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Immortalization of Hypothalamic GnRH Neurons by Genetically Targeted Tumorigenesis

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Summary

By genetically targeting tumorigenesis to specific hypothalamic neurons in transgenic mice using the promoter region of the gonadotropin-releasing hormone (GnRH) gene to express the SV40 T-antigen oncogene, we have produced neuronal tumors and developed clonal, differentiated, neurosecretory cell lines. These cells extend neurites, express the endogenous mouse GnRH mRNA, release GnRH in response to depolarization, have regulatable fast Na^+ channels found in neurons, and express neuronal, but not glial, cell markers. These immortalized cells will provide an invaluable model system for study of hypothalamic neurosecretory neurons that regulate reproduction. Significantly, their derivation demonstrates the feasibility of immortalizing differentiated neurons by targeting tumorigenesis in transgenic mice to specific neurons of the CNS.

Introduction

Study of the cellular and molecular biology of mammalian CNS neurons has been impeded by the cellular complexity of the brain and the postmitotic nature of mature differentiated neurons. The paucity of naturally occurring CNS neuronal tumors (Rubinstein et al., 1984) and the difficulty of transforming or immortalizing highly differentiated CNS neurons experimentally (Laerum et al., 1984; Cepko, 1989) have inhibited the development of CNS neuronal cell lines. Hypothalamic neurosecretory neurons producing gonadotropin-releasing hormone (GnRH) control reproductive function in mammals by regulating the secretion and synthesis of gonadotropic hormones. Unfortunately, the low number and scattered localization of GnRH neurosecretory neurons in the rostral hypothalamus make study of their cell biology difficult (Silverman, 1988). The finding that the mouse GnRH gene was expressed appropriately when introduced into transgenic mice (Mason et al., 1986b) and corrected the sterile phenotype of hypogonadal mutant mice (which carry a deletion of the GnRH gene [Mason et al., 1986a]) supported the feasibility of using transgenic technology to target oncogene expression to the GnRH neurons.

Transgenic mice have been used successfully to produce tumors in specific tissues (Jenkins and Copeland, 1989; Hanahan, 1989; Rosenfeld et al., 1988), such as the pancreas (Hanahan, 1985) and anterior pituitary (Windle et al., 1990), by specifically targeting expression of the potent oncogene SV40 T-antigen (Tag) with regulatory domains of the insulin and glycoprotein hormone α subunit genes, respectively. Cell lines derived from such tumors sometimes maintain the differentiated phenotype of the targeted tissue (Efrat et al., 1988a; Windle et al., 1990; Ornitz et al., 1987).

We have produced specific tumors of GnRH-secreting neurons by introduction of a hybrid gene composed of the GnRH promoter coupled to the coding region for Tag into transgenic mice. Clonal cell lines derived from these tumors express GnRH mRNA and secrete GnRH in response to depolarization. They express neuronal markers, including several associated with presynaptic vesicles and membranes, but do not express glial markers. These cells extend lengthy neurites that appear to end in growth cones or to contact other cells. Thus, by targeting oncogenesis to a specific, small population of neurons using the regulatory region of a gene which is expressed late in the differentiation of that cell lineage, we have succeeded in immortalizing hypothalamic GnRH neurons that maintain many differentiated functions in culture.

Results

Introduction of a GnRH-Tag Transgene Results in Brain Tumors

Nine transgenic mice carrying the hybrid GnRH-Tag gene (Figure 1) were obtained. None of the mice were fertile, preventing the derivation of lines of mice. In both female and male mice, the gonads and accessory sex organs were underdeveloped, suggesting that expression of the transgene interfered with sexual maturation. Seven of the mice were sacrificed following the appearance of neurological signs consistent with development of brain tumors. In four animals, tumors were observed in the hindbrain localized in the subarachnoid space between the cerebellum and brainstem and, in one case, superior to the parietal cortex. Cells derived from one of the hindbrain tumors expressed Tag mRNA in culture (data not shown). However, these cells did not express GnRH mRNA and were epithelial in appearance; they were not studied further. These tumors may have been derived from choroid plexus, a tumor type observed commonly in transgenic mice carrying the Tag gene (Brinster et al., 1984).

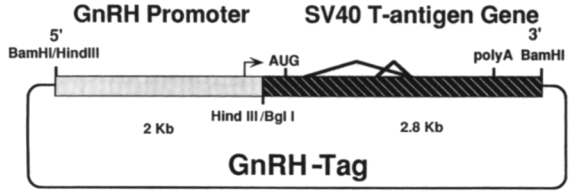

Figure 1. The GnRH-Tag Hybrid Gene

The GnRH-Tag gene contains 2.3 kb of the rat GnRH gene 5' flanking region including the mRNA start sites linked to the entire coding region of Tag from BglI to BamHI (Hanahan, 1985). The Tag gene is spliced to produce two mRNAs that give rise to small t- and large T-antigens, both of which are thought to be required for efficient transformation and immortalization (Choi et al., 1988). The region of 5' flanking DNA used in this construction (supplied by KeWen Dong and J. L. Roberts) was later determined to contain a deletion of a 731 bp EcoRI fragment at −441 bp from the start site (J. Adelman, personal communication; Bond et al., 1989). It thus contains DNA from −2987 to −1172 appended to −441 to +104.

In two other transgenic mice, anterior hypothalamic tumors were observed. In one of these mice (GT-1), following a coronal cut at the rostral boundary of the optic chiasm, a large tumor was observed extending from the dorsal border of the optic chiasm to below the internal capsule. The anterior commissure was displaced by tumor growth. Portions of this tumor were taken for mRNA analysis and tissue culture. Northern blot analysis demonstrated that the tumor expressed high levels of GnRH and Tag mRNAs and that this expression was specific to the tumor tissue (Figure 2).

