Human neurons express type I GnRH receptor and respond to GnRH I by increasing luteinizing hormone expression

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Abstract

Gonadotropin-releasing hormone receptor I (GnRHR I) has been localized to the limbic system of the rat brain, although the functional consequences of GnRH signaling through these receptors is unknown. In this paper, we characterize the expression of GnRHR I in the human hippocampus and cortex, and the functionality of GnRHR I in human neuroblastoma cells. Robust GnRHR I immunoreactivity was detected in the cell body as well as along the apical dendrites of pyramidal neurons in the CA2, CA1, and end plate, but was clearly lower in the subiculum of the hippocampus. Immunolabeling was also evident in cortical neurons, including those located in the entorhinal cortex and occipitotemporal gyrus but was not observed within the granular layer of the dentate gyrus. No differences in immunohistochemical staining were observed between control and Alzheimer's disease brain. GnRHR I mRNA and protein (mature, immature, and other variant) expression was detected in human neuroblastoma cells (M17, SH–SY5Y) and rat embryonic primary neurons and varied with differentiation and GnRH treatment. Since GnRHR I was expressed by extrapituitary cells, and hypothalamic GnRH I secretion markedly increases post-menopause/andropause, we treated human M17 neuroblastoma cells cultured in serum-free conditions with GnRH I for 6 h and measured LH expression. M17 neuroblastoma cells express LHβ mRNA, while immunoblot analysis indicated the presence of three LH variants (approximately 30, 47, and 60 kDa) that were upregulated by low concentrations of GnRH I, but down-regulated at higher GnRH I concentrations. LH expression was also found to increase in differentiating embryonic rat primary cortical neurons. Our results demonstrate that neurons expressing GnRHR I are functional, responding to GnRH I by upregulating LH production. Post-reproductive surges in GnRH I secretion may explain the accumulation of LH in pyramidal neurons of the aged human and rat.

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Introduction

Luteinizing hormone (LH) is secreted from gonadotropes of the anterior pituitary into the bloodstream following pulsatile stimulation by hypothalamic gonadotropin–releasing hormone I (GnRH I; Larsen *et al.* 2002). LH has also been immunolocalized to the cytoplasm of neurons of the cerebral cortex and hippocampus of the human and rat brains (Hostetter *et al.* 1981, Emanuele *et al.* 1983, Bowen *et al.* 2002), and is found in the cerebrospinal fluid (Bagshawe *et al.* 1968).

The exact origin of extrapituitary intracellular LH is unclear. One possible explanation is that pyramidal neurons sequester LH from extracellular sources (e.g. blood). This is supported by the findings that pyramidal neuron LH is elevated approximately twofold in Alzheimer's disease (AD) compared with age-matched control brains (Bowen *et al.* 2002), an increase that correlates with a twofold increase in serum LH (Bowen *et al.* 2000, Hogervorst *et al.* 2001, Short *et al.* 2001). Another intriguing possibility is that aging neurons, like fetal and cancer cells, might be capable of synthesizing LH (Whitfield & Kourides 1985, Krichevsky *et al.* 1995, Yokotani *et al.* 1997). This latter idea is supported by the findings that (1) mRNA for LH has been localized to pyramidal neurons of the cerebral cortex and hippocampus of the aging rat brain (Lee *et al.* 2004), (2) GnRH receptor I (GnRHR I) is localized to extrapituitary cells in the rodent

brain including the hippocampus, amygdala, entorhinal cortex, and subiculum, with lower levels in the septum, frontal cortex and hypothalamus (Reubi & Maurer 1984, Badr & Pelletier 1987, Haour *et al.* 1987, Reubi *et al.* 1987, Jennes *et al.* 1988, 1995, 1996, 1997, Leblanc *et al.* 1988, Badr *et al.* 1989, Ban *et al.* 1990, Thompson & Moss 1992, Crumeyrolle-Arias *et al.* 1994, Pierpaoli & Lesnikov 1997, Lu *et al.* 1999, Granger *et al.* 2004), and (3) the pulsatile release of GnRH I from the hypothalamus is dramatically elevated following menopause (Gill *et al.* 2002, Gore *et al.* 2004). The increased binding of GnRH I to gonadotrope GnRHR I results in dramatic elevations in the synthesis and secretion of gonadotropins following menopause and andropause (Gharib *et al.* 1990, Couzinet & Schaison 1993, Woller *et al.* 2002). The presence of GnRHR I on neurons of the brain implies that GnRH I also might signal through these neurons to increase LH synthesis and secretion.

Understanding the source of neuronal LH has implications for our understanding of neoplasia (Whitfield & Kourides 1985, Krichevsky *et al.* 1995, Yokotani *et al.* 1997) and AD (Bowen & Atwood 2004). With regards to AD, LH promotes the amyloidogenic processing of the amyloid-β precursor protein to generate amyloid-β, the major component of amyloid plaques that deposit in AD (Bowen *et al.* 2004). In addition, the GnRH I analog, leuprolide acetate, which suppresses serum LH, has been shown to decrease amyloid load in the brains of normal and aged amyloid-β precursor protein (AβPP)-transgenic mice, and maintain cognitive performance in aged AβPP-transgenic mice (Bowen *et al.* 2004, Casadesus *et al.* 2006).

Given the above observations, we examined whether neurons express GnRHR I, and like pituitary gonadotropes, respond to GnRH I by expressing LH. We report that GnRHR I is localized to the cell body and apical dendrites of pyramidal neurons in the human hippocampus, entorhinal cortex, and occipitotemporal gyrus, and that neurons are capable of *de novo* LH synthesis. GnRH I may therefore play a role in regulating biochemical events associated with post-reproductive neuronal senescence.

Materials and Methods

Antibodies and reagents

The high-affinity LH polyclonal antibody generated against the β-subunit of the LH glycoprotein was from Dr A F Parlow at the National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA, USA. The GnRHR I monoclonal antibody, F_1G_4, directed against the N-terminal amino acid residues 1–29 was from Dr Anjali Karande (Indian Institute of Science, Bangalore, India). The GnRHR I polyclonal antibody, C-18, directed against 18 C-terminal residues, and the GAPDH polyclonal antibody were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Neuron specific enolase (NSE) was purchased from

Signet Laboratories (Dedham, MA, USA). Horseradish peroxidase linked goat anti-mouse, goat anti-rabbit, and donkey anti-goat IgG were from Santa Cruz Biotechnologies. Goat affinity purified antibody to mouse IgG was from MP Biomedicals (Aurora, OH, USA) and the mouse peroxidase anti peroxidase (PAP) was from Steinberger Monoclonals Inc. (Lutherville, MD, USA). Isotype-matched antibody IgG1 was from Santa Cruz Biotechnologies. The low range pre-stained (approximately 20–120 kDa) molecular weight markers were from Bio-Rad Laboratories.

Primary rat cortical neurons (E18) were purchased from Brain Bits (Springfield, IL, USA). Opti-Mem, Dulbecco's modified Eagle's media (DMEM), modified Eagle's media (MEM), and F12 Nutrient Mixture B27 supplement were from Invitrogen. The media supplement, insulin–transferrin–selenium, was purchased from Sigma Laboratories. GnRH I peptide was from Peninsula Laboratories (San Carlos, CA, USA). Protease inhibitor cocktail (aprotinin, pepstatin, leupeptin, and phenylmethylsulphonyl fluoride) was from Roche Diagnostics. The Luteinizing Hormone Detection Kit was from Hope Laboratories (Belmont, CA, USA).

