

Identification and Structural Characterization of the Neuronal Luteinizing Hormone Receptor Associated with Sensory Systems*

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The luteinizing hormone receptor (LHR) is a G protein-coupled receptor involved in regulation of ovarian and testicular functions. Here we show that the receptor is present also in specific areas of the peripheral and central nervous system and may thus have a broader functional role than has been anticipated. Full-length LHR mRNA and two receptor protein species of M_r 90,000 and 73,000, representing mature and precursor forms, respectively, were expressed in adult and developing rat nervous tissue, starting at fetal day 14.5. The receptor was capable of ligand binding because it was purified by ligand affinity chromatography, and human chorionic gonadotropin and LH were able to displace 125 I-labeled human chorionic gonadotropin binding to fetal head membranes in a dose-dependent manner. Finally, two 5'-flanking sequences (~2 and 4 kb) of the rat LHR gene were shown to direct expression of the *lacZ* reporter to specific areas of the peripheral and central nervous system in fetal and adult transgenic mice, especially to structures associated with sensory, memory, reproductive behavior, and autonomic functions. Importantly, the transgene activity was confined to neurons and colocalized with the cytochrome P450 side chain cleavage enzyme. Taken together, these results indicate that the neuronal LHR is a functional protein, implicating a role in neuronal development and function, possibly by means of regulating synthesis of neurosteroids.

The G protein-coupled receptors (GPCRs)¹ constitute one of the largest gene families in the mammalian genome and are involved in almost all major physiological events from embryonic development to neurotransmission (1, 2). Like other GPCRs, the leucine-rich repeat-containing GPCRs (LGRs) are

integral membrane proteins with seven transmembrane domains but diverge structurally in having a large extracellular N-terminal domain that is involved in ligand binding (3, 4). In mammals this GPCR subfamily contains glycoprotein hormone receptors for luteinizing hormone (LH), chorionic gonadotropin (CG), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) (designated LH receptor (LHR), FSHR, and TSHR, respectively) as well as several recently identified novel receptors, most of which are still orphans (5–9). These novel LGRs appear to have a wide and divergent distribution, which implicates roles in reproductive, renal, cardiovascular, and brain functions as well as in tissue and organ development. In contrast, the more canonical LGRs, the LHR, FSHR, and TSHR, have been thought to display a more restricted tissue distribution, being essential in regulating development and function of the gonads (LHR and FSHR) and the thyroid gland (TSHR) (10–12). LHR, however, appears to be expressed in extragonadal tissues as well because increasing evidence suggests that the receptor may be present also in the reproductive tract (13–15), placenta (13), and umbilical cord (16). In addition, the receptor has been shown to be expressed in the nervous tissue because the rat, bovine, and human brains were reported to contain mRNA transcripts for LHR (17). Nevertheless, the existence of LHR protein in the nervous tissue has remained controversial and, furthermore, the developmental changes in the neuronal LHR expression have not been investigated thoroughly.

Here we demonstrate that the LHR mRNA is present in rat nervous tissue and is translated into receptor protein that is capable of specific high affinity ligand binding. Two receptor species of M_r 90,000 and 73,000 were expressed in the brain, representing mature and precursor receptor forms, respectively. In addition, rat LHR promoter fragments of 2,060 and 3,970 bp were shown to direct expression of the *lacZ* reporter to spatially restricted areas of the peripheral and central nervous system in fetal and adult transgenic mice, particularly to regions involved in olfaction and other sensory functions. The transgene activity colocalized with the cytochrome P450 side chain cleavage enzyme (P450_{sc}) to the same neurons, suggesting a putative regulatory role for the LHR in neurosteroid synthesis. The LHR, therefore, has a wider tissue distribution and possibly more diverse functional roles than has been anticipated previously.

EXPERIMENTAL PROCEDURES

Animals and Tissue Preparation—Sprague-Dawley rats were used for preparing tissues for RT-PCR, receptor purification, and ligand binding assays. To time the developmental stage of the fetuses, females were housed with males, and the following morning was designated day 0.5 of pregnancy (0.5 days postcoitum, dpc), when the vaginal plug was detected. Immature female rats (25–27 days old) were rendered pseudopregnant as described previously (18). The pregnant and pseudopreg-

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¹ The abbreviations used are: GPCR(s), G protein-coupled receptor(s); CG, chorionic gonadotropin; dpc, days postcoitum; FSH, follicle-stimulating hormone; h, human; LGR, leucine-rich repeat-containing GPCR; LH, luteinizing hormone; NeuN, neuronal nuclei; P450_{sc}, cytochrome P450 side chain cleavage enzyme; R, receptor; RT, reverse transcription; TSH, thyroid-stimulating hormone; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

nant females as well as adult males (90 days) were killed by inhalation of carbon dioxide, and the tissues were removed immediately, frozen in liquid nitrogen, and stored at -80°C . The use of animals was approved by the University of Oulu committee for the care of experimental animals.

RT-PCR—Four oligonucleotide primers of 29–36 nucleotides were designed based on the rat LHR sequence (GenBank accession M26199 (19)), as depicted in Fig. 1A. Primers 1 (nucleotides -27 to $+9$ from translation initiation site) and 2 (nucleotides $+2116$ to $+2084$) were used for RT-PCR and primers 3 (nucleotides $+65$ to $+97$) and 4 (nucleotides $+1887$ to $+1858$) for the subsequent nested PCR.

Total RNA was extracted from rat tissues using TriZol reagent (Invitrogen), and the RT-PCR was performed as described previously (20), using modified conditions. The reaction was carried out in a total volume of $50\ \mu\text{l}$ using $1\ \mu\text{g}$ of total RNA as template and $0.2\ \mu\text{M}$ primers 1 and 2. The reaction mixture contained 50 units of Superscript II reverse transcriptase (Ambion), $200\ \mu\text{M}$ deoxynucleotide triphosphates (Finnzymes), $1.5\ \text{mM}$ MgCl_2 , 10 units of avian myeloblastosis virus reverse transcriptase (Promega), and 2.5 units of recombinant *Taq* DNA polymerase (Invitrogen) in addition to PCR buffer (Invitrogen). After RT reaction (42°C , 60 min), the samples were run in PCR for 30 cycles (95°C for 1 min, denaturation; 60°C for 2 min, annealing; 72°C for 5 min $+1$ min/cycle, extension). For the second amplification round, $1\ \mu\text{l}$ of the product from the first amplification was used as a template with the slightly intended primers 3 and 4 in the same PCR conditions as described above. The $10\text{-}\mu\text{l}$ PCR products were analyzed on a 1% agarose gel containing ethidium bromide.

