Dissecting Autocrine Effects on Pulsatile Release of Gonadotropin-Releasing Hormone in Cultured Rat Hypothalamic Tissue

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The control of reproductive function is manifested centrally through the control of hypothalamic release of gonadotropin-releasing hormone (GnRH) in episodic events or pulses. For GnRH release to occur in pulses, GnRH neurons must coordinate release events periodically to elicit a bolus of GnRH. We used a perifusion culture system to examine the release of GnRH from both intact hypothalami and enzymatically dispersed hypothalamic cells after challenge with GnRH analogs to evaluate the role of anatomical neuronal connections on autocrine/paracrine signals by GnRH on GnRH neurons. The potent GnRH agonist des-Gly^{10}-D-Ala^{6}-GnRH N-ethylamide, potent GnRH antagonists D-Phe^{2}-D-Ala^{6}-GnRH and D-Phe^{2,6}-Pro^{3}-GnRH or vehicle were infused, whereas GnRH release from hypothalamic tissue and cells were measured. PULSAR analysis of GnRH release profiles was conducted to evaluate parameters of pulsatile GnRH release. Infusion of the GnRH agonist resulted in a decrease in mean GnRH ($P < 0.001$), pulse nadir ($P < 0.01$), and pulse frequency ($P < 0.05$) but no effect on pulse amplitude. Infusion of GnRH antagonists resulted in an increase in mean GnRH ($P < 0.001$), pulse nadir ($P < 0.05$), and pulse frequency ($P < 0.05$) and in GnRH pulse amplitude only in dispersed cells ($P < 0.05$). These results are consistent with the hypothesis that GnRH inhibits endogenous GnRH release by an ultrashort-loop feedback mechanism and that treatment of hypothalamic tissue or cells with GnRH agonist inhibits ultrashort-loop feedback, whereas treatment with antagonists disrupts normal feedback to GnRH neurons and elicits an increased GnRH signal. Exp Biol Med 229:56–64, 2004

Key words: GnRH agonist; GnRH antagonist; autocrine; ultrashort-loop

Central control of reproductive function is located among the gonadotropin-releasing hormone (GnRH) neurons scattered throughout several regions in the rostral hypothalamus and preoptic area. The signal sent to the anterior pituitary gonadotrophs by GnRH neurons is episodic or pulsatile in every model system studied to date (1). The pattern of release can vary with sex, species, and physiological state but is consistently composed of distinct pulses of GnRH (1). In the adult rat, only 1000–1600 of the neurons are immunopositive for GnRH (2), and these GnRH neurons are scattered throughout several structures within the rostral hypothalamus and preoptic area (3). For physiological pulses of GnRH to be measured, a significant number of the 1000–1600 GnRH neurons must undergo coordinated exocytosis (4, 5), although a recent study measuring electrical activity in GT1-7 cells suggests that not all of these GnRH-secreting cells participate in every pulse event (6).

The most straightforward explanation of coordinated GnRH release would involve a network of GnRH neurons connected through synapses. In a classic study, Blake and Sawyer (7) reported that surgical isolation of the rat hypothalamus from afferent input did not prevent pulsatile GnRH release, demonstrating that the cellular hardware necessary for generating GnRH pulses resided within the hypothalamus. Pulsatile GnRH release has also been reported in cultured GT1-7 cells (8) and embryonic olfactory placode cultures of GnRH neurons from monkey (9) and rat (10), illustrating that GnRH neurons have the capacity for pulsatile release independent of other neural input.

Using conventional histological methods, synapses have been documented between GnRH neurons in both rat (11, 12) and monkey (13), although the functional significance of these in coordinating pulsatile GnRH release seems minimal given that less than 8% of GnRH neurons in rats and monkeys are connected (3). Furthermore, recent dye-coupling studies have confirmed that very few (<2%) GnRH neurons are connected directly by synapses in embryonic mouse GnRH neurons (14, 15). Direct evidence of functional synaptic contact between GnRH neurons is largely limited to observations of gap junctions in the GT1-7 cell line (16, 17).

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Another possibility for coordinating GnRH neurons would be the existence of a complex interneuronal network that coordinates GnRH release through one or more of the dozens of neurotransmitters that have been shown to stimulate or inhibit GnRH (1). The best efforts to date have failed to construct a synaptic map that connects GnRH neurons together in a functional network, although this may be due to the complexity of the hypothetical interneuronal network. Furthermore, two groups using *in vitro* model systems have provided indirect evidence that synaptic contact between GnRH neurons is not necessary for coordinated GnRH release (4, 8).

