Age-Related Changes in Hypothalamic Gonadotropin-Releasing Hormone and N-Methyl-D-Aspartate Receptor Gene Expression, and their Regulation by Oestrogen, in the Female Rat

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Key words: GnRH, NMDA receptor, ageing, oestrogen, hypothalamus, preoptic area, ovariectomy, female rat.

Abstract

During reproductive ageing, the oestrous cycles of female rats become irregular and eventually cease. The mechanisms for reproductive senescence in rodents are believed to involve changes in hypothalamic neurones, including gonadotropin-releasing hormone (GnRH) cells and their afferent inputs. In addition, effects of oestrogen on hypothalamic function may vary in animals of different ages. These issues were addressed using young (aged 4–5 months), middle-aged (12–14 months) and old (24–26 months) female Sprague-Dawley rats. Animals were ovariectomized and given oestrogen or vehicle replacement. They were killed and the preoptic area-anterior hypothalamus (POA-AH) and the medial basal hypothalamus-median eminence (MBH-ME) were dissected out, RNA extracted, and RNase protection assay used to quantify gene expression of several hypothalamic molecules. In the first experiment, GnRH RNA levels were measured in the POA-AH. No effects of ageing or oestrogen were observed on GnRH gene expression. This finding suggests that ageing and oestrogen may affect GnRH release from neuroterminals independently of de novo biosynthesis, and that this may involve other neurones that affect GnRH neurosecretory function. In the second experiment, we investigated changes in N-methyl-D-aspartate (NMDA) receptor subunit mRNA levels. These receptors play an important regulatory role in mediating effects of glutamate on GnRH function, and are themselves regulated by oestrogen and ageing. NMDA receptor subunit mRNA levels. These receptors play an important regulatory role in mediating effects of glutamate on GnRH function, and are themselves regulated by oestrogen and ageing. NMDA receptor subunit (NR) 1, 2a and 2b mRNA levels were quantified in the POA-AH and MBH-ME, the sites of GnRH perikarya and neuroterminals, respectively. In general, oestrogen had inhibitory effects on NR1 and NR2a, and differential effects on NR2b subunit mRNA levels. NMDA receptor subunit mRNA levels also changed during ageing: age-related decreases in NR1 mRNA occurred in the MBH-ME, and an age-related increase in NR2b mRNA occurred in the POA-AH. Taken together, these results demonstrate subunit- and region-specific changes in hypothalamic NMDA receptor subunit gene expression with oestrogen and ageing. These alterations could have implications for the physiological effects of glutamate on its NMDA receptor, and impact the regulation of reproductive and other neuroendocrine and autonomic functions by hypothalamic glutamatergic inputs.

Reproductive ageing in female rodents is characterized by a transition from regular oestrous cycles to irregular cycles and eventually a complete cessation of reproductive cyclicity (1, 2). However, unlike menopause in women which is driven by a loss of ovarian follicles and a subsequent precipitous decline in oestrogen concentrations, reproductive senescence in rodents occurs largely independently of ovarian follicular changes (3, 4). Thus, it is believed that the mechanisms involved in the causation of reproductive senescence probably involve changes in hypothalamic function and/or changes in pituitary responses to hypothalamic input (5).
A number of hypothalamic neuroendocrine changes occur during reproductive ageing that may play a role in, or be causal to, reproductive senescence. The gonadotropin-releasing hormone (GnRH) neurones, which are responsible for the regulation of reproductive development at the onset of puberty (6–8) and the maintenance of reproductive function in adulthood (9), are likely candidates for this role. There is mounting evidence that age-related changes in GnRH neurones occur before or coincidentally with the transition to acyclicity in support of this role. First, pulsatile GnRH release changes during ageing (10–13). Second, the preovulatory GnRH/luteinizing hormone (LH) surge is significantly attenuated in middle-aged rats, prior to reproductive failure (14–17). Third, the afternoon increase in GnRH gene expression that occurs in young rats on the day of the preovulatory GnRH/LH surge (9) is not observed in middle-aged rats (18). Fourth, expression in GnRH neurones of the immediate early gene c-fos, an indicator of activated gene transcription, is diminished in GnRH cells of middle-aged compared to young rats on the day of the GnRH/LH surge (19–21). Thus, changes occur in the GnRH neurones of middle-aged rats that are still cycling that may play a role in the transition to reproductive acyclicity.

