Developmental changes in galanin in lumbosacral sympathetic ganglionic neurons innervating the avian uterine oviduct and galanin induction by sex steroids

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Abstract

We recently found lumbosacral sympathetic ganglionic galanin neurons innervating the quail uterine oviduct. Galaninergic innervation of the uterine muscle may be essential for avian oviposition, as galanin evoked oviposition through a mechanism of induction of vigorous uterine contraction. The questions arising from these findings are: what changes occur in galanin expression in the sympathetic ganglionic galanin neuron during development, and what is the hormonal factor(s) that induces galanin expression in this neuron? Therefore, the present study examined the developmental changes in galanin of the quail sympathetic ganglionic neuron and uterus, and the effect of administration of ovarian sex steroids on galanin induction. Immature birds reared under long-day photoperiods from 4 weeks of age demonstrated progressive increases in galanin levels both per unit ganglionic protein (concentration) and per ganglia (content) concurrent with ganglionic development during weeks 4–13. The uterine galanin content and uterine weight also increased progressively during the same period, but the galanin concentration in the uterus at 4 weeks was high due to the much smaller tissue mass. Immunocytochemical analysis with anti-galanin serum showed that immunoreactive ganglionic cells were few and small at 4 weeks and increased progressively thereafter. Administration of oestradiol-17β to immature birds at 3 weeks of age for 1 week increased both the galanin concentration and content in the ganglia without ganglionic growth. A marked increase in galanin-immunoreactive ganglionic cells was detected following oestradiol treatment. In contrast, progesterone increased ganglionic galanin levels, but the effects were low. Expression of the mRNAs encoding oestrogen receptor-α and -β (ERα and ERβ) in the ganglionic tissue was verified by RT-PCR/Southern blot analysis. Immunocytochemical staining with anti-ER serum further revealed an intense immunoreaction restricted to the nucleus of ganglionic neurons.

These results suggest that ovarian sex steroids, in particular oestradiol-17β, contribute as hormonal factors to galanin induction, which takes place in the lumbosacral sympathetic ganglionic neurons innervating avian uterine oviduct during development. Oestradiol may act directly on this ganglionic neuron through intra-nuclear receptor-mediated mechanisms to induce galanin.

Introduction

Oviposition means expulsion of the egg from the oviduct and is a common phenomenon in vertebrates other than eutherian mammals. It is well known that avian oviposition as well as mammalian parturition are regulated, at least partly, by a neurohypophysial hormone, arginine vasotocin (AVT), and ovarian hormones and prosta- glandins (PGs), through mechanisms of the induction of uterine contractions (Munsick et al. 1960, Rzasa & Ewy 1970, Herteledy 1972, Wechsung & Houvenaghel 1976, Olson et al. 1978, Toth et al. 1979, Takahashi et al. 1992). Avian neurohypophysial hormones are AVT and mesotocin. In birds, the oxytocic effect of AVT on uterine contractions is greater than that of mesotocin (Saito & Koike 1992) as well as oxytocin (Munsick et al. 1960). In contrast to the hormonal action, limited information is available on the neuronal mechanism controlling oviposition in birds as well as other vertebrates. Oviposition in birds is conducted by vigorous contractions of the uterine oviduct. Morphological evidence indicating abundant nerve terminals in the uterine muscle suggests the presence of a neuronal control mechanism for avian oviposition. Some functional substances that are secreted from nerve terminals may be involved in evoking oviposition, as a neurotransmitter or a neuromodulator.
When studying the regulation of avian oviposition, poultry have served as an excellent model, since oviposition occurs almost daily in the domestic quail and hen. We recently isolated an oviposition-inducing peptide from the quail oviduct (Li et al. 1996). This isolated peptide, a 29-residue peptide, including an amidated threonine at the C-terminus, was identical with avian galanin (Li et al. 1996), which has been previously isolated from the chicken intestine (Norberg et al. 1991). Avian and mammalian galanins differ at several positions in the C-terminal part (Tatemoto et al. 1983, Kaplan et al. 1988, Räkäeus & Carlquist 1988, Norberg et al. 1991, Sillard et al. 1991, Li et al. 1996). In vitro and in vivo experiments (Li et al. 1996) demonstrated that avian galanin acts directly on the uterine muscle to evoke oviposition through a mechanism of the induction of vigorous uterine contractions. Immunocytochemical analysis using anti-galanin serum revealed that immunoreactive fibres were distributed in muscular layers of the quail uterus (Li et al. 1996). However, no cell bodies that express avian galanin were detected in the uterus, as well as other oviduct regions (Li et al. 1996). Northern blot analysis verified no expression of the mRNA of avian galanin in the quail uterus (Kohchi & Tsutsui 2000). More recently, we identified the neurons that project into the uterine musculature and produce avian galanin using immunocytochemical, retrograde labelling and molecular biological methods (Sakamoto et al. 2000). In mature birds, lumbosacral sympathetic ganglionic neurons innervating the uterine muscle actively produced avian galanin and a large number of receptors for avian galanin was restricted to uterine muscles (Tsutsui et al. 1997, 1998, Sakamoto et al. 2000). Thus, it is possible that galanin acts directly on uterine muscles, by its secretion from the terminals of sympathetic ganglionic neurons projecting to the uterus, to induce uterine contractions. This is the first neuronal mechanism controlling oviposition reported in a vertebrate.

