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Expression of gonadotropin-releasing hormone receptors and autocrine regulation of neuropeptide release in immortalized hypothalamic neurons

(gonadotropin-releasing hormone receptor transcripts/binding sites/cytoplasmic calcium/episodic secretion)

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ABSTRACT **The hypothalamic control of gonadotropin secretion is mediated by episodic basal secretion and midcycle ovulatory surges of gonadotropin-releasing hormone (GnRH), which interacts with specific plasma membrane receptors in pituitary gonadotrophs. Similar GnRH receptors and their mRNA transcripts were found to be expressed in immortalized hypothalamic neurons, which release GnRH in a pulsatile manner *in vitro*. Activation of these neuronal GnRH receptors elicited dose-related intracellular Ca^{2+} concentration responses that were dependent on calcium mobilization and entry and were inhibited by GnRH antagonists. Exposure of perifused neurons to a GnRH agonist analog caused a transient elevation of GnRH release and subsequent suppression of the basal pulsatile secretion. This was followed by dose-dependent induction of less frequent but larger GnRH pulses and ultimately by single massive episodes of GnRH release. The ability of GnRH to exert autocrine actions on its secretory neurons, and to promote episodic release and synchronized discharge of the neuropeptide, could reflect the operation of the endogenous pulse generator and the genesis of the preovulatory GnRH surge *in vivo*.**

The major regulator of reproduction in mammals, gonadotropin-releasing hormone (GnRH), is produced by neuronal cells located in the preoptic area and adjacent sites in the rostral portion of the hypothalamus and secreted into the hypophyseal portal vessels at the median eminence (1, 2). The secretion of GnRH occurs in an episodic manner due to the activity of a hypothalamic GnRH pulse generator (3). Recently, pulsatile neuropeptide secretion was found to be an intrinsic property of GnRH neuronal networks and to depend on voltage-sensitive Ca^{2+} influx for its maintenance (4–7). The activity of the GnRH pulse generator is influenced by several factors, including endothelin, *N*-methyl-D-aspartate, opiates, γ-aminobutyrate, and α-adrenergic input, as well as estrogens and androgens (8–16). In addition, GnRH has been proposed to exert an inhibitory action on its own secretion (17–19); however, the mechanism and circuitry of such an ultrashort loop feedback effect have not yet been defined. We now report that GnRH acts directly on immortalized GnRH neuronal cells to regulate its own secretion and that this autocrine action of the neuropeptide is associated with activation of calcium-mobilizing GnRH receptors expressed in its cells of origin.

MATERIALS AND METHODS

Binding Studies. Plasma membrane receptors for GnRH were analyzed by binding studies with ^{125}I-labeled des-Gly^{10}[D-Ala^6]GnRH *N*-ethylamide (Hazleton Laboratories America, Vienna, VA). The radioligand (150 pM) and nonradioactive peptides were added in 100-μl aliquots to monolayers of GT1-7 cells (generously provided by Richard Weiner, University of California, San Francisco) cultured in 12-well Falcon plates at 24°C. After incubation to equilibrium for 90 min at room temperature, the cells were washed three times with ice-cold phosphate-buffered saline/0.1% bovine serum albumin and then solubilized in 1 M NaOH containing 0.1% SDS and analyzed for bound radioactivity in a γ-spectrometer.

Analysis of GnRH Receptor mRNA. For Northern blot analysis, total RNA was extracted from the cells as described (20) and electrophoresed on a denaturing 1% agarose gel containing formaldehyde. The fractionated RNA was blotted and baked onto a Nytran membrane (Schleicher & Schuell) and Northern blot analysis was performed by hybridizing the membrane with a [^{32}P]dCTP-labeled murine GnRH receptor DNA probe prepared by random hexamer-primed synthesis from the entire murine cDNA template (21). Hybridization was carried out for 16 h at 42°C in the presence of dextran sulfate (10%), followed by one wash with 2× standard saline citrate (SSC) for 30 min at room temperature and two consecutive washes with 1× SSC at 55°C. The dried blot was then exposed to Kodak X-Omat/AR film for 16 h.

