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Age-related Changes in the Serotonin 2A Receptor in the Hypoglossal Nucleus of Male and Female Rats

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Abstract

Hypoglossal motoneuron output to the genioglossus muscle contributes to upper airway patency. Serotonin (5HT) plays an important role in regulating hypoglossal motoneuron excitability via serotonin 2A receptors ($5HT_{2A}$). The purpose of this study was to investigate whether there are age-associated changes in $5HT_{2A}$ receptor expression in the hypoglossal nucleus of male and female rats. The brains of young, middle-aged and old F344 rats were sectioned, reacted immunocytochemically for the presence of $5HT_{2A}$ receptor, and the staining density quantified. The estrus stage of female rats was determined and circulating sex hormone levels measured and correlated with $5HT_{2A}$ levels. The results show that there was significantly greater $5HT_{2A}$ receptor immunoreactivity in the hypoglossal nucleus of female than of male rats. With increasing age, there was an increase in $5HT_{2A}$ receptor immunoreactivity in the hypoglossal nucleus of female rats, whereas no age-associated changes were observed in male rats. Previous studies have shown a reduction in 5HT-dependent respiratory plasticity and an age-associated decrease in 5HT in the hypoglossal nucleus in male but not female rats. Data from the present study suggest that aging male rats fail to compensate adequately for reduced 5HT in the hypoglossal nucleus by upregulating the expression of the $5HT_{2A}$ receptor.

Keywords

Control of breathing; Respiratory motoneurons; Estrogen; Progesterone; Sex hormones

1. Introduction

Serotonin (5-hydroxytryptamine or 5-HT) plays a pivotal role in the control of breathing (for review, see Bonham, 2005;Bianchi et al., 1995;McCrimmon et al., 1995;Richter et al., 2003). Caudal raphe neurons are activated during hypoxia and release 5HT in the vicinity of respiratory premotor and motoneurons (Erickson and Millhorn, 1994;Teppema et al., 1997). Serotonin has an excitatory effect on respiratory motoneurons, mediated principally by $5HT_2$ receptors (Kubin et al., 1992;Lindsay and Feldman, 1993;Arita et al., 1995;Jelev et al., 2001;Fenik and Veasey, 2003;Brandes et al., 2006).

Hypoglossal motoneurons innervate muscles of the tongue that contribute to upper airway patency during inspiration (Remmers et al., 1978;Miki et al., 1989;Horner, 1996;Saboisky et al., 2006). Serotonergic input to the hypoglossal nucleus originates in neurons in the nucleus

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raphe pallidus and obscurus and the parapyramidal region (Manaker and Tischler, 1993). Studies have shown that 5HT modulation of hypoglossal motoneuron output is mediated by one or more postsynaptic $5HT_2$ receptor subtypes (Schwarzacher et al., 2002;Volgin et al., 2003). The most prominent 5HT2 receptor subtype in hypoglossal motoneurons is 2A (Zhan et al., 2002), and application of selective 2A antagonists to the hypoglossal nucleus reduces hypoglossal nerve respiratory activity by >60% (Fenik and Veasey, 2003). As serotonergic neurons are tonically active during wakefulness, and decrease their firing rate during sleep (Jacobs and Azmitia, 1992), withdrawal of 5HT excitatory drive during sleep is thought to reduce upper airway patency and contribute to sleep apnea (Kubin et al., 1996)

There are age-associated changes in 5HT modulation of respiratory plasticity in both male and female rats (Zabka et al., 2001a,b). Long term facilitation (LTF) is a 5HT-dependent plasticity in hypoglossal and phrenic motor output in response to intermittent hypoxia (for review see Mitchell and Johnson, 2003). The $5HT_2$ receptor is critical to this form of respiratory plasticity as LTF can be abolished by ketanserin, a $5HT_2$ receptor antagonist (Kinkead et al., 1998;Ling et al., 2001;Fuller et al., 2001). LTF is significantly reduced in middle-aged male rats by comparison with young rats (Zabka et al., 2001a). In contrast, female rats show enhanced LTF in middle-age (Zabka et al., 2001b), suggesting that this form of respiratory plasticity is sexually dimorphic.

Sex hormones may play a role in regulating respiratory plasticity. LTF in respiratory motoneurons in response to intermittent hypoxia, and 5HT levels in respiratory motor nuclei fluctuate with the estrus cycle (Zabka et al., 2001b;Behan et al., 2003). In geriatric female rats that have ceased to cycle regularly, there is a decline in hypoglossal and phrenic LTF by comparison with middle-aged rats (Zabka et al., 2003), and gonadectomized young male rats also show reduced LTF in hypoglossal motor output (Behan et al., 2003).