Interestingly, both avian galanin and its mRNA in lumbosacral sympathetic ganglionic neurons innervating the uterine muscle may be highly expressed in mature birds, unlike in immature birds (Sakamoto et al. 2000). With these findings as a background, the following questions were addressed in the present study. First, what change occurs in galanin in this neuron during development? Secondly, what is the hormonal factor(s) involved in the induction of galanin during development? We have previously demonstrated that ovarian sex steroids, i.e. oestradiol-17β and progesterone, induce galanin receptors localized in the quail uterine oviduct (Tsutsui et al. 1998). Therefore, this study examined the effect of these sex steroids on galanin expression in the quail. Based on a previous study (Tsutsui et al. 1998), administration of oestradiol-17β or progesterone to immature birds was performed to increase each circulating steroid level similar to that of the maximal level observed in normal mature birds exposed to long day photoperiods. Here we show that oestradiol-17β is a potent inducer of galanin in the lumbosacral sympathetic ganglionic neuron. To reveal the mode of oestrogen action, we further investigated the expression of oestrogen receptors (ER) in this neuron.

Materials and Methods

Animals and tissue samples

Female Japanese quail (Coturnix japonica) at various ages during development were used for the present investigation. They were housed in a temperature-controlled room (25 ± 2 °C) under daily photoperiods of 16 h light : 8 h darkness (long day; lights on at 0700 h), and were given quail food and tap water, and allowed to feed ad libitum. When newly hatched quails are exposed to long day photoperiods, they reach sexual maturity at around 3 months of age (Follett 1984). In sexually mature females ovulation occurs 6–8 h after the ovulatory surge of luteinizing hormone (LH) and the egg subsequently spends about 24 h in the mature oviduct before it is laid (Follett 1984). All birds were isolated in individual cages until termination by decapitation between 1000 and 1200 h. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Hiroshima University (Higashi-Hiroshima, Japan).

The reproductive system of mature birds (poultry) consists of a single left ovary and oviduct (Sturkie 1986). It has been previously confirmed that lumbosacral sympathetic ganglionic neurons located on the uterine side (left side) project their axons to the quail uterine muscular layer (Sakamoto et al. 2000). In detail, application of neurobiotin to the uterine muscle resulted in the appearance of retrogradely labelled neurons in the lumbosacral sympathetic ganglia, mainly LS2–LS5, which were located only in the uterine side (Sakamoto et al. 2000). The LS1, LS6 and LS7 ganglia also showed a few labelled neurons, whereas no neurons were labelled in the LS8 ganglion or the thoracic sympathetic ganglia (Sakamoto et al. 2000). Therefore, the lumbosacral sympathetic ganglia (LS1–LS7) located on the uterine side at different developmental stages (4–13 weeks of age) were carefully dissected and used for ganglionic tissue samples. Because LS2 ganglion of the uterine side was a major site of retrogradely labelled neurons (Sakamoto et al. 2000), this ganglion was further subjected to immunocytochemical studies. The quail oviduct differentiates at around 5 weeks of age and gives rise to several regions, i.e. the infundibulum, magnum, isthmus, uterus and vagina (Li et al. 1996, Tsutsui et al. 1997, 1998). Therefore, the uterine samples at 6, 10 and 13 weeks of age were obtained from the differentiated uterus, but the samples from 4 weeks of age consisted of the whole oviduct.
Experimental schedules

In the first series of experiments, the uterine side LS1–LS7 of female quails at 4, 6, 10 and 13 weeks of age (25 birds in each age group and each divided into five samples) were used to determine developmental changes in the concentration and content of galanin in the sympathetic ganglionic neurons. Other female birds at 4, 6, 10 and 13 weeks of age (six birds in each age group and one sample from one quail) were also used for quantification of galanin located in the uterus. Galanin levels were quantified by a competitive ELISA method. The uterine side LS2 of female birds at 4, 6, 10 and 13 weeks of age (four birds in each age group) was used to detect galanin immunocytochemically.

