Evidence that lamprey GnRH-III does not release FSH selectively in cattle

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Abstract

Experiments were performed to test the hypothesis that lamprey GnRH-III (IGnRH-III) selectively releases FSH. Primary cultures of bovine adenohypophyseal cells were treated with mammalian GnRH (mGnRH) and IGnRH-III (10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} M) or control media in Experiment 1. All doses of mGnRH and the two highest doses of IGnRH-III stimulated ($P < 0.001$) a non-selective release of LH and FSH. In Experiments 2–4, Latin Square designs were utilized *in vivo* to examine whether physiological and hormonal milieu regulate putative selective effects of IGnRH-III. In Experiments 2 and 3, ovariectomized cows with basal levels of estradiol only (Experiment 2) or in combination with luteal phase levels of progesterone (Experiment 3) were injected with mGnRH and IGnRH-III (0.055, 0.11, 0.165 and 1.1 µg/kg body weight (BW) and saline. All doses of mGnRH released ($P < 0.001$) LH and FSH, but only the highest dose of IGnRH-III stimulated ($P < 0.001$) a non-selective release of both LH and FSH (Experiment 3). For Experiments 4A and 4B, intact, mid-luteal phase cows were injected with mGnRH and IGnRH-III (1.1 µg/kg BW; Experiment 4A), IGnRH-III (1.1 and 4.4 µg/kg BW; Experiment 4B) and saline. As before, mGnRH released ($P < 0.001$) both LH and FSH at all doses. In contrast, IGnRH-III at the highest dose released ($P < 0.001$) LH but not FSH. These findings suggest that IGnRH-III may act as a weak competitor for the mGnRH receptor and do not support the hypothesis that it selectively releases FSH in cattle.

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Introduction

Synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are regulated by the hypothalamic peptide, gonadotropin-releasing hormone (GnRH) (Knobil 1980). However, secretion of FSH is less dependent upon GnRH control and is instead regulated largely by GnRH-independent factors that include gonadal activins, inhibins and follistatins (Clarke *et al.* 1983, Pau *et al.* 1991, Kovacs *et al.* 1993).

In addition to the roles of GnRH and gonadal hormones in controlling the secretion of FSH, the existence of a separate, highly specific FSH-releasing hormone (FSH-RH) of hypothalamic origin has been proposed. Experimental evidence to support this theory has included the identification of separate fractions of FSH-RH and LH-releasing

hormone activity in sheep hypothalamic extracts (Dhariwal *et al.* 1965), as well as selectively impaired release of FSH after lesioning of the dorsal anterior hypothalamic area (Lumpkin & McCann 1984) and posterior/mid infundibulum (Marubayashi *et al.* 1999). Moreover, GnRH-independent pulses of FSH have been detected by determining concentrations of FSH in the hypothalamic–hypophyseal portal circulation (Padmanabhan *et al.* 2003).

Biochemically, several alternative forms of GnRH have been identified in vertebrates (Dubois *et al.* 2002) and have been examined as candidates for selective FSH-RH activity. One of these, identical to chicken GnRH-II, and its receptor have been identified in mammals (Lescheid *et al.* 1997, Millar *et al.* 2001). Although not isolated in mammals to date, an alternative form of GnRH, lamprey GnRH-III (IGnRH-III) (Sower *et al.* 1993), has been

reported to selectively stimulate the release of FSH in rodents (Yu *et al.* 1997) and in cattle during the luteal phase of the estrous cycle (Dees *et al.* 2001). However, several other studies, both *in vitro* (Yu *et al.* 1997, Lovas *et al.* 1998, Montaner *et al.* 2001, Kovacs *et al.* 2002) and *in vivo* (Kovacs *et al.* 2002), have questioned the ability of IGnRH-III to selectively release FSH in rodents. Clearly, the existence of a distinct mammalian FSH-RH could have far-reaching implications in both medicine and agriculture. Therefore, the objectives of studies reported herein were to assess the ability of IGnRH-III to preferentially release FSH in an array of physiological contexts in cattle.

Materials and Methods

All experimental procedures involving animals in these studies were approved by the Institutional Agricultural Animal Care and Use Committee of The Texas A&M University System.

Synthesis of IGnRH-III

IGnRH-III was synthesized by the Protein Facility at Louisiana State University. The peptide was found to be 100% pure by HPLC and exhibited a mass spectrophotometric peak of 1259.5 (molecular mass of IGnRH-III is 1259 Da).

