Endocrine, *vol. 9, no. 3, 233–241, December 1998* 0969–711X/98/9:233–241/$10.25 © 1998 by Humana Press Inc. All rights of any nature whatsoever reserved.

Expression of Activin and Follistatin in the Rat Hypothalamus

Anatomical Association with Gonadotropin-Releasing Hormone Neurons and Possible Role of Central Activin in the Regulation of Luteinizing Hormone Release

Leigh A. MacConell,[1] Amy E. Widger,[2] Sara Barth-Hall,[2] and Veronica J. Roberts[2]

[1]*Peptide Biology Laboratory, The Salk Institute, La Jolla, CA; and*
[2]*Department of Reproductive Medicine, University of California, San Diego, La Jolla, CA*

The central role of activin in the regulation of the reproductive axis remains largely unexplored. Evidence suggests that activin may play a role in controlling gonadotropin-releasing hormone (GnRH) release. We assessed potential neuroanatomical associations between activin- and GnRH-neuronal systems via examination of the distribution of activin βA-subunit and activin binding protein (follistatin) protein and mRNA signals relative to GnRH neurons in the adult rat brain. Activin βA-subunit-immunostained fibers were distributed throughout the hypothalamus and GnRH-positive perikarya, and fibers were in close association with βA-subunit-immunoreactive fibers. Follistatin mRNA-expressing cells were also identified throughout the hypothalamus with GnRH fibers often observed juxtaposed to follistatin cell bodies. Colocalization of either the βA-subunit or follistatin within GnRH neurons was not detected. The functional significance of central activin in the regulation of the reproductive axis was also demonstrated. The intracerebroventricular infusion of rh-activin A significantly increased luteinizing hormone, but not follicule-stimulating hormone, serum levels in adult male rats. Taken together, the present results support an interaction between activin and GnRH neuronal systems in the rat hypothalamus, and suggest activin may act within the brain to regulate the reproductive axis.

Key Words: Activin; follistatin; gonadotropin-releasing hormone.

Introduction

The classical regulator of normal reproductive function is the hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH). GnRH is released in a pulsatile manner from approx 1300–1500 neurosecretory cells of the medial basal hypothalamus into the hypothalamic portal vasculature *(1)*. Through the portal system, GnRH travels to the anterior pituitary where it selectively stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the gonadotropes, thus playing a pivotal role in the regulation of the reproductive axis.

Activin was originally isolated from the gonads, its most abundant source, and named for its selective stimulatory effect on FSH secretion from the anterior pituitary *(2)*. Activin is a dimeric glycoprotein composed of two distinct, disulfide-linked, 14-kDa β-subunit polypeptide chains (βA and βB), belonging to the transforming growth factor β (TGF-β) superfamily of growth and differentiation factors *(3)*. Three activin isoforms, the βA/βA homodimer (activin A), the βB/βB homodimer (activin-B), and the βA/βB heterodimer (activin AB) have been purified, characterized, and shown to exert a broad range of biological effects *(4–7)*. In addition to autocrine/paracrine control of FSH synthesis and release from the anterior pituitary, activin is an autocrine/paracrine mediator within the gonads in the control of steroid production *(8)*, follicular maturation *(9,10)*, and spermatogenesis *(11,12)*. Although the peripheral actions of activin on the hypothalamic–pituitary–gonadal (HPG) axis are the focus of numerous studies, its central effects in the regulation of reproductive functions remain largely unexplored.

Evidence suggests that activin may also regulate gonadotropin secretion indirectly at the level of the brain through modulation of the GnRH system. Substantial numbers of activin βA- and βB-subunit immunoreactive (ir) fibers *(13,14)*, as well as activin receptor type II and IIB (ActRII and IIB) mRNAs *(15)* are found in the medial septal (MS)/medial preoptic (MPOA) regions of the rat brain where GnRH neurons are relatively abundant. Further, mRNA encoding the activin binding protein, follistatin (FS), is expressed in areas of the rat brain associated with GnRH and activin β-subunit-producing neurons *(16)*.

Results from in vivo and in vitro studies also support a role of activin in the regulation of GnRH. Lee and Rivier *(17)* have shown that repeated sc injections of activin A

Received June 9, 1998; Revised September 1, 1998; Accepted September 1, 1998.

Author to whom all correspondence and reprint requests should be addressed: Dr. L. A. MacConell, The Salk Institute, 10010 N. Torry Pines Rd., La Jolla, CA 92037, E-mail: macconell@salk.edu

