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Endocrinology 2008 149:4596-4604 originally published online May 22, 2008; doi: 10.1210/en.2008-0403

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Hypothalamic GnRH neurons are essential for initiation and regulation of reproductive function. In addition to pituitary gonadotrope stimulation, activity of GnRH through its receptor (GnRHR) has been suggested to include autocrine regulation of the GnRH neuron. Two hypogonadal mouse strains, the Gnrh1 mutant (hpg) mice and Gnrhr mutant mice were used to investigate the potential role of GnRH signaling in the proper development and maintenance of GnRH neurons. Immunocytochemical analysis of heterozygous hpg mice revealed a GnRH neuron population that was normal in size and distribution, indicating no effect from reduced Gnrh1 gene dosage on the neurons themselves. To visualize GnRH neurons in homozygous GnRH-deficient hpg mice, heterozygous hpg mice were crossed with GnRH-green fluorescent protein (GFP) transgenic mice with targeted expression of the GFP reporter gene in GnRH neurons. Analysis of forebrains of homozgyous hpg/GFP-positive mice immunostained for GFP revealed a normal population size and appropriate distribution of GnRH neurons in hpg mice, with immunoreactive neuronal processes present at the median eminence. Similarly, adult mice deficient in functional GnRHR possessed a full complement of GnRH neurons in the basal forebrain that was indistinguishable from the distribution of GnRH neurons in their wild-type counterparts. Moreover, hpg/GFP neurons retained the ability to generate spontaneous bursts of action potential firing activity, suggesting that GnRH peptide is not required for this function. These data establish that autocrine-paracrine GnRH-signaling is not a prerequisite for the developmental migration of GnRH neurons into the brain or for the projection of GnRH neurosecretory axons. (Endocrinology 149: 4596–4604, 2008)

GnRH-1 DECAPEPTIDE IS A key central regulator of reproductive function. GnRH neurons develop outside the central nervous system in the embryonic nasal placodes and undergo a series of distinctive developmental events before their ultimate role in pituitary gonadotrope regulation (1, 2). The migration of GnRH neurons from the nasal placode into the basal forebrain followed by the projection of neurosecretory axons to the median eminence (ME), where the hormone is released, are critical processes necessary to ensure proper central regulation of reproductive function (3). GnRH neuronal development relies upon cooperative signaling from surrounding tissues during a short developmental window. Regulation of GnRH neuron development is dependent on factors within the nasal code environment (e.g. activator protein-2α and fibroblast growth factors) (4, 5) that first locally affect fate specification of GnRH neurons, and then on combinations of factors [e.g. nasal embryonic LHRH factor (NELF), γ-aminobutyric acid (GABA), netrins] that directly affect GnRH neuron migration and axon targeting of the ME (6–9).

A potential autocrine role of GnRH itself in the development and function of GnRH neurons has been proposed from studies of the GnRH-deficient hypogonadal (hpg) mouse (10). A deletion of the Gnrh1 gene results in hpg mice that do not synthesize GnRH decapeptide (10). Previous examination of the distribution of GnRH neurons in the GnRH-deficient hpg mouse model was dependent upon detection of a short 5’ segment of GnRH mRNA because the deletion prevented transcription of the complete coding sequence and the production of detectable levels of peptide (10, 11). GnRH mRNA levels as measured by in situ hybridization in adult hpg mouse brains were greatly reduced, detecting only 16–20% of the normal GnRH neuronal population size (10, 11). The reduction in GnRH neuron number in hpg mice was attributed to either of two explanations. One hypothesis was that the truncated GnRH mRNA produced in the presence of the gene deletion was rendered unstable, resulting in accelerated degradation of the transcript with reduced cellular levels