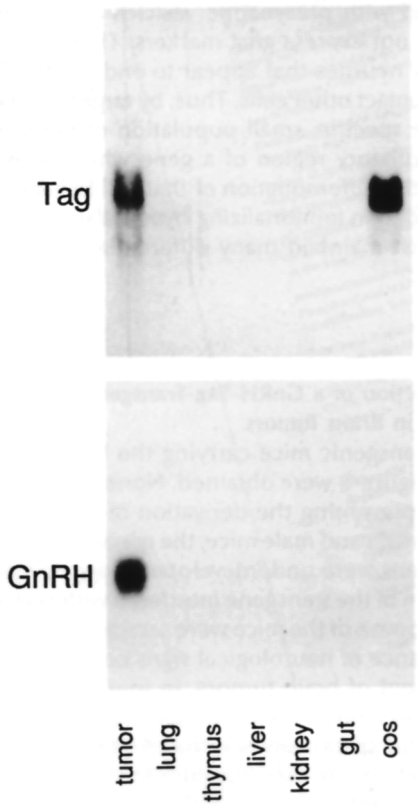

Figure 2. GnRH and Tag mRNAs Are Specifically Expressed in the GT-1 Mouse Hypothalamic Tumor

A Northern blot of several tissues of the GT-1 mouse hybridized to the Tag gene or rat GnRH cDNA showing specific expression of both mRNAs in tumor tissue. The two spliced mRNAs for small t- and large T-antigens are approximately 2450 nucleotides and 2200 nucleotides in length, respectively, and the mRNA for GnRH is approximately 500 nucleotides in length.

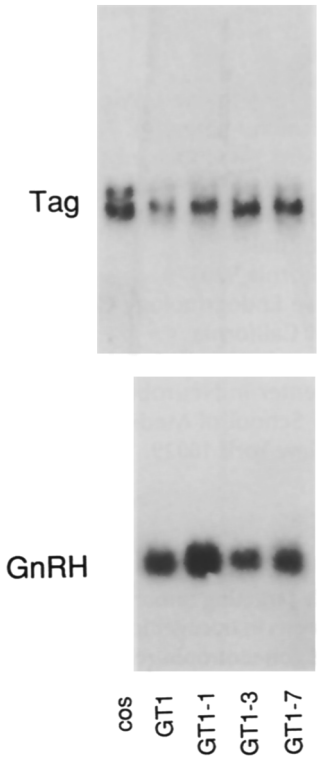

Figure 3. GT-1 Cell Population and Clonal Cell Lines Show Consistent Expression of Both Tag and GnRH mRNAs

A Northern blot of GT-1 cell population and clonal cell lines (GT1-1, GT1-3, and GT1-7) showing consistent expression of both Tag and GnRH mRNAs. COS cell mRNA was included as a positive control for Tag mRNA (Mellon et al., 1981).

Derivation of Immortalized Neuronal Cell Lines

The dispersed GT-1 tumor cells were extremely heterogeneous and included cells with neural and glial phenotypes, both of which proliferated in culture. Following 6 months of repeated passage using differential plating on plastic culture dishes to separate glia and other cell types, characteristic cultures of a pure cell population (GT-1) were established and cloned by serial dilution (GT1-1, GT1-3, and GT1-7). These cell lines have been propagated continuously for 18 months with no apparent change in phenotype and can be stored in liquid nitrogen. Northern blot analysis (Figure 3) demonstrated the presence of GnRH and Tag mRNA in the pure culture and in the individual cell lines. Growth rates for these cell lines vary from doubling every 36 hr (GT1-3 and GT1-7) to every 3–4 days (GT1-1).

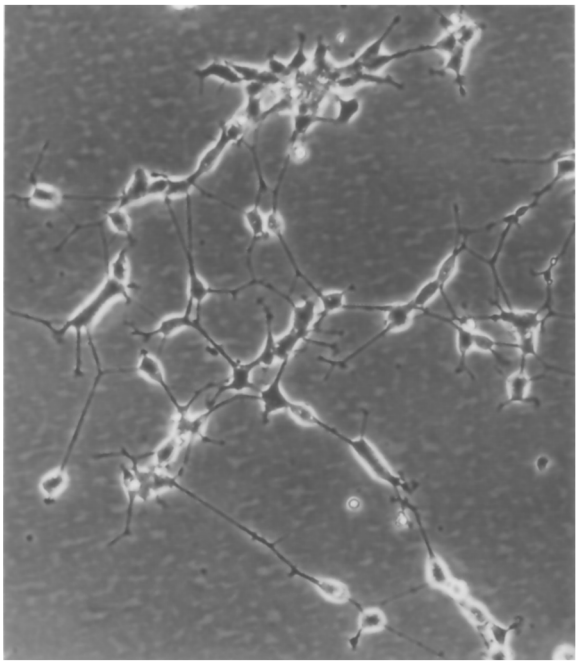

Figure 4. GT Cells Develop Neurites in Culture

A phase-contrast micrograph demonstrating the neuronal phenotype of GT1-3 cells, including neurite formation, growth cones, and cell-cell contacts; Magnification: 175×. Cells were cultured on plastic tissue culture plates in Opti-MEM without serum for 3 days.

GT cells attached to plastic culture dishes exhibit a distinct neuronal phenotype, including the extension of multiple lengthy neurites that often contact distant cells or end in apparent growth cones (Figure 4). This neuronal phenotype is more profound when cells are cultured in the absence of serum to inhibit cell division.

