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# Neurons from Fetal Rat Brains Contain Functional Luteinizing Hormone/Chorionic Gonadotropin Receptors[1]

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## ABSTRACT

**Adult and neonatal rat brains contain functional LH/hCG receptors. These findings have led us to hypothesize that the fetal rat brain may also contain these receptors. To test this hypothesis, we isolated neurons from 19-day-old fetal rat brains and cultured them in chemically defined serum-free medium. The reverse transcription polymerase chain reaction amplified an expected 256-base pair size LH/hCG receptor fragment that could hybridize with a full-length LH/hCG receptor cDNA in Southern blotting. Northern blotting demonstrated that neurons contained a major 2.6 kilobase (kb) and a minor 4.3 kb transcript. Immunocytochemistry demonstrated that the neurons contained LH/hCG receptor immunostaining. Western immunoblotting showed that neurons contained an 80-kDa receptor protein that increased to a maximal level on Day 3 of culture and then gradually decreased until the 9th day of culture. Culturing neurons for 3 days in the presence of highly purified hCG resulted in a dose-dependent increase in the outgrowth of neurite processes and total cellular protein and a decrease in DNA fragmentation as compared to values in the corresponding controls. At the maximally effective hCG concentration, the number of neurite-bearing cells was increased by 53% and the total cellular protein by 60%, and DNA fragmentation decreased by 31%. In summary, this is the first study to demonstrate the presence of LH/hCG receptors and neurotrophic effects of hCG in fetal rat brain neurons. These findings imply that locally produced gonadotropins may possibly play a role in the growth and development of the fetal brain.**

## INTRODUCTION

Fetal brain development is presumably regulated by neurotrophic factors that include nerve growth factor, fibroblast growth factor, insulin-like growth factor-I, brain-derived neurotrophic factor, neurotrophin-3, neurotrophin-4/5, and others [1–10]. The possibility of whether hCG and LH are also neurotrophic factors has never been addressed. It is now a relevant question because fetal brain, like the adult and neonatal brain [11, 12], may also contain LH/hCG receptors. In addition, previous studies have demonstrated that treatment of rats with hCG after spinal cord resection resulted in a greater recovery than in the control animals [13] and that the adrenal medulla transplanted into the lateral ventricle of the brain grew better when hCG was administered [14]. For these reasons, we have tested the hypothesis that fetal rat brain neurons contain LH/hCG receptors and that exogenous hCG could act as a neurotrophic agent in primary neuronal cultures established from fetal rat brains and maintained in a serum-free, chemically defined medium.

## MATERIALS AND METHODS

### Materials

The following items were obtained as gifts: highly purified hCG (CR-127; 14 900 IU/mg) from the National Hormone and Pituitary Program supported by NIDDK, NICHHD, and USDA (Rockville, MD); polyclonal hCG/LH receptor antibody raised against a synthetic N-terminus amino acid sequence of 15–38 and the corresponding synthetic receptor peptide from Dr. Patrick Roche at the Mayo Clinic (Rochester, MN); and a full-length porcine LH/hCG receptor cDNA from Dr. Hugues Loosfelt at Hopital de Bricetre (Bricetre, France). The following items were purchased: trypsin, DNase, and soybean trypsin inhibitor from Sigma Chemical Co. (St. Louis, MO); all cell culture supplies from GIBCO-BRL (Grand Island, NY); polyclonal anti-neuron-specific enolase and anti-glial fibrillary acidic protein antibodies from DAKO Corp. (Carpenteria, CA); random-priming cDNA labeling kits, AMV (avian myeloblastosis virus) reverse transcriptase and *Taq* DNA polymerase, and in vitro transcription kits from Promega Corp. (Madison, WI); nusieve agarose from FMC Bioproducts (Rockland, ME); micro-fast Track mRNA isolation kits from Invitrogen Corp. (San Diego, CA); Immobilon-P membranes from Millipore Corp. (Bedford, MA); rainbow protein molecular weight standards and enhanced chemiluminescence detection system kits from Amersham Corp. (Arlington Heights, IL); and miniblot system and the kits for protein determination by Bradford's method from Bio-Rad Laboratories (Melville, NY). The sources of other items are the same as previously described [11, 12, 15].

