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Development of the gonadotropin-releasing hormone (GnRH) neuronal system in the mouse

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DEVELOPMENT OF THE GONADOTROPIN-RELEASING HORMONE (GNRH) NEURONAL SYSTEM IN THE MOUSE

by

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ABSTRACT

DEVELOPMENT OF THE GONADOTROPIN-RELEASING HORMONE (GNRH) NEURONAL SYSTEM IN THE MOUSE

by

Izhar Livne

Adviser: Dr. Marie J. Gibson

Gonadotropin-releasing hormone (GnRH) neurons are derived from the olfactory placode and emigrate into the forebrain during embryogenesis to reside in the septal, preoptic and anterior hypothalamic areas. The majority of GnRH neurons are neurosecretory and project to the median eminence. In the median eminence GnRH is released to induce secretion of the gonadotropins, LH and FSH, from the anterior pituitary and acts as the central regulator of the developing and mature pituitary-gonadal axis.

I have analyzed the cellular associations of GnRH cells during their migration and how the migration of these neurons is correlated with their biochemical and morphological differentiation. Ultrastructural observations revealed that these neurons migrate through the nasal septum within axonal fascicles of the olfactory and vomeronasal nerves. These axonal fascicles serve as a conduit through which GnRH neurons migrate into the forebrain. This study also revealed that the capability to process GnRH to its bioactive

form is acquired during the transition from the nasal septum to the forebrain.

During their migration in the nasal septum many GnRH neurons express the growth associated protein GAP-43 which is associated with axonal growth in many neuronal systems. However, once in the forebrain, most GnRH neurons cease to express GAP-43 and, unlike other neuronal populations, GnRH axons do not accumulate GAP-43 as they extend towards their target.

Since I observed that the differentiation of GnRH neurons is correlated with their migratory stage, I tested whether interrupting their migration would interfere with their capability to complete their maturation. For these experiments I used the mutant hypogonadal (hpg) mouse which has an undeveloped reproductive tract due to a truncated GnRH gene. I transplanted normal fetal nasal septum tissue, containing the migratory population of GnRH neurons, into the brain of adult hpg. Some of the transplanted neurons continued their migration in the host brain and their axons grew through the host parenchyma to terminate at their normal target where they secreted bioactive GnRH. Thus, heterochronic transplantation of migratory GnRH neurons does not prevent these neurons from completing their differentiation and establishing their functional connections.

The truncated GnRH gene is transcribed in the hpg brain but the neuropeptide is not synthesized and GnRH neurons in the mutant do not perform their principal neuroendocrine function. I, therefore, asked whether the mutant neurons can elaborate and maintain their normal axonal connection with their primary secretory target - the

median eminence. Using a combined methodology of in situ hybridization and retrograde tracing with Fluoro-Gold I established that the mutant neurons, that are devoid of their major neurosecretory product can, nevertheless, elaborate and maintain their axonal projections to the median eminence. I concluded that the capability of GnRH neurons to recognize and interact with their target is independent of their neurosecretory function.

Finally, I have demonstrated that testosterone administration to neonatal hpg males can restore functional copulatory behavior in the adult. This finding indicates that the GnRH deficiency in the neonatal hpg results in reduced androgen secretion. Therefore, it can be inferred that GnRH secretion is already functional and critical for stimulation of the pituitary-gonadal axis in the early postnatal period.

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INTRODUCTION

Gonadotropin - releasing hormone (GnRH) is the central regulator of the hypothalamic-pituitary-gonadal axis. GnRH is a decapeptide [1, 2] which is synthesized from a larger precursor by a CNS neuronal population: the GnRH neurons. These neurons are scattered throughout the mammalian forebrain and their number is estimated to be 675 in the mouse [3]. The major concentration of GnRH cells is distributed throughout the medial septum, diagonal band of broca, preoptic area, and anterior hypothalamus [4, 5, 6, 7]. These neurons project to a variety of CNS regions but their major terminal field is at the median eminence (ME, [8, 9]) where GnRH is released in a pulsatile fashion [10, 11] and delivered via the hypophyseal portal system to the anterior pituitary. In the anterior pituitary the neurohormone stimulates the pulsatile release of the gonadotropins, LH and FSH [12]. Other circumventricular organs innervated by GnRH cells are the organum vasculosum of the lamina terminalis (OVLT, [13]) and the subfornical organ [5].

GnRH neurons differentiate from the olfactory placode of the mammalian embryo and enter the forebrain with the central roots of the nervus terminalis and vomeronasal nerves, to reside in their adult distribution areas [14, 15, 16, 17]. In the mouse, most of these neurons emerge from the mitotic cycle on embryonic day 10.5 (E10.5) and commence expression of the GnRH gene by E11.5 [14]. On E12.5 the number of cells synthesizing the GnRH protein in the nasal septum is similar to the number of GnRH neurons in the adult

forebrain [3, 14]. Immunocytochemistry with an antibody for the pro-GnRH precursor was used to follow these neurons as they emerge from the olfactory placode and form cords of migratory cells in the nasal septum [15, 16]. Subsequent to their emigration from the olfactory placode, on days E12.5-16.5, the cells penetrate into the medial ventral forebrain and migrate caudally to reside in the septal, preoptic and anterior hypothalamic areas [16].

GnRH is essential for the development of a functional reproductive system. In a GnRH deficient mutant, the hypogonadal (hpg) mouse, the reproductive organs fail to develop and remain juvenile in the adult [18]. The hpg homozygous phenotype is due to a large truncation in the gene encoding the GnRH precursor [19]. The truncated gene consists of exons I and II only (exon II contains the GnRH coding region and the N-terminal portion of GnRH-associated peptide), while exons III and IV which code for the remainder of the GnRH-associated peptide and for the 3' untranslated region, respectively, are deleted [19]. A low level of transcription from the truncated gene is detected in cells at the OVLT area of the hpg [19]. The GnRH peptide, however, is not detectable in these animals [18]. These findings suggest that despite their failure to synthesize their unique neuropeptide, GnRH cells pursue their normal migration from the olfactory placode to the forebrain.

The reproductive deficiency of the hpg mouse can be corrected by introducing the wild type gene into the germ line of the mutant mouse [20]. Partial reversal of the the hpg phenotype was also accomplished by daily multiple injections of GnRH to adult mutant mice [21]. The exogenous GnRH induced an increase in GnRH binding

sites in the pituitary, elevation in pituitary LH content and increased testicular and seminal vesicle weight [21, 22]. A fuller correction of the reproductive deficiency is accomplished by transplanting normal fetal brain tissue containing GnRH neurons into the third ventricle of mutant adult hosts [23]. Implantation of fetal septal-preoptic area tissue into the third ventricle of adult hpg males results in gonadal growth, increased production of gonadotropins, steroidogenesis, and gametogenesis [24]. The transplanted GnRH neurons also induce pulsatile release of LH in castrated hpg males indicating that the grafted neurons are integrated into the 'pulse generator' neuronal circuitry of the host [25]. However, grafted hpg males showing physiological recovery fail to display masculine sexual behavior in response to receptive females, or to impregnate normal females [26]. Hpg females with a graft show elevated gonadotropin levels and show positive feedback in response to exogenous progesterone but do not respond to combined administration of estradiol and progesterone [27, 28]. Some of the females enter persistent estrus following transplantation and respond to ejaculation of a normal male with an LH surge and reflex ovulation which can result in normal pregnancy and delivery [29, 30]. A few of the hpg females become cyclic following delivery and show spontaneous ovulation [27]. Reversal of the reproductive deficiency in hpg mice is dependent upon innervation of the host's ME by GnRH fibers from the graft [24]. The GnRH axons project from the graft to the ME in a reproducible outgrowth pattern. In many animals the axons emerge from the graft at the level of the medial basal hypothalamus and arch

ventro-laterally through the arcuate nucleus to terminate in the lateral aspects of the ME [24]. This projection appears to recapitulate one of the pathways taken by GnRH axons in the normal animal [24, 31, 32]. Other axons emerge from the ventral aspect of the third ventricle graft and grow directly to the ME [24]. The reproducible pattern of these axonal projections by transplanted GnRH cells suggests that they are following guidance signals retained in the adult hpg brain. This guidance is not dependent on the neurons of the arcuate nucleus since cytotoxic ablation of this neuronal population does not prevent the axons from exhibiting their outgrowth pattern [33]. Furthermore, neither the tanycytes, nor the astrocytes appear to be the cellular substrate subserving this stereotypic arcuate nucleus-ME outgrowth as the distribution of these cell populations is not limited to the region of GnRH axonal outgrowth [32]. Rather, the ME itself is the most likely source for a diffusional signal directing the GnRH axonal outgrowth [34]. This conclusion is based on the finding that when GnRH neurons are cotransplanted with basal hypothalamic tissue their axons grow into the cografted basal hypothalamus and form ME-like structures [34].

We have utilized anatomical, physiological and behavioral approaches to characterize the development of the GnRH neuronal system in the mouse. These experimental approaches were designed to address the following questions:

1) What are the cellular associations that GnRH neurons make during their migration in nasal septum and the forebrain?

2) Are GnRH cells fully differentiated as they start their migration from the olfactory placode or is full biochemical differentiation dependent on signals from their residence area in the brain or on target derived signals?

3) Do GnRH neurons require signals from the embryonic brain to complete their biochemical and morphological differentiation?

4) Can GnRH cells resume their migratory activity following transplantation into an adult host brain and can their axons navigate to their normal target through the host tissue?

5) Is GnRH synthesis and secretion critical for the capability of GnRH neurons to establish and maintain their normal axonal projections?

6) Is GnRH secretion essential for the neonatal activation of the pituitary-gonadal axis which results in stimulation of androgen secretion and masculinization of copulatory behavior?

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CHAPTER 1.

Biochemical differentiation and intercellular interactions of migratory gonadotropin-releasing hormone (GnRH) cells in the mouse.

ABSTRACT

GnRH cells are first detected in the olfactory placode on gestational day 11.5 (E11.5) in the mouse. Between E12.5-E15.5 they migrate across the nasal septum and by E16.5 take up residence in their adult distribution regions within the forebrain. In the present study, we used immunocytochemistry at the light and electron microscopic level to study the biochemical and morphological differentiation of GnRH neurons and the cellular associations that they make during this migratory process.

On E12.5 when the majority of GnRH cells are still in the nasal septum, only 15% of the population can process the pro-GnRH precursor to the amidated decapeptide. Two days later (E14.5) when most of the cells have advanced into the forebrain, 79% contain mature GnRH. In keeping with these light microscopic observations, ultrastructural analysis indicated that E12.5 GnRH neurons are only lightly immunostained and the reaction product is confined to the outer nuclear envelope and the rough endoplasmic reticulum. By E14.5 the migratory cells in the nasal septum have more immunologic reaction product in the rough endoplasmic reticulum and some of the Golgi cisternae are also immunopositive. Neurosecretory granules, some of which are immunoreactive, also appear at this stage.

We had anticipated that the expression of GAP-43 would coincide with axonal elongation and pathfinding in GnRH neurons. Instead, GAP-43 was associated with the migratory cells but it's expression declined rapidly after these neurons had entered the forebrain and

it was also absent from their growing axons. Hence, on E12.5-E14.5, 56% of GnRH cells in the nasal septum are immunopositive for GAP-43, while only 12% of the forebrain population at the same stage express the protein.

While GnRH neurons migrate in the nasal septum they remain within the confines of the olfactory and vomeronasal axonal fascicles. Immunoreactive cells maintain close apposition with each other and also with the axons and their associted glia which ensheath these fascicles. Once in the forebrain, GnRH neurons no longer maintain close association with each other, nor do they follow any defined anatomical structure.

These findings indicate that although GnRH cells express their unique neuropeptide early in their ontogeny, their differentiation continues and is coordinated with their migration. The migration of these neurons across the nasal septum appears to rely on axonal fascicles of the olfactory and vomeronasal nerves. In the forebrain, however, GnRH cells must utilize alternative guiding mechanisms to complete their migration.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) neurons differentiate from the olfactory placode in the mammalian [1, 2, 3, 4] and avian embryo [5, 6, 7]. In the mouse, expression of the GnRH gene commences at gestational day 11.5 (E11.5), a day after most of these cells withdraw from the mitotic cycle [8], and by E12.5 the number of GnRH immunopositive cells in the nasal septum is equivalent to the adult GnRH population in the forebrain [8]. Between E12.5-E13.5 the majority of the GnRH population appears as cords of migratory cells in the nasal septum [1, 8]. Subsequently, on days E12.5-16.5, the cells penetrate into the medial ventral forebrain with the central roots of the terminalis and vomeronasal nerves, and migrate caudally to reside in the septal, preoptic and anterior hypothalamic areas [1].

The current developmental studies, using light and electron microscopic immunocytochemistry, have two major aims. The first of these is to determine the state of biochemical differentiation of GnRH cells in their early migratory stages. This was accomplished by utilizing different antisera to GnRH which can distinguish between the pro-GnRH precursor and the mature bioactive decapeptide which has been cleaved and α-amidated at its C-terminal glycine [9]. Evidence obtained by these light microscopic observations were confirmed ultrastructurally by determining the subcellular compartments along the synthetic and secretory pathway that contained immunoreactive material. As a complementary approach we studied the expression of the growth-

associated protein GAP-43 in the migratory GnRH cells. GAP-43 is a phosphoprotein whose expression is associated with neuronal development, regeneration, and synaptic plasticity and it is believed to be involved in axonal outgrowth and regeneration (for review: [10]). We anticipated that expression of this protein would mark the transition from migratory to a post-migratory phase associated with axonal elaboration.

The second aim was to identify the cellular components of the pathway along which GnRH cells migrate within the nasal septum and at their entry into the central nervous system (CNS). We characterized at the electron microscopic level the association between the migrating neurons themselves, the olfactory/vomeronasal derived axons and their ensheathing glia. These studies represent an initial analysis of the kinds of cues that GnRH cells might utilize to transit from the periphery into the CNS.

MATERIALS AND METHODS

Reagents for Immunocytochemistry

The two rabbit antisera for GnRH were raised and characterized as follows: LR-1 was raised against [D-Lys 6] GnRH conjugated with glutaraldehyde to ovalbumin. In radioimmunoassay the determinants are amino acids 3, 4, 7, 8, 9, and 10 (R. Benoit, personal communication) and absorption with the GnRH decapeptide completely eliminated staining [11]. Immunocytochemical electron microscopy reveals that this antiserum recognizes the epitope (amino acids 6-10) in both the pro-GnRH precursor and the

amidated decapeptide as it stains the rough endoplasmic reticulum (RER) and Golgi cisternae as well as neurosecretory granules [12]. El 14 recognizes only the mature, amidated decapeptide in vitro [13] and in situ as its immunostaining is limited to the post-Golgi compartment [12]. In adult rats both antisera produced a similar staining pattern of GnRH cell bodies and terminals and a comparable number of neurons were stained with each antiserum [12]. The anti-GAP-43 monoclonal antibody 9-1E12 was raised and characterized by Schreyer and Skane [14, 15]. It reacts with both newly synthesized and post-translationally modified GAP-43.

Animals

Mice were housed under controlled conditions of lighting (14L:10D, lights off at 1500 h) and temperature (24-25 ^{o}C). Food and water were available ad libitum. Breeding was achieved by placing 2 females (C3H/HeHX101H) with a fertile male of the same strain. The presence of a vaginal plug on the next morning indicated copulation and this morning was designated as gestational day 0.5 (E0.5). Females with vaginal plug were separated from the males to ensure they were not impregnated at a later date. Gestation period in our colony is 20-21 days.

Tissue Preparation

The timed pregnant females were sacrificed by an overdose of chloral hydrate at E12.5-16.5 and the embryos were removed from the uterus and placed in sterile saline. Embryonic stage was confirmed using the morphologic criteria of Theiler [16]. Embryos'

heads were fixed by immersion in 4% paraformaldehyde-phosphate buffer solution (0.1 M phosphate buffer, PH 7.3 was used throughout unless indicated other wise)for 4-6 hours followed by overnight equilibration in 30% sucrose. The heads were than snap frozen on dry ice, and 20 µm sections in the sagittal and frontal planes were cut with a cryostat and collected on gelatin double-subbed slides. Sectioned tissue was stored desiccated up to 3 months in -20 ^{o}C. Adult (2-3 month old) males were deeply anesthetized with chloral hydrate and intracardially perfused with saline followed by 4% paraformaldehyde. The brains were sectioned on a vibratome at 40 µm and coronal sections were processed for immunocytochemistry along with the embryonic sections. For each age group at least 3 animals were processed from 2 different litters.

