

N-Methyl-D-Aspartate Receptor Subunit Expression in GnRH Neurons Changes during Reproductive Senescence in the Female Rat

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During reproductive senescence in females, the function of GnRH neurons becomes compromised, and this may play a role in the transition from normal estrous cycles to acyclicity. One hypothalamic component of this dysregulation is an alteration in the stimulatory effects of glutamate, acting via N-methyl-D-aspartate receptors (NMDARs), on GnRH release. The present study examined whether GnRH neurons express the subunits necessary to make functional NMDARs, and how subunit expression may change during aging in association with compromised reproductive physiology. Colocalization of the three NMDAR subunits that are most abundant in the hypothalamus (NR1, NR2A, or NR2B) with GnRH perikarya was determined in female rats at different stages of the reproductive life cycle: young (3–4 months) rats with regular estrous cycles, middle-aged (8–10 months) rats with regular estrous cycles, middle-aged rats with irregular estrous cycles, and middle-aged acyclic rats in persistent estrus. The number, percent, and localization of GnRH perikarya expressing NR1,

NR2A, or NR2B were mapped and quantified by double label immunofluorescence microscopy. Overall, each of the NMDAR subunits was present in a majority of GnRH neurons. There were no age- or reproductive status-related changes in coexpression of NR1 or NR2A subunits in GnRH neurons. However, coexpression of the NR2B subunit, which affects several functional channel characteristics, was significantly lower in young compared with middle-aged rats, irrespective of reproductive status. This may result in an age-related increase in the ratio of the NR2B to the NR1 and NR2A subunits on GnRH neurons. These data indicate that the majority of GnRH neurons express the proteins needed to receive direct NMDAR-mediated glutamatergic input, and that a change in the stoichiometry of the NMDAR pentamer occurs during aging that precedes, and may have consequences for, altered neuroendocrine function. (*Endocrinology* 143: 3568–3574, 2002)

THE PREOVULATORY GnRH surge is responsible for pituitary production of the gonadotropins FSH and LH, and, subsequently, ovulation (reviewed in Ref. 1). In middle-aged rats, GnRH and LH release are significantly reduced and the preovulatory GnRH/LH surge becomes attenuated (2). At this time, a transition from normal reproductive function to reproductive senescence occurs; this is characterized by a period of lengthened, irregular estrous cycles followed by acyclicity, or persistent estrus (3). Thus, changes in the neuroendocrine axis have been implicated in the onset of reproductive senescence, independent of ovarian function (4).

Activation of N-methyl-D-aspartate (NMDA) receptors (NMDAR) in neuroendocrine brain regions is believed to be critically important to the regulation of the GnRH neurosecretory system (reviewed in Ref. 5). During reproductive development, activation of NMDARs plays a role in regulating the onset of puberty (6, 7). In adulthood, NMDAR activation causes a rapid stimulation of GnRH release (8, 9) and gene expression (10, 11), and is important for the preovulatory GnRH/LH surge (12). During aging, the ability of NMDA agonists to stimulate GnRH/LH release and gene

expression is diminished (13–15), and the expression of hypothalamic NMDAR mRNA is altered (16). These hypothalamic changes have been shown to precede overt physiological changes such as altered estrous cyclicity (17, 18). Although changes in NMDAR-mediated input likely play a role in the age-related decline of the preovulatory GnRH surge, the molecular and cellular mechanisms by which this may occur are unknown.

The NMDAR (NR) is a pentamer composed of the obligatory NR1 subunit in combination with at least one NR2 subunit family member (NR2A, NR2B, NR2C, or NR2D), of which NR2A and NR2B are abundant in adult hypothalamus (19–23). Previous studies of the hypothalamus, hippocampus, and cortex have found that expression of the different NMDAR subunits changes during development and aging, suggesting that alterations in NMDAR subunit composition and stoichiometry may underlie functional changes (24–27). However, which NMDAR subunits are expressed by GnRH neurons is still controversial, as is the percent of GnRH neurons that express the obligatory NMDAR subunit NR1. Initial studies detected only low levels of the NR1 subunit on GnRH neurons (28–31), although subsequent studies have demonstrated the presence of NMDAR subunit mRNA and protein on GnRH perikarya and neuroterminals (16, 32–35).