GT Cells Express Neuronal but Not Glial Markers

The expression of several well-characterized neuron- and glia-specific mRNAs was investigated by Northern blot analysis using poly(A)$^+$ RNA from GT1-7 cells. Expression was compared with poly(A)$^+$ RNA derived from total mouse brain. The glycolytic enzyme enolase occurs in three forms: nonneuronal enolase, expressed in glial cells; a muscle-specific form; and neuron-specific enolase (NSE; Forss-Petter et al., 1986), localized strictly to central and peripheral neurons. The mRNA for the neuronal form was present in higher concentration in GT cells than in total brain (Figure 5). Neurofilament proteins are neuron-specific intermediate filament proteins that serve as a structural matrix in axons, dendrites, and perikarya (Lewis and Cowan, 1985). The two mRNAs for the 68 kd neurofilament protein are also present in GT cells (Figure 5).

In contrast, GT cells fail to express glial marker RNAs (Figure 5). They do not express glial fibrillary acidic protein (GFAP), the glial-specific form of intermediate filament (Lewis et al., 1984). GT cells also lack detectable mRNA for two components of brain myelin, myelin basic protein (Roach et al., 1983) and myelin proteolipid protein (Milner et al., 1985), which are characteristic of glial cells in the CNS (Lemke, 1988).

The neuroendocrine function of GT cells appears to be limited to GnRH expression. Hybridization with probes for somatostatin (Montminy et al., 1984), proopiomelanocortin (Eberwine and Roberts, 1984), corticotropin-releasing hormone (Imaki et al., 1989), and growth hormone-releasing hormone (Mayo et al., 1985) detected mRNAs corresponding to these hypothalamic neuroendocrine hormones in control brain poly(A)$^+$ mRNA, but failed to detect mRNAs in GT1-7 poly(A)$^+$ mRNA, indicating the highly specific nature of the GT cells for expression of GnRH (data not shown).

GT Cells Express Genes for Proteins Specific to Synaptic Membranes

Several genes that encode proteins specific to the presynaptic membrane have recently been identified (Trimble and Scheller, 1988). Two related proteins, VAMP-1 and VAMP-2, are associated with synaptic vesicle membranes and are differentially expressed in

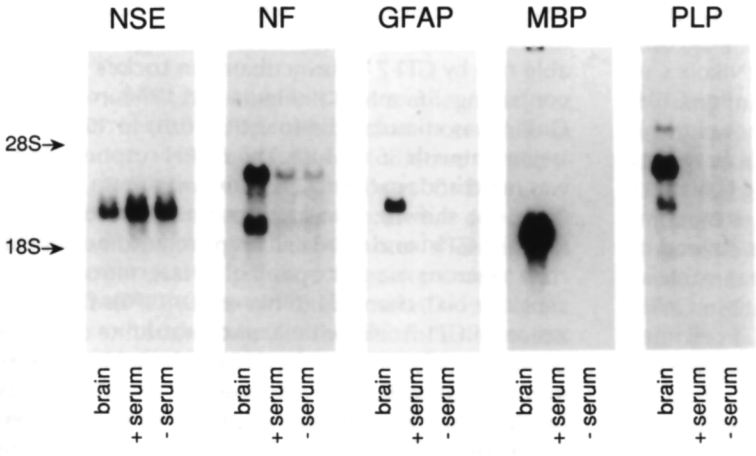

Figure 5. GT Cells Express Neuron-Specific mRNAs and Fail to Express Glia-Specific mRNAs

Northern blot of GT1-7 poly(A)$^+$ RNA demonstrating expression of neuron-specific enolase (NSE) and the 68 kd neurofilament protein (NF) mRNAs and the lack of detectable expression of glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), and myelin proteolipid protein (PLP) mRNAs. Poly(A)$^+$ RNA (5 μg) from GT1-7 cells cultured in DME with serum (+) or Opti-MEM without serum (−) is compared with 3 μg of poly(A)$^+$ RNA from mouse brain. Expression of neuronal markers does not differ with and without serum, though the cell morphology becomes more neuronal in the absence of mitosis (without serum).

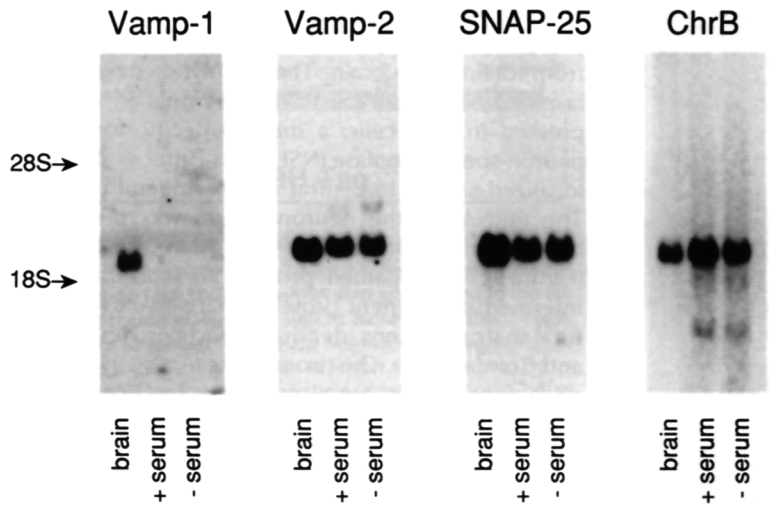

Figure 6. GT Cells Express Synapse-Specific and Neurosecretory-Specific mRNAs

Northern blot of GT1-7 poly(A)$^+$ RNA demonstrates expression of VAMP-2 and SNAP-25 mRNAs, lack of expression of VAMP-1 mRNA, and a high level of expression of chromogranin B mRNA (ChrB). Poly(A)$^+$ RNA (5 μg) from GT1-7 cells cultured in DME with serum (+) or Opti-MEM without serum (−) is compared with 3 μg of poly (A)$^+$ RNA from mouse brain.

