarding the autocrine/paracrine role of GnRH in early forebrain GnRH neuron development contribute to an expanded view of GnRH. This shift moves the field from the former prevailing view that the sole role of forebrain GnRH is to act as a gonadotropin-releasing hormone to the newly emerging view that GnRH plays multiple additional roles, including an early developmental role in neuron development and later roles as a neuromodulatory factor.

Chapter 4: INVOLVEMENT OF NETRIN1a, NETRIN2 AND HEDGEHOG IN GnRH3 SYSTEM DEVELOPMENT

Abstract

GnRH3 tangential soma migration and fiber-targeting are complex developmental events. GnRH3 fiber distribution in the CNS is extensive and GnRH3 soma migration occurs through multiple regions and over long distances. For these reasons, control of GnRH3 system development is complex and involves multiple factors. To better understand this process, we utilized the Tg(GnRH3:EGFP) line to study the participation of Netrin1a, Netrin2 and Shha in GnRH3 neuron development.

knock-down of Netrin1a induced abnormalities in GnRH3 fibers that normally extend toward or past the midline. These include the commissures formed between the GnRH3 soma clusters, as well as the fiber tract that innervates the retina. No additional effects on fiber projections to the hypothalamus or trunk were observed. Over-expression of Netrin1a in the pineal gland induced enhanced targeting of GnRH3 fibers to that region. In addition to fiber abnormalities, knock-down of Netrin1a also perturbed normal migration of GnRH3 soma into the hypothalamus, confining the soma to the olfactory region and the TN. These findings indicate that Netrin1a regulates GnRH3 neuron development, both on the level of midline fiber attraction and at later timepoints as a soma chemoattractant.

Netrin2 knock-down conferred arrested development at the 22-26 somite stage and death of the embryo. Early immergence and location of GnRH3 soma in the olfactory area of these embryos at 24 hpf was not affected by Nerin2 knock-down.

Disruption of the Shha pathway via cyclopamine immersion of larvae at different timeframes or by use of ZF smo mutants did not result in GnRH3-specific effects. Early exposure to cyclopamine (8-24 hpf) exhibited non-GnRH3-specific morphological abnormalities, as did smo mutants. These included the lack of an olfactory area, resulting in total absence of GnRH3 neurons. Later timeframe exposures to cyclopamine (24-48, 48-72 hpf) caused mild morphological abnormalities but did not effect GnRH3 development.

Taken together, these results show a specific and multifaceted role of Netrin1a in GnRH3 system development, but no specific GnRH3 developmental role for Netrin2 or Shha.

Introduction

Among the plethora of factors that regulate the development and migration of the forebrain GnRH system, secreted guidance factors play a central role (Rogers et al., 1997; Wierman et al., 2004; Wray, 2001). Contrary to radial migration of neurons, which is dependant on radial glial cells for guidance, tangential migration of neurons is based on attachment of neurons to one another or to preexisting fibers (Sobeih and Corfas, 2002).

GnRH neurons migrate along the vomeronasal nerve. Moreover, GnRH soma migrate along their own preexisting fibers. This migration of GnRH3 neurons in ZF is characterized by a continuous stream of soma migrating during development from the proliferative region in the olfactory region to the hypothalamus (Abraham et al., 2008; Palevitch et al., 2007; Schwanzel-Fukuda, 1999) (see Chapter 2). This form of

migration requires tight spatiotemporal control via multiple factors for correct guidance. Such control is particularly pertinent in the case of forebrain GnRH neurons, which traverse the longest known tangential migration in the CNS through multiple regions, each containing a distinct milieu of cells and factors.

When studying factors that are involved in GnRH system development, one must consider that development of this system takes place in two distinct stages. First, as described in previous chapters, the GnRH3 fiber network is formed and a subset of network fibers project in a posterior direction towards the hypothalamus. Only after these fibers reach the hypothalamus does the second stage take place, in which the GnRH3 soma migrate along this tract. Therefore when considering factors that are involved in GnRH3 migration, we must distinguish between factors involved in early fiber-targeting and factors involved in the second stage of axophilic tangential soma migration.

In this context, secreted guidance molecules, both chemoattractants and chemorepellents, modulate both fiber-targeting and soma migration. Secreted molecules can serve as very specific guidance factors that can be fine-tuned in accordance to varying factors, including timing of release, released quantity, distance from target, gradient characteristics, and expression of receptors by target neurons. In some cases the same factor can engage in opposite functions, such as Netrin1, which served as both a chemoattractant and chemorepellent (Sobeih and Corfas, 2002).

In this chapter, we focus on two secreted factors, the netrin family and hedgehog. Netrin, the Sanskrit term for "one who guides", is a diffusible molecule that was first described as a factor that induces commissural fiber outgrowth and is

required to guide commissural fibers towards the midline (Charron et al., 2003; Serafini et al., 1994). In netrin1 mutant mice, commissural fibers fail to enter the spinal cord and are misguided (Serafini et al., 1996). Additional roles for Netrin have been described more recently including participation in soma migration, angiogenesis, cell survival and tumorigenesis (Colamarino and Tessier-Lavigne, 1995; Navankasattusas et al., 2008; Wilson et al., 2006). These Netrin1a functions have been conserved throughout evolution from *C. elegans* to mammals. In mammals, five netrins have been identified: netrin-1, netrin-3, netrin-G1, netrin-G2 and netrin-4/b-netrin. While in ZF four netrins have been identified, netrin1a, netrin1b, and the recently described netrin2 and netrin4 (Park et al., 2005; Strahle et al., 1997). Three receptors for Netrin have been identified in mammals: Dcc, UNC5 and A2b (Mehlen and Mazelin, 2003). However, in ZF only Dcc and UNC5b have been described (Hjorth et al., 2001; Lu et al., 2004). The Netrin1 signaling pathway includes a combination of tyrosine phosphorylation, phosphatidylinositol signaling and regulation by Rho GTPases. These factors act as part of a cascade to regulate fiber extension and growth cone directional movement (Round and Stein, 2007).

Interestingly, netrin provides a bifunctional guidance cue that can mediate both chemoattraction and as chemorepulsion. The determining factor for the role of netrin depends on the constellation of receptors expressed by the target cell. The current understanding is that Dcc mediates netrin1 chemoattractant properties, while a combination of UNC5 and Dcc receptors mediate chemorepulsion action (Hong et al., 1999; Round and Stein, 2007).

Netrin1 participates in GnRH1 neuron migration in mice (Schwarting et al., 2001; Schwarting et al., 2004). In these studies, Dcc$^{-/-}$ mutant mice and netrin1$^{-/-}$ mutant mice exhibited abnormal targeting of the vomeronasal nerve and GnRH1 neurons. The percentage of GnRH neurons found in their typical basal forebrain location is reduced in both Dcc$^{-/-}$ and netrin1$^{-/-}$ mutant mice while the number of GnRH neurons located abnormally in the cerebral cortex is increased (Schwarting et al., 2001; Schwarting et al., 2004). In netrin1$^{-/-}$ mice, GnRH1 neurons specifically fail to turn ventrally after passing the cribriform plate and instead continue into the cortex. Examination of Unc5h3 mutant mice did not reveal abnormalities to GnRH1 migration.

In mice, Netrin1 is expressed during development both in the ventral forebrain and in the caudal olfactory epithelium (Schwarting et al., 2004). In ZF, netrin1a expression is widespread at 24 hpf in the brain, spinal cord, and somites, and by 36 hpf is restricted to the midline and optic nerve (Fig. 21). Expression patterns at later time points have not been elucidated. The hypothesis is that Netrin1 expression in the forebrain acts as a chemoattractant for the vomeronasal nerve as well as GnRH fibers and soma. Lack of Netrin1 expression in the forebrain is assumed to be the cause for abnormal GnRH migration in netrin1 mutant animals (Schwarting et al., 2004). Concurrently, expression of netrin1 in the caudal olfactory epithelium region during development appears to have no role in GnRH neuron development or migration. However, while GnRH cells express Dcc (Schwarting et al., 2001), this expression was only present up to E12, and thereafter expression is downregulated. This fact does not seem to be in agreement with the role that netrin1 plays during later

stages of GnRH migration into the forebrain. Questions also remain regarding the role of Netrin1 in the nasal region and the effect that Netrin1 knock-down/knock-out has on GnRH fiber-targeting in general. During development Unc5b is expressed in the olfactory region in a gradient that decreases in a dorsocaudal direction (Schwarting et al., 2004). However, migration of GnRH1 neurons in Unc5h3 mutant mice is normal and therefore this factor is not likely to be involved in early GnRH migration.

The recently described Netrin2 is of interest due to its unique expression pattern in zebrafish. Netrin2 transcript is expressed during early somitogenesis in the notochord and later on in the fourth rhombomere. Subsequently, netrin2 is expressed in the hindbrain and otic vesicles. From the perspective of GnRH development, netrin2 is of interest due to its expression pattern at 48 hpf. At this time GnRH3 soma migration begins and netrin2 is expressed in bilateral clusters in the olfactory region and telencephalon (Park et al., 2005) in proximity to the path taken by GnRH3 fibers and soma.

The third factor that will be discussed in this chapter is Hedgehog (Hh). Hh is a highly conserved secreted molecule that acts in a diffusible manner as a morphogen, providing positional information during development. In this capacity, Hh controls various aspects of morphogenesis and tissue patterning (Tabata and Takei, 2004). In mammals, there are three forms of Hh, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh), while in ZF there are four forms, Sonic hedgehog (Shh) a and b, Indian hedgehog (Ihh) and Desert hedgehog (Dhh). All forms of Hedgehog appear to act through the Patched-Smoothened-Ci/Gli pathway (Charron and Tessier-

Lavigne, 2005). The form of Hh that is most studied and seems to be most relevant to CNS development is Shha.

Recent findings indicate that after major embryonic patterning is complete, Shha switches roles to serve as a guidance factor for fiber pathfinding (Varga et al., 2001). This Hh role was uncovered as a result of the finding that in mice mutant for netrin1 some commissural fibers still reach the midline, suggesting that additional factors are involved in guiding these fibers. Shha has been suspected as a candidate guidence factor, and indeed Shha can mimic Netrin1 activity and act as a fiber chemoattractant (Charron et al., 2003). The similarity in function between Hh and Netrin1 is intriguing in the context of forebrain GnRH development. In addition, Netrin1a expression might be regulated by Hh proteins (Lauderdale et al., 1998). Here, we use the Tg(GnRH3:EGFP) ZF line to examine the roles of Netrin1a, netrin2 and Shh as factors involved in the development of the ZF GnRH3 system.

Materials and methods

Experimental animals

Experimental animals were maintained and treated as specified in Chapter 2. To obtain Tg (GnRH3:EGFP) larvae, F3 and F4 Tg(GnRH-EGFP) adults were crossed with each other or with WT fish. For MO experiments 1-2 cell stage embryos were injected with various MOs at different concentrations. Larvae that were positive for EGFP were used for further observations.

For Shha experiments a mutant smoothened homolog line ($smo^{b641/b641}$) was purchased from the Zebrafish International Resource Center (Eugene, OR).

Microinjections

To achieve knock-down of Netrin1a, Netrin2 and the Netrin receptors Dcc and Unc5b, four morpholino-modified antisense oligonucleotides were utilized (MO; GeneTools, Eugene, OR, USA) (see injection method in Chapter 3). The MO used to knock-down Netrin1a, Dcc and Unc5b knock-down have been used previously in ZF studies (Lu et al., 2004; Navankasattusas et al., 2008; Suli et al., 2006; Wilson et al., 2006). MO concentrations were based both on concentrations that were effective in previous studies and on serial dilution MO injections that were conducted to verify the best concentration for optimum specific effects.

All MO were injected (1-2 nl) into 1-2 cell stage Tg(GnRG3-EGFP) line larvae. EGFP-positive larvae were selected for further inspection. Each MO treatment was repeated at least 3 times with a minimum of 100 embryos in each experiment.

MO-injected embryos were assessed for gross morphological abnormalities; only embryos that did not exhibit gross abnormalities under a dissecting microscope were selected for further assessment. The various MO parameters are as follows:

 (i) Gene Tools control MO was used as standard control. (CCTCTTACCTCAGTTACAATTTATA, 1 mM).

(ii) Netrin1a MO is a splice blocking MO designed to bind to the last 11 bp of exon 1 and first 14 bp of intron 1 of the ZF Netrin1a sequence (Wilson et al., 2006). (ATGATGGACTTACCGACACATTCGT, 0.6 mM)

(iii) netrin2 MO is directed to the netrin2 AUG translation start codon (TGGAGGCTTGGAACACAGTGAGCAT, 0.2 mM).

(iv) Dcc MO is directed to the Dcc AUG translation start codon (GAATATCTCCAGTGACGCAGCCCAT, 0.4 mM) (Suli et al., 2006).

(v) Unc5b MO is a splice blocking MO designed to bind to the last 7 bp of exon 1 and the first 18 bp of intron 1(Lu et al., 2004; Navankasattusas et al., 2008). (CATTTAACCGGCTCGTACCTGCATG. 0.12 mM)

Morphant phenotype analysis

All morpholino and immersion experiments were repeated at least three times. Microscope images of soma were scored by comparing images of injected and wild-type embryos (Fig. 13) and fell into 3 general patterns of cell location phenotypes which were labels 1-3 yielding a semi-quantitative crossing score. For statistical analysis, the number of scored larvae for each MO was used in a T-test for independent samples and the percentage of larvae in each score was calculated.

For all analyses of fiber-targeting and location as well as measurements of soma migration, only larvae that exhibited normal gross morphology as determined under a stereoscope were used (Fig. 15).

Semi-quantitative reverse transcriptase RT-PCR

1-2 cell stage embryos were injected with either Netrin1a or Unc5b MO (1–2 nl, 0.6 and 0.12 mM respectively). For each MO, two experiments were conducted. In each experiment 100 embryos were injected with Netrin1a or Unc5b MO and 100 embryos were injected with control MO. Embryos were collected at 1,2,3,4 and 5 dpf, RNA was purified using the NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany). First-strand cDNA was synthesized using the transcriptor high fidelity cDNA synthesis kit (Roche, Mannheim, Germany) with random hexamer primers and used for PCR reactions.

To amplify Netrin1a, primers targeting Netrin1a exon 1 and exon 2, which normally produce a 386 bp product, were used:

forward: CTTTCGGAGACGAAAACGAG

reverse: CTTTGCAGTAGTGGCAGTGG

To amplify Unc5b primers targeting Unc5b exon1 and exon2, which normally produce a 224 bp product, were used:

forward: CCTCCCTGCTCGGTTTACTT

reverse: CGACCAATATCCTGAGTGACG

For control, primers for beta-actin mRNA, which normally produce a 353 bp product were used:

forward: CCCAAGGCCAACAGGGAAAA

reverse: GGTGCCCATCTCCTGCTCAA

Shha pathway disruption

       To achieve Shha pathway disruption, two methods were used. Tg(GnRH3:EGFP) larvae were immersed in 50 µM cyclopamine (LC Laboratories, Woburn, MA) (5 µl/ml from 10mM ethanol-cyclopamine stock solution), for 8-24 hpf, 24-48 hpf and 48-72 hpf. Control Tg(GnRH3:EGFP) larvae were immersed at the same timeframes in 5 µl ethanol. Each immersion experiment included 20 embryos/larvae in 2 ml of egg water. Larvae were assessed for gross and GnRH3 development at 1,2,3,4 and 6 dpf.

       For Hh knock-out, $smo^{b641/b641}$ (AB) heterozygous adult ZF were purchased from the Zebrafish International Resource Center. In these mutants, Hh activity is blocked by a point mutation in the smo gene. These adults were crossed with Tg(GnRH3:EGFP) ZF to obtain $smo^{b641/b641}$-GnRH3:EGFP P0 ZF. These $smo^{b641/b641}$-GnRH3:EGFP progeny were screened for positive EGFP expression and raised to maturity. After reaching maturity $smo^{b641/b641}$-GnRH3:EGFP fish were crossed to obtain larvae that were GnRH3:EGFP positive and $smo^{b641/b641}$ mutant homozygous.

Pineal specific Netrin1a over-expression

An aanat2:EGFP:PRDM vector was supplied courtesy of Dr. Y. Gothilf,
Tel-Aviv University. This vector included the aanat2 pineal-specific promoter
sequence (1.65 kb of 5'-flanking region and a 123-bp 5-untranslated region of the
aanat2 promoter) and a pineal-restrictive downstream module (PRDM) from ZF,
fused to an EGFP sequence (Appelbaum et al., 2004). Correct expression of the
construct was verified by injecting 1-2 cell WT ZF embryos with 1-2 nl of the
construct (25 ng/µl) and observing correct EGFP pineal expression at 1,2,3 and 4 dpf.

To clone netrin1a mRNA, total RNA was purified from 10 dpf ZF using the
NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany). First-strand cDNA was
synthesized using the transcriptor high fidelity cDNA synthesis kit (Roche,
Mannheim, Germany) with random hexamer primers and used for PCR amplification.
Netrin1a prepro mRNA was PCR amplified using the Roche Expand High Fidelity
PCR System (Roche Diagnostics, Mannheim, Germany). The primers that were used
amplify a 2177 bp fragment of Netrin1a sequence (accession # NM_131029
XM_688160). Primers used also included SacI and NotI restriction sites:
forward- gagctcagagtttgcccatgtgttgg

reverse- gcggccgcctccttcacacgttcccatt

PCR product was run on a 1.5% gel, cut out, purified using the QIAquick Gel
Extraction Kit (QIAGEN, Valencia, CA) and ligated into a vector using the GeneJET
PCR Cloning Kit (Fermentas, Glen Burnie, MD) . The pJET vector containing the
netrin1a sequence was transformed into Dh5α cells and grown O/N. Cells were
purified using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and

integration of a correct netrin1a sequence was verified by sequencing. Restriction

enzymes SacI and NotI were used to digest both the pJET vector containing the

netrin1a mRNA sequence and to excise the EGFP from the aanat2:EGFP:PRDM

vector. The netrin1a mRNA sequence was then ligated to the linearized

aanat2:PRDM vector resulting in an aanat2:netrin1a:PRDM vector. The vector was

transformed and purified as noted above. Correct netrin1a sequence and integration

was verified using flanking primers

forward- agggttaaaggcgaggagag

reverse- tcaggttcagggggaggt

For netrin1a over expression experiments, Tg(GnRH3:EGFP) fish were crossed and

embryos were injected at 1-2 cell stage with one of four vector/MO combinations

(also see MO sequences above) combinations:

(i) control MO (1 mM) (ii) Netrin1a MO (0.6 mM) (iii) aanat2-Netrin1a-PRDM

vector (25 ng/µl) (iv) aanat2-Netrin1a-PRDM vector and Netrin1a MO (25 ng/µl, 0.6

mM, respectively).

Microscopy

Light and confocal microscopy were used as described in Chapter 2 with the

the following exceptions. For lateral confocal images of Tg(GnRH3:EGFP) ZF,

larvae were anesthetized using 0.4% MS-222, the eye from one side of the head was

removed using a dissecting scope and the larvae was mounted in low melting point

agarose on its side.

Results

Netrin1a involvement in GnRH3 soma location

The first emergence of GnRH3 transcripts, as well as EGFP, in Tg(GnRH3:EGFP) larvae occurs between 24-26 hpf. Normal expression appears in the form of two well defined bilateral clusters of GnRH3 soma in the olfactory region adjacent to the olfactory organs (see Chapter 2 and Fig. 13A and 14A).

In larvae in which Netrin1a or Dcc was knock-down, abnormal locations of GnRH3 soma in a significant percentage of 2 dpf larvae was observed. To quantify these abnormalities and for statistical analysis, a crossing score was developed. This 1-3 score was based on the degree of GnRH3 soma distribution and dispersion, with (1) defined as normal distribution in two well defined bilateral clusters (Fig. 13A), (2) defined as loose bilateral clustering (Fig. 13B), and (3) defined as lack of defined GnRH3 soma clusters (Fig. 13C). Each larva was ranked based on this scale.

In Tg(GnRH3:EGFP) larvae injected with either Netrin1a or Dcc MO, abnormal GnRH3 soma location was observed. A significant percentage of these larvae exhibited dispersed location of GnRH3 soma (rank 2 or 3) (Fig. 14C,D). Larvae injected with control MO, as well as larvae in which Unc5b was knock-down (Fig. 14B,E) did not exhibit a significant increase in abnormal GnRH3 soma location when compared to WT larvae (Fig. 14A).

For statistical analysis, a two-tailed T-test was performed. Larvae of each MO-injected group for each scoring level were compared with the same scoring level group in WT and in control MO-injected larvae. The number of larvae at levels 2 and 3 were found to be significantly higher ($p<0.02$) in Netrin1a and Dcc knock-down

larvae compared with WT, control and Unc5b MO-injected larvae. Moreover, the number of larvae at level 1 was found to be significantly lower (p<0.01) in Netrin1a and Dcc knock-down larvae compared with WT, control MO and Unc5b MO-injected larvae. However, no significant difference in percentage of larvae at any level was found between Unc5b knock-down larvae and WT or control MO larvae (Fig. 14). For clarity, embryo numbers were converted into percentages (Fig. 14).

Larvae injected with MOs were assessed for gross morphology to examine whether abnormal GnRH3 soma or fiber phenotypes are the result of non-specific morphological abnormalities. Most larvae treated with Dcc, Unc5b, Netrin1a and control MOs did not exhibit such morphological abnormalities (Fig. 15). The only general effect that all MO-treated fish exhibited was a slightly delayed developmental rate and slightly reduced eye diameter. Larvae that did exhibit overt morphological abnormalities were not chosen for further assessment.

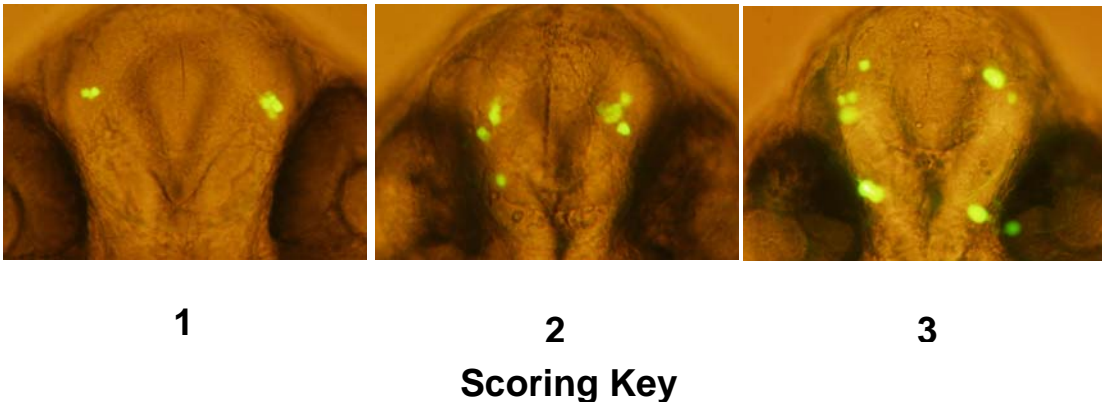**1**   **2**   **3**

Scoring Key

Figure 13.