First Published Online May 22, 2008
Abbreviations: ENU, N-ethyl-N-nitrosourea; GFP, green fluorescent protein; Gnrh1, GnRH receptor; HET, heterozygous for the Gnrh1 mutation; hpg, hypogonadal; ICC, immunocytochemistry; ME, median eminence; NGS, normal goat serum; NS, normal saline; OVL, organum vasculosum of the lamina terminalis; TBS, Tris-buffered saline; WT, wild type.
Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.
such that 80% of hpg GnRH neurons escaped detection by in situ hybridization (10, 11). An alternative possibility was that a GnRH peptide is necessary during development for GnRH neuron proliferation, migration, or survival, resulting in the loss of 80% of the population in the absence of GnRH. This potential reduction of GnRH neurons may explain the results of our recent study using targeted viral vectors that identified only a small number of hpg GnRH neurons (12). The embryonic expression of the ligand and GnRHR receptor (GnRHR) is consistent with a developmental role for GnRH (1, 2, 13); however, the role of GnRH as a candidate trophic factor on GnRH neurons in vivo remains unproven.

GnRH stimulation of pituitary gonadotropes activates the GnRHR, a G protein-coupled receptor, to release LH and FSH (1, 2, 13). Evidence of GnRHR activation in GnRH neuronal cell lines, primary GnRH neurons, and adult GnRH neurons in hypothalamic slice preparations has been shown to stimulate physiological responses, although trophic actions mediated by GnRHR have yet to be demonstrated in GnRH neurons (12). The embryonic expression of the ligand and GnRHR receptor (GnRHR) is consistent with a developmental role for GnRH (1, 2, 13); however, the role of GnRH as a candidate trophic factor on GnRH neurons in vivo remains unproven.

In mice, the earliest developmental expression of GnRH begins at embryonic d 10.5 in the nasal placode (26). The transcriptional activity of the Gnrh1 gene has been used to define the GnRH neuron because no other specific and unique marker to identify these cells has been demonstrated. The migration phase of development coincides with GnRHR expression on GnRH neurons (27), suggesting that GnRH may act as a regulatory factor during this period. Activation of the GnRHR in GnRH neuronal cell lines, primary GnRH neurons, and adult GnRH neurons in hypothalamic slice preparations has been shown to stimulate physiological responses, although trophic actions mediated by GnRHR have yet to be demonstrated in GnRH neurons in vivo (27, 28).

To determine whether GnRH indeed plays a critical role in the establishment or the maintenance of GnRH neurons in vivo, GnRH neurons were studied in different hypogonadal mouse models: GnRH-deficient hpg mice (30) and GnRHR-mutant mice (Gnrhr<sup>mut/mut</sup>) (29). These GnRH signaling-deficient models were examined for GnRH neuron numbers, distribution, and electrophysiological firing activity. Because detection of GnRH cannot be used to identify GnRH neurons in hpg mice, mice heterozygous for the hpg mutation were crossed with transgenic mice with targeted green fluorescent protein (GFP) expression in GnRH neurons (30). Mice resulting from this cross allowed the visualization of hypothalamic GnRH neurons with GnRH-GFP expression and activity, thus facilitating analysis of the mutant GnRH neuronal population and distribution even in the absence of GnRH.

Materials and Methods

Animal husbandry

All mice were maintained in a 12-h light, 12-h dark cycle, with food and water available ad libitum. Humane animal care and welfare were in accordance with guidelines established by the Harvard Medical Area Standing Committee on Animals in the Harvard Medical School Center for Animal Resources and Comparative Medicine, approved by the Institutional Animal Protocol and Care Committee of Baylor College of Medicine, or approved by the Animal Care and Use Committee of the University of Virginia.

GnRH-deficient hpg mice

Mice heterozygous for the Gnrh1 gene deletion (Gnrh1<sup>1<sup/>+<sup>-</sup></sup>; HET) in the C3H/HeJc101/H background strain were obtained from The Jackson Laboratory (Bar Harbor, ME). HET mice are fertile and generated wild-type (Gnrh1<sup>+/+</sup>; WT), HET, and homozygous (Gnrh1<sup>−/−</sup>; hpg) mice in Mendelian ratios. Genotypes of offspring were determined by PCR of genomic tail DNA with three primers: primer 1: 5′-TATGCGCTACACCTTCAAGCG (sense, intron two, upstream of the deletion); primer 2: 5′-GGGCCTTCGAGGCTTAG (antisense, intron two, within the deleted region); primer 3: 5′-GCTCCACCTCCTCCGAGCTTG (antisense, downstream of the deleted region). Primers 1 and 2 are expected to generate a PCR product of 613 bp in size from the WT allele, and primers 1 and 3 are expected to generate a 536-bp product from the mutant allele.