Along with these intrinsic changes that occur in GnRH neurones during reproductive senescence, it is also likely that changes in inputs to GnRH neurones may participate in, or play an important role in this process. The best-studied neurotransmitter in this regard is glutamate, acting through N-methyl-D-aspartate (NMDA) and non-NMDA receptors to stimulate GnRH biosynthesis and release (22–25). GnRH neurones express NMDA and non-NMDA receptors (23, 26–28). Moreover, the effects of NMDA receptor activation on GnRH neurones are steroid-sensitive, with more potent effects of NMDA receptor agonists being observed in the presence than the absence of oestradiol (29–31).

Age-related changes in glutamate regulation of neuroendocrine function have been reported. The stimulation of GnRH release from hypothalamic explants in vitro and of LH release in vivo by NMDA agonists is reduced during ageing (13, 32, 33). We recently reported that NMDA stimulation of GnRH gene expression in young rats is abolished in middle-aged rats, and that there are changes in hypothalamic NMDA receptor subunit mRNA levels and coexpression of the NMDA receptor in GnRH neurones during ageing (27).

In the present study, we quantified GnRH and NMDA receptor subunit gene expression in the hypothalamus of ageing female rats. An ovariectomized rat model was utilized in order to examine the regulation of levels of these hypothalamic molecules during ageing by oestrogen, and as an experimental model for hormone replacement in postmenopausal women.

Materials and methods

Animals

A total of 114 female Sprague-Dawley rats, purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA) were used for these studies. Animals were used at young, middle-aged and old ages (see below). All young rats were virgins, some middle-aged rats were virgins and others were retired breeders, and all old rats were retired breeders. No effect of prior breeding status was observed for any parameter in this or previous studies from our laboratory (18, 27, 34–36). Daily vaginal smears prior to surgery indicated that young rats all had regular 4–5-day oestrous cycles, middle-aged rats had irregular oestrous cycles and old rats were acyclic (mostly persistent oestrus, some persistent diestrus). All animals were housed two per cage in a temperature-controlled room with a 12:12 light:dark cycle (lights on 07:00 h). Temperature was controlled at 22–23 °C, and food and water were available ad libitum.

Surgery

All rats were bilaterally ovariectomized (OVX) under methoxyflurane anaesthesia. The post-OVX interval was set at 1 or 6 months to examine whether there were any effects of this parameter. Therefore, young rats were OVX at 3–4 months of age and utilized 1 month after OVX. A 6-month post-OVX interval could not be used in young rats as this would result in animals that were nearly middle-aged at the time of experimentation. Middle-aged rats were OVX at 6–8 months and used 6 months later (at 12–14 months), or they were OVX at 11–13 months and used 1 month later (at 12–14 months). Some old rats were OVX at 18–20 months and killed 6 months later, at 24–26 months. Other old rats were OVX at 23–25 months and killed 1 month later, at 24–26 months. The following numbers of animals were used in each group: young, 1 month OVX: 2 days vehicle (n = 7), 2 weeks vehicle (n = 6), 2 days oestrogen (n = 5), 2 weeks oestrogen (n = 6); middle-aged, 1 month OVX: 2 days vehicle (n = 5), 2 weeks vehicle (n = 6), 2 days oestrogen (n = 6), 2 weeks oestrogen (n = 7); middle-aged, 6 month OVX: 2 days vehicle (n = 7), 2 weeks vehicle (n = 5), 2 days oestrogen (n = 7), 2 weeks oestrogen (n = 7); old, 1 month OVX: 2 days vehicle (n = 3), 2 weeks vehicle (n = 3), 2 days oestrogen (n = 3), 6 month OVX: 2 days vehicle (n = 7), 2 weeks vehicle (n = 5), 2 days oestrogen (n = 8), 2 weeks oestrogen (n = 8).

Following the appropriate post-OVX interval, animals were subjected to subcutaneous implantation of a silastic capsule (inner diameter 1.96 mm, outer diameter 3.18 mm) filled with either 17β-oestradiol (10%) or vehicle (cholesterol). Surgeries were performed in animals under methoxyflurane anaesthesia. Young animals received an implant that was 1 cm in length, middle-aged animals received a 1.5-cm implant and old animals received a 2-cm implant, in order to account for differences in body weights (35, 37, 38). In this study, young, middle-aged and old rats had body weights of 29 ± 5, 361 ± 10 and 460 ± 11 g, respectively. Animals were allowed to recover for 2 days or 2 weeks after silastic capsule implantation surgery. The length of oestradiol or vehicle exposure (2 days or 2 weeks) was selected to mimic positive and negative feedback effects of oestradiol, respectively (39, 40), and based on previous studies from our laboratory (9, 41).