In the present study, we used immunofluorescence to determine colocalization of NMDAR subunits on GnRH neurons and to evaluate changes in the extent of colocalization during reproductive senescence. We quantitated the percent-

Abbreviations: MA-Irreg, Middle-aged irregularly cycling; MA-PE, middle-aged persistent estrous; MA-Reg, middle-aged regularly cycling; NMDA, N-methyl-D-aspartate; NMDAR (NR), NMDA receptors; OVLT/POA, organum vasculosum of the laminae terminalis/preoptic area.

age of GnRH perikarya that coexpress NR1, NR2A, and NR2B, and mapped the distribution of these cells in female rats at four stages of the reproductive life cycle: young regularly cycling, middle-aged regularly cycling, middle-aged irregularly cycling, and middle-aged acyclic (persistent estrus). Thus, effects of both aging and reproductive status could be examined with respect to NMDAR expression on GnRH somata.

Materials and Methods

Animals

Female Sprague Dawley rats were purchased at 3–4 months (young) and 8 months (middle-aged) from Harlan Sprague Dawley, Inc. (Indianapolis, IN), and housed two to three per cage in a temperature-controlled room (22 C) with 12 h light, 12 h dark (lights on at 0700 h). Food and water were provided *ad libitum*. Estrous cycles were monitored by daily vaginal smears for at least 4 wk to determine cycling status. Animals with two or more consecutive 4- to 5-d cycles were defined as having regular estrous cycles [young (Y) and middle-aged regularly cycling (MA-Reg)], those with two or more consecutive 6+ d cycles were defined as having irregular estrous cycles (MA-Irreg), and those with 14 d or more of cornified vaginal smears were defined as persistent estrous (MA-PE). All young rats were virgins, and all middle-aged rats were retired breeders. A previous study from our laboratory showed no difference in hypothalamic GnRH and NMDAR subunit protein or gene expression due to breeding history (16). All experiments were conducted in accordance with Guidelines for the Care and Use of Experimental Animals, using protocols approved by the institutional animal care and use committee at Mount Sinai School of Medicine (protocol no. 98–490 NA).

Tissue collection

All cycling animals were perfused on the afternoon of proestrus between 1600–1800 h, and MA-PE rats were perfused simultaneously. Animals were anesthetized with ketamine and xylazine (80 mg/kg and 5 mg/kg, respectively) and once they were deeply anesthetized (usually within 2–5 min of anesthetic/analgesic administration), they were immediately perfused with 1% paraformaldehyde (50 ml) followed by 4% paraformaldehyde (500 ml). Brains were removed and postfixed in 4% paraformaldehyde for 6 h, then transferred for storage to PBS with 0.1% sodium azide at 4 C. Brains were sectioned into 40- μ m slices on a vibratome (Ted Pella) and stored in PBS with 0.1% sodium azide until use. Five animals were used in the MA-Reg group, and six animals were used for each of the other groups.

Immunocytochemistry

Sections were chosen from the level of the organum vasculosum of the laminae terminalis/preoptic area (OVL/POA), where the majority of GnRH perikarya are located (six sections per animal for both NR2A and NR2B, and four to five sections per animal for NR1). To accurately sample colocalization throughout the OVL/POA, each of the three antibodies was assigned alternating slices as the hypothalamus was sectioned from anterior to posterior. Brain slices were washed three times for 10 min each in PBS, then incubated with 2% normal goat serum and normal horse serum for 1 h (to reduce nonspecific binding of the secondary antibody). All sections were then incubated with mouse monoclonal antibody to GnRH [1 μ g/ml concentration of HU11B, kindly provided by Dr. Henryk Urbanski (36)], together with a rabbit polyclonal antibody to either NR2A [8 μ g/ml, Upstate Biotechnology, Inc., Lake Placid, NY (37)], NR2B [0.4 μ g/ml, Novus Biologicals, Littleton, CO (38)], or NR1 [1 μ g/ml, Chemicon International, Inc., Temecula, CA (38–40)] for 72 h at 4 C. The primary antibody was omitted in control sections. Additional antibody specificity was tested by preincubating the GnRH antibody with the GnRH peptide; following preincubation and subsequent immunocytochemistry, no fluorescence was detected. Sections were washed three times for 10 min each, then incubated for 1.5 h with the appropriate secondary antibody (antimouse Texas Red for GnRH, 1:200, and antirabbit FITC for the NMDAR subunits, 1:200, both