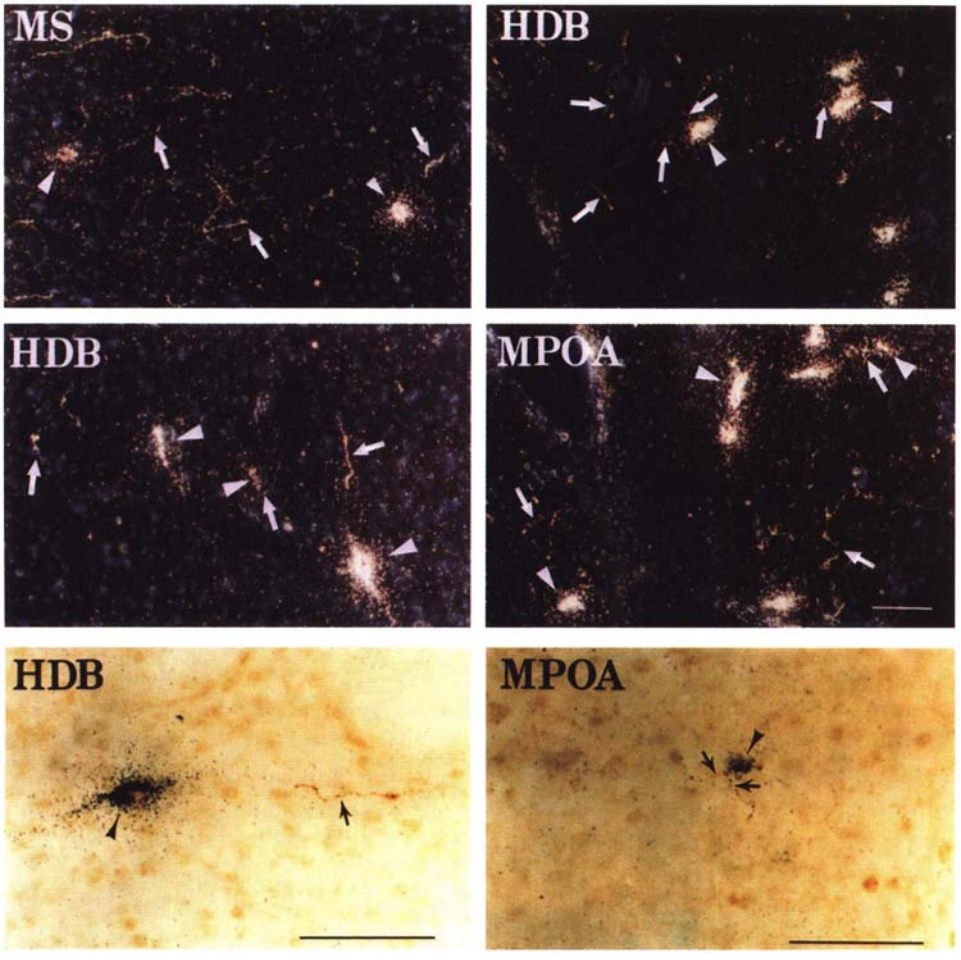

Fig. 1. Anatomical association of activin βA-subunit-ir peptide with GnRH mRNA signal in the adult male rat brain. High-magnification, dark-field photomicrographs (upper four panels) of emulsion-dipped slides show activin βA-subunit-ir fibers (arrows) and GnRH mRNA-positive perikarya (arrowheads). Anatomical overlap between the βA-stained fibers and GnRH mRNA-positive cell bodies was observed in the medial septum (MS), horizontal limb of the diagonal band (HDB), and medial preoptic area (MPOA). Note the typical appearance of activin βA-ir fibers nearby or adjacent to individual GnRH mRNA-positive cell bodies. The bottom two panels show bright-field examples of the appearance of activin βA-subunit-ir fibers (brown staining; arrows) near or adjacent to GnRH mRNA-positive perikarya (black grains; arrowheads). Bars, 100 μm.

increase GnRH mRNA levels in the MPOA region of the adult male rat brain. In addition, they showed the intracerebroventricular (icv) injection of activin A stimulates FSH secretion in the rat. Chronic icv infusion of activin A significantly elevated plasma FSH levels 2 and 3 d after the start of activin administration. In in vitro studies, González-Manchón et al. *(18)* reported that the addition of activin A to an immortalized mouse GnRH cell line (GT1-7) induces a concentration-dependent increase in GnRH secretion, and Calogero et al. *(19)* have shown that activin A stimulates GnRH release from hypothalamic explants in vitro.

Therefore, accumulating evidence suggests that activin may serve to modulate the reproductive axis at the level of GnRH neurons in the mammalian brain. The present study specifically explores the putative role of central activin in regulation of the HPG axis. Using combination histochemistry to assess potential neuroanatomical associations between central activin- and GnRH-neuronal systems in the same sections, we have addressed whether activin and/or FS is colocalized with or adjacent to GnRH neurons to clarify the potential for communication between activin and GnRH neurons. Further, by administering activin A directly into the rat brain and examining its effects on LH and FSH serum levels, we have addressed the functional importance of central activin in the modulation of the HPG axis.

Results

Anatomical Association Between GnRH mRNA-Expressing Neurons and Activin βA-ir Fibers

Activin βA-immunopositive cell bodies are localized in the brainstem, in the nucleus of the solitary tract [(NTS) *(13,14)*]. Activin βA-containing projections from this area are found in the hypothalamus where GnRH neurons are localized. To examine the anatomical relationship between activin βA-containing fibers and GnRH perikarya, immunostaining for the activin βA peptide was employed in

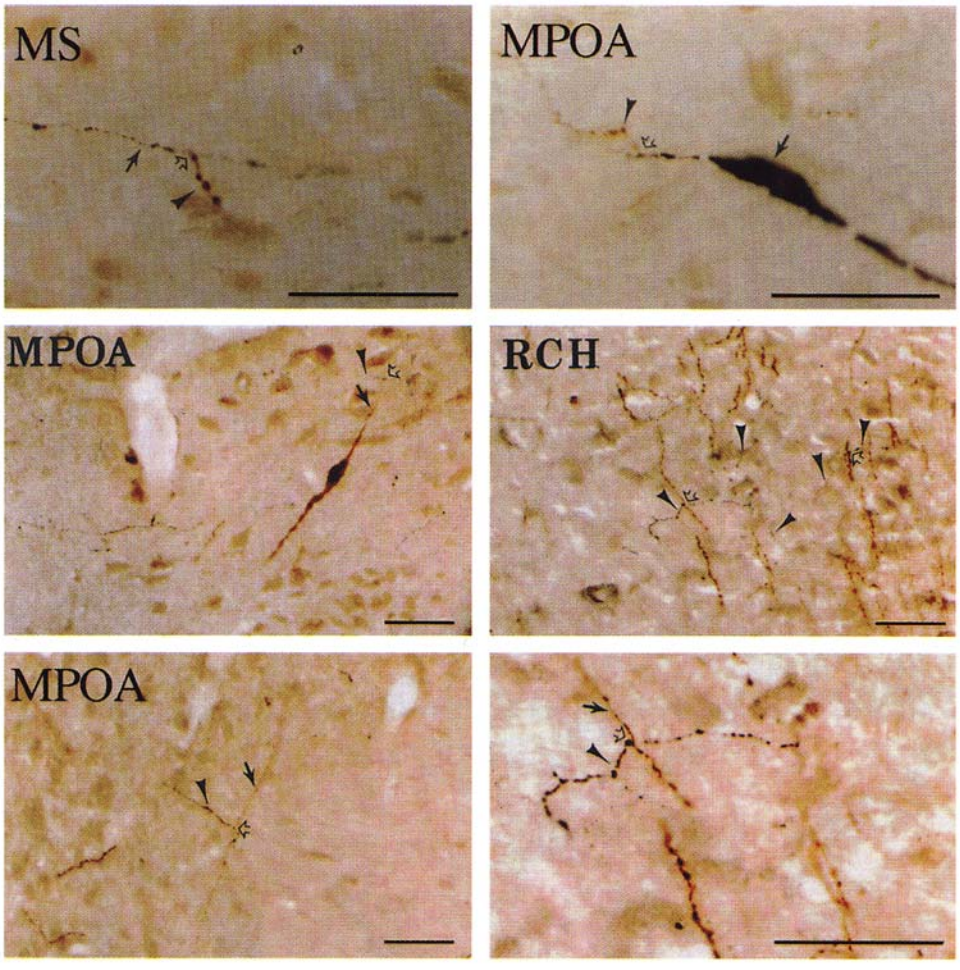

Fig. 2. Anatomical association of activin βA-subunit-ir fibers with GnRH-ir somata and fibers in the adult male rat brain. High-magnification, bright-field photomicrographs show activin βA-subunit-ir fibers (top two panels: brown staining, arrowheads; bottom four panels: blue-black staining, arrowheads) and GnRH-ir perikarya and fibers (top two panels: blue-black staining, arrows; bottom four panels: brown staining, arrows) in the medial septum (MS), medial preoptic area (MPOA), and retrochiasmatic nucleus (RCH). Typical examples of the anatomical overlap between activin βA- and GnRH-ir fibers is indicated by the open arrowheads. The bottom right panel shows an area in the middle right panel at a higher magnification. Bars, 50 μm.