GnRH-deficient GnRH-GFP mice

To facilitate the visualization of GnRH neurons in the brains of hpg mice devoid of immunoreactive GnRH, HET mice were crossed with transgenic mice engineered to specifically express enhanced GFP as a targeted reporter in GnRH neurons (30). A breeding pair [heterozygous for the Gnrh1 mutation and positive for the GnRH-GFP transgene; (HET/GFP)] was generated and used as founders to generate all subsequent GnRH-GFP reporter mice used for this study. PCR genotyping of genomic tail DNA was performed for the Gnrh1 gene as described above. The presence of the GnRH-GFP transgene was confirmed by PCR with GFP primers (forward, 5′-GACCCTATACGGCCCAAGTT; reverse, 5′-AAAGCTGTCTGCTGTTG).

Gnrh mutant mice

Homozygous Gnrhr<sup>mut/mut</sup> mice and their WT littermates were generated by crossing heterozygotes in a breeding colony maintained at Baylor College of Medicine. Genotypes were determined as previously reported (29) using PCR and primers in the flanking genomic sequence (forward, 5′-TCACATTCCTGAGCCGTC; reverse, 5′-TCACATTCCTGAGCCGTC), following Ddel digestion of the amplified products. The mice were maintained in a 129/SvEv background strain.

Immunocytochemistry and analysis of GnRH and GnRH-GFP neurons

Adult male mice (8 wk old) were anesthetized, transcardially perfused with 10–20 ml PBS (pH 7.4) using a 26-gauge needle and hand-held syringe. Testes were removed and imaged. Testes were dissected, postfixed in 4% paraformaldehyde, cryoprotected in 20% sucrose solution, 0.01% sodium azide, and cryosectioned (Microm International GmbH, Walldorf, Germany) in the coronal plane from the accessory olfactory bulbs to the tip. For quantitative bright-field microscopy, 40-μm floating sections, or 20-μm thaw-mounted sections, were pretreated with 1% hydrogen peroxide in PBS containing 0.4% Triton X-100 (PBST) for 10 min to remove endogenous peroxidase activity, then incubated with either polyclonal rabbit anti-GnRH antibody (ImmunoStar, Hudson, WI;
1.32,000 dilution) or rabbit anti-GFP (Invitrogen, Carlsbad, CA; 1:10,000) in 10% normal horse serum, 4% normal donkey serum in PBS/T for three nights at 4C. Sections were then incubated with a donkey antirabbit IgG biotinylated secondary antibody (Jackson ImmunoResearch, West Grove, PA) at 1:500 dilution using Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA), and visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Pierce, Rockford, IL). Floating brain sections were serially aligned and mounted on glass microscope slides in a rostral to caudal order. After dehydration of sections, the slides were cleared, sealed, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

For immunofluorescence microscopy, 18-μm coronal brain sections were thaw-mounted onto poly-l-lysine coated slides. Slides were incubated in polyclonal goat anti-GFP (Genetex, Inc., San Antonio, TX; 1:5000). The subsequent secondary antibody, donkey anti-goat was directly conjugated to Alexa-488 (Molecular Probes, Inc., Eugene, OR; 1:1000) to visualize GFP (green). The slides were mounted, cleared, and coverslipped with Vectashield (Vector Laboratories).

Before bright-field and epifluorescence analysis of the numbers and distribution of GnRH and GnRH-GFP neurons, slides were coded to obscure sample identification. GnRH or GnRH-GFP neurons were counted in each section, and imaged at the organum vasculosum of the lamina terminalis (OVLT) and ME. Data for total neuron numbers and neurons counted in the rostral to caudal serial sections aligned at the OVLT as a common anatomical landmark between samples were graphed using Prism statistics software (GraphPad Software, Inc., San Diego, CA).

