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ORIGINAL ARTICLE

Concentration of neuropeptide Y, galanin, β-endorphin, vasoactive intestinal peptide and gonadotropin releasing hormone in the hypothalamus of gilts during the estrous cycle

Adam J. Ziecik,[1] Stanislaw Okrasa,[2] Hanna Kalamarz,[2] Miroslaw Lakomy[3] & Robert R. Kraeling[4]

1. Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland
2. Institute of Animal Physiology, University of Agriculture and Technology, Olsztyn, Poland
3. Department of Anatomy, University of Agriculture and Technology, Olsztyn, Poland
4. Richard B. Russell Agricultural Research Center, ARS, USDA, Athens, Georgia, USA

Correspondence to: Adam J. Ziecik, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, ul. Tuwima 10, P.O. Box 55, 10-747 Olsztyn 5, Poland
TEL.: +48 89 535 7422; FAX: +48 89 535 7421
E-mail: ziecik@food.irzbz.pan.olsztyn.pl

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Abstract

Numerous reports indicate that peptides isolated from the brain such as ß-endorphin (β-END), neuropeptide Y (NPY), galanin (GAL) or vasoactive intestinal peptide (VIP), modulate secretion of gonadotropins and prolactin. The objective of the present experiment was to determine concentrations of NPY, GAL, β-END, VIP and GnRH in the preoptic area (POA), medial basal hypothalamus (MBH) and pituitary stalk-median eminence (SME) during the estrous cycle in the pig. Gilts were slaughtered on Days 5, 10, 15 and 20 of the estrous cycle. Blood samples for analyses of progesterone were taken before slaughter. Neuropeptide concentrations in brain tissues were determined using RIA. The highest concentrations of all determined peptides occurred in SME. GnRH concentration in MBH was lower ($p<0.05$) in POA and SME on Day 20 than on Day 5. NPY concentration in POA was 5–6 times greater ($p<0.05$) on Days 10 and 20 than on Day 5. Similarly, concentrations of VIP in POA were greater ($p<0.05$) on Day 10, Day 15 and Day 20 than on Day 5. The concentration of GAL in POA was higher on Days 10 and 15 ($p<0.05$) than on Days 5 and 20. The concentration of GAL in SME was lowest on Day 5 and then significantly increased on Days 10, 15 and 20. In SME, concentration of β-END increased 10 times on Days 15 and 20 when compared to Day 5 of the cycle. The correlation between concentration of GAL in the POA and MBH and progesterone concentration in the peripheral blood was positive, whereas this correlation associated with the SME was negative. These results indicate that considerable changes in various neuropeptide concentrations in different areas of the porcine hypothalamus are associated with stage of the estrous cycle and that GAL may be involved in control of the preovulatory LH surge in pigs.

Abbreviations and units

ACTH	adrencorticotrophic hormone
BSA	bovine serum albumin
β-END	β-endorphin
cpm	counts per minute
EOP	endogenous opioid peptides
g	gram
G	gravitation constant
GAL	galanin
GAP	GnRH Associated Protein
GnRH	gonadotrophin releasing hormone
GRF	growth-hormone releasing factor
h	hour
kg	kilogram
LH	luteinizing hormone
M	mol
MBH	medial basal hypothalamus
min	minutes
ml	milliliter
mM	millimol
α-MSH	a-melanocyte stimulating hormone
µg	microgram
µl	microliter
nM	nanomol
NPY	neuropeptide Y
PBS	phosphate buffer saline
pg	picogram
POA	preoptic area
r	correlation coefficient
SME	stalk-median eminence
VIP	vasoactive intestinal peptide

Introduction

Evidence has accumulated in recent years for involvement of neural peptides, such as neuropeptide Y [NPY; 1], galanin [GAL; 2] and endogenous opioid peptides [EOP; 3], in modulating gonadotropin secretion. Suprachiasmatic vasoactive intestinal peptide (VIP) plays an important role in regulation of the LH surge in rats [4] and VIP potentiated release of LH after GnRH injection in man [5].

