Prolactin Receptor Gene Expression in Specific Hypothalamic Nuclei Increases With Age

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Increasing prolactin levels or increasing responsiveness to prolactin may contribute to reproductive aging by influencing the secretory patterns of hypothalamic GnRH, pituitary gonadotropins, and/or ovarian steroids. Some studies have documented changes in the levels of prolactin in peripheral plasma. The goal of this study was to determine whether prolactin receptor mRNA levels in the brain change with aging, which may lead to increasing responsiveness to prolactin. Young (2–4 months) and middle-aged (9–11 months), demonstrating 3 consecutive estrous cycles, and old (16–19 months) and very old (20–21 months) rats, exhibiting repeated pseudopregnancies, were bilaterally ovariectomized. They were implanted with Silastic capsules containing estradiol-17β one week later, and killed 3 days after capsule implantation. Changes in prolactin receptor gene expression were assessed using in situ hybridization. The level of prolactin receptor mRNA in choroid plexus, periventricular area of the preoptic nucleus, and arcuate nucleus increased significantly by the time the animals were old. In the lateral ventromedial nucleus, prolactin receptor gene expression did not change significantly during aging, even in the oldest group of rats. These findings suggest that changes in the prolactin receptor gene may influence the ability of prolactin to exert effects and may allow older animals to be more responsive to prolactin than young rats.

AGING female rats enter reproductive infertility after a period that is marked by a transition from regular estrous cyclicity to irregular cycles and finally to acyclicity (Wise et al., 1991). Changes at each level of the hypothalamic-pituitary-ovarian axis may contribute to the reproductive decline associated with aging. Previous studies in our laboratory have shown that middle-aged rats exhibit in selected hypothalamic nuclei (a) alterations in the diurnal rhythm of several neurotransmitter systems, (b) decreased proopiomelanocortin (POMC) mRNA levels, and (c) a decline in opiate and alpha,-adrenergic receptor densities (Moore, 1987) may depend upon the status of the prolactin secretion (Clemens, 1968; Ben-Jonathan et al., 1989), recently demonstrated that prolactin receptor gene expression is detectable in individual cells of several nuclei in the hypothalamus (Chiu and Wise, 1994). The probes that we used establish that the same receptor mRNAs that previously have been shown to exist in other peripheral prolactin target tissues are expressed in specific regions of the central nervous system. The results of this earlier study also suggest that prolactin may act on brain function through the classical prolactin receptor. Thus, prolactin’s ability to induce maternal behavior (Bridges et al., 1990), decrease LH and prolactin secretion (Clemens, 1968; Ben-Jonathan et al., 1989), and increase dopamine turnover in the arcuate nucleus (Moore, 1987) may depend upon the status of the prolactin receptor in different hypothalamic nuclei.

Results from autoradiographic binding studies have demonstrated binding sites in similar areas in the hypothalamus (Barton et al., 1989). In addition, several studies have established that (a) in peripheral tissues, changes in prolactin receptor mRNA parallel changes in number of prolactin binding sites, and (b) the level of prolactin binding is responsive to hormonal manipulation and physiological state. Thus, estrogen, prolactin, and growth hormone increase both the level of receptor mRNA and number of binding sites for prolactin in liver (Robertson et al., 1990; Kelly et al., 1991). During development and pregnancy, the levels of prolactin binding and mRNA also increase in the liver; in the mammary gland, prolactin binding sites and mRNA are maximal during lactation (Kelly et al., 1991).

At the present time, it remains unclear whether changes in prolactin receptor gene expression in multiple or specific hypothalamic nuclei change with aging.

MATERIALS AND METHODS

Animals. — Virgin female Sprague-Dawley rats, 2 months or 6–8 months old, were purchased from Zivic Miller (Allison Park, PA). They were maintained in a temperature- and light-controlled environment (14-h light, 10-h dark cycle, lights on at 0400 h). Animals were given food and water ad libitum. Estrous cyclicity was monitored by daily vaginal lavage. Cycling young (2–4 month) and middle-aged (9–11 month) animals were bilaterally ovariectomized (day 0) under ether anesthesia. Repeatedly pseudopregnant, old (16–19 month) and very old (20–22 month) rats were ovariectomized after exhibiting at least two consecutive 20–22 days of leukocytic smear patterns. On day 7, animals were implanted with Silastic capsules containing...
PROLACTIN RECEPTOR mRNA IN THE AGING BRAIN