**Immunocytochemistry of Gnhrh mutant mice**

Eight-week-old male and female Gnrhrmut/mut mice, previously generated by N-ethyl-N-nitrosourea (ENU)-mutagenesis bear a T ↔ C transition resulting in the missense L117P mutation (29), were transcardially perfused as above but without post-processing brain tissue in sucrose. Brains from Gnrhrmut/mut and WT animals were embedded in 5% agarose, cryoprotected, and cut coronally at 50 μm using a vibrating microtome (Leica VT1000S, Wetzlar, Germany). Sections were placed free-floating in containers with Nitex (Wildlife Supply Co., Buffalo, NY) mesh bottoms in 0.05 M PBS, pH 7.4. Each brain was cut into three sequential containers so that each antisera/container used provided a full representation of sections from one third of the adult brain. Sections were pretreated at 4C as follows: 0.1 M glycine in PBS for 30 min, 0.5% sodium borohydride in PBS for 15 min, and 5% normal goat serum (NGS) with 0.5% Triton X-100 and 1% hydrogen peroxide in PBS for 30 min. Adjacent sections were then incubated in different antisera directed toward GnRH, either LR-1 rabbit antiserum (1:1000; PA121 obtained from Affinity Bioreagents, Golden, CO) in 1% BSA with 0.5% Twx for 3 nights at 4C. After primary incubation, sections were washed at room temperature four times for 15 min each in 1% NGS with 0.02% Twx and then incubated with donkey antirabbit IgG biotinylated secondary antibody (Jackson ImmunoResearch) at 1:500 dilution in 1% NGS with 0.32% Twx for 2 h at room temperature. Sections were subsequently rinsed four times in 0.02% Twx-PBS followed by incubation in peroxidase-conjugated streptavidin (Jackson ImmunoResearch; diluted 1:500) for 1 h, washed for 1 h in Tris-buffered saline (TBS), and developed with 0.025% 3,3′-diaminobenzidine in TBS with 0.2% nickel ammonium and 0.02% hydrogen peroxide for 5 min. After immunocytochemistry, sections were washed in TBS, mounted on gelatin-subbed slides, dehydrated, and coverslipped with Permount (Fisher Scientific). GnRH-immunoreactive neurons were counted from two containers from each brain immunoreacted for one of two GnRH antisera giving a spread of 150 μm between each analyzed section within either antisera. GnRH neurons were counted on a BH-2 photomicroscope (Olympus America, Inc., Center Valley, PA) and analyzed as a function of their rostral to caudal distribution to fully characterize the GnRH neuronal pop-

**Electrophysiological recordings of hpg/GFP neurons**

To eliminate the effect of steroid milieu on GnRH neurons during electrophysiological recordings, adult (>2 months) female hpg/GFP mice were ovarioctomized under isoflurane (Burns Veterinary Supply, Westbury, NY) anesthesia. Postoperative analgesia was provided by a long-acting local anesthetic (bupivacaine, 0.25%, 7.5 μl per site; Abbott Laboratories, North Chicago, IL). Recordings were performed 5–9 d after surgery. Brain slices from WT/GFP, n = 4) and hpg/GFP, n = 7) mice were prepared as previously described (31). Briefly, all solutions were bubbled with a 95%/5% CO2 mixture throughout the experiments and for at least 15 min before exposure to the tissue. Mice were decapi-

**Results**

**Extracellular recording data collection and analysis**

Data were recorded using MultiClamp 700B amplifier (Axon Instruments, Foster City, CA) running Clampex 9.2 software (Axon Instruments) in voltage-clamp mode with a pipette holding potential of 0 mV. Data were filtered at 10 kHz and digitized with a Digidata 1332A (Axon Instruments). Extracellular firing activity (events) was analyzed with the MiniAnalysis program (Synaptosoft Inc., Decatur, GA).

Data are reported as mean ± SEM. Events were counted and binned at 10-sec intervals, and were analyzed for mean firing rate. Firing rate (Hz) was determined in two ways: total events detected during 6 min (duration of the shortest recording) from each cell divided by 360 sec, and total number of events detected from each cell divided by the duration of the recording. No differences arose from these two approaches and the latter is reported.