Immunohistochemistry

Human hippocampal tissue was collected at autopsy from clinically and pathologically confirmed cases of AD using National Institute for Aging (NIA) and Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria (Khachaturian 1985, Mirra *et al.* 1991; Table 1). Tissues were formalin-fixed, embedded in paraffin, sectioned at 4 μm thickness and placed on SP Brand Superfrost Plus slides (Cardinal Health, Dublin, OH, USA).

Tissue sections were deparaffinized with xylene and then hydrated through a series of ethanols. Endogenous peroxidase activity was eliminated with a 30-min incubation in 3% H_2O_2 in methanol. Following placement of the sections in citrate buffer (1 mM, pH 6·0), slides were autoclaved at 121 °C for 30 min and then rinsed well. Non-specific binding sites were blocked with 10% normal goat serum in tris buffered saline (TBS; 20 mM Tris, 150 mM NaCl, pH 7·6) for 30 min before application of GnRHR I monoclonal antibody F_1G_4 (1:250 dilution) overnight at 4 °C. After a series of washes, tissue sections were incubated with both the goat affinity purified antibody to mouse IgG (1:50 dilution, 30 min) and the mouse PAP (1:250 dilution, 60 min). Immunostaining was then developed using diaminobenzidine (DAKO Corporation, Carpinterie, CA, USA) for 10 min, slides mounted using Permount (Fisher Chemicals, Fair Lawn, NJ, USA) and staining analyzed under an inverted microscope (Zeiss Axiophot, Thornwood, NY, USA). To control for non-specific primary or secondary antibody binding, control tissue sections were treated with isotype-matched IgG_1 primary antibody or secondary antibody respectively. Sections were scored by three investigators for hippocampal (CA2, CA1, end plate, and subiculum) labeling. An Olympus Optical BX40F

Table 1 Immunohistochemical labeling of human hippocampal (CA2, CA1, end plate, subiculum) tissue for GnRHR I in Alzheimer's disease (AD) and age-matched control subjects

Subject	Age (years)	Gender	Diagnosis	Brain weight	Post-mortem delay	End plate	CA2	CA1	Subiculum
2	72	M	Control	1375 g	N/A	+	+	+ +	−
5	71	M	Control	N/A	21 h 20 min	+	+	+ +	−
11	74	F	Control	1223 g	7 h 15 min	+ +	+ +	+ +	+
14	68	M	Control	1200 g	3 h 30 min	+ +	+ +	+	+
17	71	F	Control	N/A	2 h 5 min	+ +	+ + +	+ +	+
24	65	M	Control	1370 g	5 h 54 min	+ +	+ + +	+ +	+ +
1	70	M	AD	1300 g	21 h 50 min	+	+	+	+
3	90	M	AD	1300 g	N/A	+	+	+ +	+
4	88	F	AD	1100 g	3 h 45 min	+	+ +	+	N/A
23	76	F	AD	1060 g	7 h 25 min	+ +	+ +	+	+
25	86	F	AD	1040 g	20 h 55 min	+ +	+ +	+	−
29	82	F	AD	1100 g	2 h 5 min	+	+ +	+	−

Neuronal staining: −, +, + +, + + +, or + + + +; where – represents no cellular staining and + + + + represents staining equivalent to the strongest signal observed in pituitary gonadotropes. N/A, not available; area of subiculum was infarcted and a score could not be determined.

microscope at objective 10× magnification was used to capture images using Image Pro software.

Human brain tissue homogenization and immunoblot analysis

Human frontal area 9 (frontal cortical) brain tissues were kindly provided by the Sanders-Brown Center on Aging at the University of Kentucky, Lexington, KY, and pituitary was provided by the University of Wisconsin/Veteran's Administration Brain Bank. Age, gender, and post-mortem interval (PMI) matched samples (approximately 150 mg) of seven AD cases (ages 84–93) and seven control cases (ages 83–92) were homogenized in 300 μl PBS, containing 10 μg/ml aprotinin and leupeptin, 1 μg/ml pepstatin A, 1 mM phenylmethanesulfonyl fluoride, 1% Triton-X100, 0·2% NP40, and 0·1% SDS) over ice. Samples were centrifuged at 9000 g for 10 min and the supernatant removed and stored at −80 °C for future analysis.

Following protein assay (BCA assay Pierce, Rockford, IL, USA), equal amounts of protein were loaded onto 10–20% Tricine precast gels (Novex, San Diego, CA, USA). Samples then underwent immunoblot analysis as described previously using GnRHR I monoclonal antibody, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody, and GnRHR I polyclonal (C-18) antibody.

M17 neuroblastoma cell culture, treatment, and immunoblotting

Human M17 neuroblastoma cells were maintained at 37 °C in OPTI-MEM media containing 0·5% FBS and 1% penicillin/streptomycin (P/S; 100 U/ml penicillin, 100 mg/ml streptomycin). Human SH-SY5Y neuroblastoma cells were maintained in MEM and F12 medias (1:1 ratio) containing 10% FBS and 1% P/S. For experiments, cells were plated 48 h prior to treatment at a density of approximately $5·0 \times 10^5$ cells/well in six-well plates. The following day, cultures were placed in serum-free media (DMEM

supplemented with 1% insulin/transferrin/sodium selenite supplement) for 24 h prior to treatment with 0–10 μM GnRH I peptide. Cells were then collected using lysis buffer (Tris 20 mM, NaCl 150 mM, 1% SDS, 1 mM EDTA, 1 mM EGTA, and protease inhibitors; pH 7·6), the protein concentration determined using the BCA Assay and equal amounts of protein loaded onto 10–20% tricine gels (Novex). Samples were then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories), fixed with 4% glutaraldehyde in TBS Tween-20 (TBST), blocked with 10% milk in TBST for 2 h and probed with a rabbit polyclonal antibody against human LH (dilution 1:1000). Following a series of TBST washes on the second day, the blot was incubated with the appropriate secondary antibody for 2 h at room temperature, washed again and developed with ECL reagent (Amersham). The chemiluminescent signal was captured on autoradiographs (Eastman Kodak Company, Aurora, IL, USA). The blot was then stripped using Restore stripping buffer (Pierce) and subsequently reprobed with the GnRHR I monoclonal antibody and finally a GAPDH antibody as a loading control for quantification. Images were captured, scanned, and the intensity of the autoradiograph signals (including a blank region) was determined using the NIH Image J software (http://rsb.info.nih.gov/nih-image/). Control and treatment values were corrected for blank values, normalized to their respective GAPDH band intensity and the results then expressed as a fold change over control levels.

Primary rat cortical neuron culture

Neuronal cultures were taken from frontal cortices of Sprague–Dawley rat embryonic (E18) pups. The cells were dissociated by tituration in Hibernate E media (Brain Bits), undispersed tissue allowed to settle for 1 min, and the supernatant transferred to a new tube prior to centrifugation for 1 min at 200 g. The supernatant was then removed and the cells resuspended in serum-free Neurobasal medium (without

phenol red) with B-27 supplement (Invitrogen), 25 μM L-glutamate, 1% P/S and 0·5 mM L-glutamine, counted using a hemocytometer and then equal volumes of mixed cells were plated into six-well (1×10^6 cells/well; Fisher) poly-L-lysine-coated plates (Sigma). Cortical cultures were allowed to differentiate in 5% CO_2, 85% humidity in the above media for up to 12 days and then collected for immunoblot analysis. Following protein assay, gels were loaded with equal amounts of protein. A loading control (such as tubulin and actin) was not assessed since the expression of these proteins increase with differentiation (Drubin *et al.* 1988).