Immunoprecipitation and Ligand Affinity Chromatography—Total cellular membranes were prepared from rat tissues by homogenizing with a Polytron homogenizer (Ultra-Turrax T-25, Janke & Kunkel) in buffer A (phosphate-buffered saline, pH 7.5, containing 5 mM EDTA, 5 mM *N*-ethylmaleimide, and 0.2 mM phenylmethylsulfonyl fluoride ($\sim 100\ \text{mg/ml}$) and centrifuging at $100 \times g$ for 10 min. The membrane particles were then collected by centrifuging the supernatant for 30 min at $27,000 \times g$. After washing with buffer A, the protein concentration in each sample was determined by a Bio-Rad DC assay kit using bovine serum albumin as the standard. Membranes from the pseudopregnant rat ovary (1.7 mg), 14.5-dpc fetus head (5.5 mg), 19.5-dpc fetus head (23.6 mg), adult rat thalamus (14 mg) and muscle (20 mg) were then solubilized by stirring on ice for 60 min in $500\ \mu\text{l}$ of buffer B (buffer A containing 0.5% (w/v) Triton X-100 and 20% (v/v) glycerol) and centrifuging at $100,000 \times g$ for 60 min. The soluble fraction was supplemented with 0.1% (w/v) bovine serum albumin and subjected to immunoprecipitation or ligand affinity chromatography.

Immunoprecipitation was performed using a purified anti-rabbit antibody directed against the C-terminal domain of the rat LHR (Gly⁶²⁰–His⁶⁷⁴).² After adding the antibody ($5\ \mu\text{g/ml}$) and $20\ \mu\text{l}$ of protein G-Sepharose (Amersham Biosciences), the samples were incubated overnight at 4°C with gentle agitation. The resin was washed twice with $500\ \mu\text{l}$ of buffer B, twice with $500\ \mu\text{l}$ of buffer C (buffer A containing 0.1% (w/v) Triton X-100, 20% (v/v) glycerol, and 0.36 M NaCl), and twice with $500\ \mu\text{l}$ of buffer D (buffer A containing 0.1% (w/v) Triton X-100 and 20% (v/v) glycerol). The receptors were eluted by incubating the resin for 5 min at 95°C in SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue).

Ligand affinity chromatography was performed using hCG-Affi-Gel 10, which was prepared as described previously (18). The solubilized receptors were incubated with $20\ \mu\text{l}$ of the resin overnight at 4°C , washed, and eluted as described above.

Deglycosylation of the Immunoprecipitated LHRs—Immunoprecipitated receptors from the pseudopregnant rat ovary (1.1 mg of membrane protein) and the 19.5-dpc fetus head (7.9 mg of membrane protein) were deglycosylated after elution from protein G-Sepharose with 1% (w/v) SDS, 50 mM sodium phosphate, pH 7.5. Before the enzyme reaction, the eluates were diluted 5-fold with 0.5% (w/v) Triton X-100, 50 mM sodium phosphate, pH 5.5, 50 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 2 mM 1,10-phenanthroline (Endoglycosidase H, Roche Applied Science) or 0.5% (w/v) Triton X-100, 50 mM sodium phosphate, pH 7.5, 50 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 2 mM 1,10-phenanthroline (peptide *N*-glycosidase F, Roche Applied Science). The enzymes were added at final concentrations of 60 milliunits/ml (Endoglycosidase H) and 20 units/ml (peptide *N*-glycosidase F). Samples were incubated at 30°C for 16 h, and the reaction was terminated by adding SDS-sample buffer.

Western Blot Analysis—Immunoprecipitated and ligand affinity-purified

receptors were reduced by heating for 2 min at 95°C in the presence of 50 mM dithiothreitol before SDS-PAGE (4% stacking gels and 7.5% separating gels). The separated proteins were electroblotted onto Immobilon P membrane (Millipore) and probed with the anti-rabbit antibody directed against the C-terminal domain of the rat LHR ($0.12\ \mu\text{g/ml}$).² A horseradish peroxidase-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) and enhanced chemiluminescence Western blotting detection reagents from Amersham Biosciences were used to reveal the blotted proteins. The molecular mass markers (Bio-Rad) were detected by staining with Ponceau S.

Radioligand Binding Assays—The hormone binding characteristics of the membrane-bound rat LHRs were determined by competition binding experiments. Total cellular membranes were prepared as described above, and samples from the pseudopregnant rat ovary ($6.5\ \mu\text{g}$) and the 19.5-dpc fetus head ($285\ \mu\text{g}$) were incubated with $1.8\ \text{nM}$ [^{125}I]hCG in the absence or presence of increasing concentrations of unlabeled hCG ($9\ \text{fM}$ – $70\ \text{nM}$), hLH ($9\ \text{fM}$ – $75\ \text{nM}$), or hFSH ($36\ \text{nM}$) in a final volume of $150\ \mu\text{l}$ of buffer A containing 0.1% (w/v) bovine serum albumin for 60 min at 37°C . Thereafter, the membranes were collected by centrifugation at $4,000 \times g$ for 30 min, washed twice with phosphate-buffered saline, and counted for ^{125}I radioactivity. Nonspecific binding was determined in the presence of an excess of unlabeled hCG ($350\ \text{nM}$). Analysis of the binding data was performed using the GraphPad Prism version 3.02. The hCG was radioiodinated with Na^{125}I (Amersham Biosciences) by the chloramine-T method as described previously (18). The radiolabeled hCG ($16,600\ \text{units/mg}$) and the unlabeled hormones used for competition (hCG, $6,300\ \text{units/mg}$; hLH, $11,000\ \text{units/mg}$ and hFSH, $7,000\ \text{units/mg}$) were from Sigma.

Promoter Constructs and Production and Analysis of the Transgenic Mice—The rat LHR promoter constructs (see Fig. 4A) carried genomic EcoRI-XhoI fragments of the 5'-flanking region ($-2,060$ or $-3,970$ bp from translation initiation site) of the rat LHR gene in front of the reporter gene in the *lacZ* vector pNASS β (Clontech). The constructs were released from the vector sequences by EcoRI, SalI or SalI, PstI digestion, and the gel-purified constructs were microinjected into pronuclei of (C57BL/6 \times DBA)F2 mouse zygotes (21), after which they were transferred into the oviduct of pseudopregnant NMRI mice. Founder animals were identified from the tail DNA by PCR analysis using *lacZ*-specific primers (22). 22 transgenic founder mice were mated with C57BL/6 mice for litter production, and 11 of these produced offspring expressing the transgene.