Another mechanism for coordinated GnRH release, hypothesized 30 years ago, involves autocrine (or paracrine) regulation of GnRH release by GnRH (18). This mechanism is supported anatomically by the fact that GnRH receptors are expressed in GT1-7 cells (19) and in cultured embryonic rat GnRH neurons (20). The idea that GnRH regulates GnRH release has been investigated recently using two different *in vitro* models: the GT1-7 cell line and a primary culture of 17-day fetal rat hypothalamus (20). The results of the present study support a more complex role for GnRH autoregulation, although the analysis is not unequivocal. The present study utilizes perifusion of adult rat primary hypothalamic cultures, both intact and enzyme-dispersed hypothalami, and treatment with a potent GnRH agonist des-Gly^{10}-[D-Ala^6]-GnRH N-ethylamide (AG); two potent GnRH antagonists, [D-Phe^2, D-Ala^6]-GnRH (ANT-1) and [D-Phe^{2,6}, Pro^3]-GnRH (ANT-2); and vehicle control. We analyze here, for the first time, the pulse characteristics (mean GnRH release, pulse nadir, pulse amplitude, pulse frequency) of GnRH release in the rat in two *in vitro* model systems to compare any effects of organization of hypothalamic cells into neural networks (intact tissue) with neuroendocrine function in an enzymatically dispersed hypothalamic cell preparation.

Materials and Methods

Animals. Twelve adult male (3–6 months old) Sprague-Dawley rats from Harlan Sprague-Dawley (Madison, WI) weighing 380–430 g were used in these experiments. The rats were housed for 2–4 weeks in individual cages under controlled temperature and lighting conditions (23°C; 12 hrs light from 0600 to 1800 hr daily) and were allowed free access to food and water. The rats were sacrificed by decapitation at 0900–1030 hr, and the hypothalami were removed. These experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. These studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-Whitewater.

Cell and Tissue Preparation. The hypothalamus was surgically removed from the rat brain and was bisected down the midline as previously described (4, 21). Briefly, the hypothalamus was delimited laterally by the hypothalamic fissures, anteriorly by a cut 2 mm anterior to the optic chiasm, posteriorly by the rostral portion of the mammillary bodies, and by a horizontal cut approximately 2 mm in depth from the ventral surface. Each hemihypothalamic section was immediately placed in a modified Krebs-Ringer bicarbonate buffer containing 2.2 m*M* $CaCl_2$, 154 m*M* NaCl, 5.6 m*M* KCl, 1.0 m*M* MgCl, 6.0 m*M* $NaHCO_3$, 10 m*M* glucose, 2 m*M* HEPES, 0.1% bovine serum albumin, and 0.006% bacitracin, pH 7.40. For Experiment 1, hemihypothalami were kept intact for tissue culture. For Experiment 2, each hemihypothalamus was enzymatically dispersed with 0.05% trypsin, as described previously (4). Cell dispersion and viability were assessed by methylene blue dye exclusion. Immediately following dispersion, approximately 93% of cells were single or paired and more than 98% were alive; after 8 hrs in culture, more than 95% of cells remained dispersed and alive.

Perifusion Procedures. An Endotronics Accusyst-S cell perifusion system (Cellex Biosciences, Minneapolis, MN) was used in these experiments. Medium was pumped at a flow rate of 100 µl/min through chambers containing rat hemihypothalami, as either intact hemihypothalami (Experiment 1) or dispersed cells (Experiment 2). Carbon dioxide flowed into the chamber area, stabilizing media pH at approximately 7.40 prior to media entry into incubation chambers. Experiments were conducted for 8.5 hrs, with 1 ml of perifusate fractions being collected at 10-min intervals at 4°C. Samples were frozen and stored at –70°C until assayed by radioimmunoassay (RIA). In Experiment 1, intact hemihypothalamic tissue was incubated for 3 hrs in media; challenged for 3 hrs with media containing 10 n*M* AG, ANT#1, ANT#2, or vehicle; and finally incubated for 2.5 hrs of washout with media alone. Experiment 2 used the same infusion sequence, but dispersed cells from half a hypothalamus were placed in each well and challenged with media containing 10 n*M* AG, ANT#1, or vehicle for the 3-hr infusion.

GnRH RIA. GnRH concentration in perfusate was estimated by RIA, described previously (4). Synthetic GnRH, used both as trace and standard, was purchased from Richelieu Laboratories (Montreal, PQ, Canada). Anti-GnRH antibody (R1245) was provided by Dr. T. Nett (Colorado State University, Fort Collins, CO). Sensitivity of the assay was 0.1 pg/tube at 95% binding. Intraassay and interassay coefficients of variation were 4.21% and 8.36%, respectively.

Statistical Analysis. The results from each perifusion were divided into three time blocks: (i) the 3-hr pretreatment control (PRE) block; (ii) the 3-hr treatment challenge (AG, ANT#1, ANT#2, or vehicle) block; and (iii) the 2.5-hr washout with media (POST) block. Pulsatile GnRH measurements were analyzed using the PULSAR computer algorithm (22). Pulse parameters were consistent with our previous studies (4). There were no missing or undetectable data points. Comparison between groups was done using one-way analysis of variance. The response to each treatment was compared using both linear controls (treatment vs. pretreatment, treatment vs. post-treatment)

and parallel controls (treatment vs. vehicle). Pulse parameters were: mean = average of all GnRH values in a block; nadir = starting point for an identified pulse; pulse amplitude = nadir to peak difference; pulse frequency = number of pulses per hour.