the CNS (Elferink et al., 1989). The GT cells express the mRNA for VAMP-2 but not for VAMP-1 (Figure 6), consistent with the known expression of VAMP-2 in the hypothalamus (Trimble et al., 1990). A 25 kd synaptosomal protein, SNAP-25, is known to be associated with the presynaptic membrane and is specific to neurons (Oyler et al., 1989). The mRNA for SNAP-25 is present at high levels in GT cells (Figure 6). Chromogranin B (Forss-Petter et al., 1989; also termed secretogranin I) is a neuroendocrine secretory vesicle protein found in the brain, adrenal medulla, and anterior pituitary. It is particularly abundant in the hypothalamus and is present in brain throughout development (Forss-Petter et al., 1989). GT cells contain chromogranin B mRNA at significant levels (Figure 6), consistent with secretory function. The presence of these markers of neuronal, neurosecretory, and synaptic membrane function in GT cells further supports their identity as neurons and documents their differentiated state.

Immunohistochemical and Ultrastructural Characterization

One of the clonal cell lines (GT1-3) was characterized extensively by immunocytochemistry and morphological criteria. Cells immunostain for GnRH, Tag, NSE (Figure 7), and GnRH-associated peptide (Nikolics et al., 1985; data not shown) and do not stain for GFAP (Figure 7) or tyrosine hydroxylase (data not shown). GnRH is localized in the cytoplasm of cells as well as in neurites (Figure 7A). Tag immunostaining is nuclear except in dividing cells, where staining is most intense at the periphery (Figure 7B). NSE is stained in cell bodies and neurites (Figure 7C), whereas no staining was seen for GFAP (Figure 7D). In addition, GnRH can be detected in the media of all three GT cell lines by radioimmunoassay (350 pg/24 hr per 10^6 cells for GT1-3).

The cellular morphology changes with cell cycle. Rounded, dividing cells sit above flattened, extended cells attached firmly to the dish. This is easily seen in Figure 7B, where the rounded, dividing cells stain peripherally for Tag. Both dividing and nondividing cells stain intensely for GnRH (Figure 8).

At the ultrastructural level, GT cell bodies contain both forming and mature neurosecretory granules, Golgi apparatus, and rough endoplasmic reticulum (Figure 9). Neuritic processes are frequently observed extending from parikarya containing varying numbers of neurosecretory granules (Figure 9B). Large aggregates of neurosecretory granules are consistent with the immunostaining of varicosities for GnRH at the level of the light microscope (Figure 7A). At points of neurite contact, fine structural specializations are suggestive of synaptic-like morphogenesis (Figure 9B). The condensation of neurosecretory material within smooth endoplasmic cisternae is often indicative of rapid peptide processing (Figure 9C). Thus, immunohistochemically, as well as morphologically, GT cells appear to be neuronal in origin and have distinct characteristics of neurosecretory neurons.

Depolarization Induces Secretion of GnRH

Depolarization of GnRH neurosecretory neurons in brain slices causes rapid release of the neuropeptide (Drouva et al., 1981). GnRH was released at a measurable rate by GT1-7 cells incubated in Locke's medium containing 5.6 mM K^+ (Figure 10). The release of GnRH was stimulated 4-fold ($P > 0.01$) in 15 min by treatment with 56 mM K^+. The GnRH response to K^+ was rapid and reached 72% of the maximum in 1 min (data not shown). Similar responses were observed with the GT1-1 and GT1-3 cell lines. Veratridine depolarizes neurons via the opening of fast, tetrodotoxin-sensitive Na^+ channels (Ohta et al., 1973). Depolarization of GT1-7 cells with 50 μM veratridine caused a 6-fold ($P > 0.01$) increase in GnRH release during a 15 min time period. The response to veratridine was blocked by a 30 min pretreatment of the cells with 50 μM tetrodotoxin. This blockade demonstrates the

