Elevated Luteinizing Hormone Expression Colocalizes With Neurons Vulnerable to Alzheimer's Disease Pathology

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In individuals with Alzheimer's disease (AD), there is a two-fold elevation in the serum concentrations of the gonadotropins, luteinizing hormone (LH), and follicle stimulating hormone compared to age-matched controls. Whether this plays a role in disease pathogenesis is unclear. Nonetheless, gonadotropins are known to cross the blood brain barrier and the highest density of gonadotropin receptors in the brain are found within the hippocampus. We report for the first time the localization of LH in the cytoplasm of pyramidal neurons. In addition, we find a significant increase in LH in the cytoplasm of pyramidal neurons and neurofibrillary tangles of AD brain compared to age-matched control brain. Whereas the functional consequences of increased neuronal LH are unknown, it is notable that LH is primarily localized to those neurons that are known to be vulnerable to Alzheimer's disease-related neurodegeneration. Elevated serum and cortical neuron levels of LH, coupled with the decline in sex steroid production, could play important roles in the pathogenesis of AD. © 2002 Wiley-Liss, Inc.

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A number of lines of evidence indicate that decreased sex hormone production after menopause/andropause contributes to the etiology of Alzheimer's disease (AD). First, in line with the abrupt earlier loss of gonadal function in females compared to males, the prevalence rate of AD in women is roughly twice that in men (Jorm et al., 1987; McGonigal et al., 1993). Interestingly, this skewed sex ratio appears relatively specific for AD. Other dementias, such as vascular dementia (Hebert et al., 2000) and Lewy body dementia (Yamada et al., 2001; Lopez et al., 2002), do not display a similar distribution. Second, a multitude of epidemiological studies show a positive correlation between AD and decreased estrogen levels after menopause (levels below that of men) (Manly et al., 2000). In fact, the decreased incidence (Henderson et al., 1994), and delay in the onset (Tang et al., 1996), of AD among women on hormone replacement therapies after menopause (Kawas et al., 1997) supports a protective role for estrogen in AD. Likewise, the increased incidence of AD in men with low serum testosterone also supports a role for testosterone in AD (Bowen et al., 2001; Hogervorst et al., 2001), although no retrospective studies have examined the effects of androgen replacement on cognition.

Both estrogen and testosterone production are under the control of the hypothalamic-pituitary-gonadal axis that stimulates sex steroid production by increasing the secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus, which in turn stimulates the anterior pituitary to secrete the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). A negative feedback mechanism between gonadotropin and gonadal steroid production is mediated by the inhibition of hypothalamic GnRH secretion that modulates the levels of these circulating hormones. The loss of negative feedback by estrogen on gonadotropin production after menopause (Couzinet and Schaison, 1993) results in a three- to four-fold and a four- to 18-fold increase in the concentrations of serum LH and FSH, respectively (Chakravarti et al., 1976). Likewise, men also experience a greater than two- and three-fold, increase in LH and FSH, respectively (Neaves et al., 1984). Surprisingly, the effects of increased circulating gonadotropins on the aging brain are largely unexplored. In this connection, we recently found a two-fold increase in the concentration of circulating gonadotropins in individuals with AD, above that of the already elevated concentrations of serum gonadotropins in age-matched control individuals (Bowen et al., 2000; Short et al., 2001). Because LH receptors are present
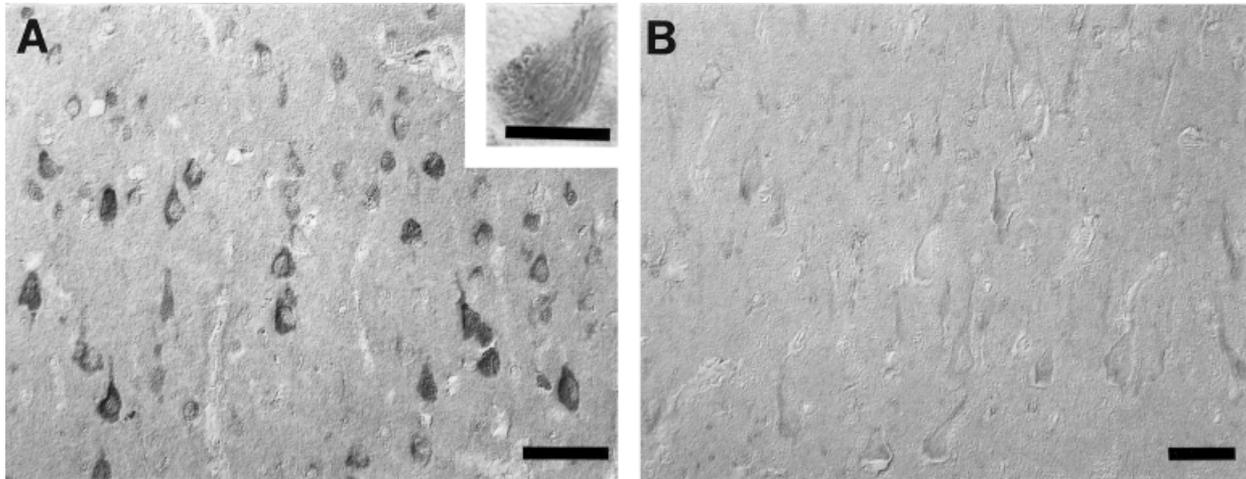
Fig. 1. The localization of LH in hippocampal tissue sections from AD (**A**) and age-matched control (**B**) brain. Increased levels of LH are found in the cytoplasm of pyramidal neurons in the hippocampus of the AD brain, with lesser staining evident in the neurons in the same region of an age-matched control brain. LH also was co-localized to neurofibrillary tangles (inset). Scale bars = 50 μm in A,B; 20 μm in inset.