from Vector Laboratories, Burlingame, CA). Sections were washed, mounted on gelatin-coated slides, and allowed to dry overnight. To reduce auto-fluorescence, all slides were dehydrated in ethanol and treated with Sudan Black (1 mg/ml, Sigma, St. Louis, MO). Slides were coverslipped with Vectashield (Vector Laboratories).

Microscopy

Sections were analyzed by two individuals working independently, both blind to treatment conditions. Both a Carl Zeiss LSM410 inverted confocal laser scanning microscope, at a magnification of $\times 1000$, and a Carl Zeiss Axiophot fluorescence microscope, at a magnification of $\times 250$, were used (Carl Zeiss Microimaging, Inc., Thornwood, NY). For analysis, every GnRH perikaryon in each section of tissue was counted and scored as either positive or negative for NR1, NR2A or NR2B. Then, each GnRH neuron was mapped onto a representation of the OVL/POA from a rat brain atlas and indicated as NMDAR subunit-positive or negative (33, 41).

Statistical analysis

For each NMDAR subunit, the percent colocalization with GnRH was calculated per group. Statistical comparisons of percent colocalization were performed by the generalized estimating equation approach via the SAS-GENMOD (Generalized Linear Model) procedure (SAS Institute Inc., Cary, NC). In all cases, significance was set at $P < 0.05$.

Results

Double-labeling of GnRH neurons with NMDAR subunits

NR1. Representative GnRH neurons that are NR1-positive or NR1-negative are shown in Fig. 1. Overall, 66% of GnRH neurons were found to coexpress NR1 (Fig. 2). In young rats, 56% of GnRH perikarya were double-labeled with NR1. In middle-aged rats, a range of 62–76% (depending upon reproductive status) of GnRH perikarya expressed NR1, with an overall average of 68% (Fig. 2). Statistical analysis indicated that there was no difference in percent colocalization of NR1 based on reproductive status or age ($P = 0.17$), although there was a trend for an increase from young (56%) to middle-aged (68%).

NR2A. Representative GnRH neurons that are NR2A-positive or NR2A-negative are shown in Fig. 1. NR2A was expressed by a majority of GnRH neurons (72% overall, Fig. 2). In young rats, 73% of GnRH neurons coexpressed NR2A, whereas in middle-aged rats, this ranged from 69–73%, with an average of 71% (Fig. 2). There were no statistical differences in percent colocalization of NR2A with GnRH neurons based on either age or reproductive status ($P = 0.92$).

NR2B. Representative GnRH neurons that are NR2B-positive or NR2B-negative are shown in Fig. 1. NR2B expression was high in GnRH neurons of adult female rats (49% overall; Fig. 2). Young rats had 37% colocalization of NR2B in GnRH neurons, whereas in middle-aged rats, this ranged from 51–63%, with an average of 55%. A significant effect of age was found ($P = 0.024$), with middle-aged rats of all reproductive statuses having significantly higher percent colocalization of NR2B in GnRH neurons than young rats.