Cytoplasmic Ca^{2+} Measurements. For intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements, GT1-7 cells were plated on 25-mm coverslips coated with poly(L-lysine) and cultured for 24 h. The cells were then washed twice and loaded with 1 μM Indo-1 AM (Molecular Probes) for 60 min at 37°C and mounted on the stage of an inverted Diaphot microscope attached to an intracellular Ca^{2+} analysis system (Nikon). All Ca^{2+} values were derived from a standard curve that was constructed by addition of known concentrations of Ca^{2+} to 10 μM Indo-1.

Perifusion of Neurons. The release of GnRH was examined in perifused neurons (Krebs–Ringer buffer; flow rate, 10 ml/h) cultured on beads (20×10^6 cells per column). The cells were loaded into a temperature-controlled 0.5-ml chamber (Endotronics, Minneapolis) and perifused for at least 1 h before testing at a flow rate of 10 ml/h to establish a stable baseline. Fractions were collected every 5 min and stored at −20°C prior to radioimmunoassays. GnRH assay was performed as described (7), with ^{125}I-labeled GnRH from Amersham, unlabeled GnRH from Peninsula Laboratories, and primary antibody donated by V. D. Ramirez (Urbana, IL). In

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Abbreviations: GnRH, gonadotropin-releasing hormone; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

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this assay, the GnRH superagonist analog, des-Gly^{10}[D-Ala^{6}]GnRH *N*-ethylamide, had <0.01% cross-reactivity at 1 μM concentration.

RESULTS

Expression of GnRH Receptors. In addition to being produced by cultured hypothalamic neurons and immortalized GT1-7 neuronal cells (4–7), GnRH binds with high affinity to specific receptors in GT1-7 cells in a dose-, time-, and temperature-dependent manner. Receptor specificity was confirmed by the ability of unlabeled GnRH (100 nM) to inhibit radioligand binding by up to 97% and the lack of displacement by unrelated peptides (100 nM), including angiotensin II, thyrotropin-releasing hormone, norepinephrine, oxytocin, and arginine vasopressin. The concentration-dependent inhibition of ^{125}I-des-Gly^{10}[D-Ala^{6}]GnRH *N*-ethylamide binding by unlabeled GnRH, and GnRH agonist and antagonist analogs, is illustrated in Fig. 1*A*. The estimated IC_{50} values for each competition curve were 12 nM for GnRH, 2 nM for the GnRH antagonist [D-pGly^{1},D-Phe^{2},D-Trp^{3,6}]GnRH, and 2.4 nM for the GnRH agonist des-Gly^{10}[D-Ala^{6}]GnRH *N*-ethylamide.

The presence of GnRH receptor mRNA in the GT1-7 cell line was demonstrated by Northern blot analysis, as shown in Fig. 1*A Inset*. The GnRH-producing neuronal cells contained receptor transcripts of the same size (3.5 and 1.6 kb) as those present in the murine pituitary and the αT3 gonadotroph cell line from which the receptor was cloned (21). The proportions of the receptor transcripts in the αT3 and GT1-7 cell lines were also similar, with a predominance of the 3.5-kb species.

GnRH-Induced Ca^{2+} Responses. Activation of neuronal GnRH receptors was associated with a rapid and prominent increase in $[Ca^{2+}]_i$. As shown in Fig. 1 *B* and *C*, single GT1-7 cells showed amplitude-modulated $[Ca^{2+}]_i$ signaling, with no indication of Ca^{2+} oscillations, in response to increasing GnRH concentrations. The Ca^{2+} response to GnRH had an EC_{50} of 14.1 nM (n = 108) and was attenuated or abolished by prior addition of the GnRH antagonist, depending on the dose relations between agonist and antagonist (Fig. 1*D*). However, addition of GnRH antagonist did not affect the basal $[Ca^{2+}]_i$ level in unstimulated neurons.