Following Netrin1a, Dcc and unc5b morpholino knock-down, Tg(GnRH3:EGFP) exhibited a variation in GnRH3 location phenotype. A scale of 1-3 in 2 dpf larvae was developed to assess the degree of soma location abnormality. (1) Level 1-normal, (2) level 2-medium, (3) level 3-severe. This scale was used to analyze soma location abnormality (Fig. 14).
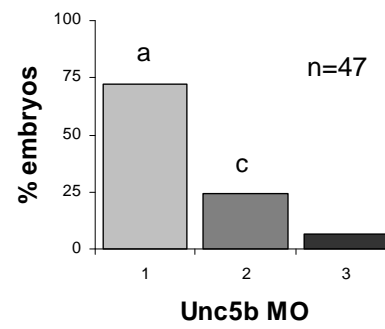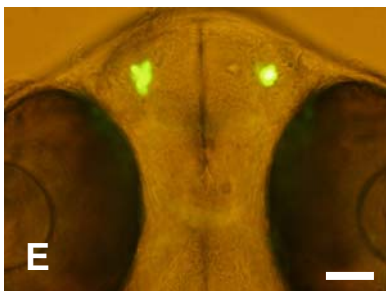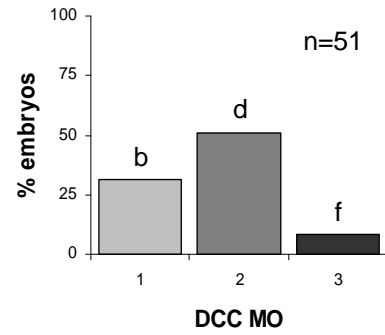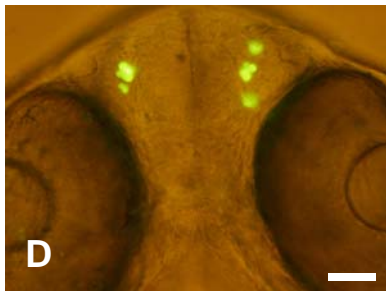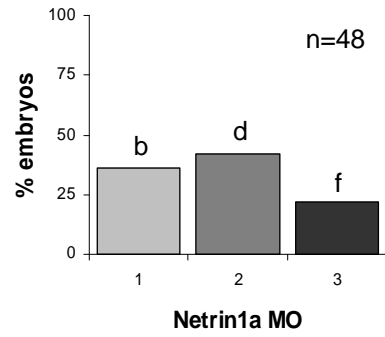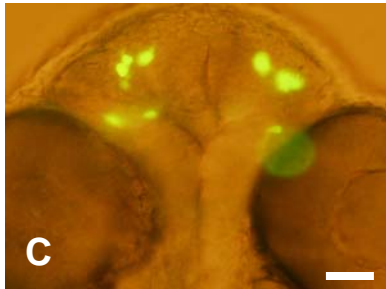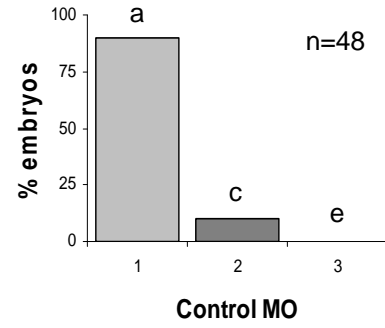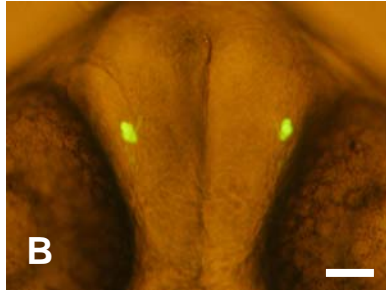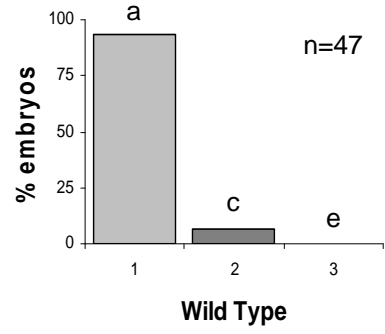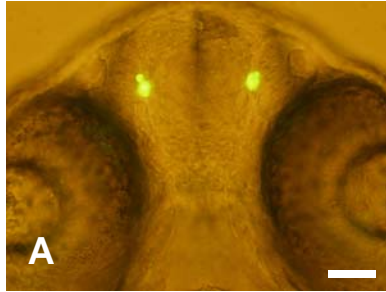
Figure 14 (previous page).

knock-down of Netrin1a and its receptor Dcc caused abnormalities to GnRH soma location within the olfactory region. (A-E) ventral view of olfactory region in 2dpf larvae treated with various MOs. To the left are representative images of the various treated larvae and to the right graphs showing the percentage of larvae from each treatment categorized to each of the three soma distribution scoring levels (see Fig. 13). Letters above bars represent statistically significant ($p < 0.02$) intra scoring level differences between treatment groups. (A) Control non-treated Tg(GnRH3:EGFP) larvae. Majority of larvae are level 1. (B) Control MO (1mM) injected larvae. Majority of larvae are level 1: no variation from control. (C) Netrin1a MO (0.6mM) and (D) Dcc MO (0.6) injected larvae. In both, significantly more larvae are level 2 and 3 and significantly fewer larvae are level 1 compared with WT and MO control. (E) Unc5b-injected larvae (0.12 mM) no significant difference in levels 1 and 2, compared with WT, and significantly fewer larvae are level 2 and 3 compared with Netrin1a and Dcc MO-treated larvae. Scale bar = 50 µm.

| Control MO | Net1a MO | Unc5b MO | DCC MO |
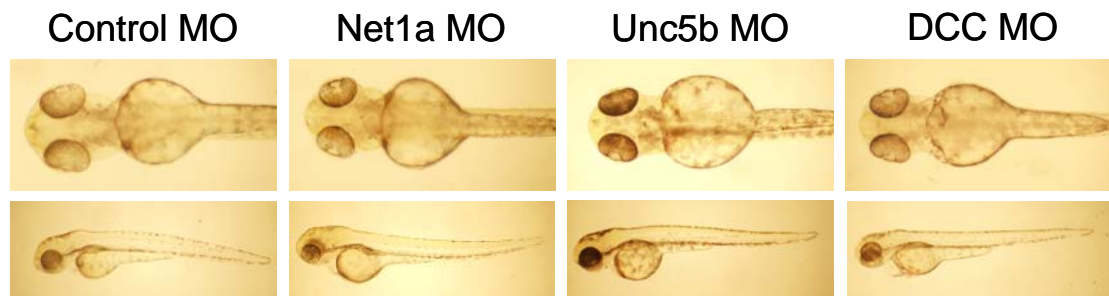
Figure 15.

MO-injected larvae were assessed at 2,4 and 6 dpf for morphological abnormalities. Above are dorsal and lateral images of control (1mM), Netrin1a (0.6 mM), Unc5b (0.12 mM) and Dcc (0.6 mM) injected larvae at 2 dpf. The majority of injected larvae did not exhibit gross morphological abnormalities following MO treatment at the stated doses. The only apparent and consistent abnormalities that were observed in some injected larvae were slightly delayed development, slightly smaller eyes and a degree of heart edema.

Netrin1a and Dcc knock-down perturbs GnRH3 soma migration

The dynamics of soma migration were examined to assess the subsequent effects of knocking-down Netrin1a or its receptor Dcc. Tg(GnRH3:EGFP) larvae that were injected with control, Netrin1a or Dcc MO were examined at 8 dpf for distance of soma migration. At this time point, GnRH3 soma migration can clearly be observed and measured. Measurements were made between the two most distant GnRH3 soma observed in n>10 larvae from each treatment group. This experiment was repeated twice.

The distance that soma in Netrin1a and Dcc MO-treated larvae migrated was significantly less than in control MO-injected larvae (Fig. 16). No significant differences were found between Netrin1a and Dcc-treated larvae, or between control MO and Unc5b MO-injected larvae. The average distance soma migrated in control MO-injected larvae was 85.6 µm (±9.2 µm), compared with a distance of 42.7 µm (±5.1 µm) and 48.8 µm (±7.7 µm) in Netrin1a and Dcc MO-injected larvae respectively, and 77.8 µm (±5.8 µm) in Unc5b MO-injected larvae (Fig. 16).

In Netrin1a and Dcc MO-injected larvae, soma remained localized to the olfactory region and the TN (Fig. 17B,C), while in control larvae soma normally migrated well into the telencephalon (Fig. 17A). No abnormalities were observed in the GnRH3 fiber tract to the hypothalamus, along which GnRH3 soma migrate (Fig. 20).
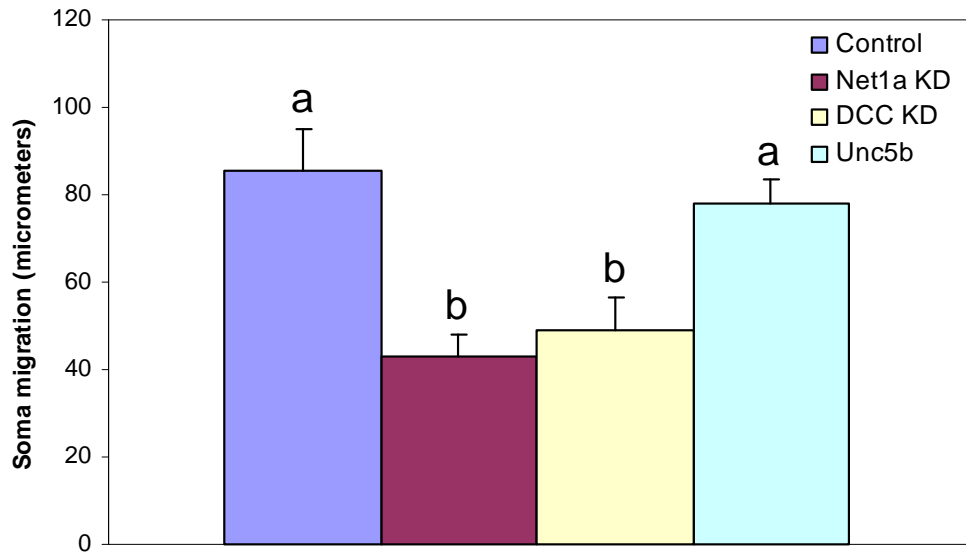
Figure 16.

Average migration distance of GnRH3 soma at 8 dpf in control MO, Netrin1a MO, Dcc MO, and Unc5b MO-injected larvae. Measurements made between the two most distant GnRH3 soma at 8 dpf. Each column represents n>10 larvae with n>2 repeats. Larvae injected with either Netrin1a or Dcc MO exhibited significantly reduced migration of GnRH3 soma from the olfactory region towards the hypothalamus in comparison with both control and Unc5b MO-injected larvae (P<0.01, T-test). No significant difference was found between soma migration in Dcc and Netrin1a MO-injected larvae.

control   Netrin1a   Dcc
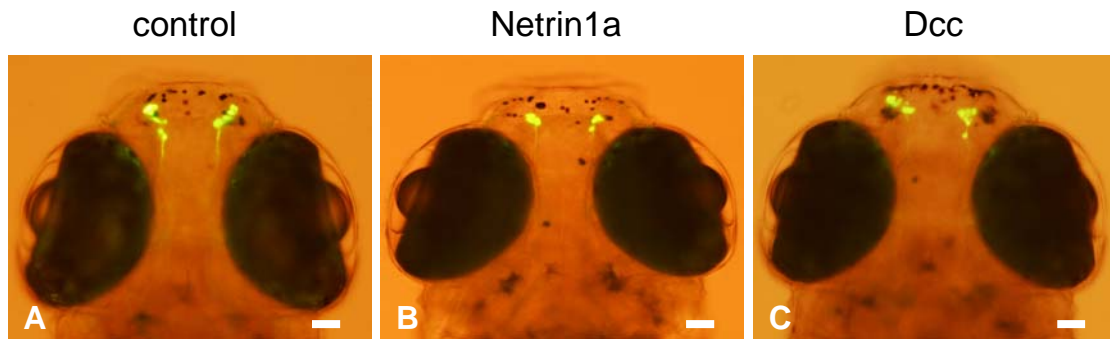
Figure 17.

Images of GnRH3 soma migration in MO-injected larvae at 8 dpf. (A-C) Ventral view of Tg(GnRH3:EGFP) at 8 dpf . (A) larvae injected with control MO (1 mM). (B) larvae injected with Netrin1a MO (0.6 mM). (C). larvae injected with Dcc MO (0.6 mM). Scale bars: 50 µm.

Netrin1a and Dcc knock-down disrupt GnRH3 fiber development

As shown in Chapter 2, during GnRH3 fiber development a subset of fibers extend along the optic tract, crossing at the optic chiasm. In doing this, these fibers cross the midline and innervate the retina. Following knock-down of Netrin1a, Dcc and Unc5b in Tg(GnRH3:EGFP) larvae, development of GnRH3 fibers was evaluated. In both Netrin1a and Dcc knock-down larvae, GnRH3 fibers developed normally along the optic tract until they reached the optic chiasm at the midline. At this point, GnRH3 fibers failed to form a normal chiasm and innervate the retina (Fig. 18B,C). In control MO-injected larvae (Fig. 18A), as well as in unc5b knock-down larvae (not shown) crossing of the midline and innervation of the retina occurred normally.

In addition to disruption of the retina innervation, larvae in which Netrin1a and Dcc were knock-down exhibited abnormal development of fibers in the olfactory region. GnRH3 fibers that extend towards the midline to form commissures in the pallium and subpallium, thus connecting the two GnRH3 clusters, were both abnormal. These commissures developed multiple branching points and extended over a wide area (Fig. 19).
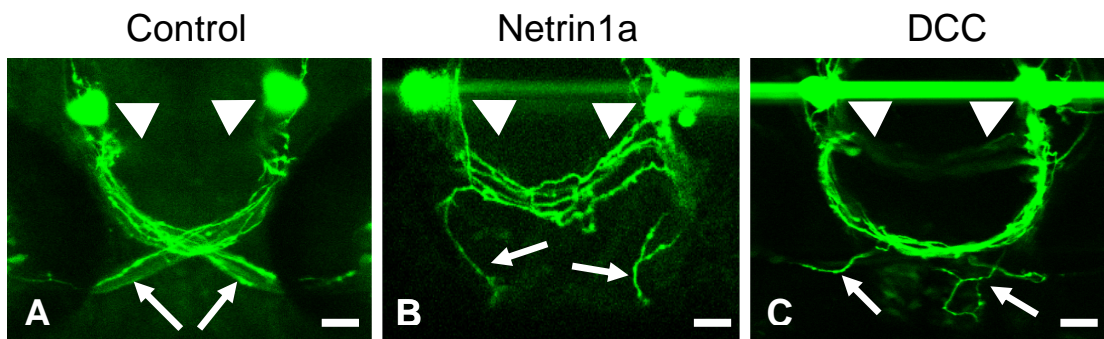
Figure 18.
Innervation of the retina via the optic chiasm is disrupted when Netrin1a and Dcc are knock-down. (A,B,C) Confocal ventral view of 2 dpf Tg(GnRH3:EGFP) larvae. Arrowheads indicate the bilateral GnRH3 soma clusters. (A) control MO-injected larvae. Arrows showing normal bilateral innervation of the retina via the olfactory chiasm. (B,C) Netrin1a and Dcc MO-injected larvae. GnRH3 fibers meet at the midline but fail to form a normal chiasm and to innervate the retina, instead extending in abnormal directions (arrows). Scale bars: 20 µm.
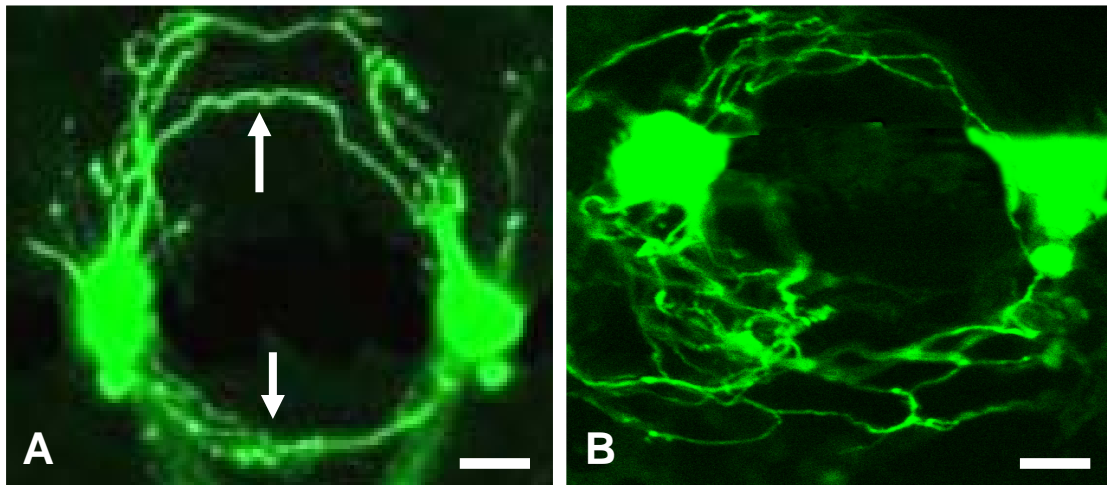
Figure 19.

Formation of the pallium and subpallium commissures between the GnRH3 neuron clusters is disrupted when Netrin1a and Dcc are knock-down. (A,B) Confocal frontal view of 2 dpf Tg(GnRH3:EGFP) larvae. (A) Control MO-injected larvae. Arrows indicate normal commissures in pallium and subpallium. (B) In Netrin1a-injected larvae commissures fibers are in disarray. Scale bars: 20 µm.

Contrary to the GnRH3 fiber abnormalities that were observed in the olfactory region and in the optic tract, both of which require fibers to extend towards and cross the midline, fibers extending in a posterior direction towards the hypothalamus developed normally in Netrin1a and Dcc knock-down larvae (Fig. 20).
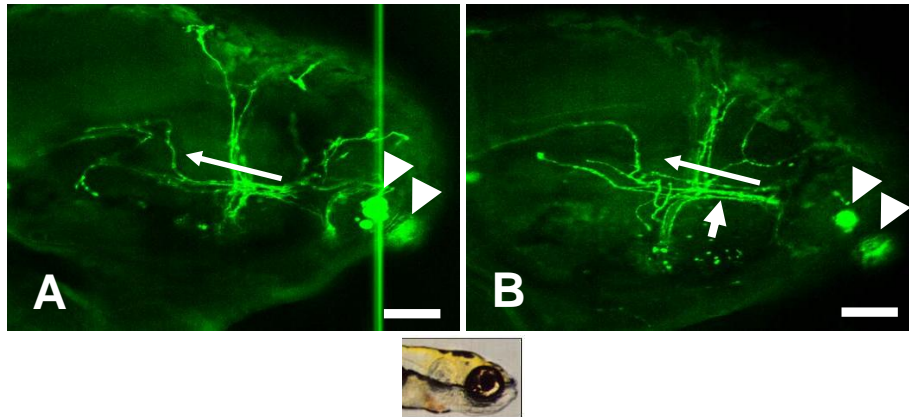
Figure 20.

knock-down of Netrin1a does not effect development of fiber projections towards the hypothalamus. (A, B) Confocal lateral view 2 dpf (eye removed). Arrowheads indicate GnRH3 soma clusters. Arrows above main GnRH3 hypothalamic fiber tract indicate location and posterior directionality of this tract into the hypothalamus. (A) Control MO-injected larvae (B) Netrin1a MO-injected larvae. Small Figure below of ZF larvae represents orientation as shown in A and B. Scale bars: 50 µm.

In conclusion, all GnRH3 fibers that extend during early development towards or across the midline, are affected by Netrin1a and Dcc knock-down, but not by Unc5b knock-down. This effect is in agreement with Netrin1a distribution, which at 36 hpf is found exclusively along the midline and apposed to the optic tract into the eyes. It is also in agreement with the distribution of Dcc that is found in the olfactory region and along the lateral aspects of the telencephalon and hypothalamus at the same areas from which the relevant GnRH3 fiber tracts extend toward the midline (Fig. 21).
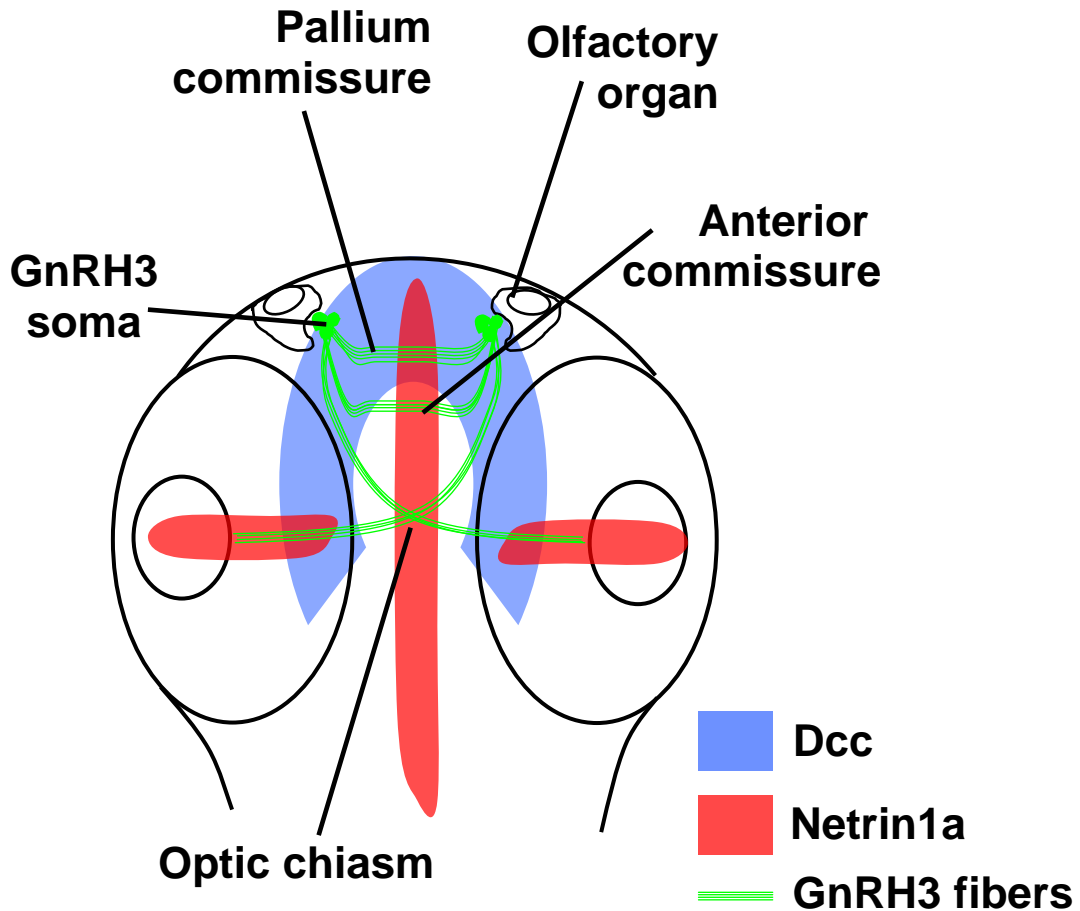
Figure 21.

Schematic of a 36 hpf Tg(GnRH3:EGFP) non-treated larvae. Netrin1a and Dcc expression location and the GnRH3 fiber tracts that are influenced by Netrin1a and Dcc knock-down are depicted. Netrin1a (red) is expressed along the midline and along the optic tract (Park et al., 2005), while Dcc is expressed in the olfactory region and along the lateral telencephalon and hypothalamus (Fricke and Chien, 2005). All three depicted fiber tracts (subpallium commissure, anterior commissure and optic tract), in addition to the pallium tract, are disrupted by Netrin1a and Dcc knock-down.