GnRH Analogs. The GnRH agonist used is a highly active stimulator of GnRH activity at the pituitary level (AG = des-Gly^{10}-[D-Ala^6]-GnRH N-ethylamide; Ref. 23). The two GnRH antagonists tested are both highly active inhibitors of GnRH activity at the pituitary level (ANT#1 = [D-Phe^2, D-Ala^6]-GnRH and ANT#2 = [D-Phe^{2,6}, Pro^3]-GnRH; Refs. 23, 24). The GnRH analogs were purchased from Sigma Chemical Co. (St. Louis, MO). None of these GnRH analogs cross-reacted in the GnRH RIA (Fig. 1).

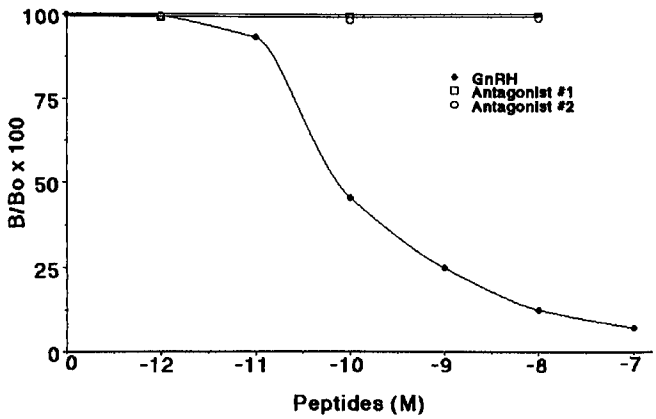

Figure 1. Competitive binding of the GnRH agonist des-Gly^{10}-[D-Ala^6]-GnRH N-ethylamide, GnRH antagonists [D-Phe^2, D-Ala^6]-GnRH (ANT#1) and [D-Phe^{2,6}, Pro^3]-GnRH (ANT#2) in the GnRH RIA. Typical displacement curves are shown, with doses of each antagonist against GnRH from 10^{-12} *M* to 10^{-7} *M*. There was no cross-reactivity observed for any of the GnRH analogs.

Results

The results of these experiments demonstrate a clear, consistent response to GnRH analog infusion both in intact hemihypothalami and enzymatically dispersed cells. In Experiment 1, vehicle, AG, and both antagonists were infused into intact hemihypothalami in parallel wells. GnRH pulsatility continued throughout the experiment in all chambers cultured. As can be seen in the individual example shown in Figure 2B, there was a decrease in mean GnRH and GnRH pulse frequency in response to agonist treatment during the 3-hr infusion period, compared with vehicle infusion (Fig. 2A). Treatment with either antagonist used resulted in an increase in mean GnRH release and in GnRH pulse frequency. In the summary of all trials shown in Figure 3, the release characteristics of pulse frequency, mean GnRH release, and pulse nadir showed a statistically significant decrease in response to agonist treatment and an increase in response to antagonist treatment. Interestingly, pulse amplitude did not change during exposure of intact hemihypothalami to agonist or either antagonist.

In Experiment 2, enzymatically dispersed rat hypothalami were challenged with vehicle, AG, and ANT#1. GnRH concentration during representative perifusions with vehicle, AG, and two examples of ANT#1 treatment are shown in Figure 4. GnRH mean, nadir, and pulse amplitude levels were substantially lower (~50% lower) in dispersed cell preparations than those observed in intact hemihypothalamic cultures (Fig. 5 vs. Fig. 3), in agreement with previous work (4, 21). This decline likely is due to physical loss of some of the GnRH neurons during the handling associated with the dispersion method. Trypsin dispersion for 15 mins may be destructive to these tissues, but our observations of cell viability before initiation and after 10 hrs of culture (>95%) suggest the tissues are not adversely affected by the enzyme treatment (4, 21). In addition, the fact that the GnRH agonist and antagonists have actions on GnRH release indicates that GnRH receptors are not digested from the cell membrane by our trypsin treatment protocol.

It should be noted that agonist and antagonists affected mean GnRH release, nadir levels, and pulse amplitude similarly when dispersed hypothalami were employed as they did when intact hemihypothalami were used, even though GnRH started at a lower baseline level prior to exposure to the agonist or antagonist when dispersed cells were employed. Furthermore, GnRH pulsatility was clearly maintained when dispersed hemihypothalami were employed (Fig. 5 vs. Fig. 3), indicating the dispersion method did not destroy the basic physiology of these cells (Fig. 4).