The resting level of Ca^{2+} prior to stimulation by GnRH influenced the pattern of the subsequent Ca^{2+} response. In general, two types of $[Ca^{2+}]_i$ signals were recorded from unstimulated GT1-7 cells. About 35% of the cells were quiescent, with a stable resting $[Ca^{2+}]_i$ level of 211 ± 13 nM (mean ± SE; n = 41). However, the majority of the GT1-7 cells (≈65%) showed episodes of prominent spontaneous fluctuations, with random frequency and amplitude, and a variable basal $[Ca^{2+}]_i$ level (from 200 to 800 nM). Three examples of such spontaneous $[Ca^{2+}]_i$ are shown in Fig. 2*A* (upper traces). Such spontaneous $[Ca^{2+}]_i$ fluctuations were abolished by addition of the Ca^{2+}-channel antagonist nifedipine (Fig. 2*B*) or when cells were exposed to Ca^{2+}-deficient medium (Fig. 2*A*, lower traces). The basal $[Ca^{2+}]_i$ level measured under extracellular Ca^{2+}-deficient conditions (free Ca^{2+}, 20 nM; lower traces in Fig. 2*A*) was significantly less than in the presence of normal Ca^{2+} [301 ± 12 (n = 74) vs. 136 ± 2 (n = 15); $P < 0.001$].

The initiation of Ca^{2+} signaling by GnRH was dependent on mobilization of intracellular Ca^{2+} stores, since the initial spike phase was only slightly reduced when Ca^{2+} was removed from the incubation medium. However, the plateau phase, when present, was completely abolished in the absence of Ca^{2+} (Fig. 2*C Left*). Also, prior addition of nifedipine abolished the plateau phase in a manner similar to that observed after omission of extracellular Ca^{2+}, while the initial spike was not altered (Fig. 2*C Right*). These data indicate that the Ca^{2+} response is initiated by Ca^{2+} mobilization and is sustained by entry of Ca^{2+} through voltage-sensitive Ca^{2+} channels.

However, a plateau phase in the Ca^{2+} response to GnRH was observed only in cells with low basal $[Ca^{2+}]_i$ levels. The majority (65%) of the cells had elevated basal $[Ca^{2+}]_i$ levels and showed a monophasic response to GnRH, usually with an exponential decrease to levels below the initial resting $[Ca^{2+}]_i$ (Fig. 3*A*, left trace). The inverse relationship between the sustained $[Ca^{2+}]_i$ response in GnRH-stimulated cells and

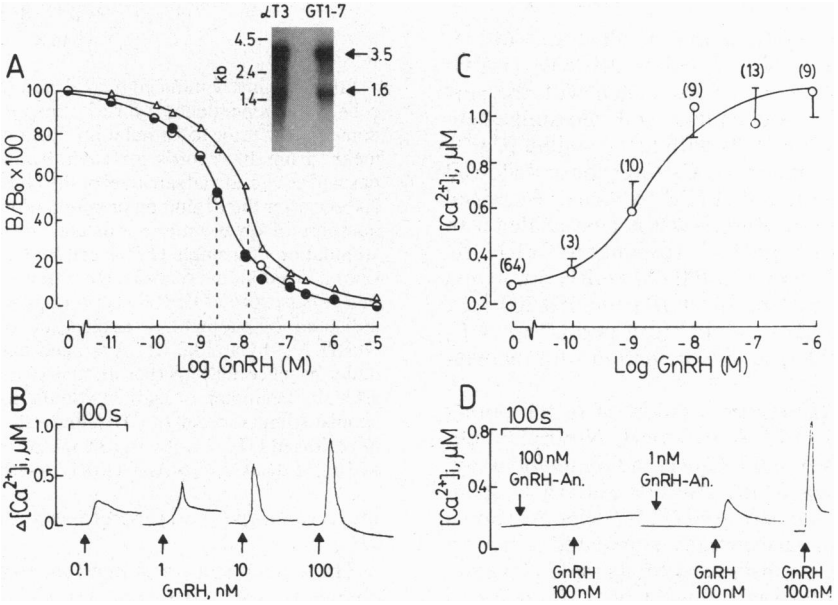

FIG. 1. Characterization of GnRH receptors in GnRH neuronal cells. (*A*) Competitive inhibition of ^{125}I-labeled GnRH agonist binding in GT1-7 cells by the unlabeled agonist (●), a GnRH antagonist (○), and native GnRH (△). Dotted lines indicate IC_{50} values. B_o refers to binding of ^{125}I-labeled agonist in the absence of competing ligands. (*Inset*) Northern blot analysis of 10 and 5 μg of total RNA isolated from murine αT3 pituitary and GT1-7 neuronal cells, respectively. (*B*) Representative $[Ca^{2+}]_i$ responses of single GT1 cells to increasing concentrations of GnRH. (*C*) Averaged data of peak Ca^{2+} responses to GnRH. Values are means ± SE of the number of observations indicated by data points. (*D*) Inhibition of GnRH-induced $[Ca^{2+}]_i$ responses by pretreatment with the GnRH antagonist (GnRH-An.).