### Cell Dispersion and Culture

The procedure originally described by Ahmed et al. [16] was used. Briefly, 19-day-pregnant Sprague-Dawley rats (Charles River Laboratories, Portage, MI) were anesthetized with diethyl ether, and fetuses were removed by laparotomy under sterile conditions and placed in ice cold isotonic buffer solution. Under a dissection microscope, whole brains were cleaned of meninges and blood vessels, and the tissues from 12 to 14 fetuses were pooled and minced with iris scissors. The tissue minces were suspended in 5.0 ml of isotonic buffer containing 0.005% trypsin and 0.004% DNase in an Erlenmeyer flask. The contents of the flask were gently mixed and incubated at 37°C in a shaking water bath. After 7 min, the contents were again gently mixed and additional DNase was added if necessary. Undissociated tissue was allowed to settle, and the supernatant was carefully removed and combined with 10 ml of defined medium (DM) containing soybean trypsin inhibitor. The su-

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pernatants were centrifuged for 5–10 min at 300 × *g*, and the cell pellet was resuspended in 10 ml of DM and filtered through sterile cheesecloth. The cell numbers in filtrate were counted in a hemocytometer. Then the cells were plated in different size culture dishes coated with poly-D-lysine. The plated cell densities were the same within but not between experiments. These details are given in the figure legends. After plating, the cells were allowed to attach for 20 min at 37°C in 5% $CO_2$ and saturated humidity. Then the cells were fed with insulin-free DM, which was changed every third day. After 3 days of culture, immunostaining for neuron-specific enolase revealed that 91% of the cells were neurons; the remaining were glial cells as determined by immunostaining for glial fibrillary acidic protein.

### *Reverse Transcription Polymerase Chain Reaction (RT-PCR)/Southern Blotting*

The RT-PCR was performed as previously described [17]. Briefly, total RNA was isolated by a single-step acid guanidinium thiocyanate-chloroform extraction method [18], and 5 μg of RNA was reverse transcribed into cDNA using primer 2 (5′-GCATCTGGTTCTGGAGCACA-3′). One seventh of the reaction mixture was amplified in 35 cycles using primers 1 (5′-AATTCACGAGCCTCCTGGTC-3′) and 2. The primers were designed from the rat LH/hCG receptor cDNA sequence at the 5′ end from 846 to 866 base pairs (bp) (primer 1) and at the 3′ end from 1101 to 1081 bp (primer 2). Both template omission and RT-omission controls were used. To determine the size of the amplified product, a 123-bp DNA ladder was run in an adjacent lane. Instead of sequencing the PCR product, we performed Southern blotting to confirm its identity. Southern blotting was performed using a full-length LH/hCG receptor cDNA labeled with [$^{32}$P]dCTP by a random-priming method using a kit.

### *Northern Blotting*

Northern blotting was performed using 10-μg aliquots of poly(A)$^+$ RNA isolated from the neurons using the commercial kits [11, 19]. Briefly, poly(A)$^+$ RNA was electrophoresed in agarose gels, transferred to nitrocellulose membranes, and hybridized for 18 h at 65°C with $^{32}$P-labeled riboprobe transcribed from LH/hCG receptor cDNA. The membranes were then washed twice at 65°C with double-strength SSC (single-strength SSC: 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0) containing 1% SDS and twice more with double-strength SSC containing 0.1% SDS. The washed membranes were exposed to x-ray film for 10 days at −80°C with intensifying screens.