combination with *in situ* hybridization for GnRH mRNA. Light microscopic analysis focused on specific brain areas well established to contain GnRH perikarya: MS, horizontal limb of the diagonal band (HDB), and MPOA *(20,21)*. As shown in Fig. 1, distinct areas of overlapping distribution between activin βA-subunit-ir fibers and GnRH mRNA-expressing neurons were readily observed in the MS, HDB, and MPOA. Close apposition between the two factors was evident with activin-ir fibers juxtaposed to or overlapping GnRH mRNA-expressing neuronal perikarya (Fig. 1). Colocalization of the activin βA-subunit peptide and GnRH mRNA in the same cell was not observed in any section examined.

Anatomical Association Between GnRH-ir and Activin βA-Subunit-ir Cells and Fibers

Double-label immunostaining was employed to examine potential GnRH/activin fiber–fiber neuronal interactions. Again, light microscopic analysis focused on hypothalamic areas where GnRH-ir neurons are found. Activin βA-subunit-ir projections were most prevalent in hypothalamic regions where GnRH-ir fibers and perikarya are abundant. As found using combination immunohistochemistry/*in situ* hybridization, close encounters between activin βA-subunit-ir fibers and GnRH-ir cell bodies were readily observed. On multiple occasions, GnRH-ir somata were surrounded by activin βA-stained fibers with short segments of activin-stained fibers overlapping or juxtapose to GnRH-ir cell bodies (Fig. 2). Activin βA-ir fibers, traversing MS, HDB, MPOA, and retrochiasmatic (RCH) regions of the hypothalamus, were also closely associated with GnRH-immunostained fibers, with activin βA-ir neuronal projections frequently crossing over GnRH positive fibers (Fig. 2).

Anatomical Association Between GnRH-ir Cells and Fibers and FS mRNA-Expressing Cells

We have previously identified FS mRNA expression within the rat hypothalamus, in an expression pattern spatially coinciding with that of GnRH. *In situ* hybridization of FS mRNA in the rat hypothalamus in combination with

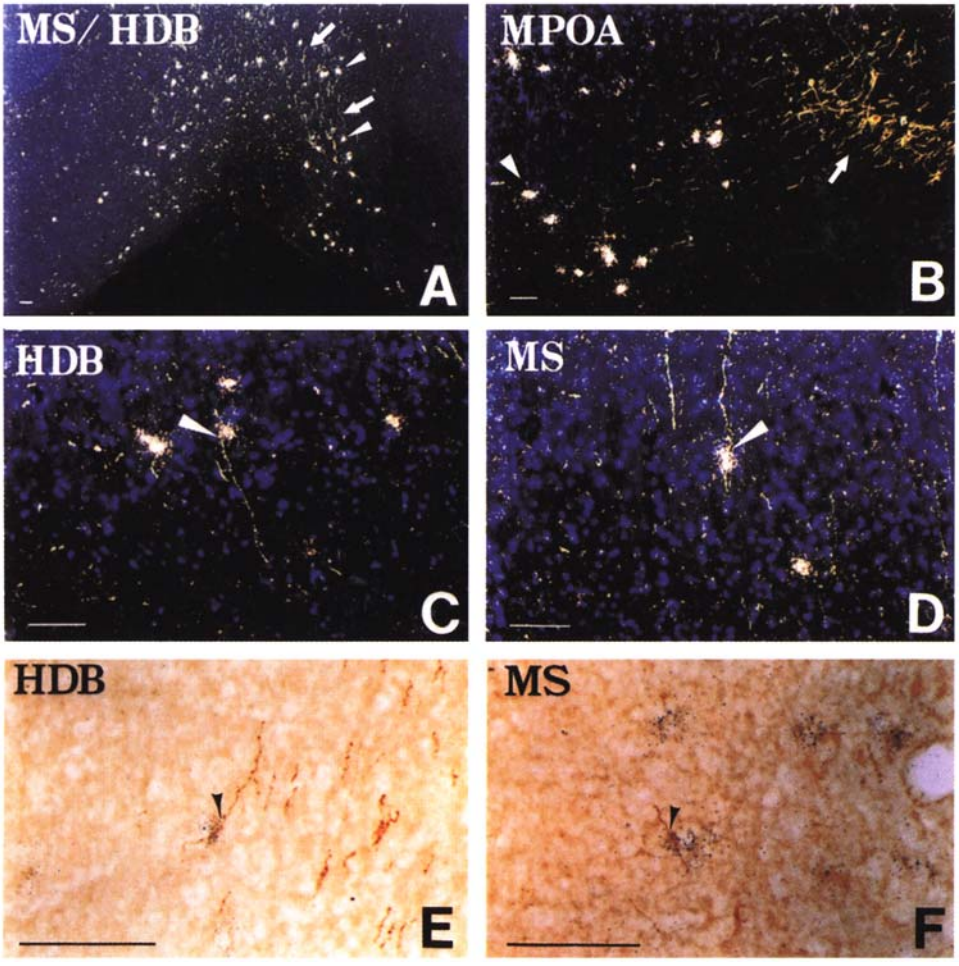

Fig. 3. Anatomical association of GnRH-ir peptide with follistatin mRNA signal in the adult male rat brain. High-magnification, dark-field and bright-field photomicrographs of emulsion-dipped slides demonstrate a close anatomical relationship between follistatin (FS) mRNA-expressing cell bodies and GnRH-ir fibers. Panels **A** and **B** show the typical dark-field appearance of numerous GnRH-ir fibers (arrows) coursing among FS mRNA-expressing perikarya (arrowheads) in the medial septum (MS), horizontal limb of the diagonal band (HDB), and medial preoptic area (MPOA). Dark-field microscopy is necessary to visualize immunoreactive fibers and mRNA signals at the magnifications used in panels A and B. Panels **C** and **D** show representative dark-field examples of GnRH-ir fibers overlapping FS cell bodies (arrowheads indicate areas of overlap), which is commonly seen in the MS and HDB. The higher-power magnifications used in panels **E** and **F** show the typical bright-field appearance of GnRH-ir fibers (brown staining) and FS mRNA-expressing cell bodies (black grains) with areas of overlap indicated (arrowheads). Bars, 100 μm.