Validation of Netrin1a and Unc5b knock-down

The efficacy and specificity of Netrin1a and Unc5b anti-splicing MOs

was validated at 1 though 5 dpf at 24 hour intervals by conducting semi-quantitative

RT-PCR (Fig. 22). At all time points, normal length Netrin1a mRNA

was absent in Netrin1a MO-injected larvae. In Unc5b MO-injected larvae, normal

length mRNA was absent in days 1,2,3 and a dramatically reduced amount of normal

length mRNA was observed in days 4,5. This result suggests that the half-life of the

Unc5b MO is shorter and some normal Unc5b transcripts are processed from 4 dpf

and on. Given these results, efficacy of Netrin1a and Unc5b MO was very high.

The specificity of Netrin1a was also validated by the fact that GnRH3 phenotypes

observed in Netrin1a MO-injected larvae were also observed in Dcc MO-injected

larvae. Since Dcc is the receptor relevant to Netrin1a-mediated attraction, their

similar knock-down effect, and the lack of similar results when conducting Unc5b

knock-down, validates the specificity of Netrin1a and Dcc. Control MO had no effect

on gross or GnRH3-specific morphology, nor did it effect Netrin1a or Unc5b

transcript processing. In all cases, only larvae that exhibited normal CNS and trunk

morphology post-injection were used for further evaluation of GnRH3 development.
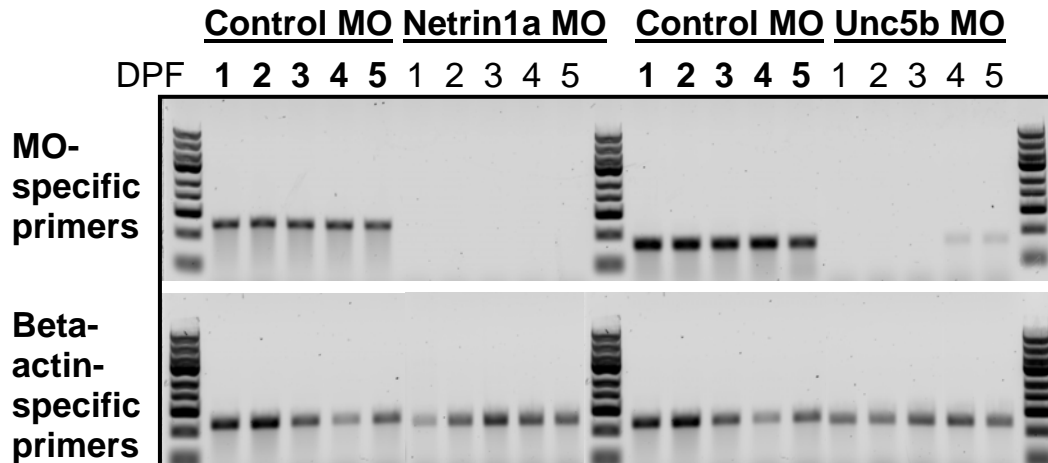
Figure 22.

Validation of Netrin1a and Unc5b MO efficacy by semi-quantitative reverse-transcriptase polymerase chain reaction. Control MO-injected and Netrin1a or Unc5b MO-injected larvae were collected at 1,2,3,4,5 dpf. Each sample included 20 larvae from which mRNA was extracted and used for producing first-strand cDNA. cDNA was PCR-amplified with specific primers for Netrin1a, Unc5b or the control beta-actin. Results show that Netrin1a MO was effective in preventing proper Netrin1a mRNA processing at all time points. Following Unc5b MO injection, normal Unc5b mRNA processing was absent in days 1,2,3 and severely decreased in days 4,5. At all timepoints beta-actin mRNA was processed normally.

Pineal-specific over expression of Netrin1a attracts GnRH3 fibers

To evaluate the role of Netrin1a as a factor involved in GnRH3 migration, Netrin1a was specifically over-expressed in the ZF pineal gland. This over-expression was conducted either with or without knock-down of normal *in vivo* expression of Netrin1a.

The aanat2:Netrin1a:PRDM vector (Fig. 23) includes 3 elements (i) the pineal specific promoter for arylalkylamine *N*-acetyltransferase (aanat2), the key enzyme in

the melatonin production pathway that is specifically expressed in the pineal gland,

(ii) the mRNA coding sequence for Netrin1a, and (iii) a pineal-restrictive downstream

module (PRDM) that acts as a repressor for non-pineal expression and as a pineal-

specific enhancer. Use of the aanat2:EGFP:PRDM construct results in a high

percentage (>80%) of pinea-specific, non-ectopic, transient expression (Appelbaum et

al., 2004).

Figure 23.

Pineal-specific Netrin1a expression construct. Consists of a 1.6 kb upstream aanat2
promoter, mRNA coding sequence for netrin1a, and a pineal-restrictive downstream
module.

In examining Tg(GnRH3:EGFP) larvae, we observed that there is a subset of

soma that form a tract towards the pineal gland. The link between GnRH and the

pineal has been previously suggested in a study that showed a close topographical

relationship between the pineal projections and midbrain GnRH-immunoreactive cells

(Mandado et al., 2001).

To validate the effectiveness and specific location of the pineal expression

construct, the EGFP expressing construct (aanat2:EGFP:PRDM) was injected into

WT embryos. Three days post-injection EGFP was specifically expressed in the

pineal region (Fig. 24A,B), indicating effectiveness of the aanat2 promoter.

When Tg(GnRH3:EGFP) fish were injected with the pineal-Netrin1a construct

(aanat2:Netrin1a:PRDM), there was a distinct increase in the amount of GnRH3

fibers that projected towards the pineal gland area when compared with control larvae

(Fig. 24C,D,E). This increase in GnRH3 innervation of the pineal region was

observed in larvae in which the pineal-Netrin1a specific construct was injected

regardless of co-injection with Netrin1a MO, and no difference was observed

between these two experiments.
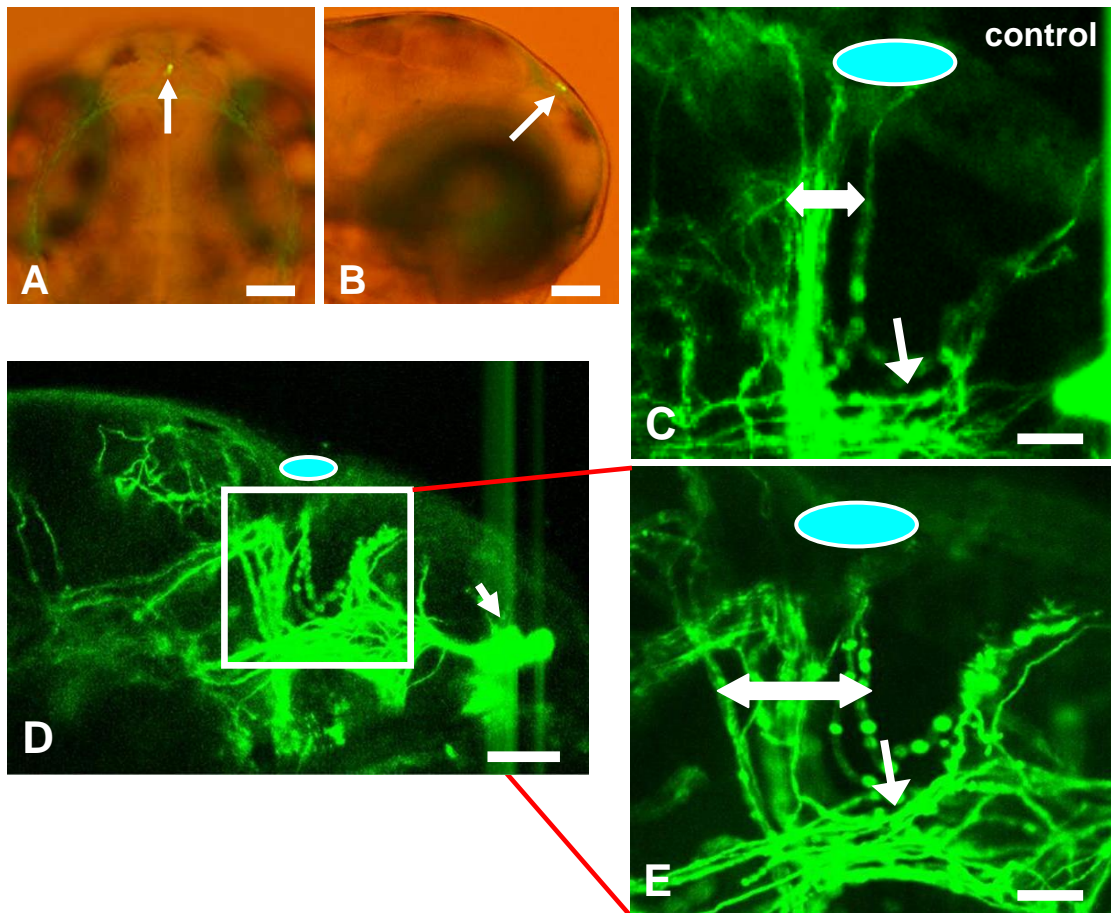
Figure 24.

Induced expression of Netrin1a in the pineal gland attracts GnRH3 fibers.
(A,B) 3 dpf larvae injected with aanat2:EGFP:PRDM construct: arrows indicate
EGFP pineal-specific expression. (A) dorsal view (B) lateral view (light microscope).
(D) lateral confocal view (anterior towards the right, dorsal towards the top, of 4 dpf
larvae injected with the aanat2:Netrin1a:PRDM construct. Square indicates borders of

enlarged area to the right (E). Arrow indicates GnRH3 soma: blue oval represents location of pineal gland. (E) enlargement of square area in (D): oval represents pineal gland, arrow indicates main fiber tract from olfactory area to the hypothalamus, two-headed arrow indicates enlarged fiber tract targeting the pineal area. (C) control 4 dpf Tg(GnRH3:EGFP) larvae, image of the exact same CNS area as shown in (E), arrow indicates main fiber tract from olfactory area to the hypothalamus, two-headed arrow indicates normal fiber tract targeting the pineal area.

Scale bars: (A,B) 50 µm; (C) 25 µm; (D,E) 10 µm.

Netrin2 is an important factor during embryogenesis

Netrin2 was hypothesized to have a specific effect on GnRH3 system development; this was based on the role of Netrin1a and on the olfactory/telencephalic location of Netrin2 transcripts at 48 hpf (Park et al., 2005). To test this, Netrin2 was knock-down using an AUG targeting Netrin2 MO. Embryos injected with Netrin2 MO developed normally up to the 22-26 somite stage (18-24 hpf), at this time point embryonic development was arrested and shortly thereafter all embryos died. In embryos that reached the most advanced stage (24 hpf), the first expression of EGFP by GnRH3 soma in the olfactory region could be observed (Fig. 25). This early EGFP expression indicated that location and development of GnRH3 neurons was normal for this developmental stage. To alleviate the concern that this effect is caused by general Netrin2 MO toxicity, multiple concentrations of Netrin2 were used (0.1, 0.2, 0.4, 0.6 µM), all concentrations yielded the same effect.

Figure 25.

Netrin2 knock-down results in arrested development and death. (A) lateral view of 24 hpf larvae injected with Netrin2 MO. Netrin2 knock-down resulted in arrested development at 18-24 hpf and subsequent death. Arrow indicates early expression of EGFP in GnRH3 neurons. Scale bar: 100 µm

Shha does not specifically effect GnRH3 development

To examine the possible role of Shha as a factor that is involved in GnRH3 soma or fiber development two models were used: (i) homozygous smo mutants that were also Tg(GnRH3:EGFP) larvae, and (ii) cyclopamine immersions at various time points.

In smo mutants, highly abnormal developmental morphology was observed. These larvae exhibited trunk abnormalities that included heart edema, curled tail, decreased trunk width, as well as eye and CNS abnormalities. The eyes merged at the

midline and the olfactory region was non-existent (Fig. 26A,B). As expected, the lack of an olfactory region conferred a lack of GnRH neurons and fibers. No EGFP olfactory or CNS expression was observed in these mutants (Fig. 26C). Trigeminal ganglia GnRH cells did however appear to be normal and expressed EGFP (Fig. 26,D).

Exposure of Tg(GnRH3:EGFP) larvae to cyclopamine at different time points resulted in differential effects. Early exposure, at 8-24 hpf, resulted in severe abnormalities that were highly similar to abnormalities observed in the smo mutants (Fig. 27). These abnormalities included malformation of CNS, small head, eyes that were fused at the midline, decreased trunk width, deformed tail and a missing olfactory region (Fig. 27A,B,C). Contrary to the smo mutant, in this case there was an absence of all tissue where the olfactory region would normally be located (Fig. 27A). Lack of the olfactory region conferred lack of GnRH3 neurons and therefore EGFP expression (Fig. 27B). Exposure of larvae at the 24-48 hpf timeframe, post-major embryogenesis events, resulted in some abnormalities including less severe CNS deformities and small eyes. However, no apparent abnormality of GnRH3 location or migration was observed (Fig. 27D). An even later exposure of larvae to cyclopamine, at the 48-72 hpf timeframe, resulted in almost no general abnormalities and no disruption of GnRH3 location or migration (Fig. 27E).
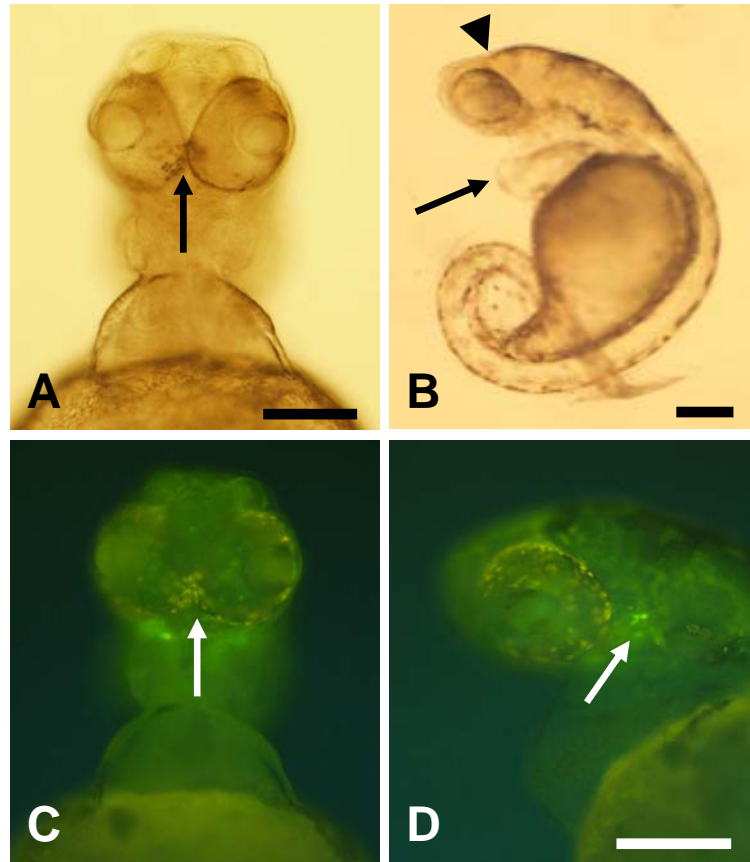
Figure 26.

Blocking the Shha pathway eliminates olfactory GnRH3 neuron development.
(A,B,C,D) Tg(GnRH3:EGFP) smo mutant larvae at 2 dpf. (A,C) ventral view (B,D)
lateral view. (A) bright light image of larvae. smo effects include reduced head and
CNS size, fusion of the eyes at the midline and lack of olfactory region (arrow). (B)
smo effects include abnormal CNS development (arrowhead), severe heart edema
(arrow) and curled tail. (C) epiflorescent image shows lack of any GnRH3 neurons in
the area that should include the olfactory region (arrow). (D) ventral view showing
correct location of GnRH3 cells in the trigeminal ganglia (arrow).
Scale bars: 100 µm.
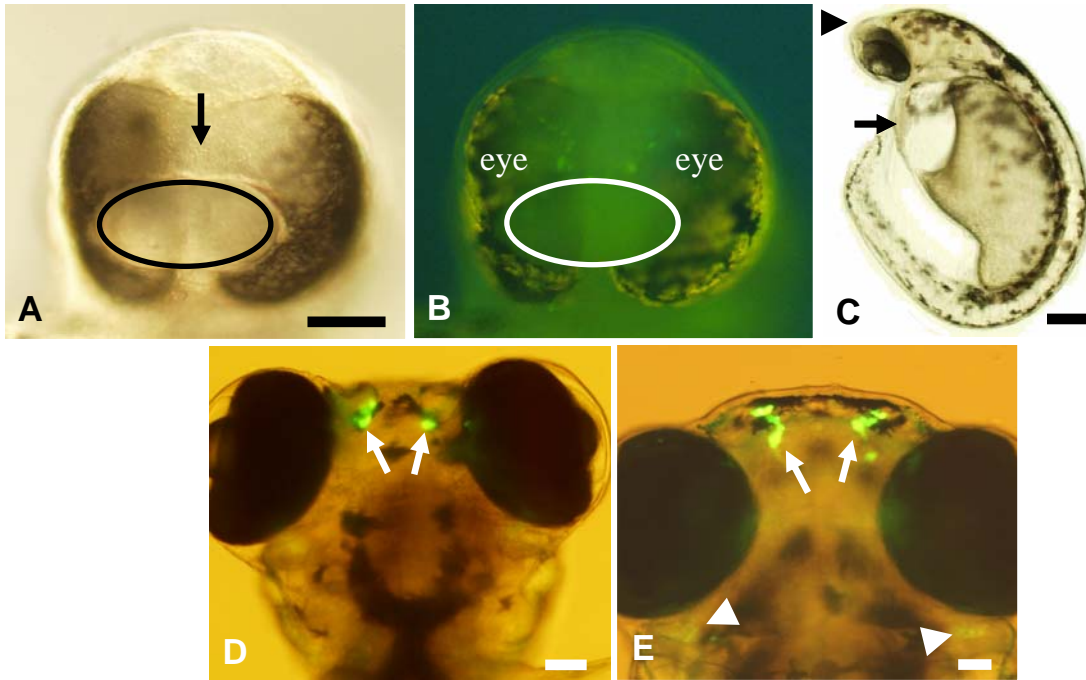
Figure 27.

Blocking the Shha pathway has temporal-specific effects on GnRH3 neurons.

(A) ventral view of 2 dpf larvae treated with cyclopamine 8-24 hpf. Eyes are fused at the midline (arrow) and missing olfactory region (oval circle).

(B) epifluorescent image of A. Oval indicates olfactory region: no GnRH3 neurons are visible. (C) lateral view of 2 dpf larvae treated with cyclopamine 8-24 hpf. Abnormalities include small and deformed CNS (arrowhead), heart edema (arrow), and deformed trunk and tail. (D) dorsal view of 3 dpf larvae exposed to cyclopamine 24-48 hpf, CNS and eye abnormalities are seen, however no significant GnRH3 neuronal abnormalities exist (arrows) (E) dorsal view of 6 dpf larvae exposed to cyclopamine 48-72 hpf. Larval development is normal, GnRH3 neuronal location and migration is also normal (arrows). Normal EGFP expression by GnRH3 cells is evident in the trigeminal ganglia (arrowheads).

Scale bars: (A) 100 µm; (C, D) 50 µm.

Discussion

Involvement of Netrin1a and Dcc in GnRH3 soma development

Netrin1a is an important attractant and/or repellant that is involved in various aspects of CNS development. Among its roles are attraction of commissural fibers towards or away from the midline, as well chemoattraction for tangential soma migration (Alcantara et al., 2000; Kawasaki et al., 2006). The role of Netrin1a in the context of forebrain GnRH neurons appears to be along the same lines, both affecting fiber development and soma location and migration. This has been demonstrated in several studies in mammals in which Netrin1 and Dcc-deficient animals have aberrant GnRH soma location (Schwarting et al., 2001; Schwarting et al., 2004). From current information, it is difficult to say whether Netrin1a affects GnRH neurons directly or acts via disrupting the targeting of the olfactory/vomeronasal nerves. In addition, there is no data regarding the activity of olfactory-expressed Netrin1a at early stages of GnRH development in the nasal area.

The first effect observed in Netrin1a and Dcc knock-down Tg(GnRH3:EGFP) larvae, was abnormal location of GnRH3 soma in the olfactory area at 1-2 dpf. GnRH3 soma dispersion was significantly wider and more scattered within the olfactory region in Netrin1a and Dcc knock-down larvae when compared with non-treated Tg larvae, control MO and Unc5b knock-down larvae. Although we did not observe overt olfactory morphological abnormalities in Netrin1a and Dcc knock-down larvae, the GnRH3 neuron dispersed location phenotype is likely a result of subtle disruption to the normal development of the olfactory region, rather than a GnRH3-specific effect. Both Netrin1 and DCC are expressed during development in

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the olfactory system in mice and rats (Astic et al., 2002; Schwarting et al., 2004; Tsim et al., 2004), and both Netrin1a and Dcc are strongly expressed in the olfactory region and CNS in 24 hpf ZF (Fricke and Chien, 2005; Park et al., 2005). Interestingly, Netrin1, can also act as a putative tumor suppressor controlling cell death commitment (Thiebault et al., 2003). Thus, Netrin1a may be acting in the olfactory region both as a guidance molecule and as a survival factor that controls cell fate and olfactory area development. This hypothesis is in agreement with our finding that Netrin1a and Dcc knock-down effect early GnRH3 cell distribution in the olfactory region. The finding that in Unc5b knock-down larvae no abnormal GnRH3 neuron distribution was observed, suggests that this role of Netrin1a is mediated by the Dcc receptor.

At later time points, once GnRH3 soma begin to migrate, at approximately 3 dpf, Netrin1a and Dcc knock-down have a significant effect on the migration process. In both Netrin1a and Dcc knock-down larvae, GnRH3 soma migration was significantly disrupted when compared to control MO and Unc5b knock-down. This disruption was manifested by a reduced distance migrated by the GnRH3 neurons. In Netrin1a and Dcc knock-down animals GnRH3 soma were located at 8 dpf in the olfactory area and the TN, while in control and Unc5b knock-down larvae, GnRH3 soma reached and entered the telencephalon. The average distance traveled by the GnRH soma was approximately double in control and Unc5b MO-injected larvae. As discussed, GnRH3 neurons and fibers migrate during development apposed to the olfactory/vomeronasal nerves. It is hypothesized that these nerves are essential for normal GnRH3 soma migration and fiber-targeting. It is also known that vomeronasal

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neurons exhibit Dcc immunoreactivity throughout natal development in rats and mice (Astic et al., 2002). In Netrin1 and in Dcc-deficient mice the majority of vomeronasal fibers fail to make their characteristic ventral turn into the forebrain and instead project to the cerebral cortex; the same is true for a large percentage of GnRH3 soma as well as fibers, strengthening the hypothesis that the vomeronasal nerve mediates GnRH migration (Schwarting et al., 2001; Schwarting et al., 2004). Given this finding, it is also possible that, in ZF, the abnormal migration of the GnRH3 soma seen in Netrin1a and Dcc knock-down larvae is a result of olfactory/vomeronasal nerve abnormalities. To clarify whether the abnormal GnRH3 soma migration is mediated directly by Netrin1a or is a result of abnormalities in olfactory/vomeronasal nerve development, we tracked the main projection of GnRH3 fibers towards the hypothalamus that are apposed to the olfactory/vomeronasal nerves. This GnRH3 tract was found to be normal in Netrin1a, Dcc, Unc5b and control MO larvae, suggesting normal olfactory/vomeronasal nerve development and thus a direct effect of Netrin1a/DCC on GnRH3 neurons that is not mediated by olfactory/vomeronasal nerve development.