### *Western Immunoblotting*

Western immunoblotting was performed as previously described [11, 20, 21]. Briefly, the neurons were homogenized for 1 min in 10 mM PBS, pH 7.4, containing 5 mM *N*-ethylmaleimide and 0.2 mM PMSF. The homogenates were centrifuged at 4°C for 15 min at 120 × *g*. Fifty-microgram aliquots of protein in supernatants were separated on discontinuous 7.5% SDS-PAGE under reducing conditions and then electroblotted to Immobilon-P membranes. Receptors were detected by using 1:1000 dilution of a polyclonal LH/hCG receptor antibody and an enhanced chemiluminescence detection system. The relative optical densities of the bands were determined in a linear range by a Bio-Rad densitometer. The receptor antibody preabsorbed with excess receptor peptide was used for a procedural control.

### *Immunocytochemistry*

Immunocytochemistry was performed by an avidin-biotin immunoperoxidase method [11, 22]. Briefly, the neurons were fixed in Bouin's solution for 5 min and immunostained with a 1:350 dilution of LH/hCG receptor antibody. The receptor antibody preabsorbed with the corresponding excess receptor peptide was used for the procedural control.

### *Counting of Neurite-Bearing Cells*

The cells were cultured for 3 days in the presence or absence of 0–250 ng/ml hCG and then examined by phase-contrast optics. For counting, the culture dishes were moved to random locations on the stage of an inverted microscope and the cells within the field of a 0.5-mm$^2$ eyepiece grid were counted from at least 5 different areas. Cells were considered as neurons according to the criteria of Guthrie et al. [23]. That is, neurons were bright in appearance and either had clearly visible processes or were a part of neuronal aggregates connected with other neurons by neurites.

### *Determination of Total Cellular Protein*

The cells were cultured for 3 days in the presence and absence of 0–250 ng/ml hCG. Then the cells were lysed with 1 ml of 0.2 N NaOH, and the protein in 100-μl aliquots was determined using Bio-Rad kits.

### *Measurement of DNA Fragmentation*

The DNA fragmentation was quantified by the diphenylamine assay [24, 25]. Briefly, cells were scraped from culture dishes, and 0.8-ml aliquots were transferred to Eppendorf tubes containing 0.7 ml ice-cold lysis buffer, which consisted of 5 mM Tris-HCl (pH 8), 20 mM EDTA, and 0.5% (v:v) Triton X-100, and left for 15 min. After centrifugation for 15 min at 13 000 × *g*, the pellet containing intact chromatin and supernatant containing DNA fragments were separated. The pellet was resuspended in 1.5 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The resuspended pellet and supernatant were transferred to conical glass tubes to which an equal volume of 10% trichloroacetic acid (TCA) was added. The 10% TCA precipitates were resuspended in 0.6 ml of 5% TCA and placed in a 100°C water bath for 15 min. After cooling to room temperature, the tubes were centrifuged at 200 × *g* for 5 min, and 0.5 ml of the resulting supernatant was transferred to round-bottomed glass tubes to which 1.0 ml of the diphenylamine reagent was added. Samples were incubated overnight at 30°C, and the absorbance was determined at 600 nm. Results, expressed as the % DNA fragmented, are determined as follows: ([absorbance of the supernatant]/[(absorbance of supernatant + absorbance of the pellet] × 100).

### *Replication of Experiments and Statistical Analysis*

All the experiments were performed in duplicate and repeated three times on different neuronal cultures. The pooled data from all the experiments were used for calculation of the means and their standard errors and also for one-way ANOVA and Tukey-Kramer multiple comparison

test [26] to determine the significant differences between the experimental groups illustrated in Figures 3–6.

## RESULTS

We first used RT-PCR to determine the possible presence of LH/hCG receptor transcripts in neurons from fetal rat brains. As shown in Figure 1A, an expected 256-bp-size LH/hCG receptor fragment was amplified from the neurons (lane 1). The omission of the template (lane 2) or reverse transcriptase (data not shown) resulted in an absence of amplification. The bottom of lane 1 shows that an amplified fragment can bind to a full-length LH/hCG receptor cDNA in Southern blot, confirming that the amplified fragment was derived from the receptor sequence.

We next used Northern blotting to determine the size of transcripts. Figure 1B shows that the neurons contained a major 2.6-kb and a minor 4.3-kb transcript of LH/hCG receptors.