Experiment 1: effects of IGnRH-III in primary adenohypophyseal cell cultures

Hypophyses were collected from steers at slaughter at the Rosenthal Meat Science Center, Texas A&M University, College Station, Texas and kept on ice until tissue processing. Adenohypophyses were dissected from the neurohypophysis and adenohypophyseal cells were dispersed enzymatically as described previously (Welsh *et al.* 1986, Tanner *et al.* 1990). Dispersed cells from two steers were combined, plated in six-well plates, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum for 4 days. On day 4, cells were incubated with serum-free DMEM overnight. On the next day, cells were treated with DMEM alone (control), DMEM containing 10^{-6} , 10^{-7} , 10^{-8} or 10^{-9} M mammalian GnRH-I (mGnRH) (Bachem Inc., Torrance, CA, USA) or DMEM containing IGnRH-III at the same concentrations as mGnRH for 4 h. Each treatment was applied to six wells. At the end of the 4 h incubation, culture media were harvested, and stored at -20°C until assayed for LH and FSH. Three independent replications were performed.

Experiment 2: effects of IGnRH-III in ovariectomized cows with basal circulating concentrations of estradiol

Seven ovariectomized cows, each bearing a Silastic (Dow Corning Corporation, Midland, MI, USA) ear

implant containing crystalline estradiol 17- β (Sigma, St Louis, MO, USA), were used. This animal model has been used extensively to study the neuroendocrine control of gonadotropin secretion in cattle without the complications associated with ovarian cyclicity. In this experiment, implants provided mean concentrations ($\pm$ S.E.M.) of estradiol of 5.46 ± 0.16 pg/ml. Cows were injected i.v. with (i) physiological saline (control), (ii) mGnRH in saline (0.055, 0.11 and 0.165 $\mu\text{g/kg}$ body weight (BW)), and (iii) IGnRH-III (same doses as mGnRH) in a Latin Square design, such that each cow received one of the treatments in random order on each day of a 7 day experiment. Therefore, each treatment was applied only once to each experimental cow and no more than one treatment was applied to each cow on a given day. Blood was collected by venipuncture immediately prior to treatment (time 0) and at 10, 30, 60, 120 and 240 min after injections.

Experiment 3: effects of IGnRH-III in the presence of basal estradiol and mid-luteal phase levels of progesterone

Five ovariectomized cows, each bearing estradiol implants similar to those described in Experiment 2, were used. Cows had mean ($\pm$ S.E.M.) concentrations of estradiol of 4.88 ± 0.19 pg/ml. To mimic luteal-phase concentrations of progesterone, cows received a single controlled release drug delivery device (CIDR) (Pharmacia Animal Health, Kalamazoo, MI, USA) intravaginally 2 days before the experiment began. CIDR devices were left in place for the duration of the experiment. In addition, i.m. injections of 100 mg progesterone (Sigma) diluted in vegetable oil were administered daily. Injections of progesterone started on the day before the experiment began and continued for the duration of the study, producing mean ($\pm$ S.E.M.) concentrations of progesterone of 8.8 ± 0.5 ng/ml. Cows were treated i.v. with (i) saline (control), (ii) mGnRH in saline (0.11 or 1.1 $\mu\text{g/kg}$ BW), and (iii) IGnRH-III in saline (same doses as for mGnRH) in a Latin Square design (i.e. each cow received one treatment in random order on each day of the 5 day experiment as in Experiment 2). Therefore, each treatment was applied only once to each experimental cow and no more than one treatment was applied to each cow on a given day. Blood was collected by venipuncture immediately prior to treatment (time 0) and at 10, 30, 60, 120 and 240 min after injections.

Experiment 4: effects of IGnRH-III during the mid-luteal phase of the estrous cycle

Experiment 4A

Estrous cycles were synchronized in six cows using a 7 day treatment with CIDR devices and an injection of a synthetic prostaglandin analog, dinoprost tromethamine (Lutalyse; Pharmacia Animal Health, Kalamazoo, MI, USA) on the day of CIDR removal. On day 10, 11 or 12

after estrus, cows were injected i.v. with (i) saline (control), (ii) mGnRH in saline (1.1 µg/kg BW), and (iii) lGnRH-III (1.1 µg/kg BW) in two replicates with three cows per replicate. As in Experiments 2 and 3, releasing hormone treatments or saline were administered in a Latin Square design in a 3 day experiment and no more than one treatment was applied to each cow on a given day. Blood was collected by venipuncture immediately prior to treatment (time 0) and at 10, 30, 60, 120 and 240 min after injections. Mean concentrations ($\pm$ S.E.M.) of progesterone observed during this experiment were 4.2 ± 0.25 ng/ml.