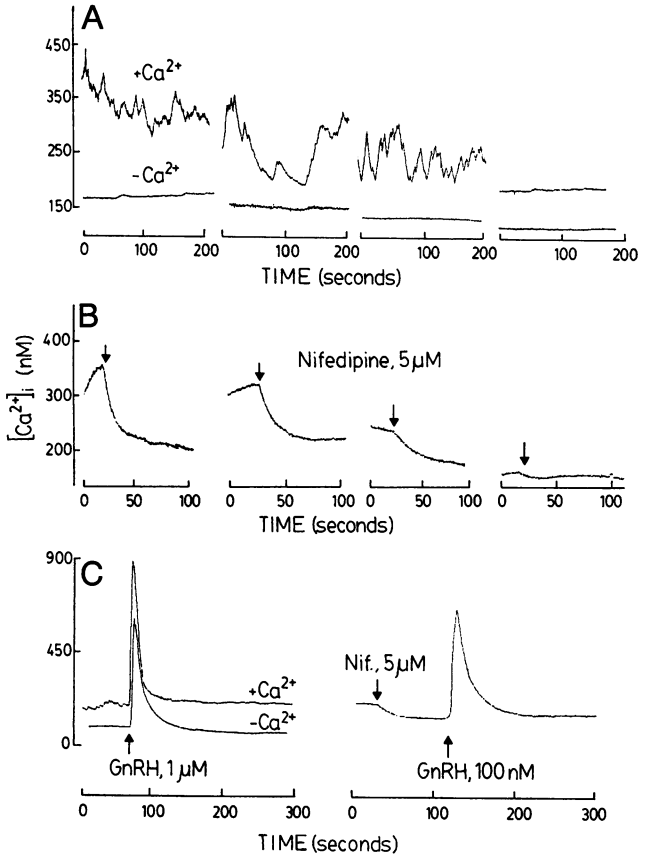

FIG. 2. Basal and GnRH-induced $[Ca^{2+}]_i$ signaling in GT1-7 cells. (*A*) Basal $[Ca^{2+}]_i$ in the presence of 1.25 mM ($+Ca^{2+}$) and 20 nM extracellular Ca^{2+} ($-Ca^{2+}$). (*B*) Inhibitory effects of the Ca^{2+}-channel antagonist nifedipine on basal $[Ca^{2+}]_i$. (*C*) (*Left*) Ca^{2+} response to GnRH in the presence and absence of extracellular Ca^{2+}. (*Right*) Effects of prior addition of nifedipine (Nif.) on the GnRH-induced Ca^{2+} response. (*A* and *B*) Data are representative recordings of typical experiments from 200 records. (*C*) Data are computer-derived means of 10 records for each group.

the level of $[Ca^{2+}]_i$ prior to stimulation is illustrated in Fig. 3*B*. In cells showing basal $[Ca^{2+}]_i$ below 240 nM, GnRH-induced mobilization of Ca^{2+} was associated with the sustained, extracellular Ca^{2+}-dependent and nifedipine-sensitive response. However, in cells with higher initial $[Ca^{2+}]_i$ levels, GnRH induced a monophasic Ca^{2+} response, followed by a decrease to below the initial $[Ca^{2+}]_i$ value. Sustained agonist stimulation of GnRH neurons was not associated with subsequent refractoriness of the Ca^{2+} response to GnRH. As shown in Fig. 3*C*, cells exposed to 100 nM GnRH for 30 min (left trace illustrates the Ca^{2+} profile during the first 200 sec), followed by washing and a 30-min rest period at 24°C, responded similarly to subsequent stimulation with the neuropeptide (right trace).