To determine whether fetal rat brain neurons also contain LH/hCG receptor protein, we performed Western immunoblotting. The results showed that the neurons do contain an 80-kDa immunoreactive receptor protein (Fig. 1C, lane 3) that was not detected when the receptor antibody was preabsorbed with excess receptor peptide (lane 4).

Because primary cultures contain 9% glial cells, we used immunocytochemistry to determine the cellular localization of LH/hCG receptors. Figure 2A shows that indeed neurons, which were identified by their characteristic morphology of neurite processes as well as by immunostaining for neuron specific enolase, contain LH/hCG receptor immunostaining. Primarily the cell body, but not the neurite outgrowths, contained the receptor immunostaining. A considerable amount of receptor immunostaining was present in and around the nucleus in the cell body. Whether this apparent perinuclear immunostaining is due to the natural thickness of the cells in the nuclear region and/or intrinsic receptor distribution is not known. However, previous studies have demonstrated that functional LH/hCG receptors are also present in part in the nucleus of the target cells [27–31]. Preabsorption of the receptor antibody with excess corresponding receptor peptide resulted in an absence of immunostaining (Fig. 2B). Glial cells also contained receptor immunostaining, but the difference between them and neurons has not been determined.

To determine possible receptor-level changes during culture, we performed Western immunoblotting using equal amounts of protein. The results shown in Figure 3 demonstrate that LH/hCG receptor protein was detectable at the beginning of culture. The levels, however, significantly increased after 3 days of culture and then progressively declined by Day 9 of the culture. Because of this data, all the functional studies were performed on cells cultured for 3 days.

We examined the effect of hCG on neuronal survival by counting the cells with neurite processes. As shown in Figure 4, culturing neurons with highly purified hCG resulted in a modest significant increase in the number of neurite-bearing cells at 100 and 250 ng/ml hCG as compared to the control. The addition of 0.33 μM insulin alone in DM caused a greater increase in the number of neurite-bearing cells (data not shown). However, insulin was not required for the hCG effect. In fact, the presence of insulin has resulted in a blunting of the hCG effect (data not shown).

We also determined the effect of hCG on neuronal survival by measuring total cellular protein. As shown in Figure 5, culturing neurons with hCG resulted in a modest significant increase in total cellular protein at 250 ng/ml hCG as compared to the control value.