Embryos processed for detection of the migrating GnRH cells in the electron microscopic level were fixed in 4% paraformaldehyde with 0.1% glutaraldehyde for 2-4 hours, and embedded in 8% gelatin. Sagittal vibratome sections were then cut at 60-80 µm and immunocytochemistry was performed on free floating sections.

Immunocytochemistry

Slides with E12.5 and E14.5 sections, and free floating sections through the diencephalon of adult males were washed twice and incubated in 0.5% hydrogen peroxide for 30 min followed by 2 washes. Subsequently the sections were washed for 1 hour in 5% non-fat dry milk containing 0.1% Triton X-100. Alternate sections from each stage were then incubated overnight with either one of the rabbit anti GnRH antisera: LR-1 diluted 1:5000, or El-14 diluted

1:4000. The antigen-antibody complex was visualized with an avidin-biotin horseradish peroxidase conjugate (Vector Laboratories; Burlingame, CA). The enzymatic reaction was carried out with either DAB or nickel sulfate intensification of the DAB reaction product. Finally the free floating sections were mounted on slides, dehydrated, cleared with Histoclear, and coverslipped with Permount (Fisher Scientific; Fair Lawn, NJ). All immunopositive cells sectioned through the nucleus were counted on alternate sections reacted with either EL 14 or LR-1. The proportion of El 14 positive cells was expressed as a percentage of the LR-1 positive population.

Sections from E12.5-E16.5 embryos were processed for double label immunocytochemistry to visualize colocalization of GnRH and GAP-43 in the same cells. The sections were processed as detailed above and incubated overnight with LR-1 diluted 1:3000 and the antigen-antibody complex was visualized with a goat anti-rabbit IgG conjugated to FITC (Vector). Thereafter the sections were incubated with 9-1E12, a monoclonal antibody against GAP-43 diluted 1:5000 which was visualized with a biotinylated horse anti-mouse IgG and AMCA-streptavidin (Jackson Immuno Research; West Grove, PA). The double stained slides were coverslipped with GelMount (Biomedia; Foster City, CA) and analyzed with a fluorescence microscope equipped with fluorescin and UV filters to detect the FITC and AMCA signals, respectively. All immunopositive cells sectioned through the nucleus were counted in every section. The proportion of GnRH neurons which were GAP-43 positive was expressed as a percentage of the total number of GnRH cells. Kruskal-Wallis one-way ANOVA and Mann-Whitney U test were

used to determine the significance of differences among the groups. Control slides were incubated with pre-immune goat or horse serum instead of one of the primary antibodies.

LR-1 immunocytochemistry for electron microscopy was performed as described above with the following modifications: hydrogen peroxide treatment was replaced with glycine buffer wash followed by incubation in 0.2% sodium borohydride, and the Triton X-100 detergent was replaced with 0.05% saponin. Following the DAB reaction, small tissue fragments containing migrating GnRH cells were microdissected and osmicated for 1 hr in 0.9% NaCl containing 2% OsO_4 and 1.5% KFeCN. The fragments were then dehydrated and embedded in Epon. GnRH neurons in the nasal septum and forebrain were serially sectioned and Ultra-thin sections in intervals of approximately 500 nm were photographed with a JEOL 100S electron microscope.

RESULTS

Mature GnRH peptide in the migratory GnRH population

Cell counts of alternate sections stained with either LR-1 or EL 14 antisera revealed that at E12.5, when most of the GnRH population is in the nasal area, the EL 14 positive cells constitute about 15% (mean $\pm$ SE = 15.3 $\pm$ 3.2%; n=3) of the LR-1 positive cell population (Fig. 1). By E14.5 most GnRH cells had advanced into the forebrain. At this stage the percentage of EL 14 positive cells increased to 79% (78.9 $\pm$ 10.4%; n=3) of the LR-1 cell population (Fig. 1). In the adult male, EL 14 immunopositive cells in the septal, preoptic, and

anterior hypothalamic areas constitute 99% (98.6 ± 6.0% ; n=3) of the LR-1 positive cells. Thus the percentage of cells capable of fully processing the pro-GnRH precursor increases significantly from E12.5 to E14.5 ($P < 0.02$). The percentage of EL 14 positive cells at E14.5 is significantly lower than that of the adult ($P < 0.05$).

Arrival of GnRH axons at the median eminence (ME)

As they enter the forebrain many GnRH cells start elaborating axonal processes which by E14.5 reach the region of the prospective ME (Fig. 2). Some of the first axons in the ME are also immunopositive for EL 14 . The appearance of the first GnRH positive axons in the prospective ME coincides with arrival of the first cohort of migratory GnRH cells to the septal, and preoptic areas. At E15.5 some axonal processes reach the tegmental region and a few extend caudally to the tegmental/pons border by E16.5. A few GnRH cells are found in the basal hypothalamus and in the mammilary area of E15.5 embryos.

Expression of GAP-43 in GnRH cells

More than half of the GnRH cells in the nasal septum of E12.5 embryos are GAP-43 positive (62.3 ± 6.0%; n=3). A significantly lower percentage of GAP-43 expressing GnRH cells is observed in the forebrain of the E12.5 embryos (12.6 ± 3.2%; n=3; $P < 0.02$). A similar decline in GAP-43 expression is seen at E14.5 when 50.7 ± 18.0% of the GnRH cells in the nasal septum express GAP-43 but only 11.6 ± 3.0% of the cells in the forebrain are GAP-43 positive ($P < 0.02$; n=3; Fig. 3). By E16.5 when most GnRH cells have attained

their adult distribution, GAP-43 is no longer detectable in any of these cells (n=3).

GAP-43 staining of GnRH cells is most pronounced in the soma, but stained processes are also seen in a few cells in the vomeronasal organ (VMO) of E12.5 embryos, or in cells just outside the VMO of E14.5 embryos (Fig. 3). However, the GnRH axons that extend towards the ME by E14.5 are devoid of GAP-43. The fascicles of the olfactory nerve are robustly stained with GAP-43 at all these stages, indicating that the changes in GAP-43 immunostaining are specific to the GnRH population. Control sections in which the GAP-43 antibody was replaced with a pre-immune serum have no specific staining.

Cellular elements associated with GnRH cells.

GnRH cells migrating through the submucosa of the nasal septum of E12.5-E14.5 embryos have fusiform morphology with short processes. At the electron microscopic level, these cells appear to maintain close membrane apposition to each other, but are apparently devoid of contact specializations such as desmosomes (Fig. 4). In the nasal septum, the cells migrate within axonal fascicles and are in close apposition to the axon fibers (Fig. 4, 5A). Another cell type associated with the GnRH cells and the axonal fascicles in the nasal septum has a glial appearance (Fig. 5). These cells probably correspond to the ensheathing cells which migrate from the olfactory placode along with the axons and later form the ensheathment of the axonal fascicles. The cytoplasmic processes of these ensheathing cells frequently envelop those GnRH cells which

are located on the external surface of the axonal fascicles (Fig. 5A). Thus, the ensheathing cells appear to delimit the axonal fascicles and their associated migratory neurons from the mesenchymal tissue of the nasal septum.

GnRH cells in the forebrain of E14.5 mice are not associated with axonal fascicles and cease to maintain close apposition to each other. Rather, the cells are associated with a mixed population of neuronal and glial cells with no apparent directional orientation (Fig. 5B)

The ultrastructural examination revealed no association of GnRH cells with extracellular matrix elements along their migratory pathway in the nasal septum or the forebrain.

Cytology of GnRH cells and distribution of reaction product

GnRH cells in the nasal septum of E12.5 embryos have a large euchromatic nucleus but relatively scant cytoplasm and cytoplasmic organelles. The LR-1 immunologic reaction product is present within the outer nuclear envelope and the cisternae of the RER. The Golgi apparatus is not immunoreactive and neurosecretory granules are undetectable (Fig. 6A). By E14.5 the immunologic reaction product is denser over the outer nuclear envelope and the RER (Fig. 6B). In some of the E14.5 cells reaction product is present over some of the Golgi's cisternae (Fig. 6B, 7). Neurosecretory granules are also present within some of these cells and few of these granules appear to be immunopositive (Fig. 7). At E14.5 GnRH neurons in the forebrain appear to have a denser immunologic reaction product in comparison to the neurons that are still in the nasal septum. The nucleus of GnRH cells in the E14.5 forebrain, contrary to the nasal

septum cells, is immunostained while the nucleolus is immunonegative in both populations (Fig. 5B). The precipitation of immunologic reaction product over the nucleus, but not over the nucleolus is often observed in GnRH cells in the adult brain [17]. As has been described previously for the adult [11], a few GnRH cells in the E14.5 nasal septum have a cilium.

Most GnRH cells (70-75%) in the E12.5-E14.5 nasal septum appear polarized vis a vis their migratory direction. In 90-95% of these polarized cells most of the cytoplasm, RER and Golgi cisternae are concentrated posterior to the nucleus which is located in the leading aspect of the cell (Fig. 4). In addition a short, organelle poor process can be observed at the anterior aspect of some of the migrating neurons.

DISCUSSION

Maturation of GnRH neurons

It is now well accepted that in mice, GnRH neurons arise in the olfactory placode by E10.5 and can be recognized prior to their migration by elaboration of the pro-GnRH mRNA [8] and by translation of this mRNA [1, 2]. As the present studies indicate, the biochemical maturation of these neurons is not complete at the time of the initial expression of the GnRH gene. Although GnRH immunoreactivity can be detected with an antiserum such as LR-1 that recognizes the pro-GnRH precursor, while it is still retained in the RER [12], we have shown here that by E12.5 this precursor is not yet processed to the amidated decapeptide. This was

demonstrated by the absence of reaction product in 85% of the neurons at E12.5 with the EL 14 antiserum, which recognizes only mature GnRH [12, 13]. Although the percentage of cells which process and amidate GnRH increases dramatically, to approximately 80% of the population by E14.5, adult levels (nearly 100%) are not achieved even at this time. The incomplete processing of the pro-GnRH precursor at the early migratory stage could be due to as yet nonfunctional cleavage and amidation enzymes or to retention of the precursor within the RER compartment where it can not be further processed. Previous work on adult rats had indicated that EL 14 antigenicity was confined to a post-Golgi compartment in the cell soma and to neurosecretory granules in the axons [12]. The present ultrastructural work indicates that on E12.5 the pro-GnRH precursor is not yet transported to the Golgi apparatus and neurosecretory granules are not detected until E14.5. Thus, it can be inferred that the neurosecretory potential of GnRH neurons to release mature neuropeptide is not realized until they are well advanced in the migratory route to the forebrain.

Ultrastructural studies also revealed a maturation of the cellular elements during the two days period from E12.5 to E14.5. Contrary to previous reports [18], we observed a general increase in immunoreactivity during this time period as well as a widened subcellular distribution of reaction product. At the earliest stage, the LR-1 antiserum revealed antigen sites only in the RER (which was sparse) and its associated outer nuclear envelope. By E14.5 there was a substantial increase in the volume of the RER as well as the Golgi apparatus, and (as noted above) neurosecretory granules

were first detected at this stage. Reaction product was now found in all of these compartments. We can not conclude from our data whether pro-GnRH synthesized at E12.5 is retained in the RER to be transported to the Golgi and processed at a later stage, or is degraded and synthesized anew. The localization of immunoreactivity in the Golgi and neurosecretory granules, as reported here, was not found in a previous study [18]. These contradictory findings could be due to our modified fixation and immunocytochemistry protocols which allow for improved preservation of subcellular details.

The spatial and temporal parameters of GnRH cell migration from the olfactory placode to the diencephalon, as observed in this study, are consistent with previous reports [1, 8]. We now describe for the first time that GnRH immunoreactive axons are elaborated and extend to their primary target, the prospective ME region, as early as E14.5. Some of these axons are immunopositive for the amidated decapeptide which is the bioactive form [9]. These observations suggest that during the initial stages of differentiation of Rathke's pouch, the neurohormone is potentially available to the developing gonadotrophs. GnRH binding sites have been reported in the E12-E13 anterior pituitary of the rat [19, 20] and circulation in the hypophyseal portal system commences on E16.5 [21]. In the mouse, the primary capillaries of the hypophyseal portal system are already present in the ME by E18 [22]. These data, taken together, support the hypothesis that the rodent pituitary-gonadal axis has access and can respond to GnRH by late gestation. Indeed, the first gonadotrophs are detected in the rat at E16 [20] and the earliest

pituitary response to GnRH is reported at E17.5 [23]. In the mouse, GnRH activation of the pituitary-gonadal axis is of critical importance in sexual differentiation by postnatal day 1 [24].

We report here that in the mouse most GnRH neurons start elaborating axons only upon entering the forebrain. In the chick, however, many axons are present in the olfactory nerve during the early migratory stage (Sullivan and Silverman, manuscript in preparation). Similarly, in the rhesus macaque GnRH neurons start elaborating axons while they are still in the nasal septum [4]. Hence, the migratory stage at which GnRH cells initiate axonal outgrowth appears to be species specific, indicating that the differentiation program of these neurons might have adapted to the ontogenetic context of each species.

To complement our studies on maturation of the GnRH neuron as demonstrated by the appearance of GnRH precursor processing capabilities and axonal outgrowth and targeting, we studied the expression of GAP-43 by these neurons. Although GAP-43 function is still an open question, its expression and subcellular localization are consistent with a role in axonal outgrowth and synaptic formation and plasticity in many neuronal systems [15, 25, 26, 27]. GAP-43 staining in the olfactory epithelium of the mouse increases sharply from E11.5 to E15.5, and the olfactory nerve is also GAP-43 positive [28]. In the olfactory epithelium of the rat, GAP-43 expression peaks in the perinatal period when many olfactory neurons are engaged in axonal outgrowth to the olfactory bulb [29] and is reinduced when the adult olfactory epithelium produces a

large cohort of undifferentiated olfactory neurons in response to a lesion [30].

Surprisingly, GAP-43 expression in the GnRH neurons was not associated with axonal growth. Instead, immunoreactivity was found in the cell bodies of at least half of these neurons in the E12.5-E14.5 nasal septum. GAP-43 expression declines dramatically in the forebrain population of the same embryos and disappears by E16.5, when most of the cells have completed their migration. Thus, GnRH neurons express GAP-43 in their initial, extracranial migratory route when their neuroendocrine function is not yet fully matured. These neurons, however, cease to express GAP-43 as they enter the forebrain and approach the vicinity of their adult location. The association of GAP-43 with neuronal migration has not been previously reported. Rather, GAP-43 staining was shown to be extremely low in the cortical plate at stages of neuronal migration [31].

In the GnRH neurons, unlike other neuronal systems, GAP-43 was not associated with outgrowing axonal processes but, as noted above, confined to the cell soma. In fact, its expression declines at E14.5-E16.5, coincident with GnRH axonal outgrowth to the basal hypothalamus and the ME. GAP-43 was also undetectable in GnRH cells or their regenerating axons in fetal grafts transplanted into adult mice (Livne and Gibson, unpublished observations). The temporal and spatial expression pattern of GAP-43 in GnRH cells is, therefore, not consistent with a role in the process of axonal outgrowth or guidance. Rather, its expression is restricted to the earlier stages of migration and differentiation of GnRH cells.

Whether GAP-43 plays a role in cell-cell or cell-substrate interactions of migratory neurons is not known.

Migration of GnRH neurons

Light microscopic studies have indicated that migratory GnRH neurons in the nasal septum maintain close association with each other and with axonal fascicles of the olfactory and vomeronasal nerves [1, 2]. This was confirmed by the ultrastructural observations in this and in a previous study [18]. Most GnRH neurons maintain membrane apposition with each other in the nasal septum. However, analysis of serial sections did not reveal specialized cell junctions that might mediate intercellular communication between the migrating neurons.