In enzymatically dispersed hypothalamic cells, infusion of GnRH analogs resulted in a GnRH release profiles similar to that of intact tissue (Fig. 2 vs. Fig. 4). AG infusion in dispersed cells decreased mean GnRH, pulse nadir, and pulse frequency. Interestingly, ANT#1 infusion in dispersed cells increased mean GnRH, pulse nadir, and pulse frequency but also resulted in a modest increase in pulse amplitude (Fig. 5). The increase in pulse amplitude was statistically significant, although some individual cases did not demonstrate a marked increase in pulse amplitude (Fig. 4D).

Discussion

The ability of GnRH antagonists to affect GnRH release was examined. Specifically, several characteristics of pulsatile GnRH release were assessed, including mean GnRH release, pulse nadir, pulse amplitude, and pulse frequency. An agonist and two different antagonists were used to treat intact hemihypothalami. The agonist and one of the antagonists (ANT#1) were also used to challenge dispersed cells. The results of this study demonstrate that interruption of GnRH autoregulation by treatment with either a GnRH agonist or a GnRH antagonist has a pronounced effect on endogenous GnRH pulse frequency. This was seen with agonist and antagonist treatment of both intact tissue and dispersed cells.

The present study represents the first detailed analysis of the effects of GnRH analogs on the characteristics of pulsatile GnRH release. These results are consistent with the general

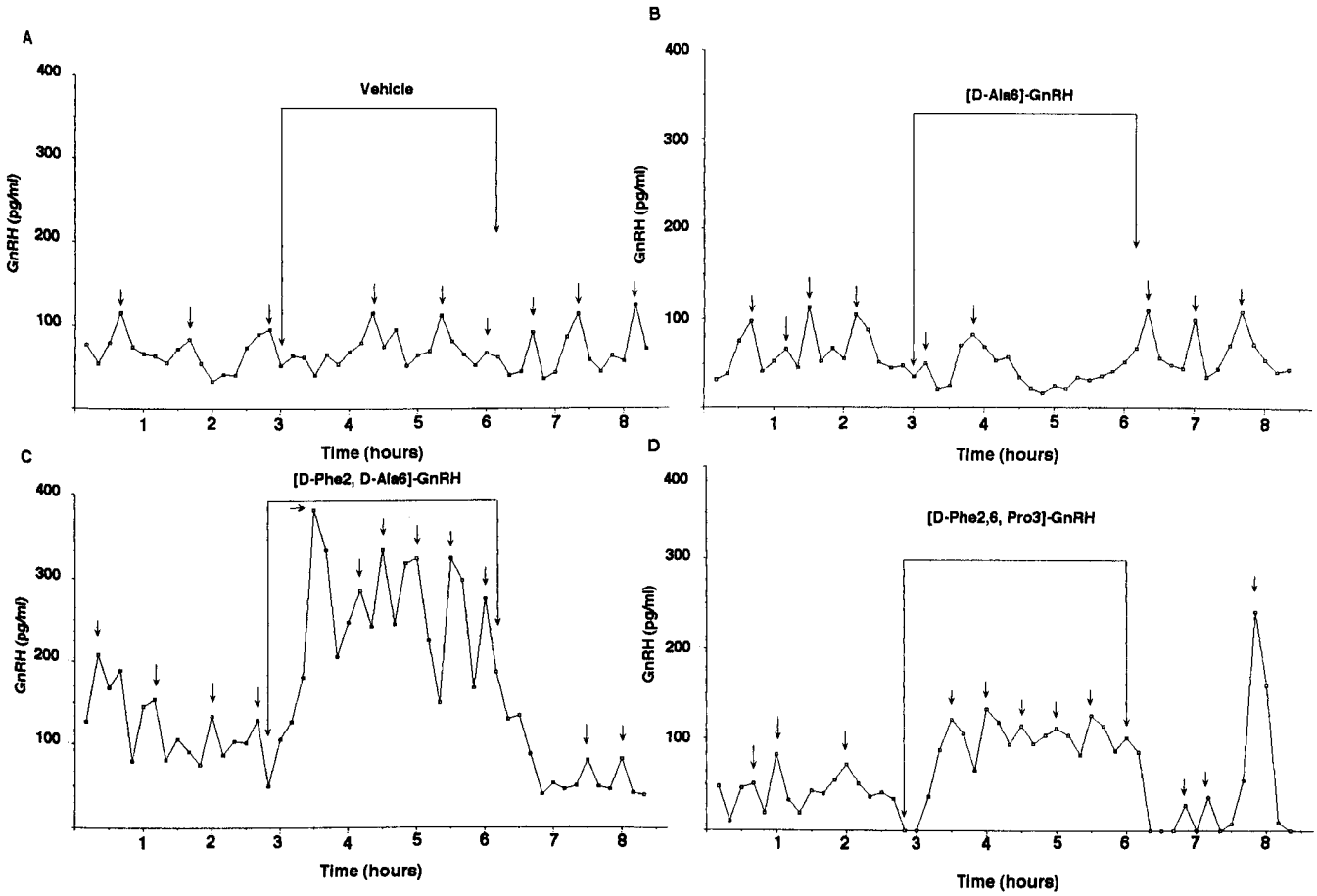

Figure 2. Representative cases of GnRH release during perifusion of intact cultured rat hemihypothalami challenged with vehicle (A), 10 n*M* GnRH AG (B), 10 n*M* GnRH ANT#1 (C), or 10 n*M* GnRH ANT#2 (D). Arrowheads above peaks indicate pulses detected by PULSAR algorithm.