**Statistics**

When appropriate, parametric statistics were used with data that satisfied Bartlett tests for equal variances. Otherwise, nonparametric statistics were used. Differences in number of GnRH neurons were analyzed by two-tailed Student’s t test or Mann-Whitney U test. Data for total neuron numbers and neurons counted in the rostral to caudal serial sections were analyzed using Prism statistics software (GraphPad Software, Inc., San Diego, CA). In electrophysiology experiments, groups were compared with Student’s t test. Differences were considered significant when P < 0.05.

**HET mice possess a normal complement of GnRH neurons**

In addition to the complete lack of detectable GnRH in the homozygous hpg mice, the HET mice have been reported to contain only 20% of the hypothalamic GnRH content of their wild-type counterparts, yet they seemed phenotypically normal and had normal fertility (10). Therefore, to fully characterize the GnRH neuronal pop-
ulation in this mouse model, studies were first carried out to compare the GnRH neuronal population in the brains of WT mice with HET littermates, heterozygous for the Gnrh1 gene deletion. The size and distribution of the GnRH neuronal population were compared between these two genotypes to determine whether a dosage effect of the Gnrh1 gene was manifest at the level of the GnRH neurons. GnRH neurons were immunostained and counted in serial coronal sections of adult male WT and HET mice. GnRH immunoreactive neurons were distributed bilaterally through the medial ventral forebrain in similar patterns in both the WT and HET hpg mouse brains. The total number of hypothalamic GnRH neurons counted in each section from the septal preoptic area to the ME was not different between WT and HET (Fig. 1A). The rostral to caudal distribution of all GnRH neurons was compared by aligning the serial sections with the OVLT as a neuroanatomical reference point. The mean number of GnRH neurons in each section rostral and each section caudal to this reference point were plotted as a histogram of GnRH neurons per section in the coronal series to compare the distribution in WT and HET mice (Fig. 1B). GnRH neuron distributions were similar, with both WT and HET mice having the highest concentration of GnRH neurons located at the OVLT and smaller numbers of GnRH neurons present as far caudally as the ME (Fig. 1, C–G). The similar patterns of rostral to caudal distribution of GnRH neurons in the WT and HET mice suggest that similar GnRH neuronal migration patterns likely occurred developmentally between these two genotypes.

**HET mice bred with GnRH-GFP transgenic mice allow detection of hpg GnRH neurons**

To facilitate the visualization of GnRH neurons in the brains of hpg mice devoid of immunoreactive GnRH, HET mice were crossed with transgenic mice engineered to specifically express enhanced-GFP as a targeted reporter in GnRH neurons (30). Breeding of HET mice with GnRH-GFP mice generated a GnRH-GFP transgene-positive Gnrh1-mutant mouse line as shown by PCR genotyping (Fig. 2A). The progeny of the resulting HET/GFP breeding pair were used for this study. hpg/GFP male mice were easily identified by their underdeveloped external genitalia and decreased testis size phenotype (Fig. 2B). Immunocytochemistry (ICC) for GFP in both HET/GFP and hpg/GFP males identified a hypothalamic population of neurons consistent in distribution and morphology with that of GnRH neurons (Fig. 2C). GFP immunostaining patterns were similar in the OVLT and ME of both HET/GFP and hpg/GFP male mice (Fig. 2, D–G).

**hpg/GFP mice reveal an intact GnRH neuronal population size and distribution**

The total number of GnRH-GFP neurons detected in hpg/GFP male mice was not different than in the HET/
GFP (Fig. 3A). In addition, there were no significant differences in the rostral to caudal distribution of GnRH-GFP neurons in brains of in hpg/GFP and HET/GFP male mice (n = 3) (Fig. 3B). The similar patterns of distribution of GnRH-GFP neurons suggest that GnRH neuronal differentiation, proliferation, migration patterns, and survival occur normally despite the absence of GnRH.