Polarization of GnRH neurons while they migrate in the nasal septum was also observed in our electron microscopic studies. In many cells the nucleus was positioned in the leading aspect of the cell while most of the cytoplasm and cytoplasmic organelles, such as the RER and Golgi cisternae, were posterior to the nucleus. A polarized distribution of organelles in migratory neurons was reported to exist in the primate cerebellum and neocortex [32, 33]. In these regions, however, most of the cytoplasmic organelles preceded the nucleus in the leading process of the migrating neurons. The migratory mechanism utilized by cerebellar and neocortex neurons appears to differ from the one utilized by GnRH neurons in the nasal septum. Unlike the cerebellar and neocortex neurons, GnRH cells do not migrate by attachment to glial fibers and do not develop long cytoplasmic processes. The different mode of

migration probably requires different morphological configuration which is reflected in a different distribution of the cytoplasmic organelles. Whether the different localization of the RER and the Golgi in the migrating neurons reflects a different functional participation of these organelles in the migratory process is not known.

The emergence of axons from the epithelium of the VMO on day 12 of gestation [34] coincides with the migration of GnRH cells out of the VMO. Thus, GnRH cells appear to comigrate with the pioneer axons of the vomeronasal nerve into the submucosa of the nasal septum and advance together towards the forebrain. A population of glial-like cells also migrate with the axonal fascicles and envelope GnRH cells located on the external surface of the axonal fascicle. These are probably glial progenitor cells which migrate from the olfactory and vomeronasal epithelia with the outgrowing axons and differentiate into ensheathing cells [35, 36, 37, 38]. The axonal fascicles and their ensheathing cells appear to form physical channels within which GnRH cells can cross the nasal septum and enter into the forebrain. The critical role of these channels in the migration of GnRH cells into the forebrain is suggested by examination of an aborted fetus with Kallman's syndrome in which the olfactory, vomeronasal, and terminal nerves fail to enter the forebrain. Coincident with this failure, GnRH cells in the aborted embryo were unable to enter the brain, and instead were clustered beneath the forebrain on top of the cribriform plate [39].

Once in the forebrain, GnRH cells are no longer associated with axonal fascicles and appear to intermingle with a heterogeneous

population of neurons and glia. Thus, in the forebrain GnRH neurons are not associated with any recognizable structural element which might serve as a physical guiding substrate. It is, therefore, plausible that these neurons rely on chemical, rather than physical cues in choosing their migratory pathway in the forebrain.

The electron microscopic data indicate that extracellular matrix elements are not associated with migratory GnRH cells in the nasal septum or the forebrain. In addition, preliminary immunocytochemical observations have indicated that the extracellular matrix molecules laminin and fibronectin are not present along the migratory route of GnRH cells in the nasal septum or the forebrain (Livne, unpublished). It seems unlikely, therefore, that extracellular matrix molecules play a role in vivo in neuronal migration from the olfactory epithelium as has been recently suggested [40].

Acquisition of the processing capability of the GnRH precursor correlates, both in the light and electron microscopic level, with the migratory stage of GnRH cells. Loss of GAP-43 expression in these cells also coincides with their passage into the forebrain. Therefore, we suggest that the migratory environment through which the cells advance regulates their differentiation. Transition from the nasal septum, where the cells migrate within axonal fascicles, into the forebrain is a possible switch to a different set of contact or diffusional signals that triggers the cells to start elaborating axonal processes. However, while still in the nasal septum, GnRH cells appear to have already acquired the information required for pursuing their differentiation into neuroendocrine cells. This is

demonstrated by transplantation of GnRH cells in the nasal septum stage into an adult brain. The transplanted cells can establish their neuroendocrine phenotype regardless of their interrupted migration and in absence of signals from the embryonic brain [41]. Conceivably, intercellular signals received by GnRH cells throughout their migratory pathway allow them to coordinate their autonomous differentiation program with their migratory phase. However, it remains to be determined whether these signals are critical for complete differentiation of GnRH cells.

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FIGURE LEGEND

Fig. 1. Parasagittal sections of embryonic stages E12.5 (A, B) and E14.5 (C, D). A. GnRH neurons (arrows) migrating out of the vomeronasal organ (VMO) are immunostained with an antibody recognizing the pro-GnRH precursor (LR-1). In an adjacent section (B) stained with an antibody to the fully processed GnRH (EL 14) no immunopositive cells can be detected in or around the VMO. On E14.5, as GnRH cells arch across the forebrain and advance ventro-caudally towards the preoptic area (POA), a comparable number of neurons is detected with either the pro-GnRH (C) or the mature GnRH (D) antibodies. Scale bar; A, 50 μm; B,C,D, 100 μm.

Fig. 2. A parasagittal section of an E14.5 brain. GnRH axons extend caudally through the basal hypothalamus (arrow) to terminate in the prospective median eminence (ME). Scale bar, 100 μm.

Fig. 3. A GnRH immunoreactive neuron (A, green fluorescence) in the olfactory placode of an E14.5 embryo is double labeled with the GAP-43 antibody (B, red fluorescence). The double exposure in B results in an orange color where the green and red fluorescence overlap. Note that the olfactory epithelium (OE) in B is also GAP-43 positive. Two GnRH neurons in the E14.5 nasal septum (C, green) coexpress GAP-43 (D, blue). The double exposure in D results in a greenish blue color where the green and blue fluorescence overlap. Note that the process extending dorsally from the right cell (C, arrowheads) is also GAP-43 positive (D, arrowheads).
The blue fluorescence around the GnRH cells (D) corresponds to the axons of the vomeronasal nerve within which GnRH neurons migrate in the nasal septum. Scale bar, 10 μm.

Fig. 4. Electron micrograph of GnRH cells in the nasal septum of E12.5 embryo. The immunopositive neurons maintain extensive membrane apposition with each other (arrowheads) as they migrate within and in parallel to axonal fascicles (Ax). In many of the GnRH neurons in the nasal septum the nucleus (N) is in the leading aspect of the cell while the cytoplasm and most cytoplasmic organelles are concentrated posterior to the nucleus. The presumed migratory direction is towards the forebrain (Fb, lower left corner). Scale bar, 1 μm.

Fig. 5. Different cellular associations are made by GnRH neurons in the nasal septum and in the forebrain. A GnRH neuron in the E12.5 nasal septum (A) is migrating within an axonal fascicle (Ax) which is delimited by ensheathing cells (En) and their processes. B. a GnRH neuron in the forebrain of E14.5 embryo is associated with a heterogeneous population of neurons (n) and glia (g) with no apparent directional orientation. Dense immunologic reaction product accumulates over most of the cytoplasm of these cells leaving only the mitochondria (M) unstained. The nucleus (N), but not the nucleolus (Nu), is also immunoreactive. G, Golgi apparatus. Scale bar, 1 μm.

Fig. 6. GnRH immunopositive neurons in the E12.5 (A) and E14.5 (B) nasal septum. On E14.5 more immunologic reaction product accumulates over the rough endoplasmic reticulum (RER). Some of the cisternae of the Golgi apparatus (G) also become immunopositive by E14.5. Scale bar, 1 μm.

Fig. 7. Subcellular distribution of GnRH immunoreactive material in the E14.5 nasal septum. Some of the Golgi cisternae (G) in A are immunopositive. Neurosecretory granules (B, arrowheads) are found in some of the cells. C. A few of these neurosecretory granules (NSG), as well as the rough endoplasmic reticulum (RER), contain immunologic reaction product. Scale bars: A, 0.5 μm., B and C, 250 nm.