The finding that in Netrin1a knock-down animals GnRH3 soma migration is curtailed and restricted to the olfactory area and TN is therefore somewhat different from findings in mammals. In mammals, GnRH soma migrate dorsocaudally to the cribriform plate and then sharply turn in a ventral direction towards in forebrain. In mice, Netrin1 and Dcc deficiency results in the inability of GnRH3 soma to turn ventrally into the forebrain, instead continuing and migrating into the cortex. However, in ZF, the GnRH3 fiber and soma trajectory is more level and the ventral

turn into the forebrain is much less pronounced. This is probably due to the difference

in brain architecture between mammals and fish. Compared with mammals, in fish

the brain is more elongated in an anterior-posterior directionality (Thomas Mueller,

2005) causing the soma path to remain in the horizontal plane. This CNS architectural

difference may cause Netrin1a expression in the fish forebrain to act as an attractant

for GnRH3 soma while they are still in the olfactory/TN area. This is different from

mammals, in which GnRH-specific Netrin1 attraction is only relevant once the soma

reach the cribriform plate and turn ventrally. Hence the result of Netrin1/Dcc

deficiency in mammals is projection of the GnRH3 soma past the ventral turn and

into the cortex, while in ZF, the same deficiency retards GnRH3 soma migration as

they are exiting the olfactory region.

Netrin1a and Dcc affect early GnRH3 fiber development

The role of Netrin1a in fiber development was limited to two subsets of fiber

tracts. One is the GnRH3 fibers that form commissures between the two GnRH3

soma clusters. These commissures extend from the olfactory area to the pallium and

subpallium. The second is the fiber tract that is parallel to the optic tract and

innervates the retina. In regard to the commissures, the general development of these

fibers was abnormal, resulting in a high degree of branching and abnormal targeting.

The GnRH3 fibers apposed to the optic tract developed normally until reaching the

optic chiasm, at which point the fibers did not continue into the retina but rather

pointed in other random directions. No abnormalities to other GnRH3 fiber tracts,

including the main tract into the hypothalamus, were observed. Interestingly, the

GnRH3 commissures and the tract innervating the retina are the only GnRH3 fiber

tracts that reach or cross the midline. Therefore abnormalities in these tracts are in

agreement with the role of Netrin1a as a factor that is involved in attraction of fibers

towards the midline in multiple species, including ZF (Brankatschk and Dickson,

2006; Suli et al., 2006). The expression pattern of both Netrin1a and Dcc at 36 and 48

hpf in ZF corroborates our results. Netrin1a localizes at 36 hpf specifically to the

midline and to projections from the midline into the eyes (Park et al., 2005), and Dcc

is robustly expressed in the olfactory area and lateral aspects of the telencephalon and

hypothalamus from 24-48 hpf (Fricke and Chien, 2005). This Netrin1a and Dcc

distribution correlates specifically to the commissure and optic chiasm abnormalities

that we observed. These findings suggest that one of the roles of olfactory Netrin1a

expression is to attract Dcc-expressing GnRH3 fibers towards and across the midline.

Given these results, it seems that olfactory Netrin1a expression, as opposed to

forebrain Netrin1a expression, specifically affects GnRH3 fiber development in the

olfactory area and telencephalon, but not the GnRH3 fiber tract that innervates the

POA-hypothalamus/pituitary.

The general attraction of GnRH3 fibers to Netrin1a is best shown by our

findings from Netrin1a over-expressed in the pineal gland. This specific over-

expression mediated enhanced GnRH3 fiber-targeting to the pineal area. In untreated

Tg animals there is a tract of GnRH3 fibers that extends toward the pineal region;

however, this tract was significantly more robust in larvae in which Netrin1a was

over-expressed in the pineal. To reach the pineal area, which is at the midline,

GnRH3 fibers must be attracted towards the midline. Indeed, as discussed, this

attraction seems to be mediated by Netrin1a. This result, coupled with the knock-down findings, establish that Netrin1a has a specific role in mediating GnRH3 fiber attraction to the midline and, as mentioned earlier, is in agreement with the distribution of Netrin1a and Dcc in the olfactory area and along the midline (Park et al., 2005; Schwarting et al., 2001; Schwarting et al., 2004). In ZF this fiber-specific role seems to be limited to fiber development in the olfactory region and telencephalon, but not to fibers that extend into the hypothalamus. It is certainly possible that GnRH3 fiber growth cones in the olfactory region express Dcc, while GnRH3 growth cones in other regions may not. This is an interesting topic for further examination.

Netrin2 expression is essential for embryogenesis

Netrin2 knock-down resulted in arrested embryonic development and death of all embryos. The arrested development occurred between 18-24 hpf, which is at the end of the segmentation period and the beginning of the pharyngula period (Kimmel et al., 1995). At this time, netrin2 is expressed in the hindbrain, especially in rhombomere 4, as well as in ventral somites in the tail. Expression is also evident in a small cluster of cells in the developing otic vesicle. Earlier, during somitogenesis Netrin2 is highly expressed in the notochord and developing somites (Park et al., 2005). These early locations of netrin2 expression suggest that it plays a role during embryo development, a role that may be crucial in ZF. Hence the lack of Netrin2 induces fatal developmental abnormalities. Phylogenetic analysis revealed that ZF netrin2 is similar to chick netrin3, mouse netrin3 and human netrin2-like.

Interestingly, mouse netrin3 is expressed in sensory ganglia, mesenchymal cells, and muscles during development but not in the CNS. Moreover, it is ineffective in influencing commissural fiber outgrowth in vitro (PuschelPuschel, 1999; Wang et al., 1999). Another interesting finding is that mouse Netrin-3 promotes myotubule formation and myogenic differentiation (Kang et al., 2004). These findings, which are also in line with our results, suggest a developmental role for mouse Netrin-3/ZF Netrin2 that is not related to CNS development.

Taken together, the findings regarding the expression pattern of ZF netrin2 and its homologues in other species, in addition to the findings described herein, indicate that Netrin2 has an important non-CNS, non-GnRH3, role during mid-late embryogenesis that is crucial for the completion of embryogenesis and survival. In the context of GnRH3 neuronal development, this conclusion is strengthened by the fact that GnRH3 development at very early stages was normal in Netrin2 knock-down larvae. Additional experimentation into the role of ZF Netrin2 during embryogenesis, preferably by conditional knock-down at later developmental time points, will be no doubt yield interesting and useful information.

Shha does not mediate GnRH3 neuronal development

Shha is a morphogen that is critical in metazoan embryonic development. However, Shha protein has the capacity to switch during development from a morphogen to a floor plate derived chemoattractant affecting commissural fiber and neuronal guidance (Charron et al., 2003; Charron and Tessier-Lavigne, 2005; Varga et al., 2001). In this regard, various aspects of Shha activity are similar to Netrin1

activity and Shha is hypothesized to collaborate with Netrin1 in mediating fiber-targeting (Charron et al., 2003). For these reasons we were interested in examining the role of Shha on GnRH3 neuronal development.

In this study we used two methods to block Shha activity. One is immersion of larvae in cyclopamine, a compound that blocks activation of the Shha response pathway by directly binding to the Smoothened G protein-coupled receptor (smo), and therefore affects the balance between active and inactive smo (Chen et al., 2002b; Taipale et al., 2000). Blocking or mutating this receptor, which is part of the Shha receptor complex (Fig. 28) prevents binding of ptc, which is activated by Shh and thus disrupts the Shh pathway (Barresi et al., 2000; Varga et al., 2001). The second method was the use of the $smo^{b641/b641}$ mutant ZF. In this mutant the smo receptor is inactivated, thus severely disrupting the Shha pathway (Fig. 27, 28).

Blocking Shha activity at early time points, while Shha is acting in its capacity as a morphogen, causes severe morphological abnormalities. We were interested in blocking Shha at these early time points, as this results in abnormal hypothalamic development and in formation of an ectopic lens in place of the pituitary (Sbrogna et al., 2003). Early disruption of the Shha pathway in Tg(GnRH3:EGFP) ZF, both by cyclopamine and in the smo mutant, induced CNS abnormalities. However, blocking Shha also abolished formation of the olfactory region. As expected in the context of the GnRH3 neurons, this resulted in a total lack of GnRH3 neuron immergence and development.

Shh signaling cascade
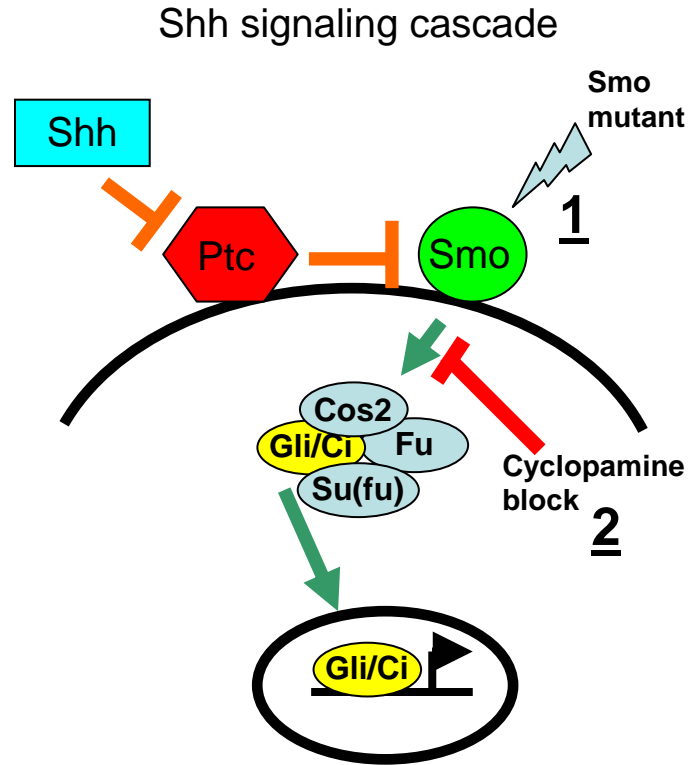
Figure 28.

Diagram of the Shh signaling pathway and the points at which Shh was blocked in this study. 1. smo receptor is mutated in the smo ZF line. 2. smo activity is blocked via cyclopamine immersion. Modified from Charron (2005).

Blocking the Shha pathway by cyclopamine immersion between 24-48 hpf resulted in mild morphological abnormalities but no apparent abnormalities to GnRH3 soma or fiber development, while cyclopamine immersion between 48-72 hpf resulted in no apparent morphological or GnRH3-specific abnormalities. These results show that while Shha activity as a morphogen has a severe but indirect effect on GnRH3 neurons, the activity of Shha as a chemoattractant at later time points has no role in GnRH3 system development. Although fibers are attracted to the midline by the combined chemoattractant effects of Netrin1 and Shh (Charron and

Tessier-Lavigne, 2005), this does not seem to be the case for all midline targeting fibers, GnRH3 fibers included. With regard to GnRH3 neurons, activity of Netrin1a seems to be sufficient for attracting GnRH3 fibers that extend towards or cross the midline.

Summary

In this chapter, we aimed to examine the spatiotemporal function of Netrins and Shha in the development of the forebrain GnRH system. For this purpose, use of the Tg(GnRH3:EGFP) line proved once again to be invaluable. The combination of the ability to knock-down, over-express and disrupt these factors, coupled with the ability to visualize the details of GnRH3 system development, were highly beneficial.

Our findings in regard to Netrin1a and its receptor Dcc suggest two interesting roles for Netrin1a in GnRH3 system development. The first role seems to be mediated by netrin1a that is expressed during early development in the olfactory area and optic nerve (Park et al., 2005; Schwarting et al., 2001; Schwarting et al., 2004). Netrin expression in these regions serves as a GnRH3 fiber attractant that mediates fiber extension to the midline, resulting in formation of commissures and fiber crossing of the midline to innervate the retina. This role of Netrin1a as a midline attractant for GnRH3 fibers, is strengthened by the fact that over-expression of Netrin1a in the pineal gland, which is situated at the midline, mediated enhanced attraction of GnRH3 fibers to the pineal area.

The second role seems to be mediated by Netrin1a that is expressed in the forebrain. This Netrin1a expression results in attraction of GnRH3 soma from the olfactory/TN area towards the hypothalamus. In this case, there seems to be a

difference between mammalian models and ZF. While in mammals, lack of Netrin1 or Dcc cause GnRH3 soma to pass the ventral turn into the forebrain and continue migrating into the cortex, in ZF this deficiency prevents GnRH3 soma from exiting the olfactory/TN area altogether. In mice, it is proposed that this soma location abnormality is caused by inappropriate targeting of the vomeronasal nerve (Schwarting et al., 2001; Schwarting et al., 2004). However, while affecting GnRH3 soma development in ZF, Netrin1a does not seem to affect GnRH3 fibers that are extending posteriorly along the vomeronasal nerve towards the telencephalon and hypothalamus. This suggests that Netrin1a expression in the forebrain does not mediate attraction of the vomeronasal nerve in ZF, but is rather a GnRH3 soma-specific attractant. The different consequence of Netrin1 knock-down/knock-out on GnRH3 soma distribution in mice and ZF may be in part a result of different brain architecture between mammals and fish. It is also important to note that the effects of Netrin1a and Dcc knock-down were identical, but we did not see any effect when Unc5b was knock-down. This suggests that GnRH3-specific Netrin1a activity is mediated by the attractive properties of Netrin1a-Dcc and that the general repulsive role of Netrin1a-Unc5b as described in mice (Schwarting et al., 2004) has no significant forebrain GnRH developmental role in ZF or mice.

Findings regarding Netrin2 suggest that this factor is crucial during the later stages of embryogenesis, possibly at the transition phase in which the ZF embryo switches from use of maternal transcripts to zygotic gene transcription. Based on the location of Netrin2 mRNA at later time points, this factor may indeed be involved in

GnRH3 development. However, to examine this, conditional knock-down at later time points of development are required.

Lastly, it appears that Shha, in its capacity as a morphogen has a severe indirect effect on GnRH3 neurons, however does not have a specific GnRH3 neuron development role in its later capacity as a chemoattractant.

These findings shed additional light on the complex development of the GnRH3 system. Although the main factors that are involved in GnRH3 development in mammals and fish seem to be conserved, our data points to some subtle variations between these two models in terms of the specific spatiotemporal role that various factors play.

Chapter 5: GnRH3 SOMA ABLATION

Abstract

Hypophysiotropic GnRH neurons are located in the preoptic area (POA) and ventral hypothalamus in sexually mature vertebrates. In mammals, these neurons originate in the olfactory region. However, in fish, the origin of the hypothalamic GnRH population is still unclear. Using the Tg(GnRH:EGFP) zebrafish line, in which GnRH3 neurons specifically express EGFP, GnRH3 neurons in the olfactory region were individually ablated during early development. The specific ablation of GnRH3 neurons was conducted via targeted laser pulses. An additional ablation method based on a toxin-delivering construct was attempted but was not effective, suggesting ineffectiveness of this method when attempting to ablate cells within the olfactory region/CNS. Post-ablation, GnRH3 neurons exhibited the ability to regenerate in the olfactory region, however, this capacity was time-dependant. When ablation took place at 2 dpf, high regeneration rates were observed, however regeneration ability significantly decreased when ablation took place at 4 or 6 dpf. This finding suggests a limited timeframe capacity for GnRH3 neurogenesis. Successful GnRH3 soma ablation during development resulted in complete lack of olfactory, TN, POA and hypothalamic GnRH3 soma in 12 week old sexually mature animals. Unilateral GnRH3 neuron ablation resulted in normal bilateral fiber extensions, but also unilateral soma location. Mature animals in which successful bilateral ablation of GnRH3 neurons was achieved exhibited arrested oocyte development, and reduced average oocyte diameter. Mature animals in which GnRH3 neurons were partially

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ablated exhibited normal oocyte development, however their fecundity was significantly reduced. These findings show that the POA and ventral hypothalamic hypophysiotropic GnRH populations in zebrafish are GnRH3 neurons that originate in the olfactory region during development. Presence of GnRH3 neurons in the reproductively mature zebrafish is a prerequisite for normal gonadal development and spawning.

Introduction

In teleost species that have two GnRH forms, forebrain GnRH is present in both the POA-hypothalamus, with fibers ending at the pituitary gland, and in the OB-TN (Okuzawa et al., 1990; Yu et al., 1988). In these species, the forebrain GnRH induces gonadotropin-releasing activity and is also thought to assimilate non-redundant functions of the lost (third) GnRH form (Amano et al., 1995; Kobayashi et al., 1997; Okubo and Nagahama, 2008). As is discussed below, this is most likely the situation in zebrafish. Two GnRH forms, GnRH2 and GnRH3, have been identified in the zebrafish brain using HPLC analysis (Powell et al., 1996). Subsequently, two genes encoding these forms of GnRH were isolated and characterized (Torgersen et al., 2002; Steven et al., 2003). It seems that in zebrafish and several other teleosts, the GnRH1 gene was lost during evolution (Kuo et al., 2005; Kah et al., 2007). As in all other investigated jawed vertebrates, in adult zebrafish, GnRH2 is localized to the midbrain tegmentum and GnRH3 is located both in the OB-TN and in the POA-hypothalamus (Steven et al., 2003). The levels of GnRH3 peptide in the adult zebrafish pituitary are 3-4 fold higher then those of GnRH2. Therefore, GnRH3 is

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considered to be the hypophysiotropic form (Powell et al., 1996; Steven et al., 2003). Our detailed investigations in larval stage zebrafish, using whole-mount ISH, a transient promoter-reporter expression system (Palevitch et al., 2007) and the Tg(GnRH3:EGFP) zebrafish line (chapter 2), suggest that hypothalamic GnRH3 neurons emerge from the olfactory region and migrate posteriorly to the hypothalamus. Thus, it seems that OB-TN and POA-hypothalamic GnRH neuronal populations share the same embryonic origin. These findings are in accordance with results in other fish species which have only two molecular GnRH forms. However, they differ from other studies suggesting that zebrafish hypothalamic GnRH neurons have a different origin. According to this hypothesis, the OB-TN GnRH population originates from the cranial neural crest while the POA-hypothalamic population, which reportedly expresses a different form of immunoreactive GnRH, emerges from the adenohypophyseal region of the developing anterior neural plate (Whitlock, 2005, Wu et al., 2006), and thus does not migrate to a final hypothalamic location.

To conclusively establish that POA-hypothalamic hypophysiotropic GnRH neurons originate in the olfactory placode, two approaches can potentially be used. Following the migrational path of specific GnRH soma from their origin in the olfactory region to the hypothalamus is an obvious strategy. This method, however, does not exclude the independent immergence of a separate hypothalamic population. The other approach is to ablate olfactory region GnRH neurons during development. This aim can be achieved be a) ablation of the entire olfactory region during development, b) use of mutant lines in which the olfactory region does not develop normally and c) specific ablation of GnRH neurons during development. The specific

GnRH neuron ablation method is the most precise and minimizes non-specific effects, but has not been implemented to date due to lack of a model that allows GnRH-specific neuron ablation in a live animal. If olfactory region GnRH ablation results in lack of POA-hypothalamic GnRH neurons in mature animals and inability to reproduce, this would provide conclusive evidence that indeed POA-hypothalamic hypophysiotropic GnRH neurons originate in the olfactory region. This working hypothesis was tested in both mouse and chick. In mouse, the Pax-6 mutant displays failed eye and olfactory placode development. When examining this mutant, no GnRH1 neurons were present in any CNS region at various developmental stages (Dellovade et al., 1998). A similar result was obtained using PKR2 mutant mice which display hypoplasia of the OB. These mutants showed absence of GnRH1 neurons in the hypothalamus (Matsumoto et al., 2006). In agreement with these studies was the finding that unilateral ablation of the olfactory placode in chick and newt embryos results in a lack of GnRH neurons on the ablated side (Akutsu et al., 1992; Murakami et al., 1992). However, contrary to these findings, several studies have suggested multiple embryonic origins of forebrain GnRH neurons in chicks (Norgren and Gao, 1994), salamanders (Northcutt and Muske, 1994) and zebrafish (Whitlock, 2005, Wu et al., 2006).

Although bilateral and unilateral ablation of the entire olfactory placode during development has been utilized, this method may well influence development of other CNS regions such as the hypothalamus. Accordingly, the inability to specifically ablate olfactory GnRH neurons has been a limiting factor in determining the origins of the POA-hypothalamic GnRH neurons.

Herein, we capitalized on the fact that in the Tg(GnRH3-EGFP) zebrafish line GnRH3 neurons can be observed during development in the olfactory region of the intact animal. This adventitious characteristic has enabled the precise, targeted ablation of GnRH3 neurons during development. Subsequently, GnRH3-ablated mature animals were studied for existence and location of GnRH3 neurons as well as for gonad morphology and reproductive capacity.

In relation to reproduction capacity, assuming hypophysiotropic GnRH neurons originate in the olfactory region and migrate to the POA-hypothalamus, ablation of these neurons would be expected to result in an effect that is similar to what is seen in the hypogonadal (hpg) mouse. In this model, the GnRH1 gene is mutated and the GnRH decapeptide is inactive, resulting in small ovaries, a lack of early follicular antrum formation and atretic or cystic interstitium (Cattanach et al., 1977). Indeed, similar reproductive system abnormalities are also seen in mouse models in which the OB development is disrupted (Matsumoto et al., 2006). In these mice the ovary is hypoplastic, mostly containing undeveloped follicles that are arrested in the preantral phase. Also similar to the hpg mouse, the interstitium is atrophic.

In this chapter, we describe targeted ablation of GnRH3 neurons in the olfactory region during development. Following ablation, several parameters were assessed, including GnRH3 neuron capacity for regeneration, distribution of GnRH3 neurons in control and GnRH3-ablated mature brains, reproductive capacity and ovarian morphology. Our findings demonstrate that the embryonic origin of

POA-hypothalamic GnRH3 neurons is the olfactory region and that the presence of this olfactory region population during development is necessary for normal gonad development and reproduction.

Materials and methods

Experimental animals

Experimental animals were maintained and treated as specified in Chapter 2. To obtain Tg (GnRH3:EGFP) larvae, F5 and F6 Tg(GnRH-EGFP) adults were crossed with each other or with WT fish. GnRH3- and control-ablated fish were raised to maturity (12-15 weeks post-fertilization) in 10 gallon tanks in accordance with the zebrafish book (Westerfield, 1995). Control-ablated and GnRH3 ablated females were spawned with WT males on three occasions at one week intervals. Embryos were collected, pooled and counted.

Laser ablation

Tg(GnRH3:EGFP) larvae at 2, 4 and 6 dpf were used for laser ablation. Approximately 60-80 Tg larvae of each time point were used for ablation. Ablations targeted two groups of cells

1) GnRH3 neurons in the olfactory region, these neurons were detected and targeted based on their EGFP expression. GnRH3 ablations were conducted either unilaterally or bilaterally

2) control ablations targeted non-EGFP expressing cells located laterally to GnRH3 neurons (not along the subsequent GnRH3 migrational path).

Ablations were conducted using 3-4 bursts of rapid laser pulses directed at each EGFP expressing GnRH3 neuron. The laser used was a Micro Point (Photonic Instruments, Inc., St. Charles, IL) with a 30/70% splitter for UV/laser. Two days and six days post-ablation larvae were assessed for regeneration of GnRH3 neurons as determined by renewed EGFP expression.

nfsB construct

A pTol2-nfsB-GnRH3-EGFP vector was constructed in collaboration with Dr. Y. Gothilf's lab at Tel-Aviv University. This construct mediates expression of the bacterial nitroreductase (NTR) enzyme in GnRH3 neurons This enzyme, in turn, can convert the prodrug metronidazole (Mtz) into a cytotoxic DNA cross-linking agent. Thus immersion of pTol2-nfsB-GnRH3-EGFP transgenic zebrafish in Mtz should cause ablation of GnRH3 neurons. To develop this construct the GnRH3 promoter (see Chapter 2) was inserted into a pTol2-nfsB-insulin-EGFP plasmid, (obtained from Dr. M.J. Parsons, Johns Hopkins University, Baltimore) (Pisharath et al., 2007), replacing the original insulin promoter. The construct was injected at a concentration of 25ng/µl into 250 WT ZF and correct transient expression of EGFP in GnRH3 cells was observed in 3 dpf larvae. Integration of the vector into larval genomic DNA was facilitated by co-injecting transposase mRNA (25ng/µl) with the pTol2-nfsB-GnRH3-EGFP construct (Kawakami, 2007; Kawakami et al., 1998).