GnRH receptor mutant mice possess an intact GnRH neuronal population

As an alternate hypogonadal mouse model, GnRH neurons in the ENU-generated Gnrhr<sup>mut/mut</sup> mice were compared with GnRH neurons in WT littermates. Homozygous Gnrhr<sup>mut/mut</sup> mice possess a GnRH receptor with a single amino acid change within the third transmembrane domain that renders the receptor nonfunctional (29). Detection of GnRH neurons using anti-GnRH PA121 demonstrated that the number of GnRH neurons found in the brains of Gnrhr<sup>mut/mut</sup> mice was not significantly different from the total GnRH neurons found in WT (Fig. 4A). Analysis of the rostral to caudal distribution of GnRH neurons counted in sections aligned at the OVLT showed near identical patterns between Gnrhr<sup>mut/mut</sup> mice and WT (Fig. 4B). GnRH neurons in mice possessing the nonfunctional GnRHR are thus present in normal numbers after an apparently normal developmental migration into the brain.

To validate the use of the recently available PA121 antibody, the comparison of total GnRH neuron numbers and their distribution in Gnrhr<sup>mut/mut</sup> mice was not significantly different from the total GnRH neurons found in WT (Fig. 4A). Analysis of the rostral to caudal distribution of GnRH neurons counted in sections aligned at the OVLT showed near identical patterns between Gnrhr<sup>mut/mut</sup> mice and WT (Fig. 4B). GnRH neurons in mice possessing the nonfunctional GnRHR are thus present in normal numbers after an apparently normal developmental migration into the brain.

To validate the use of the recently available PA121 antibody, the comparison of total GnRH neuron numbers and their distribution in Gnrhr<sup>mut/mut</sup> and WT mice were repeated using the well-characterized anti-GnRH LR1 antibody. In adjacent sections analyzed from the same brain region, the number of WT GnRH neurons was not different regardless of the anti-GnRH polyclonal antibody used.
Similarly, both antibodies detected similar numbers of Gnrhr mut/mut GnRH neurons (PA121, 127/13 neurons; LR1, 123/16 neurons; n = 5) to reveal these antibodies had equivalent sensitivity to detect GnRH neurons.

Electrophysiology of hpg/GFP neurons

The normal migration and position of GnRH neurons in hpg mice does not mandate their normal function. To begin to examine the effects of endogenous GnRH on GnRH neuronal activity, we used targeted extracellular recording to study firing activity of GnRH neurons in hpg/GFP mice. Representative examples of firing activity from WT/GFP and hpg/GFP mice are shown in Figure 5A. There were no significant differences in mean firing rate between groups (Fig. 5B; $P = 0.38$). Furthermore, GnRH neurons fired in small bursts of activity as previously described (32, 34, 35), suggesting that the lack of GnRH peptide does not preclude spontaneous generation of bursts of action potentials in GnRH neurons.

Discussion

Developmental organization of the GnRH neuronal network is a critical prerequisite for sexual differentiation, puberty, and fertility, yet a comprehensive understanding of the regulatory signals that establish this neuronal population is lacking (36). Here we report the characterization of adult GnRH neurons in two models of hypogonadal mice with primary deficiencies that abolished GnRH signaling. In contrast to the pro-migratory role of GnRH previously reported (37, 38), the migration of hypothalamic GnRH neurons into the brain occurred normally both in mice deficient in GnRH (hpg), and mice with an inactivating Gnrhr mutation. In both hypogonadal strains used in this study, the sizes of the GnRH neuronal population and their distributions throughout the basal forebrain were normal in the ages used in this study, with axonal processes appropriately projecting to the ME. These findings provide evidence that the GnRH neuropeptide and its signaling through the cognate receptor are not critical for the development or maintenance of the hypothalamic GnRH neuronal population or for the formation of the neuroendocrine afferents.

The hypothesis that GnRH may developmentally regulate GnRH neurons originated from the distinctive expression of GnRH at times and in a place not associated with GnRH endocrine function—the embryonic nasal compartment (1, 2). To date, however, a function of Gnrh1 expression coincident with the migration of GnRH neurons toward the brain has not yet been demonstrated. Embryonic GnRH expression and peptide processing have been measured (both directly and indirectly) to increase, paralleling maturational events of GnRH neurons, including cytoskeletal changes associated with migration (36, 39–41). Therefore, a plausible role for GnRH in GnRH neuronal development has been suggested and supporting evidence has been described in different models.

