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Developmental Biology

Migratory arrest of gonadotropin-releasing hormone neurons in transgenic mice

(T antigen/infertility/Kallmann syndrome/hypogonadism/hypothalamus)

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ABSTRACT **Gonadotropin-releasing hormone (GnRH) is important in reproduction, although the mechanism of central hypogonadism in humans remains unclear. Because the GnRH neuron originates from the olfactory placode and migrates to the hypothalamus during development, central hypogonadism in humans could be caused by failure in normal migration of GnRH neurons to the hypothalamus. We report that in transgenic mice expression of the simian virus 40 T antigen, driven by the promoter of human GnRH gene, resulted in central hypogonadism due to an arrest in neuronal migration during development and tumor formation along the migratory pathway. This system appears to be an important animal model of hypogonadotropic hypogonadism in humans. Additionally, olfactory bulb tumors from these animals were dispersed, and a GnRH-secreting neuronal cell line (GN cell line) was established.**

Gonadotropin-releasing hormone (GnRH) plays a vital role in reproduction and is expressed in neurons located predominately in the septal-preoptic nuclei and hypothalamus of mammals (1). Although mouse central hypogonadism has been shown to be due to a large deletion of the GnRH gene (2), the mechanism of central hypogonadism in humans remains to be elucidated. It has been speculated that the anosmia and hypogonadism in hereditary hypogonadotropic hypogonadism associated with agenesis of the olfactory bulbs, referred to as Kallmann syndrome, may be from failure of migration of GnRH neurons from the olfactory placode to the hypothalamus (1). Viral oncogenes have been used to direct tumor formation and study cell-specific expression in a variety of tissues (3). Here we report the expression of a viral oncogene, the simian virus 40 large tumor (T) antigen (Tag), driven by the promoter of human GnRH gene [−1131 to +5 base pairs (bp)] in transgenic animals. The rationale for using such a hybrid was to (*i*) examine the ability of the human GnRH promoter to direct cell-specific expression; (*ii*) examine the consequences to the organism of GnRH neuronal-specific expression of Tag; and (*iii*) establish stable cell lines expressing the fusion gene from this low-abundance neuron.

MATERIALS AND METHODS

The human GnRH gene promoter from −1131 to +5 bp (4) was inserted upstream of the Tag-coding region in the pEMP construct (5) at the *Bam*HI site. A 4-kilobase (kb) DNA fragment containing the human GnRH–Tag hybrid gene and devoid of vector sequences was injected into fertilized one-cell embryos, as described (6). Transgenic animals were identified by using Southern blot analysis and a human GnRH cDNA probe (Genentech). Total RNA from a variety of tissues was prepared as described (4), and RNA blot analysis was done by using a radiolabeled specific Tag probe derived from the 3′ end of Tag-coding region.

Tissue sections (12 μm) were obtained by using a cryostat and mounted onto slides coated with gelatin/chrome alum. The slides were placed in 4% (vol/vol) formalin (20 min), rinsed twice in phosphate-buffered saline, and placed in 10% normal goat serum/0.2% Triton X-100 for 1 hr. After two 30-min rinses in 10% normal goat serum, the slides were incubated in one of three primary GnRH antibodies (SW-1 at a dilution of 1:2500; LR5 at 1:10,000; KN1 at 1:1000) (7), or a T antigen antibody at 1:1000 (6). The sections were immu-