FIG. 1

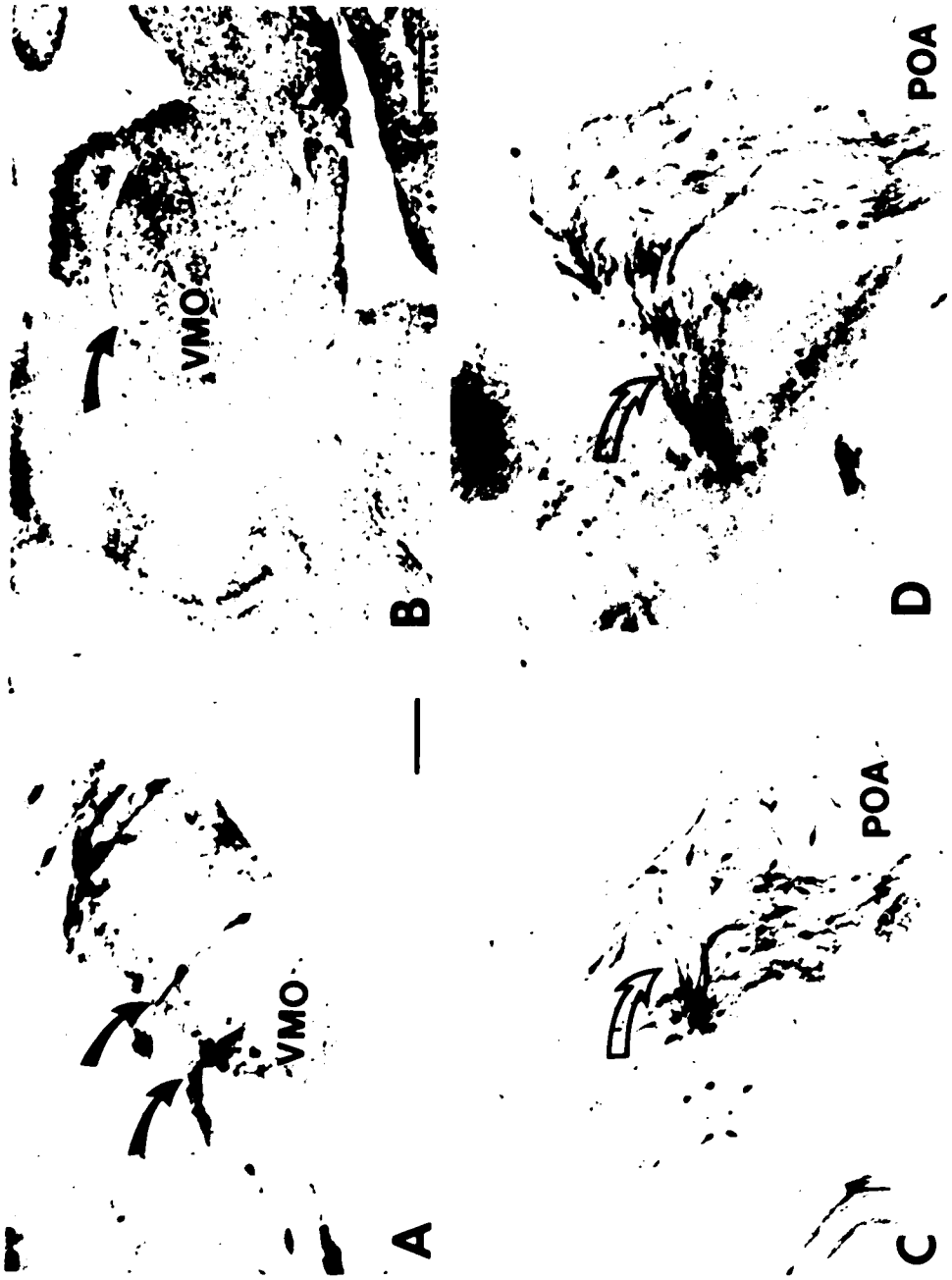


FIG. 2

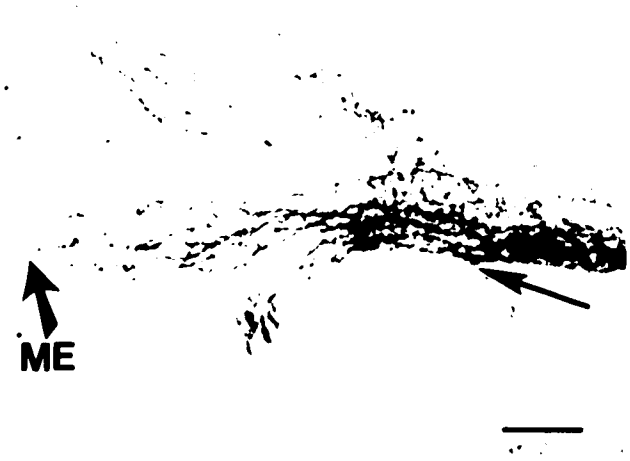


FIG. 3

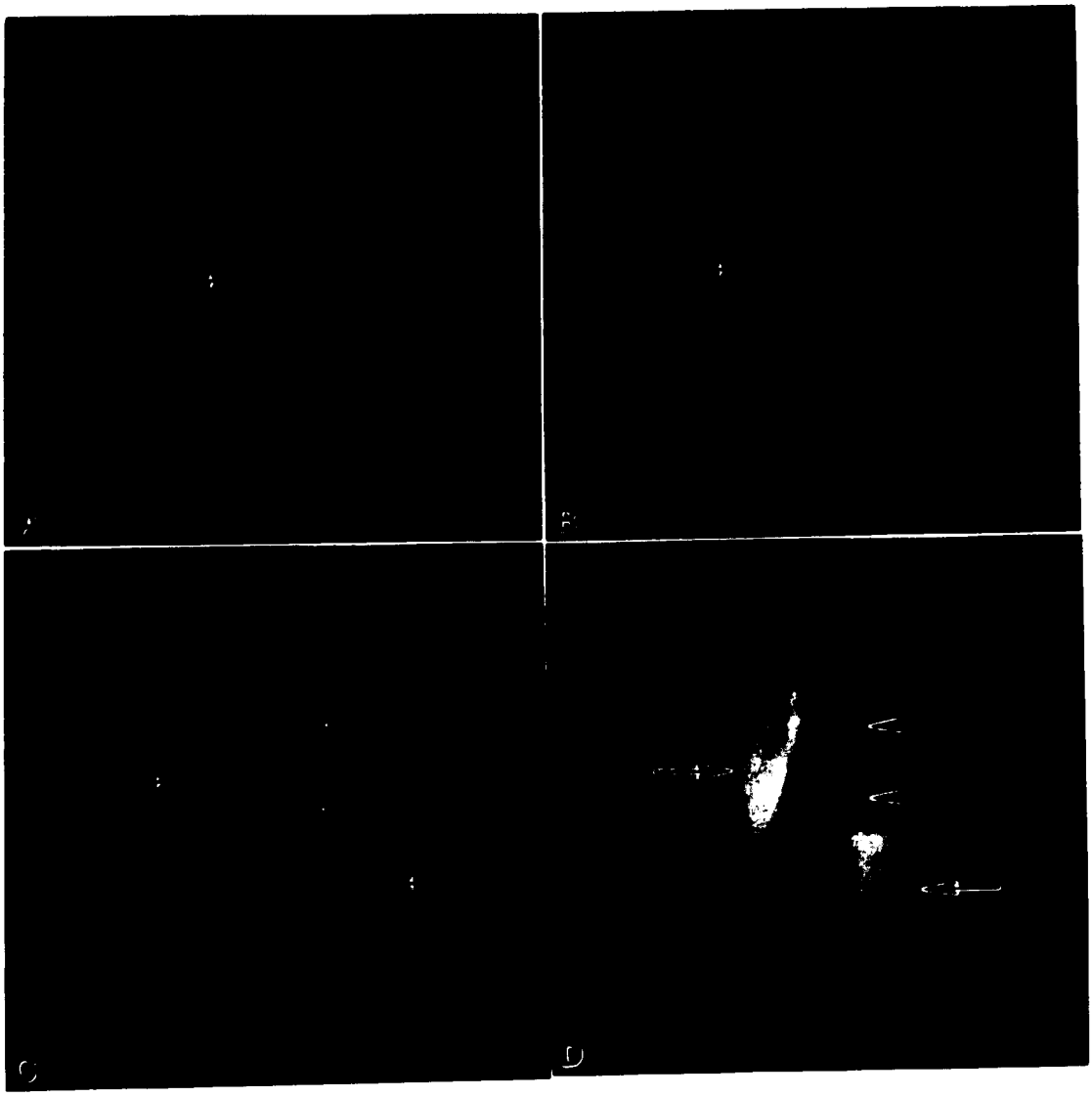


FIG. 4

FIG. 5

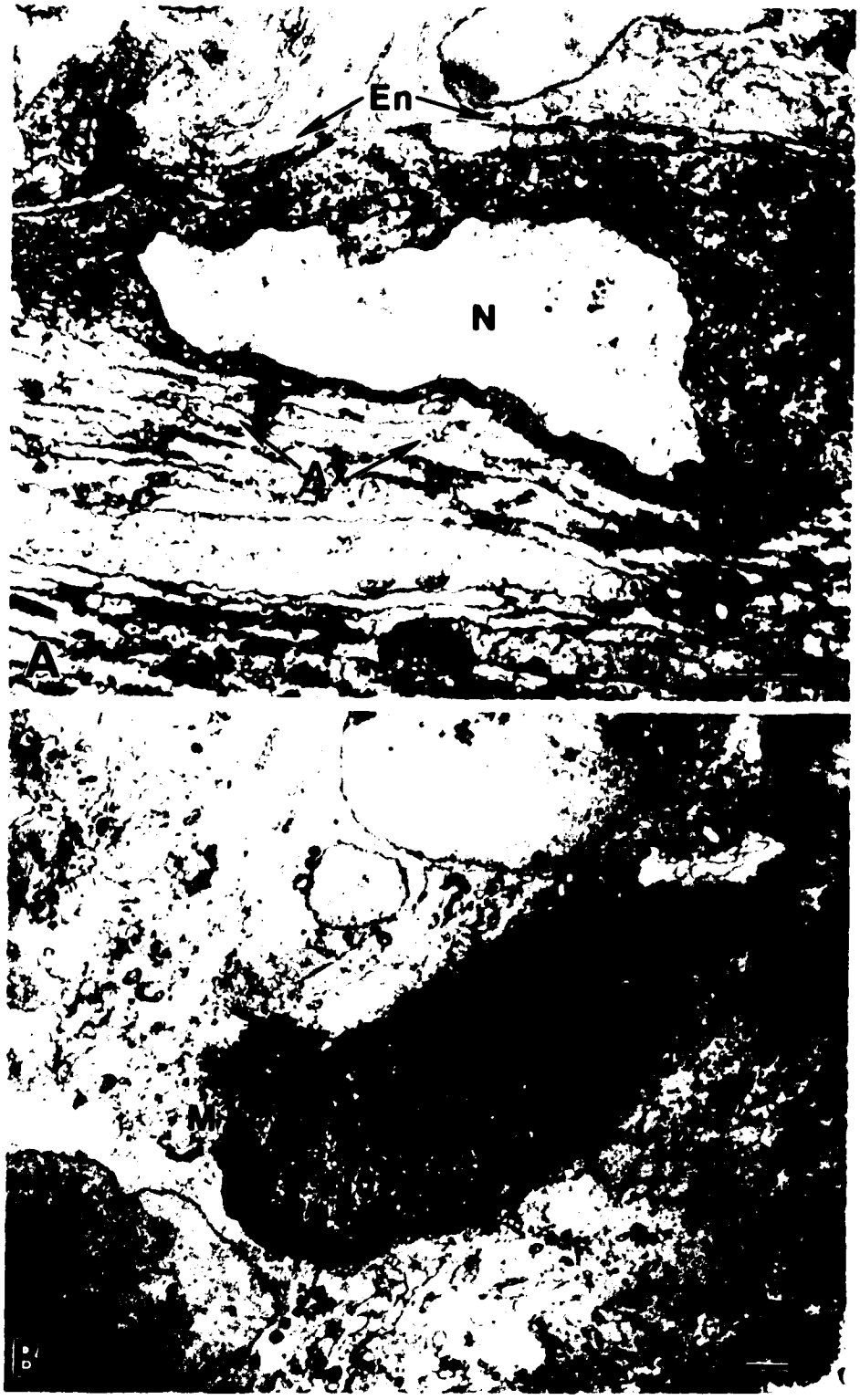


FIG. 6

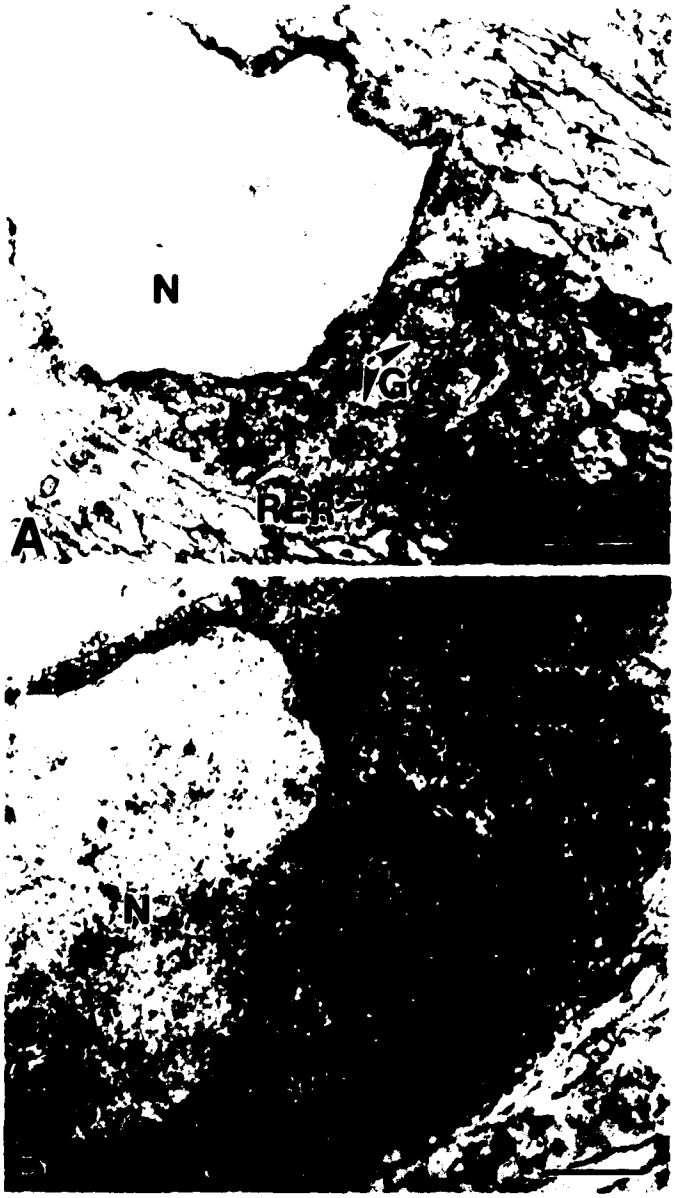
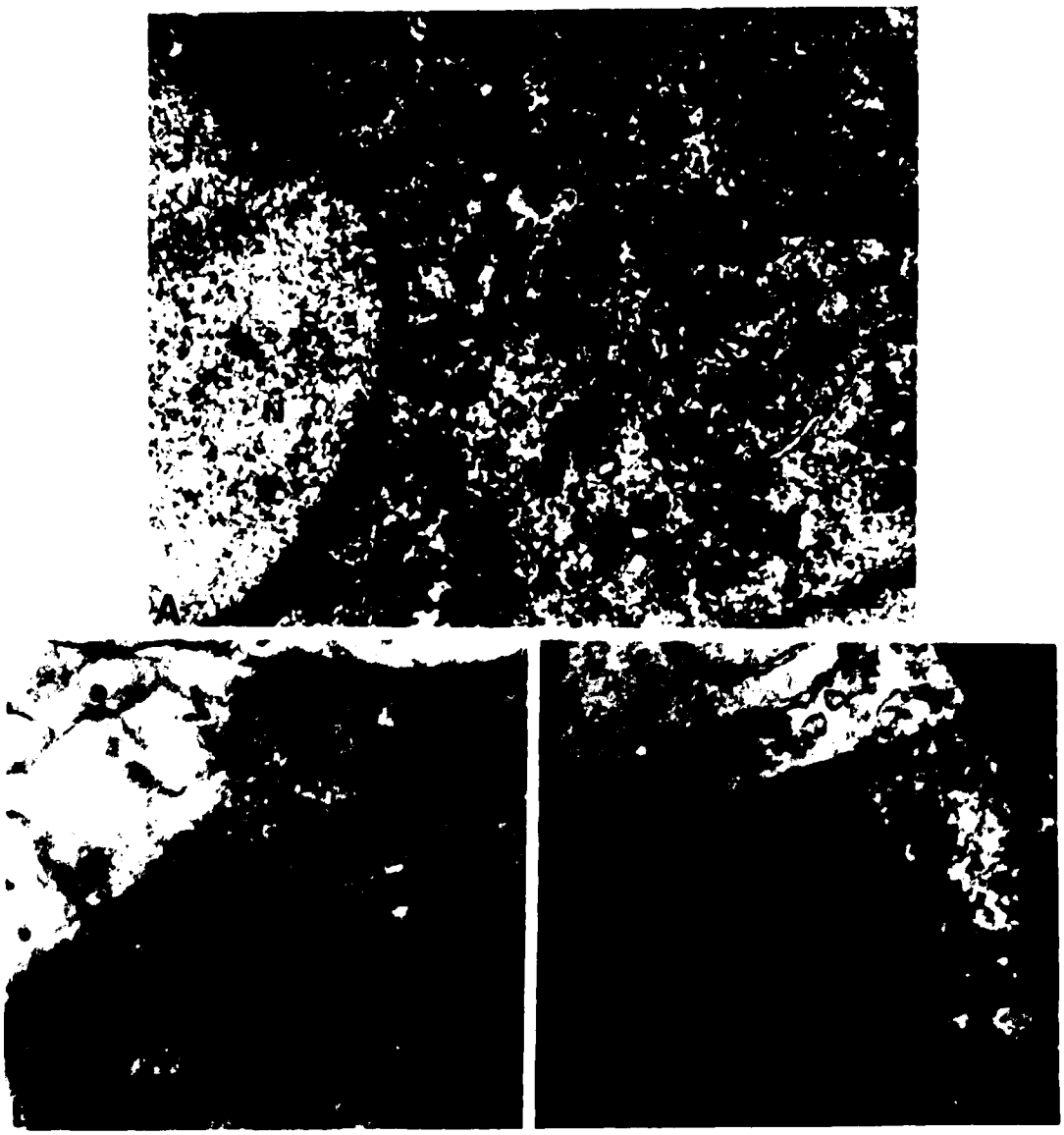


FIG. 7



CHAPTER 2.

Brain grafts of migratory GnRH cells induce gonadal recovery in hypogonadal (hpg) mice.

ABSTRACT

Gonadotropin-releasing hormone (GnRH) neurons are derived from the olfactory placode and migrate into the CNS during embryogenesis. During this migration the GnRH neuronal population follows a very specific pathway through the nasal septum and forebrain with individual neurons 'stopping' at various points along the way. Following migration, GnRH neurons elaborate axonal projections, the major one to the median eminence. The function of this neurosecretory connection can then be assessed by activation of the pituitary-gonadal axis. In previous experiments we had demonstrated that grafted post-migratory GnRH neurons could send axons to the median eminence and initiate gonadal development in hypogonadal (hpg) mice that lack GnRH. In the present experiment, grafts derived from the embryonic nasal septum, which contains the migratory population of GnRH neurons, were used to determine if the transplanted GnRH neurons could (1) continue their migration in the adult host brain, (2) elaborate axons to their normal target in the host and (3) stimulate the host pituitary-gonadal axis to induce gonadal development. Nasal tissue from normal mouse embryos was implanted into the preoptic area (n=8), anterior hypothalamus (n=3) or third ventricle (n=1) of adult hpg males. Following survival of 10 days to 10 weeks, the distribution of GnRH immunoreactive elements was assessed and testicular weight recorded. Surviving GnRH neurons were few in number and were found within the graft (n=3), the host brain (n=2) or both (n=1). Four grafts resulted in specific outgrowth of GnRH axons through the host parenchyma to

the median eminence. Three of the four animals with median eminence innervation had testicular development. These observations indicate that migratory GnRH neurons from the nasal septum retain a limited but real capacity to migrate in adult host brain. Furthermore, interruption of the normal migratory pathway of these neurons does not disrupt their potential for axonal growth, axonal pathfinding and the establishment of a functional neurosecretory connection with their normal target.

INTRODUCTION

GnRH neurons are derived from the olfactory placode, where they are first detected in the mouse on gestational day 11.5 (E11.5) [1, 2]. On subsequent days (E12.5-13.5) most of the GnRH cells appear in the nasal septum, arranged in compact clusters or 'migratory cords'. They display a fusiform morphology with short undifferentiated processes. At this stage the cells migrate within the axonal fascicles of the olfactory and vomeronasal nerves, and penetrate into the medial ventral forebrain with the central roots of the terminalis and vomeronasal nerves [1]. By E14.5 the majority of GnRH cells are dispersed rostro-caudally through the anterior forebrain and at E16.5 their distribution indicates that most cells have completed their migration and now reside in their adult distribution regions: the diagonal band, medial septum, preoptic area (POA) and anterior hypothalamic area (AHA) [1, 2].

Tissue explants containing the migratory population of GnRH cells were transplanted into the brains of hpg mutant mice. Due to an autosomal recessive mutation in the GnRH gene, the brains of these animals are devoid of GnRH, and their reproductive system remains undeveloped after birth [3]. Transplantation of late fetal or neonatal POA tissue containing GnRH cells into the third ventricle of adult male or female hpg can induce increased gonadotropin secretion, initiation of steroidogenesis and gametogenesis, and increase in gonadal weight [4, 5]. When transplanted into hpg mice, grafted GnRH cells can be clearly traced in the host by

immunocytochemistry, and any stimulation of the reproductive structures and physiology can be attributed to their functional interaction with the host median eminence (ME) [6].

This study examined the capacity of GnRH neurons derived from the nasal septum of E12-13 embryos to survive in an adult brain, express their migratory potential by entering the host tissue and fulfill their developmental program by elaborating axonal processes. The degree of differentiation was also assessed by the targeting of GnRH axons to their major terminus, the ME, and their ability to release bioactive GnRH, resulting in activation of the pituitary-gonadal axis and in gonadal development.

MATERIALS AND METHODS

Animals

Hpg and normal mice of the same stock were housed in a colony room with lights on at 01.00 h and lights off at 15.00 h. Food and water were available ad libitum. Two normal females and one male were housed in breeding cages and the females were inspected every morning for a vaginal plug. A vaginal plug indicates insemination and the day it was found was designated gestational day 0 (E0).

Neural Tissue Grafts

Pregnant females were sacrificed on E12 or E13 and the embryos were removed from the uterus and placed in 0.9% sterile saline on ice. The embryo's head was placed under a dissecting microscope,

and a tissue fragment of about 1 cubic mm was dissected from the junction of the nasal septum and the telencephalic vesicle at the midline. The fragment was then placed in a drop of ice cold saline and, using gentle suction, taken into a 23 gauge needle. Adult hpg males were anesthetized with chloral hydrate (360 mg/kg) and placed in a stereotaxic apparatus. The needle with the tissue fragment was lowered into the host brain through a hole drilled in the skull and the fragment ejected into the host's POA (n=8), AHA (n=3), or the third ventricle (n=1). according to the coordinates of Slotnick and Leonard [7]). All transplants, except the one in the third ventricle, were placed bilaterally.

Immunocytochemistry

Following a survival period of 10 days to 10 weeks the animals were deeply anesthetized with chloral hydrate and perfused transcardially with saline followed by 4% paraformaldehyde or Zamboni's fixative (1.8% paraformaldehyde with 7.5% saturated picric acid). The brains were removed, postfixed overnight and 40-50 μm coronal sections were cut on a Vibratome. Sections through the diencephalon were processed for GnRH immunocytochemistry as previously described [8] using the LR-1 antibody (gift of R. Benoit) at a dilution of 1:20,000. Finally the sections were mounted on gelatin-subbed slides, counterstained with cresyl violet, dehydrated and coverslipped. Testicular weight, at sacrifice, of at least 2 standard deviations heavier than the mean of age-matched untreated adult hpg (7.5 $\pm$ 2.5) was considered an indicator for

stimulation of the host pituitary-gonadal axis by the transplanted neurosecretory GnRH cells.

RESULTS

The graft in most of the animals was much larger than its original size at the time of implantation and occupied much of the host's diencephalon (Fig.1). This was clearly due to continued mitotic activity of the transplanted tissue, particularly of non-neuronal elements such as cartilage, a major constituent of most grafts. In 5 of the 11 animals that received intraparenchymal grafts (POA or AHA) the transplant grew into the third ventricle. In most animals the boundary between the graft and host parenchyma was indistinct and appeared to merge in several points. The implant was poorly vascularized in comparison to septal-POA grafts [6] possibly due to the fact that cartilage is a nonvascularized tissue.

Six of the hosts had GnRH positive cells or axons in their brain (Table 1). In 4 of them GnRH axons were found in the ME (Fig. 2), and 3 of these showed testicular development (L9, L28, N27-Table 1). Tne one animal that had GnRH axons in its ME but no testicular growth (L20) survived only 3 weeks post grafting, when graft induced testicular development is rarely observed [6].