Experimental design

Animals were killed by decapitation at 15:00 h. Trunk bloods were collected, allowed to clot, centrifuged, and the serum stored at −20 °C for subsequent assays. The brain was rapidly removed and placed ventral side-up into a stainless steel brain slicer (model RBM-4000C, Activational Systems, Warren, MI, USA). Two coronal cuts were made (18, 27). For the preoptic area-anterior hypothalamus (POA-AH), the caudal border was made by a coronal cut just posterior to the entry point of the optic chiasm. The rostral border was exactly 4 mm anterior, at the posterior third of the olfactory tubercle. This coronal section, 4 mm thick, was laid rostral side-up on a chilled glass plate. An isosceles triangle-shaped cut was made with its apex just under the midline of the corpus callosum and the two legs passing through the anterior commissure. The medial basal hypothalamus-median eminence (MBH-ME) dissection had as its rostral border the caudal border of the POA-AH dissection, and a second coronal cut was made exactly 3 mm caudal, at the rostral border of the mammillary bodies. This coronal section, 3 mm thick, was laid rostral side-up on the glass plate, and another isosceles triangle was cut, with the apex just below the thalamus and the two legs passing through the MBH-ME and the subcallosal nucleus at the level of the supraoptois nucleus. The POA-AH and MBH-ME dissections were snap-frozen on dry ice and stored at −80 °C.

The carcasses were examined to confirm complete removal of both ovaries and the presence of the subcutaneous implant. The uterus of each animal was also examined to determine whether oestrogen replacement was effective, with uterine hypertrophy seen only for the oestradiol-treated animals.
RNA was extracted from frozen POA-AH and MBH-ME dissections using a double-detergent lysis buffer system, as described previously (9, 18, 24, 27). Cytoplasmic and nuclear RNA from individual POA-AH or MBH-ME double-detergent lysis buffer system, as described previously (9, 18, 24, 27). RNA was extracted from frozen POA-AH and MBH-ME dissections using a RNA extraction and RNase protection assay 302 Oestrogen, NMDA receptors and GnRH neurones in ageing rats

overnight at 30°C with 5 μl probe labelled to high (GnRH cDNA, B3C, NR2a, NR2b) or low (NR1, cyclophilin) specific activity with [32P]UTP. All tubes were treated with RNase A/T1 for 1 h, and then with protease K for 15 min. Samples were phenol-chloroform extracted and precipitated and resuspended in 1.5 × Ficoll loading buffer. The samples were electrophoresed through 6% nondenaturing polyacrylamide gels that were dried and exposed to a Phosphor imaging screen (Molecular Dynamics, Sunnyvale, CA, USA) for 1–3 days for quantification. Regression analysis was performed on the amount of radioactivity in each sample compared to the amount of reference RNA to produce a standard curve, which was used to calculate the absolute amount of RNA in each sample (23, 24). For each transcript, the amount of RNA was normalized to levels of cyclophilin mRNA, for which the latter did not change across any of the groups, in order to minimize gel-loading variation (9, 24, 42).

The following DNA clones were used to make riboprobes: (i) GnRH cDNA clone, 362 bp in length, spanning the HindIII site in exon 1 to the BamHI site in exon 4, and subcloned into a pBS(+) vector (Stratagene, La Jolla, CA, USA) to measure GnRH mRNA in the cytoplasm (43); (ii) a proGnRH (B3C) genomic fragment spanning the 506 bp of the intron B-exon 3-intron C junction and subcloned into the EcoRI and HindIII sites of a pBS(+) vector, to measure GnRH primary transcript in the nucleus (24), an indicator of GnRH gene transcription (44); (iii) a cyclophilin cDNA clone, 111 bp in size, spanning the PstI and Xmor restriction sites and subcloned into a Bluescript KS(+) vector (43); (iv) an NMDA-R1 (NR1) cDNA clone, complementary to 284 bp of the N-terminus, spanning the BamHI and HindIII restriction sites and subcloned into a Bluescript KS(+) vector (kindly provided by Dr Stuart Sealfon, Mount Sinai School of Medicine, NY, USA) (23); (v) NR2a; and (vi) NR2b cDNA clones, complementary to bases 1585–2154 and 1423–1992, respectively, cloned into the Smal site of the pBluescript II SK(+) vector (kindly provided by Drs S. A. Lipton and N. J. Sucher) (45, 46).