In culture, the addition of exogenous GnRH to FNC-B4, GnRH-secreting cells derived from human olfactory epithelium, induced GnRHR-dependent changes consistent with neuronal differentiation, and included increased GnRH ex-
pression combined with down-regulation of nestin expression (37). Furthermore, changes in GnRH-stimulated FNC-B4 cells included axon growth, actin cytoskeleton remodeling and 3- to 4-fold increases in cell motility in Boyden chamber experiments (37). These findings in FNC-B4 cells strongly suggested an autocrine role for GnRH in the differentiation and migration of GnRH neurons. In vivo, a recent study in zebrafish that disrupted GnRH translation early in development by morpholino-modified antisense oligonucleotides altered the pattern of GnRH neuronal migration (38). This observation is a strikingly divergent from our findings in mice, where no deviations in the size or distribution of the adult GnRH neuron population were found despite the biosynthetic deficiency of GnRH or the loss of function of its specific receptor. This may represent a class difference of GnRH neuron development in fishes. Alternatively, the anomalous GnRH neuron development may represent the occasional non-target-related phenotype that can occur in morpholino knockdown experiments that may have simultaneously silenced an essential developmental gene for GnRH neurons (42).

It also may be possible that a part of the Gnrh1 gene or gene product spared in the deletion of exon 2 in hpg mice enables GnRH neuronal migration. This gene product would need to function independently of normal GnRHR signaling because GnRH neuronal migration occurred in Gnrhr<sup>mut/</sup>mut mice. Furthermore, a second form of GnRH (GnRH2) and a type II GnRH receptor (GnRHR2) exists in most species, and it could be argued that normal development may be mediated through an up-regulation of this second system in the absence of GnRH1 or GnRHR1. The compensatory GnRH2/ Gnrhr2 hypothesis is unlikely, however, in our model, because this system appears to be absent in mice (43).

We have provided further observations that the heterozygous deletion in the Gnrh gene in adult male HET mice resulted in the identical reproductive phenotype as their WT counterparts, confirming previous reports (10). Moreover, we can now extend these observations to indicate that a comparable number of hypothalamic GnRH neurons is identified by ICC in the forebrain of HET mice as is found in their WT male littermates. This stands in contrast to an earlier description of an 80% reduction in hypothalamic GnRH content in hpg HET (10), a discrepancy that may reflect differences in methodology. Our observation in 2- to 3-month-old HET male mice suggests little to no effect of haploinsufficiency of the Gnrh1 gene on the GnRH neuronal network or on reproductive capacity.

To circumvent the reliance on potentially unstable Gnrh1 mRNA to identify GnRH neurons in homozygous hpg mice, transgenic GnRH-GFP mice were used, providing a reporter gene to identify the hpg GnRH neurons by crossing the two mouse strains. In these mice, genetically targeted GFP in GnRH neurons identifies 99.5% of GnRH neurons with little ectopic GFP fluorescence (30). GFP-labeled neurons were observed in neuroanatomical regions consistent with appropriate GnRH neuronal distribution in the caudal olfactory bulb, medial septal area, and medial preoptic area, with GFP-positive fibers found in the ME (30). The transgenic GnRH promoter/enhancer activity in hpg/GFP mice effectively directed GFP expression to identify hypothalamic neurons that are morphologically and neuroanatomically similar to GnRH neurons. It should be noted that other cells were observed with low levels of immunodetectable GFP in the brains of all GnRH-GFP mice; these cells appeared consistent with multipolar cells located dorsal and lateral to the normal distribution of GnRH neurons identified in previous reports (44, 45). These cells were detected in all GnRH-GFP genotypes and were distinct from populations of hypothalamic GnRH neurons, thus easily avoided during cell count analysis by their location, size, morphology, and reduced intensity of GFP expression.

We substantiated that GnRH neurons can develop normally independent of GnRH signaling with the corollary experiment that examined the content and distribution of GnRH neurons in the Gnrhr<sup>mut/</sup>mut mouse generated by ENU mutagenesis (29). The Gnrhr<sup>mut/</sup>mut mouse has a hypogonadal phenotype similar to the hpg and hpg/GFP mice due to the inability of pituitary gonadotropes to respond to GnRH. These GnRH-insensitive mice provided the next logical avenue to determine whether a Gnrhr signaling mechanism was necessary to support the development, differentiation, and maintenance of GnRH neurons. The similar distribution of GnRH neurons in homozygous Gnrhr<sup>mut/</sup>mut mice and WT control littermates supported the conclusion from the hpg GnRH neuron analysis that developmental regulation and maintenance of adult GnRH neurons is independent of GnRH activation of the Gnrhr.