GnRH immunostaining of neuronal cell bodies and fibers facilitated determination of whether FS is colocalized with or in close proximity to GnRH neurons.

Again, light microscopic analysis focused on specific brain areas well established to contain GnRH somata. Although colocalization of the GnRH peptide and FS mRNA was not found, abundant anatomical overlap of GnRH-ir cell bodies and fibers and FS mRNA-expressing cells was observed in the MS, HDB, and MPOA. Figure 3 shows several cell bodies expressing high levels of FS mRNA closely surrounded by GnRH-ir cell bodies and fibers in the HDB. In the MS and HDB regions, numerous GnRH-ir fibers coursed among FS positive perikarya, and on several occasions, *en passant* associations were observed between GnRH-ir fibers and FS cell bodies (Fig. 3). Although the close anatomical relationship between GnRH and FS was maintained in the MPOA, GnRH-ir somata and fibers were more often found to be lateral to the FS mRNA perikarya with less *en passant* relationships between the two factors in this region (Fig. 3).

Effect of icv Microinfusion of Activin A on Serum LH and FSH Levels

To explore the potential role of central activin in modulation of the HPG axis, we examined whether the bilateral icv microinfusion of rh-activin A affects systemic LH and FSH levels. Blood samples were taken at –10 and 0 min before the initiation of infusion, and the mean of these two values was used as the preinfusion value (T = 0 min). Animals received 730 ng/side rh-activin A, and subsequent blood sampling was conducted every 10 min for up to 60 min. As shown in Fig. 4A, serum LH values began to

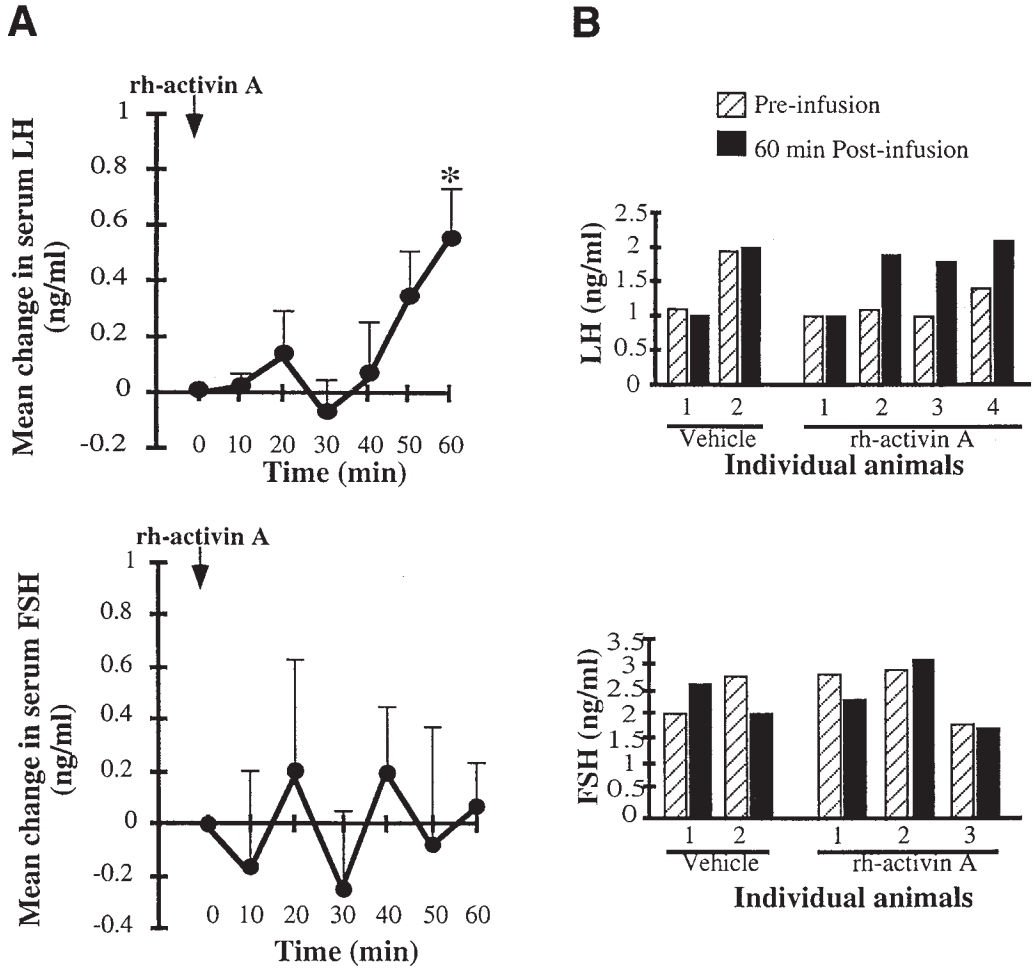

Fig. 4. Effect of centrally administered rh-activin A on LH and FSH serum levels in normal adult male rats. **(A)** To determine whether central activin influences the release of LH and/or FSH, animals received bilateral intracerebroventricular (icv) microinfusions of rh-activin A (730 ng/side). For each animal, the mean of the LH or FSH serum values obtained at 10 and 0 min prior to initiating icv microinjections was used as the preinfusion value for that animal ($T = 0$ min). Postinfusion LH and FSH serum levels, at each time-point, were compared to this preinfusion value, using the paired *t*-test to assess changes within subjects. Each point represents the mean ± SEM. $*p < 0.05$ vs T = 0 min; $n = 3$–4. **(B)** The data obtained from individual animals are shown, illustrating the change in LH and FSH serum levels within each animal before ($T = 0$ min) and after ($T = 60$ min) bilateral intracerebroventricular microinfusion of rh-activin A. Vehicle-treated animals were used to control for general surgical effects.

rise 50 min postinfusion (T = 50 min) relative to T = 0 min. A significant 51% increase in serum LH levels was observed at 60 min postinfusion ($T = 60$ min) relative to the preinfusion value ($n = 4$; $*p < 0.05$). There were no significant changes in serum FSH levels detected in response to rh-activin A, at any time-point examined, relative to preinfusion values ($n = 3$; Fig. 4). Figure 4B demonstrates LH and FSH serum levels at $T = 60$ min relative to $T = 0$ within individual subjects (vehicle-treated animals were also used to control for general surgical and infusion effects; $n = 2$).