LH secretion assay

Human M17 neuroblastoma cells were cultured in OPTI-MEM media (Invitrogen) containing 0·5% FBS and 1% P/S for 72 h or until confluence was reached. Cells were then treated in serum-free conditions with 0–10 μM GnRH I for 48 h. Media were collected and stored at −80 °C prior to enzyme immunoassay (Hope Laboratories) to quantitate media LH concentration. Two human serum samples containing low and high concentrations of LH were included as controls for the assay.

Detection of GnRHR I and LH mRNA

Total RNA was isolated from cultured M17 cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. GnRHR I and LHβ cDNA were synthesized and amplified using the SuperScript III One-Step RT-PCR system (Invitrogen). Both cDNA synthesis and PCR amplification were carried out using gene-specific primers: GnRHR I forward 5′ AGCAGACAGCTCTGGACAGACAAA 3′, reverse 5′ ACATAGTAGGGAGTCCAGCAGACA 3′. LHβ forward 5′ GCTACTGCCCCACCATGATG 3′, reverse 5′ ATGGACTCGAAGCGCACATC 3′. The PCR product was run on 4% Metaphor agarose gel (Cambrex Bio Science, Rockland, ME, USA) or 20% polyacrylamide gel (Invitrogen), and amplified DNA extracted using a QIA quick gel extraction kit (Qiagen). Extracted PCR product was cycle sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Madison, WI, USA) and automated sequencing was performed at the University of Wisconsin Biotechnology Center (Madison, WI, USA). To confirm that the sequenced PCR products were in fact human GnRHR I or LHβ cDNA and not the result of amplification of residual genomic DNA in the RNA sample, sequenced PCR products were aligned with the known GnRHR I mRNA sequence (Accession number AY392011), or the LHβ mRNA sequence (Accession number NM_000894) and checked for the absence of an intronic sequence.

Statistical analysis

For immunohistochemical labeling of AD and control tissues, scores from three investigators for hippocampal (CA2, CA1,

end plate, and subiculum) labeling of GnRHR I were averaged. Statistical analysis of the expression of GnRHR I variants between AD and age-matched control samples was conducted using a two-sample *t*-test (between groups) (Statview 5.0; SAS Institute, Inc Cary, NC, USA).

Results

### Immunolocalization of GnRHR I to human pyramidal and cerebrocortical neurons

To determine whether GnRHR I is expressed by neurons of the human brain, hippocampal and neocortical tissue sections were immunostained using the F_1G_4 monoclonal GnRHR I antibody that detects the N-terminal 29 amino acids (Karande *et al.* 1995). Immunoreactivity was clearly present throughout the hippocampus and neocortex in the cell body of large pyramidal neurons and extended along the apical dendrites of these neurons (Figs 1 and 2). GnRHR I immunostaining in the hippocampus compared favorably with GnRHR I immunostaining in gonadotropes of the human pituitary (Fig. 1).

To determine if GnRHR I expression varied throughout the hippocampus, immunolabeling was scored in four main regions of the hippocampus: CA2, CA1, end plate, and subiculum (Fig. 2; Table 1). Labeling was most prominent in the CA2 sector (Fig. 2; Table 1), was noted in the end plate and CA1 sector, but was clearly less in the subiculum when compared with the other hippocampal sectors (Table 1). Labeling was also evident in neocortical neurons, including those located in the entorhinal cortex and occipitotemporal gyrus (Fig. 2). Staining was not observed within the granular layer of the dentate gyrus or in other neuronal cell types.

To determine if there were differences in the expression of GnRHR I between control tissues and tissue from the AD brain, age-matched AD brain tissues were stained for GnRHR I. Immunolabeling of AD tissues was clearly evident in the regions described above for control tissues (Fig. 2). No significant differences in labeling intensity were observed between control and AD tissues (Table 1). However, labeling was clearly decreased on apical dendrites of pyramidal neurons in the AD compared with control brains (Fig. 2; upper versus lower panels). Tissues incubated with an isotype-matched primary antibody or with the secondary antibody alone were not labeled (not shown). These results demonstrate that like the rat, GnRHR I is present on neurons of the human hippocampus and neocortex and is not solely located within the anterior pituitary.

AD and age-matched control human cortices express GnRHR I

To examine if there were differences in GnRHR I expression between age-matched controls and AD, immunoblot analyses of total homogenate from the frontal cortex were performed using the F_1G_4 antibody (Karande *et al.* 1995). Three main GnRHR I variants were present,

## HIPPOCAMPUS   PITUITARY
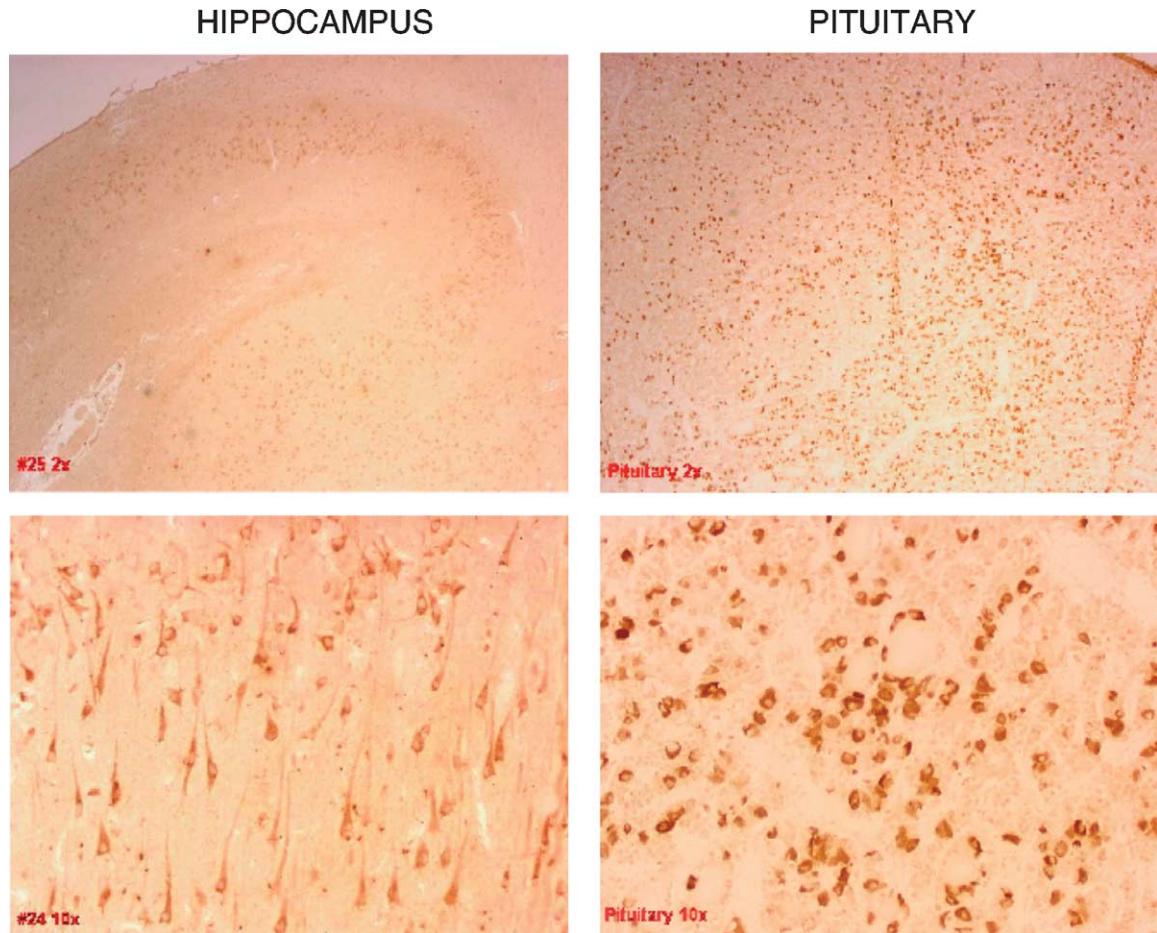
Figure 1 Immunolabeling of GnRHR I in the human hippocampus and pituitary. Human hippocampal and pituitary tissue (positive control) were immunolabeled with the GnRHR I monoclonal antibody, F_1G_4. Upper panels illustrate GnRHR I immunolabeling throughout the hippocampus and pituitary (objective magnification $2\times$). Lower panels illustrate labeling of hippocampal pyramidal neurons and pituitary gonadotropes (objective magnification of $10\times$).