In the second series of experiments, immature females at 3 weeks of age were divided into three groups (25 birds in each group and each divided into five samples) and treated with a SILASTIC brand (Dow Corning, Midland, MI, USA) plate containing oestradiol-17β, progesterone or vehicle to examine the effect of sex steroids on ganglionic galanin levels. A SILASTIC plate (5·0 × 5·0 × 2·0 mm; 10 mg crystal/plate) made of a mixture of Silipot 184 (silicone type, Dow Corning) and crystalline sex steroid (Sigma, St Louis, MO, USA) was intra-abdominally implanted as described previously (Tsutsui et al. 1998). For immunocytochemical analysis, female birds at 3 weeks of age were treated with oestradiol-17β or vehicle with a plate (four birds in each group). One week after the beginning of implantation, all birds were used for the collection of samples. It has previously been confirmed that circulating endogenous steroid levels at 3 weeks of age are very low (Tsutsui et al. 1998).

Finally, immature females (48 birds and divided into three samples) at 3 weeks and mature females (36 birds and divided into three samples) at 3 months were used to determine the expressions of ERα and ERβ mRNAs in the sympathetic ganglia (LS1–LS7) located on the uterine side. ER mRNA expression was detected by a RT-PCR/Southern blot method. For immunocytochemistry for ERα, immature females (four birds) at 3 weeks were used. To detect co-expression of ER and galanin, immunocytochemical analyses with two kinds of antibodies against ER and galanin were further conducted using mature females (four birds) at 3 months of age, as galanin-like immunoreactivity was very low in immature females at 3 weeks of age (Sakamoto et al. 2000).

Peptide extraction and ELISA of galanin

Galanin levels in the lumbosacral sympathetic ganglia (uterine side LS1–LS7) and the uterus were quantified by a competitive ELISA method using the antiserum raised against avian galanin, according to our previous method (Sakamoto et al. 2000). Females at different developmental ages (4–13 weeks) and immature females treated with oestradiol-17β, progesterone or vehicle alone were killed by decapitation. The LS1–LS7 ganglia of the uterine side or the uterus (whole oviduct at only 4 weeks) were carefully removed using fine forceps under a dissecting microscope, snap-frozen immediately in liquid nitrogen and used for peptide extraction.

Peptides were extracted according to our previous methods (Li et al. 1996, Sakamoto et al. 2000). Frozen samples were boiled for 7 min and homogenized in 5% acetic acid using a homogenizer (Ultra-Turrax T8 IKA, Labortecnik, Staufen, Germany). The homogenate was centrifuged at 16 000 g for 30 min at 4 °C. The supernatant was collected into a tube, and the resulting precipitate was further homogenized, centrifuged, and the supernatant was collected again. The two supernatants were pooled and forced through a disposable C-18 cartridge (Sep-Pak Vac 1cc, Waters, Milford, MA, USA). The retained material was then eluted with 60% methanol. The pooled eluate was concentrated in a vacuum evaporator at 40 °C, passed through disposable Ultrafree-MC Centrifugal Filter Units (Millipore, Bedford, MA, USA) and subjected to competitive ELISA, as previously described (Sakamoto et al. 2000). In brief, different concentrations of avian galanin (1–1000 pmol/ml) and adjusted tissue extracts were added with the antiserum against avian galanin (1:500 dilution) to each antigen-coated well of a 96-well microplate (ELISA plate, Corning, Inc., Corning, NY, USA) and incubated for 1 h at 37 °C. The antiserum against avian galanin was raised in a rabbit using synthetic avian galanin linked to keyhole limpet haemocyanin (Li et al. 1996). It has been previously confirmed that the antiserum cross-reacts with avian galanin on the basis of immunoblotting analysis (Li et al. 1996, Sakamoto et al. 2000). After the reaction with alkaline phosphatase-labelled goat anti-rabbit IgG, immunoreactive products were obtained in a substrate solution of p-nitrophenylphosphate, and the absorbance was measured at 415 nm on a microtitre plate reader (MTP-120, Corona Electric, Ibaraki, Japan).

Protein content in the ganglionic tissues used for ELISA were measured by the bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, USA) with BSA as a standard. The concentration of avian galanin was calculated in terms of pmol per mg ganglionic protein or per mg uterine tissue.

Immunocytochemical analysis of galanin

Females at different developmental ages (4–13 weeks) and immature females treated with oestradiol-17β were killed by decapitation. The LS2 ganglion of the uterine side was carefully dissected and used, because abundant immunoreactive cells were localized in this ganglion (Sakamoto et al. 2000). Tissues were immediately immersion-fixed in 4% paraformaldehyde in 0·1 M phosphate buffer (PB; pH 7·3) for 14–16 h at 4 °C before immunocytochemical
procedures. Subsequently, tissues were dehydrated in ethanol and xylene, and embedded in paraffin wax. Serial sections of each LS2 ganglion were cut horizontally on a microtome at 5 μm thickness. Deparaffinized sections were processed according to the ABC immunocytochemical technique described previously (Azumaya & Tsutsui 1996, Li et al. 1996, Ukena et al. 1998, Sakamoto et al. 2000).