Number and anatomical distribution of GnRH neurons and NMDARs

The number of GnRH neurons identified in each group did not change with age or reproductive status, although there

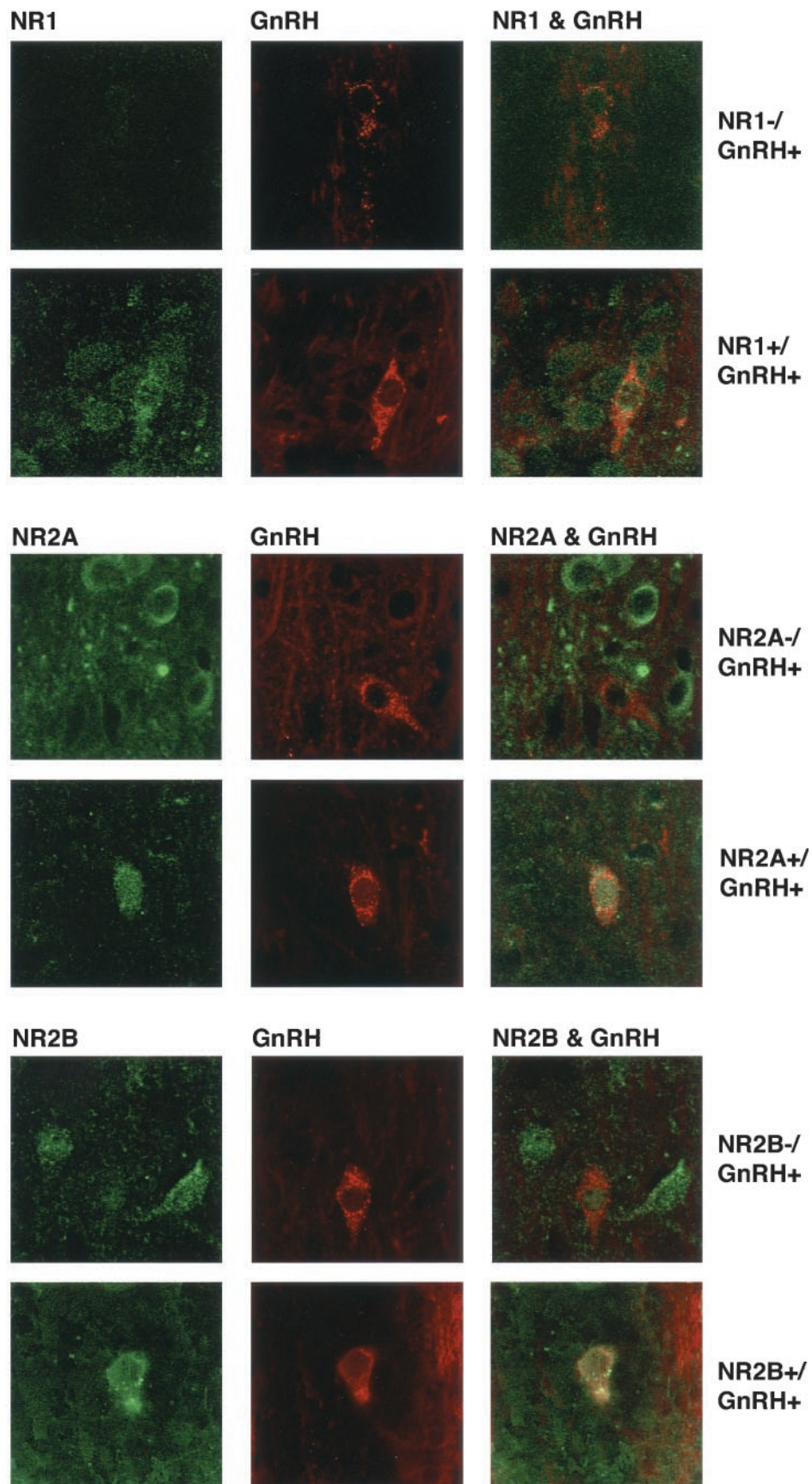


FIG. 1. Photomicrographs of representative GnRH cell bodies in the OVLT/POA of female Sprague Dawley rats. Cells were detected by double-label immunofluorescence for GnRH with NR1, NR2A, or NR2B, and imaged on a confocal laser microscope. *Top*, A GnRH neuron single- (*top*) and double-labeled (*bottom*) with NR1 is shown. *Center*, A GnRH neuron single- (*top*) and double-labeled (*bottom*) with NR2A is shown. *Bottom*, A GnRH neuron single- (*top*) and double-labeled (*bottom*) with NR2B is shown. Magnification, $\times 1000$.

was a nonsignificant tendency for MA-Irreg rats to have a higher number of GnRH neurons counted than the other groups ($P = 0.11$, Fig. 3). An average of 37 ± 5 , 39 ± 8 , 49 ± 7 , and 37 ± 6 GnRH neurons were counted per five to six sections in the OVLT/POA of Y, MA-Reg, MA-Irreg, and MA-PE, respectively.