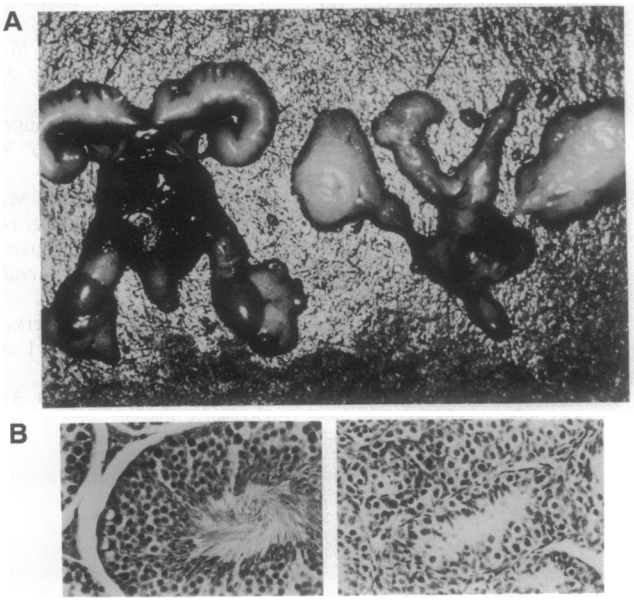

FIG. 1. (*A*) Necropsy specimens of the founder male transgenic mouse 56 (right), compared with an age- and sex-matched control (left). Note hypoplasia of the seminal vesicles (long arrow) and testes (short arrow) of transgenic animal compared with control. Hypoplasia of prostate and preputial glands was also seen. The male transgenic F_1 mice, animals 65, 79, 80, and 83, had similar histological findings. (*B*) Testicular histology of transgenic mouse 56 (*Right*) revealed small seminiferous tubules with hypoplasia of the interstitial cells compared with normal testes (*Left*). No spermatogenesis was seen in the testes of the transgenic animal. Similar histologic patterns were noted in animals 65, 79, 80, and 83. (×250.)

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Abbreviations: GnRH, gonadotropin-releasing hormone; Tag, simian virus 40 T antigen.

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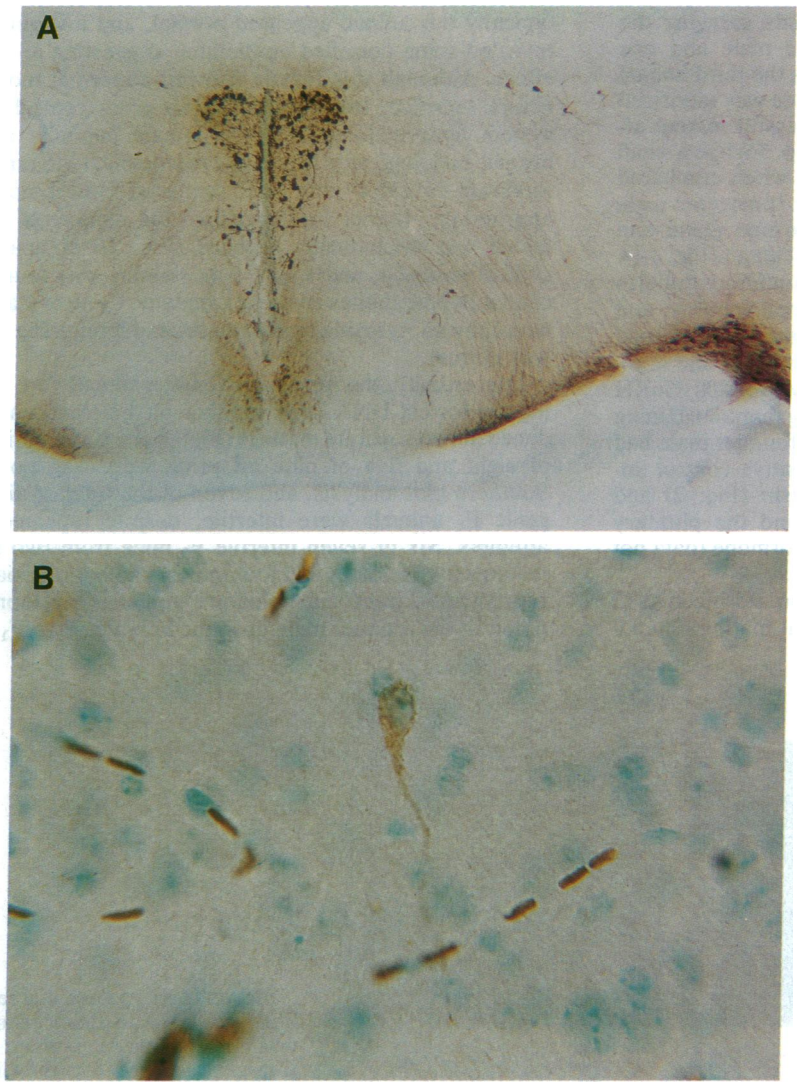

FIG. 2. (*A*) Immunocytochemical staining for neurophysin in hypothalamus of male human GnRH–Tag transgenic mouse 56. Normal architecture of the hypothalamus was found. (*B*) Mouse GnRH immunocytochemical staining of hypothalamus of female human GnRH–Tag transgenic mouse 57 at level of third ventricle. One GnRH neuron is shown (upper center). Only 20 GnRH neurons were found in the hypothalamus of this animal.

nocytochemically stained by using the avidin–biotin–horseradish peroxidase procedure, as described (8). Immunocytochemical staining for neurophysin, tyrosine hydroxylase, and luteinizing hormone was also performed by using the methods described (8).