For immunocytochemistry, endogenous peroxidase activity was eliminated from the sections by incubation with 0.3% H2O2 in absolute methanol for 10 min. After blocking nonspecific binding components with 1% normal goat serum and 1% BSA in PBS (10 mM PB and 0.14 M NaCl; pH 7.3) containing 0.3% Triton X-100 for 30 min at room temperature, the sections were immersed with the primary antisera directed against avian galanin at a dilution of 1:500 for 16–20 h at 4°C. Several concentrations of the antisera from 1:500 to 1:2000 were examined, and a solution of 1:500 proved the most satisfactory (Li et al. 1996, Ukena et al. 2000). Immunoreactive products were detected with the ABC kit ( Vectastain Elite Kit, Vector Laboratories, Inc., Burlingame, CA, USA), followed by diaminobenzidine (DAB) reaction with a slight modification of the manufacturer’s instructions.

Control for specificity of the immunocytochemistry of galanin was performed by pre-absorbing the working dilution of the primary antisera with a saturating concentration of the antigen (18 μg avian galanin/ml). The sections were incubated with this control serum in a concentration of the antigen (18 μg avian galanin/ml). The reaction was stopped by incubation at 65°C for 10 min, the cDNA was ethanol precipitated and redissolved in 5 μl distilled water. For PCR, an aliquot of the cDNA solution corresponding to 1 μg of the initial total RNA was used as a template in a 25 μl reaction mixture. The PCR mixture contained cDNA, 20 mM TBS (pH 8.0), 100 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl2, 0.2 mM deoxynucleotide triphosphate mix, 0.5 μM of each primer, and 1 U Gene Taq DNA polymerase (Wako). After denaturation at 94°C for 3 min, the mixture was subjected to 35 thermal cycling in a programmed temperature control system (PC7000, ASTEC, Fukuoka, Japan) as follows: denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and extension at 72°C for 1 min. After the thermal cycling, the mixture was additionally incubated at 72°C for 10 min. A 5 μl aliquot of each sample was electrophoresed through a 1.5% agarose gel.

To confirm the identity of the amplified fragment, the gel was applied to Southern analysis with a digoxigenin-labelled oligonucleotide probe, corresponding to the internal sequence of the target gene. Digoxigenin DNA labelling and detection were performed according to the manufacturer’s recommendations (Roche Molecular Biochemicals, Mannheim, Germany). Oligonucleotides used as PCR primer and probe for ERα and ERβ mRNA detection, based on nucleotide sequences of chicken ERα (Krust et al. 1986), quail ERβ (Foidart et al. 1999) and chicken β-actin, were as follows: ERα sense primer, 5’-GGCGAATGTGAAACAGAAA-3’ (nucleotides 968–988 in Krust et al. 1986); ERα antisense primer, 5’-AGAAAGAATGATGAAACAGAAA-3’ (nucleotides 1573–1553 in Krust et al. 1986); ERα probe, 5’-TCAT CCGAAACCGAGCAGCAG-3’ (nucleotides 1496–1476 in Krust et al. 1986); ERβ sense primer, 5’-ATTCG AATCCTGCGGCCCATCGT-3’ (nucleotides 544–567 in Foidart et al. 1999); ERβ antisense primer, 5’-GCTTTCAACACGGCTGACTTGTC-3’ (nucleotides 858–835 in Foidart et al. 1999); ERβ probe, 5’-TTT GCTCGGACGACTCACACA-3’ (nucleotides 720–700 in Foidart et al. 1999); β-actin sense primer, 5’-GAG ACCTTCAACACCCAGCAG-3’ (nucleotides 441–459, gb L08165); and β-actin antisense primer, 5’-GACAGA GTACTTGCCTCAG-3’ (nucleotides 1085–1066, gb L08165). The ERα sense and antisense primers give a 606 bp amplified fragment of the ERα gene, and the ERβ primers give a 315 bp amplified fragment of the ERβ gene. The β-actin sense and antisense primers give a 645 bp amplified fragment. RT-PCR analyses were repeated three times with independently extracted RNA samples from different birds.