GnRH neurons that were single- or double-labeled for each NMDAR subunit were mapped onto brain atlas representations at the level of the OVLT/POA. Because no age-related difference in distribution of double-labeling was observed, data are shown for GnRH neurons combined for the four groups of animals. As shown in Fig. 4, the distribution of GnRH neurons that were single- or double-labeled with each NMDAR subunit was similar, with cells found across the entire dorsal-ventral and rostral-caudal (not shown) continuum. Both single- and double-labeled GnRH neurons

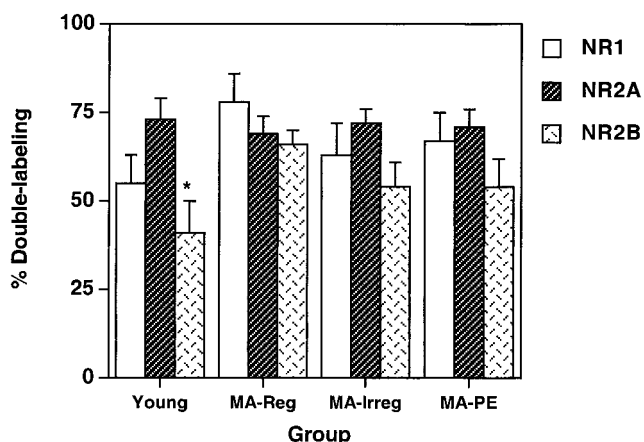


FIG. 2. Percentages of GnRH perikarya that are double-labeled with the NMDAR subunits (NR1, NR2A, NR2B). Double-label immunocytochemistry was performed for GnRH together with either NR1, NR2A, or NR2B in brain sections at the level of the OVLT/POA. GnRH neurons of young (Y), MA-Reg, MA-Irreg, and MA-PE were identified and counted, and each GnRH neuron was then scored as positive or negative for the respective NMDAR subunit. Data shown are mean \pm SEM. *, $P < 0.05$ vs. middle-aged groups.

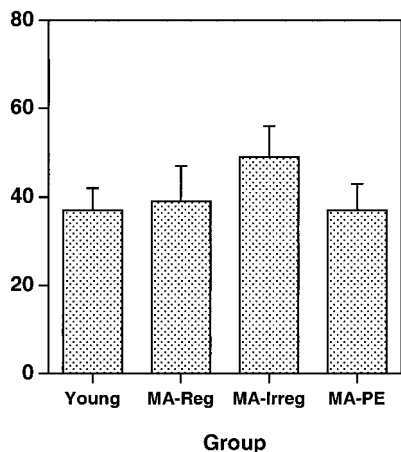


FIG. 3. Average number of GnRH neurons detected per animal in each group. Abbreviations are the same as in Fig. 2. A total of five to six sections at the level of the OVLT/POA were counted per animal. Data shown are mean \pm SEM.