including a 30 kDa GnRHR I variant, the mature 64 kDa variant (Wormald *et al.* 1985, Karande *et al.* 1995), a 136 kDa variant, and two minor variants migrating at approximately 77 and 94 kDa that might represent differentially post-translated proteins or receptor complex components (Fig. 3a; Conn & Venter 1985, Wormald *et al.* 1985, Stojilkovic & Catt 1995, Keller *et al.* 2005b). These same variants were also expressed in the pituitary, where the 94 kDa variant predominated (Fig. 3c). Quantification of the major variant bands indicated a 41, 28, and 86% decrease in the relative intensities of the 30, 64, and 136 kDa variants between control and AD brain respectively that reached significance with the 30 and 136 kDa variants when normalized to the GAPDH loading control (Fig. 3b). To determine whether the decrease in GnRHR I expression was a result of neuron loss in the AD brains, we reprobed the blot with NSE. As expected, the level of NSE was lower in the AD compared with control cortex. Normalization of the GnRHR I expression against NSE indicated that only

the 136 kDa variant was significantly decreased in the AD brain (Fig. 3b).

To confirm that there were no changes in the mature 64 kDa variant, we reprobed the blot using the C18 polyclonal GnRHR I antibody that has been shown to recognize only the mature uncomplexed form of the receptor (Bajo *et al.* 2003, Keller *et al.* 2005b). We detected one band migrating at 64 kDa, the mature form of the receptor (Wormald *et al.* 1985, Karande *et al.* 1995, Keller *et al.* 2005a; Fig. 4a). This band also was detected in homogenized pituitary tissue (Fig. 4b; Hazum *et al.* 1987). As noted above for the monoclonal antibody, GnRHR I (64 kDa) expression was not different between control and AD tissues (quantitation not shown). These results indicate that mature GnRHR I variant expression is similar in both AD and control cortical tissue, but that there is a differential expression of the 136 kDa variant between AD and age-matched control cortex. Together, these results demonstrate the localization of GnRHR I on both human hippocampal and cortical tissues.

HIPPOCAMPUS   NEOCORTEX
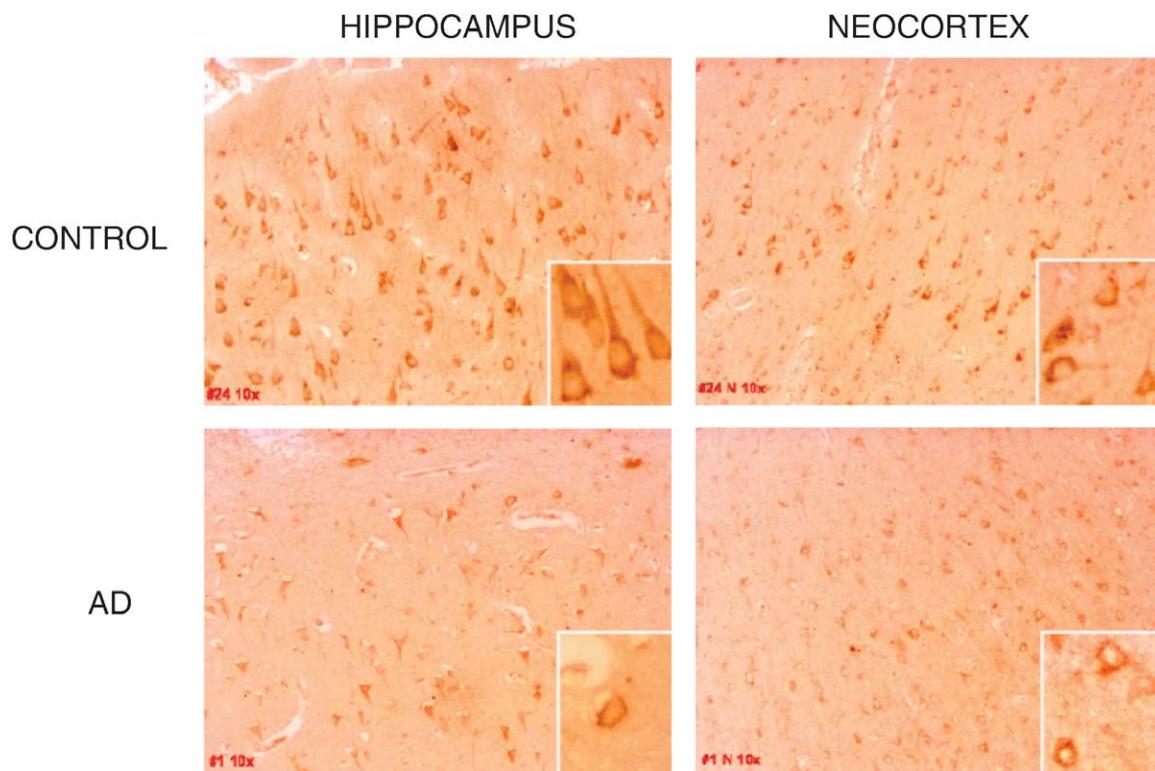
Figure 2 Immunolocalization of GnRHR I in human control and AD hippocampal and cortical neurons. Immunolabeling of control hippocampus (upper left) and neocortex (upper right) with the GnRHR I monoclonal antibody F_1G_4 demonstrates GnRHR I localization to the cell body and apical dendrites (case #24). Immunolabeling of AD hippocampus (lower left) and neocortex (lower right) demonstrates GnRHR I localization predominantly to the cell body (case #1). Insets are representative neurons from the $10\times$ pictures, taken at $40\times$ objective magnification to illustrate membrane labeling.

GnRH I induces the expression of LH in neuroblastoma cells and primary cortical neurons

Given that GnRHR I mediates signaling for the synthesis and secretion of LH by pituitary gonadotropes, we examined the functionality of neuronally expressed GnRHR I in terms of LH expression in human M17 and SH-SY5Y neuroblastoma cells cultured in serum-free media. Immunoblot analysis under reducing conditions and using the well-characterized polyclonal antibody anti-human-LHβ (Parlow 2004) indicated that neuroblastoma cells express three LH variants, including the mature 30/31 kDa full-length protein (Nureddin & Johnson 1977) and two minor variants migrating at approximately 43 and 47 kDa that might represent differentially post-translated proteins (Fig. 5a, lane 1 and d). A 60 kDa band also was detected that might represent a dimer. Since the anti-human LHβ antibody was generated against the entire β-subunit of the LH glycoprotein, the increase in expression of the mature 30 kDa protein suggests that GnRH promotes the expression of both LHβ and glycoprotein hormone alpha subunit (α-GSU). To confirm LH expression, isolated RNA was reverse transcribed and LHβ cDNA amplified using two gene-specific primers. The expected 95 bp cDNA fragment encompassing portions of exons 2 and 3 was detected

(Fig. 5c). The sequence of the amplified cDNA matched the genomic sequence (minus the intronic sequences) demonstrating that the amplified cDNA was from LHβ mRNA template.