Concentrations of GAL, NPY and β-END in SME are similar to or even higher than GnRH. Similarly, comparable concentrations of GnRH, GAL, NPY, β-END and VIP immunoreactivity were determined in POA and MBH in pigs [6]. The estradiol induced preovulatory like LH surge in ovariectomized gilts was associated with considerable changes of various neuropeptide concentrations in the hypothalamus [6].

Concentration of VIP appeared to be the most estrogen dependent since its concentration was affected in SME, MBH and POA after estrogen injection. In addition, GAL concentration, which is widely distributed in the central nervous system of pig [7], increased significantly in SME during the preovulatory like LH surge. The objectives of the present experiments were: 1) to determine concentrations of NPY, GAL, β-END, VIP and GnRH in the POA, MBH and SME during the estrous cycle in the pig and 2) to establish possible correlations between concentration of hypothalamic neuropeptides and peripheral blood progesterone concentrations in the course of the estrous cycle in pigs.

Materials and methods

Animals and Experimental Procedures.

Twenty-one gilts which displayed estrous cycles of 20 to 22 days, weighing approximately 120 kg, were maintained in separate pens and checked daily for estrus in the presence of a boar. The first day of behavioral estrus was designated Day 0 of the estrous cycle. Three days before blood collection, a cannula was inserted through the humoral cephalic vein into the anterior vena cava under general anesthesia [8]. Blood samples for analyses of progesterone were taken every 15 min within four hours before slaughter. Gilts were slaughtered on Days 5 (n=6), 10 (n=4), 15 (n=6) and 20 (n=5) of the estrous cycle. Within 5 min of death a block of brain tissue encompassing the hypothalamus was excised and divided into POA, MBH and SME as described by Sesti and Britt [9].

Preparation of brain extracts.

Brain extracts were prepared according to a method described by Ziecik et al. [6]. Briefly, tissues were homogenized in 0.5 M acetic acid at 4°C with a Ultra-Turrax and placed in boiling water for 10 min. After cooling on ice, homogenates were centrifuged for 20 min at 10,000 x G, the clear supernatant collected and then the pellet extracted two more times. The supernatants were combined and lyophilized. Extracts were reconstituted in 2 ml H_2O passed through octadecasilyl - silica cartridges (Sep Pak C 18, Waters Assoc.). After an initial wash with 10 mM HCl - 10% CH_3CN, 0.5 ml, samples were applied and the retained peptides subsequently eluted with 3 x 1 ml 10 mM HCl - 50 % CH_3CNcontaining 0.1% trifluoroacetic acid (TFA). Samples were evaporated in a vacuum centrifuge, reconstituted in assay buffer and examined for GnRH, NPY, GAL, β-END and VIP in duplicates.

Radioimmunoassays of peptides

NPY

Rabbit antiserum to synthetic porcine NPY [10] was used at a 1:2,500 final dilution in combination with [^{125}I]NPY (10,000cpm) labeled by the iodogen method of Fraker and Speck [11]. This antiserum does not bind porcine VIP, substance P, rat GRF, met-enkephalin, neurotensin, oxytocin, secretin or

vasopressin.The iodination mixture was fractionated in a Sephadex G-50 (fine) column eluted with 0.1 N acetic acid in 0.1% BSA. Synthetic porcine NPY (Peninsula Laboratories Inc., Belmont, California) was used as a standard in serial dilution of 2-500 pg/tube. The NPY standards (100 μl), tissue extracts in 100 μl of buffer and 200 μl of NPY antiserum diluted in assay buffer were incubated at 4°C for 24 h before 10,000 cpm [^{125}I]NPY was added. Incubation of the assay was continued for another 24 h. Antiserum bound tracer was separated from the unbound by a second antibody method. Sheep anti-rabbit serum at a final dilution of 1:20 was added and incubation continued for another 18 h. The assay was terminated by centrifugation (2,000 x G, 4°C, 30 min). Supernatants were aspirated to waste, and the pellets were counted in a gamma counter. The sensitivity of the NPY assay was 10-15 pg/tube at 92% binding, and the intra- and interassay coefficients of variation were 7.8% and 10.8%, respectively.