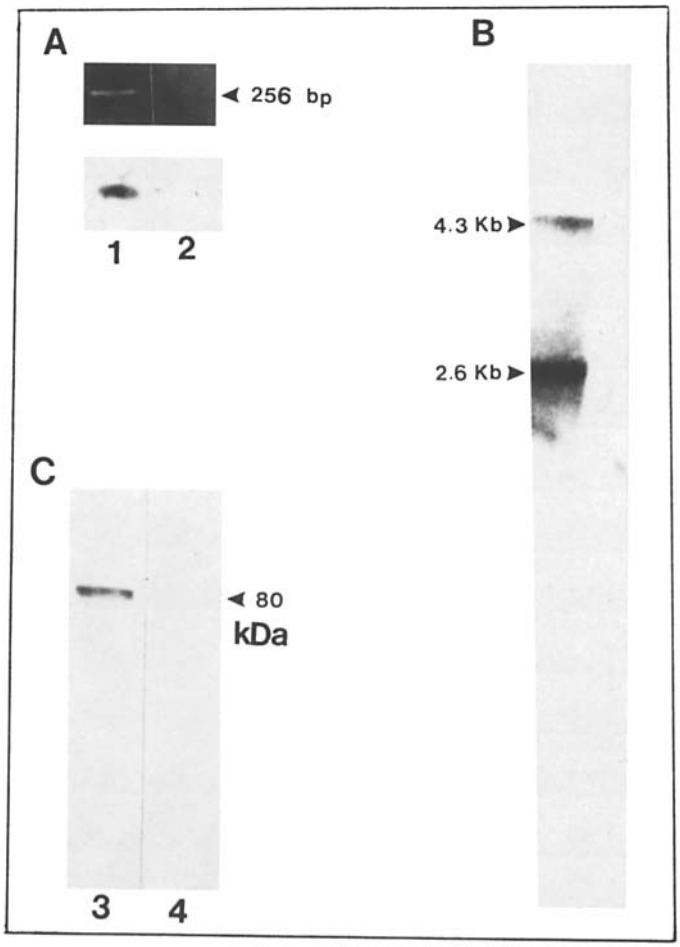


FIG. 1. RT-PCR/Southern blotting (**A**), Northern blotting (**B**), and Western immunoblotting (**C**) for LH/hCG receptors in neurons from fetal rat brains. The cells were plated at a density of 8000/mm² in 100-mm petri dishes and cultured for 3 days. Lane 2 is RNA omission control and lane 4 is a control in which the receptor antibody was preabsorbed with excess receptor peptide.

Because increased neuronal survival is expected to be inversely related to decreased apoptosis, we measured the effect of hCG on DNA fragmentation. First, there was considerable DNA fragmentation in the cells cultured for 3 days in the absence of hCG (Fig. 6). Culturing with increasing concentrations of hCG resulted in a modest significant decrease in DNA fragmentation at 250 ng/ml as compared to that in the control (Fig. 6).

## DISCUSSION

Adult and neonatal rat brains contain functional LH/hCG receptors [11, 12, 15, 32–36]. This would suggest that the fetal rat brain may also contain functional LH/hCG receptors. To determine this possibility, we first established primary cultures of neurons from different areas of fetal rat brains in a serum-free and chemically defined medium.

RT-PCR amplified a receptor fragment of the expected size that could bind to receptor cDNA, suggesting that the amplified fragment was indeed derived from the receptor transcripts in the neurons. Northern blotting demonstrated that neurons contained a major 2.6-kb and a minor 4.3-kb transcript. The major and minor transcripts found in the