We next tested whether neuroblastoma cells respond to GnRH I by increasing LH expression. Treatment of M17 cells with increasing concentrations of human GnRH I (0–10 μM) for 6 h initially increased the expression of all LH variants. Quantitation of the immunoblots indicated that the expression of the 30 and 47 kDa variants were approximately two- to threefold elevated between 1 and 10 nM, but at high GnRH I concentrations ($\geq$1000 nM), the expression of both LH variants was suppressed (Fig. 5b). To examine whether primary neurons also express LH, cortical neurons from day 18 rat embryos were differentiated over 12 days. An increase in the expression of the 30 kDa full-length LH was apparent with differentiation (Fig. 5e). These results indicate that LH mRNA and protein are normally expressed by neurons.

To determine whether changes in LH expression following GnRH I treatment was a result of GnRHR I activation, we examined GnRHR I expression in the neuroblastoma samples, using the monoclonal antibody F_1G_4 described above. Immunoblot analysis indicated that M17 neuroblastoma cells
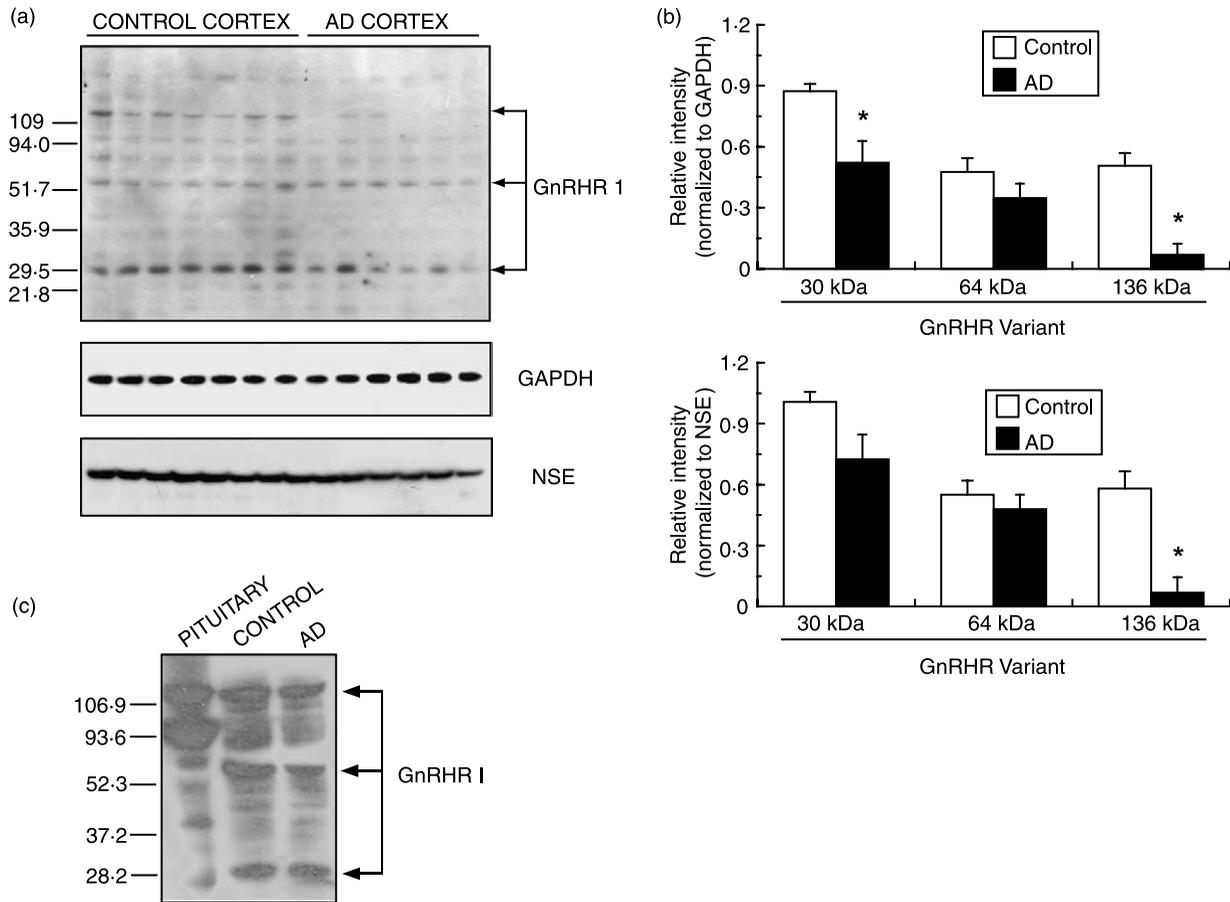
Figure 3 GnRHR I expression in the cortex of age-matched control and AD subjects. (a) GnRHR I expression was examined in homogenized age-matched AD and control cortical tissue by immunoblot analysis using the GnRHR I monoclonal antibody, F_1G_4. The blot was reprobed for tissue (GAPDH) and neuron-specific (NSE) loading controls. (b) Quantification of the three prominent GnRHR I variants revealed significant decreases in the 30 and 136 kDa in AD compared with age-matched control tissue when normalized to the GAPDH loading control (mean±S.D., $n=6$–7, *$P<0.01$). A significant decrease was maintained only for the approximately 136 kDa receptor complex in AD tissue after normalization against the NSE loading control (mean±S.D., $n=6$–7, *$P<0.01$). (c) Immunoblot analysis of pituitary and representative control and AD cortex revealed similar GnRHR I variants. Molecular weight markers are illustrated on the left of figures.