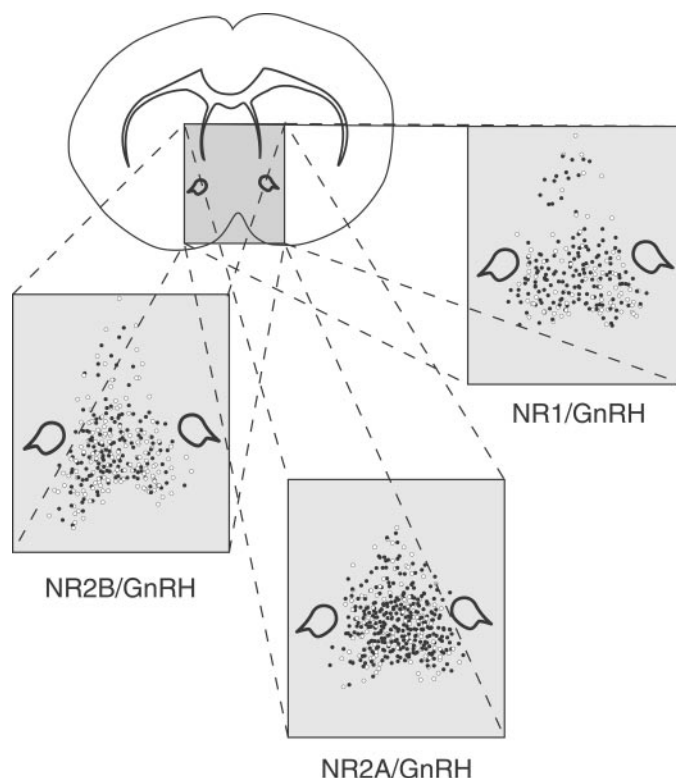


FIG. 4. Distribution of GnRH neurons of all age groups, mapped onto a single brain atlas representation at the level of the OVLT/POA. GnRH neurons that are single- (open circle) or double-labeled (filled black circle) with NR1 are shown at the right. GnRH neurons that are single- (open circle) or double-labeled (filled black circle) with NR2A are shown in the center. GnRH neurons that are single- (open circle) or double-labeled (filled black circle) with NR2B are shown on the left.

were found throughout the entire rostral-caudal and dorsal-ventral extent in animals in the four groups (Fig. 4).

To determine the distribution of GnRH neurons, independent of double-labeling, all perikarya from each experimental group were mapped onto a brain atlas representation at the level of the OVLT/POA (Fig. 5). The anatomical distribution of GnRH neurons appeared to undergo a qualitative change during aging. GnRH perikarya were predominantly localized in the OVLT and anterior POA, diagonal band of Broca, medial septum, and lateral septum, but there were fewer GnRH neurons in the septum of the MA-Irreg and MA-PE groups (Fig. 5).

Discussion

The present results show that the majority of GnRH perikarya of adult female rats express the proteins needed to form functional NMDARs. Overall, 66, 72, and 49% of GnRH somata express NR1, NR2A, and NR2B subunits, respectively. Therefore, NMDAR-mediated glutamatergic input can occur directly at the level of GnRH somata. Additional input may occur indirectly via interneurons, as numerous GnRH-immunonegative cells in the OVLT/POA contained immunoreactivity for NR1, NR2A, or NR2B. We also observed a significant change in NR2B, but not NR1 or NR2A, expression by GnRH neurons from the young to the middle-aged groups. This suggests that a change in the stoichiometry

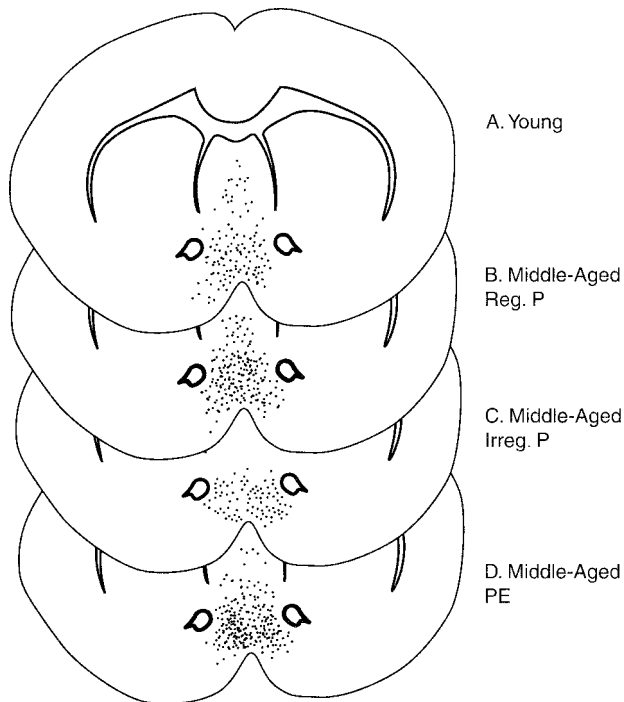


FIG. 5. Distribution of all GnRH neurons in (A) young P, (B) MA-Reg P, (C) MA-Irreg P, and (D) MA-PE rats, mapped onto a single brain atlas representation at the level of the OVLT/POA. Reg. P, Middle-aged regularly cycling proestrous rats. Irreg P, Middle-aged irregularly cycling proestrous rats.