P0 mature fish and F1 larvae were screened by finclip (see Chapter 3) or whole larvae DNA extraction, for integration of the construct into genomic DNA, by amplifying a 376 bp region of the nfsB sequence via PCR

Primers:

forward-CCAGCACGGAAGAAGGTAAA

reverse-AAATTCTGCATCGAGGATGG

Correct expression of EGFP in GnRH3 neurons was verified in F1 larvae at 1 and 2 dpf. GnRH3:EGFP positive larvae were immersed in 10mM Mtz (Sigma-Aldrich, St. Louis. MO) at 28° C between 24-48 hpf or 24-72 hpf. Post-immersion larvae were assessed for GnRH3 ablation at 36, 48, 72 and 96 hpf as determined by correct EGFP expression.

Brain observation and cryosections

Reproductively mature (12-14 weeks post-ablation) GnRH3 laser ablated and control laser ablated fish were euthanized using MS-222. Immediately thereafter the entire brain was extracted and placed O/N in 4% paraformaldehyde, then transferred to 30% sucrose-PBS solution for an additional 12 hours. Fixed brains were placed whole under a fluorescent microscope and assessed for GnRH3 neuron location. Brains were subsequently embedded in tissue freezing medium (TBS, Triangle Biomedical Science) in plastic cryomolds. Cryomolds were frozen in liquid nitrogen and placed O/N at -80° C. Frozen blocks were serially sectioned at 20-30 µM using a cryostat (Leica CM3000, Leica Microsystems Inc. Bannockburn, IL), Sections were placed onto glass slides and observed using a Zeiss Axioplan 2 microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) with appropriate filters, an Attoarc HBO100 W power source and an Olympus DP70 digital camera (Olympus, Tokyo, Japan).

Histology

Mature fish from which brains were extracted were also used to collect gonad samples. Ovaries and testis from each fish were collected and placed in 10% buffered formalin. Fixed gonads were dehydrated in a 75-90% ethanol series and embedded in glycol methacrylate plastic (JB-4 Mini Kit, Polysciences, Inc., Warrington PA, USA). Embedded tissues were cut into 5-6 µm serial sections on a microtome (Microm, HM 340, Portsmouth, NH), sections were placed onto glass slides. The sections were then stained with methylene blue/basic fuchsin. Stock solutions of 0.125% methylene blue (Polysciences Inc., Warrington PA) and 0.125% Fuchsin basic (Polysciences Inc.) were prepared with DO water. 12ml of each stock solution was mixed together with 21 ml of PBS and 15ml 95% EtOH. Slides were placed into this working solution for 6-8 min, then removed, washed for 5 min in water, dipped twice in 95% EtOH, washed with water again and dried. Inspection of sections was conducted using a compound microscope at 4-10x magnifications, a Zeiss Axioplan 2 microscope (Carl Zeiss MicroImaging, Inc.) and Olympus DP70 digital camera (Olympus, Tokyo, Japan).

Gonad analysis

Analysis of oocyte stage and diameter in control-ablated and GnRH3 ablated fish was conducted on histology sections. Sections from n>3 female fish from each category (control-ablated, partial GnRH3-ablated and complete GnRH3-ablated) were examined for stage of oocyte development. A random region of ovary measuring

approximately 1000 µM x 1000 µM was selected and oocyte diameters were measured.

Statistics

For statistical analysis one of two methods was employed, either One-Way Analysis of Variance for independent samples followed by Tukey HSD Test or a Two-Tailed T-test for independent samples.

Results

GnRH3 neuron ablation

The Tg(GnRH3:EGFP) line allows *in vivo,* whole animal observation of GnRH3 neurons. We utilized this trait to conduct physical laser ablation of GnRH3 neurons in the olfactory region during development. In all cases laser pulses easily ablated GnRH3 neurons, as evident from cell death that was visualized by immediate loss of all EGFP signal (Fig. 29, 30). The resolution of neuron ablation was high i.e., individual soma could be targeted. Three forms of ablation were conducted , including a) unilateral ablation, b) bilateral ablation, and c) control ablation (Fig. 30). Post-ablation observations did not reveal deleterious effects to surrounding tissues.

Ablations were conducted at three time points, 2, 4 and 6 dpf. When larvae were examined 2 days post laser-ablation, several phenotypes were seen. Some larvae were found to have regenerated new GnRH3 neurons in the olfactory region (Fig. 31A). In others there was unilateral regeneration of GnRH3 neurons and in some of these cases the unilateral soma projected fibers bilaterally (Fig. 31B).

Lastly, in some larvae there was no regeneration. Larvae that were determined not to undergo GnRH3 neuron regeneration were assessed at 2 and 6 days post-ablation at which times no GnRH3 neurons were present (Fig. 31C).
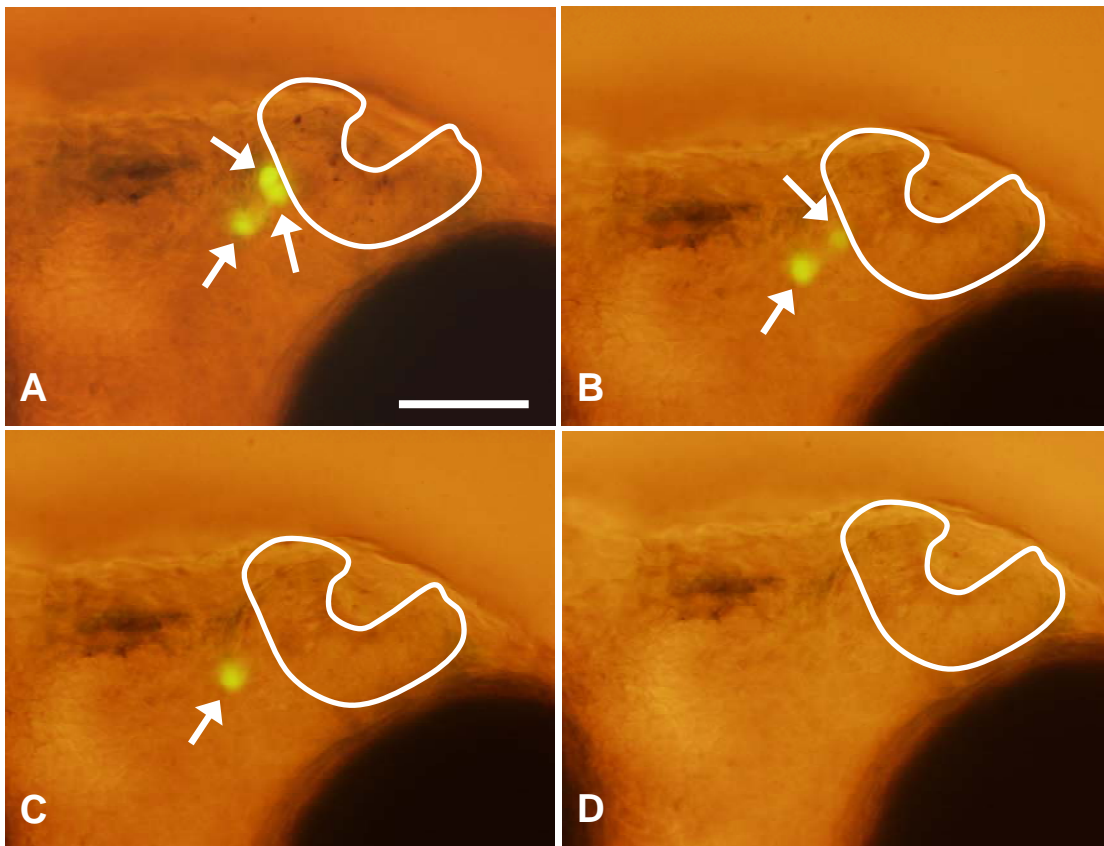
Figure 29.

Cell by cell ablation of GnRH3 neurons. Arrows indicate individual GnRH3 soma: area framed by line represents olfactory organ (A,B,C,D) Ventral view of right side GnRH3 neuron cluster in 2 dpf Tg(GnRH3:EGFP) larvae. (A) larvae prior to application of laser. GnRH3 cluster is intact and located immediately posterior-medially to the olfactory organ (B) After application of ablation laser to one soma, this soma has been ablated. No residual EGFP is evident. (C) the second soma is ablated (D) after the third GnRH3 soma is ablated this cluster has disappeared, no residual EGFP is apparent. Scale bars: 25 µm.

Figure 30.

Bilateral and unilateral ablation of GnRH3 neurons. (A,B,C) lateral view of 6 dpf Tg(GnRH3:EGFP) larvae. GnRH3 neurons were ablated using laser and photographed two minutes post laser application. Arrows indicate GnRH3 neurons. (A) prior to ablation. (B) post left side unilateral ablation. (C) post bilateral ablation. Scale bars: 50 µm.
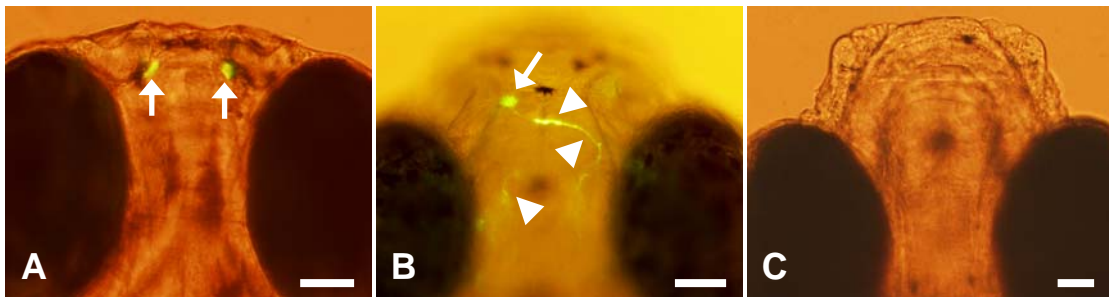
Figure 31.

Various phenotypes of GnRH3 neurons post-ablation. (A,B,C) ventral view of Tg(GnRH3:EGFP) larvae. (A) 4 dpf larvae. Two days post-bilateral ablation, GnRH3 neurons have regenerated bilaterally (arrows). (B) 6 dpf larvae. Four days post-bilateral ablation, GnRH3 soma have regenerated only on left side and abnormally extend fibers ventrally that cross the midline and innervate bilaterally (arrowheads). (C) 10 dpf. 6 days post-bilateral ablation, no regeneration of GnRH3 neurons is seen anywhere in the olfactory region or CNS. Scale bars: (A,B) 50 µm; (C) 75 µm.

Interestingly, there was a significant difference in the capacity of GnRH3

neurons to regenerate between larvae that were ablated at different developmental

timepoints. The percentage of larvae in which GnRH3 neurons bilaterally regenerated

by 2 days post-ablation, was significantly higher in larvae that were ablated at 2 dpf

(59.3% ±0.3%), when compared with larvae that were ablated at 4 (12% ±6.6%) and

6 dpf (10% ±4.4%) (Fig. 32, Table 1). The opposite was true when looking at the

percentage of larvae in which no GnRH3 neuron regeneration was observed. The

percentage of larvae in which unilateral ablation was observed was low

(10% ±4.4%), indicating that this may be a consequence of incomplete ablation rather

than unilateral regeneration.
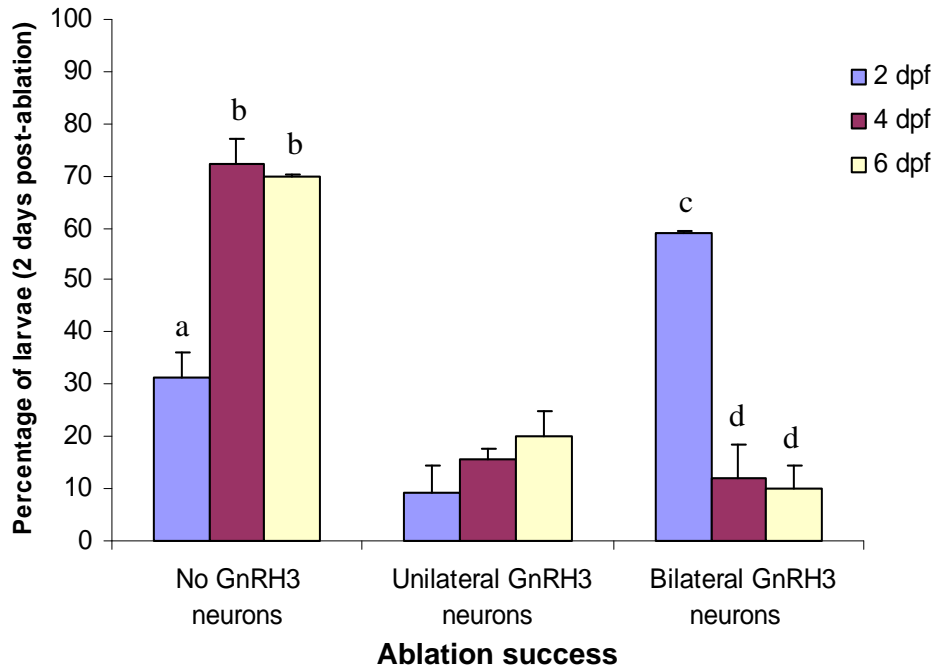
Figure 32.

GnRH3 regeneration rates were higher when ablation was conducted at 2 dpf. Laser ablations took place at 2,4 or 6 dpf, larvae were assessed at two days post-laser ablation for regeneration of GnRH3 soma. Significant differences were found between larvae in which neurons were ablated at 2 dpf versus larvae in which neurons were ablated at 4 and 6 dpf. Ablation at 2 dpf resulted in significantly more larvae that exhibited regeneration of GnRH3 soma when compared with larvae that were ablated at 4 and 6 dpf. Different letters represent statistically significant differences (T-test, two tailed p≤0.01).

	Number of ablated larvae	No GnRH3 neurons (%)	Unilateral GnRH3 neurons (%)	Bilateral GnRH3 neurons (%)
2 dpf	66	31.5 (±4.8)	9.1 (±5.2)	59.1 (±4.2)
4 dpf	83	72.3 (±4.6)	15.7 (±2.0)	12 (±6.6)
6 dpf	80	70 (±0.4)	20 (±4.8)	10 (±4.4)

Table 1

Percentages of Tg(GnRH3:EGFP) larvae that exhibited regeneration of GnRH3 neurons two days post-ablation (ablation conducted at 2,4 or 6 dpf).

Location of GnRH3 soma and fibers in sexually mature zebrafish

The Tg(GnRH3:EGFP) line is an excellent model, not only for studying GnRH3 system development (Chapter 2) but also for establishing location of GnRH3 soma and fibers in sexually mature zebrafish. Defining the location of GnRH3 neurons and fibers in normal mature Tg(GnRH3:EGFP) fish was a prerequisite to studying the effects of GnRH3 ablation in mature animals.

GnRH3 soma are comprised of several distinct populations in the CNS (Fig. 45). The first is the OB-TN population, these neurons are located bilaterally in the ventral OB, and extend in the form of intermittent soma clusters into the ventral nucleus of the ventral telencephalic area (Vv) along the TN (Fig. 33). These soma form in clusters, express high EGFP levels, and are connected by a robust fiber tract (Fig. 33,34). The second population is composed of the POA-hypothalamic GnRH3 neurons that serve the hypophysiotropic role. This group is divided into two populations: 1) soma in the anteroventral parvocellular preoptic nucleus (PPa) that are

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apposed to the anterior commissure and extends posteriorly to reach the

posteroventral parvocellular preoptic nucleus (PPp), and 2) GnRH3 soma in the

ventral periventricular hypothalamus (Hv) just above the adenohypophysis (Fig. 34)

(M.F. Wullimann, 1996). GnRH3 soma in the POA-hypothalamic region are

distinctly different from the OB-TN population in that they are well dispersed rather

than aggregating in clusters and seem to express lower levels of EGFP (Fig. 33,34).

Finally, a fourth population of GnRH3 soma are located in the facial lobe that is in

the hindbrain (not shown).
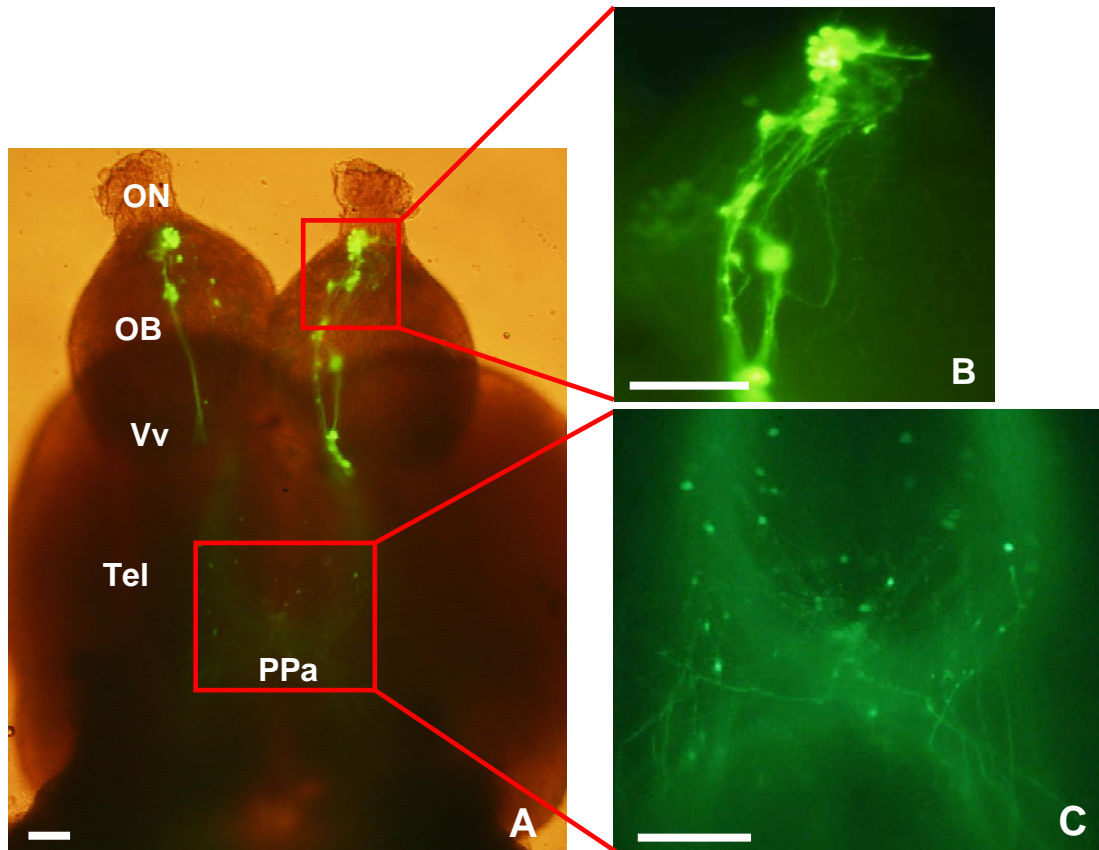
Figure 33.

GnRH3 neurons in a mature Tg(GnRH3:EGFP) zebrafish intact brain. (A) Ventral view of the ON, OB, Tel and PPa. GnRH3 soma are located in a continuum from the anteroventral OB to the Vv and are connected by fiber tracts. (B) enlarged fluorescent image of upper boxed area in (A), GnRH3 soma clusters and connecting fibers can be seen in the ventral OB and extending to the Vv. (C) enlarged fluorescent image of lower boxed area in (A). GnRH3 soma are scattered in the PPa apposed to the anterior commissure. Scale bars: (A,C) 100 µM; (B) 50µM. ON-olfactory nerve, OB-olfactory bulb, Tel-telencephalon, PPa-parvocellular preoptic nucleus, Vv-ventral nucleus of the ventral telencephalon.
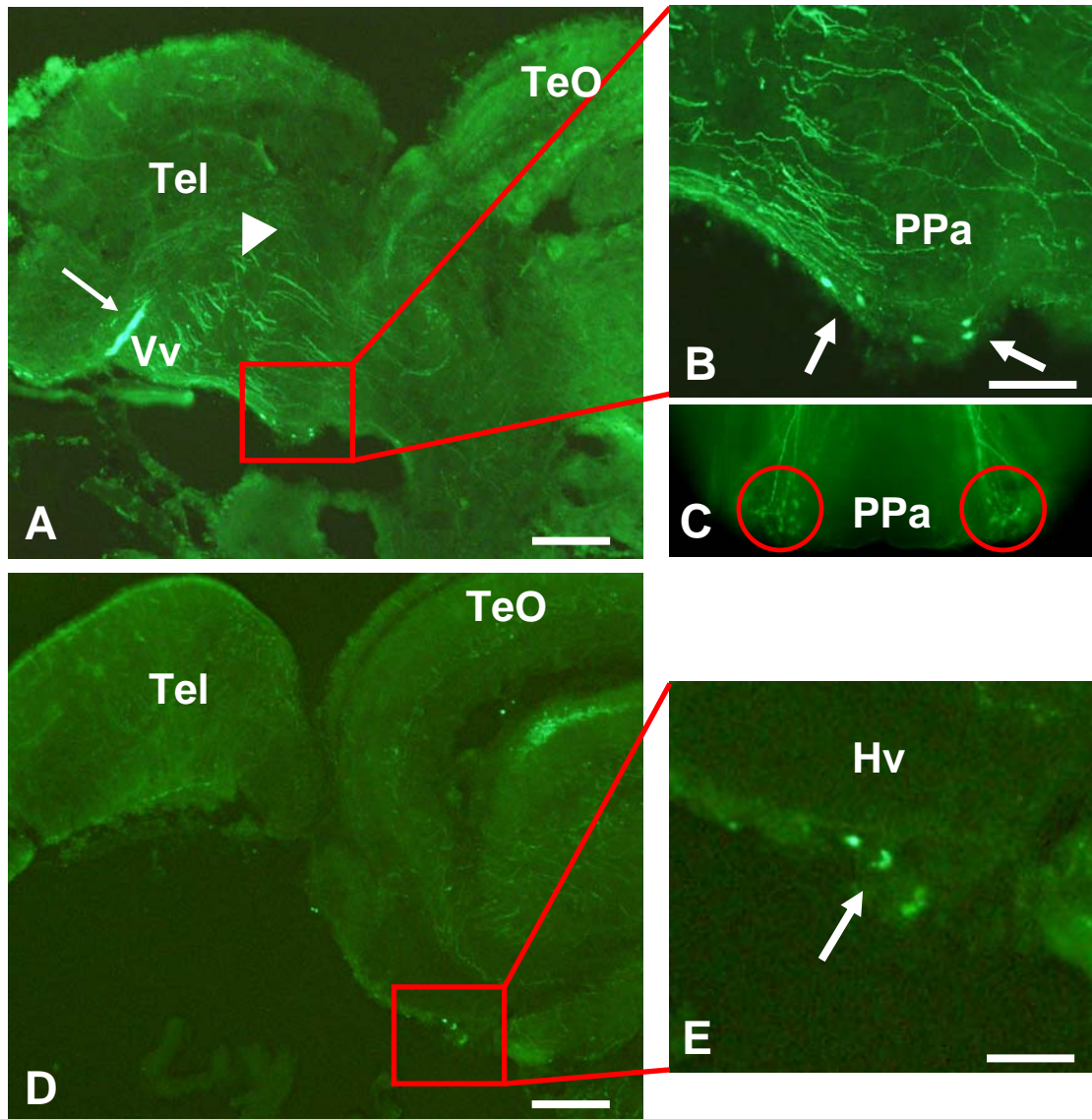
Figure 34.