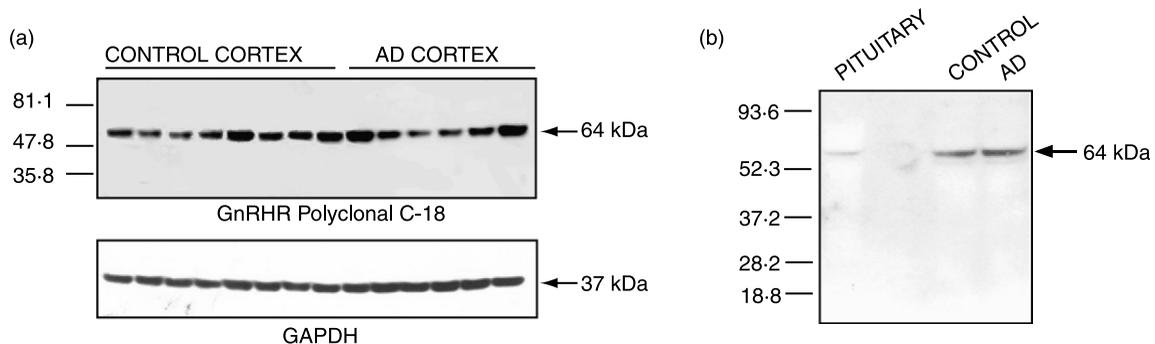
Figure 4 GnRHR I expression in the cortex of age-matched control and AD subjects. (a) The immunoblot in (Fig. 3a) was reprobed with a polyclonal antibody against the C-terminus of GnRHR I (C-18). One distinct band at approximately 64 kDa, representative of the functional GnRHR I variant, was detected. (b) The samples in (Fig. 3c) were immunoprobed with the polyclonal antibody against the C-terminus of GnRHR I (C-18). A single approximately 64 kDa band is present in both pituitary and brain. Molecular weight markers are illustrated on the left of figures.
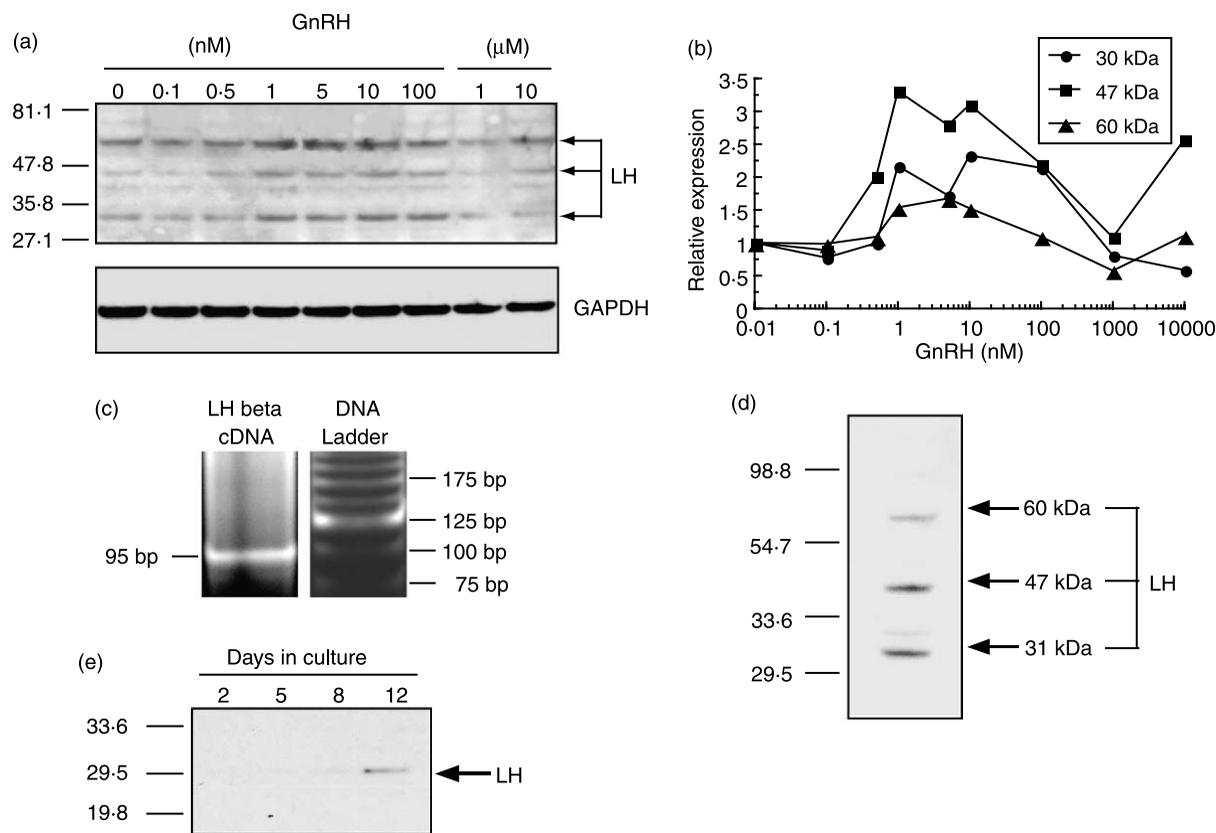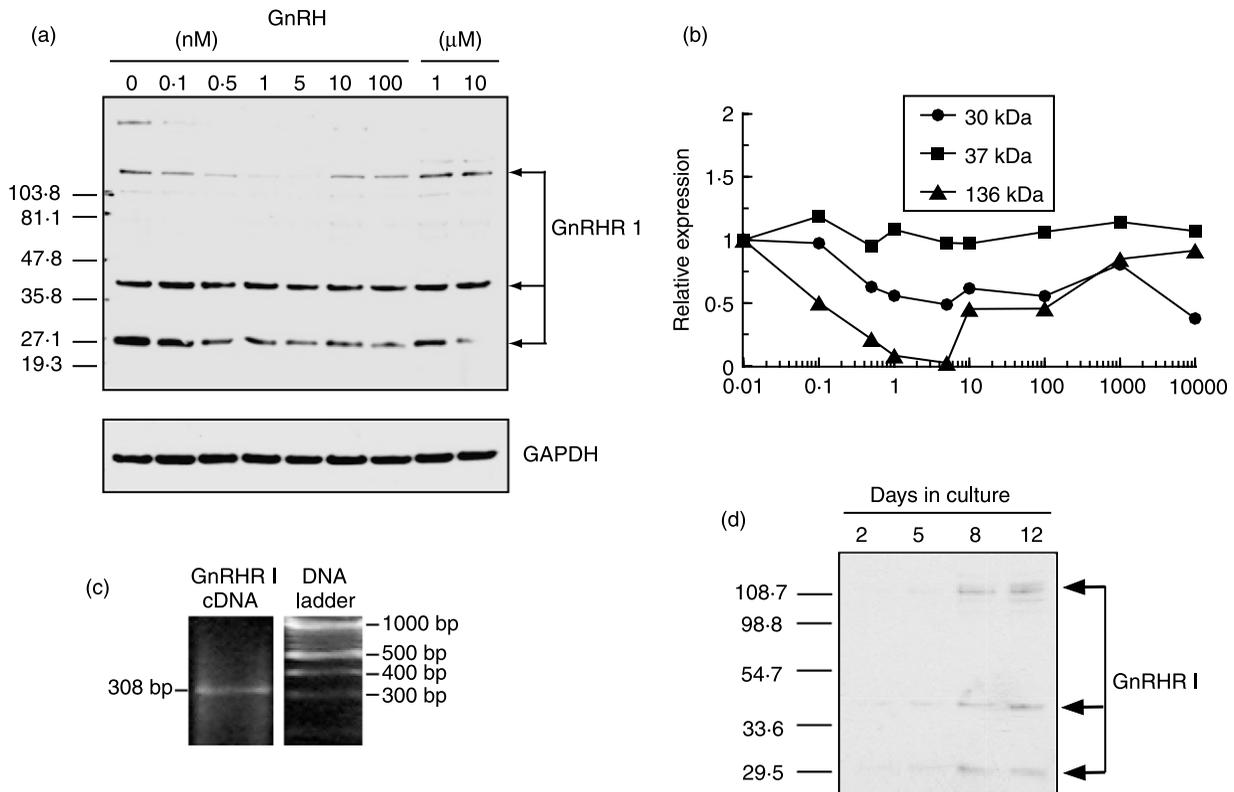
Figure 5 LH expression on neuronal cell types and its modulation by GnRH. (a) Human M17 neuroblastoma cells cultured in defined, serum-free media were treated with increasing concentrations of GnRH I (0–10 µM) for 6 h and then collected for immunoblot analysis of LH expression using the polyclonal anti-human β-LH antibody. (b) Relative intensities of the 30, 47, and 60 kDa bands were normalized to the loading control, GAPDH. Data are representative of three experiments. (c) LHβ cDNA was detected in human M17 neuroblastoma cells. (d) Human SH-SY5Y neuroblastoma cells express similar LH variants as described above for M17 cells. (e) Primary cortical neurons from embryonic day 18 rats cultured in serum-free conditions demonstrate increased LH (30 kDa) expression with differentiation. Molecular weight markers are illustrated on the left of figures.