Altered serotonergic modulation of respiratory motoneurons with age in male or female rats could result from a number of different mechanisms including a decrease in serotonergic innervation of respiratory nuclei and/or changes in receptor expression. Previous studies in our laboratory have shown that there are sex differences in 5HT levels in the hypoglossal nucleus: more in females than males (Behan et al., 2003). We hypothesized that female rats would have greater $5HT_{2A}$ receptor expression in the hypoglossal nucleus than male rats. With increasing age there is a decrease in serotonergic terminals and altered synaptic bouton morphology in the hypoglossal nucleus of male rats (Behan and Brownfield, 1999;Behan et al., 2002), whereas female rats do not show a decrease (M. Behan, unpublished observations). Additionally, middle-aged male but not female rats have reduced hypoglossal LTF by comparison with young rats (Zabka et al., 2001 a,b). Thus, we hypothesized that $5HT_{2A}$ receptor levels in the hypoglossal nucleus of male and female rats would be differentially affected by age. In the present study we measured $5HT_{2A}$ receptor immunoreactivity in the hypoglossal nucleus of young, middle-aged and old male and female rats. Additionally, we tested the hypothesis that circulating estrogen and progesterone levels correlate with $5HT_{2A}$ receptor immunoreactivity in the hypoglossal nucleus in female rats.

2. Methods

2.1. Animals and Tissue Preparation

All procedures were performed in compliance with the *NIH Guide for the Care and Use of Laboratory* Animals and approved by the Institutional Animal Care and Use Committee at the University of Wisconsin School of Veterinary Medicine. Three age groups were studied: young (3 months), middle-aged (14 months), and old (>20 months). A total of 12 male rats (4 young, 4 middle-aged, 4 old) and 15 female F344 rats (6 young [3 estrus, 3 diestrus], 6 middle-aged [3 estrus, 3 diestrus], 3 old) were used in the present study. Estrus cycle stage was determined by vaginal lavage and light microscopy (Hebel and Stromberg, 1984). Young and middle-aged female rats were followed through at least two estrus cycles. Old female rats are generally acyclic. They were followed for approximately one week to confirm their lack of cycling, and establish their estrus cycle stage.

Rats were anesthetized with sodium pentobarbital (50mg/ml i.p.). Anesthetized rats were transcardially perfused with 200ml of heparinized saline (10,000 units/liter) followed by 400ml of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (PB) (pH 7.4). Brains were removed and postfixed for 1 hour at 4°C, then cryoprotected for 24–36 h at 4°C with 20% sucrose and 5% glycerol in 0.1M phosphate buffer. Sections were cut in the horizontal plane with a freezing microtome at a thickness of 50μm. Sections that were not reacted immediately were stored in 0.1M phosphate buffer containing 0.02% sodium azide at 4°C.

2.2. Immunocytochemical Staining

All sections through the hypoglossal nucleus were reacted for the presence of $5HT_{2A}$ receptor. Free floating sections were incubated in 3% normal goat serum (NGS) in 0.1M sodium phosphate buffer (PB) for 30 minutes. Sections were then reacted with antiserum to $5HT_{2A}$ receptor (gift from M. Brownfield; Brownfield et al., 1998) in 1% NGS (1:500) for 18 hours at 4°C. The same lot of $5HT_{2A}$ antibody was used in all immunocytochemical reactions. Sections were washed in 0.3% Triton-X in 0.1M PB (2×15 mins) and incubated in 3% NGS in PB at 4°C. Sections were incubated in biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA) in 1% NGS with 0.75% Triton-X for 1 hour. Sections were washed (2×15 mins) and incubated with ABC complex (Vectastain Elite Kit, Vector Laboratories) for 1 hour. Sections were washed with 0.1 M PBS (5×5 min), and reacted with 0.04% DAB in 0.1 M PB with 0.003% hydrogen peroxide and 0.1% nickel ammonium sulfate. Sections were washed, mounted and cover slipped.