Agonist-Induced GnRH Secretion. Addition of the potent GnRH agonist, des-Gly^{10}[D-Ala^6]GnRH *N*-ethylamide, which does not cross-react in the GnRH radioimmunoassay, caused significant changes in the pulsatile pattern of spontaneous GnRH release from perifused GT1-7 cells. As shown in Fig. 3*D*, the GnRH agonist initially stimulated a modest increase in GnRH release, consistent with its rapid elevation of $[Ca^{2+}]_i$. However, this was followed by a decrease to GnRH levels below the basal release rate and abolition of the pulsatile secretory pattern. The secondary inhibitory effect of GnRH receptor activation, following its initial stimulatory action on neuropeptide secretion, was not long lasting. Instead, during continued exposure to the agonist the cells recovered from the inhibitory phase and exhibited less frequent but larger and discrete episodes of GnRH release (Fig. 3*F*).

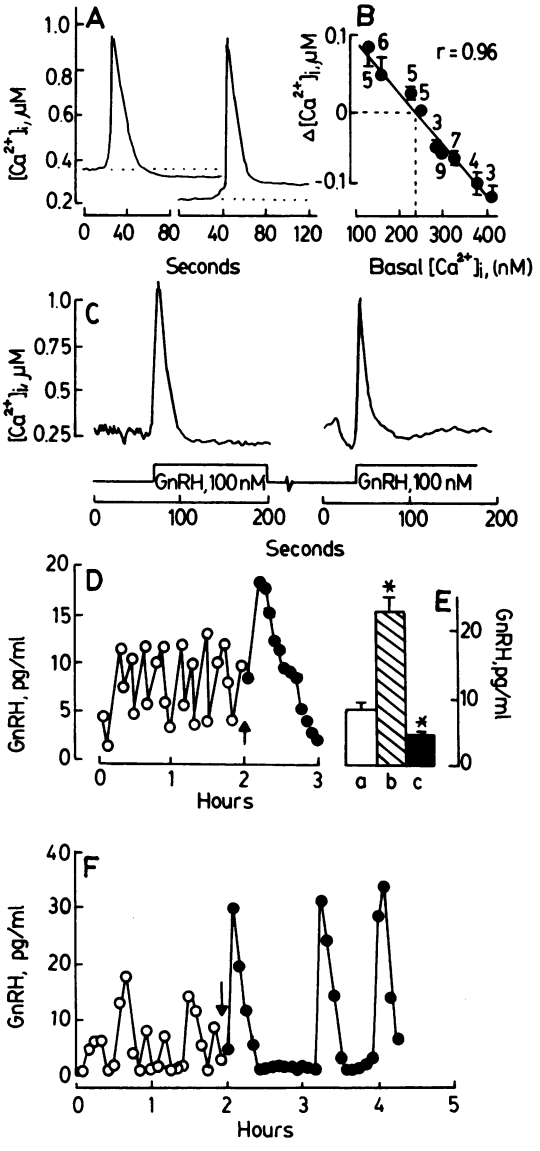

FIG. 3. GnRH-induced $[Ca^{2+}]_i$ and secretory responses in GT1-7 cells. (*A*) Dependence of Ca^{2+} response on the level of $[Ca^{2+}]_i$ immediately prior to stimulation. Data shown are computer-derived means from 10 records for each group. (*B*) Relationship between basal $[Ca^{2+}]_i$ and plateau level of the GnRH-induced $[Ca^{2+}]_i$ response 200 sec after the beginning of stimulation. Values are means ± SE of numbers of observations indicated by data points. (*C*) Repetitive stimulation of a single GT1-7 cell by GnRH. Traces are representative of 15 individual records. (*D*) Effects of agonist stimulation on the pulsatile pattern of GnRH secretion in perifused GT1-7 cells. Arrow indicates the moment of application of 10 nM des-Gly^{10}[D-Ala^6]-GnRH *N*-ethylamide. (*E*) Averaged data (n = 4) of GnRH release under basal conditions (bar a), and 10 min (bar b) and 60 min (bar c) after the beginning of agonist stimulation. (*F*) Effects of prolonged agonist stimulation on the frequency and amplitude of GnRH release in perifused GT1-7 cells. Arrow indicates the moment of application of 10 nM des-Gly^{10}[D-Ala^6]GnRH *N*-ethylamide.