express a number of GnRHR I variants, including the immature 37·7 kDa full-length protein (deduced from the amino acid sequence), a 30 kDa variant which may represent a truncated variant, and a 136 kDa variant that represents the functional form of the receptor (Conn & Venter 1985; Fig. 6a). Similar variants were observed in SH–SY5Y neuroblastoma cells (data not shown). To confirm GnRHR I expression, isolated RNA was reverse transcribed and GnRHR I cDNA amplified using two gene-specific primers. The expected 308 bp cDNA fragment encompassing parts of exons 2 and 3 was detected (Fig. 6c). The sequence of the amplified cDNA matched the genomic sequence (minus the intronic sequences) demonstrating that the amplified cDNA was from the GnRHR I mRNA template. These results indicate that GnRHR I mRNA and protein is normally expressed by neurons. Expression of the 37·7 kDa immature form of the receptor did not alter with increasing concentrations of GnRH I. However, the levels of the 30 and 136 kDa variants initially decreased with increasing GnRH I concentration before increasing at higher GnRH

concentrations (Fig. 6b). Immunoblot analysis of these samples using the GnRHR I polyclonal antibody against a C-terminal stretch of 18 amino acids revealed only one band that was representative of the immature 37·7 kDa variant (data not shown). Overall, these results indicate that transcription of the immature form of the receptor is not affected by GnRH I, but that receptor complex formation and/or post-translational modification of the GnRHR I is modulated by GnRH I.

Immunoblot analysis of differentiating rat cortical primary neurons for GnRHR I indicated that the same variants were expressed (approximately 30, 37, and 136 kDa; Fig. 6d) as for the human neuroblastoma cell lines (Fig. 6a). GnRHR I expression increased with neuron differentiation (i.e. neuritic outgrowth), and since equal amounts of protein were loaded at each time point, these results suggest that neurites may express proportionally more GnRHR I than the neuronal cell body.

Together, these results indicate that neurons, (1) express mature and post-translationally modified variants of LH, (2) respond to GnRH I by increasing the expression of LH, and

Figure 6 GnRHR I expression on neuronal cell types and its modulation by GnRH. (a) The immunoblot from Fig. 4a was stripped and reprobed with the F_1G_4 monoclonal GnRH I receptor antibody. Three major GnRHR I variants were detected: approximately, 30, 37, and 136 kDa. (b) Relative intensities of GnRHR I receptor variants were normalized to GAPDH. Decreases in the levels of the approximately 30 and 136 kDa GnRHR I variants were observed with increasing GnRH I concentrations. The data are representative of three experiments. (c) GnRHR I cDNA was detected in human M17 neuroblastoma cells. (d) The immunoblot from Fig. 5e was stripped and reprobed with the F_1G_4 monoclonal GnRH I receptor antibody. The expression of approximately 30, 37, and 136 kDa GnRHR I variants increased with neuron differentiation. Equal protein was loaded at each time point. Molecular weight markers are illustrated on the left of figures.

(3) possess functional GnRHR I as illustrated by the modulation of LH and GnRHR I expression by GnRH I.

Discussion

Expression of GnRHR I by neuronal cells

Although GnRHR I has been localized to extrapituitary regions of the rodent brain, we demonstrate for the first time the presence of GnRHR I on pyramidal neurons of the hippocampus and neocortical neurons of the entorhinal cortex and occipitotemporal gyrus of the human brain (Figs 1–4). The presence of GnRHR I throughout the neuron body as well as in the apical dendrites of pyramidal neurons, but not in neurons of the granular cell layer, suggests that GnRH I signaling plays an important role in the function of hippocampal neurons. In this respect, previous studies have shown that leuprolide acetate, a potent GnRHR I agonist, induces a long-lasting enhancement of synaptic transmission mediated by ionotropic glutamate receptors in CA1 pyramidal neurons of rat hippocampal slices (Lu *et al.* 1999). The use of GnRH analogs in modulating

synaptic transmission and neuronal metabolism in these areas of the brain requires further investigation due to its implications in learning and memory.

Immunoblot analysis of human cortical tissue confirmed the presence of three major GnRHR I variants: the mature 64 kDa receptor, a 30 kDa truncated variant and an approximately 136 kDa complex variant (Fig. 3). Although there were considerable individual variations in the expression of GnRHR I variants, the expression of the mature 64 kDa variant (Wormald *et al.* 1985, Karande *et al.* 1995, Keller *et al.* 2005a) was not different between control and AD brains (Fig. 3). Interestingly, radiation inactivation analysis of GnRHR I (a method for determining the functional molecular weight of a receptor while it is still a component of the plasma membrane) indicates that GnRHR I has a molecular mass of $136\,346 \pm 8120$ (Conn & Venter 1985). This estimate is approximately twice of that obtained by immunoblot analyses (Karande *et al.* 1995, Keller *et al.* 2005a; Fig. 3) and by photoaffinity labeling with a radioactive GnRH analog followed by electrophoresis under denaturing conditions (Wormald *et al.* 1985). The high molecular weight band present under our non-reducing conditions in both the

brain and neuroblastoma cells may therefore represent a high molecular weight complex of GnRHR I in its native state, coupled to G-proteins and other associated proteins, as has been reported for numerous other G-protein coupled receptors (reviewed by Park *et al.* 2004). The low expression level of the mature 64 kDa variant in neuroblastoma cells, and of the immature 37·7 kDa variant in the brain, suggests rapid post-translational modification and complexing of GnRHR I to other components of the receptor complex.

GnRHR I expression on transformed cell lines (Fig. 6) has previously been reported for the human olfactory FNC-B4 cell line (Romanelli *et al.* 2004), as well as numerous other cancer cell lines originating from the prostate, kidney, skin, lympho-cytes, liver, larynx, pancreas, colon, and brain (Fekete *et al.* 1989, Friess *et al.* 1991, Pati & Habibi 1995, van Groeninghen *et al.* 1998, Krebs *et al.* 2002, Moretti *et al.* 2002, Ben-Yehudah & Lorberboum-Galski 2004). Rat primary cortical neurons express the same GnRHR I variants as neuroblastoma cells (Fig. 5) indicating that neoplastic transformation does not affect GnRHR I expression. The functionality of these receptors is indicated by the GnRH I-induced decrease in the expression of the 136 kDa receptor complex in neuroblastoma cells (Fig. 6b). A similar decrease in the expression of the 136 kDa receptor complex (-86%) was noted in the AD brain (Fig. 3b), a time when LH/ follicle stimulating hormone (FSH) and presumably GnRH I levels are high (Bowen *et al.* 2000). Since continuous GnRH I stimulation of the GnRHR I is known to 'desensitize' receptor signaling, our results suggest that GnRH I may promote the uncoupling of GnRHR I from G-proteins and other proteins of the receptor complex (Fig. 6; Park *et al.* 2004). Alternatively, GnRH I may promote the internalization and degradation of the receptor complex (Adams *et al.* 1986).