2.3. Data Analysis

Digital photographs (16 bit; 65,536 grey levels) of brain sections were taken using a SPOT camera (Diagnostic Instruments; optical resolution 1600 × 1200 pixels; 7.4μm square pixels) mounted on a Nikon Omniphot microscope, and quantitative image analysis was performed with ImagePro Plus software. All images were obtained under identical bright field illumination. The paired hypoglossal nuclei could be seen clearly in each horizontal section (Fig. 1). Staining in the hypoglossal nucleus was darker than in the overlying dorsal vagal motor nucleus. This, combined with localization of the central canal and fourth ventricle, allowed the dorsal vagal motor nucleus to be identified and excluded from the analysis. Two photographs were taken of each section: one of the paired hypoglossal nuclei and one of a region with homogeneous light $5HT_{2A}$ staining. A rectangular sample area was defined (62,500 μm^2) and optical density, which measures the darkness of each pixel, was measured in rostral, middle and caudal samples bilaterally (Fig. 1). To control for background labeling, optical density was measured in a region of homogeneous light staining, a fiber tract that could be easily identified in each horizontal section. This value was subtracted from each measurement of $5HT_{2A}$ density in that section (Fig. 1). Data were analyzed using either a one-way or two-way repeated measures ANOVA (SigmaStat, Version 2.0, Jaendel, San Rafael, CA). Differences were considered significant at $P < 0.05$.

2.4. Measurement of Sex Hormone Levels

Prior to perfusion, blood was drawn from the abdominal aorta in female rats for measurement of sex hormone levels. Blood samples were centrifuged, and serum was immediately frozen and stored at −80°C. After collection of all serum samples, 17β estradiol (the most potent mammalian estrogenic hormone) and progesterone levels were analyzed by ELISA (IBL, Hamburg, Germany; 17β estradiol, Cat. # RE50421, sensitivity 4.6 – 3000 pg/ml; progesterone, Cat. #RE52231, sensitivity 0.05 – 36 ng/ml). Serum levels of estradiol and progesterone and the progesterone-to-estradiol (P/E) ratio of individual rats were related to the magnitude of $5HT_{2A}$ immunoreactivity via linear regression (SigmaStat, Version 2.0, Jaendel, San Rafael, CA). A variable was considered to significantly contribute to the model if $P< 0.05$.

3. Results

3.1. $5HT_{2A}$ Immunoreactivity in Male and Female Rats

Dense $5HT_{2A}$ immunoreactivity was present throughout the hypoglossal nucleus by comparison with the surrounding neuropil (Fig. 1). When considered as a group, female rats had significantly more $5HT_{2A}$ immunoreactivity in the hypoglossal nucleus by comparison with male rats ($P<0.001$) (Fig. 2). This sex difference was present in the middle-aged ($P = 0.009$) and old groups of rats ($P = 0.001$), but not in the young group ($P = 0.068$). Sex differences were present in rostral ($P = 0.022$), middle ($P = 0.001$) and caudal ($P < 0.001$) regions of the hypoglossal nucleus, and in dorsal ($P = 0.004$) and ventral ($P = 0.003$) subdivisions.

3.2. Age-related changes in $5HT_{2A}$ in Male and Female Rats

There was a statistically significant increase in $5HT_{2A}$ immunoreactivity in the hypoglossal nucleus of old female rats by comparison with young ($P <0.001$) and middle-aged females ($P = 0.002$) (Figs. 2, 3). Significant differences were found in all three regions: rostral (old vs. young, $P = 0.001$; old vs. middle-aged, $P = 0.016$), middle (old vs. young, $P = 0.001$; old vs. middle-aged, $P = 0.004$) and caudal (old vs. young, $P = 0.001$; old vs. middle-aged, $P = 0.001$). Significant differences were also found in dorsal (old vs. young, $P = 0.006$; old vs. middle-aged, $P = 0.034$) and ventral (old vs. young, $P = 0.004$; old vs. middle-aged, $P = 0.036$) subdivisions of the hypoglossal nucleus. In young and middle-aged female rats, $5HT_{2A}$ levels varied with the estrus cycle: greater in diestrus than in estrus, although this was not statistically significant (Fig. 4). This trend was also seen in rostral, middle and caudal regions of the hypoglossal nucleus. In contrast to female rats, there was no significant effect of age on $5HT_{2A}$ immunoreactivity in any region of the hypoglossal nucleus in male rats (Fig. 2).