**LH radioimmunoassay (RIA)**

LH concentrations in serum samples were determined in a single RIA in duplicate samples by double-antibody RIA (47). The LH RP-3 standard from the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), kindly provided by Dr A. Parlow, was used. The in-tras assay coefficient of variation was 6.9%.

**Oestradiol RIA**

Oestradiol concentrations in serum were measured by RIA of duplicate samples using the DSL ultrasensitive oestradiol RIA kit (DSL-4800, Diagnostic Systems Laboratories, Inc., Webster, TX, USA), according to the manufacturer’s instructions. The assay sensitivity was 7 pg/ml, and intra-assay coefficient of variation was 4%.

**Statistical analysis**

Differences in GnRH and NMDA receptor subunit mRNA levels, and LH and oestradiol concentrations, were analysed as described previously (35). Two initial assumptions were tested before statistical analysis was performed. In all cases, an F-test on equality of variances (two groups) or Bartlett’s test (multiple groups) was performed to ensure that there was homogeneity of variance. Second, normality of data was determined for each variable. Then, comparisons were made using a two-factor analysis of variance on each variable (age, hormonal treatment, duration of oestrogen replacement or length of ovariectomy). Because no effect of length of ovariectomy was seen, data were collapsed for the middle-aged and old groups, and the effects of the other three variables were examined by ANOVA. Post-hoc comparisons were made, when appropriate, using Fisher’s protected least significant difference (PLSD) analysis. In all cases, P<0.05 was considered statistically significant.

**Serum oestradiol concentrations in ageing female rats**

In order to confirm the efficacy of oestrogen replacement, serum oestradiol concentrations were measured by RIA. As shown in Table 1, oestradiol concentrations were at or near the level of assay sensitivity in OVX rats that received implantation of the vehicle, while oestradiol levels were significantly elevated in OVX rats receiving oestrogen replacement. No effect of duration of oestrogen or vehicle replacement (2 days or 2 weeks) was observed. For all three ages, concentrations of oestradiol were significantly higher in oestradiol-treated compared to vehicle-treated animals (P<0.0001). While levels were somewhat higher in oestro-gen-treated middle-aged and old rats compared to young rats, differences were not significant among groups.

**Serum LH concentrations in ageing female rats**

Serum LH levels were measured by RIA in OVX female rats receiving oestradiol or vehicle. As shown in Table 2, LH levels varied significantly by age (P<0.0001) and hormone treatment (P<0.0001), but not by duration of replacement (2 days or 2 weeks). Significant interactions among these variables were also detected by ANOVA and are shown in Table 2. A significant interaction of age and the duration of replacement was found (P<0.001), as was an interaction of hormone and duration of replacement (P<0.01). A surprising observation of a difference between old animals receiving 2 days versus 2 weeks of vehicle was made; although we are not certain of the explanation, this may be attributable to a difference in postsurgical recovery.

**GnRH RNA levels**

GnRH cytoplasmic mRNA

GnRH mRNA levels were quantified in the cytoplasmic fraction of POA-AH dissections. As shown in Fig. 1(a), GnRH mRNA levels were similar in rats of the three ages and among different treatment groups. It was found by ANOVA that there was no significant effect of age (P = 0.70), hormone treatment (P = 0.12) or duration of replacement (P = 0.52) on

<table>
<thead>
<tr>
<th>Table 1. Serum Oestradiol Levels in Ageing Ovariectomized Female Rats.</th>
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<tbody>
<tr>
<td>Age</td>
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<tr>
<td>--------------------------------</td>
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<tr>
<td>Young (4–5 months)</td>
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<tr>
<td>Middle-aged (12–14 months)</td>
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<tr>
<td></td>
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<tr>
<td>Old (24–26 months)</td>
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Data are expressed as mean ± SEM. Oestradiol levels were measured by radioimmunoassay in serum samples of rats of different ages and hormone replacement. *P<0.0001 versus corresponding vehicle for all age groups.