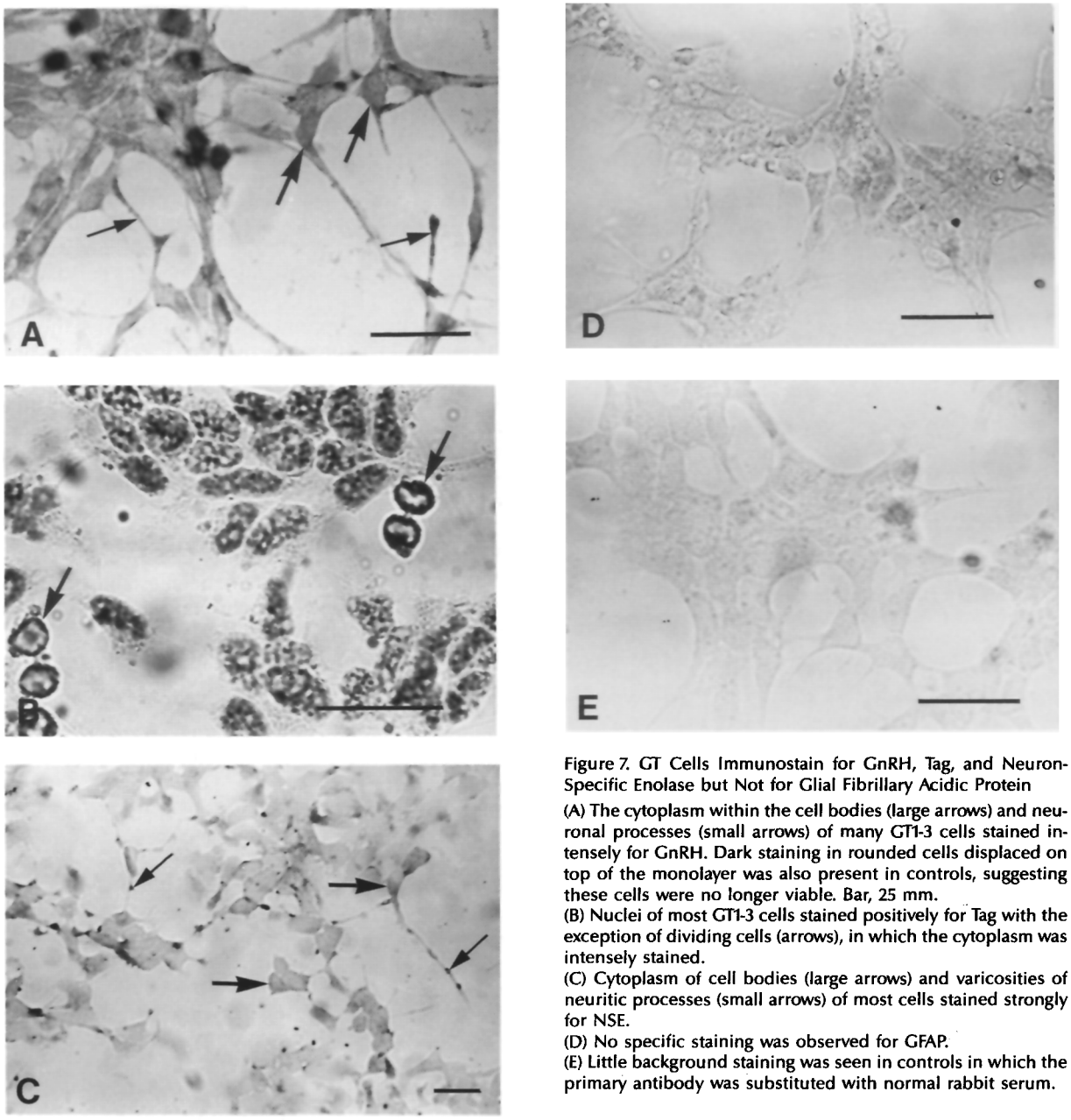

Figure 7. GT Cells Immunostain for GnRH, Tag, and Neuron-Specific Enolase but Not for Glial Fibrillary Acidic Protein

(A) The cytoplasm within the cell bodies (large arrows) and neuronal processes (small arrows) of many GT1-3 cells stained intensely for GnRH. Dark staining in rounded cells displaced on top of the monolayer was also present in controls, suggesting these cells were no longer viable. Bar, 25 mm.

(B) Nuclei of most GT1-3 cells stained positively for Tag with the exception of dividing cells (arrows), in which the cytoplasm was intensely stained.

(C) Cytoplasm of cell bodies (large arrows) and varicosities of neuritic processes (small arrows) of most cells stained strongly for NSE.

(D) No specific staining was observed for GFAP.

(E) Little background staining was seen in controls in which the primary antibody was substituted with normal rabbit serum.

presence of fast Na^+ channels that are necessary for the occurrence of propagated action potentials. In contrast, the action of K^+ was not affected by tetrodotoxin, indicating that K^+ stimulates GnRH secretion via a mechanism different from that of veratridine. Studies with hypothalamic slices showing that high K^+ releases GnRH via voltage-dependent Ca^{2+} channels are consistent with this result (Drouva et al., 1981). Tetrodotoxin alone also significantly decreased basal GnRH release, linking basal secretion to spontaneous Na^+ channel activity. These findings demonstrate that hormone release can be regulated in these cultured neurosecretory neurons by depolarization.

Discussion

By targeting expression of an oncogene using the regulatory regions of the GnRH gene, we have produced tumors in transgenic mice and derived clonal GnRH-secreting cell lines that are immortal, differentiated hypothalamic neurons. Expression of Tag directed by the 5′ flanking DNA of the rat GnRH gene produced uniform sterility in nine transgenic founder mice, indicating a specific effect of the transgene on reproductive function. However, a number of these mice developed brain tumors that were not of hypothalamic origin, indicating that the transgene was also ectopically expressed. Nevertheless, the development of immortal cell lines representing GnRH hypotha-

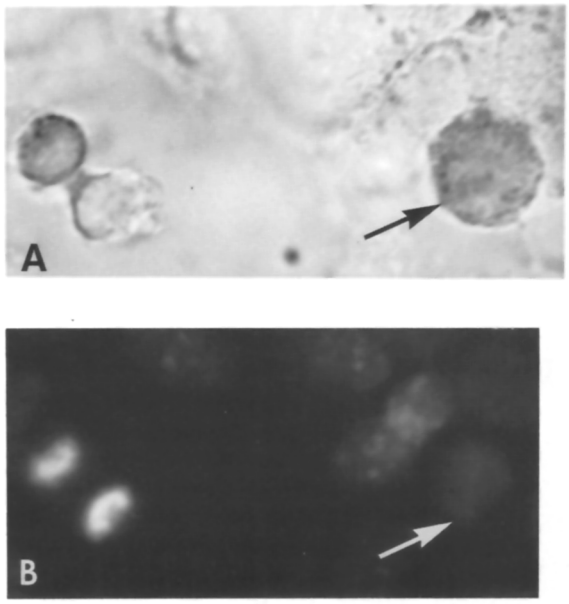

Figure 8. GT Cells Contain GnRH during Cell Division

GT1-3 cells were immunostained for GnRH and then fluorescently stained for DNA using Hoechst (10 μM bisbenzimide) dye.
(A) Transmission light microscopy reveals 3,3'diaminobenzidine tetrahydrochloride-labeled GnRH in the peripheral cytoplasm of two apposed, postmitotic cells and one enlarged dividing cell (large arrow). Magnification: 2680×.
(B) Epifluorescence microscopy of the same field with wide band UV excitation reveals the chromatin configuration in the dividing cells. The GnRH-immunoreactive, postmitotic cells appear to be in telophase. However, in the enlarged cell, the DNA is not condensed and the fluorescence is diffuse (large arrow). Magnification: 2680×.

lamic neurons required only the recovery and successful culture of one tumor caused by appropriately targeted oncogenesis.