3.3. Sex hormone levels and $5HT_{2A}$ Immunoreactivity

Levels of 17 β estradiol (pg/ml) and progesterone (ng/ml) were correlated with $5HT_{2A}$ immunoreactivity in the hypoglossal nucleus in young, middle-aged and old female rats (Fig. 5A). Linear regression showed no statistically significant relationship between $5HT_{2A}$ receptor immunoreactivity and sex hormone levels in female rats ($n = 15$). However, when $5HT_{2A}$ receptor immunoreactivity in the ventral half of the hypoglossal nucleus was analyzed in young and middle-aged female rats ($n = 12$; this region contains motoneurons that innervate protrusor tongue muscles; Aldes, 1995), there was a weak but significant relationship between $5HT_{2A}$ and P/E ($R^2 = 0.384$; $P = 0.032$). The relationship between $5HT_{2A}$ receptor immunoreactivity in the ventral half of the hypoglossal nucleus and P/E in rats in diestrus (young and middle-aged, $n = 6$) was also not statistically significant ($R^2 = 0.647$; $P = 0.054$) (Fig. 5B). The relationship between $5HT_{2A}$ receptor immunoreactivity in the ventral half of the hypoglossal nucleus and P/E in rats in estrus (young and middle-aged, $n = 6$) was not significant ($R^2 = 0.603$; $P = 0.070$) (Fig. 5C).

4. Discussion

The main findings of this study are: (1) there is sexual dimorphism in $5HT_{2A}$ receptor immunoreactivity in the rat hypoglossal nucleus; (2) there is an age-associated increase in $5HT_{2A}$ receptor immunoreactivity in the hypoglossal nucleus of female but not male rats.

4.1. Sexual Dimorphism in the Serotonergic System

Several studies have reported greater circulating levels of 5HT in women by comparison with men (Ashcroft et al., 1964;Wirz-Justice et al., 1977;Ortiz et al., 1988). In the mammalian nervous system sex differences have been reported in the morphology of 5HT neurons and in 5HT synthesis, turnover and receptor binding (Carlsson and Carlsson, 1988;Nishizawa et al., 1997;Rubinow et al., 1998;Zhang et al., 1999;Cordero et al., 2001). Most studies have focused on the cerebral cortex, hippocampus, hypothalamus and dorsal raphe nuclei with a view to understanding the role of 5HT in depression, learning and memory. However, sex differences are also present in the medulla. Previously we reported that levels of 5HT in the hypoglossal nucleus of female rats, as measured by ELISA, were double that of male rats (Behan et al., 2003). Although the number of serotonergic neurons in the medullary raphe that project to the hypoglossal nucleus is similar in male and female rats (Barker and Behan, 2006), synaptic terminal density is greater in the hypoglossal nucleus of female rats (M. Behan, unpublished observations). In the present study we show that $5HT_{2A}$ receptor immunoreactivity in the hypoglossal nucleus is greater in female than in male rats (Fig. 2). Thus, by several measures there appears to be a more robust serotonergic innervation of the hypoglossal nucleus in female than in male rats. As 5HT has an excitatory effect on upper airway motoneurons mediated by $5HT_{2A}$ and $5HT_{2C}$ receptor subtypes (Fenik and Veasey, 2003), sex differences in 5HT input to, or $5HT_{2A}$ receptor expression in hypoglossal motoneurons may contribute to the susceptibility of males to breathing disorders such as obstructive sleep apnea (OSA). Nonetheless, recent data shows that there is a negligible serotonergic drive to hypoglossal motoneurons during natural sleep in rats (Sood et al., 2005). The prevalence of OSA is greater in men than in women (Young et al., 1993;Bixler et al., 1998,2001). After menopause, estrogen and progesterone appear to have a neuroprotective effect on sleep disordered breathing in women (Bixler et al., 2001;Young et al., 2003;Shahar et al., 2003).

4.2. Estrogen and Progesterone in the Serotonergic System

The female rat estrus cycle is 4–5 days in duration and consists of 4 stages: proestrus (~12 hrs), estrus (~12 hrs), metestrus (~21 hrs), diestrus (~57 hrs) (Freeman 1994). Levels of plasma estradiol are low in estrus, higher in diestrus. There are two peaks in plasma progesterone levels, a lower peak in metestrus and a higher peak late in proestrus. During normal hormonal fluctuations, there are variations in 5HT synthesis, release, reuptake and turnover (Biegon et al., 1980;Bethea et al., 2000;Lu et al., 2003; Pekins-Thompson et al., 1998) and in 5HT receptor expression (Biegon et al., 1980;Moses et al., 2000; Birzniece et al., 2002; Kugaya et al., 2003) in rats and primates. Additionally, treatment with estrogen or progesterone has been shown to alter $5HT_{1A}$ mRNA levels in the dorsal raphe of non-human primates (Pecins-Thompson and Bethea, 1999).