Fetuses and tissue samples from the adult mice were collected as described above and fixed in 0.2% (v/v) glutaraldehyde, 5 mM EGTA, 2 mM MgCl_2 , 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) at 4°C for 1–24 h depending on the sample size. The fixed samples were washed three times for 15 min with the washing buffer (5 mM EGTA, 2 mM MgCl_2 , 0.01% (w/v) deoxycholate, 0.02% (v/v) Igepal CA-630 (Sigma) in 0.1 M phosphate buffer, pH 7.3) and stained overnight in 0.1% (w/v) 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal, MBI Fermentas), 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 5 mM $\text{K}_4\text{Fe}(\text{CN})_6 \times 3\ \text{H}_2\text{O}$ in the washing buffer. After staining, the samples were washed and postfixed overnight in 4% (w/v) paraformaldehyde in phosphate-buffered saline and observed under a stereoscope as whole mounts or, alternatively, were frozen or embedded into paraffin. The frozen samples were sectioned at $5\ \mu\text{m}$ and counterstained with safranin. The paraffin-embedded tissue samples were sectioned at $10\ \mu\text{m}$ and used for immunohistochemistry. A mouse monoclonal antibody for neuronal nuclei (NeuN, Chemicon International) and a polyclonal rabbit antibody for bovine S-100 protein (DakoCytomation) were used to detect neurons and glial cells, respectively. A polyclonal rabbit antibody for the P450scc (Chemicon International) was used to detect steroidogenic cells. Subsequent biotin-streptavidin-peroxidase complex detection systems for the S-100, NeuN, and P450scc antibodies were used according to the instructions of the manufacturers.

RESULTS

Detection of the LHR mRNA in Developing and Adult Rat Tissues—To evaluate the overall spatial and temporal tissue expression pattern of the LHR, total RNA was isolated from adult and fetal rat tissues and subjected to RT-PCR, followed by nested PCR for amplification of the products. The primers used (Fig. 1A) were chosen to flank the entire coding region of the LHR to amplify cDNAs with full-length open reading frames. To assess the expression in the fetal brain, the head, middle trunk, and lower trunk of the 14.5-dpc and the 19.5-dpc fetuses were isolated to separate the brain from the developing

² J. T. Aatsinki and H. J. Rajaniemi, unpublished data.

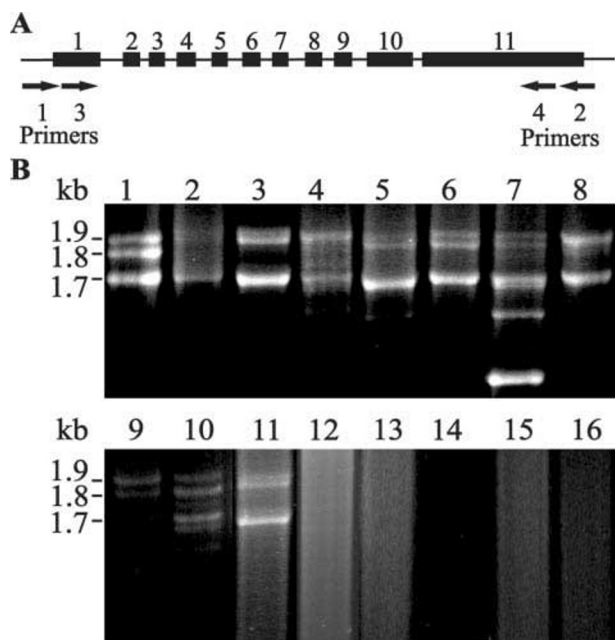


FIG. 1. Characterization of the LHR mRNA expression in rat tissues. A, oligonucleotide primers used in the RT-PCR analysis of the rat LHR mRNA. The positions of the primers are related to the diagram of the rat LHR gene. Exons 1–10 and the 5'-end of the exon 11 code for the extracellular domain, whereas the rest of exon 11 encodes the transmembrane and the C-terminal intracellular domains of the receptor. B, RT-PCR analyses of the total RNA. The analyses were performed with primers flanking the entire coding region of the LHR to amplify the full-length cDNAs followed by nested PCR with the intended primers, as described under "Experimental Procedures." Reactions without RNA and ovarian RNA without RT reactions were used as negative reaction controls. The samples were from adult rats unless otherwise indicated. Lane 1, testes; lane 2, 19.5-dpc fetal lower trunk; lane 3, 19.5-dpc fetal middle trunk; lane 4, 19.5-dpc fetal head; lane 5, 14.5-dpc fetal head; lane 6, trigeminal ganglion; lane 7, pituitary; lane 8, thalamus; lane 9, olfactory bulb; lane 10, placenta; lane 11, ovary; lane 12, spinal cord; lane 13, muscle; lane 14, liver; lane 15, PCR (ovary) without RT; lane 16, RT-PCR without RNA. The major amplified mRNA products are indicated on the left.

gonads, which in male fetuses start to express the full-length LHR mRNA at 15.5 dpc (23).

Fig. 1B shows the expression pattern of the LHR transcripts isolated from the various adult and fetal tissues. Three major products of 1.7, 1.8, and 1.9 kb were obtained from the adult testis (lane 1), ovary (lane 11), and placenta (lane 10), whereas muscle (lane 13) and liver (lane 14) showed no amplification of the LHR mRNA. The 1.9- and 1.7-kb species correspond to the full-length LHR and the most common receptor isoform, respectively (24–26), whereas the 1.8-kb species most likely represents a hybrid of the 1.9- and 1.7-kb products (27). Because of the nested primers used in the second amplification run, the final products were slightly (~200 bp) smaller than the original cDNA. The three major mRNA products were also obtained from the adult nervous tissue (e.g. from trigeminal ganglion, thalamus, olfactory bulb, and pituitary; lanes 6, 8, 9, and 7, respectively), but the spinal cord showed no amplification of the LHR mRNA (lane 12). The three major products were also amplified from the heads of the 14.5-dpc (lane 5) and the 19.5-dpc (lane 4) fetuses. However, the amount of the LHR mRNA transcripts in the nervous system appears to be very low because the transcripts were detected only after the second amplification round. Interestingly, several smaller mRNA transcripts were also observed, possibly representing developmental stage- or tissue-specific isoforms resulting from alternative splicing of the primary transcript (23–25, 27–29). This was especially evident in the pituitary (lane 7), which exhibited