GnRH3 neurons are located in the preoptic area and periventricular hypothalamus. (A,B,D,E) sagittal cryosections of Tg(GnRH3:EGFP) mature zebrafish brains, anterior towards the left. (A) GnRH3 soma are located in the Vv (arrow) and PPa (boxed area): GnRH3 fibers are located in various regions (arrowhead). (B) enlarged image of boxed area in (A), GnRH3 soma in the PPa (arrows). (C) ventral view of PPa, anterior towards the top (whole disconnected telencephalon), circles surround clusters of GnRH3 soma in the PPa (D) GnRH3 soma are located in the Hv (boxed area). (E) Enlarged image of boxed area in (C), GnRH3 soma in the Hv (arrow). Scale bars: (A,D) 250 µM; (B,E) 50 µM. Tel-telencephalon, TeO-optic tectum,

PPa-parvocellular preoptic nucleus, Vv-ventral nucleus of the ventral telencephalon, Hv-ventral zone of the periventricular hypothalamus.

GnRH3 fibers are located widely in the CNS (Fig. 33,34,35,36), areas in which GnRH3 fibers are localized include the olfactory bulbs, telencephalon, optic tectum, cerebellar corpus, hypothalamus, and hindbrain. Particularly robust fibers extend from the GnRH3 clusters in the OB towards the telencephalon and midbrain, and from the PPa towards the Hv and pituitary (Fig. 35). These fibers clearly take two tracts, one turning in a ventral direction towards the Hv and pituitary (Fig. 35 B), and the other continuing posteriorly.
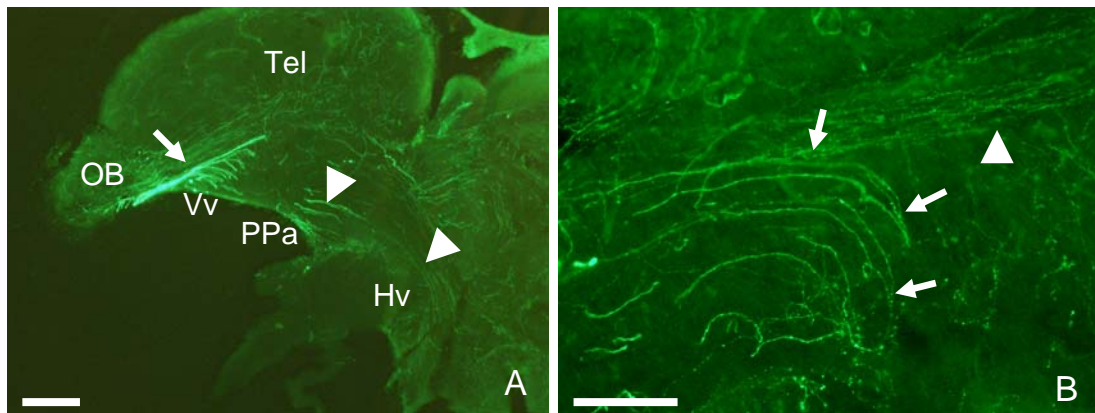
Figure 35.

GnRH3 fibers are localized to many CNS regions. (A,B) sagittal cryosections of Tg(GnRH3:EGFP) mature zebrafish brains. These sections of the forebrain show GnRH3 fibers localized to the OB, Tel, PPa and Hv. (A) a major fiber tract is seen exiting the ventral olfactory bulb and entering the telencephalon (Vv) (arrow) additional tracts extend from the PPa and turn ventrally towards the Hv (arrowheads). (B) a close-up of GnRH3 fibers extending from the PPa and turning ventrally towards the Hv (arrows) and a subset of fibers that do not turn ventrally and target the midbrain and hindbrain (arrowhead). Scale bars: (A) 250µM; (B) 50µM.

Tel-telencephalon, TeO-optic tectum, PPa-parvocellular preoptic nucleus, Vv-ventral nucleus of the ventral telencephalon, Hv-ventral zone of the periventricular hypothalamus.

GnRH3 neurons in ablated mature zebrafish

Fish in which GnRH3 neurons were successfully ablated as determined by observation of larvae at 6 days post-ablation, as well as comparison with control-ablated larvae, were raised to sexual maturity at 12-14 weeks post-fertilization. Localization of GnRH3 neurons and fibers in mature fish was analyzed in comparison with control-ablated fish in which only GnRH3 adjacent neurons were ablated at the larval stage.

When observing GnRH3-ablated fish brains as a whole organ as well as after cryosectioning, most contained some degree of GnRH3 neurons (Table 2). One fish contained normal GnRH3 neuronal distribution, however, most contained isolated neurons or fibers. These neurons and fibers were mostly in the OB, rather than in the PPa or Hv.

	Number of ablated fish	Male/female	No GnRH3 neurons	Few GnRH soma or fibers	Normal GnRH3 distribution
GnRH3 ablated fish	16	4/12	3 (18.75%)	12 (75%)	1 (6.25%)
Control ablated fish	18	8/10	0	0	18 (100%)

Table 2

Analysis of GnRH3 presence in brains of control-ablated and GnRH3-ablated Tg(GnRH3:EGFP) zebrafish. Fish were analyzed for GnRH3 neuron existence based on detection of EGFP in the whole-brain and by cryosections. Male/female

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determination was based on ovarian morphology.

Of the 16 GnRH3-ablated Tg(GnRH3:EGFP) mature fish that were examined three fish exhibited no forebrain EGFP expression whatsoever when examining the intact brain (Fig. 36, Table 2) and brain cryosections (Fig. 37, Table 2). All three fish were female. Most GnRH3-ablated fish (12), in which no EGFP signal was detected at 6 days post-ablation, contained drastically reduced numbers of GnRH3 neurons in the mature brain (Table 2). The existence of GnRH3 neurons in these fish, despite the fact that at 6 dpf they were examined and no EGFP expression was detected, suggests that smaller GnRH3 neurons that were undetectable at larval stages were not ablated and persisted to maturity. Interestingly, the GnRH3-ablated group contained a higher percentage of females (75%) compared to the control-ablated group (56%) (Table 2). Successfully GnRH3-ablated mature fish contained no GnRH3 neurons in the OBs, Vv, Tel, POA or ventral hypothalamus, however the facial lobe GnRH3 population remained intact. This lack of GnRH3 neurons and fibers was determined by examining both intact brains (Fig. 36) as well as cryosections (Fig. 37). In comparison, control-ablated Tg(GnRH3:EGFP) mature fish brains contained a normal distribution of GnRH3 soma and fibers (Fig. 36, 37).
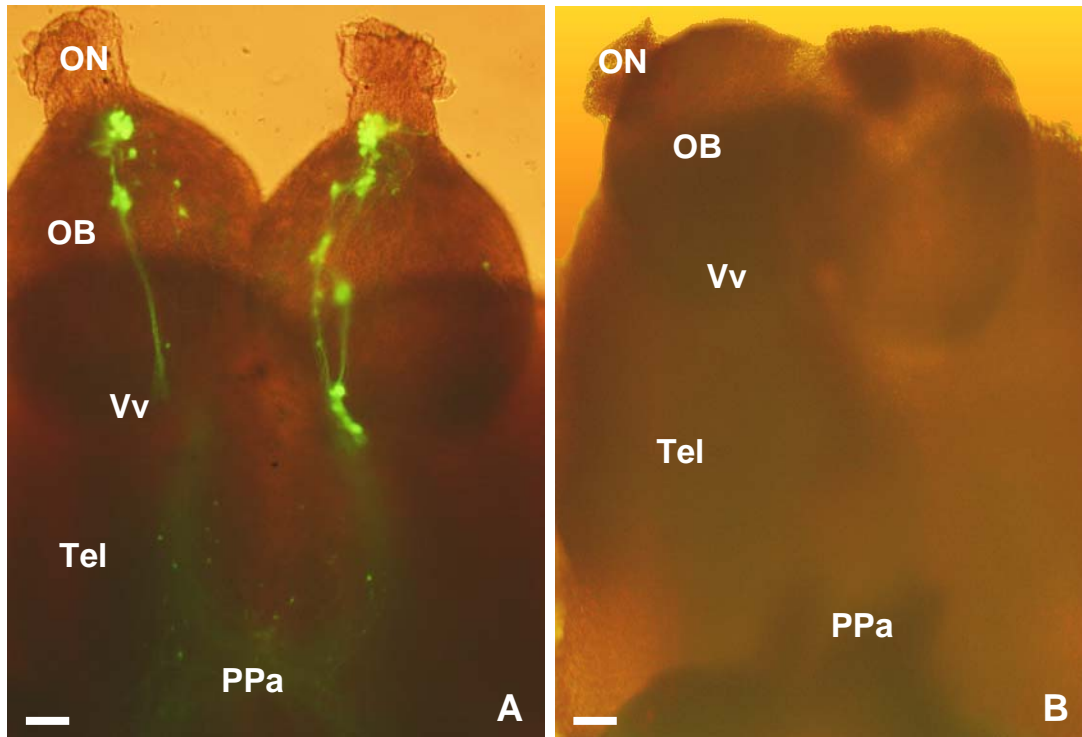
Figure 36.

Intact brains of mature Tg(GnRH3:EGFP) zebrafish in which GnRH3 neurons were successfully ablated at the larval stage do not contain GnRH3 neurons. (A,B) ventral images of Tg(GnRH3:EGFP) brains at 12 weeks post-fertilization. (A) Control ablated fish. GnRH3 neurons and fibers are clearly visible in the OB, Vv, Tel, and PPa. (B) successfully GnRH3-ablated fish. No GnRH3 soma or fibers can be seen as evident from lack of EGFP expression. OB-olfactory bulb, ON-optic nerve, Tel-telencephalon, PPa-parvocellular preoptic nucleus, Vv-ventral nucleus of the ventral telencephalon. Scale bars: (A,B) 150 µM.

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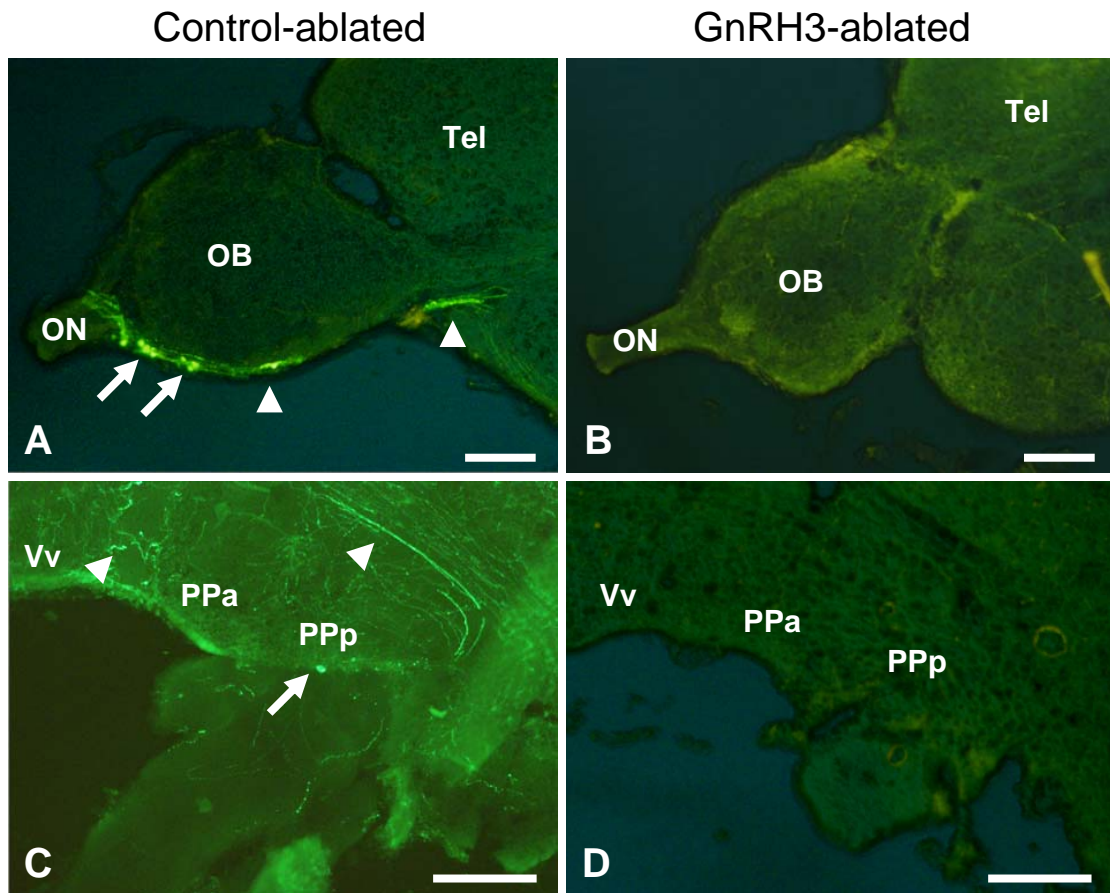
Figure 37.

Successful GnRH3 neuron ablation in larvae results in lack of GnRH3 neurons in
mature fish. (A,B,C,D) sagittal images of control ablated and GnRH3 ablated
Tg(GnRH3:EGFP) brains. (A,C) control ablated fish exhibit normal distribution of
GnRH3 soma (arrows) and fibers (arrowheads) in the OB, Tel, Vv, PPa and PPp.
(B,D) successfully GnRH3 ablated brains contain no GnRH3 soma or fibers.
OB-olfactory bulb, ON-optic nerve, Tel-telencephalon, PPa-parvocellular preoptic
nucleus (anterior), PPa-parvocellular preoptic nucleus (posterior), Vv-ventral nucleus
of the ventral telencephalon. Scale bars: (A,B,C,D) 150 µM.

Another interesting phenomenon is found in Tg(GnRH3:EGFP) mature fish in which successful unilateral ablation was conducted. In these fish, the unablated side develops normally while no soma can be seen in the OB of the ablated side. However, the ablated side is innervated by fibers that extend from the unablated side, especially posteriorly to the Vv (Fig. 38). This suggests that while unilateral ablation of GnRH3 neurons in the olfactory region prevents the appearance and migration of soma to the OB, Vv and PPa of that side, correct bilateral fiber innervation is enabled via existing soma in the unablated side.
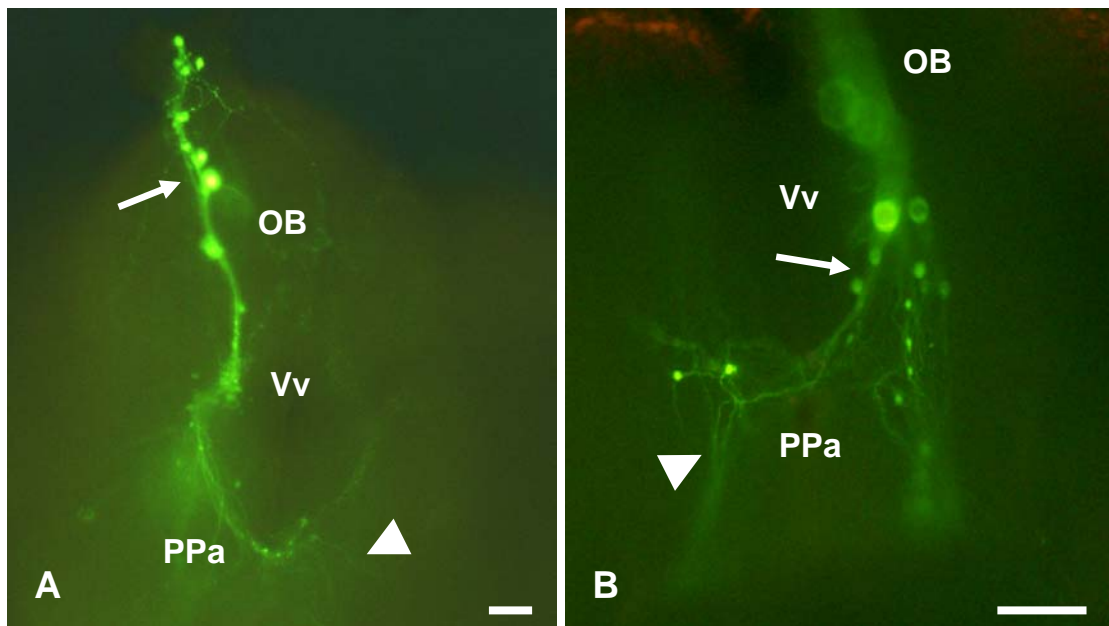
Figure 38.

Unilateral ablation of soma persists; however fibers from the unablated side cross the midline to innervate the opposing ablated side. (A,B) ventral view of Tg(GnRH3:EGFP) whole brains from two fish (forebrain). (A) left side ablation (B) right side ablation. (A,B) GnRH3 neurons are missing due to ablation on one side, and are normally positioned in the opposing unablated side (arrows). Despite ablation, fibers extend from the unablated side and are present in the Vv and PPa of both sides (arrowheads). Scale bars: (A,B) 150 µM. OB-olfactory bulb, PPa-parvocellular preoptic nucleus, Vv-ventral nucleus of the ventral telencephalon.

GnRH3-ablated zebrafish exhibit reduced fecundity and abnormal gonads

Prior to sacrificing ablated mature fish, female reproductive capacity was examined by placing females with WT male fish and examining fecundity. This experiment was repeated three times at one week intervals. While the average fecundity of control-ablated fish was 122 ($\pm$53) eggs per female, females in which GnRH3 was ablated exhibited significantly reduced fecundity averaging 24 ($\pm$17) eggs per female (Fig. 39), representing a more than 5 fold reduction in reproductive capacity. As discussed above, 12 out of 16 of the fish that were assumed to be successfully ablated were not entirely devoid of GnRH3 neurons, which presumably allowed these fish to spawn albeit with reduced fecundity. In accordance with this, in each spawning round only 15-40% of GnRH3-ablated females spawned, compared with 70-80% of control-ablated females. This suggests that 1) the three females that subsequently were found to have no GnRH3 neurons did not spawn at all and 2) Females in which a reduced number of GnRH3 neuron existed exhibited significantly reduced fecundity.
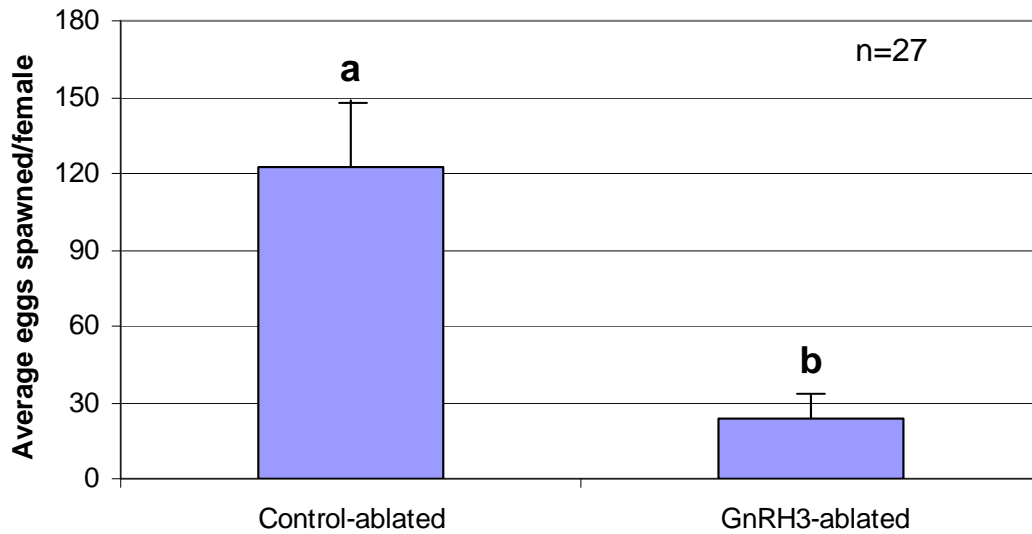
Figure 39.

Partial GnRH3 neuron ablation reduces fecundity. Reproductively mature
Tg(GnRH3:EGFP) zebrafish females (7-9 individuals), either GnRH3-ablated or
control-ablated, were placed in spawning tanks with WT males (2 females and 1 male
in each tank). Fish were allowed to spawn for six hours, eggs from each group were
collected, pooled and counted. The average number of eggs spawned per female was
significantly higher in the control ablated group than in the GnRH3-ablated group
(T-test, Two-Tailed $p<0.01$).

Following the fecundity experiment, fish were sacrificed and gonads (in
addition to brain) were extracted and sectioned. In fish that were verified as having no
GnRH3 neurons in the brain, the gonads exhibited abnormal morphology when
compared with control-ablated fish and GnRH3 ablated fish in which some remaining
GnRH3 neurons were detected in the brain. Zebrafish oocyte development occurs in 5
stages: I (primary growth), II (cortical alveolus or pre-vitellogenic),
III (vitellogenic), IV (maturation), and V (mature egg) (Ge, 2005). The morphological
difference between fish that had no GnRH3 neurons and control fish was manifested

in the lack of stage III (vitellogenic) and post-stage III oocytes (Fig. 40). No significant difference in oocyte development was found in GnRH3-ablated mature fish that were found to have a decreased number of GnRH3 neurons in the brain.

The effect of lack of GnRH3 neurons was further elucidated by examining oocyte diameter in the three categories of fish: 1) females in which GnRH3 neurons were completely missing from the brain as evident from whole brain and brain cryosection observations, 2) females in which GnRH3 neurons were targeted for ablation at larval stage, but contain in the mature brain a drastically decreased number of GnRH3 neurons, and 3. females in which control non-GnRH3 ablation was conducted.