Previously we found that 5HT levels in the rat hypoglossal nucleus were higher in diestrus than in estrus (Behan et al., 2003). Consistent with this, in the present study there was a trend towards higher levels of $5HT_{2A}$ receptor immunoreactivity in the hypoglossal nucleus in diestrus by comparison with estrus in young and middle-aged rats (Fig. 4), suggesting that receptors can upregulate very rapidly. Correlations between sex hormone levels (P/E) and $5HT_{2A}$ receptor levels in rats in estrus or diestrus are weak (Fig. 5B, C), and a larger data set is needed to clarify these potential relationships. Whether estradiol and progesterone influence 5HT synthesis, release and/or reuptake in the hypoglossal nucleus and thereby regulate $5HT_{2A}$ receptor expression, or whether they act directly on hypoglossal motoneurons is not known. Estrogen receptors are present on both hypoglossal and medullary raphe neurons, and it likely that these neurons also express progesterone receptors (Behan and Thomas, 2005;Mitra et al., 2003;Bethea, 1993).

Serotonin is known to play a critical role in respiratory plasticity (for review, see Mitchell and Johnson, 2003). Consistent with the finding that hypoglossal 5HT and $5HT_{2A}$ receptor levels are greater in diestrus than in estrus, hypoglossal LTF also varies with the estrus cycle: greater in diestrus than in estrus (Zabka et al., 2001b). Thus, respiratory plasticity in hypoglossal motor output in female rats in response to intermittent hypoxia (as measured by LTF) appears to be influenced by circulating sex hormone levels via the serotonergic system. Although the physiological relevance of LTF is not yet clear, it may play a protective role, particularly during sleep when serotonergic activity is low. In a recent study in rats, ventilatory LTF in response to intermittent hypoxia was observed during non-REM sleep, but not during wakefulness (Nakamura et al., 2006). Ventilatory instability during sleep, resulting in intermittent hypoxia, could activate serotonergic neurons to upregulate breathing and upper airway tone via LTF of hypoglossal motoneuron output and an increase in upper airway muscle activity (Suratt et al., 1988;Babcock and Badr, 1998;Morelli et al., 2004). Interestingly, upper airway resistance during sleep varies with the menstrual cycle: lower in the luteal phase than in the follicular phase (Driver et al., 2005). Thus, it is possible that serotonergic modulation of upper airway motoneurons might also vary with the menstrual/estrus cycle.

The relative influence of estrogen and progesterone on $5HT_{2A}$ receptor expression in the hypoglossal nucleus is not clear. Our data suggest that $5HT_{2A}$ receptor immunoreactivity is correlated with circulating hormone levels especially in the ventral half of the hypoglossal nucleus where motoneurons that innervate protrusor muscles are located (Aldes, 1995), but the nature of this relationship differs between diestrus and estrus (Fig. 5B, C). Hypoglossal LTF also differs in diestrus and estrus in young and middle-aged female rats (Zabka et al., 2001b), and in a recent study of young and geriatric female rats, there was a significant correlation between hypoglossal LTF and the progesterone-to-estradiol ratio (Zabka et al., 2003). Ultimately, the relative influence of estradiol and progesterone in regulating upper airway patency may depend on a critical ratio of these two hormones that changes across the estrus cycle as well as throughout life.

4.3. Aging and the Serotonergic System

Age-associated changes have been described in serotonergic input to the hypoglossal nucleus in male rats including a reduction in the number of 5HT terminals and an increase in axons with aberrant morphology (Behan and Brownfield, 1999;Behan et al., 2002). Hypoglossal LTF also diminishes with aging in male rats (Zabka et al., 2001a;2005), suggesting that sertonergic function is impaired. An age-associated reduction in 5HT in the hypoglossal nucleus without a compensatory $5HT_{2A}$ receptor upregulation could contribute to the reduction in hypoglossal LTF in aging male rats. Whether sex hormones might directly influence medullary raphe or hypoglossal neurons in male rats is not yet known, although androgen and estrogen receptors are present on hypoglossal neurons (Behan and Thomas, 2005).

4.4. Conclusion

Several lines of evidence suggest that there is a relationship between circulating sex hormone levels and the serotonergic system. Dynamic changes that occur in sex hormone levels can influence a 5HT-dependent form of respiratory plasticity in hypoglossal motoneurons, and may also have an effect on levels of 5HT and the $5HT_{2A}$ receptor in the hypoglossal nucleus. With increasing age and a decline in hormone levels in male rats, or the loss of regular cycles in female rats, the relationship between the serotonergic system and sex hormones may be weakened, and respiratory challenges that require adaptive responses may result in functional impairment, especially in male rats.