findings of a number of groups who previously investigated autoregulation by GnRH (25–31). The unique aspects of the present study include detailed analysis of changes in various parameters of pulsatile GnRH release in response to treatment with various GnRH analogs, and our use of both intact hemihypothalamic tissue preparations and enzymatically dispersed rat hypothalami. The latter provides an opportunity to access the role of an organized interneuronal network in coordinating the activity of hypothalamic GnRH neurons given that our method disrupts virtually all cell-cell connections for the duration of the experiment.

There are a number of neuromodulators that influence specific parameters of pulsatile GnRH release. In one exhaustive study, Gearing and Terasawa (32) demonstrated that catecholamine manipulation primarily influenced GnRH pulse amplitude *in vivo* in the rhesus monkey. In a similar study, it was reported that during the steroid-induced LH surge, increases in neuropeptide Y (NPY) pulse frequency correlated tightly with increases in GnRH pulses (33). NPY pulse amplitude remained unchanged, however, whereas GnRH pulse amplitude increased dramatically during the gonadotropin surge, suggesting NPY may act as a frequency trigger and catecholamines may play a more direct role in influencing GnRH pulse amplitude (33). The precise role of GnRH in influencing specific characteristics of pulsatile GnRH release remains unclear, given the results of two studies that partially support (20) or do not support the GnRH autoregulation hypothesis (34).

The results of our study demonstrate that interruption of GnRH autoregulation by treatment with a GnRH antagonist has a pronounced effect on endogenous GnRH pulse frequency. This is an important observation, given that many previous studies investigating the effects of GnRH autoregulation have not assessed parameters of pulsatile GnRH release (25–28).

Of the studies that have assessed pulsatile release parameters, three of five are consistent with the GnRH autoregulation hypothesis. In one study, rat hypothalamic cultures were challenged with two superactive agonists, resulting in a decrease in GnRH pulse frequency and amplitude, consistent with the GnRH autoregulation hypothesis (30). In our study, treatment with a GnRH agonist suppressed mean GnRH pulse frequency and pulse nadir but had no effect on pulse amplitude in either intact hemihypothalami or dispersed cells. Another recent study testing potential autocrine regulation by GnRH used two distinct model systems (perifusion of GT1-7 cells and perifusion of a primary culture of 17-day fetal rat hypothalamus; Ref. 20).