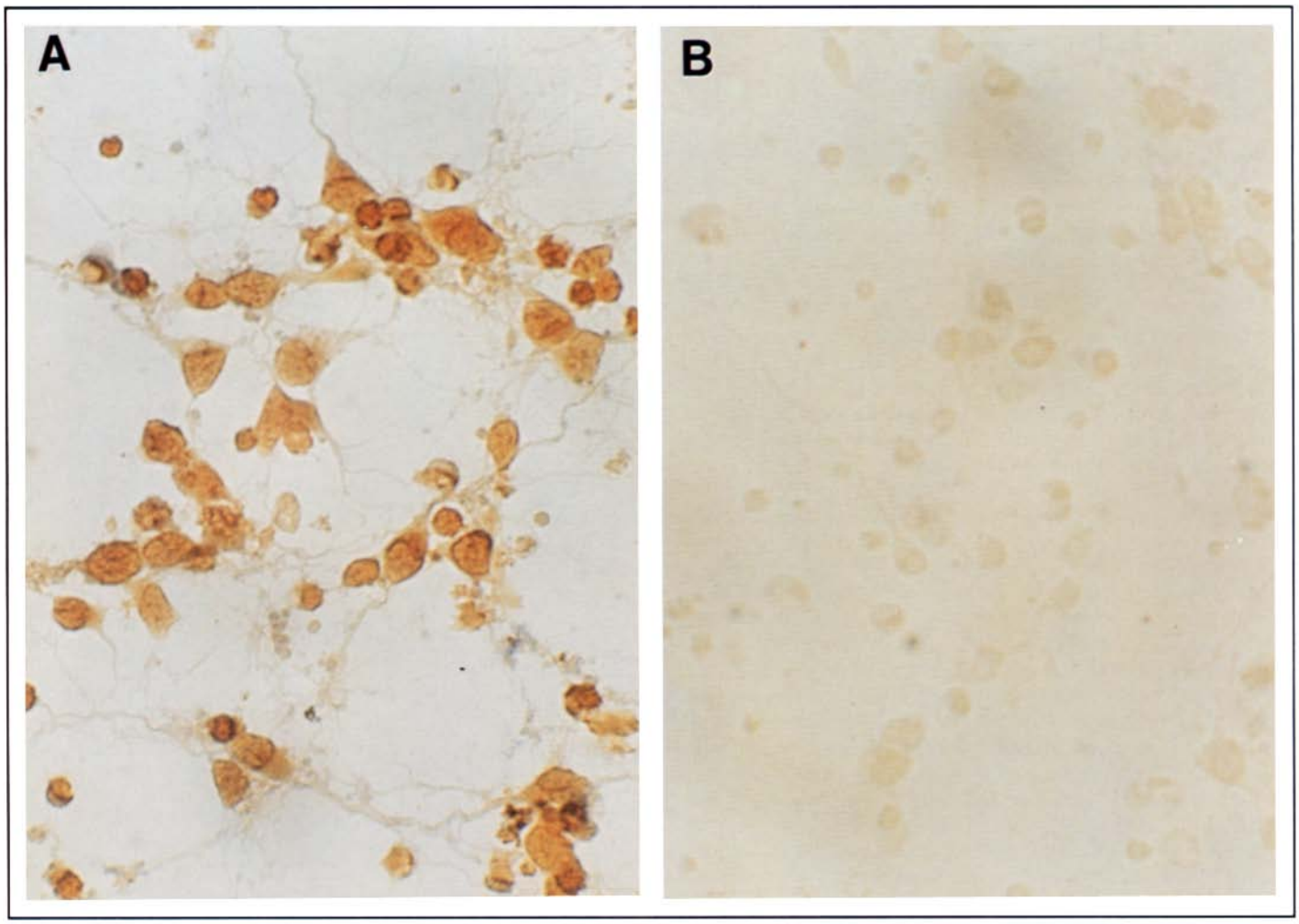


FIG. 2. Immunocytochemistry for LH/hCG receptors in neurons from fetal rat brains. The cells were plated at a density of 2000/mm² in two-well chamber slides and cultured for 3 days. Unabsorbed receptor antibody was used in **A**, and the receptor antibody preabsorbed with the excess corresponding receptor peptide was used in **B**. ×600.

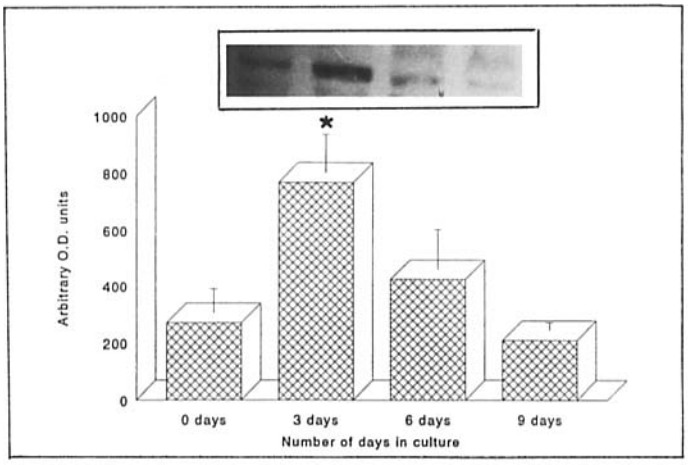


FIG. 3. The LH/hCG receptor protein levels in neurons from fetal rat brains. The cells were plated at a density of 8000/mm² in 100-mm petri dishes and cultured at 3-day intervals for 9 days. The inset shows a representative Western immunoblot with the sequence of lanes corresponding to the bars. The asterisk indicates a significant difference as compared to Day 0 ($p < 0.01$).

neurons were the same as those found in glial cells from neonatal rat brains and whole adult rat brain except that the latter also contained an additional minor 1.8-kb transcript [11, 12]. GT1-7 neurons, on the other hand, contain a major 1.8-kb and minor 2.6-kb and 4.3-kb transcripts [15]. Despite the presence of more than one transcript, fetal rat brain neurons, like whole adult rat brains, GT1-7 neurons, and glial cells from neonatal rat brains, contain a single 80-kDa receptor protein [11, 12, 15].