RT-PCR/Southern blot analyses of ERα and ERβ mRNAs

To determine the expression of mRNA encoding for ERα or ERβ in the lumbosacral sympathetic ganglia (LS1–LS7) located in the uterine side, RT-PCR analysis was performed using immature (3 weeks) and mature (3 months) females according to our previous method (Ukena et al. 1998). Total RNA (including ribosomal RNA and mRNA) from the ganglia was isolated by the guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987), and it was reverse transcribed using oligo(deoxythymidine) primer and RT in a 30 μl reaction volume for 2 h at 37°C. The reaction mixture was composed of 5 μg total RNA, 50 mM Tris–HCl-buffered saline (TBS; pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate mix, 0.25 μg oligo(deoxythymidine)12–18 (Amersham Pharmacia Biotech, Amersham, Bucks, UK), 15 U ribonuclease inhibitor (Wako, Osaka, Japan) and 150 U Moloney murine leukemia virus transcriptase (Life Technologies, Burlington, Canada). After the reaction was stopped by incubation at 65°C for 10 min, the cDNA was ethanol precipitated and redissolved in 5 μl distilled water. For PCR, an aliquot of the cDNA solution corresponding to 1 μg of the initial total RNA was used as a template in a 25 μl reaction mixture. The PCR mixture contained cDNA, 20 mM TBS (pH 8.0), 100 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl2, 0.2 mM deoxynucleotide triphosphate mix, 0.5 μM of each primer, and 1 U Gene Taq DNA polymerase (Wako). After denaturation at 94°C for 3 min, the mixture was subjected to 35 thermal cycling in a programmed temperature control system (PC7000, ASTEC, Fukuoka, Japan) as follows: denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and extension at 72°C for 1 min. After the thermal cycling, the mixture was additionally incubated at 72°C for 10 min. A 5 μl aliquot of each sample was electrophoresed through a 1.5% agarose gel.

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Immunocytochemical analysis of ERα

The uterine side LS2 of immature birds at 3 weeks of age was embedded in OCT compound (Miles, Elkhart, IN, USA), and quickly frozen in liquid nitrogen. Cryostat
sections (15 µm thickness) were air-dried on slides and fixed with 4% paraformaldehyde in 0.1 M PB (pH 7.3), methanol and acetone in series at 4 °C for 10 min each. Endogenous peroxidase activity was eliminated from the sections by incubation with 0.3% H₂O₂ in absolute methanol for 10 min. The sections were immunostained for ER using an ER immunostaining kit (Abbott GmbH, Wiesbaden-Delkenheim, Germany). The usefulness of the present ER immunostaining kit for the detection of ER in female quail and hen tissues has been established (Ohashi et al. 1991, Yoshimura et al. 1995, 2000). An anti-human ERα monoclonal antibody (H222) raised in rats was used in this study, and it recognized 66 kDa ERα in the avian oviduct (Sato et al. 1994). After blocking nonspecific binding components with normal goat serum, the sections were immersed with ER antibody at 4 °C for 40–44 h. The primary immunoreaction was followed by a 2-h incubation with anti-rat IgG. The sections were then incubated with peroxidase anti-peroxidase (PAP) complex for 1 h at room temperature. Immunoreactive products were detected by immersing the sections in a DAB chemistry of ERα and after blocking nonspecific binding components. After a rinse in PBS, sections were incubated for 4 h with rhodamine-labelled goat anti-rabbit IgG, diluted at 1:1000. After rinsing, ERα immunostaining was then performed on the same sections as described above. Immunoreactivity against galanin or ERα was studied using a Nikon fluorescence microscope or an Olympus BH-2 microscope.

To detect colocalization of ER and galanin in the ganglionic cell, double-staining was further conducted in this study. Sections were first immersed with antiserum against avian galanin (1:500 dilution) at 4 °C for 16–20 h after blocking nonspecific binding components. After a rinse in PBS, sections were incubated for 4 h with rhodamine-labelled goat anti-rabbit IgG, diluted at 1:1000. After rinsing, ERα immunostaining was then performed on the same sections as described above. Immunoreactivity against galanin or ERα was studied using a Nikon fluorescence microscope or an Olympus BH-2 microscope.

Statistical analyses

Results for galanin levels in the ganglia and uterus during development and after steroid treatment were expressed as the mean ± s.e.m. and were analysed for significance of difference by Duncan’s multiple range test or Kruskal–Wallis test followed by Mann–Whitney U test after verification of equality or inequality, respectively, of variances among the groups compared (Bliss 1967). Significant differences (P<0.05) in the number and soma area of galanin-like immunoreactive cells among different developmental stages or between oestradiol-17β- and vehicle-treated groups were analysed by Duncan’s multiple range test or Student’s t-test (Bliss 1967).

Results

Changes in ganglionic and uterine galanin levels during development

The lumbosacral sympathetic ganglia (LS1–LS7) located on the uterine side developed progressively from 4 to 13 weeks of age (P<0.05 or 0.01, 4 vs 6, 10 and 13 weeks; 6 vs 10 and 13 weeks; Fig. 1a). Avian galanin in these ganglia was measured during ganglionic development by a competitive ELISA using the antiserum raised against avian galanin. The concentration of avian galanin on a unit protein basis of ganglia was very low at 4 weeks of age and tended to increase during 4–10 weeks of age (Fig. 1b). The ganglionic galanin concentration increased significantly at 13 weeks of age (P<0.01, 13 vs 4, 6 and 10 weeks; Fig. 1b). As shown in Fig. 1c, a similar developmental change in the galanin content per ganglia was observed during the same period (P<0.01, 13 vs 4, 6 and 10 weeks).