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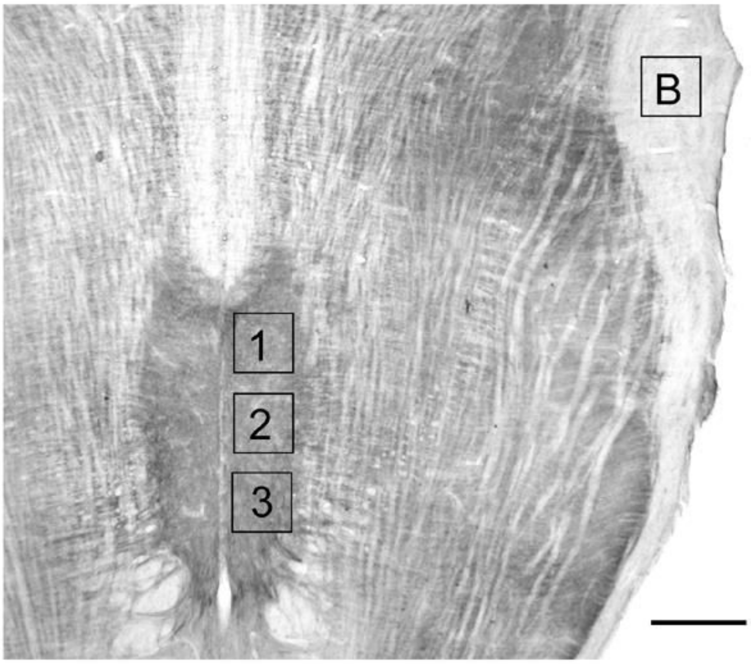

Figure 1.
Photomicrograph of a horizontal section through the medulla stained for $5HT_{2A}$ immunoreactivity. Rostral (1), middle (2) and caudal (3) sample areas are shown together with the location of the sample area analyzed for background label (B). Scale bar = 750 μm.

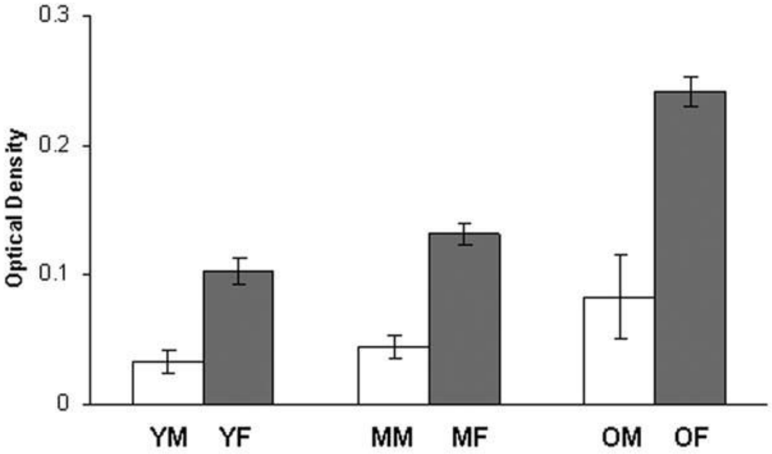

Figure 2.

Quantitative analysis of $5HT_{2A}$ receptor immunoreactivity in the hypoglossal nucleus of young, middle-aged and old male and female rats. Receptor level was measured by optical density (OD), averaged within each rat and then across rats in each group. Middle-aged and old females has significantly more $5HT_{2A}$ receptor immunoreactivity than males. Old females also has significantly more $5HT_{2A}$ receptor immunoreactivity than young (P=0.001) and middle-aged (P=0.002) females. There were no significant differences between young, middle-aged and old male rats. YM, young male, n=4; MM, middle-aged male, n=4; OM, old male, n=4; YF, young female, n=6; MF, middle-aged female, n=6; OF, old female, n=3. Values are means ± S.E.