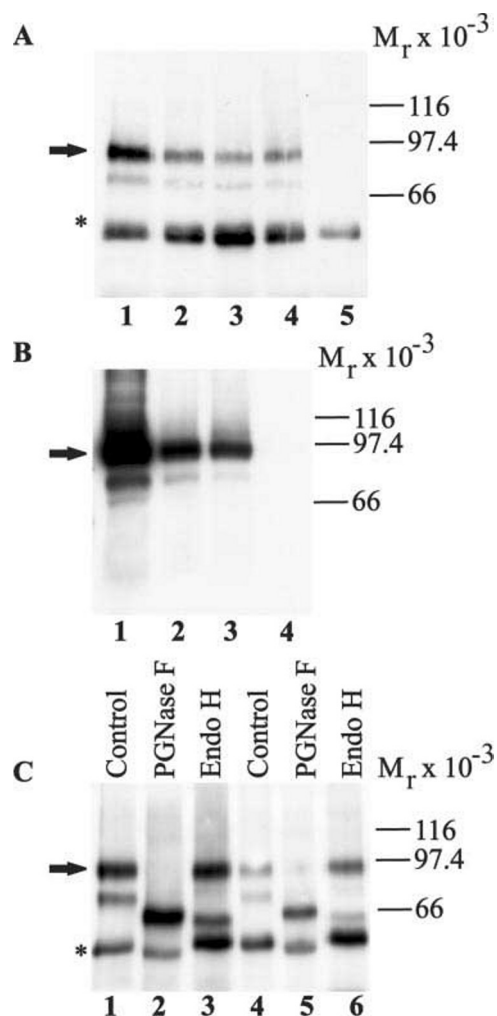


FIG. 2. Characterization of the LHR forms expressed in the fetal and adult rat nervous tissue. Solubilized cellular membranes from the pseudopregnant rat ovary (A1, B1, C1–3), 19.5-dpc fetal head (A2, B2, C4–6), 14.5-dpc fetal head (A3), adult thalamus (A4, B3), and muscle (A5, B4) were subjected to immunoprecipitation (A, C) or ligand (hCG) affinity chromatography (B) as described under "Experimental Procedures." Immunoprecipitated samples (C) were subjected to glycosidase treatment for 16 h at 30 °C with 20 units/ml peptide N-glycosidase F (lanes 2 and 5) or 60 milliunits/ml Endoglycosidase H (lanes 3 and 6). The control samples contained only buffer (lanes 1 and 4). The samples were analyzed by Western blotting using a polyclonal antibody directed against the C terminus of the receptor. The asterisk indicates immunoglobulin heavy chains, and the arrow points to the M_r 90,000 LHR. Molecular mass markers are indicated on the right.

unique sized mRNAs compared with the other brain areas. Taken together, the LHR appears to be expressed at the mRNA level in several regions of the developing and adult nervous system in a manner similar to that of the LGR5–8 (5–9).

Detection of LHR Protein in Rat Nervous Tissue—The results shown suggest that the full-length LHR mRNA is expressed in both the adult and fetal rat nervous tissue, albeit at a low level. To determine whether the receptor is also expressed at the protein level, Triton X-100-solubilized membranes were subjected to immunoprecipitation using a LHR-specific polyclonal antibody directed against the C-terminal domain of the protein. The immunoprecipitated samples were then analyzed by Western blotting using the same antibody for detection.

As shown in Fig. 2A, the LHR-specific antibody immunoprecipitated two bands with apparent molecular masses of 90,000 and 73,000 from the pseudopregnant rat ovary (lane 1), which were not detected when the antibody was replaced with a nonimmune serum (data not shown). This indicates that they

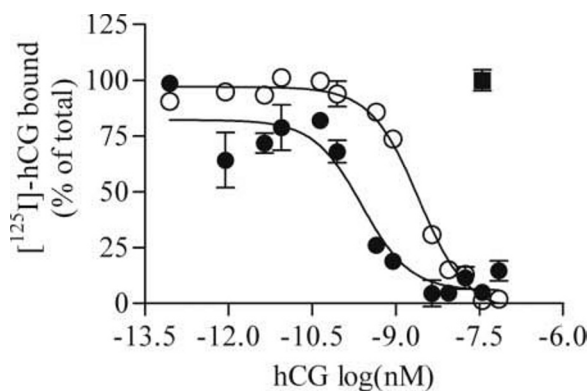


FIG. 3. **Competitive displacement of [125 I]hCG binding to the 19.5-dpc fetal rat head membranes.** Membranes (285 μ g/150 μ l) were incubated for 60 min at 37 $^{\circ}$ C with 1.8 nM [125 I]hCG and the indicated concentrations of unlabeled hCG (●), hLH (○), or hFSH (■), as described under "Experimental Procedures." Nonspecific binding was determined in the presence of an excess (350 nM) of unlabeled hCG. Binding in the absence of the unlabeled hormone was regarded as 100%. Data are representative of four separate experiments with similar results; each point represents the mean \pm S.E. of triplicate values.

specifically represent the expressed LHR. Furthermore, the two bands were not immunoprecipitated from muscle (lane 5), in accordance with the absence of the LHR mRNA in this tissue (see Fig. 1B, lane 13). The two LHR species of M_r 90,000 and 73,000 were also immunoprecipitated from the 14.5-dpc (lane 3) and the 19.5-dpc fetus heads (lane 2) as well as from the adult thalamus (lane 4). Importantly, the two receptor species were also purified by ligand affinity chromatography (Fig. 2B), indicating that the LHR expressed in the nervous system is capable of ligand binding.

The data presented above show that two different LHR forms were immunoprecipitated from the rat ovaries and nervous tissue. To find out whether these two forms differ in their post-translational modifications, *i.e.* carbohydrates, the immunoprecipitated receptors were subjected to glycosidase treatments. Endoglycosidase H reduced the molecular mass of the M_r 73,000 LHR to about 62,000 (Fig. 2C, lanes 3 and 6), indicating that it represents the full-length receptor bearing high mannose-type oligosaccharides because Endoglycosidase H selectively removes unprocessed high mannose type oligosaccharides from glycoproteins (30). The resistance of the M_r 90,000 receptor species to the Endoglycosidase H treatment suggests that this species contains complex-type *N*-linked oligosaccharides (Fig. 2C, lanes 3 and 6). This interpretation was supported by the fact that peptide *N*-glycosidase F, an enzyme that can remove all types of *N*-linked oligosaccharides from glycoproteins (30), was able to digest the M_r 90,000 receptor (Fig. 2C, lanes 2 and 5). Taken together, these results demonstrate that the LHR protein is expressed in the adult and fetal rat nervous system. The M_r 90,000 and 73,000 receptor forms contain either fully or partially processed oligosaccharides, respectively, thus representing mature and precursor forms of the receptor. Furthermore, the data indicate that the receptor protein is processed in a similar manner in the ovaries and in the neuronal tissue.

Characterization of the Ligand Binding Ability of the Rat LHR Expressed in the Fetal Nervous Tissue—The ability of the neuronal LHR to bind ligand was tested directly in competition binding experiments by incubating crude membrane particles from the 19.5-dpc fetal rat heads with [125 I]hCG in the presence of an increasing amount of unlabeled hCG, hLH, or hFSH. As expected, both hCG and hLH inhibited binding of [125 I]hCG in a dose-dependent manner with half-maximal inhibition of binding obtained at concentrations of 0.10 ± 0.05 (mean \pm

S.E.) nM and 2.63 ± 0.46 nM, respectively (Fig. 3). In contrast, hFSH was not able to displace [125 I]hCG binding, confirming the hormone binding specificity of the neuronal LHR. The inhibitory dissociation constant (K_i) obtained for hCG (0.08 ± 0.04 nM) was in good agreement with that reported previously for rat gonadal LHRs (12, 31). The maximal binding capacity (B_{max}) for hCG, determined from the homologous competition experiments, was 31.5 ± 1.1 fmol/mg membrane protein in the fetal heads compared with 3.68 ± 0.22 pmol/mg in the pseudo-pregnant rat ovaries.