RESULTS AND DISCUSSION

Three transgenic mice, identified by Southern analysis, were derived from fertilized ova that had been microinjected with a chimeric gene containing the protein-coding region of the Tag driven by the promoter of human GnRH gene (−1131 to

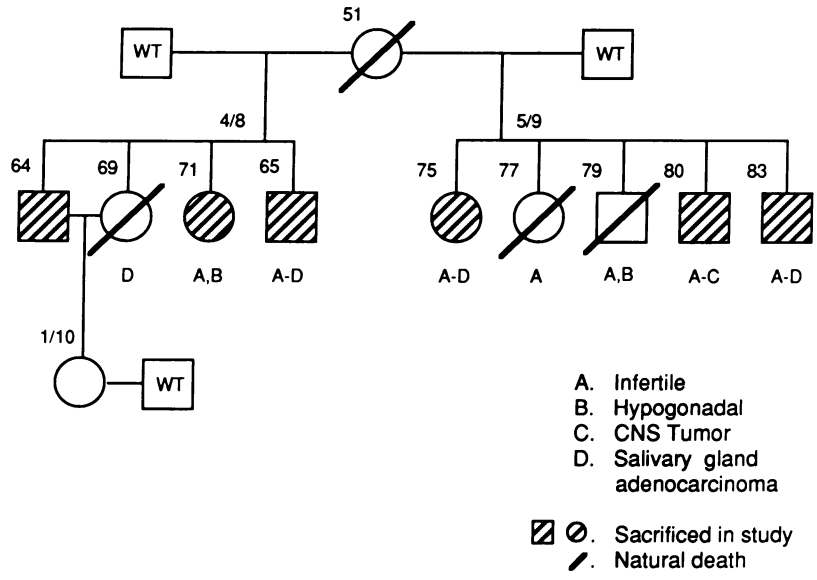

FIG. 3. Partial pedigree of transgenic mice carrying the human GnRH–Tag fusion gene. This transgene contains the human GnRH promoter region from −1131 to +5 bp (4) fused to the simian virus 40 early region (5). The hybrid gene was excised from the plasmid vector, purified, and injected into fertilized one-cell embryos. Transgenic mice were identified by Southern blot analysis of DNA extracted from tail segments. WT, wild type.

+5 bp) (4). Of these three founder animals carrying the human GnRH–Tag hybrid gene, two (one male and one female) animals did not reproduce, whereas the third animal had two successful matings. The founder male was sacrificed at 13 weeks of age after repeated unsuccessful mating attempts. Phenotypically, the founder male (no. 56) had a small phallus and cryptorchidism. At necropsy, when compared with an age- and sex-matched control, the transgenic male had hypoplasia of the epididymis and prostate gland and aplasia of the seminal vesicles and preputial gland (Fig. 1*A*). Histology of the testes revealed small seminiferous tubules with normal architecture but without spermatogenesis; and the interstitial cells were hypoplasic compared with normal testes (Fig. 1*B*). Immunocytochemical staining of the brain revealed no GnRH neurons, compared with ≈800 GnRH neurons in control mice (8). In addition, Tag immunostaining was not seen in this animal. However, the founder male had normal hypothalamic architecture with positive control immunostaining for hypothalamic neurophysin (Fig. 2) and tyrosine hydroxylase (data not shown); and the pituitary gland was immunopositive for luteinizing hormone (data not shown).

A female founder mouse (no. 57) was also sacrificed at 13 weeks of age after unsuccessful attempts at mating. Phenotypically this animal appeared normal, and a vaginal smear revealed some cornified epithelium, suggesting an estrogen effect. Although this female showed abnormal movements before sacrifice, this animal had no gross central nervous system abnormalities, and the female genitalia appeared normal histologically. Immunocytochemical staining of the forebrain revealed only ≈20 scattered GnRH neurons in appropriate anterior brain regions; the number of cells detected was dramatically reduced (Fig. 2*B* compared with control animals), and sparse Tag staining was found in the caudal hypothalamus (data not shown). Control staining for neurophysin, tyrosine hydroxylase, and luteinizing hormone was normal.