GnRH neurons were found in the host parenchyma of 3 animals (see Table 1) at a distance of more than 2 cell diameters from the graft/host border (Fig. 3). The displacement of these cells indicates an active migration out of the grafted tissue. Some of these neurons had elaborated a dendrite and a long bifurcating axon growing

medially in the direction of the third ventricle. The small number of GnRH cells surviving in the transplanted animals (1-10 per animal) did not permit us to conclude whether these cells were migrating preferentially towards their normal residence areas, or were displaced at random. Only in 1 animal (L30) did some transplanted GnRH cells maintain the compact arrangement formed by migratory GnRH cells in the nasal septum (Fig. 4A). Most cells, however, were spaced apart from each other both in the graft and the host tissue (Fig. 4B).

GnRH axons were found in 6 of the grafted animals. Although GnRH axons grew out of the grafts in different directions, the majority took a ventromedial route, crossing the host parenchyma in the direction of the third ventricle (Fig.5). These axons did not fasciculate though they traveled in parallel. Once at the ventricular wall, GnRH axons turned caudalward, following the periventricular pathway, which is a major projection of GnRH axons in the rodent [9], terminating at the lateral aspects of the ME (Fig. 2). Evidence that a GnRH cell resident in the host generated a functional ME innervation is derived from one animal (L28) in which only one immunoreactive neuron was identified. This GnRH neuron was resident in the host tissue and its axon terminated in the ME and supported testicular growth.

DISCUSSION

We report here that migratory GnRH cells, transplanted into an adult brain within E12-E13 nasal septum tissue, are capable of full

differentiation. GnRH neurons from grafts in the POA or AHA elaborated dendrites and axons and the latter innervated the host ME within three weeks post transplantation. By 4 weeks this innervation induced testicular development in some of the recipient hpg males indicating that the transplanted GnRH neurons processed the GnRH precursor to the amidated bioactive decapeptide.

A few GnRH cells migrated a short distance from the graft into the host brain and, at least in 1 animal, a cell that migrated into the host parenchyma innervated the ME. The view of the adult brain as a permissive medium for migration is consistent with the report of Sotelo and Alvarado-Mallart [10]. These authors have demonstrated that Purkinje cells from embryonic cerebellum placed into the cerebellum of adult pcd mutant mice are capable of migrating into their proper position in the host cerebellum and establishing synaptic connections with their normal target areas. It has also been reported that embryonic motorneurons transplanted into an adult mouse CNS can migrate as much as 4 mm [11]. In our study, however, GnRH cells migrated only a few cell diameters out of the graft. This could be due to some inhibition imposed by the host parenchyma. The migration of transplanted GnRH cells into the host is not restricted to GnRH cells in the migratory phase. When septal-POA grafts, containing post migratory GnRH cells from E17-21 donors, are placed into the third ventricle, a small number (1-10) of GnRH cells appear to migrate into the host hypothalamus [12]. However this migratory process is apparently incompatible with survival of the cells, since the migrating population can be detected in animals surviving 5-10 days post grafting [12], but only rarely at

30 days or longer survival periods (A. J. S.-unpublished observations).

The number of GnRH cells found in animals with nasal septum transplants was much smaller than the average number found in the nasal septum of E13 embryos [1, 2]. Similarly only a few GnRH cells were detected in E14.5 rat nasal tissue transplants placed into adult rat third ventricle, although nasal tissue fragments from as many as 8 embryos were pooled into each graft [13]. However, when the nasal tissue was cotransplanted with medial basal hypothalamic (MBH) tissue many more GnRH cells survived in the graft and projected to the MBH cotransplant [13]. It appears that transplantation of GnRH cells in their early migratory phase results in either cell death or phenotype shift and, presumably, only those capable of migrating or projecting out of the grafted nasal tissue are rescued. The poor vascularization of the graft might affect the survival of GnRH cells that fail to migrate into the host. Indeed most grafts contained tissues, such as cartilage, that are not normally vascularized. However, we cannot exclude the possibility that, due to a misplaced dissection, the bulk of GnRH cells in the nasal septum were not included in the transplanted tissue.

A few of the GnRH cells migrated into the host parenchyma and extended long axons, some of which terminated in the ME, thereby inducing gonadal growth. These findings demonstrate that at least some GnRH cells in their early migratory phase, before their entry into the forebrain, are fully commited to their morphological and biochemical phenotype and are capable of expressing it when transplanted into an adult brain. Similarly, cortical neurons were

shown to become committed to a limbic phenotype during their migratory phase, before arriving at the cortical plate [14]. We have recently demonstrated that the majority of GnRH cells in the nasal septum do not process the GnRH precursor to the bioactive decapeptide by E12-13 (I. L., M. J. G. and A. J. S.-manuscript in preparation), indicating that at this early migratory phase the cells do not yet express their fully differentiated biosynthetic activity. Following transplantation into an adult brain these cells must assume the enzymatic capability for producing the bioactive decapeptide, as demonstrated by stimulation of testicular growth. Therefore, it appears that uninterrupted migration of GnRH cells is not required for elaboration of their proper projections and assumption of their neuroendocrine function.

Correction of the reproductive deficiency of the hpg mouse was previously demonstrated by transplanting normal fetal septal-POA tissue containing GnRH neurons into mutant adult hosts [5]. Implantation of fetal POA tissue into the third ventricle of adult hpg males results in increased gonadotropin production, testicular development and seminal vesicle growth. These changes are driven by stimulation of the pituitary-gonadal axis which in turn is dependent on innervation of the host's ME by GnRH fibers from the graft [6]. Unlike previous studies, however, here we have transplanted GnRH cells from the nasal septum. These transplants are not known to contain any cell population, other than GnRH cells, that would directly innervate or interact with the ME. Also, we found that the ME innervation can be supplied exclusively by a GnRH cell outside the graft. Thus, we have demonstrated in this

study that the capability of GnRH cells to recognize and innervate their proper target in the adult host brain is not likely to be imparted by signals from other cells in the transplanted tissue. It is, therefore, a plausible hypothesis that the recognition of the ME as a target for axonal outgrowth is an intrinsic property of the GnRH cells. This hypothesis is consistent with Perlow et al. [15] who reported that GnRH cells in transplants taken from the accessory olfactory bulb are capable of innervating the ME and inducing physiological recovery in hpg males, although these cells do not normally innervate the ME.

We report for the first time that GnRH axons from transplants placed in the POA or AHA can grow a long distance through the host parenchyma to innervate the ME, located 2-3 mm caudal to the graft. This observation indicates that the tropic signals directing the outgrowth of GnRH axons to the ME must operate over long distances. The host ME as well as cografted ME has been proposed to attract axons from grafted GnRH cells [16, 17], thus implicating the ME as a source of a chemotropic signal. However, the effective range of chemotropic factors on axonal outgrowth was reported to be 100-300 μm in explants from vertebrate embryos [18]. Therefore, it is unlikely that a diffusible gradient emanating from the ME is the only signal directing GnRH axonal outgrowth from the transplants. Guidance by local signals which are retained by the adult host tissue should therefore be invoked to account for the accurate navigation of the GnRH axons. Such local guidance cues are probably responsible for the trajectory displayed by many GnRH axons which extend medially from the transplant, through the host POA-AHA, in

the direction of the third ventricle. Since this axonal pathway does not represent GnRH projections found in the normal mouse [9], it might indicate that the guidance cues directing GnRH axonal outgrowth in the host are widely distributed, allowing ectopically located GnRH axons to home in towards the third ventricle. That broadly distributed tropic cues operate in the embryonic vertebrate CNS was demonstrated by Harris [19] who studied the axonal projections from ectopically transplanted retinae towards the tectum in Xenopus. We propose that a similar guidance mechanism is also operating in the mammalian CNS and is maintained through adulthood. An alternative explanation to the observed projection would be that the ME provides a trophic support, which is critical for GnRH cells, and this support results in favorable survival of the cells that had innervated the ME at random. This explanation, however is not supported by a previous study from this laboratory which established that 25-83% of GnRH cells surviving in POA transplants placed into the third ventricle of adult hpg males do not innervate the ME [20]. In addition GnRH cells derived from fetal POA grafts and placed into the lateral ventricle also survive although they do not form connections with the host ME [21].

We have demonstrated here that heterochronic and heterotopic transplanted GnRH cells which are prevented from pursuing their normal migration and have to establish connections in an adult brain are, nevertheless, capable of producing their fully processed neuropeptide product and of recognizing their proper target in the host brain. We, therefore, conclude that a migratory population of GnRH cells, in the nasal septum, is already fully committed and

capable of pursuing its morphological and physiological differentiation in the adult brain.

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FIGURE LEGEND

Fig. 1. Low power micrograph showing the grafted nasal septum (G) within the host AHA. 140X.

Fig. 2. Dark (A) and light (B) field micrographs of GnRH axons emerging from the third ventricle (3V) and terminating at the host ME (arrows). A=350X. B=175X.

Fig. 3. A: GnRH cells (arrow) in the host (H) outside the graft (G). 350X. B: A GnRH cell that has migrated further into the host AHA. 560X.

Fig. 4. A: Two clusters of GnRH cells (arrows) in the graft in a similar formation to the migratory cords seen in the embryonic nasal septum. 280X. B: A GnRH cell in the host displaying a mature morphology with dendritic and axonal processes (arrows) typical to most grafted GnRH cells. 280X.

Fig. 5. A low power dark field micrograph depicting GnRH axonal processes emanating from the graft (G) and projecting across the host AHA towards the third ventricle (3V). 140X.

FIG. 1

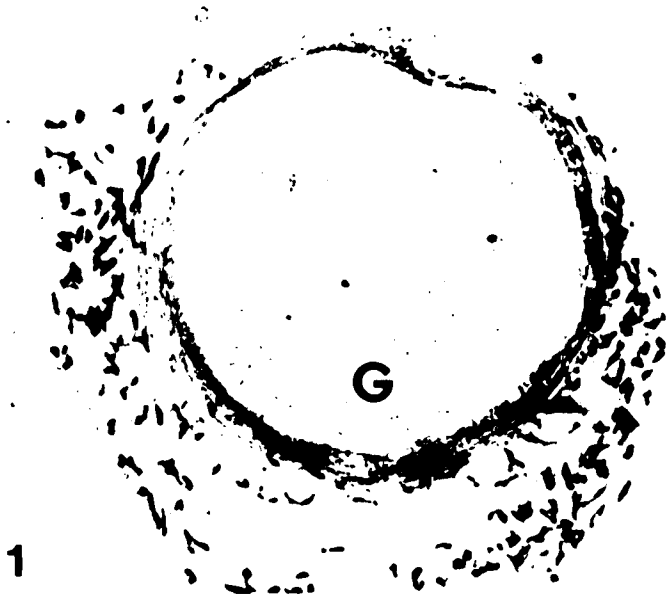

FIG. 2

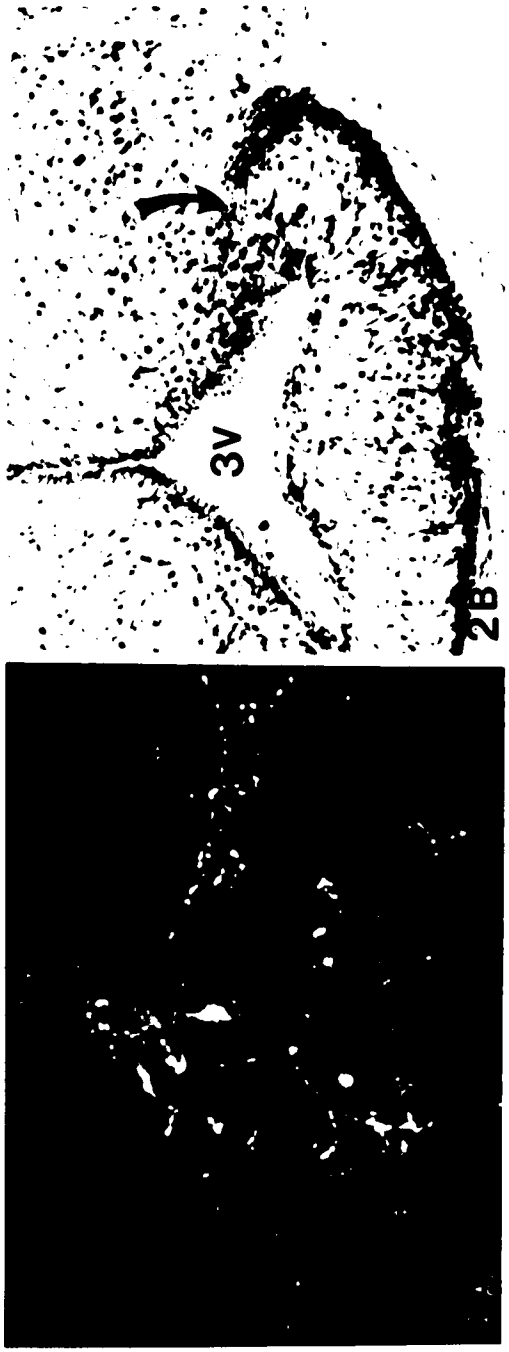

FIG. 3

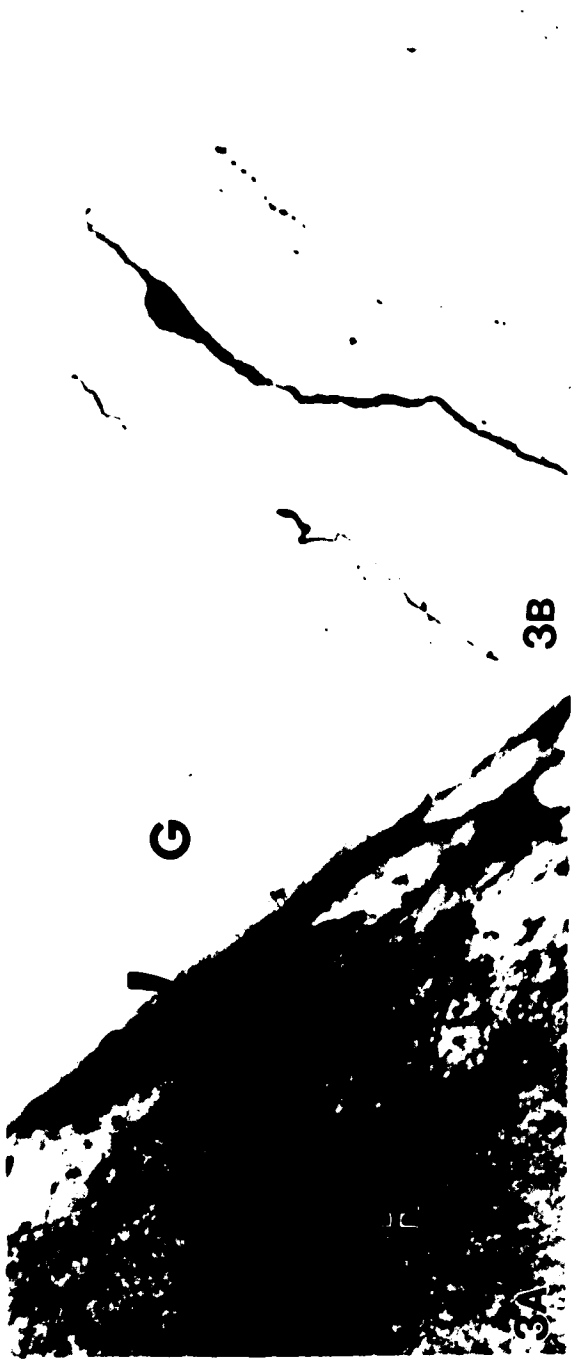

FIG. 4

FIG. 5

Table 1. Transplants of E12-E13 nasal septum tissue into the brain of adult hpg males: Testicular weight and GnRH immunopositive elements in the host.

Animal No.	Age of Donor	Post Grafting Survival (weeks)	Testis Weight (mg)*	GnRH Immunopositive Elements
L20	E13	3	5.9	6 cells in graft and many positive fibers in the host extending medially from the graft, and approaching the third ventricle. Many fibers in the ME.
L30	"	3	7.3	12 cells in the graft and fibers in the host extending medially from the graft, and approaching the third ventricle.
L35	"	10	11.1	No immunopositive staining.
L09	"	10	13.8	1 cell in the graft/host border and fibers in the ME.
L50	"	1.5	7.4	No immunopositive staining.
L55	"	1.5	11.8	No immunopositive staining.
L51	E12	4	4.3	No immunopositive staining.
L28	"	4	23.6	1 cell in the host dorsal to the graft, and a few fibers in the ME.
N31	E13	7.5	8	2 cells in the host medial to the graft and fibers extend medially from the graft towards the third ventricle.
N32	"	7.5	9	No immunopositive staining; the grafted tissue is dead.
N26	"	8	7	No immunopositive staining.
N27	"	8	45.5	7 cells in the graft and 3 in the host. Many fibers extend medially from the graft in the direction of the third ventricle, and many axons terminate in ME.