on neurons, we decided to examine whether there were changes in the level of LH in the brains of control and AD individuals. We report an increase in LH immunoreactivity in the cytoplasm of pyramidal neurons of AD compared to age-matched control brains. Our findings are discussed in the context of physiological changes of gonadotropin concentration and their correlations with aging and disease.

MATERIALS AND METHODS

Tissue Sections

Brain tissue was obtained at autopsy from clinically and pathologically confirmed cases of AD using NIA and CERAD criteria (Khachaturian et al., 1985; Mirra et al., 1991) as well as from controls without dementia. Hippocampal and neocortical tissue from six AD cases (ages 65–89 years; postmortem interval 5.5–16 hr; 3 females, 3 males) and seven controls (ages 48–74 years; postmortem interval 7–24 hr, 4 females, 3 males) were fixed in methacarn (methanol:chloroform:acetic acid; 6:3:1) overnight at 4°C. After fixation, tissue was embedded in paraffin and 6 μm sections placed on silane-coated (Sigma, St. Louis, MO) slides.

Immunocytochemistry

Tissue sections were deparaffinized with xylene and then hydrated through graded ethanol. Endogenous peroxidase activity was eliminated with a 30-min incubation in 3% H_2O_2 in methanol. Sections were treated with 70% formic acid for 5 min at room temperature. Non-specific binding sites were blocked with 10% normal goat serum in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.6) for 30 min before application of either polyclonal LH (1:100 dilution), monoclonal LH (1:5 dilution; Endocrine Technologies Inc., Newark, CA) or a monoclonal to amyloid-β (1:1000 dilution, 4G8; Senetek,

Maryland Heights, MI). Immunostaining was developed by the peroxidase-antiperoxidase procedure (Sternberger, 1986) using 3-3'-diaminobenzidine (Dako, Glostrup, Denmark) as the co-substrate. Comparisons were made with adjacent sections in which the primary antibody was omitted.

Immunocytochemical Staining Quantitation

Three adjacent fields of hippocampus were viewed with a Zeiss Axiophot microscope with an attached Zeiss Axiocam digital camera and Zeiss Image Analysis system. The intensity of the immunoreaction in each field was determined by measuring the average optical density (OD) of the cytoplasm and nucleus of pyramidal neurons as described previously (Nunomura et al., 2001). The OD for each field was corrected for background by subtracting the OD of an adjacent area that did not contain positive staining.

RESULTS

Immunocytochemical analysis of tissue sections from the CA1 region of the hippocampus with both polyclonal and monoclonal antibodies specific for LH indicated an increase in LH immunoreactivity in the cytoplasm of hippocampal neurons from AD compared to age-matched control subjects (Fig. 1). LH immunoreactivity was present in neurofibrillary tangles, however, no staining was observed in other neuronal cell types or in amyloid plaques. Quantitative analysis revealed a greater than two-fold elevation ($P < 0.005$) in LH in cortical neurons from AD compared to age-matched control brain (Fig. 2). Some elderly controls with significant pathology showed intermediate levels of neuronal LH (not shown). Attesting to the specificity of our findings, both polyclonal and monoclonal antibodies revealed similar results, and we
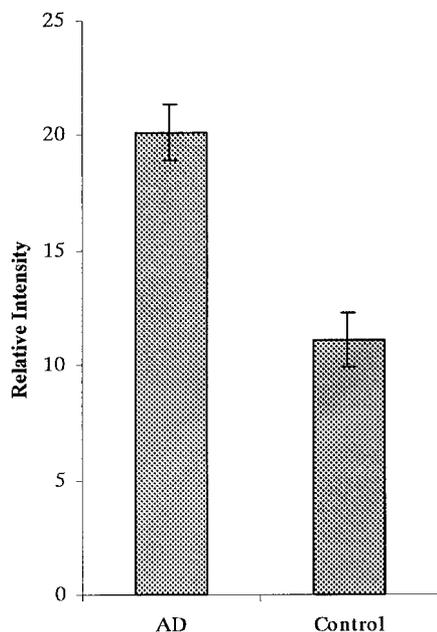
Fig. 2. LH levels in pyramidal neurons are elevated in AD brain compared to control brain. Immunocytochemical staining for LH was quantitated for pyramidal neurons from AD and age-matched control (mean ± SE; $P < 0.005$) brains.

found no specific labeling upon omission of these primary antibodies from the protocol.