of the NMDAR, rather than in the absolute number of NMDARs, may be responsible for or play a role in the altered effects of NMDA agonists on GnRH neurons in middle-aged animals compared with young animals (13, 14).

The NR1 protein is an obligatory component for a functional NMDAR, together with at least one member of the NR2 subunit family, with which it forms a heteromeric pentamer (19). It has previously been demonstrated that, of the NR2 family members, NR2A and NR2B are most abundant in adult hypothalamic regions (20, 42). The present results, which demonstrate that high percentages of GnRH cells express the three NMDAR subunits, suggest that the NR1 subunit occurs in combination with the NR2A and/or NR2B subunit to form functional NMDARs on GnRH perikarya and surrounding cells. Although technical limitations prevented us from performing triple-labeling experiments, the observed high expression of the various NMDAR subunits in GnRH neurons suggests the likelihood that at least some GnRH neurons express both NR1 and NR2 subunits, and therefore possess functional NMDARs.

There have been differences in reports from several laboratories, including earlier work from our own laboratory, in the percentages of GnRH neurons that express the NMDAR. These differences are likely attributable to the technique used to detect the NMDAR and/or the GnRH neurons. Several earlier *in situ* hybridization studies reported that fewer than 5% of GnRH neurons express the NR1 subunit, and that expression of the NR2B-D subunits is similarly low (23, 28, 29). However, NMDARs are quite abundant in central nervous system tissues, including the hypothalamus/POA (20),

and it is difficult to determine the precise localization of NMDAR subunit mRNA in specific cells by *in situ* hybridization, as NMDAR mRNA appears to be distributed almost ubiquitously throughout these tissues. The use of newly available NMDAR antibodies in combination with a monoclonal antibody to GnRH enables the most specific determination of colocalization of NMDAR subunits in GnRH neurons by dual label immunofluorescence, and we believe that this is the optimal method for performing these studies.

Although immunofluorescence offers superior single-cell signal specificity, the strength of the signal depends on the specific antibody being used. In a previous study (16), our laboratory found a lower overall percent of GnRH/NR1 colocalization than the percent reported here. Because a monoclonal NR1 antibody was used in the earlier study, whereas a polyclonal NR1 antibody was used in the present study, it is likely that the increased number of potential binding sites present in the polyclonal antibody boosted the signal strength, increasing the number of cells identifiable as NR1-immunoreactive. Additional differences between the two NR1 antibodies may be due to antibody cross-reactivity, as the antibody used in the present study has been shown to recognize all major NR1 splice variants (38). Optimization of the dual label immunofluorescence technique in our laboratory has also improved our ability to detect double-labeled GnRH neurons. Recently, two other laboratories have reported that NR1 is expressed in 50% (35) to 80% (34) of GnRH neurons, consistent with the findings of the present study.

The present study also reports a lower number of total GnRH neurons than previous studies (43). However, the number of GnRH neurons detected depends on the specific primary antibody and on the method of immunovisualization used: whereas immunofluorescence provides a better method of resolving colocalization, peroxidase staining is several times more sensitive. Previous studies have shown that detection of individual GnRH neurons depends on the level of GnRH peptide expression, such that more GnRH neurons are detectable on the evening of proestrus than on the morning of proestrus (44). This suggests that the population of GnRH neurons directly responsible for the GnRH surge is the most detectable population. Although the reason for an increase in detectability is unknown, it may be related to those cells with enhanced synthesis or accumulation of peptide, which can subsequently be used for the preovulatory GnRH surge. Although the population of GnRH neurons that we sampled—collected at late proestrus—was reduced due to the use of immunofluorescence and may therefore not represent the entire GnRH population, we believe that it is an accurate representation of the total active population. Moreover, the primary GnRH antibody we used has been extensively characterized (45).