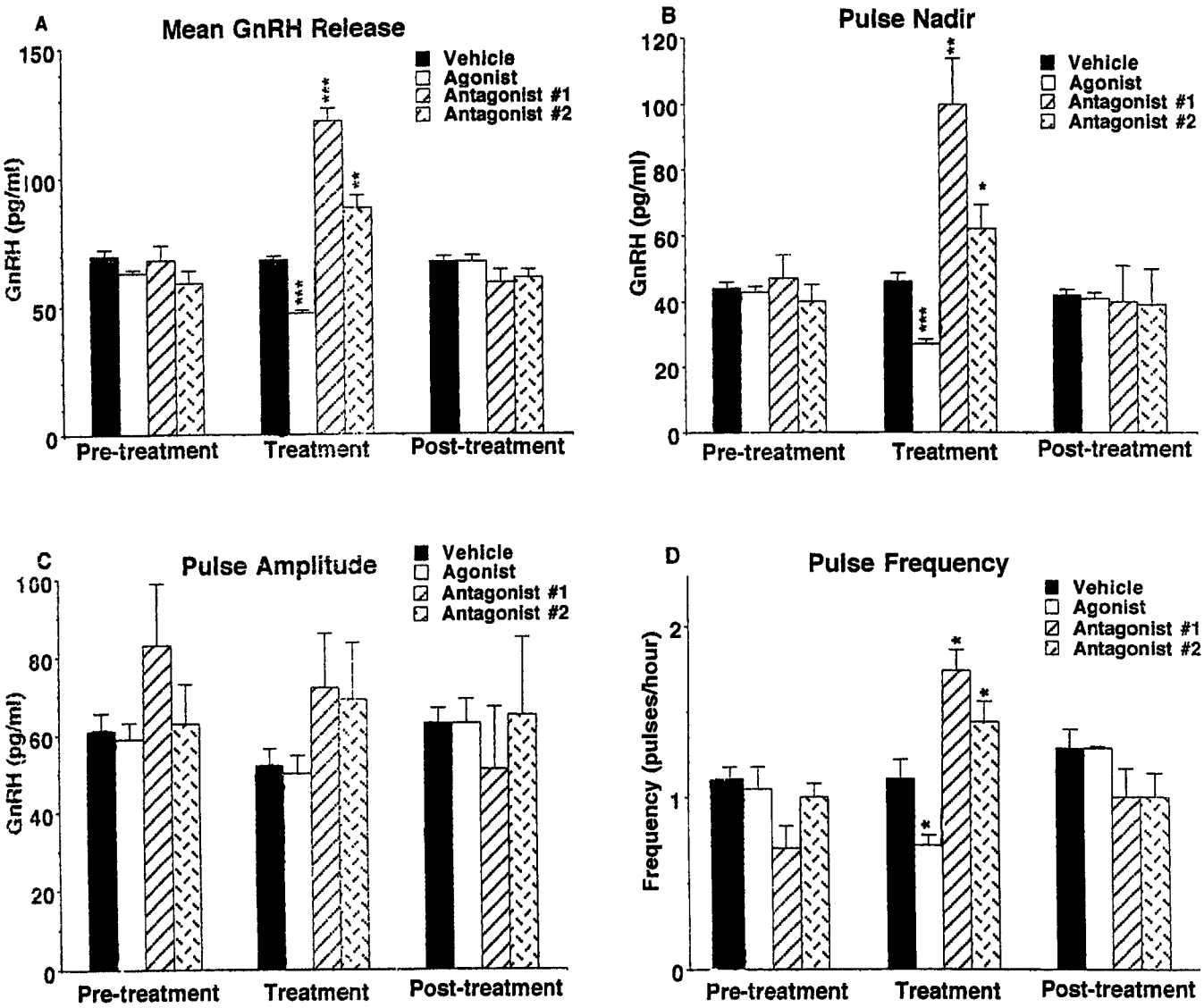

Figure 3. Summary data for GnRH pulse analysis before (Pre-Treatment), during (Treatment), and after (Post-Treatment) vehicle or analog infusion. The characteristics of pulsatile GnRH release assessed were mean GnRH release (A), pulse nadir (B), pulse amplitude (C), and pulse frequency (D). $N = 6$ for vehicle and AG; $N = 9$ for both antagonists tested.

In that study, treatment of GT1-7 cells and cultured fetal hypothalamic cells with the GnRH agonist des-Gly^{10}-[D-Ala^{6}]-GnRH N-ethylamide decreased pulse frequency and increased pulse amplitude significantly, whereas treatment with the GnRH antagonists SB-75 or [D-Glu]-GnRH resulted in cessation of GnRH pulses but a gradual increase in baseline release of GnRH (20).

These results only partially support the model of an operative ultrashort loop feedback mechanism in GnRH regulation because GnRH antagonist infusion would be expected to increase pulsatile GnRH release. However, although the study did include pulse analysis, the control group included KCl-induced pulses, which result in GnRH release via depolarization of the cell membrane (4, 5). Although useful in assessing cell viability, this treatment profoundly confounds interpretation of agonist or antagonist effects on parameters of pulsatile GnRH release. Regardless of how the controls were handled, the analog infusion data presented by Krsmanovic *et al.* (20) differ qualitatively from the results of the present study using higher doses of both agonist and antagonist. This difference may be a function of the doses used or the particular antagonists used by the two groups (the agonist was the same in both studies) or may represent a physiological difference between the model systems used (GT1 cells vs. primary hypothalamic culture).

Two previous studies that carefully evaluated parameters of pulsatile GnRH release as an end point have been conducted. Both studies were done with sheep but were conducted independently. An *in vivo* study with rams reported that treatment with GnRH agonists and antagonists had no effect on endogenous GnRH release (34), inconsistent with the GnRH autoregulation hypothesis. However, in