GAL

Tissue concentrations of GAL were determined by a sensitive and specific radioimmunoassay (Peninsula Laboratories Inc., Belmont, California). RIAs were performed in 0.1 M sodium phosphate buffer (pH 7.4), 0.05 M NaCl, 1% BSA, 0.01 NaN_3 and 0.1% Triton X-100. One hundred μl of standard or tissue extract and 100 μ1 of antiserum (RAS 7153) were added to polystyrene culture tubes. Approximately 10,000 cpm of ^{125}I-GAL (porcine, iodinated by the iodogen method) were added to each reaction mixture and incubations continued at 4°C for an additional 24 h. After incubation, 100 μl sheep anti-rabbit serum (1:20) and 100 μl normal rabbit serum (1:200) were added. Precipitates were allowed to form for 2 h at room temperature before 500 μl of assay buffer was added. Precipitates were isolated by centrifugation at 2,000 x G for 20 min. The standard curve was linear for concentrations ranging from 3 to 100 pg/100 μl and the detection limit was 3 pg/100 μl at 90% binding. Anti-galanin serum (RAS 7153) did not cross-react with porcine secretin, VIP or NPY. The intra- and interassay coefficients of variation were 8.1% and 13.7%, respectively.

VIP

Specific antiserum to porcine VIP (1-28; gift of Prof. Franco Sanchez Franco, Centro Nacional Investgaciones Clinicas, Madrid, Spain) was used at a 1:90,000 final dilution. Anti-VIP serum cross-reacted 100% with human, porcine and rat VIP and did not cross-react with substance P, endothelin-1, secretin, galanin or somatostatin. Synthetic porcine VIP (Peninsula Laboratories Inc., Belmont, California) was used as standard and for iodination. VIP was iodinated by the iodogen method. The iodination mixture was fractionated in a Sephadex G-50 (fine) column eluted with 0.1 M acetic acid in 0.01% BSA. Incubation tubes contained 100 μl of assay buffer, 100 μl of unknown or standard solution and 100 μl [^{125}I]VIP (10,000 cpm) was added 24 h later and samples incubated for another 24 h at 4°C. To perform a second antibody separation, 100 μl sheep anti-rabbit serum (1:20) and 100 μl normal rabbit serum (1:200) were added for 2 h at room temperature, then 500 μl of RIA buffer was added. Tubes were centrifuged at 2,000 x G for 30 min at 4°C and the pellet counted in a scintillation well gamma counter. The standard curve was linear for concentrations ranging from 3 to 100 pg/tube and the detection limit was 3 pg/100 μl. Intra- and interassay coefficients of variation were 5.5% and 11.8%, respectively

GnRH

GnRH concentration in tissue extracts was determined by a RIA procedure using rabbit antiserum from Peninsula Laboratories Inc., Belmont, California. This anti-GnRH serum (RIN 7201) did not cross-react with GAP, GRF and ACTH. Synthetic GnRH (acetate salt) was radioiodinated by the iodogen method of Fraker and Speck [11]. The iodinated GnRH was separated from free iodine by ion exchange chromatography using a QAE-Sephadex column [12]. Antiserum diluted in 200 μl of 0.1% BSA-PBS was added to 200 μl of unknown or standard samples prepared in PBS supplemented with bacitracin (2 x 10^{-5} M), followed by 100 μl ^{125}I-GnRH in 0.1% BSA-PBS (approximately 20,000 cpm) and were incubated for 24 h at 4°C. Free labeled GnRH was separated from bound using a second antibody against rabbit γ-globulin (produced in our Institute). Following a 2 h incubation with 200 μl of second antibody (diluted 1:24), 1 ml 6% polyethylene glycol solution was added and 1 h later samples were centrifuged at 3,000 x G for 20 min, and the supernatant was decanted. Bound ^{125}I-GnRH was estimated by counting the pellet. Sensitivity of the assay was 7.8 pg/ml at 93% binding. Intra- and interassay coefficients of variation for GnRH determinations were 8.4% and 12.3%, respectively.