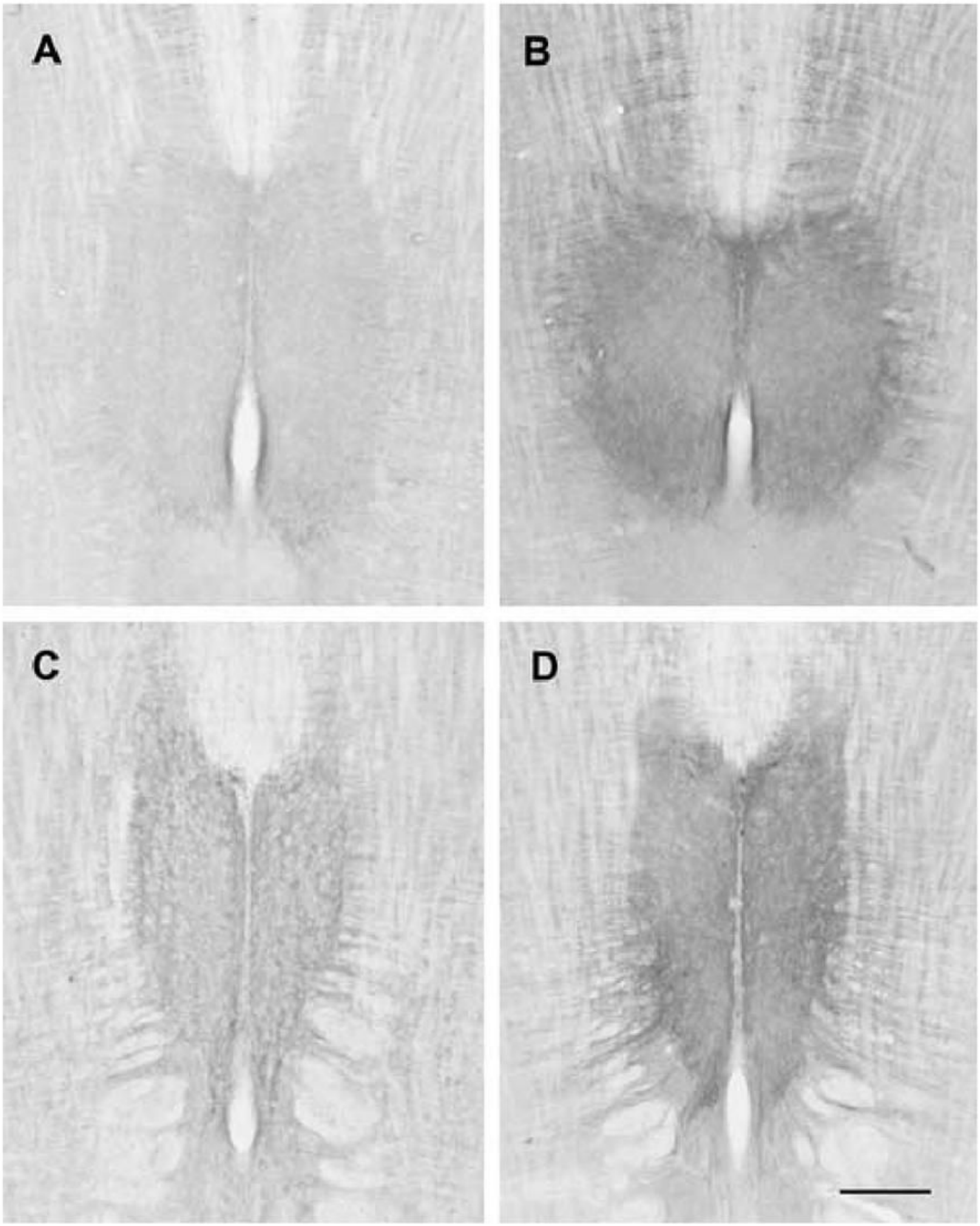

Figure 3.

Photomicrographs of horizontal sections through the hypoglossal nucleus of young and old female rats stained for $5HT_{2A}$ immunoreactivity. A. Young female, dorsal section. B. Old female, dorsal section. C. Young female, ventral section. D. Old female, ventral section. Sections from young and old rats were reacted at the same time. Images were captured using identical illumination parameters; no further adjustments were made. Scale bar = 750 μm.