Uterine weight also increased progressively with age (P<0.01, 4 vs 6, 10 and 13 weeks; 6 vs 10 and 13 weeks; Fig. 2a). In contrast, the concentration of uterine galanin on a unit weight basis at 4 weeks of age was significantly higher (P<0.01) than those at 6, 10 and 13 weeks of age (Fig. 2b). However, the content of galanin per whole uterus at 4 weeks of age was low, due to the much smaller tissue mass, and increased significantly during 4–13 weeks of age (P<0.05 or 0.01, 4 vs 6, 10 and 13 weeks; 6 vs 10 and 13 weeks; Fig. 2c).

Changes in galanin-immunoreactive cells during ganglionic development

As shown in Fig. 3a, galanin-like immunoreactivity was found in the LS2 ganglion at 4 weeks of age, but immunoreactive cell bodies were few and relatively small. Ganglionic immunoreactive cell bodies increased during 4–13 weeks of age (Fig. 3a, c, e and g). At 13 weeks of age, an intense immunoreaction for galanin was observed in the relatively large cell bodies, which were distributed throughout the whole LS2 ganglion (Fig. 3g). Pre-absorbing the antiserum (1:500 dilution) with an excess of avian galanin at a concentration of 18 µg/ml resulted in a complete absence of galanin-like immunoreactivity in all of the positively stained cells in the LS2 ganglion at each age (Fig. 3b, d, f and h).

Immunocytochemical experiments were repeated four times using different birds at each developmental stage and their results were subjected to the following morphological analyses. Based on serial sections immunostained for avian galanin, we counted the number of immunoreactive cells per section of the LS2 ganglion at each age and expressed this in terms of the number per square millimetres (mm²). Figure 4a summarizes developmental changes in the number of ganglionic galanin-immunoreactive cells per
Figure 1  Developmental changes in protein content in quail lumbosacral sympathetic ganglia (LS1–LS7, uterine side) (a), galanin concentration per mg ganglionic protein (b) and galanin content per ganglia (c). Each column and vertical line represents the mean ± S.E.M. (five samples in each age: one sample from five quails). *P<0·05, **P<0·01 vs 4-week group. †P<0·05, ††P<0·01 vs 6-week group or 10-week group. Duncan’s multiple range test.

Figure 2  Developmental changes in quail uterine weight (a), galanin concentration per mg uterine tissue (b) and galanin content per uterus (c). Each column and vertical line represents the mean ± S.E.M. (six samples in each age: one sample from one quail). *P<0·05, **P<0·01 vs 4-week group. ††P<0·01 vs 6-week group. Duncan’s multiple range test.
Figure 3 Immunocytochemical staining with the antiserum to avian galanin (a, c, e, g) or with the antiserum pre-incubated with a saturating concentration of synthetic avian galanin (b, d, f, h) in the quail lumbosacral sympathetic ganglion (LS2, uterine side) at 4 weeks (a and b), 6 weeks (c and d), 10 weeks (e and f) and 13 weeks of age (g and h). (a)–(h) are of the same magnification (bar represents 50 μm). Similar results were obtained in repeated experiments using four different birds at each age and used for quantitative analyses (see Fig. 4).
Effects of sex steroids on galanin levels and galanin-immunoreactive cells in the immature ganglion

To investigate whether sex steroids are involved in galanin induction, avian galanin levels in the LS1–LS7 ganglia located on the uterine side were measured following treatment with oestradiol-17β or progesterone using immature birds at 3 weeks of age. Administration of both oestradiol-17β and progesterone alone did not influence the ganglionic protein content (Fig. 5a). In contrast, oestradiol-17β administration induced a significant increase ($P<0.01$) in the ganglionic galanin concentration on a unit protein basis compared with the control group (Fig. 5b). Progesterone administration also induced an increase ($P<0.05$) in the galanin concentration, but the stimulatory effect of progesterone was much lower ($P<0.05$) than that of oestradiol-17β (Fig. 5b). Treatment with oestradiol-17β or progesterone resulted in a similar change in the ganglionic galanin content per ganglia ($P<0.01$, vehicle vs oestradiol-17β; $P<0.05$, vehicle vs progesterone; Fig. 5c).

Morphological changes in galanin-immunoreactive cells in the LS2 ganglion of the uterine side were also analysed following oestradiol-17β administration, as the effect of oestradiol-17β was more noticeable. As shown in Fig. 6a, oestradiol-17β administration markedly increased ($P<0.01$) the number of ganglionic galanin-immunoreactive cells per unit area compared with the control group. However, the cross-sectional soma area of immunoreactive cells remained unchanged following oestradiol-17β administration (Fig. 6b).