Differentiated Characteristics of GnRH Neuronal Cell Lines

The cell lines derived from the GT-1 tumor show many characteristics of differentiated cells. Their overall morphology is neuronal, with the spontaneous extension of neurites that end in growth cones or contact distant cells. During cell division, they retract these extensions and divide as rounded cells, reextending neurites after division. In the absence of serum, and hence cell division, the arbors of neurites are maintained and become more elaborate. Many specific characteristics of neurosecretory cells are maintained; these include the Golgi apparatus, rough endoplasmic reticulum, secretory granules, processing of the GnRH precursor, regulation of secretion, and expression of specific neural and neuroendocrine markers.

The GT cell lines represent an important experimental model for the study of neuroendocrine regulation. The secretion of GnRH from these cells is stimulated by depolarization, a hallmark of neurosecretory neurons (Rubin, 1970). Tetrodotoxin blockage of the veratridine-stimulated GnRH release from GT cells demonstrates the presence of fast Na^+ channels necessary for the occurrence of propagated action potentials. The ability of tetrodotoxin alone to inhibit basal release of GnRH is consistent with these neurons generating spontaneous action potentials via changes in Na^+ conductance. In addition, numerous whole animal and in vitro brain slice experiments have demonstrated that norepinephrine can regulate GnRH secretion (Weiner et al., 1987). Preliminary studies with GT cells show dose-dependent norepinephrine stimulation of GnRH secretion (R. I. W., unpublished data). Thus, GT cells represent a model for studying the regulation of the neuronal activity of GnRH neurons and provide a system in which to unravel the complex regulation of these neurons by numerous neurotransmitters and neuromodulators. Since the cells also contain GnRH-associated peptide, they will be valuable in studying the biosynthesis and processing of the pro-GnRH molecule and its products.

Prospects for Immortalization of CNS Neurons

Numerous PNS cell lines have been established from neuroblastomas and pheochromocytomas (e.g., RT-4 [Imada and Sueoka, 1978] and PC12 [Dichter et al., 1977]); however, previous attempts to obtain cell lines of differentiated CNS neurons by various means have met with little success. Transformation of cultured embryonic cerebrocortical cells with retroviruses produced pluripotent or glial cells (Cepko, 1988; Frederiksen et al., 1988; McKay, 1989; Giotta et al., 1980; Giotta and Cohn, 1981). SV40 infection of primary cultures of hypothalamic tissue resulted in peptide-producing cell lines that were not fully differentiated (de Vitry, 1978; de Vitry et al., 1974). Tissue-specific expression of Tag under the control of the glucagon promoter in transgenic mice specifically induced tumors of the α cells of the pancreas. Although glucagon is normally also produced in neurons in the brain, no CNS tumors were observed (Efrat et al., 1988b). Tag expression driven by the promoter for the phenylethanolamine N-methyltransferase gene (PNMT, an enzyme in the catecholamine synthetic pathway) was found in the retina but not in the brain (Baetge et al., 1988). Tumors of the retina were obtained; however, neither the tumors nor the cell lines derived from them expressed the endogenous PNMT gene (Hammang et al., 1989, Soc. Neurosci., abstract). In addition, transgenic mice bearing Tag under the control of the promoter from the gene of another hypothalamic peptide hormone, growth hormone-releasing factor, produced nonspecific thymic hyperplasia but failed to transform cells of the hypothalamus (Botteri et al., 1987). These findings supported the view that differentiation of CNS neurons occurs postmitotically and that mitosis and neuronal differentiation may be incompatible (Efrat et al., 1988b; Rubenstein et al., 1984; Cepko, 1988). In contrast, our finding that mitosis occurs in GT cells in concert with GnRH expression, which is clearly a marker of differentiation, and with