The concentration dependence of GnRH agonist-induced changes in the oscillatory pattern is shown in Fig. 4; increasing agonist concentrations caused a progressive decrease in frequency and increase in amplitude of the GnRH pulses, leading to less frequent but increasingly prominent peaks and a net increase in GnRH release. Fig. 4*A* illustrates the pulsatile response in the controls, with an average interpulse period of 8.5 ± 2.3 min (n = 3). The frequency and amplitude

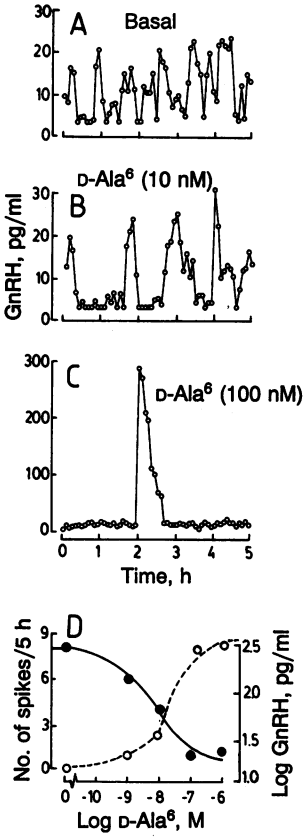

FIG. 4. Dose-dependent effects of GnRH agonist stimulation on the pattern of secretory responses in perifused GT1-7. (*A*) Controls. (*B* and *C*) GnRH agonist, in the doses indicated, was present throughout the recording period. (*D*) Relationship between frequency (●) and amplitude (○) of GnRH secretory pulses in cells exposed to increasing concentrations of des-Gly^{10}[D-Ala^6]GnRH *N*-ethylamide (D-Ala^6).

of the basal pulsatile release of GnRH were not affected by addition of two potent GnRH antagonists (data not shown). In cells exposed to 10 nM GnRH agonist, the interpulse period increased to 75 ± 5.2 min (n = 3; Fig. 4*B*). At 100 nM agonist, the interpulse period was 120 ± 8.1 min (n = 3; Fig. 4*C*). Despite this decrease in pulse frequency, the mean GnRH secretion during 5 h of agonist treatment increased from 12 ± 0.5 (basal) to 19 ± 1, 24 ± 2, 35 ± 8, and 39 ± 1 pg/ml, for 1, 10, 100, and 1000 nM des-Gly^{10}[D-Ala^6]GnRH *N*-ethylamide, respectively. This increase in net GnRH release was due to the progressive increase in peak amplitude, which increased from 10–20 pg/ml during basal pulsatile release to 250–300 pg/ml for the infrequent surge-like peaks observed at high agonist concentrations. The inverse relationship between the frequency and amplitude of GnRH pulses in cells exposed to increasing GnRH concentrations is shown in Fig. 4*D*.

DISCUSSION

These findings provide direct evidence for operation of an ultrashort loop feedback mechanism in the control of hypothalamic neurohormone secretion, a concept that was first proposed by Hyyppa *et al.* (17) in studies on the control of follicle-stimulating hormone secretion. Such a mechanism was supported by the observation that intracerebroventricular administration of hypothalamic peptides influenced anterior pituitary hormone release, sometimes in a manner opposite to their direct actions, as reported for somatostatin (22) and growth hormone-releasing hormone (23). The injection of relatively large doses of GnRH into the third ventricle was reported to increase plasma luteinizing hormone levels (24), presumably due to transport of GnRH to the vicinity of the hypophyseal portal vessels and thence to the pituitary gland. However, injection of lower doses of GnRH was found to cause a transient reduction, rather than an increase, in plasma luteinizing hormone levels (18).

More conclusive evidence for the existence of negative feedback was provided by the demonstration that GnRH concentration in hypophyseal portal plasma was reduced by treatment of ovariectomized rats with a GnRH agonist (19). Also, GnRH was found to inhibit its own secretion from medial basal hypothalamic fragments *in vitro* (25). However, the manner in which GnRH exerts such inhibitory effects remained unclear. Such inhibition was not observed in median eminence explants, indicating that axoaxonic synaptic autofeedback was not involved (25). It was postulated that feedback may occur by means of recurrent collaterals of GnRH axons that synapse on dendrites or perikarya of GnRH neurons (18). An alternative hypothesis proposed that negative feedback could be mediated via axodenritic/axosomatic synapses on adjacent GnRH or other types of neurons (25), and histological evidence for such connections has also been reported (2).