Experiment 4B

Because of an apparent change in sensitivity to lGnRH-III in mid-luteal phase cows in Experiment 4A (see Results) compared with ovariectomized cows (Experiments 2 and 3), higher doses of lGnRH-III were tested in this experiment. Three intact cows with estrous cycles synchronized using a single injection of Lutalyse administered during the mid-luteal phase were used. Cows were injected i.v. with (i) saline (control) or (ii) lGnRH-III in saline (1.1 or 4.4 µg/kg BW). Injections were administered in a Latin Square design in a 3 day experiment and no more than one treatment was applied to each cow on a given day, as in previous experiments. Because synchronization of estrus using only a prostaglandin can result in a wide distribution in time of onset of estrus compared with other methods, the stage of follicular development at the time of corpus luteum regression (Bó *et al.* 2002) could potentially influence the regulation of FSH. To minimize these effects, two cows were used on days 10–12 and one cow was used on days 11–13 after estrus. Blood was collected by venipuncture immediately prior to treatment (time 0) and at 10, 30, 60, 120 and 240 min after injections. Mean concentrations ($\pm$ S.E.M.) of progesterone observed during the experiment were 4.5 ± 0.41 ng/ml.

RIAs

Concentrations of LH in culture media collected from each well (Experiment 1) and in serum (Experiments 2–4) were determined with a validated RIA as described previously (McVey & Williams 1991). Intra- and inter-assay coefficients of variation averaged 5.9 and 10.6% respectively. Concentrations of FSH in culture media (Experiment 1) and serum (Experiments 2–4) were determined using an RIA described previously by Krystek *et al.* (1985). In this assay, a highly purified ovine FSH (oFSH) (AFP 5679C; National Hormone and Pituitary Program (NHPP), Harbor-UCLA Medical Center, Torrance, CA, USA) was used as both the reference preparation and as iodinated tracer. Antiserum produced in rabbits immunized against oFSH was utilized as the primary antiserum. This antiserum shows similar cross-reactivity

between partially purified preparations of oFSH (oFSH S8 and S9; NHPP) and bovine FSH (bFSH B1; NHPP) and does not cross-react with other pituitary hormones (Krystek *et al.* 1985). Sensitivity of the assay averaged 0.05 ng/ml. Intra- and interassay coefficients of variation averaged 5.7 and 9.6% respectively.

Because of the heterogeneity of FSH molecules (Padmanabhan *et al.* 1992) and varied sources of reference hormones, iodinated tracers and antiserum in RIAs for ruminant FSH, the above RIA plus two additional FSH assay systems were compared in Experiment 3. Assay 2, referred to hereafter as the Bolt assay, used a bFSH preparation (AFP 5332B; NHPP) as both the reference preparation and as iodinated tracer (Bolt & Rollins 1983). The antiserum (US Department of Agriculture (USDA) 5-0122) utilized in this assay was raised against bFSH- β and reacts with intact molecules of FSH, but does not react significantly with the α -subunit of bFSH or other pituitary hormones (Bolt & Rollins 1983). Sensitivity of the single assay performed was 0.05 ng/ml. The intra-assay coefficient of variation averaged 7.2%. The third RIA system utilized highly purified bFSH (AFP-5332B; NHPP) as the reference preparation and iodinated tracer. The antiserum (AFP7711690; NHPP) was raised against intact bFSH. Sensitivity of the single assay performed was 0.06 ng/ml. The intra-assay coefficient of variation averaged 5.6%.

Serum concentrations of estradiol were determined in extracted samples in a single assay as reported previously (Talavera *et al.* 1985). Concentrations of progesterone were determined in selected samples in a single assay with the Coat-A-Count direct assay (Diagnostic Products, Los Angeles, CA, USA) as reported previously from this laboratory (Fajersson *et al.* 1999). Sensitivity for estradiol and progesterone assays was 3.7 pg/ml and 0.05 ng/ml respectively. The intra-assay coefficients of variation for these assays averaged 2.7 and 4.2% respectively.