The receptor protein levels increased from the beginning of the culture to Day 3 and then decreased by Day 9 of the culture. Why this increase and decrease occurred, and what could have sustained the increased receptor levels, are not known. In any case, we cultured the neurons for 3 days for the functional studies.

We used three endpoints to determine whether exogenous hCG would be neurotrophic in the neurons from fetal rat brains. The first was an increase in neurite outgrowth; the second was an increase in total cellular protein; and the third was a decrease in DNA fragmentation. Culturing the neurons with highly purified hCG for 3 days, when the receptor levels are maximal, resulted in an increase of neurite outgrowth and total cellular protein and a decrease of DNA fragmentation as compared to values in the corresponding controls. These changes were modest, but they

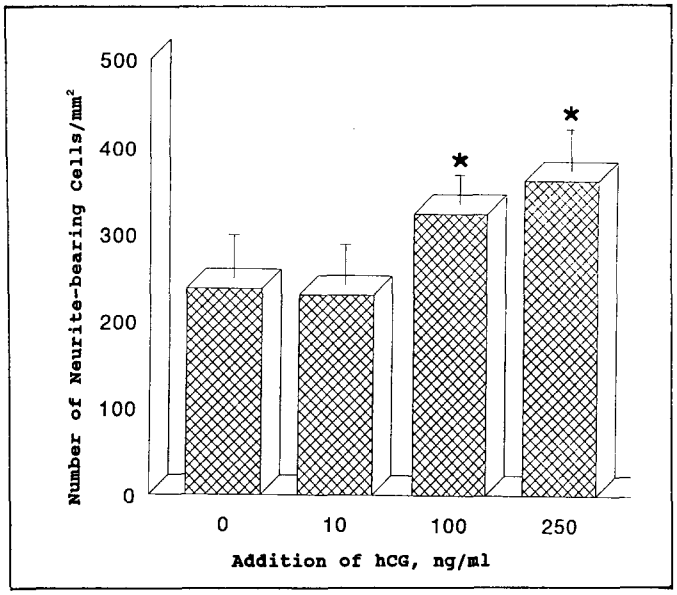


FIG. 4. The dose dependency of hCG effect on the number of neurite-bearing neurons from fetal rat brains. The cells were plated at a density of 1000/mm² in 12-well 22-mm cluster dishes and cultured for 3 days in the presence and absence of increasing concentrations of hCG. The asterisks indicate significant differences as compared to the control ($p < 0.05$).

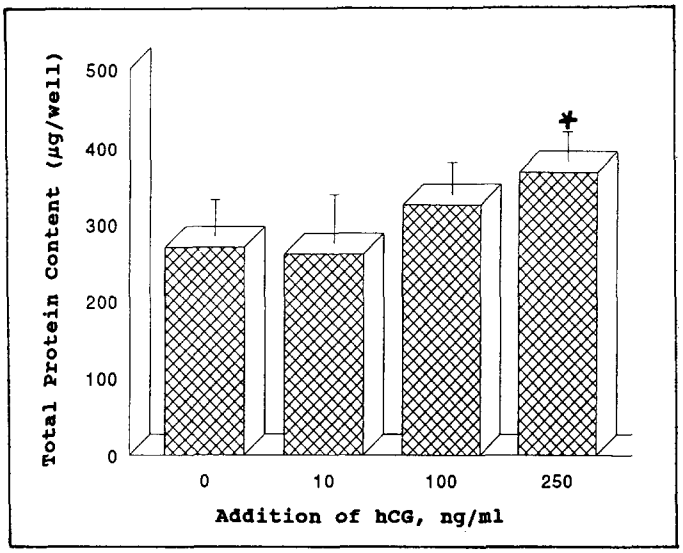


FIG. 5. The dose dependency of hCG effect on total cellular protein content in neurons from fetal rat brains. The cells were plated at a density of 8000/mm² in 12-well 22-mm cluster dishes and cultured for 3 days in the presence and absence of increasing concentrations of hCG. The asterisk indicates a significant difference as compared to the control ($p < 0.05$).

could be important because of the possibility that these effects may be augmented by other factors.