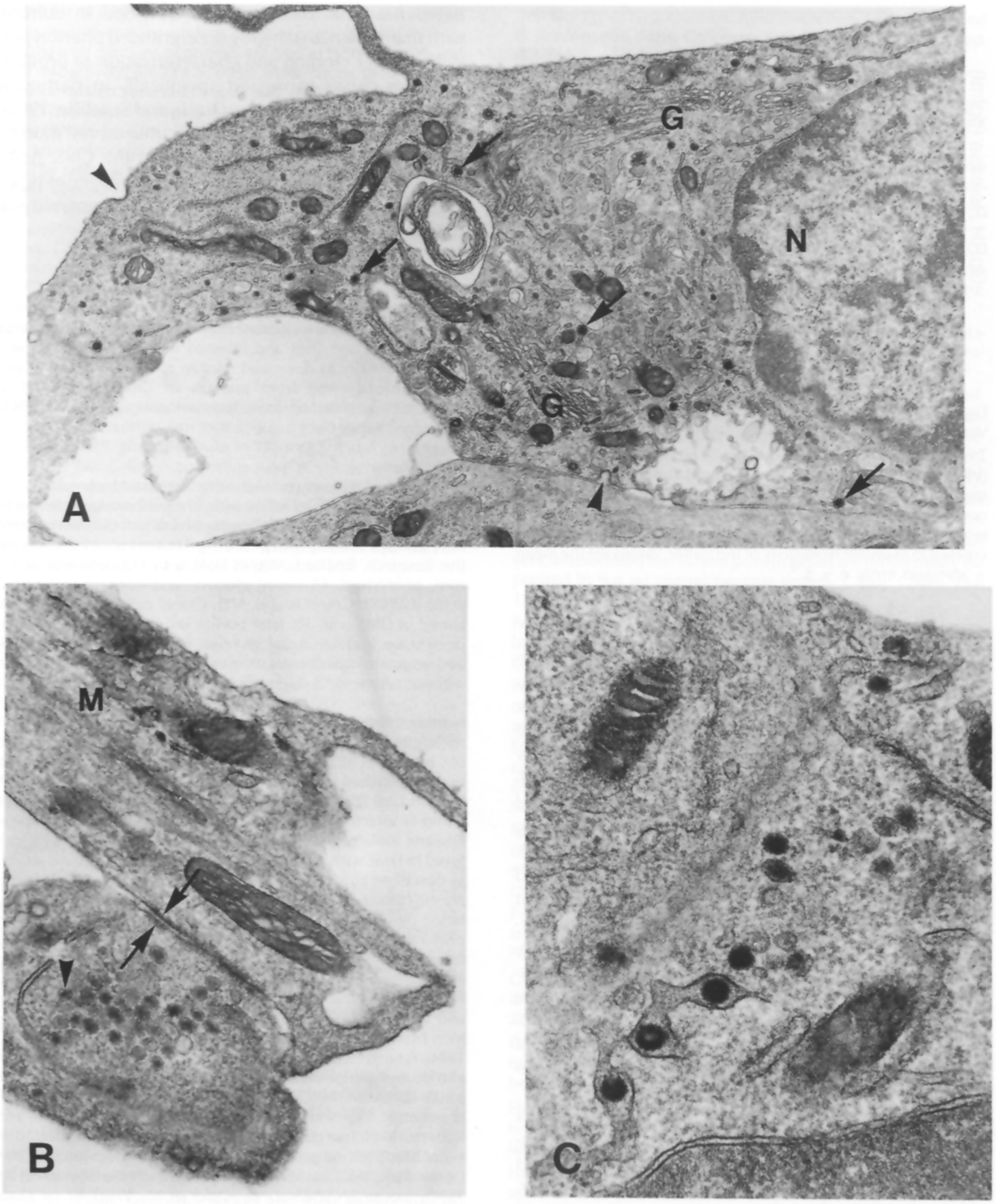

Figure 9. Cultured GT Cells Display Characteristics of Neurosecretory Neurons by Transmission Electron Microscopy

(A) A large nucleus (N) and prominent Golgi apparatus (G) occupy this GT1-7 cell body cut parallel to the culture surface. Secretory granules (arrows), cisternae of the endoplasmic reticulum, and mitochondria continue into a major dendritic process. Clathrin-coated invaginations (arrowheads) sometimes appear along the plasmalemma. Magnification: 16,320×.

(B) Evidence of synaptic-like morphogenesis at points of contact between microtubule-containing processes (M) includes aggregations of dense-core granules, symmetrical thickening of apposed, parallel membranes bordering a cleft (arrows), and occasional coated vesicles (arrowhead). Magnification: 36,580×.

(C) A region of perikaryal cytoplasm shows typical neurosecretory granules condensing in a cistern of smooth endoplasmic reticulum. Magnification: 52,800×.

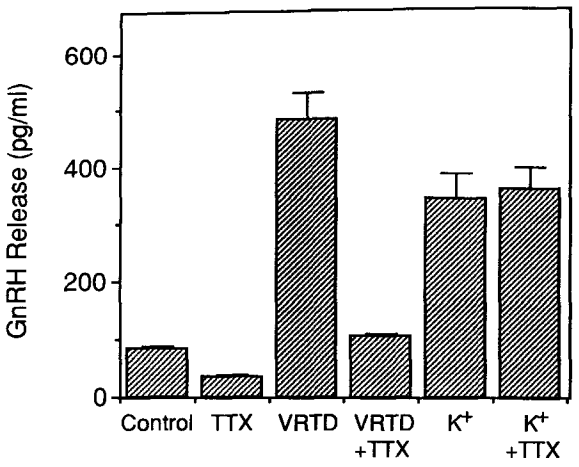

Figure 10. Depolarization of GT Cells with 56 mM K^+ or 50 μM Veratridine Stimulates Release of GnRH

Treatment of GT1-7 cells with 56 mM K^+ or 50 μM veratridine (VRDT) for 15 min dramatically increased GnRH release into the medium, as detected by radioimmunoassay. The veratridine but not the K^+ stimulation was blocked by pretreatment with tetrodotoxin (TTX) for 30 min. The Na^+ concentration was decreased to maintain isotonicity of the buffer. Values are the mean ± standard error, n = 3.

maintenance of many other characteristics of differentiated neurons indicates that it is possible to immortalize at least some types of differentiated neurons.

The presence of a GnRH-Tag transgene in the mouse genome throughout development may allow expression of the oncogene during early stages of cellular differentiation. Early in fetal development, GnRH neurons become immunopositive while remaining capable of division (Schwanzel-Fukuda and Pfaff, 1989). Thus, the use of transgenic mice may allow targeted oncogene expression in these neurons throughout differentiation, providing the potential for transformation within the appropriate developmental window. In addition, cells that dedifferentiate and lose expression of GnRH will also lose the expression of the GnRH-Tag transgene. Accordingly, coupling of oncogene expression to the control of a specific marker of differentiation for this population of neurons may select specifically for differentiated cells.

The possibility exists, however, that GnRH neurons represent the exception rather than the rule for transformation of CNS neurons. Schwanzel-Fukuda and Pfaff (1989) have concluded that GnRH neurons migrate into the brain from the olfactory placode and continue to divide after expressing GnRH (see also Wray et al., 1989). The developmental origin of the olfactory placode is unclear, and the possibility exists that it is derived from the neural crest instead of the neural tube. Tumors of tissues derived from the neural crest occur more commonly, and such cells may be immortalized more easily.