LHR Promoter-driven Transgene Expression in the Fetal and Adult Mouse Nervous System—To examine regional and cell type-specific expression of the LHR in the fetal and adult nervous tissue in more detail, we generated transgenic mice, which carried the *Escherichia coli lacZ* gene under the control of the rat LHR promoter fragments of 2,060 and 3,970 bp in length (Fig. 4A). These two promoter fragments were chosen because the basal activity of the rat LHR gene is known to involve ~ 180 nucleotides, and sequences further upstream possess elements directing the cell- and tissue-specific basal promoter activity (32–34). The promoter constructs were microinjected into fertilized mouse eggs, and the progenies were tested for transgenicity from the tail DNA with the *lacZ*-specific PCR screening. The expression of the *lacZ* gene was examined by whole-mount staining of the fetuses and adult tissues with the chromogenic substrate X-gal.

The 2,060- and 3,970-bp LHR promoter fragments were able to direct expression of the *lacZ* gene in three and eight transgenic mouse lines, respectively. All of these expressed β -galactosidase in the gonads in a similar manner,³ indicating that the two promoter fragments contain all of the necessary elements required for β -galactosidase expression in cells and tissues that are known to express the LHR. A representative mouse line expressing the *lacZ* gene under the 3,970-bp LHR promoter was chosen for further analyses.

Whole-mount staining of the developing mouse fetuses revealed that the transgene was first expressed in the sensory ganglia (*e.g.* in trigeminal ganglia) in the 11.5-dpc fetuses (Fig. 4B). The nontransgenic littermates showed no β -galactosidase activity (data not shown). During the next 3 days, the expression levels increased throughout the developing nervous system, and in the 14.5-dpc fetuses (Fig. 4C), the β -galactosidase activity was clearly apparent in cranial and spinal ganglia and in the peripherally and centrally outgrowing nerve fibers. Distinct expression was also apparent in the developing olfactory bulb and the thalamic and brain stem areas. The sympathetic trunk and the autonomic nerves heading, *e.g.* to the stomach wall showed expression as well. In the 16.5-dpc fetuses (Fig. 4D), the expression had decreased in the sensory ganglia but was still clearly detectable in the peripherally and centrally outgrowing nerves. This was particularly clear in the 1-day-old neonates (Fig. 4E). No differences among female and male fetuses were observed. These results indicate that during organogenesis, the transgene expression was clearly dependent on the developmental stage of the fetus, the most distinct expression of β -galactosidase appearing in the ganglia of the sensory and autonomic systems and in the olfactory bulb.

To gain a more comprehensive view of the transgene expression in the brain, isolated whole brains of fetal and adult mice were examined. β -Galactosidase expression was apparent in the thalamic and brain stem structures of the 14.5-dpc fetuses (Fig. 5A) but seemed to have decreased by 16.5 dpc (Fig. 5B), except in the olfactory bulb. In the adult, the most distinct

³ P. M. Apaja, U. E. Petäjä-Repo, and H. J. Rajaniemi, unpublished data.

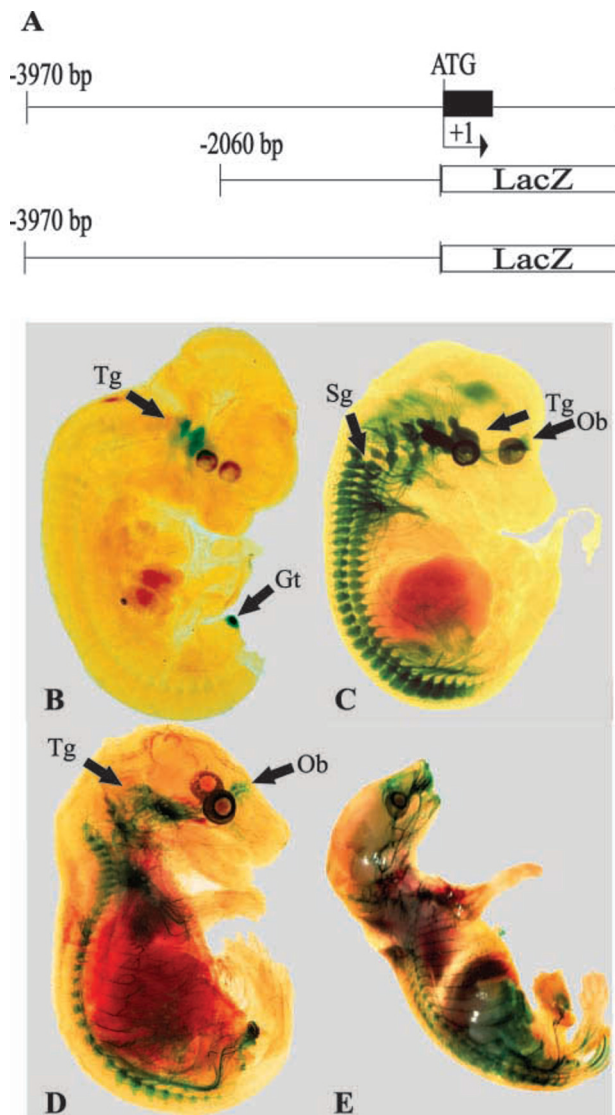


FIG. 4. Expression of the LHR promoter-driven transgene in mouse fetuses. A, LHR promoter-*lacZ* reporter gene constructs. The constructs contained either 2,060 kb or 3,970 kb of the 5'-flanking region of the LHR gene in front of the *lacZ* gene. The translation initiation site (ATG) and the first exon-intron boundary of the LHR gene are presented at the top. B, β -galactosidase expression in mouse fetuses. Whole-mount fetuses of age 11.5 dpc (B), 14.5 dpc (C), and 16.5 dpc (D), and a 1-day-old neonate (E) were analyzed for β -galactosidase activity of the rat LHR promoter-*lacZ* construct by the *in situ lacZ* enzyme assay, as described under "Experimental Procedures." The intestinal staining in the 1-day-old neonate is caused by endogenous β -galactosidase activity. Gt, genital tubercle; Ob, olfactory bulb; Sg, spinal ganglion; Tg, trigeminal ganglion.