β-*END*

β-endorphin-like immunoreactivity (β-END-IR) in tissue extracts was established by the RIA procedure previously described by Ostrowska et al. [13], except that a second antibody was used to separate free labeled β-END from bound, as in the GnRH

assay. Rabbit antiserum against β-END (RAS 8616 N), which exhibited equimolar cross reactivity (100%) with β-END and β-lipotropin and did not cross-react with γ-endorphin, met-enkephalin, ACTH and α-MSH, was obtained from Peninsula Laboratories Inc., Belmont, California. Human β-END was used for iodination and standards. Sensitivity of the assay and the intra- and interassay coefficients of variation were 20 pg ml^{-1} (at 92% binding), 5.6% and 10.8%, respectively.

Progesterone

Serum concentration of progesterone was determined by radioimmunoassay described by Stupnicki et al. [14]. The assay sensitivity was 0.63 nM/l and the intra-assay coefficient of variation was 8%.

All experiments were performed in accordance with *Guiding Principles for the Care and Use of Research Animals* and the internal instructions of the Institute's Director.

Statistical analysis.

One-way analysis of variance was used to compare differences of progesterone concentrations in blood collected on different days of the estrous cycle. The changes in each neuropeptide content in tissues were subjected to two-way analysis of variance with time and region as the factor. Significant changes in concentration between region for a given time and content between times within region were determined by Duncan's multiple range test or Bonferroni's test. The effects of progesterone concentration in blood on neuropeptides concentration in areas of hypothalamus were subjected to regression analysis. All calculations were performed using the statistical package GraphPad PRISM (GraphPad Software, San Diego, CA, USA)

Results

Fig. 1. presents mean level of progesterone during 4 hours of blood collections. The highest concentrations were recorded in the middle of the cycle on Day 10 and the lowest during the periovulatory stage of the estrous cycle, i.e. on Day 20. Progesterone values for Day 5 (early luteal phase) and Day 15 (late luteal phase) were intermediate.

The highest concentrations of all determined peptides occurred in SME: NPY, GnRH, VIP and GAL were 3–8, 18–20, 30–32 and 18–120 times higher respectively than in MBH and POA ($p< 0.01$) with the highest concentration being β-END like activity (270–1300 higher than in MBH and POA). GnRH concentration did not change during the estrous cycle in MBH, but was lower in POA and SME on Day 20 (μg/g tissue) than on Day 5 (0.048 ± 0.003 vs. 0.040 ± 0.004 and 0.074 ± 0.04 vs 0.40 ± 0.02, respectively; $p<0.05$). NPY concentrations on Days 10 and 20 in POA were 5–6 times greater than on Day 5 ($p<0.05$). Similarly, concentrations of VIP (ng/g) were greater on Days 10, 15 ($p<0.05$) and Day 20 than on Day 5 in POA. Concentration of GAL (μg /g) in POA was greater on Days 10 and 15 ($p< 0.05$) than on Days 5 and 20 (0.12 ± 0.01 and 0.12 ± 0.02 vs. 0.05 ± 0.007 and 0.03 ± 0.005, respectively). Concentration of GAL (μg/g) in MBH was lowest on Day 20 (0.07 ± 0.007) and highest on Day 15 (0.19 ± 0.03; $p<0.05$). The concentration of GAL in SME was lowest on Day 5 (5.57 ± 0.97) and then significantly increased on Days 10, 15 and 20. The concentration of β-END was constant in POA and MBH during the whole estrous cycle, however in SME, concentration of β-END was 10 times greater on Day 15 and 20 than on Day 5 (Fig. 2).