Discussion

Previous studies support a role of activin in the control of GnRH synthesis and release. Calogero et al. have reported that activin treatment of hemihypothalami, explanted from male rats, stimulates GnRH release in a biphasic manner *(19)*. Using the immortalized GnRH cell line (GT1-7), we have recently reported that GnRH protein and mRNA levels are upregulated on treatment with activin A *(22)*. In addition, we have demonstrated the expression of ActR (types I, IB, and II) mRNAs by this GnRH cell line, which further supports that activin contributes to the regulation of the GnRH neurons *(22)*. Previous anatomical studies have demonstrated that activin βA-subunit-ir fibers traverse the MS, HDB, and MPOA regions of the hypothalamus where GnRH neurons are found *(13,14)*, and that patterns of FS mRNA expression in the MS and HDB mirror that of the GnRH neuronal distribution *(16)*. Although ActR mRNA expression has also been localized in the MPOA where GnRH neurons are found, expression of the ActR mRNAs by the GnRH neurons themselves has not been

detected in vivo *(15,23)*. It is possible that levels of ActR mRNA expression by scattered, individual GnRH cells in the brain are below histochemical detection limits or are masked in the presence of the myriad of central and/or gonadal feedback molecules found in vivo.

In the present study, we utilized combination histochemical techniques to determine whether the activin βA-subunit or FS is expressed in or adjacent to GnRH neurons to elucidate the potential mode of interaction between activin and GnRH neurons. Since *in situ* hybridization provides high sensitivity and resolution in the identification of relevant cell bodies, this technique was employed in combination with immunohistochemistry to answer specific questions regarding apposition with perikarya and colocalization. Significant anatomical overlap between activin and GnRH cell systems was observed. Neuronal fibers expressing the activin βA-subunit were found proximal to GnRH-mRNA-positive somata. This finding was confirmed using double-label immunohistochemistry, which revealed potential points of contact between activin-ir fibers and GnRH-ir soma and fibers. In addition, FS and GnRH cell systems were closely associated, since GnRH-ir fibers were frequently observed to crossover FS mRNA-expressing cell bodies directly. Interestingly, no GnRH mRNA-expressing neuron was double-labeled for the activin βA-subunit peptide in any section examined. Double-label immunohistochemistry confirmed this finding and failed to detect colocalization of the two peptides. Similarly, FS mRNA expression was not detected in a single GnRH-ir cell. Although synaptic contact cannot be determined utilizing the present histological methods, it seems reasonable to speculate that the close intercellular relationship between activin-containing fibers and GnRH-ir cell bodies and fibers represents the potential for communication between these factors, since activin is a secreted peptide hormone.

Although it has not been determined whether GnRH receptors are expressed in FS-positive cell bodies, the ability of GnRH to stimulate FS has been shown both in vitro and in vivo. Continuous GnRH treatment of proestrous and metestrous pituitaries in a perifusion system significantly elevated FS mRNA levels *(24)*. In another study, fast frequency GnRH pulses applied to adult male rats were associated with an increase in pituitary FS mRNA levels *(25)*. It may be reasoned that this relationship also exists in the brain, and hypothalamic GnRH-containing fibers may act on FS-expressing perikarya to regulate FS synthesis and release as a local feedback mechanism.

We have also explored the functional significance of central activin in the modulation of HPG axis function. The icv microinfusion of rh-activin A in adult male rats elicited a significant increase in serum LH levels 60 min following initiation of infusion relative to preinfusion levels. There was no detectable change in serum FSH levels in response to rh-activin A. Lee and Rivier have shown that chronic icv administration of activin A elicited a significant increase in plasma FSH levels on days 2 and 3 with no effect on LH secretion *(17)*. Since LH levels were measured 5 and 8 h postinfusion on these days, a more immediate LH response may have been missed as we recorded an elevation in serum LH by 60 min postinfusion. It is also possible that chronic icv administration may elicit a delayed pituitary effect on FSH release not observed in our acute study. It may be speculated that the activin-induced increase in circulating LH levels, observed in our study, may be exerted at the level of the pituitary. However, a pituitary effect is unlikely, since one would expect a concordant rise in serum FSH levels, since the best-known function of activin is as a potent and selective FSH secretogogue.

Whether central activin stimulates GnRH release in vivo has not been established in the present study. It is possible that the rise in LH levels following centrally administered activin A is mediated by a facilitation of hypothalamic GnRH release. As stated previously, activin A has been shown to stimulate GnRH production and release from hypothalamic explants and a GnRH cell line *(18,19)*. If centrally administered activin stimulates LH secretion via activation of GnRH release, it is not clear why there was not a concordant increase in FSH serum levels. Studies have shown that varying GnRH pulse frequency or amplitude differentially regulates the biosynthesis *(26)* and release *(27)* of the gonadotropins. Higher frequencies of GnRH pulsatility increase LHβ mRNA expression as well as LH secretion. Lower pulse frequencies favor FSHβ mRNA expression and FSH secretion. Thus, its possible that our mode of activin delivery produced a GnRH pulse with a contour that favored LH, but not FSH release.

In summary, the present study encompasses anatomical and functional approaches toward better understanding the site of action and modulation of activin as a central regulator of the HPG axis. Combination histological techniques demonstrate that although neither activin-βA nor FS expression is colocalized within GnRH neurons, the activin and GnRH cell systems overlap extensively in the rat hypothalamus. In addition, the present physiological data suggest that activin may regulate pituitary LH secretion via an action within the brain.