Statistical analysis

In Experiment 1, LH and FSH data were analyzed by ANOVA using the general linear models procedure (PROC GLM) of the Statistical Analysis System (SAS 8.1; SAS Institute Inc., Cary, NC, USA). Sources of variation were treatment, well(treatment), replication, and treatment $\times$ replication interaction. A significant replicate effect was observed; thus, treatment effects were analyzed for each replication. The least significant difference was used to compare means when significant differences were detected by ANOVA.

In Experiments 2–4, LH and FSH data were analyzed by ANOVA using the PROC GLM procedure of SAS for a Latin Squares design. Sources of variation were cow, day and treatment. The least squares means procedure was used to compare means when significant differences were detected. Adenohypophyseal responses in each

Figure 1 Fold changes in mean concentrations ($\pm$ S.E.M.) of LH (top) and FSH (bottom) released into the media by primary adenohypophyseal cell cultures relative to controls (Experiment 1). Data from one representative replication, which included six wells per treatment, are shown. All doses of mGnRH stimulated the release of LH and FSH into the media. The two highest doses of IGnRH-III stimulated a non-selective release of both LH and FSH. * $P < 0.001$ compared with controls.

experiment were also analyzed by examining maximum (peak) release and areas under the response curves for LH and FSH. Because results of these analyses resulted in identical interpretations to those obtained with the

ANOVA of mean concentrations following releasing hormone challenges, only the results of the latter analyses are presented in the Results.

Results

Experiment 1: effects of IGnRH-III in primary adenohypophyseal cell cultures

Differences in the magnitude of LH and FSH released into the media among replicates resulted in a significant replicate effect ($P < 0.001$). However, analysis of treatment effects within each replicate resulted in similar conclusions in all three replicates, as the interaction resulted from differences in the magnitude of change among replicates and not in direction. All doses of mGnRH stimulated ($P < 0.001$) the release of LH and FSH. However, only the two highest doses of IGnRH-III (10^{-7} and 10^{-6} M) stimulated ($P < 0.001$) a non-selective release of both LH and FSH. Results from one representative replication are shown in Fig. 1.

Experiment 2: effects of IGnRH-III in ovariectomized cows with basal circulating concentrations of estradiol

All doses of mGnRH (0.055, 0.11 and 0.165 μ g/kg) tested induced ($P < 0.001$) release of both LH and FSH. However, none of the IGnRH-III doses tested stimulated release of either LH or FSH (Fig. 2).

Figure 2 Temporal changes and overall mean concentrations ($\pm$ S.E.M.) of LH (top) and FSH (bottom) in ovariectomized cows with basal serum concentrations of estradiol and injected i.v. at time 0 with saline (control), mGnRH or IGnRH-III (Experiment 2) in a Latin Square arrangement. All doses of mGnRH increased overall mean concentrations of LH and FSH (insets). None of the doses of IGnRH-III stimulated the release of either gonadotropin. * $P < 0.001$ compared with saline ($n = 7$ cows per treatment).

Experiment 3: effects of lGnRH-III in the presence of basal estradiol and mid-luteal phase levels of progesterone

Both doses of mGnRH (0.11 and 1.1 µg/kg) stimulated ($P < 0.001$) release of LH and FSH (Fig. 3). However, only the highest dose of lGnRH-III (1.1 µg/kg) stimulated ($P < 0.001$) a non-selective release of both LH and FSH (Fig. 3). A comparison of three FSH RIA systems, widely available for use by other investigators, produced comparable results and demonstrated that the higher dose of lGnRH-III induced ($P < 0.005$) a non-selective release of FSH (Fig. 4). No stimulation of release of FSH was observed with the lower dose.

Experiment 4: effects of lGnRH-III during the mid-luteal phase of the estrous cycle

Experiment 4A

In intact mid-luteal phase cows, lGnRH-III at 1.1 µg/kg did not stimulate ($P > 0.1$) the release of either LH or FSH (Fig. 5). As expected, mGnRH at the same dose (1.1 µg/kg) released ($P < 0.001$) both LH and FSH (Fig. 5).

Experiment 4B

In intact, mid-luteal phase cows, a dose of 4.4 µg/kg lGnRH-III stimulated ($P < 0.001$) the release of LH, but not FSH (Fig. 6). Similar to experiment 4A, a dose of 1.1 µg/kg lGnRH-III did not stimulate the release of either LH or FSH (Fig. 6).