* The mean ± SD of testes weight in untreated adult hpg is 7.5 ± 2.5 mg.

CHAPTER 3.

Gonadotropin-releasing hormone (GnRH) neurons in the hypogonadal mouse elaborate normal projections despite their biosynthetic deficiency.

ABSTRACT

The hypogonadal mutant mouse (hpg) is characterized by failure of gonadal growth after birth. This phenotype is caused by a truncated GnRH gene. The truncated gene is transcribed in regions corresponding to the normal distribution of GnRH cells. However, the neuropeptide is undetectable in the homozygous animals. This finding suggests that in spite of their failure to synthesize their unique peptide, GnRH cells pursue their normal migration from the olfactory placode to their normal residence areas. We initiated this study to determine whether the mutant GnRH cells can establish and maintain their normal axonal connections with their major target, the median eminence. Adult hpg males were given intraperitoneal injections of the fluorescent tracer Fluoro-Gold. This tracer does not cross the blood-brain-barrier and hence is taken up only by CNS neurons whose axons terminate on fenestrated capillaries, such as the capillaries of the median eminence. Cells transcribing the truncated GnRH gene were visualized by *in situ* hybridization with a probe to the intact portion of the gene. In 3 hpg males 64.1 $\pm$ 5.6% of GnRH transcribing cells contained Fluoro-Gold particles, while 55.8 $\pm$ 6.4% of the cells in 3 normal males had taken up the tracer. Thus, we have established that the mutant neurons, that are devoid of their major neurosecretory product can, nevertheless, elaborate and maintain their axonal projections to their primary secretory target. We conclude that the capability of GnRH neurons to recognize and interact with their target is not dependent upon their neurosecretory function.

INTRODUCTION

GnRH neurons are derived from the olfactory placode, where they are first detected in the mouse on gestational day 11.5 [1, 2]. On subsequent days these cells migrate into the forebrain to reside in their adult distribution regions: the diagonal band, medial septum, preoptic area (POA) and anterior hypothalamic area (AHA) [3]. As GnRH cells approach their residence areas they elaborate axonal projections to their major secretory target, the median eminence (ME, [4]. In the adult male rat 50-70% of GnRH cells in the septal-POA-AHA regions project to the ME, as indicated by retrograde tracer studies [5, 6], while in the male mouse approximately 65% of GnRH cells in those regions project to circumventricular organs [7].

The hypogonadal mouse (hpg) carries an autosomal recessive mutation characterized by an undeveloped reproductive tract in the adult mutant [8]. The homozygous phenotype is due to a large truncation in the gene encoding the GnRH precursor. The truncated gene consists of exons I and II only (exon II contains the GnRH coding region), while exons III and IV are deleted [9]. A low level of transcription from the truncated gene is detected in cells near the organum vasculosum of the lamina terminalis (OVLT) of the hpg brain, a region which contains many GnRH neurons in the normal mouse [9]. However, the decapeptide is undetectible in these animals [8]. These findings suggests that in spite of their failure to synthesize their unique neuropeptide, GnRH cells pursue their normal migration from the olfactory placode to the forebrain.

To further evaluate the role of GnRH synthesis and secretion in the differentiation of GnRH cells we have asked whether GnRH transcribing cells can elaborate their projections to their major terminal field in the ME in the absence of their principal secretory product. This question was addressed by peripheral injections of the fluorescent retrograde tracer Fluoro-Gold into adult hpg males. These males were subsequently perfused and their brains processed for *in situ* hybridization histochemistry to detect GnRH transcribing cells. Peripherally administered Fluoro-Gold can be captured only by CNS neurons whose axons terminate outside the blood-brain barrier, such as GnRH terminals in the ME [10]. Hence, capture of Fluoro-Gold by GnRH expressing cells would indicate that these cells are capable of elaborating axonal projections to their normal target regardless of their biosynthetic and secretory deficiency.

MATERIALS AND METHODS

Animals

Hpg and normal mice of the same strain (C3H/HeHX101H) were housed in a colony room with lights on at 01.00 h and lights off at 15.00 h. Food and water were available *ad libitum*. Three adult hpg males and three normal adult males were injected intraperitoneally with 40 mg/kg Fluoro-Gold (Fluorochrome, Englewood, CO) in saline. Each animal received 3 injections at 5 day intervals.

Tissue preparation

One week following the last injection the animals were deeply anesthetized with chloral hydrate and perfused intracardially with saline followed by 40 ml 4% paraformaldehyde in 0.1 M phosphate buffered saline PH 7.2 (PBS). Following perfusion the brains were removed from the skull, post-fixed in the same fixative for 2 hr and equilibrated in 10% sucrose in PBS for 2 hours and 20% sucrose overnight. Finally the brains were snap-frozen in isopentane on dry ice and kept at -70 ^{o}C. Coronal sections were cut on a cryostat at 20 μm, thaw mounted on polylysine coated slides and stored at -70 ^{o}C until further processed.

In situ hybridization

Sections through the septal-POA-AHA were processed for *in situ* hybridization using a modification of Young's protocol [11]. Briefly, the sections were immersed in 4% paraformaldehyde in PBS followed by 2 washes in PBS. Subsequently the sections were placed in 0.25% acetic anhydride in 0.1 M triethanolamine/saline PH 8.0, dehydrated and delipidated in chloroform. Following rehydration, the sections were air-dried and covered for 2 hr with prehybridization buffer containing 4 X SSC (1 X SSC is 0.15 M NaCl/0.015 M sodium citrate, PH 7.2), 50% formamide, 10% dextran sulfate, 500 mg/ml sheared single stranded salmon sperm DNA, 250 mg/ml tRNA, 1 X Denhardt's and 10 mM dithiothreitol. The prehybridization buffer was then replaced with a new buffer containing 500,000-1,000,000 cpm/25 μl of [^{35}S]dATP labeled probe and the sections were covered with parafilm and incubated in 37 ^{o}C for 18-20 hr. The post-hybridization washes included 2

washes in 1 X SSC/50% formamide at 40 °C, and 2 washes in 1 X SSC at room temperature. Dithiothreitol (5mM) was included in all the washes. Finally the slides were dipped in nuclear emulsion (NTB2, Kodak) diluted 1:1 in water and stored in 4 °C . Following exposure period of 4-8 weeks the slides were developed with Kodak D19 and sections analyzed under bright field, dark field, and fluorescence microscopy with a UV filter to detect silver grain aggregates and Fluoro-Gold particles, respectively. In the normal animals a cell was considered labeled when the grain density above it was at least 4 times the density over adjacent cells. In the hpg animals, the GnRH hybridization signal was greatly reduced compared to the normal animals and accordingly a cell was considered labeled if the grain density above it was at least twice the density over adjacent cells.

Probe labeling

Two oligodeoxynucleotide probes were synthesized on an Applied Biosystems 380B DNA synthesizer: a 45-base probe (MS2) complementary to the GnRH coding region in exon II of the mouse gene and a 50-base probe (MS1) complementary to the GnRH-associated peptide coding region in exon III [9]. The labeling reaction was performed by incubating the probe (15 pM) with 120-130 pM [^{35}S]dATP (NEN, >1000 Ci/mmol), and 30 U of terminal deoxyribonucleotidyl transferase (BRL), in 37 °C for 1.5 hr. The probe was then purified on a Nensorb-20 column (NEN).

RESULTS

GnRH transcribing cells in the POA and AHA of 3 hpg males concentrated peripherally administrated Fluoro-Gold (Fig. 1A, 1B). The Fluoro-Gold positive cells comprised 64.1 ± 5.6% of the total population of GnRH transcribing cells in the hpg males. In the 3 normal males 55.8% ± 6.4 of the GnRH transcribing cells had concentrated Fluoro-Gold particles in their somata (Fig. 1C, 1D). The mean number of cells with hybridization signal in the hpg males was 42 cells/animal. In the normal males, on the other hand, 269 cells/animal had hybridization signal. In confirmation of Mason et al. [9], the distribution of the GnRH transcribing cells in the hpg males was similar to the pattern observed in the normal animals.

Adjacent sections from the brain of a normal male hybridized with probes MS1 or MS2 had a comparable number of cells with hybridization signal. Sections from the hpg brain, on the other hand, had hybridization signal only when hybridized with probe MS2 which corresponds to exon II which is spared by the mutation. Sections hybridized with the exon III complementary probe (MS1), had no detectable signal.

DISCUSSION

GnRH transcribing cells in the POA and AHA of hpg males were found to concentrate Fluoro-Gold using a combined *in situ* hybridization-Fluoro-Gold methodology. This finding indicates that mutant GnRH cells elaborate and maintain axonal connections with their major target, the ME, in the absence of their principal secretory product. Thus, we have demonstrated that these neuropeptidergic cells can send their axons to their normal target and maintain their innervation of this region independently of synthesis and secretion of their unique product. However, we can not rule out the possibility that another neuropeptide is synthesized by GnRH cells, allowing for the mutant cells to maintain functional neurosecretory activity. The neuropeptide galanin was shown to be expressed by 20% of the GnRH cells around the OVLT of the male rat [12], and some of these cells appear to project to a circumventricular organ [13]. Delta sleep-inducing peptide is another neuropeptide reported to be colocalized with GnRH in axonal terminals in the guinea pig ME [14]. It remains, however, to be demonstrated whether these neuropeptides colocalize with GnRH in the mouse and whether the colocalizing cells actually project to the ME.

Since the ME is not the only circumventricular organ innervated by GnRH cells in the septal-POA-AHA continuum [3], it is possible that the Fluoro-Gold was taken up by GnRH cells projecting to other neurohemal organs; the OVLT and the subfornical organ. However,

the rodent ME is the major neurohemal target of GnRH cells, as demonstrated by retrograde tracer studies [5, 6]. Therefore, in the hpg mouse, the ME is likely to be the primary site of GnRH terminals and thus the site of Fluoro-Gold uptake.

The small number of GnRH transcribing cells detected by *in situ* hybridization in the hpg male (about 16% of the GnRH cells detected in a normal brain) is probably due to the small number of GnRH mRNA molecules per cell, rendering the signal in most cells below detectability level. The distribution of silver grains in the hpg brain is not limited to the nuclear area suggesting that at least some of the GnRH transcripts represent a cytoplasmic mRNA species. Whether the pre-mRNA is fully processed in the mutant cells, cannot be safely deduced from our data.

We have previously reported that upon transplantation of fetal tissue into hpg mice, the grafted GnRH cells innervate the host ME in a pattern reminiscent of the normal innervation [15, 16]. The recapitulation of the specific innervation pattern by axons from transplanted cells was attributed to the existence of a vacant terminal field in the lateral median eminence. According to this hypothesis the vacant field, which is not occupied by the mutant GnRH axons, is available for the ingrowing axons from the transplant [16]. This hypothesis, however, is not supported by the present findings which suggest that the mutant GnRH system in the hpg mouse occupies at least part of its terminal field in the ME. Thus, innervation of the ME by the mutant cells does not appear to exclude additional innervation of this target by transplanted GnRH cells. Although most GnRH axons terminate in the lateral aspects of

the rodent ME [16], this terminal field appears to be loosely organized and a defined synaptic organization is not apparent [17]. Rather, most GnRH terminals in the ME are associated with tanycytes and glial processes which often separate the terminals from the pericapillary space [18]. Since these terminals do not form synaptic contacts with a defined target, competition for a limited post-synaptic space is not likely to play a major role in the choice of termination site by GnRH axons. As an alternative explanation to the stereotypic innervation pattern displayed by axons from the transplanted cells we propose that signals which operate in the fetal brain to guide ingrowing GnRH axons into the ME are retained by the adult brain. These signals can, therefore, operate in the adult to direct the outgrowth of GnRH axons from the transplant to the host ME. This hypothesis is supported by a previous study of fetal transplants placed in the AHA of the adult hpg. GnRH axons emanating from these transplants navigate across 2-3 mm distances, through the parenchyma of the adult host brain, to innervate the ME [19].

Our findings suggest that failure to synthesize a major secretory neuropeptide does not interfere with the cell's ability to elaborate and maintain its normal projections. It remains, however, to be demonstrated whether the mutant cells also receive their normal complement of synaptic input and hence are integrated into their functional neuronal circuitry. That a biochemically and secretory deficient neuroendocrine cell is, nevertheless, properly connected has been shown in the Brattleboro rat. This mutant has a single base deletion in the vasopressin gene and consequently fails to

synthesize the neurohormone, although the gene is correctly transcribed and spliced [20]. The Brattleboro rat was shown to maintain a normal pattern of circadian variation in the level of vasopressin mRNA in the suprachiasmatic nucleus [21].

The distribution pattern and proportion of the GnRH cells projecting outside the blood-brain-barrier in the hpg male is similar to those in the normal male. These findings are consistent with the report of Mason et al. [9] and support the conclusion that the migration and functional organization of the GnRH cell population is not affected by their biosynthetic deficiency.

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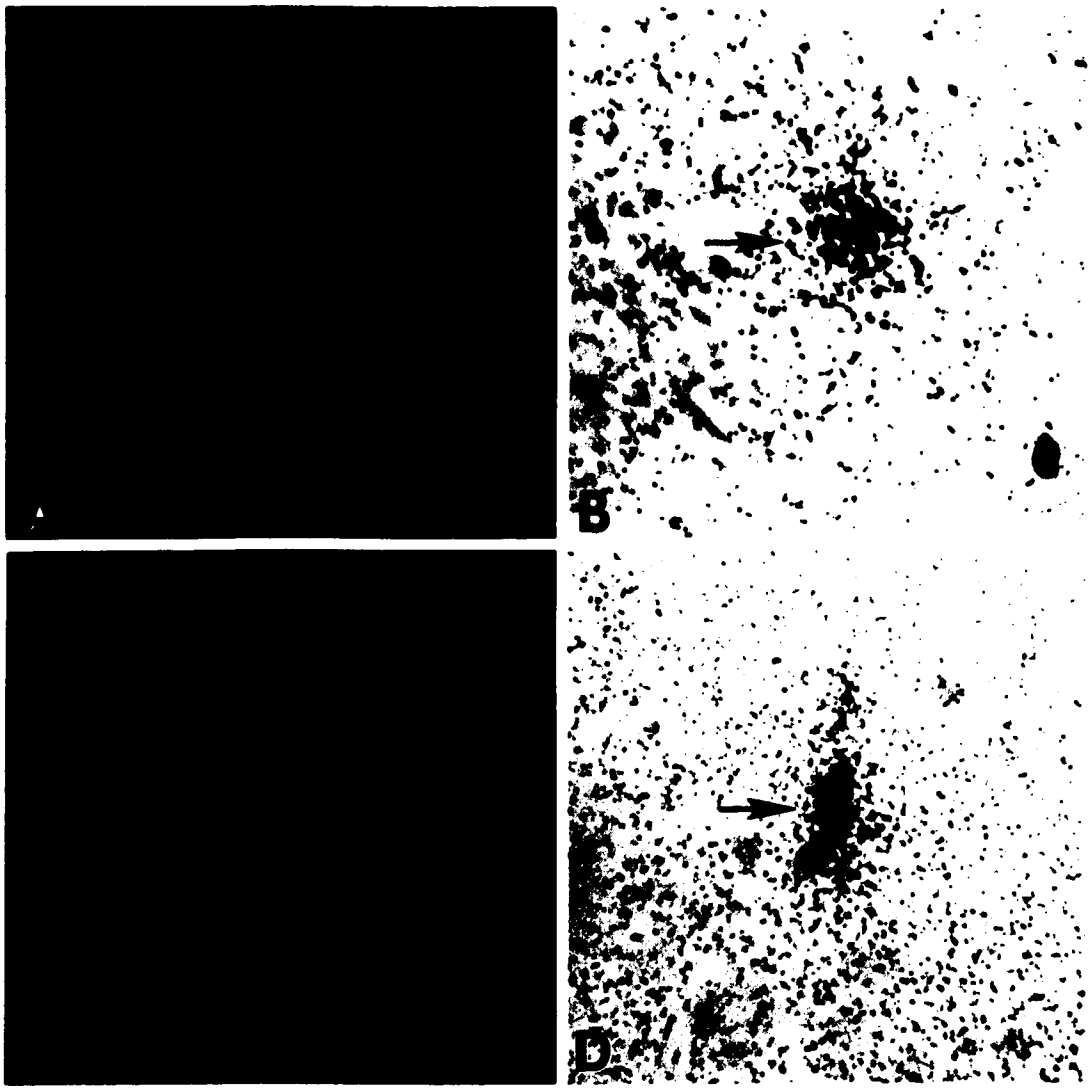
FIGURE LEGEND

FIg. 1. Retrograde Fluoro-Gold uptake by GnRH expressing cells in an hpg and a normal male mice. A neuron in the preoptic area of an hpg male mouse contains Fluoro-Gold particles in its cytoplasm (A). The hybridization signal over this cell (B) indicates that it is expressing the GnRH gene. Similarly, in the preoptic area of a normal male mouse a cell containing Fluoro-Gold particles (C) is expressing the GnRH gene (D). Bar; A, B = 10 μm, C, D = 20 μm.