### TABLE 2. Serum Luteinizing Hormone (LH) Levels in Ageing Ovariectomized Female Rats.

<table>
<thead>
<tr>
<th>Age</th>
<th>Hormone treatment</th>
<th>LH (ng/ml)</th>
<th>P-value if significant</th>
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</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>Young</td>
<td>Vehicle (2 days)</td>
<td>13.9 ± 1.7</td>
<td>0.05 versus Y 2d Veh</td>
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<td>Oestrogen (2 days)</td>
<td>9.5 ± 2.1</td>
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<td></td>
<td>Vehicle (2 weeks)</td>
<td>12.5 ± 0.4</td>
<td>0.0001 versus Y 2d E and Y 2w Veh</td>
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<tr>
<td></td>
<td>Oestrogen (2 weeks)</td>
<td>1.2 ± 0.1</td>
<td>0.0001 versus Y 2d E and Y 2w Veh</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>Vehicle (2 days)</td>
<td>10.1 ± 1.5</td>
<td>0.005 versus Old 2d Veh; 0.05 versus Y 2d Veh</td>
</tr>
<tr>
<td></td>
<td>Oestrogen (2 days)</td>
<td>3.7 ± 0.5</td>
<td>0.0001 versus MA 2d Veh; 0.0005 versus Y 2d E</td>
</tr>
<tr>
<td></td>
<td>Vehicle (2 weeks)</td>
<td>9.0 ± 3.0</td>
<td>0.0001 versus MA 2w Veh</td>
</tr>
<tr>
<td></td>
<td>Oestrogen (2 weeks)</td>
<td>2.2 ± 0.5</td>
<td>0.0001 versus MA 2w Veh</td>
</tr>
<tr>
<td>Old</td>
<td>Vehicle (2 days)</td>
<td>4.0 ± 0.6</td>
<td>0.0001 versus Y 2d Veh</td>
</tr>
<tr>
<td></td>
<td>Oestrogen (2 days)</td>
<td>2.2 ± 0.3</td>
<td>0.0001 versus Y 2d E</td>
</tr>
<tr>
<td></td>
<td>Vehicle (2 weeks)</td>
<td>9.6 ± 1.5</td>
<td>0.01 versus Old 2d Veh</td>
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<tr>
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<td>Oestrogen (2 weeks)</td>
<td>2.4 ± 0.7</td>
<td>0.0005 versus Old 2w Veh</td>
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</table>

Data are expressed as mean ± SEM. LH levels were measured by radioimmunoassay in serum samples of rats of different ages and hormone replacement. Y, young; MA, middle-aged; Veh, vehicle; E, oestrogen; 2d, 2 days; 2w, 2 weeks.

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**FIG. 1.** (a) Gonadotropin-releasing hormone (GnRH) mRNA levels (normalized to cyclophilin mRNA) in the preoptic area-anterior hypothalamus of young, middle-aged and old ovariectomized (OVX) rats given vehicle (light grey bar) or oestrogen (dark grey bar) replacement for 2 days (top) or 2 weeks (bottom). No significant effects of age, oestrogen replacement or duration of replacement were observed. (b) GnRH primary transcript levels in young, middle-aged and old OVX rats given vehicle (light grey bar) or oestrogen (dark grey bar) replacement for 2 days (top) or 2 weeks (bottom). Again, no significant effects of age, oestrogen replacement or duration of replacement were observed.

GnRH cytoplasmic mRNA levels, and no interactions among these variables.

GnRH nuclear primary transcript
GnRH primary RNA transcript levels, an index of GnRH gene transcription (44), were measured in the nuclear fraction of POA-AH dissections. As shown in Fig. 1(a), GnRH primary transcript levels were similar among rats of different ages and treatments. Statistical analysis demonstrated no significant effect of age although there was a trend for age-related differences (P = 0.06). No significant effects of hormone treatment (P = 0.32) or duration of replacement (P = 0.24) were found.

NMMA receptor subunit mRNA levels

**POA-AH:** NR1 mRNA levels in the POA-AH did not vary significantly by age (P = 0.13) or duration of replacement (P = 0.07), but were significantly affected by hormone treatment (P < 0.001; Fig. 2). No significant interactions among these variables were detected. For hormone treatment, post-hoc analysis demonstrated that levels of NR1 mRNA were significantly higher in vehicle than oestrogen-treated animals (P < 0.001). NR2b mRNA levels in the POA-AH were not significantly affected by age (P = 0.87), but were significantly affected by hormone treatment (P = 0.05; Fig. 3). There were also significant interactions of age with hormone treatment (P < 0.001), and of age, hormone treatment and duration of replacement (P < 0.05) were also detected. Thus, oestrogen had a significant stimulatory effect on NR2b mRNA levels in the POA-AH of old rats, and this was greater in rats receiving 2 days compared to 2 weeks of treatment (P < 0.05).