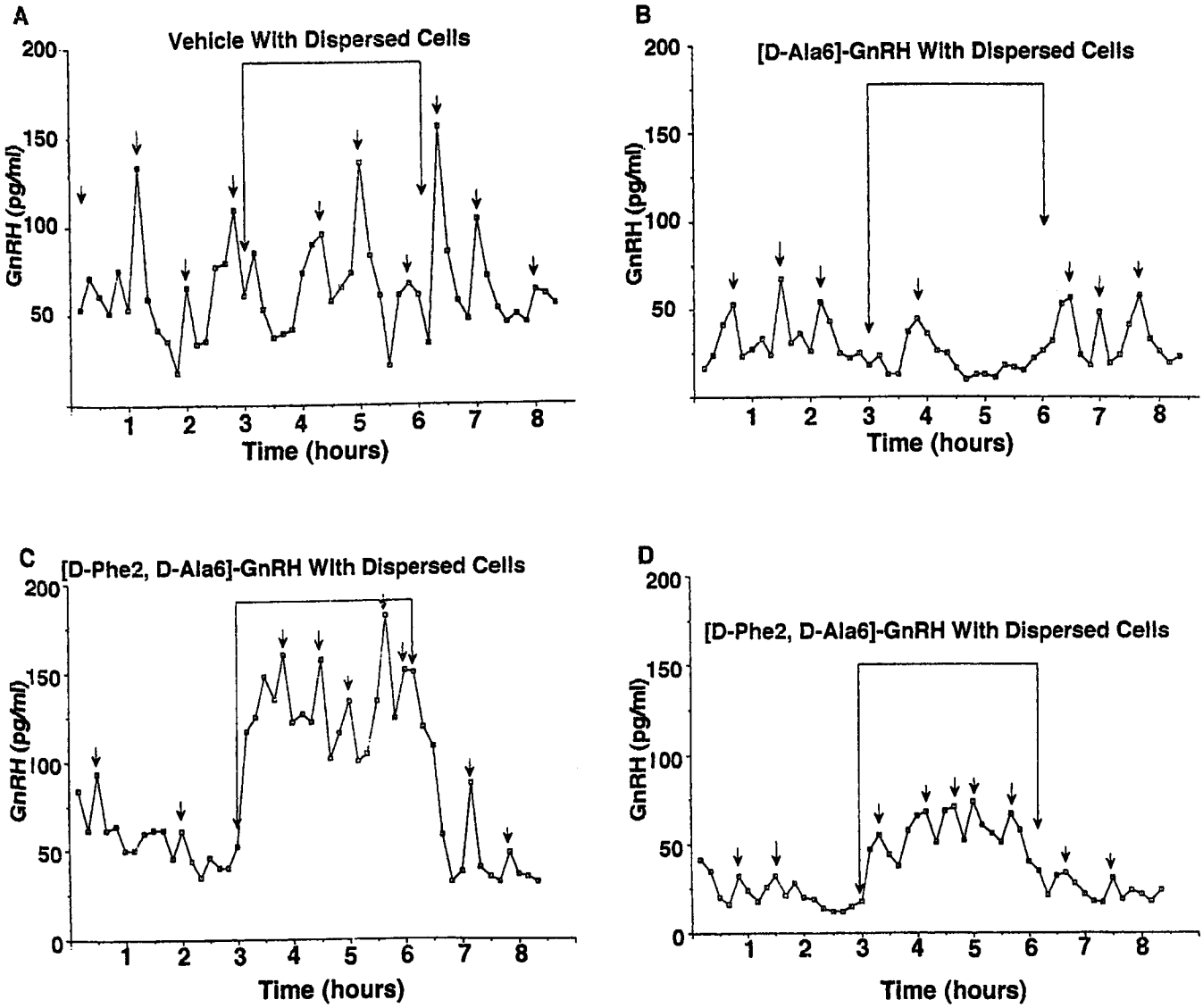

Figure 4. Representative cases of GnRH release during perifusion of enzymatically dispersed rat hypothalami challenged with vehicle (A), 10 n*M* AG (B), and two cases treated with 10 n*M* GnRH ANT#1 (C, D).

a more recent and thorough *in vivo* study in ewes (31), treatment with GnRH antagonist resulted in an increase in GnRH pulse frequency (~2-fold increase), comparable with that observed in our study with rats.

The study by Padmanabhan *et al.* (31) also reported a doubling of pulse amplitude in their midluteal ewes but not in their artificial luteal phase or ovariectomized ewes. The hypothalamic tissue used in the present study were harvested from gonad intact male rats, confounding comparisons between the two sheep studies and ours. Our results did show a significant but modest increase in pulse amplitude in response to antagonist treatment only in dispersed cell preparations. There was no effect of agonist treatment on pulse amplitude in either intact tissue preparations or dispersed cells.

It remains difficult to interpret the importance of analog treatment on pulse amplitude in our study and the Padmanabhan study, given that amplitude is affected only in some specific model systems. That amplitude is affected only in the dispersed cell model might suggest a paracrine rather than synaptic GnRH signal playing a role in the control of GnRH pulse amplitude. Further work is needed to determine the importance of enzymatic dispersion, sex, or reproductive status on the responsiveness of pulse amplitude to GnRH analog treatment.

A third *in vivo* study in rats was conducted through *in vivo* pituitary portal blood collection (29). This study reported that GnRH agonist reduced GnRH pulse frequency and amplitude, although their analysis was limited to pulse analysis of only a 70-min prechallenge and 110-min postchallenge blocks of time, making precise pulse frequency analysis difficult.