The following correlations between neuropeptide concentration in SME, MBH and POA and peripheral blood progesterone concentrations were found (Fig. 3 A-E): GAL in POA ($r=0.697$; $p<0.001$; Fig 3 A); GAL in MBH ($r=0.462$; $p<0.05$; Fig. 3B); GnRH in SME ($r=0.574$; $p<0.01$; Fig 3C); NPY in SME ($r=0.451$; $p<0.05$; Fig. 3D); GAL in SME ($r=-0.478$; $p<0.05$; Fig. 3E).

Discussion

Concentrations of various neuropeptides in some areas of hypothalamus to some extent depend on the estrous cycle phase in pigs. The greatest fluctuations of neuropeptide concentrations (NPY, VIP, GAL, GnRH) during the estrous cycle in pigs were found in the preoptic area of hypothalamus. We confirmed our earlier results [6] that concentrations

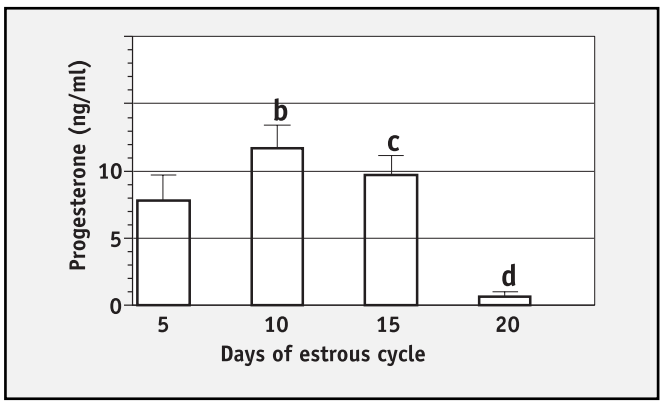

Fig. 1. Mean serum progesterone level during 4 hours of blood collection in gilts on Days 5, 10, 15 and 20 of estrous cycle. Values are means ±SEM. Means with various superscripts differ significantly ($p<0.01$).