Expression of oestrogen receptors in the ganglion

To understand the mode of oestrogen action on galanin induction, we further investigated the expression of ERs in the ganglionic tissue. The expression of mRNA encoding for ERα or ERβ in the lumbosacral sympathetic ganglia (LS1–LS7, uterine side) was examined in both immature (3 weeks of age) and mature (3 months of age) quails by RT-PCR analysis. The initial amount of RNA used in the RT-PCR was adjusted spectrophotometrically. RT-PCR for β-actin was performed as a control experiment (Fig. 7c). As shown in Fig. 7a, a single band of 606 bp corresponding to chicken ERα size, but not genomic DNA size was detected in the sympathetic ganglia as well as the diencephalon. The expression of ERα mRNA in the ganglia was greater in mature birds than in immature birds (Fig. 7a). Serial Southern hybridization confirmed that this band was ERα mRNA specific (Fig. 7b). In addition, RT-PCR/Southern blot analysis for ERβ identified a single band of 315 bp corresponding to quail ERβ mRNA in the ganglia as well as the diencephalon (Fig. 7c and d). The ganglionic expression of ERβ mRNA was also greater in mature birds than in immature birds (Fig. 7c and d). Furthermore, the

![Figure 4](image-url) Quantitative analyses for developmental changes in number of galanin-immunoreactive cells per unit area in the quail lumbosacral sympathetic ganglion (LS2, uterine side) (a) and soma area of galanin-immunoreactive cells (b). Each column and vertical line represents the mean ± S.E.M. (four samples from four different birds at each age). Data derived from randomly selected sections in each sample. **$P<0.01$ vs 4-week group, ††$P<0.05$, †††$P<0.01$ vs 6-week group or 10-week group. Duncan’s multiple range test.
expression of ERα mRNA tended to be greater than that of ERβ mRNA in immature birds (Fig. 7).

In the final experiment, ganglionic localization of ER was immunocytochemically examined with the anti-human ERα monoclonal antibody raised in rats. Immunoreactive cells were scattered throughout the LS2 ganglion of immature birds at 3 weeks of age (Fig. 8a). As shown in Fig. 8b, a most intense immunoreaction was concentrated in the large cell nuclei. Controls in which normal rat serum was substituted for the antibody against ERα showed no immunoreactivity in the LS2 ganglion (Fig. 8c and d). Interestingly, the antibody against ER recognized galanin-immunoreactive cells in this ganglion of mature birds (Fig. 9a and b).

Figure 5 Effects of oestradiol-17β (E2) or progesterone (P4) on protein content in quail lumbosacral sympathetic ganglia (LS1–LS7, uterine side) (a), galanin concentration per mg ganglionic protein (b) and galanin content per ganglion (c). Each sex steroid was administered to immature quails at 3 weeks by a SILASTIC plate for 1 week. Control quails were implanted with only SILASTIC adhesive. Each column and vertical line represents the mean ± S.E.M. (five samples: one sample from five quails). *P<0.05, **P<0.01 vs vehicle group, †P<0.05 E2 group vs P4 group. Kruskal-Wallis test followed by Mann-Whitney U test.

Figure 6 Quantitative analyses for effects of oestradiol-17β (E2) on number of galanin-immunoreactive cells per unit area in the quail lumbosacral sympathetic ganglion (LS2, uterine side) (a) and soma area of galanin-immunoreactive cells (b). Each column and vertical line represents the mean ± S.E.M. (four samples from four different birds in each group). Data derived from randomly selected five sections in each sample. **P<0.01 vs vehicle group. Student’s t-test.
The efferent innervation of galaninergic neurons of the lumbosacral sympathetic ganglia to uterine muscles may play an important role in avian oviposition (Sakamoto et al. 2000), because avian galanin acts directly on the quail uterus to cause contractions of the uterine musculature and consequently evokes oviposition (Li et al. 1996, Tsutsui et al. 1997, 1998). The present results provide a detailed profile of developmental changes in avian galanin levels not only in the neuronal somata located in the quail lumbosacral sympathetic ganglia (LS1–LS7) of the uterine side but also in the uterus containing the neuronal terminals. Ganglionic galanin was detectable in immature quails at 4 weeks of age, but the level was extremely low. Such birds responded to long day photoperiods by showing both increases in the concentration and content of ganglionic galanin, concomitant with ganglionic development. From the results of the present immunocytochemical analysis, the following three explanations for the changes in ganglionic galanin during development may be possible: (1) the number of ganglionic cells expressing galanin increased, (2) the soma area of ganglionic cells expressing galanin increased, and (3) the galanin expression per ganglionic cell increased. In addition, the developmental change in ganglionic galanin levels was associated with an increase in the uterine galanin content. The unexpected high concentration of uterine galanin in immature birds at 4 weeks of age may be due to the much smaller tissue mass. We have previously demonstrated that lumbosacral sympathetic ganglionic neurons localized in the LS1–LS7 ganglia (mainly LS2 ganglion) of the uterine side project to uterine muscles and produce avian galanin in mature quails (Sakamoto et al. 2000). Taken together, avian galanin may increase in both somata and terminals of the lumbosacral sympathetic ganglionic neurons innervating the uterine muscle during development. Such an increase in avian galanin may be essential for avian oviposition.