Changes in GnRH pulse frequency and/or pulse amplitude can have specific effects on pituitary function

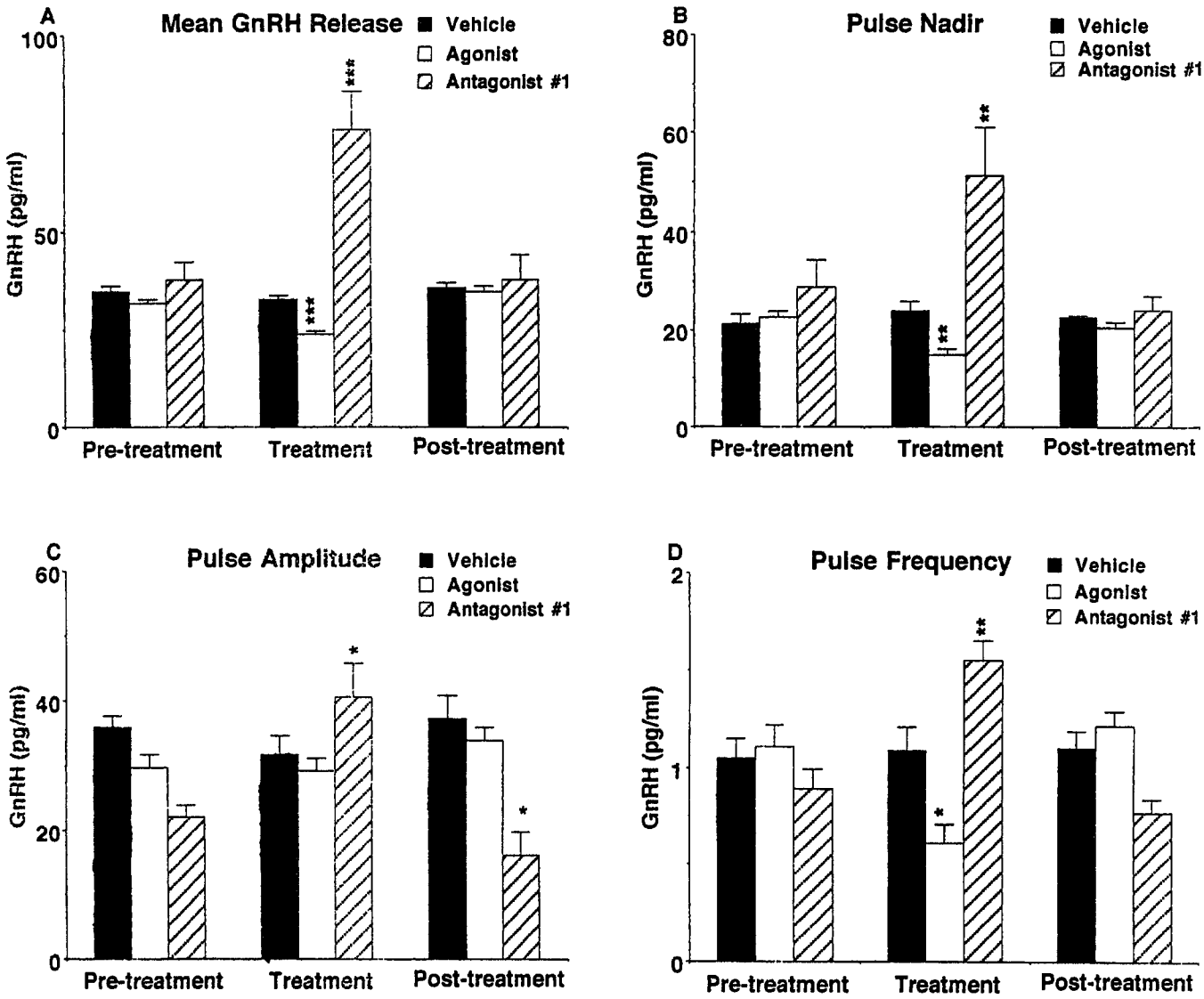

Figure 5. Summary data for GnRH pulse analysis before (Pre-Treatment), during (Treatment), and after (Post-Treatment) infusion of vehicle or analogs into enzymatically dispersed rat hypothalamus. The characteristics of pulsatile GnRH release assessed were the same reported as those in Figure 3. $N = 6$ for vehicle and AG- and ANT#1-treated cultures of enzymatically dispersed cells.

(35–37). GnRH has been implicated in various regulatory effects on GnRH receptors in the pituitary (38) as well as effects on gene transcription (39, 40) and c-*fos* production (41) in GT1-7 cells. GnRH has also been shown to increase electrical activity in the median eminence (42). We think that detailed assessment of the regulation of parameters of pulsatile GnRH release may prove critical to the eventual understanding of how GnRH neurons coordinate exocytosis to elicit pulses.

Our present study supports the model that GnRH is regulated by a negative ultrashort-loop feedback mechanism, the GnRH autoregulation hypothesis. Although this hypothesis is not supported by every study that investigated GnRH effects on GnRH secretion (20, 34), it is consistent with the general findings of most of these studies (25–31). Agonist treatment of both intact hemihypothalami and enzymatically dispersed cells in culture resulted in suppression of GnRH, whereas antagonist treatment resulted in disruption of the negative feedback loop. The present study does represent the first detailed analysis of the effects of both GnRH agonist and antagonists on the parameters of pulsatile GnRH release in the rat, providing one more step toward the development of an understanding of how GnRH pulses are initiated and regulated.

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