FIG. 1



CHAPTER 4.

Reversal of reproductive deficiency in the hpg male mouse by neonatal androgenization.

ABSTRACT

Some aspects of reproductive function in the GnRH deficient hypogonadal (hpg) mutant mouse can be restored by transplanting normal fetal brain tissue containing GnRH cells into the CNS of adult hpg. However, hpg males showing physiological response to the graft fail to display sexual behavior and are infertile. We hypothesized that the reproductive deficit of these males is due to insufficient perinatal exposure to testicular androgens as a consequence of the GnRH deficiency. To test this hypothesis we androgenized hpg males by injecting them neonatally with testosterone propionate (TP). Controls consisted of hpg males, not androgenized neonatally, and of normal males. All three groups were given a TP implant in adulthood and their copulatory behavior and reproductive capability recorded. In addition, other hpg males, not androgenized neonatally, received fetal brain transplants containing GnRH neurons and were also tested for copulatory behavior and reproductive capability before and after receiving a TP implant.

Three of 8 neonatally androgenized hpg males expressed the full repertoire of male sexual behavior, including intromission and ejaculation, and sired several litters. Three of 7 control hpg males, which were not androgenized neonatally but implanted with TP in adulthood, also displayed mounting and intromission but there was no evidence of ejaculation and these males failed to impregnate normal females. Of the 8 hpg males which responded to a fetal transplant with testicular growth only 1 displayed mounting

behavior. However, when given a TP implant 4 of 8 hpg males with grafts displayed mounting and intromissions.

We conclude that perinatal androgens, which are required to masculinize copulatory behavior, are deficient in the hpg and therefore are probably dependent on GnRH secretion. Accordingly, full expression of adult reproductive behavior must be dependent on a functional hypothalamic-pituitary-gonadal axis in the neonatal male mouse.

INTRODUCTION

The hypogonadal (hpg) mouse is a mutant with a large truncation in the GnRH gene, resulting in failure to synthesize GnRH [1]. The homozygous phenotype is characterized by an undeveloped reproductive tract (testes and seminal vesicle weight in the adult hpg male are 2% and <1%, respectively, of the normal male) and low levels of the gonadotropins, LH and FSH [2]. Some aspects of the reproductive deficit in both hpg males and females can be restored by transplanting normal fetal preoptic area (POA) tissue containing GnRH cells into the third ventricle of adult hpg mice [3]. Recovery in the recipient animals (POA/hpg) is manifested by gonadal growth, increased production of gonadotropins, steroidogenesis, and gametogenesis [4]. Physiological response to the graft is evident in some POA/hpg animals by one month post-transplantation and is dependent on innervation of the median eminence by GnRH axons from the graft [4]. However, POA/hpg males showing physiological recovery have failed to display masculine sexual behavior in response to receptive females, or to impregnate normal females.

Perinatal androgens are known to be critical for complete masculinization of sexual behavior in rodents [5]. In mice both prenatal [6] and postnatal [7] testosterone levels are higher in the male than in the female. Testosterone is, therefore, an appropriate candidate for induction of masculine developmental pattern in rodents. In addition to their organizational effect on copulatory behavior, perinatal androgens induce a permanent masculine

pattern in androgen sensitive neural and muscular structures. One of these structures, the sexually dimorphic spinal nucleus of the bulbocavernosus (SNB) and its target perineal muscles, can be permanently modified by perinatal androgen manipulation in the rat [8]. The perineal muscles innervated by the SNB motor neurons are involved in control of some copulatory penile reflexes [9]. The mouse SNB is also sexually dimorphic and sensitive to neonatal androgen manipulation [10].

We hypothesized that the behavioral and reproductive deficit of POA/hpg males is due to insufficient perinatal exposure to testicular androgens as a result of the GnRH deficiency. To test this hypothesis we evaluated the effect of neonatal androgenization, by testosterone propionate (TP) injection on postnatal day 1 or 2, on the sexual behavior and reproductive capability of hpg males treated in adulthood with TP implants. In addition, POA/hpg males were tested for sexual and reproductive capability in the presence or absence of TP.

MATERIALS AND METHODS

Animals

All mice were housed under controlled conditions of lighting (14L:10D, lights off at 1500 h) and temperature (24-25 C). Food and water were available ad libitum. Males in all the experimental groups remained gonadally intact.

Experimental Groups

Neonatally Androgenized hpg (And/hpg). Males and females heterozygous for the hpg mutation were housed in breeding cages and delivered pups were given a subcutaneous injection of 100 μg TP in 0.02 ml sesame oil on postnatal day 1 or 2. This protocol effectively masculinized sexual behavior in female mice [11]. At 2-3 months of age, males that received a postnatal TP injection were anesthetized with chloral hydrate (360 mg/kg) and laparotomized. Those with undeveloped testes were diagnosed as And/hpg (n=8) and thereupon received a subcutaneous implant of 2 cm Silastic tubing (id, 0.04 in; od, 0.085 in; Dow-Corning, Midland, MI) containing TP, which supports plasma levels of about 110 ng/ml [12].

Nonandrogenized Control hpg (Cnt/hpg). Seven hpg males that were not injected neonatally with TP were given a TP implant at age 6-9 months.

Normal Control males. Normal (C3H/HeHX101H) males (n=4) also received TP implants at age 2-3 months and were observed for sexual behavior with receptive females.

Nonandrogenized hpg with grafts (POA/hpg). Adult hpg males that were not injected neonatally with TP received grafts of fetal septal-POA tissue containing GnRH cells. The transplant was placed in the third ventricle as previously described [13]. Five-six months post-implantation, 17 POA/hpg animals were housed with normal cycling females for 5 weeks to assess their reproductive capability, and subsequently observed in 2 tests of sexual behavior. Four of these POA/hpg males and an additional 4 POA/hpg (3-5 months post-implantation) were given TP capsules as above, paired with

cycling normal females for 3 weeks and tested twice for sexual behavior.

Behavioral and Reproductive Tests

Weekly tests of sexual behavior commenced 2 weeks after TP implantation. The tests were carried out by placing a receptive female into the male's cage for a 2 hr interval, beginning at lights off (1500 hr), under dim red light. Female mice were ovariectomized and implanted with a 10 mm silastic tubing filled with 1 μg 17β-estradiol/ml SO. Behavioral estrus in these females was induced by injections of estradiol benzoate (1 μg at 0900 hr a day prior to the test), and progesterone (500 μg at 0900 hr on the day of the test. Receptivity of these females was confirmed by placing them with normal males at the conclusion of the test. Only tests with receptive females (those scoring average or better according to "McGill's receptivity scale" [14]) were included in the test results. Each male was tested 2-4 times and the number of mounts and intromissions (as described by McGill [14]) was recorded. For each male the percent of tests in which mounts and intromissions were displayed was recorded. The mean for each group was derived from these individual percentages. Kruskal-Wallis one-way ANOVA was used to determine the significance of differences among the groups. To evaluate the reproductive capability of And/hpg, Cnt/hpg and POA/hpg, the males from these groups were housed with 2 normal cycling females for at least 3 weeks .

Radioimmunoassay (RIA)

At the conclusion of the experiment, the animals were anesthetized with an overdose of chloral hydrate. Blood was taken from the orbital plexus of anesthetized mice using a heparinized glass pipette. Plasma was harvested from the blood after centrifugation and stored at -20 C until assayed using the RIA kit for LH and FSH provided by the NIDKK. Pituitaries were collected from animals in each group, snap frozen on dry ice, and extracted as previously described [3]. The pituitary extracts were processed for LH and FSH RIA along with the plasma samples. All samples were assayed in duplicates in one assay. The intraassay coefficient of variation, calculated as the mean of the variation of all sample duplicates in the assay was 3.4% for FSH, and 12.7% for LH.

Histochemistry and Immunocytochemistry

Testicular weight, at sacrifice, of at least 2 standard deviations heavier than the mean of age-matched untreated hpg (7.5 $\pm$ 2.5 mg) was considered an indicator for stimulation of the host pituitary-gonadal axis by transplanted neurosecretory GnRH cells in POA/hpg males. The testes were fixed by immersion in Bouin's fixative, and embedded in paraffin. Embedded testes were cut at 6 μm and sections were stained with hematoxylin-eosin. Testicular spermatogenic state was determined by comparing the percentage of tubules containing elongated spermatids for animals in each group. Most (n=7) animals from the And/hpg group were perfused intracardially with saline and 4% paraformaldehyde, and their brains collected and postfixed in the same fixative overnight. The brains were sectioned on a vibratome at 40 μm, and coronal sections

from the septal-anterior hypothalamic extent were immunocytochemically stained for GnRH according to the procedure described previously [13]. This was essential to confirm that all And/hpg were devoid of GnRH cells and, therefore, were indeed homozygous for the hpg mutation. Pituitaries from the perfused And/hpg, as well as from intact hpg and normal males, were cryoprotected in 30% sucrose, and 20 μm cryostat sections were immunocytochemically stained with LH or FSH antisera to visualize the gonadotrophs.

RESULTS

Reproductive capability

Three of 8 And/hpg males expressed the full repertoire of male sexual behavior including intromission and ejaculation, and sired several normal-sized and viable litters (Table 1). Three of 7 Cnt/hpg and 4 of 8 POA/hpg males displayed mounting and intromission behaviors when given TP in adulthood. However, the Cnt/hpg and POA/hpg males did not ejaculate, and failed to impregnate normal females. And/hpg and Cnt/hpg testes were significantly heavier than the testes of untreated hpg, but also significantly lighter than normal testes ($P < 0.05$, Table 1). Spermatogenic status was estimated by the percentage of seminiferous tubules containing elongated spermatids in one testicular cross section. Similar spermatogenic recovery was found in the testes of And/hpg and Cnt/hpg (in the range of 85-95%). In testes of normal males nearly 100% of the tubules contained elongated spermatids (Fig. 1).

Sexual Behavior

There were no significant differences in mounting and intromission behavior among the And/hpg, Cnt/hpg, and normal groups (Table 2). However both the And/hpg and Cnt/hpg groups displayed a trend toward lower levels of intromission than did the normal males. Two of the 4 normal males ejaculated during the behavioral tests. Although And/hpg males did not ejaculate during the tests, vaginal plugs were found in receptive females which were paired overnight with 3 of the 8 And/hpg, indicating that ejaculation had occured, and as noted above these animals sired litters. Cnt/hpg, on the other hand, neither displayed ejaculatory behavior, nor left vaginal plugs in females. Only one of the 17 untreated (with no TP implant) POA/hpg males displayed mounting behavior and none displayed intromission. In contrast, 4 of 8 POA/hpg with a TP implant mounted and intromitted, including the animal which had mounted in tests prior to receiving a TP capsule. However, none of the POA/hpg, whether TP-treated or not, impregnated normal cycling females during 3 weeks of continuous pairing. Testes and seminal vesicle weights were obtained in 11 of the untreated POA/hpg, and there was significant testicular development in 8 (82.9 $\pm$ 13.9 mg) and seminal vesicle development in 7 (107.0 $\pm$ 34.6 mg) of these animals, suggesting the presence of bioactive circulating androgens in many of the untreated POA/hpg males.

Plasma and Pituitary Gonadotropins

Plasma LH levels for all the groups were at or below the level of detection. Pituitary LH and FSH levels were low in all the hpg groups compared to the normal males. As most And/hpg mice were perfused for GnRH immunocytochemistry, their pituitaries were not available for RIA. Nevertheless, it can be appreciated that plasma and pituitary FSH in a single fertile And/hpg male were not elevated above the levels of Cnt/hpg (Table 1).

Immunohistochemistry for GnRH and Gonadotropins

And/hpg brains reacted with GnRH antiserum were completely devoid of GnRH cells or processes in the septal and preoptic areas, thereby confirming that these animal were homozygous hpg. Immunocytochemical examination of anterior pituitaries reacted with LH or FSH antisera revealed that And/hpg gonadotrophs were not markedly different in distribution or density from those of untreated hpg, or normal males.

DISCUSSION

Demonstration of full recovery of sexual behavior and reproductive capability in neonatally androgenized hpg males is in agreement with the critical role assigned to perinatal gonadal steroids. These steroids are essential for organization of the neural

circuits regulating male sexual behavior in the adult [5]. Circulating testosterone is higher in male mice than in females prenatally [6] as well as postnatally [7]. It appears, however, that neonatal androgenization is not critical for normalizing some aspects of masculine sexual behavior in the hpg male (i.e. mount and intromission) since display of these parameters is not significantly different between And/hpg and Cnt/hpg given TP in adulthood. This is in agreement with Edwards and Burge [11] who demonstrated that mounting behavior of female mice treated neonatally with oil and given TP in adulthood was not significantly different from the mounting display of females treated neonatally with TP, or from oil treated males.

Spermatogenic recovery also appears to be independent of neonatal androgenization as demonstrated by a comparable percentage of tubules containing elongated spermatids in And/hpg and Cnt/hpg testes. Similarly, the accessory glands and seminal vesicles of the adult hpg male are independent of neonatal androgens in their capability to respond to TP [15].

Neonatal androgenization is critical for complete reproductive function manifested by the capability of And/hpg males to both ejaculate and impregnate females. There was no evidence of ejaculation or fertility in Cnt/hpg males, although we can not exclude the possibility that unobserved ejaculatory responses occurred in this group. Neonatal androgens affect male copulatory behavior, presumably by permanent modification of the SNB and its target perineal muscles which are involved in the control of penile copulatory reflexes [9]. The SNB system in the mouse is sensitive to

neonatal androgens [10] which appear to act via androgen receptors on the perineal muscles [16, 17]. Neonatal androgens are also known to affect the organization of the hypothalamus as demonstrated by the hypothalamic sexually dimorphic nucleus which is larger in the male rat than in the female [18]. The size and cell number in this nucleus are sensitive to perinatal androgen manipulation. In the mouse, however, there is no apparent sexually dimorphic structure in the hypothalamus [19]. Hence, we can not determine whether there is a morphological difference between And/hpg and Cnt/hpg brains. However, the absence of a significant difference in mount and intromission behavior between these groups suggests that sexual motivation and arousal are not deficient in the Cnt/hpg group. It is therefore concluded that the critical role of neonatal androgen in restoring reproductive capability to the hpg male is in the motor component of masculine sexual behavior, presumably at the level of the SNB and the perineal muscles.

The requirement for neonatal TP treatment to complete the masculinization of the hpg male suggests that the perinatal rise in androgen secretion, thought to be the endogenous masculinizing signal, is indeed deficient in the hpg male. This in turn supports the hypothesis that perinatal androgen secretion in the male mouse must be GnRH dependent. That perinatal testicular steroidogenesis is regulated by the hypothalamic-pituitary axis has been previously suggested by the reduction of circulating levels of LH, FSH, and testosterone following passive immunization with GnRH antiserum of 5 day old rats [20]. Furthermore, Warren et al. [21] have reported that testes from prenatal and postnatal rats increased their

testosterone production when incubated with hCG. However, it has not been conclusively demonstrated that the perinatal testosterone rise in the male rodent is driven by the hypothalamic-pituitary axis, as now implied by our findings.