These studies demonstrate the feasibility of using targeted oncogenesis to transform specific differentiated neurons that can be established in culture, with maintenance of highly differentiated phenotypes. With further cloning and characterization of promoters from genes expressed specifically in particular neurons or neuronal layers (Travis and Sutcliffe, 1988), the potential exists for obtaining cultured cell models for additional neuronal cell types of the CNS. Additional studies in which expression of oncogenes is targeted to specific neurons will test the generality of this approach.

Experimental Procedures

Transgenic Mice

The GnRH-Tag gene was excised from the plasmid vector using BamHI restriction sites and injected into fertilized 1-cell embryos essentially as described (Hogan et al., 1986). The F_2 embryos were derived from matings of CB6F_1/J (C57Bl/6J × BALB/cJ) mice obtained from Jackson Laboratory (Bar Harbor, ME).

Cell Culture

After surgical removal, tumors were dispersed by incubation at 37°C in Hanks buffered saline with 10 mg/ml collagenase and 10 μg/ml DNAase for 30 min. Following trituration, cells were plated onto plastic culture plates or Matrigel-coated plates (Collaborative Research, Bedford, MA) in DME with 15% fetal bovine serum, penn/strep, 4.5 mg/ml glucose, and nonessential amino acids (GIBCO, Grand Island, NY). Clonal cell lines were maintained in DME with 5% fetal bovine serum, 5% equine serum, penn/strep, and 4.5 mg/ml glucose. Cells for light microscopy and secretion experiments were cultured in Opti-MEM (GIBCO) without serum for 2 days on plastic culture dishes.

Northern Blot Analysis

mRNA from both tissues and cells was prepared by the method of Chirgwin et al. (1979), and Northern blot analysis was carried out as described (Maniatis et al., 1982) using 10 μg of total RNA in each lane (Figures 2 and 3) or 5 μg of poly(A)$^+$ RNA (Figures 5 and 6) selected by oligo(dT)-cellulose chromatography (New England Biolabs, Beverly, MA). Cells for poly(A)$^+$ RNA were cultured in DME with 5% fetal bovine serum and 5% equine serum, as described above, or without serum in Opti-MEM for 1 day on plastic culture dishes. The probes were generated by nick translation (Meinkoth and Wahl, 1984; Figures 2 and 3) or random priming (Feinberg and Volgelstein, 1983, 1984; Figures 5 and 6) from plasmids containing either the rat GnRH cDNA (Seeburg and Adelman, 1984), Tag coding region, rat NSE cDNA (Forss-Petter et al., 1986), mouse neurofilament protein cDNA (Lewis and Cowan, 1985), mouse GFAP cDNA (the 3′ untranslated region from the HindIII site at bp 1369 to the 3′ end; Lewis et al., 1984), rat myelin basic protein cDNA (Roach et al., 1983), rat myelin proteolipid protein cDNA (Milner et al., 1985), rat somatostatin cDNA (Montminy et al., 1984), rat proopiomelanocortin (a genomic fragment containing the third exon; Eberwine and Roberts, 1984), rat corticotropin-releasing hormone cDNA (Imaki et al., 1989), rat growth hormone-releasing hormone cDNA (Mayo et al., 1985), rat Vamp-1 and Vamp-2 cDNAs (Elferink et al., 1989), mouse SNAP-25 cDNA (Oyler et al., 1989), or rat chromogranin B cDNA (Forss-Petter et al., 1989).

Radioimmunoassay

GnRH was measured by a double antibody radioimmunoassay using rabbit anti-GnRH antiserum 42 obtained from G. Niswender (Nett et al., 1973). The antibody is highly specific to GnRH. Data were analyzed for statistical differences using Student's t-test.

Immunocytochemistry

GT1-3 cells cultured on Matrigel were fixed for 10 min in PBS containing 4% paraformaldehyde and 0.1% glutaraldehyde and

treated for 10 min with 0.2% Triton X-100 for immunostaining of GnRH, neuron-specific enolase, GFAP, or controls. Cells were incubated with dilutions of primary antibodies (1:5000, 1:1000, and 1:500 respectively) for 2 hr at room temperature, then coupled to a biotinylated second antibody and an avidin–biotin–horseradish peroxidase complex (Vectastain ABC slide technique; Vector Laboratories Inc., Burlingame, CA) with 3,3'diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) as the chromagen. The rabbit antibody to GnRH (LR 1) was obtained from Dr. Robert Benoit. The antigenic determinants consist of amino acids 2–4 and 7–10 of GnRH (Schwanzel-Fukuda and Pfaff, 1989). The antibody to GFAP was obtained from Lab Systems (San Mateo, CA) and neuron-specific enolase was obtained from Chemicon International, Inc. (El Segundo, CA). For staining of Tag, GT1-3 cells were fixed for 10 min in methanol, acetone followed by 10 min in 1% Triton X-100. The rabbit antibody against Tag was obtained from Dr. Doug Hanahan and used at a dilution of 1:5000.

Electron Microscopy

GT1-7 cells were grown on Matrigel-coated Lux Thermanox coverslips (E & K Scientific, San Jose, CA) and fixed with 4% paraformaldehyde, 0.1% glutaraldehyde in 150 mM sodium cacodylate buffer (pH 7.4). Cells on coverslips were postfixed with 2% osmium tetroxide, stained with 2% uranyl acetate during ethanolic dehydration, embedded in Epon, and sectioned with an ultramicrotome at 60 nm. Thin sections retrieved on uncoated copper grids were counterstained with uranyl acetate and lead citrate and examined in a Philips EM300 at 60 kV.

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