**MBH-ME:** NR2b mRNA levels in the MBH-ME were not significantly affected by age (P = 0.09), hormone treatment (P = 0.16) or length of replacement (P = 0.26; Fig. 4). However, there were significant interactions among these variables. Significant interactions of age with hormone (P < 0.005), of age with duration of replacement (P < 0.001), and of age, hormone and duration of replacement (P < 0.05) were detected. In young rats given 2 weeks of hormone replacement, NR2b mRNA levels were significantly higher in oestrogen- than vehicle-treated animals (P < 0.05). Old rats given 2 weeks of oestrogen had significantly lower NR2b mRNA levels compared to old animals given 2 weeks of vehicle (P < 0.05).

Discussion

The present results demonstrate significant effects of ageing, oestrogen and the duration of hormone replacement on NMDA receptor subunit mRNA levels in the hypothalamus. However, GnRH mRNA and primary transcript did not change with ageing or oestrogen treatment. Although GnRH release from neuroterminals in the median eminence occurs during reproductive ageing (10–13), our results suggest that this probably occurs in the absence of de novo biosynthesis. Effects of changes in NMDA receptors may therefore be exerted at the endpoint of GnRH release as opposed to biosynthesis.

The NMDA receptor is a heteromorphic pentamer comprising the obligatory NR1 subunit together with at least one member of the NR2 family of subunits (45). The most abundant members of the NR2 family found in hypothalamus are NR2a and NR2b (48). Thus, the most likely combinations of NMDA receptor subunits in hypothalamus are NR1 together with NR2a and NR2b. The stoichiometry of the NMDA receptor varies depending upon the number of each of these subunits that combines to form the pentamer. This has functional consequences, as the NR1 subunit plays a role in ligand binding, while different NR2 family members mediate ion gating properties and removal of the Mg2+ block (45). Therefore, the stoichiometry of the NMDA receptor may affect the binding of glutamate to the receptor, signal transduction and intracellular signalling.

Effects of ageing and oestrogen on NMDA receptor subunit mRNA levels in POA-AH and MBH-ME

In the present study, we measured NR1, NR2a and NR2b mRNA levels in POA-AH, the site of GnRH perikarya, and in the MBH-ME, the site of GnRH neuroterminals. These data provide information as to the regulation of the synthesis of these receptors and relate to the availability of translated proteins to form the mature pentameric complex. NR1, NR2a and NR2b mRNA levels were abundant in both brain regions, although they differed in absolute levels. NR1 mRNA levels were approximately 2.5 times higher in MBH-ME than POA-AH, NR2a mRNA levels were approximately five times higher in POA-AH than MBH-ME, and NR2b mRNA levels were similar between the two regions. These differences in mRNA levels, if reflected by similar changes in protein, would predict substantial differences in the composition of the NMDA receptor pentamer. In the POA-AH, the ratio of NR2a to NR1 would be much higher than in the MBH-ME, while NR2b levels would be anticipated to be in relatively constant proportions. This could affect the physiology of the NMDA receptor and result in different effects of glutamate binding in the POA-AH and MBH-ME.

Effects of ageing and oestrogen replacement on NMDA receptor subunit mRNA levels were observed that were regionally specific. For NR1 mRNA, levels in both POA-AH and MBH-ME were significantly lower in rats given oestrogen replacement than in vehicle-treated rats. No age-related changes in NR1 were seen in the POA-AH. However, an age-related decrease was observed in the MBH-ME, particularly in rats exposed to 2 weeks of treatment compared to those receiving 2 days of treatment. This suppressive effect of oestrogen on NR1 mRNA levels is consistent with another study performed in juvenile female rats, in which oestrogen caused a decrease in NR1 mRNA levels in the anteroventral periventricular area of the hypothalamus, as measured by in situ hybridization (49). This region is encompassed by the

POA-AH dissection in the present study. However, a different laboratory, using Northern hybridization, did not detect an effect of oestrogen on NR1 mRNA levels (50), but that group used a very different treatment paradigm that may account for differences. Nevertheless, it appears that NR1 gene expression is a potential level for oestrogen feedback onto the neuroendocrine system, and may be a route by which oestrogen affects GnRH neurones at the level of their perikarya and neuroterminals.