During aging, the effect of NMDA agonists on GnRH gene expression and release is attenuated (15, 16). In the present study, NR2B protein expression was significantly up-regulated in the GnRH perikarya of middle-aged animals, irrespective of reproductive status, and NR1 expression showed a small, albeit nonsignificant increase during reproductive aging. Importantly, the attenuated response of GnRH neurons to NMDAR activation is first observed in middle aged rats that are still cycling, comparable to the period when

changes in expression of the immediate early gene *c-fos* in GnRH neurons during the LH surge, and the magnitude and timing of the preovulatory LH surge (17, 18), are first seen. Therefore, the onset of these functional changes coincides with the change in coexpression of NMDAR subunits by GnRH neurons but precedes overt changes in estrous cyclicity.

The NR2B subunit mediates certain NMDAR channel properties; for example, its presence in the NMDAR pentamer confers longer excitatory postsynaptic potentials compared with the NR2A subunit (46, 47). *In vivo* up-regulation of NR2B has recently been shown to result in significantly altered neuronal and behavioral phenotypes (48). The present study extends the significance of altered NMDAR subunit stoichiometry to the neuroendocrine axis. Although it may seem surprising that an increase (as opposed to a decrease) in expression of the NR2B subunit in GnRH perikarya occurs at middle age, this may be a compensatory mechanism for reductions in glutamatergic input, similar to the mechanism involved in up-regulation of NMDAR subunit expression following long-term deafferentation (49). Moreover, this increase in NR2B protein in GnRH perikarya may seem contradictory with our previous report of a decrease in NR2B mRNA levels in the POA-AH with aging (16). Nevertheless, it is important to note that our previous study on NR2B mRNA measured its levels in many cells other than GnRH neurons, and additionally, changes in mRNA and protein may not occur in parallel. We speculate that the observed increase in NR2B protein specifically on GnRH somata is responsible for, or plays a role in, the changes in GnRH neuron responsiveness to NMDAR agonists during reproductive aging.

Previous studies have demonstrated no changes, or only small decreases, in the number of GnRH neurons during aging (16, 44, 50, 51). In the present study, no significant change in the number of GnRH neurons was observed with age or reproductive status. However, a qualitative change in the distribution of immunoreactive GnRH neurons, independent of double-labeling with NMDAR subunits, was observed. Middle-aged rats that had irregular cycles (MA-Irreg) or had become acyclic (MA-PE) had qualitatively fewer GnRH neurons in more dorsal neuroendocrine regions, particularly the medial septum, when compared with the regularly cycling young (Y) and middle-aged (MA-Reg) animals. An alteration of the distribution (and to a lesser extent number) of GnRH neurons in the groups most closely associated with the transition to acyclicity may correlate with this physiological process. A study from the laboratory of Rubin and King (44) demonstrated changes in the distribution of GnRH neurons during the preovulatory LH surge in young compared with middle-aged rats, and the authors suggest that there may be subpopulations of GnRH neurons driving the preovulatory LH surge that become compromised during reproductive aging. The GnRH neurons of the medial septal region may represent one such quiescent population and are an important target for future studies.

In summary, the present study shows that the majority of GnRH neurons in adult female rats express the subunits necessary to assemble functional NMDARs. Furthermore, we show that the NR2B subunit, which mediates certain

receptor channel properties, is up-regulated in GnRH neurons at a time that coincides with changes in the functional response of GnRH neurons to NMDAR agonists. Although NR2B up-regulation occurs before alterations in the estrous cycle, early signs of hypothalamic-pituitary-gonadal dysregulation, such as the attenuated preovulatory GnRH/LH surge, are already present at this time. Altered subunit stoichiometry of NMDARs on GnRH neurons may be a cellular mechanism involved in the neuroendocrine dysregulation leading up to reproductive senescence.

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