staining was found in the cortical cell layers of the cerebrum and in the cerebellum, thalamic region, and brain stem areas, like the colliculus inferior and olivary nuclei (Fig. 5, C and D). Examination of the 1.5-mm sagittal slices of the adult brain revealed further that β -galactosidase expression in the cortical cell layers of the cerebrum was localized particularly to the olfactory (piriform and entorhinal cortex), auditory, visual, and somatosensory cortices (Fig. 6, A–D), and the expression appeared to follow the direct connections between the olfactory bulb and entorhinal cortex. In addition, the expression was apparent in the posterior hippocampus, such as CA 4, dentate gyrus, and subiculum (Fig. 6, A–D). Interestingly, the spinal cord was negative, except for a few scattered cells (Fig. 6H), unlike the trigeminal nerves, which showed strong expression (Fig. 6D). Taken together, these results indicate that the trans-

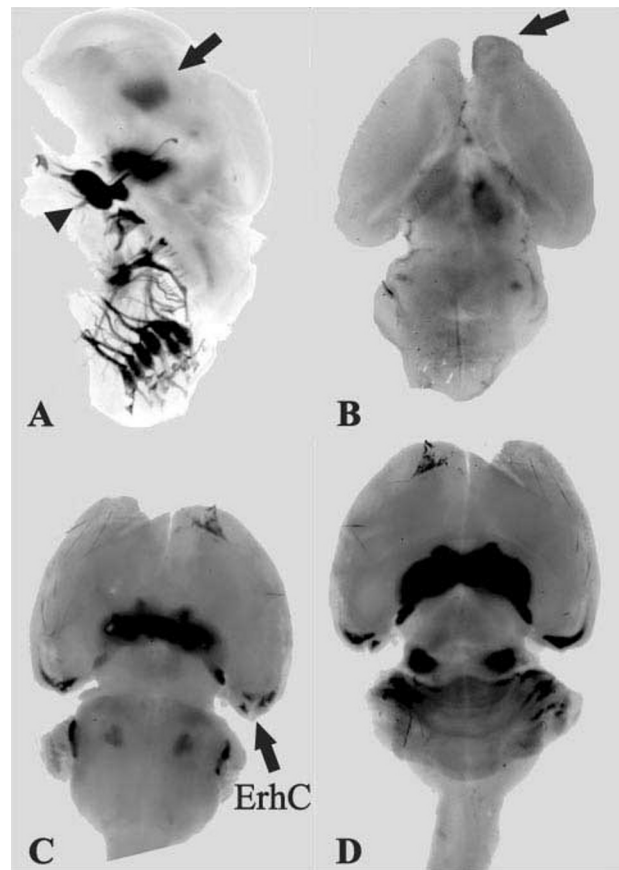


FIG. 5. Distribution of the LHR promoter-driven transgene expression in the mouse brain. The dissected brains of transgenic fetuses and adult mice were stained as described in the legend for Fig. 4. In the 14.5-dpc fetal mouse brain (A, lateral view), the expression could be localized to the developing thalamic area (arrow) as well as to the trigeminal ganglia (arrowhead). The same expression pattern was detected in the 16.5-dpc fetal brain (B, ventral view) but at a lower level. The developing olfactory bulb (B, arrow) showed strong expression. The adult mouse brain (C, ventral view; D, dorsal view) showed distinct staining in the cortical cell layers of the cerebrum and in the cerebellum, thalamic area, and specific areas of the brain stem. ErhC, entorhinal cortex.

gene expression in the brain continues at a varying level until adulthood and is then restricted to specific brain areas involved in olfaction and other sensory functions.

Colocalization of the LHR Promoter-driven Transgene Activity with the Cytochrome P450 Side Chain Cleavage Enzyme Immunoreactivity in Neurons—To assess the LHR promoter-driven β -galactosidase expression in the transgenic mice nervous system at the cellular level, thin sections (5 μ m) were analyzed microscopically. In the trigeminal and spinal ganglia, the β -galactosidase activity was restricted to the ganglia themselves and to the peripherally and centrally outgrowing nerves (Fig. 7, A–D), suggesting that the transgene was expressed in neurons but not in the glial cells. To assess this possibility further, sections of the X-gal-stained paraffin-embedded adult brain were stained for either the glia-specific S-100 (Fig. 7, G and H) or the neuron-specific NeuN (Fig. 7, E and F). No specific S-100 immunoreactivity was detected in cells that showed β -galactosidase activity in the trigeminal ganglion (Fig. 7G) or colliculus inferior (Fig. 7H), whereas NeuN immunoreactivity and β -galactosidase activity colocalized to the same cells (Fig. 7, E and F). This demonstrates that the β -galactosidase positive staining was solely confined to nerve cells.

The gonadotropins LH and hCG regulate steroidogenesis in the ovaries and testes by regulating the expression of steroido-

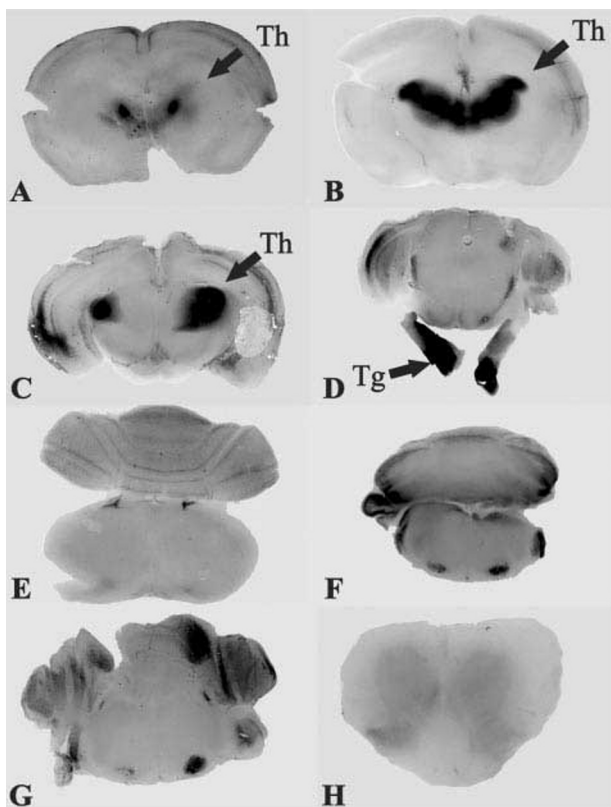


FIG. 6. **Localization of the LHR promoter-driven transgene expression in coronal sections of the adult mouse brain.** 1.5-mm slices proceeding from the frontal part of the brain to the spinal cord are presented as a panel starting from the upper left corner. The brain sections were stained for β -galactosidase activity as described in the legend for Fig. 4. Distinct staining was detected in the cortical cell layers, particularly in the olfactory cortex areas (A–D). Thalamic nuclei (A–C), trigeminal ganglia (D), lateral areas of the brain stem (F), and cerebellum (E–G) also showed expression, while spinal cord (H) was negative. Tg, trigeminal ganglion; Th, thalamus.