Interestingly, the remainder founder female (no. 51), having the lowest DNA copy number of the transgene, reproduced in two separate matings (Fig. 3). In these matings, four of eight and five of nine offspring were Tag positive on Southern blot analysis, and seven of the total of nine transgenic F_1 animals were infertile, despite repeated mating attempts. Six of seven infertile F_1 mice from two separate matings (two females and four males) had hypoplasia of their reproductive tract and immature gonadal development, as noted in the founder male described above. Two F_1 females

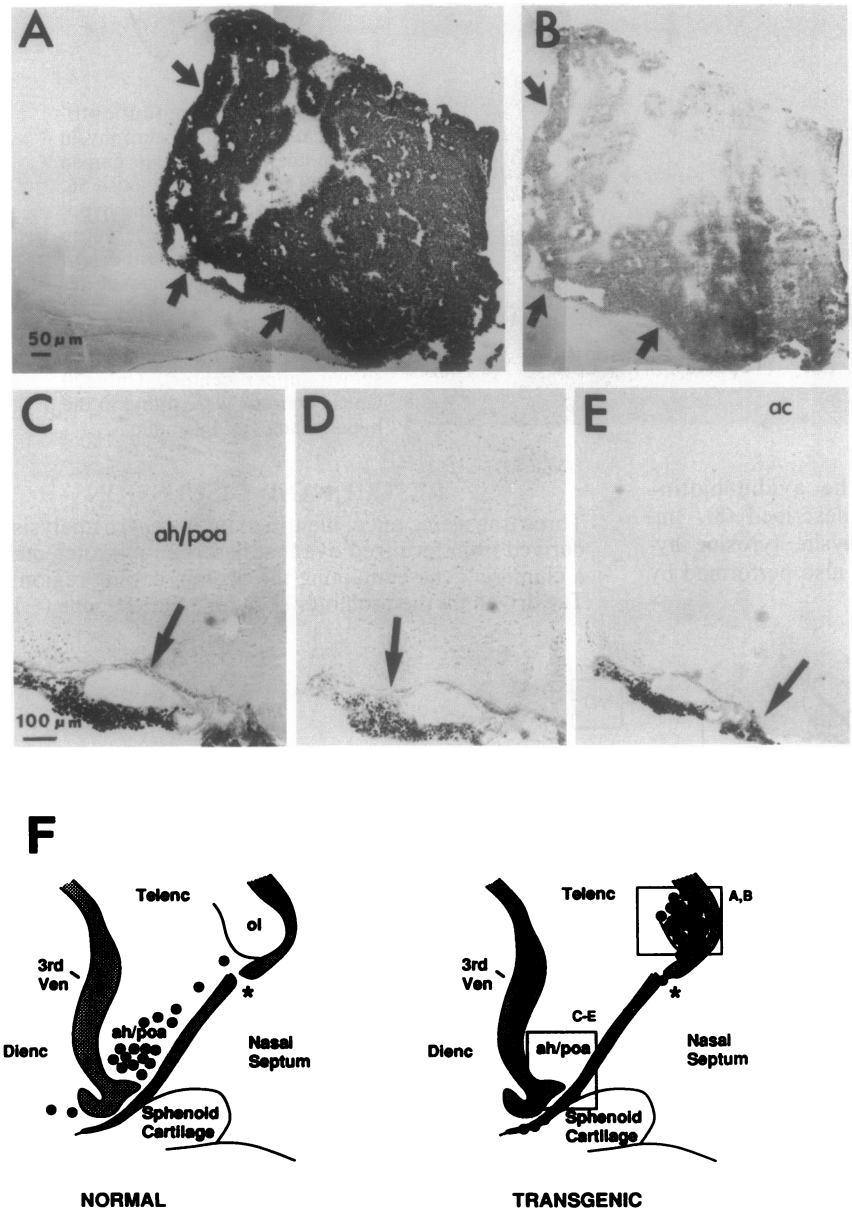

FIG. 4. Serial parasagittal sections (rostral is to right) at low magnification through brain of transgenic mouse 65, showing cells immunostaining for T antigen (*A*) and GnRH (*B*). Cells expressing the construct infiltrated the olfactory bulb but did not cross into the forebrain proper (arrows). (*C–E*) Serial parasagittal sections of same mouse brain at level of anterior hypothalamus/preoptic area (ah/poa) immunostained for T antigen (*C* and *E*) and GnRH (*D*). No GnRH cells were detected within the brain at their normal locations. Cells expressing the construct were located caudal to the major cell mass shown in *A* and *B* ventrally along, but outside, the brain (arrows). (*F*) Schematic representation of anterior brain, adapted from Wray *et al.* (8), showing the normal location (*Left*) of GnRH neurons in the anterior hypothalamus and preoptic area (ah/poa), compared with location in transgenic animals (*Right*). Note abnormal collection in boxed area representing neurons shown in *A* and *B* and lack of cells in ah/poa in lower boxed area representing *C–E*. *, Point at which migrating GnRH neurons enter forebrain during development. Telenc, telencephalon; Dienc, diencephalon; Ven, ventricle.