When assessing average oocyte diameter in these three categories of females, average oocytes diameter was significantly reduced in category 1 fish when compared with categories 2 and 3, but no significant difference was found between categories 2 and 3 (Fig. 41). This suggests that even a drastically reduced numbers of GnRH3 neurons is sufficient to induce ovarian oogenesis, in which all oocyte stages are present. However, as discussed above, the fecundity of these fish was significantly reduced.
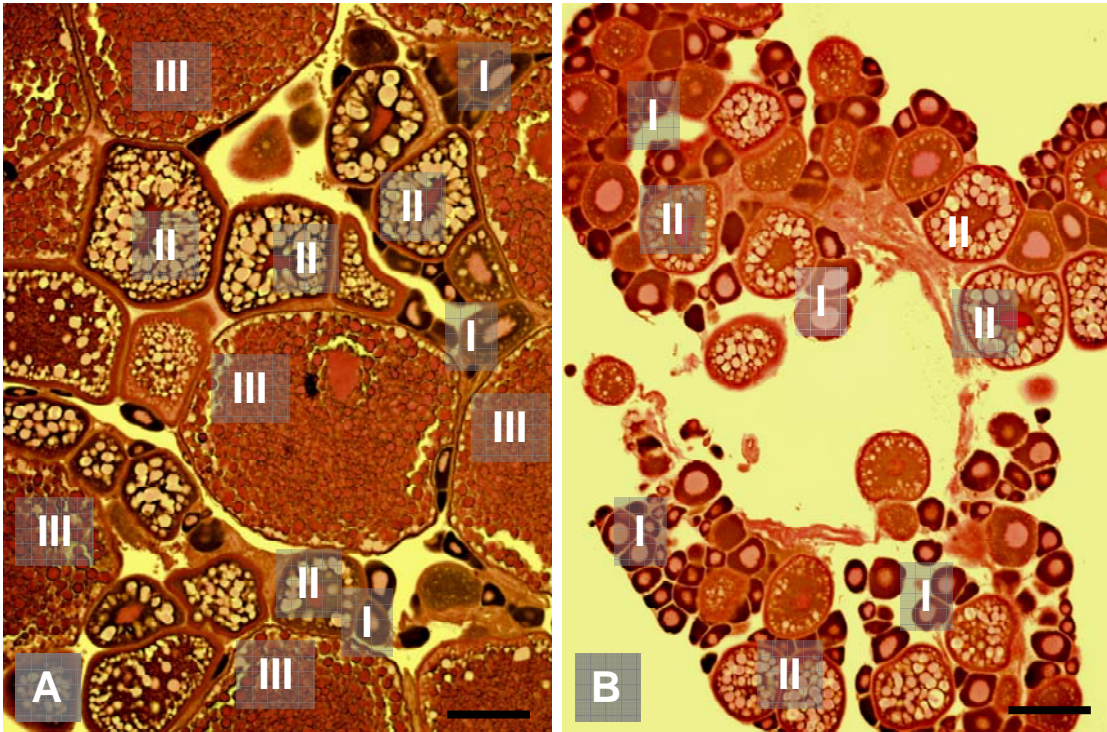
Figure 40.

Gonadal development is impaired in zebrafish that lack GnRH3 neurons. (A) control-ablated zebrafish ovary. Oocytes at all stages of development are present throughout the ovary. (B) GnRH3-ablated zebrafish ovary. Oocytes are arrested at stage II of development; no stage III oocytes are present. I- primary growth, II- pre-vitellogenic, III- vitellogenic/maturation. Scale bars: (A,B) 200 µM.
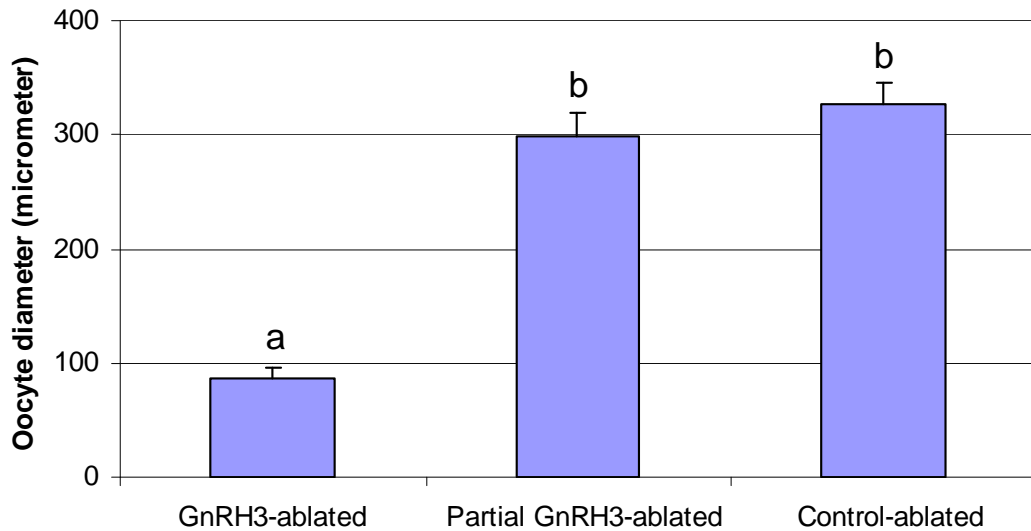
Figure 41.

Lack of GnRH3 neurons result in a significant decrease in average oocyte diameter. Oocyte diameters of a random gonad region in fish (n=3) from each groups were measured. Average oocyte diameter for each specimen was calculated. The average diameter of oocytes in fish that contained no GnRH3 neurons (as determined by examination of the brain) was significantly lower than fish in which a decreased number of GnRH3 neurons were present in the CNS and from control ablated fish. No significant difference was found between the average oocyte diameter of the latter two groups. (One-way ANOVA, $p<0.01$).

Genetic-toxin ablation of GnRH3 neurons

Concurrent to utilizing laser ablation to ablate GnRH3 neurons, we also attempted to employ a genetic-toxin method for GnRH3 neuron ablation. This method involved the use of a pTol2-nfsB-GnRH3-EGFP vector in which the nitroreductase (NTR) enzyme (nfsB) and EGFP are driven by the GnRH3 promoter and expressed in GnRH3 neurons (Fig. 42). When larvae are exposed to the prodrug metronidazole (Mtz), the NTR enzyme converts it into a cytotoxic DNA cross-linking agent, thus

ablated GnRH3 neurons. The advantage of this method is that it allows for

conditional ablation of GnRH3 neurons at various time points.
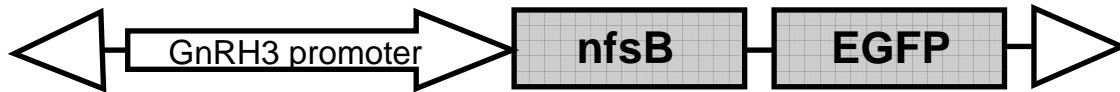
Figure 42.

pTol2-nfsB-GnRH3-EGFP expression construct. Triangles represent Tol-2 elements,

a 2.4-kb sequence upstream of the gonadotropin-releasing hormone (GnRH3)

decapeptide coding region was used as promoter (see Chapter 2), nfsB-nitroreductase

enzyme coding sequence, EGFP-green fluorescent protein coding sequence.

When injecting the pTol2-nfsB-GnRH3-EGFP vector into WT zebrafish

embryos, we observed correct EGFP expression in GnRH3 neurons, suggesting that

the construct acts properly and expresses NTR and EGFP specifically in GnRH3

neurons. The same correct expression pattern of EGFP was observed in F1 progeny,

suggesting correct incorporation of the vector into genomic DNA. To verify this we

conducted semi-quantitative RT-PCR using NTR specific primers. The results

showed that both mature P0 and F1 larvae were positive for the vector (Fig. 43),

demonstrating that the pTol2-nfsB-GnRH3-EGFP vector had integrated appropriately

into the genome.
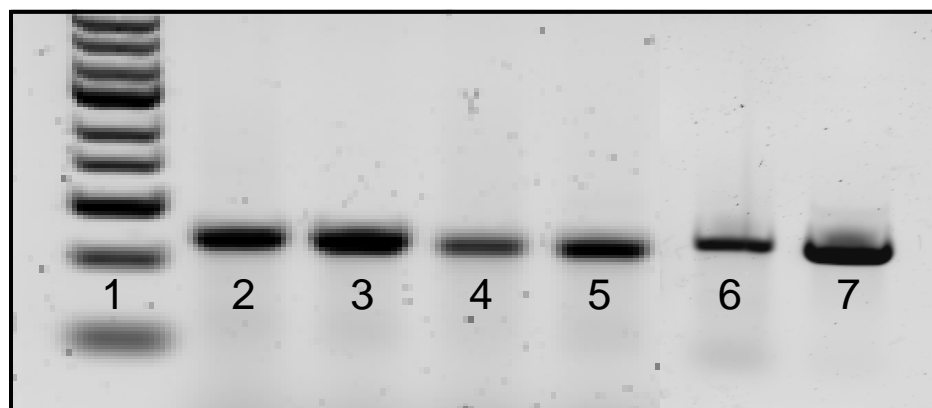
Figure 43.

pTol2-nfsB-GnRH3-EGFP vector is present in P0 and F1 zebrafish. Semi-quantitative RT-PCR was conducted using NTR-specific primers and P0/F1 genomic DNA. Lane 1-DNA ladder, Lanes 2,3,4,5 - P0 mature fish that were injected with the vector as embryos (finclip), lanes 6-F1 larvae, progeny of P0 mature fish, lane 7-positive control using the pTol2-nfsB-GnRH3-EGFP vector as template. In all lanes, the correct 376 bp product is present.

Tg(pTol2-nfsB-GnRH3-EGFP) F1 positive larvae, as determined by correct EGFP expression in GnRH3 neurons and by RT-PCR, were immersed in the prodrug Mtz at various time points and concentrations. Timeframe of exposures included 24-48 and 24-72 hpf. None of these exposure timeframes resulted in ablation of GnRH3 neurons as evident from continuous correct expression of EGFP in GnRH3 neurons (Fig. 44).
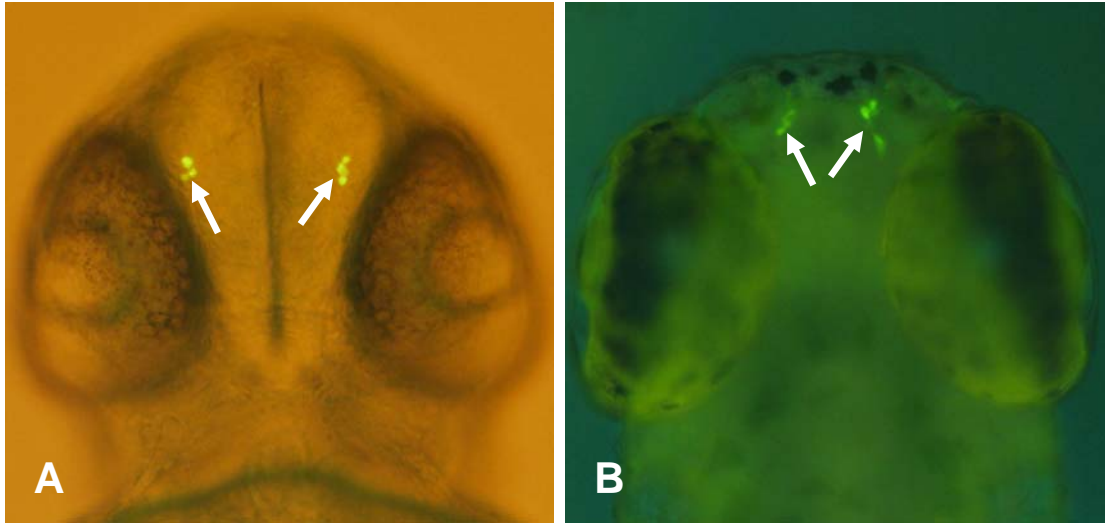
Figure 44.

Immersion of pTol2-nfsB-GnRH3-EGFP in Mtz does not ablate GnRH3 neurons.
(A) ventral view of 2 dpf larvae immersed in 10mM Mtz 24-48 hpf. (B) lateral view of 6 dpf larvae immersed in 10mM Mtz 24-72 hpf. Both larvae exhibit normal location of GnRH3 soma as evident form EGFP expression (arrows).

This result suggests that the genetic-toxin NTR-Mtz approach to conditionally ablating GnRH3 neurons is not effective. Although this method has successfully been used in other zebrafish organs, it seems not to work for ablation of GnRH3 neurons, possibly due to their location in the olfactory region/CNS.

Discussion

The two-fold goal of ablating GnRH3 neurons during development was 1) to establish whether the POA-hypothalamic hypophysiotropic GnRH neurons are indeed GnRH3 neurons that originate in the olfactory region during development, and 2) to study the effects resulting from lack of GnRH3 neurons. In addition to answering these questions, we obtained interesting findings regarding GnRH3 neuronal regeneration capacity, as well as location of GnRH3 neurons in the reproductively mature normal brain.

The olfactory region has a limited capacity to regenerate GnRH3 neurons

One of the interesting phenomenon seen post-ablation of GnRH3 neurons is the ability of the olfactory region to regenerate these neurons. While regeneration does occur, this capacity declined over time. The ability to regenerate GnRH3 neurons post-ablation was significantly higher when ablation took place at 2 dpf when comparing to ablation at 4 and 6 dpf.

The olfactory epithelium in adult mice contains self-regenerating sensory neurons that have the ability to form new neuronal populations (Choi et al., 2008). In general, olfactory neurons seem to have a half-life of several weeks, at which point they are replaced by new neurons. The source of these neurons is stem cell populations in the olfactory epithelium (Calof and Chikaraishi, 1989; Romanelli et al., 2004). The olfactory region possesses the capacity to generate not only olfactory neurons but also GnRH neurons well into development (up to 20 weeks of gestation in humans) (Kim et al., 1999). It is speculated that the olfactory region contain a

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reservoir of neurons able to generate GnRH cells. This is similar to what is seen in Tg(GnRH3:EGFP) zebrafish, in which there is a steady stream of GnRH3 neurons migrating from the olfactory region through 15 dpf.

These findings suggest that there are progenitor/stem cell populations in the olfactory region that give rise to migrating GnRH3 neurons well into the later developmental stages. The olfactory epithelium retains the ability to readily regenerate these progenitor GnRH3 populations if they are ablated during the 2 dpf timeframe but this ability significantly declines with time. When early GnRH3 olfactory neuron progenitor populations are completely destroyed after a certain time point, no GnRH3 neurons are subsequently found in the sexually mature brain.

GnRH3 location in mature zebrafish

The Tg(GnRH3:EGFP) zebrafish is the first two-GnRH fish transgenic model system in which GnRH neurons are marked by a reporter protein. The specificity and robustness of the EGFP signal allows clear observation of GnRH3 soma and fibers in the reproductively mature animal. This model is highly relevant to mammals and humans due to the fact that zebrafish, like mammals, have only two GnRH forms.

In mature animal brains, GnRH3 soma are divided into four distinct populations, the OB-TN, POA, ventral hypothalamus and facial lobe (Fig. 45). These findings are in agreement with multiple previous studies of GnRH in teleosts (Lethimonier et al., 2004). The locations of GnRH3 in mature zebrafish encompass the location of both GnRH1 and GnRH3 in fish that have three forms of GnRH. This organization of GnRH3 neurons is similar to what is seen in Masu salmon

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(Oncorhynchus masou) (Amano et al., 1991), a teleost that also has two GnRH forms. Similar to what is found in zebrafish, GnRH neurons in adult humans are diffusely scattered in the olfactory bulb, olfactory nerve, olfactory cortex, and TN, as well as in the POA-hypothalamus (Kim et al., 1999). The small soma population in the facial lobe is in agreement with previous ISH-based data (Steven et al., 2003), these cells are most probably associated with GnRH3-expressing cells found in the trigeminal ganglia (Chapter 2), as these regions are connected (Kerem et al., 2005; Xue et al., 2006). Location of GnRH soma in the facial lobe/trigeminal ganglia indicates possible involvement of GnRH3 neurons in smell, taste and tactile stimuli, which may well be relevant to reproduction (Kerem et al., 2005; Kiyohara and Caprio, 1996). There is a distinct difference in the level of EGFP expression between the OB-TN population and the POA-hypothalamic populations (POA and Hv). This difference may reflect differential control over GnRH3 transcription. Such a difference would be warranted given that these two populations play different roles while being controlled by the same GnRH3 promoter. This suggests that control over GnRH3 transcription is mediated by trans-acting promoter binding elements that are GnRH3 population specific.

These findings clearly demonstrate that in zebrafish both the OB-TN GnRH population and the hypophysiotropic POA-hypothalamus populations are indeed GnRH3-expressing neurons as shown previously (Steven et al., 2003), and are controlled by the same promoter.

The distribution of GnRH3 fibers is also in line with prior accounts in fish and mammals (Chen and Fernald, 2006; Gonzalez-Martinez et al., 2004b; Pandolfi et al.,

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2005). One aspect of GnRH3 fiber location that could be observed very clearly in the

Tg(GnRH3:EGFP) zebrafish is the robust subsets of fibers extends posteriorly from

the PPa. Similar to the case in mammals, this fiber tract dissociates into two tracts,

one turning ventrally to innervate that Hv and pituitary, and the other coursing

posteriorly towards the hindbrain (Fig. 45).
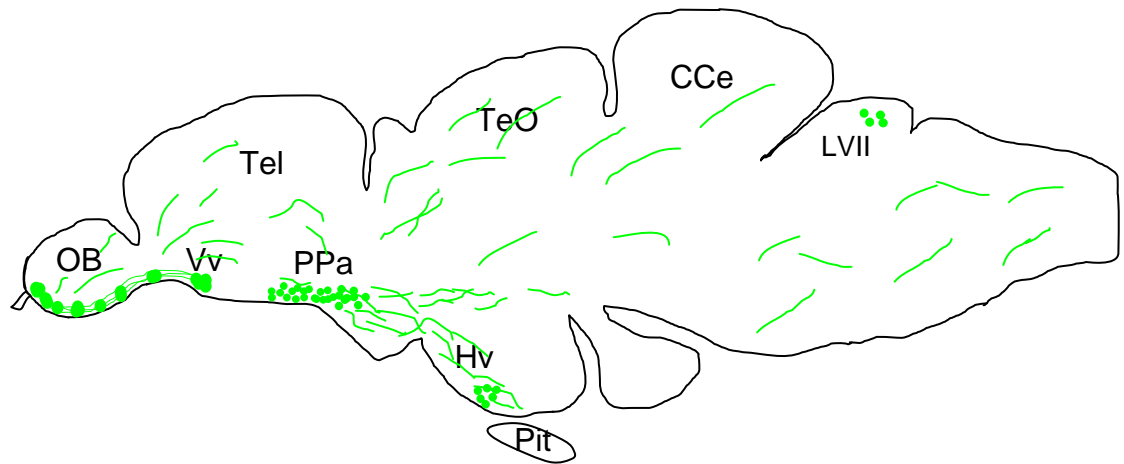
Figure 45.

A sagittal schematic of a mature zebrafish brain. Green dots represent GnRH3 soma, green lines represent GnRH3 fibers. Four distinct GnRH3 populations are located within the CNS 1) in the anteroventral OB extending towards the Vv and presumed TN. 2) in the PPa, 3) in the Hv and 4) in the LVII. GnRH3 fibers are located in many brain regions including the OB, Tel, Vv, TeO, Hv, midbrain and hindbrain. Especially prominent tracts extend out of the OB towards the Tel and TeO, as well as from the PPa, making a ventral turn into the Hv/pituitary. OB-olfactory bulb, Tel-telencephalon, TeO-optic tectum, Vv-ventral nucleus of the ventral telencephalon, PPa-parvocellular preoptic nucleus, Hv-ventral zone of the periventricular hypothalamus, CCe- cerebellar corpus, LVII- facial lobe, Pit- pituitary.

Downstream effects of GnRH3 neuron ablation

Targeted physical ablation of olfactory GnRH neurons during development is not feasible in mammals, even in GnRH-GFP transgenic lines, due to lack of transparency. For this reason, the use of the Tg(GnRH:EGFP) zebrafish line for this purpose has been proven to be very beneficial. Even when using this line, the percentage of fish in which all GnRH3 neurons were permanently ablated was extremely low (~1.5%), pointing both to the robustness of the GnRH3 system and to the difficulty to target each and every individual neuron. Nonetheless, successful ablation of all GnRH3 neurons has allowed insight into various aspects of GnRH development and function.

The embryonic origin of POA-hypothalamic hypophysiotropic GnRH neurons has not been unequivocally determined in all species (Norgren and Gao, 1994; Northcutt and Muske, 1994; Whitlock, 2005). Studies in which the entire olfactory region has been ablated point to the olfactory area as the embryonic source of forebrain GnRH neurons, however total olfactory ablation may influence development of brain areas beyond the olfactory area.

Successful early ablation of GnRH3 neurons in the olfactory region results in the total lack of GnRH3 neurons in the brain, including in the POA-hypothalamic areas. This finding clearly demonstrates that the embryonic origin of these GnRH populations is in the olfactory region, and not, as suggested by some, in the adenohypophyseal region of the developing anterior neural plate (Whitlock, 2005, Wu et al., 2006).

This finding is strengthened by the result that lack of GnRH3 neurons in the brain results in abnormal gonadal development and reproductive impairment. In this regard, we must distinguish between fish in which GnRH3 neurons were partially ablated, and fish in which GnRH3 neurons were totally absent from the brain. Females in which GnRH3 neurons were partially or unilaterally absent exhibited seemingly normal oogenesis in which all oocyte stages were represented in the ovary. The delivery of GnRH3 to the pituitary may be mediated by fibers which extend bilaterally into the POA region even when GnRH3 soma are only unilaterally present. Interestingly, a similar result was obtained in Xenopus in which unilateral olfactory ablation resulted in bilateral innervation by GnRH fibers (Setalo, 1996). However, despite the apparent ability to normally deliver GnRH3 to the pituitary and despite the presence of all oocyte stages in the gonad of these partially ablated fish, their reproductive capacity was significantly mitigated as was reflected by reduced fecundity. The explanation for this decrease in fecundity may be two-fold, 1) abnormalities to the OB-TN GnRH3 population and possibly to eye innervations may result in reduced capacity to process pheromonal or visual reproductive cues, and 2) although all oocyte stages are present in the gonads, various other less apparent aspects of gonadal function may be impaired.

Contrary to the situation in fish in which the GnRH3 populations were partially intact, in fish that had no GnRH3 neurons the effect on gonadal development was clear. These fish exhibited incomplete gonadal development in which oocytes were arrested at the pre-vitellogenesis stage. This result is similar to ovarian morphology that is found in the hpg mouse (Pask et al., 2005) or in mice that lack

GnRH1 neurons due to abnormal olfactory development (Matsumoto et al., 2006). In these mice, ovarian follicles are arrested at the early antral stage. This indicates that the lack of GnRH3 neurons exerts a similar effect to the lack of hypophysiotropic GnRH decapeptide in the hpg mouse and lack of GnRH1 neurons in mammals.

Interestingly, despite the wide distribution of GnRH3 soma, and especially fibers in the mature brain, lack of these soma and fibers seemed to affect little more than reproduction, similar to what is observed in the hpg mouse. This suggests that the additional roles that are no doubt played by GnRH3 are either subtle or that a redundancy mechanism allows for other factors to substitute non-reproductive GnRH3 roles.

Application of the genetic-toxin approach

The ability to conditionally ablate hypophysiotropic GnRH neurons at various time points during development and transition to puberty is highly desirable. Our attempt to develop a zebrafish line in which this is possible by using the nfsB-Mtz system was not successful. However, a transgenic system was implemented successfully using a very similar construct to ablate zebrafish pancreatic cells (Pisharath et al., 2007). In addition, our RT-PCR results and the fact that EGFP is correctly expressed in GnRH3 neurons of F1 larvae, demonstrates that the pTol2-nfsB-GnRH3-EGFP construct was correctly integrated into the genome. Despite these points, ablation of GnRH3 neurons did not occur following immersion of larvae in Mtz. It is not clear why this methodology was not successful, possibly because the penetration of the Mtz antibiotic into the olfactory region/CNS is not

sufficient to induce ablation. It is noteworthy that recent attempts to utilize this

system for ablating other CNS neuron populations have also been unsuccessful

(personal communication, Dr. Y. Gothilf). Future use of similar systems, possibly

with a different antibiotic, should be attempted in an effort to develop a conditional

GnRH3 neuron ablation technique.

Summary

      This is the first successful attempt to specifically ablate forebrain GnRH

neurons. The results from this experiment clearly demonstrate the origin of

hypophysiotropic hypothalamic GnRH neurons.

      Ablation of olfactory region GnRH3 neurons during early development has

afforded various insights into developmental aspects and reproductive roles of

GnRH3 in zebrafish. The time-sensitive capacity of GnRH3 neurons to regenerate in

the olfactory region is important in the context of GnRH3 neuron development, as

well as in the more general context of neurogenesis during late development.