We also observed an age-related decrease in NR1 mRNA levels that was specific to the MBH-ME. These results suggest that NR1 inputs to GnRH neuroterminals in the MBH-ME may decline with age. However, different results have been reported in ovarian-intact rats. Zuo et al. (13) found that NR1 mRNA levels in both the POA and arcuate nucleus/median eminence (ARC-ME) decreased from young to middle-aged rats in intact rats. Our laboratory reported in intact female rats that NR1 mRNA levels did not change in POA-AH, and increased in MBH-ME, during reproductive ageing (27). Thus, there appear to be substantial differences in the gene expression of this molecule between ovarian-intact and ovariectomized rats. The present experiment differed from our previous one (27) only on the basis of ovarian status, as animals were purchased at similar times, utilized at the same ages, housed and sacrificed identically and subjected to the same brain dissections, RNA extractions and assays. The experimental model of ovariectomy plus oestrogen, while an important one, does not necessarily reflect changes in ovarian-intact animals because the ovaries of these latter animals produce many factors other than oestrogen that may influence neuroendocrine function. Thus, while studies on oestrogen replacement in the ovariectomized rat are extremely important for understanding the specific role of oestrogen in the regulation of neuroendocrine physiology, it is ultimately necessary to perform studies on intact animals that are allowed to undergo natural reproductive senescence (18, 27, 34).

NR2a mRNA levels were not affected by chronological age, but were affected by oestrogen treatment, and this was specific to the POA-AH. Moreover, the effects of oestrogen on

![Fig. 3. NMDA-R2a (NR2a) mRNA levels in the preoptic area-anterior hypothalamus (POA-AH) (A) and medial basal hypothalamus-median eminence (MBH-ME) (B) of young, middle-aged and old ovariectomized rats. Animals were given vehicle (light grey bar) or oestrogen (darker grey bar) for 2 days (top) or 2 weeks (bottom). In the POA-AH, overall NR2a mRNA levels were significantly higher in vehicle-treated than oestrogen-treated rats. This effect interacted with age and length of replacement, with significant differences seen for the middle-aged rats given 2 days or 2 weeks of oestrogen, and in old rats given 2 weeks of oestrogen replacement. In the MBH-ME, no significant differences in NR2a mRNA levels were observed for age, hormone or length of replacement. *P < 0.005 versus corresponding vehicle. †P < 0.005 versus corresponding 2 days of treatment.](image-url)
NR2a mRNA levels varied among the three age groups in this brain region. There, NR2a mRNA levels were lower in oestrogen- than vehicle-treated rats and this was most pronounced in middle-aged rats in which animals receiving 2 days or 2 weeks of oestrogen had significantly lower levels of this mRNA than animals receiving vehicle. In old rats, 2 weeks of treatment was necessary to see a difference in NR2a mRNA levels. Young rats appeared to be insensitive to oestrogen effects on NR2a mRNA levels. Thus, effects of oestrogen on NR2a are confined to the two oldest age groups and indicate a differential age-related sensitivity of this molecule to oestrogen replacement. For the MBH-ME, no significant differences in NR2a were seen on the basis of ageing or oestrogen replacement.

Effects of ageing and oestrogen were seen for the NR2b subunit. With regard to ageing, in the POA-AH, NR2b mRNA levels were significantly higher in old than young or middle-aged rats. Interactions of age, hormone and length of replacement were observed, with old rats given oestrogen having higher NR2b mRNA levels than old rats given vehicle. In the MBH-ME, interactions among age, hormone and replacement were seen, with significant effects observed in the 2 week treatment group. In young rats, oestrogen caused significant increases in NR2b mRNA levels, while in old rats, oestrogen caused significant decreases. *P < 0.05 versus corresponding vehicle. †P < 0.005 versus corresponding 2 days of treatment.