Materials and Methods

Anatomical Studies

Animals and Tissue Preparation

Male Sprague-Dawley rats (9–10 wk old) were purchased from Harlan Sprague-Dawley (San Diego, CA) and kept under standard housing, feeding, and lighting conditions (23°C, 12 h light, 12 h dark cycle with lights on at 0600 h). Rats were deeply anesthetized with 35% chloral hydrate (1.0 mL/100 g body wt, ip) and perfused transcardially with 100 mL ice-cold saline followed by 700 mL ice-cold 4% paraformaldehyde in borate buffer (38.14 g sodium tetraborate/L dH_2O, pH 9.5). Brains were removed

and postfixed for 1 h in the same fixative, then stored at 4°C in a 10% sucrose/0.02 *M* potassium phosphate-buffered saline solution (KPBS: 0.45 g KH_2PO_4, 3.8 g $K_2HPO_4H_2O$, 9 g NaCl/L dH_2O, pH 7.4) 12 h before frozen coronal sections were cut on a sliding microtome. Areas of the brain within which GnRH neurons and fibers are found were blocked and every 30-μm coronal section from the median eminence to the most rostral areas localizing GnRH neurons was saved. Free-floating sections were processed for immunohistochemical analysis followed by slide mounting onto Superfrost/Plus slides (Fisher Scientific, Tustin, CA). For dual localization of proteins and mRNAs, slide-mounted sections were processed for the *in situ* hybridization of mRNAs of interest. All procedures involving the use of animals were performed in accordance with federal, state, and local laws and institutional and NIH regulations.

Primary Antisera

Antibodies raised in rabbit were generated against the activin βA-subunit by coupling a synthetic anticyclized acetyl porcine activin βA (81-113)-NH_2 fragment to human α-globulins via *bis*-diazotized benzidine. The affinity-purified polyclonal antiserum recognizes porcine, bovine, human, ovine, rat, and rhesus monkey activin βA-subunit. Preparation *(28)* and characterization *(13,14,29)* of the activin βA-subunit antiserum has been described.

GnRH immunostaining was performed using polyclonal antibodies raised in rabbit and generated against [D-Lys ^{6}]GnRH conjugated to ovalbumin and glutaraldehyde. The LR_1 antiserum recognizes residues 3–5 and 7–10 of the native decapeptide, but not the GnRH precursor. Its purification and characterization have been previously described *(30,31)*.

Generation of RNA Probes

Antisense ^{33}P-labeled RNA probes specific for rat GnRH and FS were generated as previously described *(16)*. The rat FS probe consists of a 630-bp *Hin*dIII fragment that corresponds to amino acids 114–315 of the mature 315 amino acid rat FS protein. The specificity of the FS riboprobe has been ascertained by Northern analysis, and details of the cloning and specificity are described *(32)*. The antisense GnRH riboprobe is a 330-bp *Hin*dIII fragment corresponding to rat hypothalamic GnRH, the cloning and specificity of which are described *(33)*.

Double-Label Immunohistochemistry

We employed double-label immunostaining to visualize the putative anatomical relationship between GnRH- and activin βA-containing fibers in the same section. Free-floating sections were incubated for 10 min in 0.3% hydrogen peroxide to quench endogenous peroxidase activity, rinsed with KPBS, and treated with 1% sodium borohydride for 8 min to reduce free aldehydes. Sections were incubated for 1 h at room temperature, followed by 36 h at 4°C, with βA-antiserum diluted at 1:1000 in KPBS plus 0.3% Triton-X (Sigma, St. Louis, MO) and 2% normal goat serum (Sigma). Sections were then rinsed in KPBS followed by a 1-h incubation with biotinylated goat antirabbit secondary (Vector Labs, Burlingame, CA) at room temperature. KPBS-washed tissue was incubated in an avidin–biotin–horseradish peroxidase (HP) complex (Vector Labs, Burlingame, CA) for 1 h at room temperature. Secondary antiserum and avidin–biotin–HP incubations were repeated for 30 min each. The peroxidase reaction was visualized as either a blue-black reaction product with a 3–5 min incubation in a mixture of 3,3' diaminobenzidine tetrahydrochloride and nickel (DAB-Ni [0.03% DAB/dH_2O plus 4.0 mg $C_6H_{12}O_6$, 0.8 mg NH_4Cl, and 0.06 mg glucose oxidase {all from Sigma Chemical Co.}/mL nickel ammonium sulfate solution [NAS: 50 g nickel ammonium sulfate/L 0.1 *M* sodium acetate buffer, pH 6.0; 13.6 g sodium acetate/L dH_2O]) or as a brown reaction product with a 3- to 5-min incubation in a mixture of 0.03% DAB and 0.015% H_2O_2 in 0.1 *M* Tris-HCl, pH 7.4.

KPBS-washed sections were then processed for GnRH immunostaining. Incubation with LR_1 antiserum diluted at 1:10,000 in KPBS plus 0.3% Triton-X and 2% normal goat serum was carried out for 1 h at room temperature, followed by 16 h at 4°C. As described above, the avidin–biotin–HP method was used for localization of GnRH peptide. The peroxidase reaction was visualized as either a blue-black or brown reaction product as described above.

Combination Immunohistochemistry/In Situ Hybridization

Immunohistochemistry. Immunohistochemical localization was used in combination with *in situ* hybridization to examine the distribution of either the GnRH peptide and FS mRNA or the activin βA-subunit peptide and GnRH mRNA in the same section. First, peptides were detected using standard immunohistochemical techniques as described above with modifications to obtain RNase-free conditions. BSA (which does not contain RNase; Sigma Chemical Co.) was substituted for normal goat serum; heparin (antiprotease and RNase inhibitor; 2.5 mg/mL; Sigma Chemical Co.) was included in the primary and secondary incubations; and reagents were made with diethyl pyrocarbonate-treated water (DEP C: 1 mL/L dH_2O; Sigma Chemical Co).

Free-floating sections were incubated for 1 h at room temperature, followed by 36 h at 4°C, with βA-antiserum diluted at 1:1000 or 16 h at 4°C with LR_1 antiserum diluted at 1:10,000. Primary antisera incubations were carried out in KPBS plus 0.3% Triton-X, 2% BSA, and 2.5 mg/mL heparin. For GnRH, the brown reaction product was developed during a 3- to 5-min incubation in the DAB mixture, and for the activin βA-subunit, a blue-black reaction product for optimal visualization of fibers was obtained using the DAB-Ni mixture, both as described above. Sections were mounted onto Superfrost/Plus slides, and tissue was viewed under a light microscope to assure that immunopositive staining was obtained prior to proceeding. Sec-

tions were then processed for the detection of either GnRH or FS mRNAs.