DISCUSSION

We report for the first time localization of LH, a gonadotropin that mediates sex steroid synthesis by the gonads, in the cytoplasm of pyramidal neurons in the brain (Fig. 1). In addition, we find that there is an increase in cytoplasmic LH immunoreactivity in pyramidal neurons of the AD brain compared to age-matched control brain (Fig. 1). Notably, LH is concentrated only in those neurons vulnerable to AD-related neurodegeneration, including those neurons containing neurofibrillary tangles. Such increased LH in neurons of the AD brain is consistent with the elevation in the peripheral circulation of LH in individuals with AD compared to age-matched controls (Bowen et al., 2000; Short et al., 2001).

The presence of elevated levels of LH in the cytoplasm of neurons in individuals with AD may be due to one or more of the following: 1) increased intracellular LH expression; 2) a decreased intracellular LH degradation; or 3) increased LH sequestration from extracellular sources. If LH is expressed by pyramidal neurons of the cortex, such an increase in LH expression may be in response to GnRH, such as is observed in cells of the pituitary (Conn et al., 1981; Kamel and Krey, 1982). Tissues other than the pituitary (e.g., leukocytes) also have been shown to produce gonadotropins and express GnRH receptors (Costa et al., 1990; Chen et al., 1999). The possibility that LH is sequestered by neurons is supported by findings that go-

nadotropins are known to cross the blood brain barrier (Lukacs et al., 1995) and that the highest density of gonadotropin receptors in the brain is found within the hippocampus (Lei et al., 1993; Al-Hader et al., 1997a,b). The possible sequestration of LH by neurons in the AD brain may reflect alterations in the transport and/or metabolism of LH, however the mechanisms leading to such alterations remain to be determined.

Whereas there is significant epidemiologic data to suggest that estrogen appears to provide important trophic/protective support for hippocampal neurons, the negative feedback of the sex hormones on gonadotropin production indicates that any causative role of the sex hormones in the disease cannot be assessed without also examining the effects of the gonadotropins. Indeed, it might well be a combination of the decline in serum estrogen/testosterone together with the elevation in gonadotropins that contribute to the pathogenesis of AD. A decline in serum estrogen/testosterone alone does not support a role of sex hormones in dementia because AD-like changes are not observed during the 12–14-year period before pubescence when circulating concentrations of sex steroids are low. It should be pointed out that a role of elevated gonadotropins in the etiology of AD, is suggested by data from studies on individuals with Down's syndrome (DS), the majority of whom develop AD-like neuropathology if they live into the fourth decade (Oliver and Holland, 1986; Mann, 1988). Males with DS are at significantly higher risk of developing AD and at an earlier age than their female counterparts (Schupf et al., 1998). It is thought that this is due to some hormonal aberration seen in DS, although the concentration of sex hormones in both sexes is similar to those in the general population (Hasen et al., 1980; Hsiang et al., 1987). It is known, however, that the concentrations of gonadotropins, are significantly higher in DS and that this phenomenon is more pronounced in males with DS than their female counterparts (Hsiang et al., 1987). This elevated gonadotropin secretion, perhaps combined with the overexpression of the chromosome 21 amyloid-β protein precursor gene, would not only explain the resulting high prevalence of AD-like neuropathology in DS patients, but also the reversal of the female predilection seen in the general population.

Whereas epidemiological data leaves little doubt that the prophylactic use of estrogen delays the onset of AD (e.g., Henderson et al., 1994; Tang et al., 1996; Kawas et al., 1997), several studies evaluating estrogen as a potential therapy for AD have not shown any benefit (Henderson et al., 2000; Mulnard, 2000; Mulnard et al., 2000; Wang et al., 2000; Seshadri et al., 2001). Another study using higher doses of the estradiol patch did, however, produce beneficial results (Asthana et al., 2001). These contradictory results could be explained by higher serum concentrations of estradiol or they could just as easily be attributed to the greater suppression of gonadotropin production.

LH, FSH, and human chorionic gonadotropin (hCG), members of the TGF superfamily, have been

shown to play a critical role in brain development and neuron differentiation (Konishi et al., 1999). During fetal development hCG, which binds to LH receptors, is present at concentrations 1,000 times higher than during young adulthood. With the onset of menopause/andropause, LH concentrations increase to consistently higher levels (Andersson et al., 1998; Boyar et al., 1972). Interestingly, the increase in LH is higher and occurs earlier in women, who are more susceptible to AD than men. The increase in gonadotropins after menopause/andropause suggests that the neuron hormonal environment reverts back to one more akin to that of the fetal brain, which has been shown to display many similarities to AD brains, namely hyper-phosphorylated tau (Goedert et al., 1993) and increased presenilin expression (Berezovska et al., 1997). The importance of LH as a fundamental and early pathogenic driver in AD is perhaps indicated by the upregulation of neuronal LH in elderly controls with substantial neurofibrillary changes. Many of these cases might be expected to have gone on to develop AD. The effects of high concentrations of gonadotropins, potent neurotropic and mitogenic agents on neuronal metabolism and function warrant further investigation. If future studies support a role for elevated levels of LH in AD, then the use of GnRH receptor agonists, such as leuprolide, or GnRH antagonists, such as cetrorelix, might prove therapeutic.

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