The hCG concentrations required for the neurotrophic effects are rather high. Yet these effects could be potentially physiologically relevant because of the possibility that the fetal rat brain, like the adult rat brain [37–40], may make LH. In such a case, the local concentrations may reach high enough levels to exert neurotrophic effects. The present findings may also possibly be relevant to the growth of the human fetal brain. For example, the major growth of the human fetal brain occurs during the first trimester of pregnancy when maternal circulatory hCG levels are very high. Although this does not necessarily mean that fetal circulatory hCG levels are correspondingly high, there could be locally produced hCG or LH or similar peptides, and/or some of the fetal circulatory hCG might get into cerebrospinal fluid. In these cases, local concentrations may again reach high enough levels to affect the growth of the fetal central nervous system. These provocative possibilities require verification through further experiments.

The affinity of fetal rat brain neuronal LH/hCG receptors is not known. Assuming that it is the same as in gonadal tissues, the requirement for high hCG doses for a response would suggest a substantial saturation of receptor sites before the physiological response can occur. This kind of phenomenon is not unusual, as it is rather commonly seen with some responses in most receptor systems.

Previous studies have demonstrated that nerve growth factor, fibroblast growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 are neurotrophic [1–10]. Human CG, like nerve growth factor, has a cystine-knot structural motif [41]. This places both of these molecules in the structural superfamily of cystine-knot growth factors that may have some similar biological functions. The present study suggests that the neurotrophic effect is one such example for hCG and nerve growth factor. It will be interesting to determine whether treatment with LH/hCG can up-regulate nerve growth factor or any of these other neurotrophic factors and/or whether nerve growth factor or any of the other neurotrophic factors can synergistically function with LH/hCG.

The neurotrophic effects of hCG may be mediated directly via neuronal LH/hCG receptors. Alternatively, the effects could be mediated indirectly via LH/hCG receptors in glial cells that are present in small numbers in the cultures. Finally, it is also possible that both direct and indirect mechanisms may be involved.

In summary, we conclude that neurons from fetal rat brains contain LH/hCG receptors and that exogenous hCG can exert neurotrophic actions on them. These seminal findings suggest that LH and hCG can potentially regulate fetal brain growth and development.

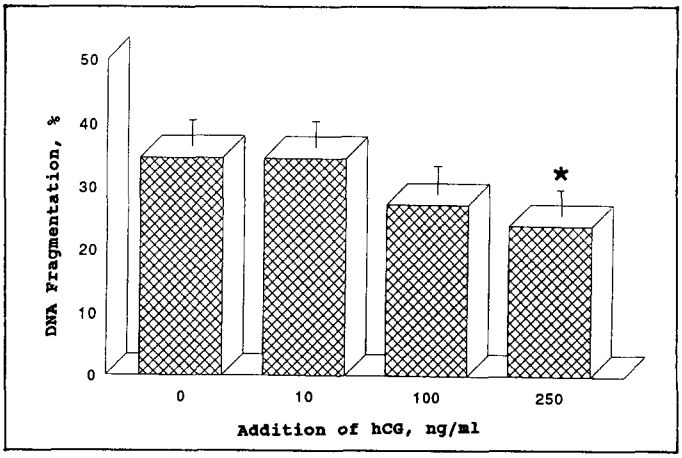


FIG. 6. The dose dependency of hCG effect on DNA fragmentation in neurons from fetal rat brains. The cells were plated at a density of 8000/mm² in 35-mm culture dishes and cultured for 3 days in the presence and absence of increasing concentrations of hCG. The asterisk indicates a significant difference as compared to the control ($p < 0.05$).

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