Discussion

Using both *in vitro* and *in vivo* approaches, we were unable to demonstrate that lGnRH-III can selectively stimulate the release of FSH in cattle. In all experimental models reported herein, when lGnRH-III induced the release of FSH it also induced the release of LH. Moreover, lGnRH-III was less potent for stimulating release of either gonadotropin than mGnRH. In the intact cow, in which the ovarian peptides inhibin and follistatin exert inhibitory control over release of FSH, lGnRH-III was even less effective for inducing release of FSH. In that model, the stimulation of LH secretion by lGnRH-III during the mid-luteal phase was not accompanied by release of FSH. In contrast, mGnRH was always effective for inducing the

Figure 3 Temporal changes and overall mean concentrations ($\pm$ S.E.M.) of LH (top) and FSH (bottom) in ovariectomized cows with basal serum concentrations of estradiol and mid-luteal phase concentrations of serum progesterone (Experiment 3). Cows were injected i.v. at time 0 with saline (control), mGnRH or lGnRH-III. Both doses of mGnRH increased overall mean concentrations of LH and FSH (insets). Only the dose of 1.1 µg/kg lGnRH-III stimulated a non-selective release of both gonadotropins and increased the overall mean concentrations of LH and FSH. * $P < 0.001$ compared with saline ($n = 5$ cows per treatment).

Figure 4 Comparison of three RIA systems for evaluating FSH release in Experiment 3. Both the 'Bolt' (β -subunit) and 'Parlow' assays gave results similar to those observed with the 'Dias' assay which was used throughout all other experiments. * $P < 0.005$ compared with controls, ** $P < 0.001$ compared with controls ($n = 5$ cows per treatment).

release of both gonadotropins, independently of physiological or hormonal status. These results are in contrast to those of Dees *et al.* (2001), who reported selective release of FSH by low-dose IGnRH-III and failure to release LH during the mid-luteal phase of cattle. Our results showing no selective effect of IGnRH-III on FSH secretion are supported by recent findings in the rat (Kovacs *et al.* 2002).

The amino acid sequence of IGnRH-III exhibits a 60% homology to that of mGnRH (type I). The two molecules differ in sequence at amino acid positions 5–8, which confers distinct conformational characteristics that may account for differences in biological activity (Watts *et al.* 2001). Yu *et al.* (2000) reported selective FSH-releasing activity in fractions of gel-purified hypothalamic extracts that could be neutralized by antisera against IGnRH and proposed that the putative FSH-RH fractions were IGnRH-III or a closely related peptide. However, Montaner *et al.* (2001) were unable to detect selective FSH-releasing activity in rat and hamster hypothalamic extracts purified by HPLC. At least two other reports have failed to demonstrate selective FSH release by IGnRH-III in rat pituitary cells (Lovas *et al.* 1998, Montaner *et al.* 2001).

In the present study, we observed that IGnRH-III induced gonadotropin release from bovine primary adenohypophyseal cells at concentrations of 10^{-7} and 10^{-6} M, but not at lower concentrations. Importantly, there was no selective release of FSH by IGnRH-III, similar to that reported by Yu *et al.* (2002) in primary rat adenohypophyseal cells. Therefore, there is general agreement among studies that, in primary cultures of adenohypophyseal cells, IGnRH-III does not stimulate the release of gonadotropins at low concentrations, but at higher concentrations it stimulates a non-selective release of both LH and FSH. In contrast, *in vitro* studies using hemipituitaries suggested that IGnRH-III could preferentially release FSH (Yu *et al.* 1997, 2002). Whether the divergent effects of IGnRH-III in primary adenohypophyseal cell cultures vs hemipituitaries were due to differences in the physiological state of gonadotropes is not clear. Yu *et al.* (2002) proposed that the lack of selective responsiveness of cultured adenohypophyseal cells to IGnRH-III is due to the absence of gonadal steroids, resulting in a down-regulation of putative IGnRH-III receptors.

Because reports on *in vivo* studies testing the effects of IGnRH-III on gonadotropin secretion have been inconsistent, a careful examination of IGnRH-III action during different physiological/endocrinological states and at different doses seems prudent. Dees *et al.* (2001) reported that doses of 0.25 and 0.5 mg IGnRH-III selectively stimulated the release of FSH during the luteal phase (days 9–14) of the estrous cycle in cows. However, doses of 2 or 8 mg IGnRH-III released both LH and FSH during this period. In contrast, 0.5 mg IGnRH-III released only LH during the follicular phase of the estrous cycle and was less potent in this effect than mGnRH (Dees *et al.* 2001). These findings are interpreted to mean that low doses of IGnRH-III have the ability to selectively stimulate FSH release during periods of high circulating progesterone. The report did not discuss how this occurs in the face of expected increases in circulating inhibin that are associated with the development of second-wave follicle dominance during this time period (see review by Padmanabhan *et al.* 2002).