genic enzymes (35). To find out whether the neuronal LHR might also be involved in regulation of neurosteroid synthesis, we tested the possibility that the LHR promoter-driven transgene activity might colocalize with enzymes involved in steroid biosynthesis. To this end, the X-gal-stained paraffin-embedded mouse brain sections were stained for P450scc immunoreactivity, an enzyme that catalyzes the initial reaction in the steroidogenic pathway and has been shown to localize to steroidogenic gonadal cells (36, 37) and to steroidogenic neurons and glial cells in the nervous tissue (38, 39). The antiserum recognized both testicular Leydig cells (Fig. 8C) and specific neurons of the trigeminal ganglion (Fig. 8A) and colliculus inferior (Fig. 8B), and the staining was typical for mitochondrial proteins, with punctuate cytoplasmic staining and nuclear exclusion. Most importantly, the β -galactosidase activity colocalized with the P450scc immunoreactivity to the same neurons (Fig. 8, A and B). This coordinate expression in the nervous system suggests that the LHR might indeed be involved in regulation of neurosteroid synthesis.

DISCUSSION

The glycoprotein hormone receptors for LH, FSH, and TSH are LGRs that have been shown to display very restricted tissue expression patterns. In the present study, we demonstrate that the LHR is not expressed solely in the reproductive organs but is also present as a functional protein in the nervous system. The expression appears to commence prenatally in both the peripheral and central nervous system and continue

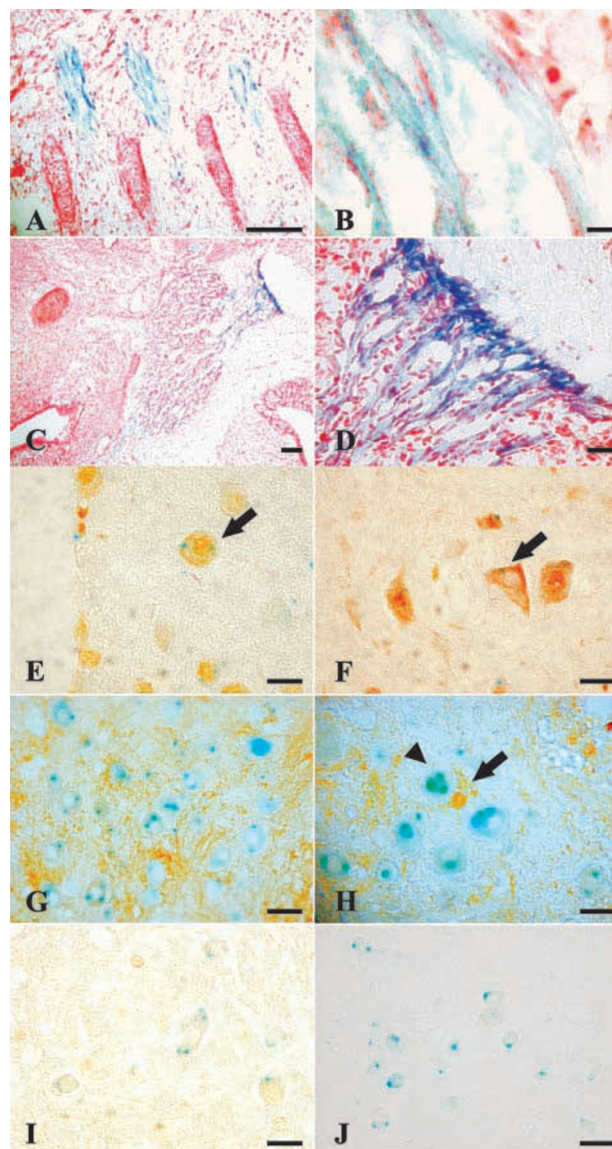


FIG. 7. **Expression of the LHR promoter-driven transgene in neurons.** Whole-mount β -galactosidase staining of the transgenic 14.5-dpc fetal (A–D) and adult mouse brain (E–J) was performed as described in the legend for Fig. 4. Cryostat sections (5 μ m) of the fetal brain were prepared and counterstained with safranin. Intense blue staining was observed in the developing spinal (A and B) and trigeminal (C and D) ganglia. The paraffin-embedded adult brain was sectioned (10 μ m) and stained either with a monoclonal antibody directed against the neuronal nuclei protein (NeuN, E, F) or a polyclonal antibody directed against the glia-specific S-100 protein (G and H), as described under "Experimental Procedures." The NeuN immunoreactivity (E and F; brown) and β -galactosidase activity (E and F; blue) colocalized to the same cells (arrow), whereas the S-100 protein immunoreactivity (G and H; brown, arrow) and β -galactosidase activity (G and H; blue, arrowhead) showed differential distribution. When the NeuN and the S-100-specific antibodies were replaced with the normal mouse (I) or rabbit serum (J), respectively, only the neuron-specific β -galactosidase activity was detected. E and G, trigeminal ganglion; F, H, I, and J, colliculus inferior. Scale bars: A and C, 100 μ m; B and D, 50 μ m; and E–J, 20 μ m.

through adulthood, especially in specific areas of the brain involved in sensory functions. The LHR, thus, resembles other LGRs (LGR4–8), which display a wide tissue distribution (5–9), suggesting that this may be a characteristic property of the LGR subfamily.

Expression of the LHR in the nervous system was demonstrated both at the mRNA and protein level. Various adult rat brain areas and the heads of the 14.5- and 19.5-dpc fetuses showed expression of the full-length LHR mRNA and several

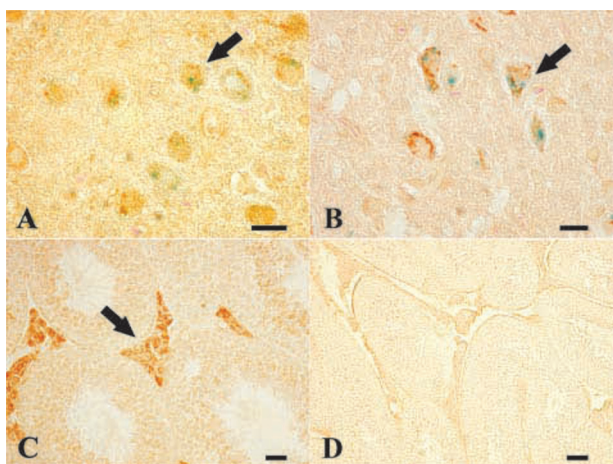


FIG. 8. Expression of the LHR promoter-driven transgene in steroidogenic neurons. A and B, whole-mount β -galactosidase staining of the adult mouse brain was performed as described in the legend for Fig. 4. The paraffin-embedded brain was sectioned (10 μ m) and stained with a polyclonal antibody directed against P450scc, as described under "Experimental Procedures." The P450scc immunoreactivity (brown) and β -galactosidase activity (blue) colocalized to the same neurons (arrow) in the trigeminal ganglion (A) and colliculus inferior (B). C and D, paraffin-embedded testicular sections (10 μ m) of an adult nontransgenic male mouse were stained with the polyclonal P450scc antibody (C) or normal rabbit serum (D) to show the specific P450scc staining in steroidogenic Leydig cells (arrow). Scale bars: A and B, 20 μ m; C and D, 50 μ m.