(no. 71 and no. 75) had hypoplasia of all layers of the uterus and degenerative changes in many of the larger ovarian follicles (data not shown). The seventh F_1 animal (no. 77) died unexpectedly before the reproductive tract and brain could be examined. Olfactory lobe tumors were found in four F_1 progeny (animals 65, 75, 80, and 83). Two of these animals (nos. 75 and 65) were examined extensively. The F_1 female (no. 75) had a large tumor along the ventral aspect of the brain (outside the brain tissue proper) that extended from the olfactory bulbs to the caudal hypothalamus. This tumor was stained with the Tag antibody but was not immunopositive for GnRH. The F_1 male (no. 65) had a large mass visible directly over the olfactory bulbs that extended rostrally into the nasal region. This mass contained an abnormal collection of Tag- and GnRH-immunopositive neurons (Fig. 4). Northern (RNA) blot analysis of male (no. 65) and several F_1 progeny revealed expression of Tag mRNA but not in the liver, heart, bowel, or spleen of these animals (data not shown).

Two tumors (from male 65 and female 75) were dispersed with collagenase by using standard methods (9) and cultured. The cells were found to grow in clusters and were relatively large compared with GnRH cells normally found in the brain. Cells derived from male 65 immunostained for GnRH and Tag (Fig. 5), whereas those of the female (no. 75) stained robustly for Tag but were not immunopositive for GnRH. These results are consistent with the *in vivo* data presented above. Cells derived from male 65 have been frozen and regrown several times, and each batch has restained positive for both Tag and GnRH. Northern (RNA) blot analysis of these dispersed cell cultures confirmed that they contained Tag mRNA (data not shown).

Four F_1 progeny (animals 69, 65, 75, and 83) developed adenocarcinoma of the salivary gland. The tumor from animal 65 was immunocytochemically examined and stained positive with both GnRH and Tag antibodies. Although GnRH is expressed in the lactating mammary gland (10), expression in the salivary gland is unknown. Thus, the exact mechanism of salivary gland tumorigenesis in these animals is unclear.

Thus, ≈1100 bp of human GnRH 5′-flanking DNA was sufficient to direct Tag expression to the GnRH neuron. Two of the three founder animals were infertile, presumably due to either an absence or a substantially reduced number of GnRH neurons in the hypothalamus. The third founder animal reproduced and yielded 9 transgenic offspring from a total of 17 mice in two separate matings. Seven of the 9 transgenic mice were infertile, 6 were hypogonadal associated with hypoplasia of the reproductive tract and gonads, and 4 animals had olfactory lobe tumors and a depletion of hypothalamic GnRH neurons. The pattern of inheritance of the reproductive disorder in this pedigree can best be described as autosomal dominant with variable penetrance. Because hypothalamic depletion of GnRH neurons was associated with an abnormal collection of Tag-positive GnRH