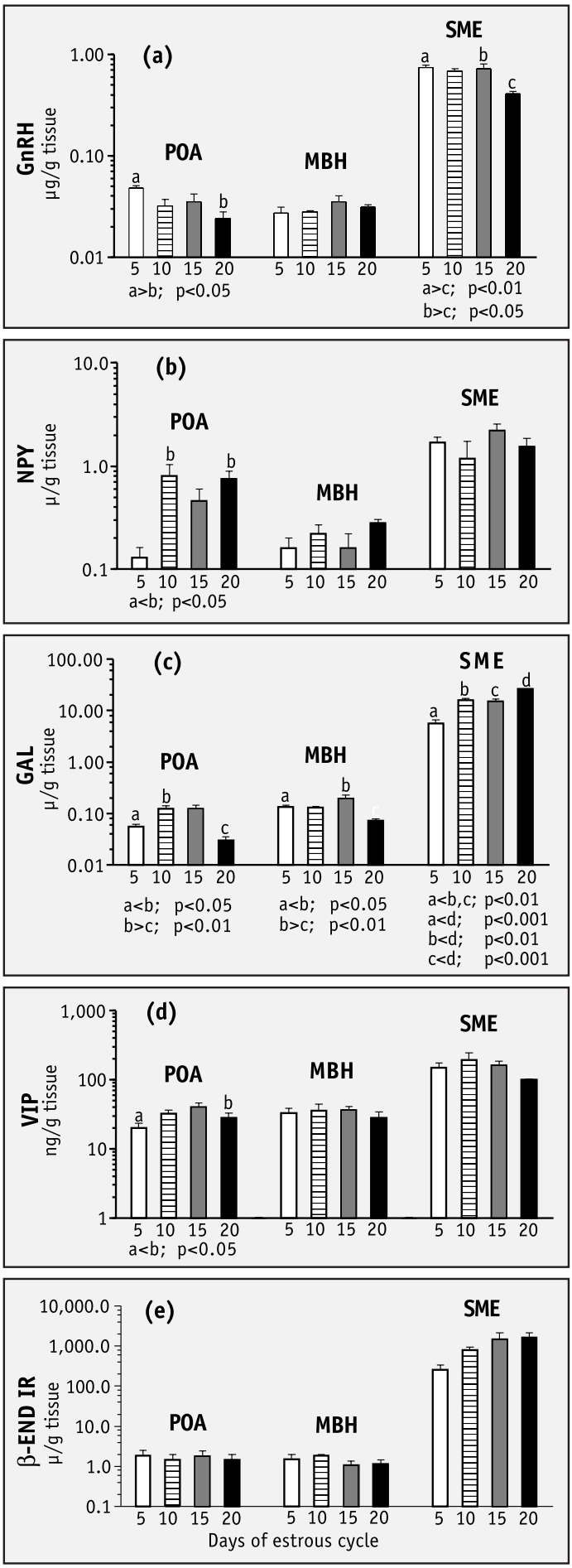

Fig. 2. Concentrations of (**a**) GnRH, (**b**) neuropeptide Y (NPY), (**c**) galanin (GAL), (**d**) vasoactive intestinal peptide (VIP), and (**e**) β-endorphin (β-END) in preoptic area (POA), medial basal hypothlamus (MBH) and stalk median eminence (SME) in gilts during the estrous cycle in pigs.

of NPY, GAL and β-END were very high in SME of pig hypothalamus suggesting that these neuropeptides, like GnRH, can be released into the portal blood and affect secretion of pituitary gonadotropins and other tropic hormones in pigs. Concentrations of GnRH in the POA, MBH and SME were similar to those reported by Sesti and Britt [9] in lactating and weaned sows and in ovariectomized and estradiol primed gilts [6]. The low concentration of this neuropeptide in SME during the periovulatory stage of the estrous cycle may reflect a decrease in the readily releasable pool of GnRH during a preovulatory LH surge. Similar changes in GnRH content were found in POA suggesting that this region of the porcine hypothalamus may be involved in induction of the LH surge. In our previous study [6], we found very dynamic changes in VIP concentration; a temporary increase in POA and MBH during the negative feedback phase and their considerable elevation in SME during the positive feedback phase of estrogen-induced LH surge in ovariectomized gilts. VIP immunoreactive neurons are anatomically positioned to release VIP into the hypophysial portal system to subsequently modulate gonadotropin secretion in pigs. We did not find, however, a significant decline VIP content in SME during the periovulatory phase of the estrous cycle compared to other stages of the estrous cycle. It does not rule out a possibility that VIP is also involved in control of the LH surge in pigs as in other mammals [4], but confirmation of such a hypothesis requires more studies, including recording of the timing and amplitude of GnRH and LH secretions. Contrary to VIP, we confirmed an earlier report [6] that GAL concentration was very high in SME around the time of preovulatory LH surge. This neuropeptide seems to play a role in the estradiol-induced LH surge in ovariectomized gilts as well as in intact animals. GAL also stimulated GnRH release from SME of ovariectomized gilts [15] and calves [16] in vitro. Interestingly, the correlation between concentration