Expression of LH by neurons

We report that primary neurons and neuroblastoma cells express the gonadotropin LH, and that LH expression is modulated in a dose-dependent manner by GnRH I (Fig. 5). The expression of LH in neurons grown in serum-free media (Fig. 5) indicates that neuronal LH was not sequestered from the serum but synthesized *de novo*. LH expression increased with differen-tiation in primary cortical neurons, suggesting that LH expression is a normal function of differentiated neurons. In neuroblastoma cells, GnRH I promoted a similar pattern of expression for all the three major LH variants (30, 47, and 60 kDa); LH expression increased with increasing GnRH I concentrations, but at high GnRH I concentrations the expression of LH decreased. The increase in the expression of LH variants was first detectable at 0·5 nM GnRH I (Fig. 5), which is within the affinity range of GnRH I and GnRH I analogs for GnRHR I ($K_d = 0\cdot1$–1 nM) in the hippocampus of the rat (reviewed by Vadakkadath Meethal & Atwood 2005). GnRH I did not alter the expression of the 37·7 kDa variant of GnRHR I over this short treatment period (6 h) at any concentration (Fig. 6) indicating that a mechanism independent of the regulation of GnRHR I expression mediated the

decrease in LH expression (it is unlikely that GnRH I was acting through the 30 and 136 kDa variants, since their expression decreased with increasing GnRH I concentration when LH expression was increasing). The decline in cellular LH at high GnRH I concentrations over this short time (6 h) is unlikely to be explained by GnRH I-induced secretion of LH, since we did not detect LH secretion into media (data not shown), although it cannot be ruled out that neuroblastoma cells secrete low levels of LH. It is therefore likely that desensitization of GnRH I signaling was induced by continuous GnRH I treatment that has previously been shown with other G protein-coupled receptors to involve rapid uncoupling of the receptor from the G-protein and loss of downstream signaling events (and which is followed by subsequent sequestration of the receptor from the plasma membrane, internalization, and proteolytic degradation; Cheng & Leung 2000).

At least 39 isoforms of human LH have been identified (Nureddin & Johnson 1977, Stanton *et al.* 1993). The LH variants detected in human neuroblastoma cells (30, 47, and 60 kDa) under non-reducing immunoblot analysis have been detected in post-menopausal (30, 35, and 43 kDa; Iles & Chard 1991, Kurowska & Szewczuk 1999) and pre-ovulatory urine (29, 49, 67, 80, and 95 kDa; Kurowska & Szewczuk 1999), and in the human pituitary (29, 49, 60, 67, and 95 kDa; Stanton *et al.* 1993, Kurowska & Szewczuk 1999).

The expression of LH by neurons is corroborated by the presence of translatable levels of LH mRNA and LH protein in pyramidal neurons of the cerebral cortex and hippocampus of the rat and human (Lee *et al.* 2004). Levels of neuronal LH were undetectable in adult (3–5-month old) animals but increased with age at 1 and 2 years of age. Previous studies have demonstrated the presence of membrane-associated LH and human chorionic gonadotropin (hCG, which has 83% amino acid sequence homology and shares a common receptor to LH, which they bind with similar affinity) on a number of different human cancer cell lines including retinoblastoma cells cultured in serum-free conditions (Rosen *et al.* 1980, Acevedo *et al.* 1992, Krichevsky *et al.* 1995), (Whitfield & Kourides 1985, Krichevsky *et al.* 1995, Yokotani *et al.* 1997).

Functional consequences of GnRH I induction of LH expression by neurons

The presence of GnRHR I on reproductive and non-reproductive tissues suggests GnRH I has physiological affects on tissues of the body other than the pituitary, including the hippocampus (Kakar & Jennes 1995, Cheng & Leung 2000, Harrison *et al.* 2004). The high binding affinity of GnRH I for its neuronal receptor and the activation by GnRH I of neuronal signal transduction pathways in the rat brain (Jennes *et al.* 1995, 1997) supports the functionality of extrapituitary GnRHR I. It is not clear whether GnRH I produced by the hypothalamus or by other tissues binds to and signals through these receptors throughout the body. At least for the brain, GnRHR I signaling is likely mediated through GnRH I produced by GnRH I neurons that extend throughout different regions of

the brain (Tobet *et al.* 1996, Quanbeck *et al.* 1997, Kim *et al.* 1999, Reed *et al.* 2002). The short serum half-life of GnRH I (approximately 2–3 min; Redding *et al.* 1973, Fauconnier *et al.* 1978) would support autocrine release of GnRH I within the brain, although it has been shown in rats that GnRH I can cross the blood brain barrier (BBB) (Dvorska *et al.* 1992).

The increased secretion of GnRH I by GnRH I neurons following menopause (Hall *et al.* 2000, Gore *et al.* 2004) may explain the increased expression of LH in aging pyramidal neurons of the cerebral cortex and hippocampus of the rat (Lee *et al.* 2004). In addition, the elevation in gonadotropins (Bowen *et al.* 2000, Hogervorst *et al.* 2001, Short *et al.* 2001) and presumably GnRH I secretion would explain the accumulation of LH detected in pyramidal neurons of the AD compared with age-matched control brain (Bowen *et al.* 2002). LH released by pituitary gonadotropes could also transfer through perivascular pathways into the brain. The identification of LH/hCG receptors on both endothelial and smooth muscle cells suggests a route by which LH might cross from the vasculature into the brain (Toth *et al.* 2001). The contribution of *de novo* synthesized and extracellular LH to total intracellular LH remains to be determined.

The functional consequences of intracellular neuronal LH production are unclear. If neuronal LH was secreted or bound to its receptor intracellularly during its processing through the estrogen receptor (ER) and/or transport to the cell membrane, then a number of scenarios are possible. First, the expression of membrane-associated glycoproteins LH/hCG is a phenotypic characteristic of human cancer cells; hCG is a common serum marker used to determine the progression of cancer and its regression following chemotherapy (Stenman *et al.* 2004). Thus, the elevation in LH expression in pyramidal neurons in the AD brain (Bowen *et al.* 2002) is suggestive of a cellular transformation from a differentiated to a mitotic phenotype. In this respect, it is becoming increasingly apparent that pyramidal neurons of the AD brain, those most vulnerable to AD neuropathology, are also those which display numerous markers of cell cycle re-entry (Raina *et al.* 2000, Herrup *et al.* 2004). Since both GnRH I and LH/hCG have mitogenic properties, it is plausible that these mitogens may drive differentiated neurons back into the cell cycle. Secondly, in this connection we have shown that LH promotes the amyloidogenic processing of AβPP (Bowen *et al.* 2004), a cell cycle-related protein that releases Aβ when neurons are driven into the cell cycle (McPhie *et al.* 2003). Likewise, LH dose-dependently increases tau phosphorylation and the activity of glycogen synthase kinase-3β (GSK-3β) (Casadesus *et al.* 2006), a key regulator of tau phosphorylation and neurofibrillary tangle formation. In this context, leuprolide acetate (a GnRH I analog), which suppresses serum LH levels, decreases amyloid plaque load in normal and aged AβPP-transgenic mice and maintains the cognitive performance of AβPP-transgenic mice (Bowen *et al.* 2004, Casadesus *et al.* 2006). It is unclear at this stage whether leuprolide acetate is acting through the suppression of LH to alter amyloid production and cognition (Bowen *et al.* 2004, Casadesus *et al.* 2006), or directly through

GnRHRs (Figs 1–4 and 6) to modulate synaptic transmission (Lu *et al.* 1999). Irrespective, these data support the use of leuprolide acetate in the treatment of AD; leuprolide acetate has been shown in clinical trials to stabilize cognition in women with mild-to-moderate AD over a 1 year period (LaPlante *et al.* 2006). Thirdly, neuronal LH might impact neurosteroid production, since we have recently demonstrated that LH promotes neurosteroid production in human neuroblastoma cells and in differentiated rat primary neurons (Liu *et al.* 2006). Future studies will therefore be important in determining the exact role of neuronal LH.

In summary, this paper has demonstrated the presence of GnRHR I on hippocampal and cortical neurons of the human brain, differentiated rat embryonic primary neurons and human neuroblastoma cells. Our findings implicate GnRH I in the normal functioning of the brain, although the physiological significance of our unexpected finding that GnRH can induce neuronal LH synthesis remains to be fully investigated. Therefore GnRH I surges associated with the dysregulation of the hypothalamic-pituitary-gonadal (HPG) axis with menopause and andropause may mediate neurodegenerative events either directly or indirectly via LH production.

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