***In Situ* Hybridization.** GnRH or FS mRNAs were localized using standard *in situ* hybridization protocols *(16)*. Briefly, slide-mounted sections were incubated at room temperature for 30 min in neutral buffered 10% formalin and rinsed in KPBS. For maximum probe penetration, sections were digested for an additional 30 min in proteinase K (0.001%) in digestion buffer (0.1 *M* Tris, 0.05 *M* EDTA, pH 8.0) at 37°C. Dehydrated (ascending ethanol concentrations) and vacuum-dried sections were hybridized for 12–16 h at 60°C to the antisense ^{33}P-labeled RNA probe specific for rat FS or GnRH. After hybridization, nonspecific binding was reduced with ribonuclease-A treatment (RNase; 10 mg/mL at 37°C for 30 min) and a low-salt/high-temperature wash (15 mmol/L NaCl–1.5 mmol/L sodium citrate with 1 mmol/L dithiotheitol at 65°C for 30 min). Dehydrated sections were exposed to β-max Hyperfilm (Amersham, Arlington Heights, IL) for 3–7 d. After film exposure, slides were defatted, dipped in nuclear track emulsion (NTB-2, Kodak, Eastman-Kodak, Rochester, NY), and exposed at 4°C for 3–6 wk. After developing (Kodak D19) and fixation (Kodak, Ektaflo), slides were stained with *bis*-benzimide (Sigma Chemical Co.), which provides a fluorescent blue ribosomal counterstain, and cover slips were mounted with DPX mountant (Gallard Schlesinger, Long Island, NY).

Intracerebroventricular Microinfusion Study

Animals and Surgery

Subjects were normal adult male rats (9–10 wk old) obtained from Harlan Sprague-Dawley and kept under standard housing, feeding, and lighting conditions (23°C, 12 h light/12 h dark cycle, lights on at 0600 h).

Animals were stereotaxically fitted with 26-gage (ga) bilateral cannulae (Plastics One, Roanoke, VA) as previously described *(34)*. Briefly, rats were deeply anesthetized with an ip injection of ketamine hydrochloride and xylazine (ketamine hydrochloride: 75 mg/kg body weight; xylazine: 10 mg/kg body weight). An additional 250 μg of atropine sulfate (in a volume of 0.2 mL) was administered intramuscularly to alleviate potential respiratory congestion.

The following stereotaxic coordinates were used: the tooth bar was set at 3.0 mm below the interaural line, and bilateral cannulae were aimed at the lateral ventricles (LV): 0.7 mm posterior to bregma, 1.5 mm lateral to midline, 3.6 mm ventral to the skull surface. Coordinates were placed using the atlas of Paxinos and Watson *(35)*.

After a 1-wk recovery period, rats were anesthetized with the ketamine/xylazine cocktail and were surgically fitted with an indwelling silastic catheter in the right external jugular vein and advanced to the right atrium for blood collection as described *(36)*. Sterilized cannulae were filled with sterile heparinized saline (25 IU/mL) for insertion. Atrial cannulae were exteriorized at the nape of the neck and kept patent by occasional slow infusion of heparinized saline. Animals remained anesthetized for the remainder of the experiment and were allowed to stabilize for 1 h before initiating blood sampling. Animal care and procedures followed institution, local, state, federal, and NIH guidelines.

Intracerebral Microinjections and Systemic Blood Sampling

Recombinant human activin A (rh-activin A) was prepared as described *(37)*, and stored, as well as injected, in a neutral buffer (0.15 *M* NaCl/0.05 *M* Tris-HCl, pH 7.4). Animals were given bilateral microinjections (1.0 μL/side) of either rh-activin A (730 ng/side) or the neutral buffered vehicle (0.15 *M* NaCl/0.05 *M* Tris, pH 7.5) as described *(38)*. Briefly, infusions were applied via 100 μL Hamilton syringes connected by polyethylene 20 tubing to 33-ga internal cannulae, which, when inserted into the animals, extended 0.5 mm past the end of the implanted guide cannulae. Infusions were automated by Razel pumps, which delivered the solutions at a rate of 66.7 nL/min over a time period of 15 min. Injection cannulae were kept in place for an additional 2 min to ensure diffusion of the infusate away from the tip of the cannulae.

Atrial blood samples (0.5 mL) were collected 10 and 0 min prior to initiation of the microinjections, and every consecutive 10 min after the start of infusion for up to 60 min. An equal volume of heparinized saline was replaced after each blood sampling. Blood samples were allowed to remain at room temperature for approx 30 min, centrifuged, and separated serum stored at –20°C until radioimmunoassay (RIA) analysis of FSH and LH concentrations.

On completion of the experiment (1 h following initiation of the microinjection), animals were killed with an overdose of ketamine/xylazene and perfused though the heart with ice-cold saline followed by 4% paraformaldehyde in borate buffer as described above. The brains were removed and stored in 10% formalin until histological analysis of guide cannulae placement.

Results were analyzed using the paired *t*-test to compare changes in LH and FSH serum levels within activin-treated animals before and after infusion (n = 3–4). For each animal, serum samples obtained at 10 and 0 min prior to initiating microinjections were averaged, and this mean was used as the preinfusion value for that animal. Postinfusion LH and FSH serum levels, at each time-point, were compared to the preinfusion value. Vehicle-treated animals were used to control for general surgical effects (n = 2).

Radioimmunoassays (RIAs)

Serum LH and FSH were measured in duplicate by previously characterized RIAs *(39,40)* using the LH standard, NIDDK-rLH-RP3, and antibody anti-rLH-S-11 (1:150,000 dilution), and the FSH standard, NIDDK-rFSH-RP-2, and

antibody anti-rFSH-S-11 (1:25,000 dilution). All samples were measured in the same assay.

Acknowledgments

We thank the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases at NIH (Rockville, MD) for providing the rh-activin A as well as the LH and FSH standards and antibodies used for RIAs. We also thank Wylie Vale (Salk Institute, San Diego, CA) for providing the activin-βA antiserum and Robert Benoit (Montreal General Hospital, Montreal, Canada) for the LR_1 antiserum (bleed 20-6-79). John P. Adelman (Oregon Health Sciences University, Portland, OR) generously provided the rat GnRH cDNA, and Nicholas Ling (Neurocrine Biosciences, Inc., La Jolla, CA) the rat FS cDNA. We are appreciative of Jeff Wong for conducting the RIAs in the laboratory of Samuel Yen (University of California, San Diego, CA). We thank Paul Sawchenko for critical reading of the manuscript and Cornelia Bentley for her helpful advice. This work was supported by a grant from the National Science Foundation (IBN-9604071) to V. J. R.