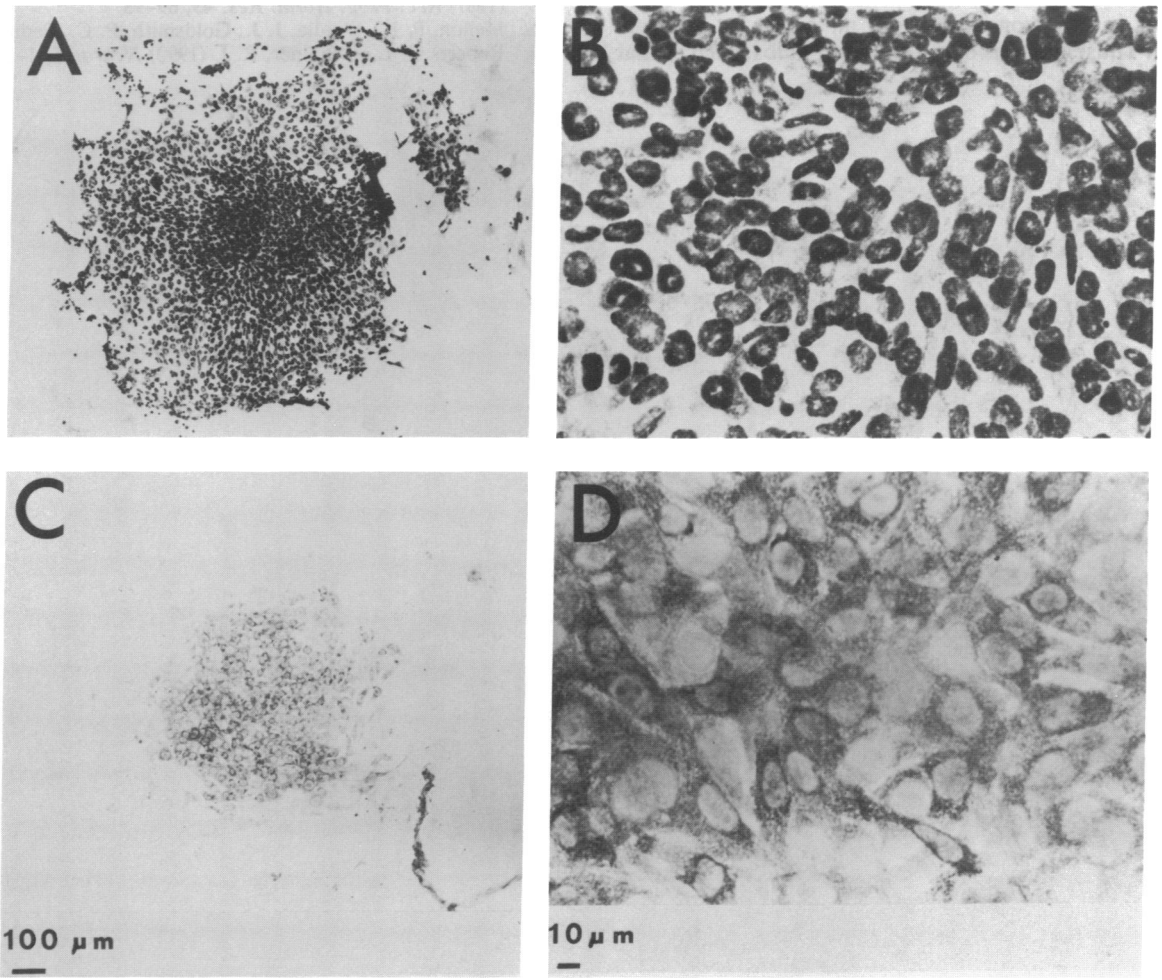

FIG. 5. Cultured cells obtained from the tumor shown in Fig. 3 dispersed with collagenase by using standard methods (9). Low magnification (*A*) and high magnification (*B*) of cultured cells stained with Tag showing extensive nuclear staining. Low magnification (*C*) and high magnification (*D*) of cells stained with GnRH showing staining in the cytoplasm. Note mitotic figures detectable in the cells stained with Tag. Double labeling indicated Tag and GnRH were within the same cells.

neurons along the migratory pathway from the olfactory placode to the hypothalamus (1, 8) in some animals, Tag expression during a critical phase of development probably resulted in a migratory arrest of these neurons before they entered the forebrain.

The phenotypic differences between animals, as well as the variability in the extent of hypothalamic depletion of GnRH neurons, may be from differences in the developmental stage at which Tag is expressed in these neurons. Expression of Tag early in development of the GnRH neuron could result in migratory arrest at the olfactory placode with no apparent immunostaining for GnRH in anterior brain sections, whereas expression in late developmental stages could result in some GnRH neurons in the hypothalamus, such as the female founder animal (no. 57). Expression of Tag at different stages of GnRH neuronal development would explain the lack of complete penetrance of the hypogonadal disorder in this pedigree.

In conclusion, neoplastic transformation of the GnRH neuron can arrest migration of the neuron during development and central hypogonadism. Previous investigators (1, 8) have hypothesized that hypogonadism may be caused by failure of GnRH neuronal migration from data extrapolated from studies of normal neuronal development. However, our data directly validate the physiological outcome of altered GnRH neuronal migration in mammals. Because central hypogonadism in humans is many times not associated with an abnormality in olfaction, developmental abnormalities of the GnRH neuron, such as neoplastic transformation, could also result in hypogonadism from an arrest in neuronal migration. Thus, agenesis of the olfactory bulbs, such as that seen in some cases of Kallmann syndrome, may represent only one disorder in a spectrum of disorders of GnRH neuronal migration that result in central hypogonadism. These mice should provide a useful model of central hypogonadism and could lead to better understanding of disorders associated with puberty and reproduction in humans.

Note Added in Proof. Recently Mellon *et al.* (11) reported similar findings using the rat GnRH promoter.

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