smaller amplified transcripts, most likely representing receptor isoforms. The receptor protein was expressed concomitantly with the full-length receptor mRNA, and two species of M_r 90,000 and 73,000 were detected. The M_r 90,000 receptor species contained fully processed *N*-linked oligosaccharides and thus represents the mature receptor. A similar sized receptor species was also present in the ovaries, in agreement with previous reports by us and others (18, 40–42). The smaller, M_r 73,000 LHR species, on the other hand, is most likely a biosynthetic intermediate of the larger one because it contained high mannose type oligosaccharides. In contrast to these findings, Rao and co-workers (17, 43, 44) have previously identified a LHR form of M_r 80,000 in the rat and human brain as well as in isolated rat neuronal and glial cells. This apparent discrepancy might be explained by the different methods used to detect the receptors (Western blotting *versus* immunoprecipitation). In the present study, the neuronal LHR species were detected only after immunoprecipitation, and direct Western blotting of the solubilized membranes revealed no specific bands (data not shown), possibly because of the very low level of receptors in the nervous tissue.

Both mature and immature LHR species were detected in the rat nervous tissue and ovaries with indistinguishable molecular masses, suggesting that the receptor is processed in these tissues in a similar manner. Immature and mature forms of the LHR have also been identified, when the protein is expressed in heterologous expression systems⁴ (42, 45). The high amount of the M_r 73,000 receptor species most likely reflects inefficient conversion of the receptor precursor to the mature form, as we have reported previously for the human δ -opioid receptor (46). Of interest, inefficient processing of the LGRs may be a common property among this group of GPCRs because the ovarian FSHRs have also been shown to exist in both immature and mature forms (47). Whether inefficient processing of the LGRs provides a mean to control receptor

numbers at post-translational level or has some other functional significance is an interesting possibility and remains to be determined in the future.

Several lines of direct and indirect evidence demonstrated that the neuronal LHR is a functional protein and capable of ligand binding. 1) The rat LHR-specific antibody was able to immunoprecipitate the M_r 90,000 receptor species from the fetal and adult rat nervous tissue. The fully processed oligosaccharides of this receptor species provide indirect evidence that the protein has folded correctly and has been transported from its site of synthesis in the endoplasmic reticulum to the cell surface. 2) The M_r 90,000 receptor species was purified by ligand affinity chromatography. 3) Membranes prepared from the 19.5-dpc fetus heads exhibited high affinity binding for [¹²⁵I]hCG. 4) Both hCG and hLH were able to displace [¹²⁵I]hCG binding to the fetal head membranes in competition binding experiments unlike hFSH, demonstrating that the neuronal receptor displays similar hormone binding specificity as gonadal LHRs (12, 31). All of these data suggest that functional LHRs with ligand binding ability appear in the nervous tissue early during fetal development, preceding those expressed in the gonads. In the testes and ovaries, [¹²⁵I]hCG binding sites are first detected in the 15.5-dpc fetuses and the 7-day-old neonates, respectively (29, 48).

A thorough examination of the spatial and temporal expression of the neuronal LHR was performed by using transgenic mice that expressed the *lacZ* reporter under the control of the rat LHR promoter. The two promoter fragments of 2,060 and 3,970 bp used were both functional *in vivo* and directed β -galactosidase expression to specific regions of the peripheral and central nervous system. Importantly, no sex differences were observed, which is in contrast to the findings reported for the developing gonads, which exhibit clear temporal differences in the LHR gene activity in males and females (23, 29). During organogenesis, the LHR promoter activity was first detected in the 11.5-dpc fetuses, and during the following 3 days the activity became apparent in specific peripheral and central nervous system structures, like the developing olfactory bulb, sensory and autonomic ganglia, and thalamic, hypothalamic, and brain stem areas. The expression persisted in the adults, confining to the cortical cell layers of the cerebrum, olfactory bulb, cerebellum, thalamus, posterior hippocampus, as well as the colliculi inferior and olivary nuclei of the brain stem. In comparison, Huhtaniemi and co-workers (49) observed recently that the mouse LHR promoter fragment of 2.1 kb directed *lacZ* reporter gene expression to adult brain areas that were only partially overlapping to the areas shown in the present study. These differences may be the result of the species differences or differences in the length and usage of the 5'-flanking sequences.

Translation of the neuronal LHR mRNA into protein was shown to take place in the 14.5-dpc rat fetuses, and the receptor gene promoter in the transgenic mice was active even earlier, at 11.5 dpc. This speaks for the fact that the LHR might have a role in regulation of neuronal growth and differentiation. This possibility is supported by the finding that administration of hCG has been shown to result in neurite outgrowth of cultured rat neuronal cells (44). The LHR gene was also active in distinct areas of the adult brain, indicating that the receptor might be involved in regulation of brain functions after birth as well, particularly in the sensory and autonomic systems and the hippocampal and cerebellar areas that are related to reproductive behavior, memory and motor coordination.

An interesting possibility is that the LHR might take part in regulation of neurosteroid production by regulating the expression of steroidogenic enzymes and the steroidogenic acute regulatory protein, as it does in the gonads (35). The neuro-

⁴ E. M. Pietilä, H. J. Rajaniemi, and U. E. Petäjä-Repo, unpublished data.

steroids are known to act as signaling factors that coordinate environmental cues to reproductive behavior and regulate neuronal growth and differentiation (50, 51). This possibility was supported by our finding that the LHR promoter-driven transgene activity was found to colocalize with the P450_{scc} to the same neurons of the colliculus inferior and trigeminal ganglion. In addition, the expression pattern of the transgene activity appeared to be very similar to that of other proteins involved in neurosteroid synthesis, such as cytochrome P450c17 (38, 39) and steroidogenic acute regulatory protein (52).

In conclusion, the present results demonstrate that the LHR gene is active in restricted areas of the developing and adult nervous tissue, resulting in expression of multiple receptor mRNA transcripts and synthesis of receptor protein exhibiting specific high affinity ligand binding. This spatially restricted LHR expression pattern in the developing and adult nervous tissue suggests a previously unanticipated role for this LGR in neuronal development and function. An intriguing possibility is that the neuronal LHR may have a role in regulation of neurosteroid production.

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