In the present study, hormonal administration to immature birds at 3 weeks of age was then performed to identify the hormonal factor(s) inducing the increase in galanin during development. It has been confirmed that plasma oestradiol–17β and progesterone concentrations in the immature quails before 4 weeks of age are negligible (Tsutsui et al. 1998). Although oestradiol–17β and progesterone individually induced an increase in galanin in the lumbosacral sympathetic ganglia (LS1–LS7, uterine side), the stimulatory effect of oestradiol–17β was much greater than that of progesterone. Accordingly, it is probable that ovarian sex steroids, in particular oestradiol–17β, contribute as hormonal factors to galanin induction in the lumbosacral sympathetic ganglionic neuron during development. This hypothesis may be partly supported by our previous results showing an increase in circulating levels of these steroids in the quail during development (Tsutsui et al. 1998). In addition, plasma steroid levels in steroid-treated immature quails were in the proximity of the maximal levels observed in mature birds exposed to long day photoperiods (Tsutsui et al. 1998), suggesting that the levels may be within the physiological range. Thus, the increase in these circulating sex steroids during development may be a possible cause of the induction of ganglionic galanin. However, sex steroids did not influence the growth of lumbosacral sympathetic ganglionic neurons. Accordingly, the steroid effect on ganglionic neurons may be specific for galanin induction. In addition, other factors might also contribute to galanin induction, as the ganglionic galanin level in mature birds was higher than that in immature birds treated with oestradiol–17β.
To understand the mode of steroid action on ganglionic galanin induction, we further analysed the expression and localization of ERs in the ganglia. The present RT-PCR analysis together with Southern hybridization revealed expression of ERα and ERβ mRNAs in the immature ganglia. The expression of ERα mRNA, which tended to be higher than that of ERβ mRNA, was localized immunocytochemically in ganglionic cells. It is well known that ER, one of several nuclear proteins, functions as a ligand-activated transcription factor (Evans 1988, Katzenellenbogen 1996). The present immunocytochemical study also revealed an intense immunoreaction for ERα in the large nuclei of ganglionic neurons in the immature bird. Ganglionic neurons may express ER prior to galanin, because galanin-immunoreactive cells were few in immature birds. In contrast, most galanin-immunoreactive cells possessed ER-immunoreactivity in mature birds. Consequently, it is possible that oestradiol acts directly on ganglionic neurons through intranuclear receptor-mediated mechanisms to induce galanin expression. This genomic action of oestradiol possibly enables ganglionic galanin induction, which occurs during development. On the other hand, progesterone also induced an increase of ganglionic galanin, although the stimulatory effect was much lower. Further study is required to draw a firm conclusion concerning progesterone action on ganglionic galanin induction.

A similar effect of ovarian sex steroids on the induction of galanin mRNA has been reported in the rat central nervous system (Rossmanith et al. 1996). According to Rossmanith et al., oestradiol-17β was the primary ovarian signal inducing galanin mRNA expression in gonadotrophin-releasing hormone (GnRH) neurons. In addition, progesterone facilitated the action of oestradiol-17β on galanin gene expression in GnRH neurons (Merchenthaler et al. 1991, Rossmanith et al. 1996). It has also been reported that galanin neurons exhibit oestrogen receptors in the rat hypothalamus (Horvath et al. 1995). Thus, it may be that these sex steroids act as important hormonal factors to induce galanin in both the central and peripheral nervous systems. In addition to these nervous systems, it has been reported that oestradiol induces galanin gene expression in the mouse pituitary in an ERα-dependent manner (Shen et al. 1999). On the other hand, we have recently demonstrated that oestradiol-17β and progesterone induce galanin receptors in the quail uterus (Tsutui et al. 1998). Accordingly, actions of ovarian sex steroids may be essential for the evoking of oviposition through mechanisms inducing both galanin and its receptors in birds.

Figure 8  Immunocytochemical staining with the antibody to human ERα raised in rats (a and b) or with the normal rat serum (c and d) in the quail lumbosacral sympathetic ganglion (LS2, uterine side) at 3 weeks of age. (a) and (c) are of the same low magnification (bar represents 50 μm). (b) and (d) are of the same high magnification of blocked areas in (a) and (c). The arrow indicates the stained large cell nucleus (b). Similar results were obtained in repeated experiments using four different birds in each group.

Figure 9  Immunocytochemical staining with the antibody to human ERα (a) or with the anti-galanin serum (b) on the same section of the quail lumbosacral sympathetic ganglion (LS2, uterine side) at 3 months of age. (a) and (b) are of the same magnification (bar represents 20 μm). Similar results were obtained in repeated experiments using four different birds.
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References