The present data demonstrate that immortalized GnRH neurons express GnRH receptors that mediate autocrine regulation of neuropeptide secretion *in vitro*. In GT1-7 cells, Northern blot analysis revealed two mRNA species (1.6 and 3.5 kb) that are similar to those observed in αT3 gonadotrophs and in the mouse pituitary gland. Both transcripts were shown to encode functional GnRH receptors when expressed in *Xenopus laevis* oocytes (21). Thus, in addition to being expressed in gonadotroph cells in the anterior pituitary gland (26) and αT3 immortalized murine gonadotrophs (21, 27), as well as in the central nervous system and gonads in the rat (28–31), GnRH receptors are also present in GnRH-producing cells and may mediate the proposed feedback control of neuropeptide secretion. Furthermore, our studies have revealed that GnRH can exert both stimulatory and inhibitory actions in GnRH neuronal cells, depending on its concentration and duration of action.

The initial stimulatory action of GnRH on its secretion from GT1 cells is consistent with the coupling of their GnRH receptors to mobilization of intracellular Ca^{2+}, as in other cell types expressing these receptors. However, the present data clearly indicate the transient nature of this early stimulatory response and the subsequent inhibition of spontaneous pulsatile GnRH secretion. Since the GnRH-induced elevation of $[Ca^{2+}]_i$ in GT1 cells was frequently followed by a decrease to below the initial level, it is possible that the suppression of pulsatile GnRH release is initiated by agonist-induced inhibition of Ca^{2+} entry, an action also observed in GnRH- and thyrotropin-releasing hormone-stimulated pituitary cells (32, 33). In accord with this, GnRH neurons *per se* constitute the basic elements of the GnRH pulse generator (4–7) and express voltage-sensitive calcium channels that serve as the Ca^{2+} entry pathway responsible for elevation of $[Ca^{2+}]_i$ and initiation of Ca^{2+}-dependent pulsatile GnRH secretion (7). Also, activation of GnRH receptors leads to abolition of such pulsatility in a manner comparable to that observed after application of EGTA and nifedipine (7).

The complexity of GnRH action in GT1 cells was further demonstrated by the delayed stimulatory effect of GnRH on its own secretion. The absence of long-lasting desensitization of the Ca^{2+} response of the GnRH neuron, observed in these experiments, is consistent with the reversible nature of the inhibitory action of GnRH. Such bidirectional effects of GnRH on Ca^{2+} signaling and secretory responses lead to changes in the pulsatile pattern of GnRH release, with more discrete secretory episodes of decreased frequency and increased amplitude and an overall increase in the release of

GnRH. Thus, the feedback effects of GnRH include both positive and negative components that exert an integrated regulatory action on its neurons and contribute to the operation of the GnRH pulse generator. On the other hand, the finding that GnRH antagonists did not alter the intrinsic pattern of GnRH release argues against the participation of negative feedback control in the basal pulsatile mode of GnRH secretion.

In conclusion, the expression of GnRH receptors in GnRH-producing hypothalamic cells, and their actions on Ca^{2+} mobilization and Ca^{2+} entry pathways, provides a mechanism for bidirectional autoregulation of GnRH secretion. The receptor-mediated actions of GnRH on the neuronal network of GnRH cells (6, 7), with decreases in the frequency and increases in the amplitude of GnRH pulses, lead to switching of the pattern of neuropeptide secretion from the basal pulsatile mode to one of episodic secretion and surge-like release as observed during the preovulatory period (34). Several other neuropeptides are known to modulate the activity of the GnRH pulse generator and may participate in the control of the preovulatory GnRH surge (8–16). There is also abundant evidence that estrogen promotes the development of the midcycle luteinizing hormone surge (35–37). However, the present data indicate that the autocrine feedback action of GnRH on the GnRH neuron can regulate the pattern of neuropeptide release in the absence of other cell types. The operation of such an autoregulatory process *in vivo* would clearly be relevant to the control of the episodic mode of gonadotropin secretion and the genesis of the midcycle luteinizing hormone surge that triggers ovulation.

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