The successful ablation of all GnRH3 neurons in the brain, which was a consequence

of olfactory region GnRH3-specific ablation, demonstrates that, as in mammals,

GnRH3 neurons in the POA-hypothalamus originate in the olfactory region during

development. The fecundity and gonadal development consequences of lack of

GnRH3 neurons substantiate this migrational hypothesis. These findings are in

agreement with results obtained in mammals that lack GnRH1.

These results further our understanding of GnRH3 function in fish as well as mammals, and substantiate the importance and effectiveness of the Tg(GnRH3:EGFP) zebrafish line in studying GnRH development and function.

Chapter 6: GENERAL DISCUSSION

This chapter briefly summarizes the major findings detailed in this thesis and discusses the overall conclusions. In addition, it will address our current understanding of GnRH3 system development and regulation based on results described herein, as well as results obtained by our collaborators at Tel-Aviv University. Lastly, future directions regarding the study of GnRH development and reproduction will be addressed.

The Tg(GnRH3:EGFP) zebrafish line

In zebrafish it is possible to endogenously express markers in specific tissues thereby enabling observation of tissue/organ development in the whole animal. When this tool coupled to knock-down or OE of specific genes, it allows *in vivo* analysis of downstream morphological, developmental and physiological effects. In contrast to the use of ISH and ICC, in which development is only seen as a snapshot in time, endogenous expression of proteins such as EGFP or RFP is highly specific and temporally sensitive. In comparison to transgenic mammals, zebrafish afford the advantage of optic transparency, and hence the ability to fully take advantage of endogenous marker expression.

Given the small number of GnRH neurons in the sexually mature CNS and the fact that these neurons are scattered in a loose continuum (Rubin and King, 1994; Tsai et al., 2005), study of GnRH development and function has been challenging.

Use of transgenic animals in which GnRH neurons are marked by reporter proteins, has significantly advanced the study of the GnRH system. Several lines of GnRH-GFP transgenic mice have been developed (Han et al., 2004; Spergel et al., 1999; Suter et al., 2000). However, observation of GnRH in these lines is only possibly after sacrificing the animal and sectioning the brain. In comparison, use of the transgenic GnRH3:EGFP zebrafish affords direct observation of GnRH3 neurons in the intact live animal. This ability allows real-time high resolution analysis of GnRH3 fiber and soma development, and of the factors controlling these events in the whole-animal context. The ability to study GnRH development in the intact animal is especially important given that long range chemoattractants and chemorepellents are involved in the development of forebrain GnRH neurons, these factors may be lost when the CNS-olfactory region are not kept intact.

Similar to the case in mammals, and unlike many other fish species, the zebrafish has two GnRH forms (GnRH3, GnRH2). In addition, the migratory path of hypophysiotropic GnRH neurons in teleosts is very similar to mammals. For these reasons, we set out to develop a Tg(GnRH3:EGFP) zebrafish line in which EGFP is specifically and robustly expressed in GnRH3 soma and fibers. Combining the use of the Tg(GnRH3:EGFP) zebrafish line with the various traits of zebrafish, as well as the molecular tools and mutants available, has greatly aided us in establishing a better understanding of GnRH system development, as well as the factors that control this process. This line is especially advantageous in observing early GnRH3 fiber development, which is particularly difficult to follow using other models and methods.

In summary, we have shown that the Tg(GnRH3:EGFP) zebrafish line specifically and robustly expressed EGFP in GnRH3 neurons and can be used to study various aspects of the GnRH3 system. Establishment and use of this line allows unprecedented ability to visualize GnRH3 system development, as well as location in the mature CNS. It is also an excellent tool for studying the regulation of GnRH3 system development.

Early development of the GnRH3 system

Migration of forebrain GnRH neurons is a well studied biological process. Despite this, due to the inherent complexity of this migration, many unknown aspects remain regarding its regulation. The path of forebrain GnRH neurons is long and traverses multiple CNS and non-CNS regions, each containing a distinct milieu of cells, extracellular proteins and factors. This requires regulation of forebrain GnRH development to be multifaceted and spatiotemporally-specific. Another drawback to studying GnRH neurons is the fact that to date the only specific marker for GnRH neurons is GnRH itself. This is one of the reasons why there is still no clear consensus regarding the embryonic origins of these neurons, especially in the chick and fish models (Norgren and Gao, 1994; Northcutt and Muske, 1994; Whitlock, 2005).

Observations in the transgenic line have shown that POA-hypothalamic GnRH3 neurons indeed originate in the olfactory region and migrate to the telencephalon and hypothalamus. Conclusive evidence for this was also obtained by

ablation of GnRH3 soma in the olfactory region during development, which resulted in lack of GnRH3 neurons in the POA-hypothalamus and underdeveloped gonads.

Using the Tg(GnRH3:EGFP) line, we have delineated the development of GnRH3 fibers, as well as migration of GnRH3 soma. In the past, the focus of forebrain GnRH system development has been on soma migration. Observing GnRH3 development in the transgenic line, it is evident that GnRH3 system development is divided into two distinct phases. First, prior to soma migration, GnRH3 fibers extend from the soma in the olfactory region, forming a complex fiber network. This network included several tracts that target the midline as well as tracts that extend posteriorly. Of special interest are the tract that targets the pineal region and the tract that innervates the retina. These tracts may be involved respectively in coordinating reproduction with the biological clock and visual cues. One of the major tracts, which is also the hypophysiotropic tract, extends towards the hypothalamus and turns ventrally to innervate the pituitary. This tract subsequently serves as a scaffold for axophilic GnRH3 soma migration. The assumption is that this tract is apposed to the TN/VNN tracts. Migration of GnRH3 soma, as described in the Tg(GnRH3:EGFP) line, is similar to GnRH migration as described in other vertebrates, including teleosts. Migration begins at 3 dpf and continues throughout 15 dpf with the first soma reaching the POA region at 12-13 dpf. This migration seems to occur as a continuous stream, with new neurons being born in the olfactory region and migrating posteriorly. Many important questions remain regarding this process, such as: How do individual soma act and interact? When does the POA-hypothalamic population

differentiate from the OB-TN population? When does migration from the olfactory region cease?

Answers to these questions should be explored using the Tg(GnRH3:EGFP) zebrafish line developed in this study.

Factors mediating GnRH3 system development

Study of forebrain GnRH development is important both from a reproduction perspective and to further basic understanding of neuronal tangential migration in the developing and mature CNS. Given the nature of GnRH3 system development, multiple factors are involved in regulating this migration process in a spatiotemporal-specific manner (Cariboni et al., 2007). Factors that have been studied using the Tg(GnRH3:EGFP) line by our collaborators at Tel-Aviv University and by us include GnRH3, Netrin1a and its receptors, NELF, and Cxcl12 (previously SDF-1) and its receptor Cxcr4. As NELF and Cxcl12/Cxcr4 are not an integral part of this thesis, they will only be mentioned briefly in the general context of their role in regulating GnRH3 neuronal establishment using the Tg(GnRH3:EGFP) line and not addressed in depth.

One of the basic concepts delineated in this thesis is that development of the forebrain GnRH system in vertebrates should be divided into two phases. The first is fiber development and the second is soma migration. These two phases are interdependent, yet distinct, and are undoubtedly regulated differentially. Even within these two phases subdivisions are warranted. For instance, it is clear that targeting of GnRH3 fiber tracts towards the midline, as seen in formation of commissures and

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tracts that cross the midline to form chiasms, are differentially regulated from fibers that conduct posterior tangential targeting of the midbrain and hindbrain. This is demonstrated in the case of Netrin1a, which we show is important for mediating midline targeting, but not essential for posterior targeting of fiber tracts (Fig. 46). Similarly, it is most likely that there is differential migrational regulation of the various GnRH soma populations, especially in species such as humans and zebrafish that have only one forebrain GnRH form. The regulation of the OB-TN population migration and organization undoubtedly differs from regulation of the POA-hypothalamic populations. This is true despite the fact that both populations originate in the olfactory region.

Interestingly, some factors involved in GnRH3 system development influence both soma migration and axon targeting. Although each factor has a unique role, this dual influence seems to be a result of one of two mechanisms:

1. Regulation of forebrain GnRH neuronal development during the early stages of immergence, differentiation and development in the olfactory region. Disruption of such factors results in global downstream fiber and soma developmental abnormalities.

2. Control of development at later stages, after fiber scaffold development and soma migration become distinct events. Also in this context, one factor can influence both fiber development and soma migration. However, at this stage the effect on fibers and soma is specific, more subtle, and manifested in a spatiotemporally-divergent manner. This may be an example of evolutionary efficiency in which one factor is utilized for several temporally separated events.

The former paradigm seems to include GnRH3 and NELF, the latter seem to include Netrin1a/Dcc and Cxcl12/Cxcr4.

GnRH3 and NELF knock-down result in generalized abnormalities which include abnormal distribution of GnRH3 soma in the olfactory region, lack of GnRH3 soma migration, abnormal proliferation of soma, and either total a lack of or generalized abnormalities to fiber development. In contrast to these factors, Netrin1a/Dcc, as well as Cxcl12/Cxcr4, have a more selective effect on specific fiber tracts (Fig. 46) and at later time points, on soma development (Fig. 47). Netrin1a/Dcc has an effect only on fibers that target or cross the midline, while Cxcl12/Cxcr4 has an effect on fiber tracts that extend towards the hypothalamus (Fig. 46). In regard to soma development, Netrin1a/Dcc knock-down results in the inability of soma to migrate past the TN region, this does not seem to be a consequence of earlier fiber abnormalities as the relevant fiber tract that targets the POA-hypothalamus develops normally. In the case of Cxcl12/Cxcr4, knock-down results in aberrant location of soma within the olfactory region as well as in certain areas of the forebrain (Fig. 47). As Cxcl12/Cxcr4 knock-down disrupts development of the POA-hypothalamic fiber tract it is not clear whether this is a soma specific effect or a result of earlier fiber development abnormalities. The effect of Cxcl12/Cxcr4 knock-down on GnRH3 soma is in line with its earlier role in GnRH3 hypothalamic fiber tract development, aberrant targeting of this tract that serves as a soma migration scaffold would indeed result in ectopic distribution of soma within the brain.

Interestingly, these results are similar but not identical to findings regarding the involvement of NELF, GnRH, Netrin1 and Cxcl12 in mammals (Kramer and

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Wray, 2000b; Romanelli et al., 2004; Schwarting et al., 2001; Schwarting et al., 2004; Toba et al., 2008). The similarity in the function of these factors, vis-à-vis GnRH development in mammals versus zebrafish, points to the conservation of the roles of these factors, while the dissimilarities point to the inherent differences between these two models. One of the possible reasons for differences in this regard is the significant difference in CNS architecture between mammals and fish. At the same time, some differences may simply be a consequence of research focus. For example, the work with Netrin1 in mammalian has focused on GnRH1 soma location while fiber development, especially in the olfactory epithelium (OE) region, has not been widely addressed, despite the fact that Netrin1 is expressed in the OE. This is probably due to the difficulty in visualizing early development of GnRH1 fibers in the OE, which in the Tg(GnRG3:EGFP) model can be clearly observed.
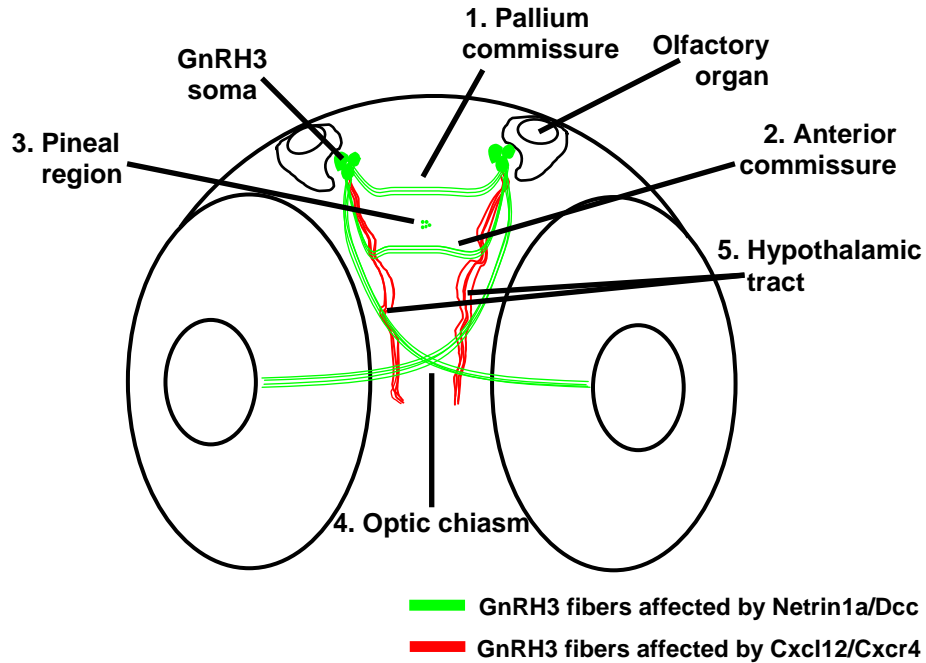
Figure 46.

Effect of Netrin1a/Dcc and Cxc12/Cxc4 on GnRH3 fiber development. Schematic illustration of major fiber tracts extending from GnRH3 somata at 36 hpf. Five major tracts are depicted from a ventral view:

1. Pallium and subpallium commissures. 2. Anterior commissure. 3. Innervation of the pineal region. 4. Innervation of the retina via the optic tract. 5. Bilateral innervation of the hypothalamus.

Netrin1a/Dcc **Cxcl12/Cxcr4**
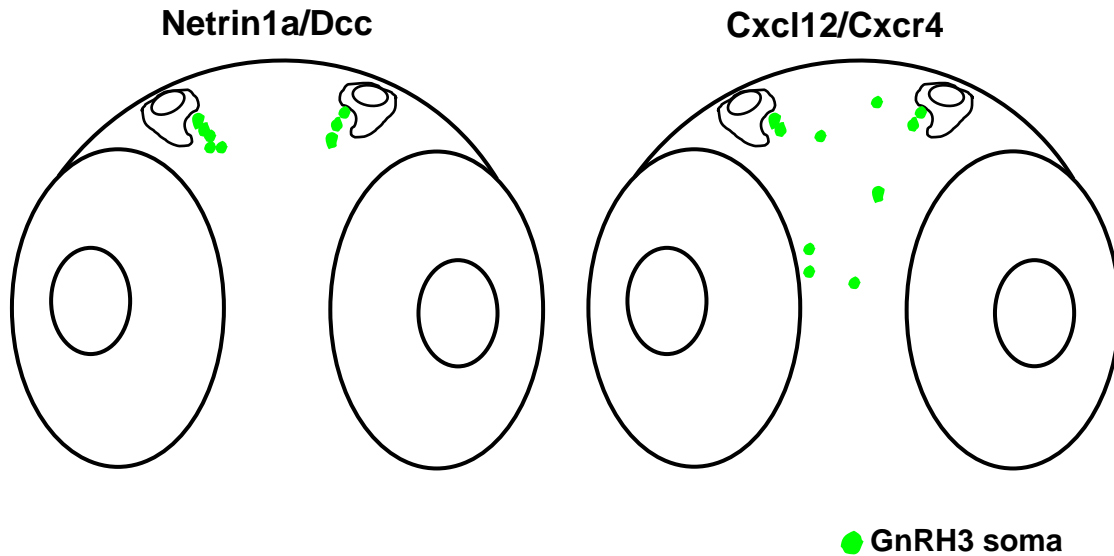
● **GnRH3 soma**

Figure 47.

Effect of Netrin1a/Dcc and Cxc12/Cxc4 knock-down on GnRH3 soma development at 7 dpf. Netrin1a/Dcc knock-down results in restriction of GnRH3 soma to the OB-TN region. Cxc12/Cxc4 knock-down results in aberrant location of GnRH3 soma in the olfactory region, telencephalon and hypothalamus.

To summarize, we have studied GnRH3 development using an innovative approach that takes into account the complexity of this system and the interdependence between different developmental events that comprise its establishment. Our research has laid the foundation for the further use of the Tg(GnRH3:EGFP) line to facilitate additional detailed high resolution studies of GnRH3 development in the whole animal context.

Ablation of GnRH3 neurons

The question of the origin of the hypothalamic GnRH neurons has long been a controversial issue. In recent years zebrafish has immerged as a model system in the forefront of this research (Gopinath et al., 2004; Whitlock et al., 2003; Wu et al., 2006). Conclusive evidence as to whether the origin of hypothalamic GnRH neurons is the olfactory region could be provided by specific ablation of GnRH neurons in the olfactory region during development and observation of whether hypothalamic GnRH neurons are present at later time points and whether these animals undergo normal gonadal development. However, a method and model system for such specific GnRH neuron ablation has not been available. Similar approaches have been employed in various species, either by physical ablation of the entire olfactory region or by using mutant lines in which development of the olfactory region is disrupted (Dellovade et al., 1998; Matsumoto et al., 2006). For the most part, these experiments resulted in subsequent lack of GnRH neurons in the hypothalamus and impaired reproductive capacity. However, ablation of the entire olfactory region or global disruption of its development through use of mutant lines may result in detrimental effects to other CNS regions, including the hypothalamus. In addition, despite these studies, others have suggested and shown evidence for multiple embryonic origins of forebrain GnRH neurons (Norgren and Gao, 1994; Northcutt and Muske, 1994, Whitlock, 2005, Wu et al., 2006).

Use of the Tg(GnRH3:EGFP) line coupled with laser ablation technology has for the first time, allowed specific ablation of GnRH3 neurons in the olfactory region during early development. When these neurons are completely ablated, there is a total

lack of GnRH3 neurons in the brain, including in the POA-hypothalamus. In addition, successful ablation results in incomplete development of the ovaries in which oocytes do not undergo final oocyte maturation and consequently, the animal is not able to reproduce. These results are similar to findings seen in mammals in which the GnRH1 gene is non-functional (Cattanach et al., 1977), or when olfactory region development is severely disrupted (Matsumoto et al., 2006). Thus, our ablation experiment has conclusively shown that POA-hypothalamic hypophysiotropic GnRH3 neurons arise in the olfactory region and migrate to the POA-hypothalamus.

Interestingly, partial ablation of GnRH3 neurons, in which all but several neurons are present in the sexually mature brain, results in normal oocyte development and the presence of all oocyte stages in the ovary. This suggests that even a small number of GnRH3 hypothalamic neurons is sufficient to induce normal oocyte development. This ability is probably mediated by the fact that unilateral soma have the capacity to extend fibers that form bilateral innervations. However, these animals displayed significantly reduced reproductive capacity. This phenomenon may be a consequence of subtle abnormalities in gonad development and/or the consequence of the reduction in OB-TN GnRH3 neurons numbers which may be necessary for mediating pheromonal cues.

Examining sexually mature Tg(GnRH3:EGFP) animal brains has also yielded interesting information regarding the normal distribution of GnRH3 soma and fibers in the brain. The findings regarding this distribution are in agreement with prior information form mammals and various fish species.

Summary

Development of the Tg(GnRH3:EGFP) zebrafish line has facilitated a deeper understanding of forebrain GnRH system development, as well as some of the individual factors that are involved in this complex developmental process. Moreover, this first-ever promoter-reporter transgenic zebrafish line for GnRH3 lays a foundation for future dissection of GnRH system development and function.

GnRH3 fibers and soma undergo a fascinating process of migration, targeting and innervation of various CNS and non-CNS regions. After GnRH3 neurons initially immerge in the olfactory region, GnRH3 fibers extend to form a complex scaffold that extensively innervates the CNS. This scaffold consists of distinct subsets of commissures and posteriorly coursing tracts. This developmental event is completed by approximately 48 hpf. At the same time, GnRH3-expressing cells immerge in the non-CNS trigeminal ganglia and pronephric ducts. The hypothalamic/pituitary GnRH3 fiber tract serves as a scaffold for the subsequent migration of GnRH3 soma from the olfactory region, through the ventral telencephalon to the POA-hypothalamus. This soma migration event begins at 3-4 dpf and ultimately results in four distinct GnRH3 CNS populations in the OB/TN, POA, ventral hypothalamus and facial lobe.

The process of GnRH3 system development is regulated by various factors that control soma differentiation, proliferation, and migration as well as fiber extension and targeting. Each of these processes is differentially controlled by multiple factors. Our findings show that GnRH3 serves as an early

autocrine/paracrine factor involved in regulating GnRH3 neuron proliferation and is necessary for normal fiber development. At later timepoints, Netrin1a acts through its receptor Dcc as a midline chemoattractant for GnRH3 commissural fiber tracts. Thereafter Netrin1a also plays a chemoattractive role that is necessary for GnRH3 soma migration into the

POA-hypothalamus. In looking into the role of Netrin2 and Shha, we did not find that these factors play a specific role in GnRH3 system development.

Through GnRH3 neuron-specific ablation experiments we have studied the GnRH3 embryonic origins and reproductive role. Our findings have shown that the POA-hypothalamic hypophysiotropic GnRH3 population originates in the olfactory region and that GnRH3 is necessary for normal gonad development and reproduction.

Future directions

The prospects for using the Tg(GnRH3:EGFP) line for studying various aspects of GnRH3 system development, CNS development, and sexual development are almost limitless. The inherent properties of the zebrafish as a model system and the simplicity by which GnRH3 neurons can be observed in our line make this an ideal model for perusing many avenues of research.
Several possible lines of research include:

1. Further study of factors regulating the development of the GnRH3 system via use of knock-down, OE, and new technologies such as the immerging synthetic zinc finger nuclease-mediated knocknock-outut (Ekker, 2008), as well as conditional expression and ablation studies. These studies could also

use the Tg(GnRH3:EGFP) line to revisit factors that have been previously looked at, however with a focus on their role in fiber development.

2. The use of Tg(GnRH3:EGFP) larvae and adults to isolate various GnRH3 neuron populations at different developmental stages. This focus can be accomplished by use of flow cytometry. Isolated GnRH3 neurons from different time points and different populations can be used for microarray-based and functional genomics studies that can be the basis for exploring many GnRH-related issues.

3. Study of puberty, exploring the morphological and genetic changes that GnRH3 neurons undergo during puberty. This can be investigated by observing structural changes to GnRH3 fibers and analysis of gene up- and down-regulation in GnRH3 neurons before, during and after puberty.

4. GnRH3 neurons can easily be targeted for patch-clamp recordings both in intact larvae as well as in mature extracted brains, thus enabling a variety of electrophysiological studies.

5. A slightly modified line, using a GFP protein that has a short half-life, can be used. Such a line would allow the use of GFP expression as a quantitative reporter mirroring changes in GnRH3 protein levels. This line could also be used to study up- and down-regulation of GnRH protein levels in response to various chemical and behavioral stimuli.

6. The Tg(GnRH3:EGFP) line can be used together with other lines that express various CNS markers, as a model for toxicological studies into the effects of developmental neurotoxins. Transgenic zebrafish larvae can be easily used in

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high throughput screening studies looking for neurotoxins that affect various aspects of early CNS development. Such a study would be relevant to understanding human neurodevelopmental issues.

7. Lastly, the study of interactions between GnRH3 neurons and other neuronal populations that influence GnRH activity would be extremely interesting. This broad topic can be simplified by development of additional promoter-reporter transgenic lines in which other reporter proteins such as RFP are used. In such lines, factors such as kisspeptin, GnRH2, dopamine and others would be marked, subsequently these lines could be crossed with the Tg(GnRH3:EGFP) line to obtain double promoter-reporter lines.

As this list suggest, the options are indeed limitless and very exciting.

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