180 μg/ml of E2 dissolved in sesame oil (20mm in length for young rats, 30mm in length for middle-aged, old, and very old rats), which produced circulating E2 levels of approximately 20 pg/ml serum (Wise et al., 1981). All rats (n = 4–9 per age group) were killed between 0900–1200 h on day 9. The ovaries were examined at the time of ovariectomy; the pituitary and uterus were examined at the time of sacrifice. Any animal that was not healthy or showed any indication of tissue hyperplasia, hypertrophy, or the presence of a tumor was not included in the analysis. Brains were removed, frozen on dry ice, and stored at −70 °C until sectioned (20μm) in a cryostat. Brain sections were thaw-mounted onto gelatin-coated slides and stored at −70 °C until the in situ hybridization was performed. Coronal sections from A7250 to A6482, A5660 to A4892, and A4700 to A3548 (Konig and Klippel, 1963) which include regions of the anterior-, mid-, and posterior-hypothalamus were used in this study. Guidelines for the care and use of these animals have been approved by the Institutional Animal Care and Use Committee, University of Maryland at Baltimore.

Riboprobe preparation.—Riboprobes complementary to four regions of the prolactin receptor mRNA were prepared as described previously (Chiu and Wise, 1994). We used this combination of riboprobes because we were unable to visualize prolactin receptor mRNA using a single probe or any other combination of two or three probes. The first probe complemented nucleotides −17 to 230, and the second probe encompassed nucleotides 437 to 618 of the short and long prolactin receptor cDNA (Kelly et al., 1991). The third probe complemented nucleotides 940 to 1182 of the short receptor only. The fourth probe uniquely complemented nucleotides 1399 to 1667 of the long prolactin receptor. The cDNA for probes 1 and 2 were subcloned from the short prolactin receptor; probes 3 and 4 were transcribed from cDNA (gift of P.A. Kelly; Kelly et al., 1991). Riboprobes were transcribed using 40μM α-UTP and 10μM α-thio-UTP. The specific activity of riboprobes generated was 2.2 × 10⁶ cpm/μg. Unincorporated label was removed with Boehringer Mannheim Quick-Spin columns.

In situ hybridization.—In situ hybridization was performed as described previously under RNase-free conditions with diethylpyrocarbonate-treated solutions and baked glassware (Chiu and Wise, 1994). In brief, the brain sections were post-fixed in 4% paraformaldehyde, rinsed in phosphate buffer, water, acetic anhydride in diluted triethanolamine buffer, and 2× SSC (1× SSC = 150 mM NaCl and 15 mM trisodium citrate), and incubated with probe in hybridization buffer. The hybridization buffer contained a total concentration of 0.2 μg/ml riboprobe (0.05 μg/ml of each probe), 60% formamide, 0.1 M sodium chloride, 2 mg/ml tRNA, 10% dextran sulfate, 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 1 μM DTT. In a preliminary experiment, we performed saturation analysis to determine the optimal concentration of riboprobe. Only at 0.2 μg/ml were we able to detect a signal that was 5 times over background. Slides were incubated at 55 °C overnight, treated with RNase, and washed. The stringent wash was performed in 0.1× SSC at 60 °C for one hour. Slides were then dehydrated and dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY) which had been previously diluted 1:1 with distilled water, exposed for two weeks, and developed with conventional photographic methods. Sections were counterstained with 0.4% toluidine blue and 2 μg/ml ethidium bromide.

Quantitation.—A BioQuant IV Image Analysis system (R & M Biometrics, Nashville, TN) was used to quantitate the level of mRNA. Only exposed silver grains were computer-enhanced and detected by the image analysis system by choosing the appropriate gray level threshold. Therefore, the measurement was not affected by the counterstain used to identify individual cells. The hybridization signal was measured as the number of enhanced pixels. We determined the size of the window to be placed over a cell so that all grains associated with an individual cell were included in the measurement. A cell was included in the analysis only if (1) its nucleus was visible within a cluster of silver grains, (2) the cluster did not overlap with the cluster of another labeled cell, and (3) the mean area of enhanced grains was at least 5 times background in another part of the brain section. In the choroid plexus, labeled cells were so densely packed that it was impossible to distinguish the signal associated with an individual cell. Therefore, in this brain region we measured the level of gene expression by counting the number of enhanced pixels in a fixed field that covered the choroid plexus. When the number of enhanced pixels was recorded with a 40 × objective, each pixel was equivalent to 0.246μm².