Testicular histology indicates that complete spermatogenesis was restored in And/hpg and Cnt/hpg males with TP implants. The major endocrine regulators of Sertoli cell function, and thereby of spermatogenesis, are FSH and testosterone. Hence, it is conceivable that the external TP combined with endogenous circulating FSH is sufficient to initiate and support spermatogenesis in both the And/hpg and Cnt/hpg. Since sperm quantity and quality was not evaluated, we can not exclude the possibility that the reproductive failure of the Cnt/hpg males was due to deficiency in sperm production or maturation. However, we do not favor this explanation since it was reported that reduced fertility in male mice treated neonatally with cyproterone acetate is not associated with deficient sperm production [22]. In addition Kolho et al. have demonstrated that male rats made infertile by neonatal treatment with a GnRH antagonist had no apparent abnormalities in sperm count, morphology, or motility and their ejaculatory behavior was deficient although their mounting and intromission appeared to be unaffected [23, 24].

Although selected And/hpg males attained complete reproductive function, not all animals showed this level of recovery. This is not due to lower testosterone levels in the non-responsive animals since there was no correlation between seminal vesicle weight, (which reflects the level of plasma testosterone), and reproductive recovery.

The failure to respond to neonatal testosterone might be due to differential exposure to a masculinizing factor in utero. Thus, it is conceivable that only hpg males which undergo sufficient prenatal masculinization can respond to the neonatal testosterone treatment. Since similar ratios in the Cnt/hpg group (3 of 7) and the POA/hpg with TP implant (4 of 8) displayed male sexual behavior, it is tempting to speculate that only hpg males positioned next to a normal or a heterozygous male in utero receive sufficient androgen exposure to induce prenatal masculinization. It has been demonstrated that male mice who develop between 2 males in utero are exposed to higher levels of testosterone than males with no male contiguity [25].

Although Cnt/hpg males received the TP implant later than the And/hpg (6-9 and 2-3 months of age, respectively), it is unlikely that the age difference between these groups has a critical role in their ability to respond to the exogenous hormone. Animals from both groups responded to the implant with similar degree of gonadal growth, initiation of spermatogenesis and sexual behavior. The only consistent difference between these groups is the capability of the And/hpg to exhibit functional ejaculation and to impregnate normal females. This capability was not demonstrated by the Cnt/hpg males. In addition, some of the And/hpg males remained sexually active and fertile at 11 months of age indicating that the behavioral and physiological response to exogenous TP is not diminished at this stage.

In our early observations adult hpg males remained sexually inactive even after receiving fetal POA grafts that induce testicular

growth and testosterone production. We have replicated these observations in this study and established that even POA/hpg males which responded to the graft with considerable testicular and seminal vesicle growth failed to display masculine sexual behavior. When given TP implant, however, some POA/hpg animals displayed mounting and intromission behavior, but failed to ejaculate or impregnate normal females. The requirement for TP supplementation by POA/hpg males to display sexual behavior implies that the low level of plasma LH in grafted hpg [4] is not sufficient to stimulate testosterone production to a level that will support activation of masculine behavior. This explanation is consistent with the low levels of plasma testosterone measured in POA/hpg males (about 30% of the normal level [3]).

Manifestation of the complete repertoire of male sexual behavior in the And/hpg males suggests that while GnRH may facilitate sexual behavior in the male rat [26, 27], it does not have a critical role in expression of mounting, intromission, and ejaculation behaviors by the male mouse. Similarly, while GnRH may play a role as a facilitator of female lordosis behavior in the rat [28, 29] it appears to be dispensable for expression of normal levels of lordosis in hpg female mice primed with estrogen and progesterone [30].

In agreement with a previous report [15], we have demonstrated that neonatally androgenized hpg males implanted with a TP capsule in adulthood are capable of expressing the complete repertoire of male copulatory behavior. In addition, we have demonstrated that neonatal androgenization can reverse the reproductive defects of the GnRH deficient hpg mutant. These findings suggest that perinatal

androgen secretion, which is required for masculinization of copulatory behavior, is dependent on a functional hypothalamic-pituitary-gonadal axis driven by GnRH.

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FIGURE LEGEND

FIGURE 1. Elongated spermatids (arrow) are present in seminiferous tubules of both And/hpg (A) and Cnt/hpg (B). Higher number of elongated spermatids is found in tubules from a normal male (C), while no spermatogenesis is seen in an untreated hpg (D). X 470

FIG. 1

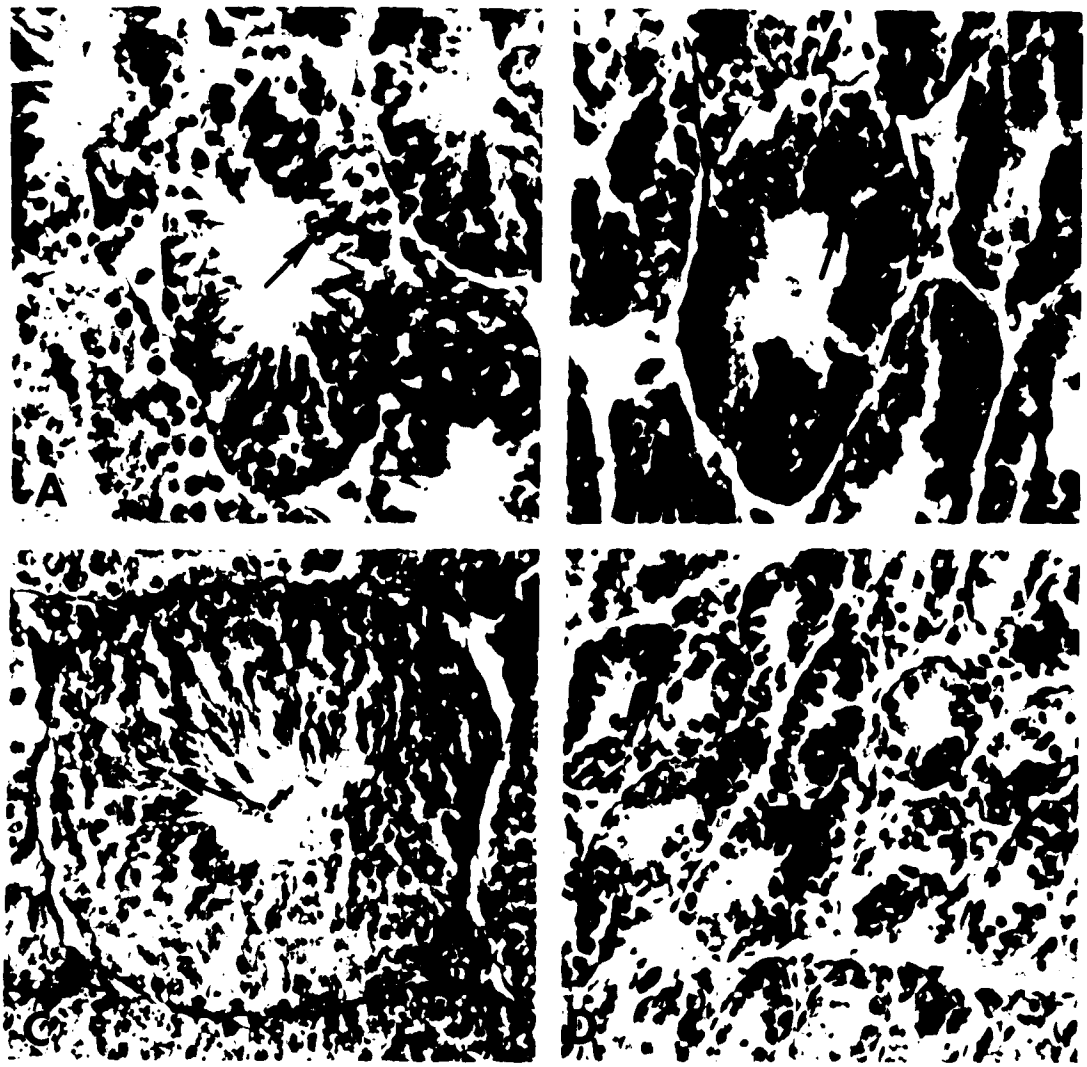

TABLE 1. Fertility, reproductive organ weight, and gonadotropin levels of And/hpg and control groups.

Group	Number of litters sired	Testis Wt[b] (mg)	Seminal Vesicle Wt.[b] (mg)	Plasma FSH[b] (ng/ml)	Pituitary FSH[b] (ng/pit.)	Pituitary LH[b] (ng/pit.)
And/hpg	10 (8)[a]	43.6 ± 3.5 * (5)	230.2 ± 69.0 (4)	8.24 (1)	16.68 (1)	0.80 (1)
Cnt/hpg	0 (7)	27.3 ± 0.9 * (3)	NR[c]	17.44 ± 0.84 (3)	23.30 ± 2.58 (3)	0.99 ± 0.24 (3)
Untreated hpg	NR	6.8 ± 0.4 (4)	2.3 ± 1.1 (2)	15.81 ± 0.18 (2)	30.77 ± 9.55 (2)	2.71 ± 1.21 (2)
Normal	NR	172.5 ± 7.9 (3)	289.9 ± 33.2 (3)	41.59 ± 4.72 (3)	192.53 ± 23.16 (3)	42.53 ± 5.98 (3)

[a]The numbers in parentheses indicate animals per group. [b]Values are mean ± SE.

[c]NR = not recorded.

* $P < 0.05$ vs. untreated hpg and normal groups.

TABLE 2. Masculine sexual behavior of And/hpg, Cnt/hpg and normal males.

Group	% of Tests with Mounting Display [b]	Mounting Freq. (times/hr) *	% of Tests with Intromission Display	Intromission Freq. (times/hr)*	Mount Latency (min)	Intromission Latency (min)
And/hpg Sexually Active# (4)[a]	68.7 ± 12.0%	4.7 ± 2.0	50.0 ± 0.0%	9.0 ± 4.1	7.6 ± 7.4	26.6 ± 9.0
Cnt/hpg Sexually Active# (3)	66.7 ± 0.0%	3.4 ± 1.6	38.9 ± 5.6%	10.0 ± 4.0	12.7 ± 5.8	28.8 ± 10.4
Normal (4)	83.3 ± 16.7%	6.6 ± 2.6	83.3 ± 16.7%	18.3 ± 4.7	12.0 ± 5.3	20.0 ± 6.7

[a]The numbers in parentheses indicate animals per group. [b]All values are means ± SE.

Only animals showing intromission were designated as sexually active.

* Data from test with highest frequency.

SUMMARY

The studies described above were initiated to expand our understanding of the guiding mechanisms which direct the migration of GnRH neurons from the olfactory placode to the forebrain. In addition the biochemical and morphological differentiation of these neurons was investigated to determine whether these processes are correlated with the migration of GnRH cells. I have demonstrated here that GnRH neurons migrate in the nasal septum within the axonal fascicles of the olfactory and vomeronasal nerves. The axonal fascicles and their glial ensheathing cells appear to serve as conductive channels for the migrating neurons to the forebrain (Chapter 2; [1]). These cellular contacts may also regulate the biochemical differentiation of GnRH cells as demonstrated by the finding that most of these neurons do not process the pro-GnRH precursor to the mature decapeptide while in the nasal septum. Furthermore, many GnRH neurons in the nasal septum express the developmentally regulated gene GAP-43. This expression coincides with GAP-43 expression in the olfactory receptor neurons and their axonal fascicles within which GnRH neurons are migrating (Chapter 2; [1]). The morphological differentiation of GnRH neurons is also correlated with their transition from the nasal septum into the forebrain. Only upon entering the forebrain do most of these neurons initiate axonogenesis (Chapter 2; [1]).

In the forebrain GnRH neurons are no longer associated with axonal fascicles and have to continue their migration to the septal-preoptic and anterior hypothalamic areas (AHA) without following overt cytoarchitectonic elements. Thus it can be postulated that, once in the forebrain, these neurons must rely on biochemical signals to direct their migratory course. Since extracellular elements do not appear to be specifically associated with GnRH neurons in the forebrain, a diffusional signal is a likely signal in which the neurons can navigate according to a concentration gradient. The source and the nature of such a signal may be detected by co-culture methodology.

Although the transition into the forebrain is correlated with biochemical and morphological maturation of the GnRH cells an embryonic-brain derived signal is probably not critical for these neurons to elaborate their mature phenotype. This conclusion is based on the finding that GnRH cells in their early migratory stage can complete their differentiation upon transplantation into an adult brain (chapter 3; [2]). It is, therefore, plausible that intercellular signals received by GnRH cells throughout their migratory pathway allow them to coordinate their autonomous differentiation program with their migratory phase but are not critical for their maturation. An alternative explanation to the differentiation of GnRH cells outside the embryonic brain is that these neurons respond to a signal from the adult host brain, hence, dependence of a brain derived signal is not ruled out by our study. Future studies, in which explants of early migratory GnRH cells will be kept in a chemically defined culture medium, could indicate whether these cells can

complete their differentiation in the absence of brain derived factors.

The same transplantation paradigm revealed that GnRH axons often emerge from the nasal septum graft and cross the host AHA towards the third ventricle. These axons then grow caudally along the ventricular wall to terminate on the infundibular sulci of the median eminence (ME) (chapter 3; [2]). This outgrowth pattern does not recapitulate any of the GnRH axonal projections to the ME [3]. Furthermore, the axonal trajectory does not follow existing axonal fascicles. Rather, GnRH axons emerging from the graft grow directly towards the third ventricle while navigating across several mm of host tissue. The direct projection of these axons suggests that they may be guided by a diffusional signal. The source of this signal may be the ME itself as indicated by a cotransplantation study [4]. Ectopic transplantation studies in which GnRH neurons will be grafted into an area caudal to the ME may indicate whether GnRH axons grow directly from the graft towards the ME regardless of their position in the host brain.

Synthesis and secretion of the GnRH neurohormone is not a crucial factor in the capability of the GnRH neurons to pursue their normal migratory path. Thus, GnRH expressing cells in the adult hpg mouse are found in their normal residence area around the organum vasculosum of the lamina terminalis [5]. We have shown here that these biosynthetic and secretory deficient neurons are, nevertheless, capable of innervating their major secretory target: the ME (chapter 4; [6]). We conclude from this finding that elaboration and maintenance of normal axonal projections by these neuroendocrine

cells is independent of their neuroendocrine function. We can not, however, exclude the possibility that GnRH neurons synthesize and secrete another neuropeptide and, hence, maintain some functional relation with the ME. Two neuropeptides are known to be coexpressed by GnRH cells; galanin in in the rat [7] and delta sleep-inducing peptide in the guinea pig [8]. In future experiments we will try to determine whether these neuropeptides are colocalized with GnRH expressing cells in the hpg mouse.

GnRH axons containing the bioactive decapeptide arrive at the prospective ME by embryonic day 14 (E14; chapter 2; [1]) while GnRH binding sites are detected in the rat pituitary by E12-13 [9, 10]. The first gonadotrophs are detected in the rat at E16 [10] and the earliest pituitary response to GnRH is reported at E17.5 [11]. These data, taken together, support the hypothesis that the rodent pituitary-gonadal axis has access and can respond to GnRH by late gestation. I have, therefore, asked whether GnRH assumes its critical role in stimulation of the pituitary-gonadal axis at the perinatal stage. At this developmental stage, circulating testicular androgens play a crucial role in masculinizing the sexual behavior of the rodent [12, 13, 14]. I have utilized the hpg mouse to ask whether the GnRH deficiency of this mutant is responsible for the incomplete masculinization of its sexual behavior. To answer this question I injected testosterone propionate to hpg male pups on postnatal days 1 or 2 (chapter 5; [15]). Some of the neonatally androgenized males displayed the full complement of masculine sexual behavior when supplemented with testosterone propionate in adulthood. Moreover, these males were reproductively competent and sired several litters.

Thus, I have demonstrated that the deficient reproductive behavior of hpg males is, probably, a consequence of insufficient testosterone exposure in the perinatal period. This finding indicates that GnRH assumes its critical role for stimulation of the pituitary-gonadal axis in the early postnatal period. In agreement with this conclusion, it was reported that passive immunization with GnRH antiserum reduces the circulating levels of LH, FSH, and testosterone in 5 days old rats [16]. The case for the critical role of GnRH in activating the neonatal pituitary-gonadal axis can be further supported by demonstrating that the pituitary content and circulating levels of gonadotropins in the neonatal hpg are lower than in their heterozygous littermates.

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