In the present study, the absence of any deficit in the size or the rostral to caudal distribution of the GnRH neuronal population implies that GnRH is not necessary for GnRH neurons to properly differentiate, migrate into the appropriate areas of the brain, extend axons to the ME, or survive into adulthood. The possibility remains that GnRH may act cooperatively to facilitate the activity of other factors. However, unlike the genetic removal or inhibition of other regulators (e.g. FGFR signaling, GABA, and cholecystokinin) which resulted in significant (20–30%) reductions in size of the GnRH neuronal population or disruption of its organization (46–48), the absence of GnRH signaling did not indicate any partial contribution to GnRH neuronal development. Rather, the completion of GnRH neuron development in the absence of GnRH narrows a potential role of GnRH to post-migratory neurosecretory phases of GnRH neuron differentiation and function.

Beyond effects on migration, autocrine or paracrine GnRH regulation of hypothalamic GnRH neuron function has been proposed to contribute to the regulation of pulsatile GnRH secretion through autocrine or paracrine feedback signaling (49). GnRH is postulated to regulate GnRH neuronal intrinsic pulsatility in vivo, in a GnRH neuronal cell line, in primary GnRH neurons, and in brain slices (13, 28, 50–52). Models of Gnrhr-mediated GnRH autoregulation of pulsatile secretion have been proposed to be both inhibitory or stimulatory (28). Here we show hpg/GFP neurons are capable of firing spontaneous bursts of action potentials, a fundamental component of GnRH neuron function (53). The mean firing rate in the adult hpg/GFP neurons was not significantly different from WT/GFP neurons. hpg/GFP neurons further exhibited individual variability in burst firing similar to wild-type GnRH neurons in previous reports (32, 34, 35).
Although GnRH peptide has been reported to alter the activity of GnRH neurons in both stimulatory and inhibitory directions (28, 51, 54), the decapetide itself is apparently not critical for the genesis of electrical activity by these cells.

Interpretation of the electrophysiological data obtained in ovariectomized female hgp mice, whose male counterparts were used in the ICC studies, indicated that spontaneous neuronal activity occurred in GnRH-deficient neurons. We do not anticipate that this is a gender-specific event, and we expect that male hgp/GFP neurons would be similarly capable of spontaneous firing. Nonetheless, it is possible that sexual dimorphism may exist in WT GnRH neurons and future experiments will explore these questions in hgp GnRH neurons. Moreover, the investigation of electrophysiological responses of hgp/GFP neurons to relevant neuropeptide and hormonal stimuli may reveal as yet undetected effects of GnRH peptide on the electrophysiological properties of GnRH neurons.

In summary, normal GnRH neuronal development occurs in two strains of hypogonadal mice lacking the GnRH decapeptide or expressing inactive GnRH, respectively. These results indicate that in the absence of GnRH autocrine or paracrine actions, the normal complement of GnRH neurons, become specified in the nasal placode and these neurons migrate normally into the medial basal forebrain to establish appropriate neurosecretory contacts with the ME.

Acknowledgments

We extend gratitude for the collaborative assistance from Monica Justice at the Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX. Also, our appreciation is extended to Rona Carroll for the constructive comments and critical review during the manuscript preparation.

Received March 24, 2008. Accepted May 14, 2008.

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This work was supported by National Institutes of Health (NIH) Grants R21 HD050412 (to U.B.K.), R01 HD19938 (to U.B.K.), NIH MH63954 (to S.M.), NIH M57542 (to S.M.), NIH R01 HD 54861 (to S.M.M.), NIH T32 HD07382 (P.C.), NIH F32 HD056765 (to P.C.) and NIH DC00934 (to S.T. and G.A.S.).

Disclosure Statement: The authors have nothing to disclose.

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