Statistics.—Mean ± SEM were determined for area of enhanced silver grains (μm²) covering individual cells. Statistical analysis of mean areas with respect to age was determined by one-way analysis of variance (ANOVA). If the ANOVA detected a significant effect of age, Duncan’s new multiple range test was applied to determine which age groups differed from each other. A p-value of less than .05 was considered statistically significant.

RESULTS
To determine the effect of aging on prolactin receptor gene expression, we measured levels of mRNA by in situ hybridization in several brain regions. Prolactin receptor mRNA was detectable in the periventricular area of the preoptic nucleus, medial preoptic nucleus, arcuate nucleus, lateral ventromedial nucleus, and choroid plexus of all age groups. This confirms our previous work localizing prolactin receptor gene expression in the lactating and ovariectomized, estradiol-treated young rat (Chiu and Wise, 1994). In addition, we observed increases in prolactin receptor mRNA levels with increasing age in several of these hypothalamic nuclei. The age-related changes were limited to the level of expression per cell or per brain region; there were no significant changes in the number of cells that expressed the prolactin receptor gene (Table 1).

In the periventricular area of the preoptic nucleus (Figure 1), ANOVA revealed a significant effect of age on prolactin receptor mRNA levels (p < .02). The old (16–19 month) and very old (20–22 month) rats exhibited mean levels of prolactin receptor mRNA per cell that were 3 and 2 times
Table 1. Number of Cells That Expressed Prolactin Receptor mRNA in Subjects Young to Very Old

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Young</th>
<th>Middle-Age</th>
<th>Old</th>
<th>Very Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pe-MPN</td>
<td>48.4 ± 4.88</td>
<td>50.4 ± 3.7</td>
<td>43.5 ± 5.8</td>
<td>48.2 ± 3.2</td>
</tr>
<tr>
<td>AN</td>
<td>39.2 ± 3.6</td>
<td>47.0 ± 7.1</td>
<td>37.8 ± 3.6</td>
<td>46.8 ± 7.1</td>
</tr>
<tr>
<td>VMN</td>
<td>20.9 ± 1.9</td>
<td>23.6 ± 1.6</td>
<td>21.5 ± 2.0</td>
<td>22.0 ± 1.5</td>
</tr>
</tbody>
</table>

Note: Cells were defined as expressing the receptor mRNA if the area covered by grains was greater than 5 times background.

*Mean ± SE.

Figure 1. Periventricular of the medial preoptic nucleus (Pe-MPN) for young (Y), middle-aged (M), old (O), and very old (OO) animals. Mean areas covered by silver grains per individual cell were analyzed as described in Materials and Methods. Asterisks designate statistically significant differences compared to young controls. *p < .05.

Figure 2. Arcuate nucleus (AN) for young (Y), middle-aged (M), old (O), and very old (OO) animals. Mean areas covered by silver grains per individual cell were analyzed as described in Materials and Methods. Asterisks designate statistically significant differences compared to young controls. *p < .05.

Figure 3. Lateral ventromedial nucleus (LVMN) for young (Y), middle-aged (M), old (O), and very old (OO) animals. Mean areas covered by silver grains per individual cell were analyzed as described in Materials and Methods. There was no difference between the level of prolactin receptor mRNA in any age group.

Figure 4. Choroid plexus for young (Y), middle-aged (M), old (O), and very old (OO) animals. Mean areas covered by silver grains over a fixed field that covered the choroid plexus were analyzed as described in Materials and Methods. Asterisks designate statistically significant differences compared to young controls. *p < .05.

DISCUSSION

We have shown that age-related changes in prolactin receptor gene expression in several hypothalamic nuclei can be detected by in situ hybridization. This is the first evidence that prolactin receptor mRNA levels increase with age in several hypothalamic nuclei. An age-related decline in prolactin binding has been reported in rabbits (Di Carlo et al.,...
the number of binding sites parallel changes in mRNA levels; whole hypothalamic tissue chunks were used. Third, the studies examined prolactin binding in rabbit membrane preparations using ovine prolactin as a ligand. Second, however, in other instances the two do not occur at the same time or are not similar in magnitude (Kelly et al., 1991). This lack of correspondence reflects the fact that prolactin receptors are regulated at several steps in its synthesis, such as during transcription, translation, and degradation (Kelly et al., 1991). Alternatively, discrepancies between changes in mRNA and protein may reflect differences in our ability to quantify accurately changes in the level of mRNA and protein. To our knowledge there are no studies on prolactin binding in the rat brain during aging. Therefore, it remains to be determined with more sensitive methods than used in earlier studies whether levels of prolactin binding parallel receptor mRNA levels in the hypothalamus.