In our experiments using the ovariectomized, estradiol-implanted cow without any progesterone treatment, doses of IGnRH-III of up to $0.165 \mu\text{g/kg}$ (equivalent to ~ 0.075 mg) failed to elicit measurable release of either gonadotropin. In contrast, doses of mGnRH as low as $0.055 \mu\text{g/kg}$ (equivalent to ~ 0.025 mg) effectively released both LH and FSH. Using the ovariectomized, estradiol-implanted cow, with exogenous progesterone treatment to simulate luteal-phase concentrations of progesterone, we observed that doses of $1.1 \mu\text{g/kg}$ (equivalent to ± 0.5 mg) IGnRH-III stimulated a non-selective release of both LH and FSH. Thus, similarly to the studies reported by Kovacs *et al.* (2002) using the ovariectomized, estradiol/progesterone-treated rat, IGnRH-III did not stimulate preferential release of FSH in our studies with cattle. Moreover, during the mid-luteal phase (days 10–12) in the intact cow, doses

Figure 5 Temporal changes and overall mean concentrations ($\pm$ S.E.M.) of LH (top) and FSH (bottom) in intact cows during the mid-luteal phase of the estrous cycle (Experiment 4A). Cows were injected i.v. at time 0 with saline (control), mGnRH or IGnRH-III. Mammalian GnRH increased overall mean concentrations of LH and FSH (insets). IGnRH-III did not stimulate release of either gonadotropin. * $P < 0.001$ compared with saline ($n = 6$ cows per treatment).

of 1.1 $\mu\text{g/kg}$ IGnRH-III failed to release either LH or FSH. However, doses of 4.4 $\mu\text{g/kg}$ (equivalent to ± 2 mg) IGnRH-III released only LH. Therefore, the ability of IGnRH-III to stimulate the release of gonadotropins was impaired during this period. It is generally accepted that ovarian follicular inhibins and follistatins suppress FSH secretion (Padmanabhan *et al.* 2002) without apparent effects on secretion of LH (Rivier *et al.* 1986); nonetheless, a decrease in the number of binding sites for GnRH induced by inhibin has been reported (Wang *et al.* 1988). Thus, if IGnRH-III has lower binding affinity for GnRH receptors, potential decreases in numbers of GnRH receptors on gonadotropes induced by ovarian hormones could also account for significantly diminished responsiveness to IGnRH-III. IGnRH-III is also less potent compared with mGnRH (type I and II) for inducing inositol-phosphate production in COS-1 cells expressing GnRH receptors I and II (Neill 2002). Therefore, while it remains possible that IGnRH-III acts on a distinct receptor other than GnRH-I or -II, as proposed by Yu *et al.* (2000); no sequence for GnRH receptor forms, other than types I and II and a non-functional GnRH receptor-like homologue, has been observed in the human genome (Neill *et al.* 2001).

FSH molecules are largely heterogeneous and may vary in immunological and biological activities (Padmanabhan *et al.* 1992). To examine whether the discordant results obtained in our experiments and those performed by Dees *et al.* (2001), both involving cattle, could be accounted for by potential differences in immunological assay methodology, we used three distinct RIA systems validated for use in bovine samples. All three RIAs used to determine concentrations of FSH produced similar results. Therefore, it is not likely that contrasting observations between our experiments and those performed by Dees *et al.* (2001) are due to differences in the ability of particular RIAs used to detect circulating FSH.

In summary, results from our experiments do not provide evidence that IGnRH-III can selectively release FSH in cattle. The identification of such a factor would have important implications in both animal agriculture and human medicine.

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Figure 6 Temporal changes and overall mean concentrations ($\pm$ S.E.M.) of LH (top) and FSH (bottom) in intact cows during the mid-luteal phase of the estrous cycle (Experiment 4B). Cows were injected i.v. at time 0 with saline (control) or IGnRH-III. IGnRH-III at a dose of 4.4 μ g/kg increased overall mean concentrations of LH but not FSH (insets). IGnRH-III at a dose of 1.1 μ g/kg had no significant effect on release of either gonadotropin. * $P < 0.001$ compared with saline ($n = 3$ cows per treatment).

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