NR2a mRNA levels varied among the three age groups in this brain region. There, NR2a mRNA levels were lower in oestrogen- than vehicle-treated rats and this was most pronounced in middle-aged rats in which animals receiving 2 days or 2 weeks of oestrogen had significantly lower levels of this mRNA than animals receiving vehicle. In old rats, 2 weeks of treatment was necessary to see a difference in NR2a mRNA levels. Young rats appeared to be insensitive to oestrogen effects on NR2a mRNA levels. Thus, effects of oestrogen on NR2a are confined to the two oldest age groups and indicate a differential age-related sensitivity of this molecule to oestrogen replacement. For the MBH-ME, no significant differences in NR2a were seen on the basis of ageing or oestrogen replacement.

Effects of ageing and oestrogen were seen for the NR2b subunit. With regard to ageing, in the POA-AH, NR2b mRNA levels were significantly elevated in old compared to young and middle-aged rats. In the MBH-ME, a trend for an increase was also seen in old rats given 2 days of oestrogen or vehicle treatment. Oestrogen also exerted effects on NR2b mRNA levels, and these were most pronounced for the old rats. In this age group, 2 days of oestrogen increased NR2b mRNA levels in the POA-AH, and 2 weeks of oestrogen decreased these levels in the MBH-ME. Taken together, these alterations in NMDA receptor subunit mRNA levels would be predicted to result in an NMDA receptor pentamer that differs depending upon the age and hormonal status of the animal. In the POA-AH, in general, oestrogen decreased NR1 and NR2a mRNA levels. NR2b mRNA levels were increased by oestrogen only in the old group. In the MBH-ME, effects of oestrogen were less pronounced than in the POA-AH, although some effects were observed in specific age groups. These alterations in receptor stoichiometry may be a mechanism by which the NMDA receptor can alter the sensitivity to oestrogen. Effects of NMDA receptor agonists on GnRH release are enhanced in
Effects of ageing and oestrogen on GnRH mRNA and primary transcript in POA-AH

No significant changes in GnRH mRNA or primary transcript RNA levels were observed in OVX rats during ageing or following oestrogen replacement. These results on ageing were somewhat surprising as we had previously reported in ovarian-intact female Sprague-Dawley rats, using the same quantitative techniques, that GnRH mRNA levels increase during reproductive ageing, while GnRH primary transcript levels decrease (18). Because the ovary produces several hormones other than oestrogen, studies using the OVX/oestrogen replacement model cannot address the roles of these other ovarian substances that may impact upon GnRH gene expression. As stated above, effects of ageing on levels of NMDA receptor subunits differ depending upon the ovarian status of the animal, and the same appears to be the case for the GnRH system.

The lack of an effect of oestrogen on GnRH RNA levels in the present study is consistent with our previous reports in young and middle-aged OVX rats (9, 41). Moreover, no effects of the post-OVX interval (1 versus 6 months) were seen in the middle-aged and old rats, either on GnRH or NMDA receptor RNA levels. Thus, a 1-month post-OVX interval is probably sufficient to induce any of the changes seen in the present experimental model, such that additional time after OVX does not have any further effects.

Studies from other laboratories have also evaluated effects of ageing and oestrogen on GnRH gene expression. One group, using in situ hybridization, reported that GnRH mRNA decreases with ageing from young to middle-aged stages in OVX, oestrogen-primed rats (51). Differences between that study and ours may be attributable to the use of different techniques. The in situ hybridization study of Rubin and colleagues, while semiquantitative, would be more likely to provide specific anatomical information about changes in GnRH gene expression than the present experiments performed by RNase protection assay. It is quite likely that a subset of GnRH neurones undergoes a decrease in gene expression with ageing that is regionally specific.

The present study provides evidence for changes in NMDA receptor subunit gene expression in the hypothalamus of ageing rats, and the regulation of these subunits by oestrogen. These observations potentially have much broader implications than on the reproductive neuroendocrine axis. Indeed, NMDA receptors in POA, organum vasculosum of the lamina terminalis, anterior hypothalamus and median eminence undoubtedly impact upon other neuroendocrine axes, which are also glutamate-sensitive (52–55). With respect to the reproductive axis, although GnRH gene expression was not altered by oestrogen and ageing, it is quite likely that activation of NMDA receptors has effects on pulsatile GnRH release, and that this phenomenon is subject to age and oestrogen regulation. Indeed, reports that NMDA stimulates GnRH release from the median eminence, and that this undergoes alterations with ageing, support this hypothesis. Future studies will be directed at studying the regulation of GnRH neuroterminals by the NMDA receptor in ageing rats.

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