Several studies suggest that prolactin receptors are homologously regulated by the ligand. Prolactin treatment increases the number of its binding sites in the rat liver (Amit et al., 1985), lung (Amit et al., 1985), and kidney (Barash et al., 1986). Prolactin receptor mRNA is also upregulated by the ligand (Robertson et al., 1990). The prolactin that is synthesized in the anterior pituitary gland may influence hypothalamic prolactin receptors because long portal vessels leaving the hypothalamus contain approximately 5000–6000 ng/ml of prolactin, about 160-fold greater than concentrations of prolactin in the peripheral circulation (Oliver et al., 1977). In addition, the median eminence and arcuate nucleus are completely accessible to retrograde blood flow from the pituitary gland because they lie outside of the blood brain barrier (Benson et al., 1989). Lastly, the choroid plexus may concentrate prolactin via receptor-mediated mechanisms (Bridges et al., 1990) which are under the influence of prolactin (Mangurian et al., 1992) and thus deliver prolactin to many brain regions (Bridges et al., 1990).

Changes in prolactin receptor mRNA levels during aging may be important, because an increase in pituitary prolactin secretion is associated with the transition to reproductive senescence, and prolactin may feed back at the level of the hypothalamus to regulate LH and its own secretion. Serum LH and prolactin levels are suppressed by high levels of prolactin, which then may inhibit ovulation and reduce fertility. Infertility is caused by high serum prolactin associated with lactation, implants of a prolactin-secreting tumor or an ectopic pituitary gland under the kidney capsule, exogenous prolactin, or drugs that elevate the hormone (Wise, 1986). Prolactin may act directly in the rat medial preoptic to affect GnRH (Azad et al., 1990) release and maternal behavior via norepinephrine containing neurons (Bridges et al., 1982).

In the arcuate nucleus, prolactin may be acting via dopamine neurons to influence pituitary hormone secretions. Serum prolactin levels peak in 2 hours after subcutaneous administration and are associated with changes in dopamine turnover (Ben-Jonathan et al., 1989). In addition, prolactin antibodies blunt potassium-induced dopamine and norepinephrine release from perfused medial basal hypothalamic fragments (Ben-Jonathan et al., 1989). Prolactin may also influence the opioidergic system since it increases opioidergic activity, decreases opiate tone, and reduces the number of naloxone binding sites (Weiland and Wise, 1989).

In the ventromedial nucleus, iontophoretically applied prolactin increases electrical activity of neurons in this region (Chan et al., 1983; Haskins and Moss, 1983). Therefore, prolactin receptors in the lateral ventromedial nucleus may be mediating prolactin’s influence on dopamine secretion (Mechanick et al., 1987), diurnal prolactin surges induced by cervical stimulation (Smith and Gala, 1978) or estrogen (Pan and Gala, 1985), or sexual behavior (Harlan et al., 1983). The lack of any significant alterations in prolactin receptor gene expression in this nucleus corroborates several studies that find no age-related changes in sexual behavior (Cooper and Linnoila, 1977; Peng et al., 1977), which might be mediated by prolactin (Harlan et al., 1983). In addition, the prolactin receptor may not be an important element in the age-related decline of other functions mediated by the ventromedial nucleus.

In summary, we observed age-related increases in the level of prolactin receptor gene expression in specific regions of the hypothalamus in which we and others have found multiple neuroendocrine and neurochemical changes during the transition to reproductive senescence. These data allow us to speculate that changes in the prolactin receptor may play a role in neuroendocrine aging and the ability of prolactin to act in multiple regions of the aging brain.

Acknowledgments

This work is supported by National Institutes of Health grant AG-02224 to Dr. Wise. We thank Ms. Katherine Rosewell and Ms. Klara Margaretten for their excellent technical assistance and expertise.

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Received April 3, 1995
Accepted November 2, 1995