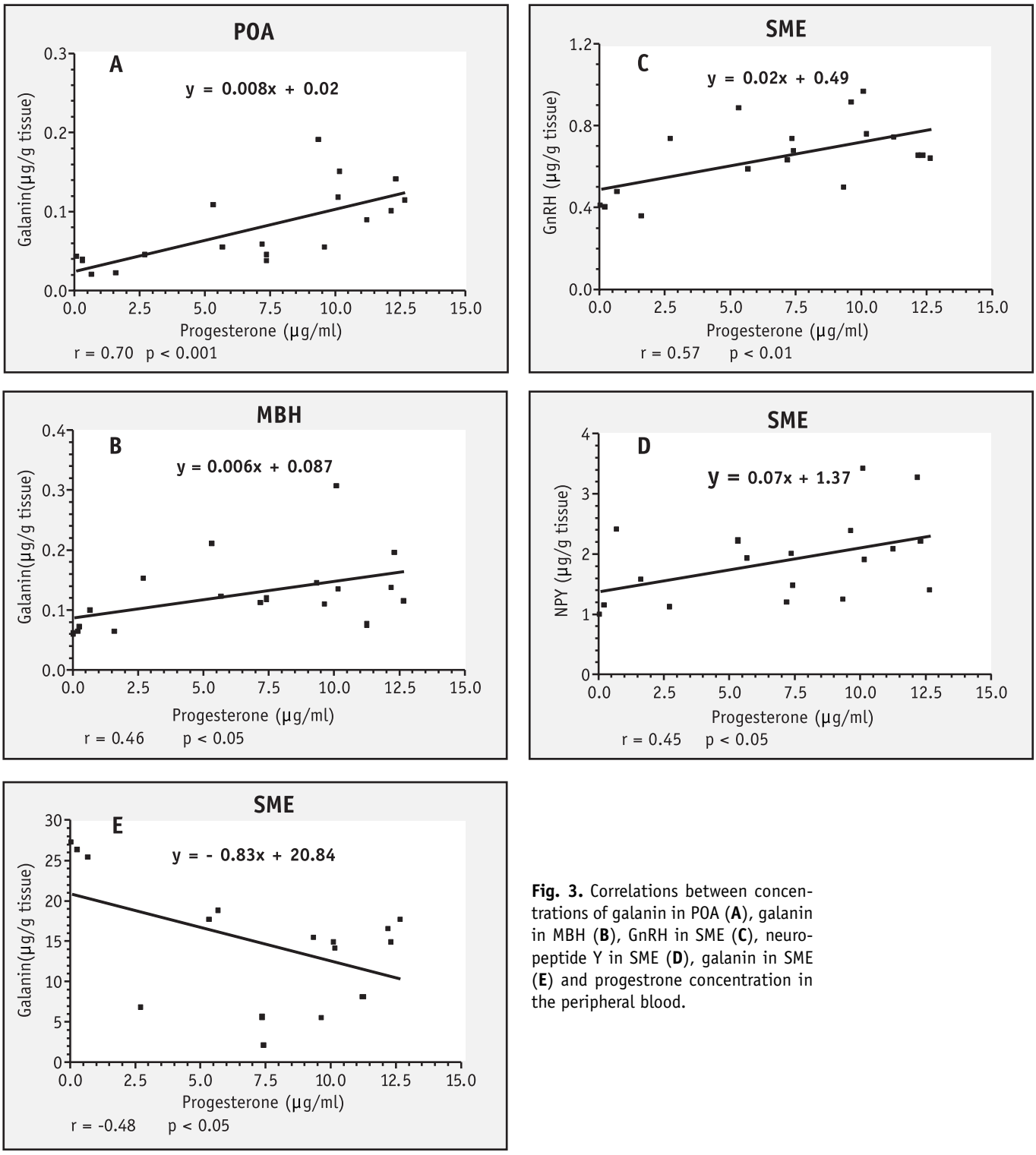

Fig. 3. Correlations between concentrations of galanin in POA (**A**), galanin in MBH (**B**), GnRH in SME (**C**), neuropeptide Y in SME (**D**), galanin in SME (**E**) and progestrone concentration in the peripheral blood.

of GAL in the POA and MBH and progesterone concentration in the peripheral blood was positive, whereas this correlation was negative in the SME. We suggest that GAL involvement in control of GnRH and then LH secretion is regulated by both progesterone and estradiol [6]. Also, progesterone is responsible for storage of both GnRH and NPY in SME of the pig hypothalamus.

Acknowledgments

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