References

1. Wray, S., Grant, P., and Gainer, H. (1989). *Proc. Natl. Acad. Sci. USA* **86,** 8132–8136.
2. Ying, S., Becker, A., Baird, A., Ling, N., Ueno, N., Esch, F., et al. (1986). *Biochem. Biophys. Res. Commun.* **135,** 950–956.
3. Mason, A. J., Hayflick, J. S., Ling, N., Esch, F., Ueno, N., Ying, S., et al. (1985). *Nature* **318,** 659–663.
4. Ling, N., Ying, S. A., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., et al. (1986). *Nature* **321,** 779–782.
5. Mason, A. J., Berkemeier, L. M., Schmeizer, C. H., and Schwall, R. H. (1989). *Mol. Endocrinol.* **3,** 1352–1358.
6. Nakamura, T., Asashima, M., Yuzuru, E., Takio, K., Uchiyama, H., Moriya, N., et al. (1992). *J. Biol. Chem.* **267,** 16,385–16,389.
7. Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., et al. (1986). *Nature* **321,** 776–779.
8. Hsueh, A. J. W., Dahl, K. D., Vaughan, J., Tucker, E., Rivier, J., Bardin, C. W., et al. (1987). *Proc. Natl. Acad. Sci. USA* **84,** 5082–5086.
9. Woodruff, T. K., Lyon, R. J., Hansen, S. E., Rice, G. C., and Mather, J. P. (1990). *Endocrinology* **127,** 3196–3205.
10. Findlay, J. K. (1993). *Biol. Reprod.* **48,** 15–23.
11. Mather, J. P., Attie, K. M., Woodruff, T. K., Rice, G. C., and Phillips, D. M. (1990). *Endocrinology* **127,** 3206–3214.
12. Kaipia, A., Parvinen, M., and Toppari, J. (1993). *Endocrinology* **132,** 477–479.
13. Roberts, V. J., Barth, S., Meunier, H., and Vale, W. (1996). *J. Comp. Neurol.* **364,** 473–493.
14. Sawchenko, P. E., Plotsky, P. M., Pfeiffer, S. W., Cunningham, E. T., Jr., Vaughan, J., Rivier, J., et al.(1988). *Nature* **334,** 615–617.
15. Cameron, V. A., Nishimura, E., Mathews, L. S., Lewis, K. A., Sawchenko, P. E., and Vale, W. W. (1994). *Endocrinology* **134,** 799–808.
16. MacConell, L. A., Barth, S., and Roberts, V. J. (1996). *Endocrinology* **137,** 2150–2158.
17. Lee, S. and Rivier, C. (1997). *Biol. Reprod.* **56,** 969–975.
18. González-Manchón, C., Bilezikjian, L. M., Corrigan, A. Z., Mellon, P. L., and Vale, W. (1991). *Neuroendocrinology* **54,** 373–377.
19. Calogero, A. E., Burrello, N., Ossino, A. M., Polosa, P., and D'Agata, R. (1998). *J. Endocrinol.* **156,** 269–274.
20. Samson, W. K., McCann, S. M., Chud, L., Dudley, C. A., and Moss, R. L. (1980). *Neuroendocrinology* **31,** 66–72.
21. Witkin, J. W., Paden, C. M., and Silverman, A. (1982). *Neuroendocrinology* **35,** 429–438.
22. MacConell, L. A. and Roberts, V. J. (1997). Program of the 79th Annual Meeting of the Endocrine Society, Minneapolis, MN, p. 364.
23. MacConell, L. A. and Roberts, V. J. (1994). Program of the 24th Annual Meeting of the Society for Neuroscience, Miami, FL, p. 90.
24. Silverman, R. C., Silverman, A. J., and Gibson, M. J. (1989). *Brain Res.* **501,** 260–268.
25. Kirk, S. E., Dalkin, A. C., Yasin, M., Haisenleder, D. J., and Marshall, J. C. (1994). *Endocrinology* **135,** 876–880.
26. Haisenleder, D. J., Dalkin, A. C., Ortolano, G. A., Marshall, J. C., and Shupnik, M. A. (1991). *Endocrinology* **128,** 509–517.
27. Haisenleder, D. J., Ortolano, G. A., Yasin, M., Dalkin, A. C., and Marshall, J. C. (1993). *Endocrinology* **132,** 1292–1296.
28. Vaughan, J. M., Rivier, J., Corrigan, A. Z., McClintock, R., Campen, C. A., Jolley, D., et al. (1989). *Methods Enzymol.* **168,** 588–617.
29. Meunier, H., Rivier, C., Evans, R. M., and Vale, W. (1988). *Proc. Natl. Acad. Sci. USA* **85,** 247–251.
30. Shivers, B. D., Harlan, R. E., Morrell, J. I., and Pfaff, D. W. (1983). *Nature* **304,** 345–347.
31. Witkin, J. W. and Silverman, A. (1985). *Peptides* **6,** 263–271.
32. Shimasaki, S., Koga, M., Buscaglia, M. L., Simmons, D. M., Bicsak, T. A., and Ling, N. (1989). *Mol. Endocrinol.* **3,** 651–659.
33. Bond, C. T., Hayflick, J. S., Seeburg, P. H., and Adelman, J. P. (1989). *Mol. Endocrinol.* **3,** 1257–1262.
34. Duvauchelle, C. L., Levitin, M., MacConell, L. A., Lee, L., and Ettenberg, A. (1992). *Brain Res.* **576,** 104–110.
35. Paxinos, G. and Watson, C. (1986). The rat brain in stereotaxic coordinates (2nd ed.). Academic Press, San Diego, CA.
36. Ettenberg, A., MacConell, L. A., and Geist, T. D. (1996). *Psychopharmacology* **124,** 205–210.
37. Schwall, R. H., Nikolics, K., Szonyi, E., Gorman, C., and Mason, A. J. (1988). *Mol. Endocrinol.* **2,** 1237–1242.
38. Plotsky, P. M., Kjær, A., Sutton, S. W., Sawchenko, P. E., and Vale, W. (1991). *Endocrinology* **128,** 2520–2525.
39. Yen, S. S. C., Llerena, O., Little, B., and Pearson, O. H. (1968). *J. Clin. Endocrinol.* **28,** 1763–1767.
40. Anderson, D. C., Hopper, B. R., Lasley, B. L., and Yen, S. S. C. (1976). *Steroids* **28,** 179–196.