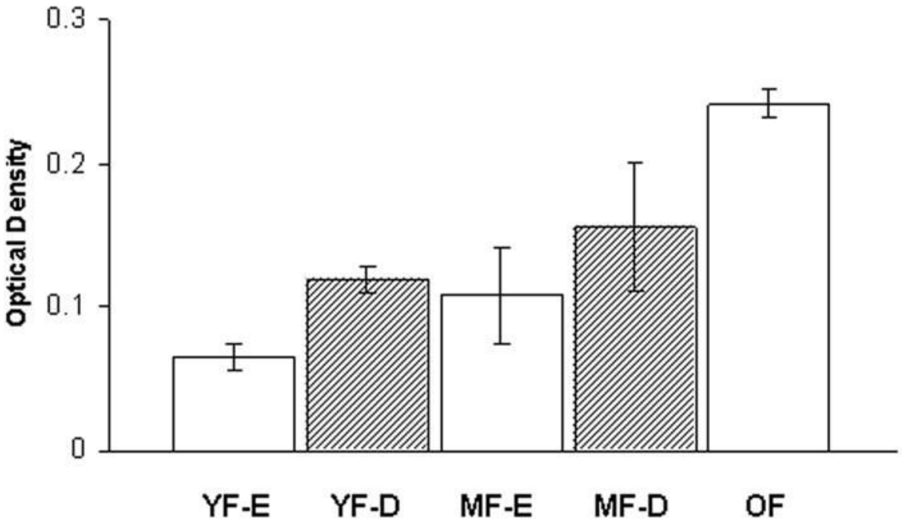

Figure 4.

Quantitative analysis of $5HT_{2A}$ receptor immunoreactivity in the hypoglossal nucleus of female rats in estrus and diestrus. Receptor level was measured by optical density (OD), averaged within each rat and then across rats in each group. Although there is a trend towards an increase in diestrus, this was not significant. YF-E, young female in estrus, n=3; YF-D, young female in diestrus, n=3; MF-E, middle-aged female in estrus,, n=3; MF-D, middle-aged female in diestrus, n=3; O, old female, n=3. Values are means ± S.E.

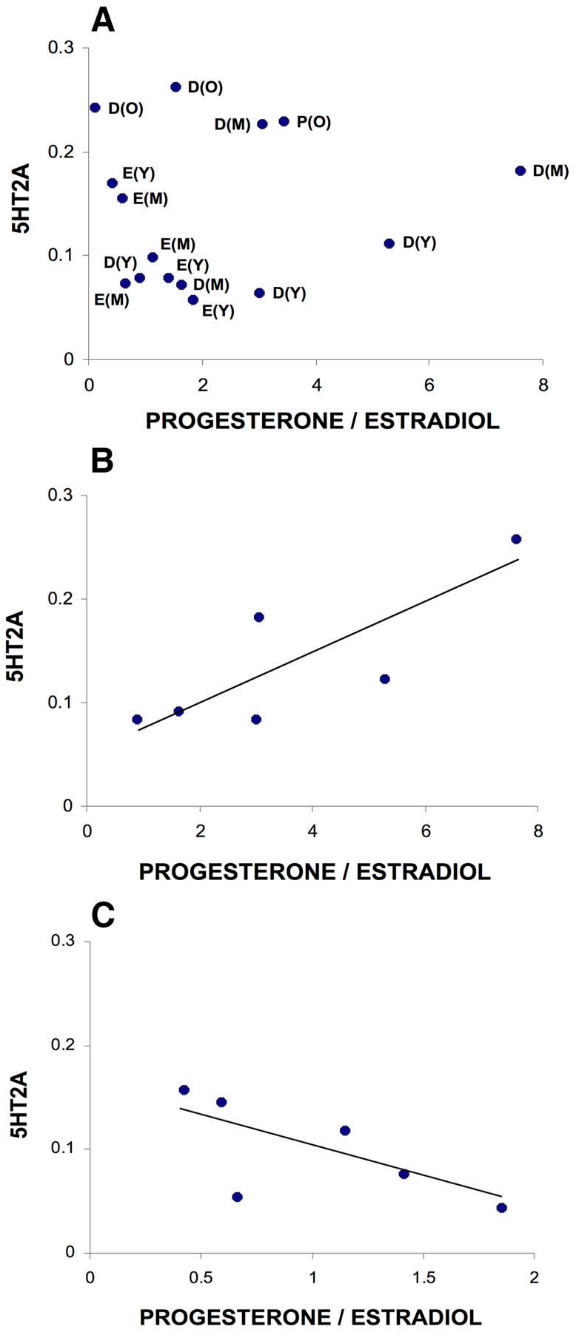

Figure 5.

Relationship between $5HT_{2A}$ immunoreactivity in the hypoglossal nucleus and sex hormone levels. A. The magnitude of $5HT_{2A}$ immunoreactivity (optical density) in the hypoglossal nucleus is plotted against progesterone/estradiol for all15 female rats. The age and estrus stage of each rat is indicated. B. Relationship between the magnitude of $5HT_{2A}$ immunoreactivity in the ventral half of the hypoglossal nucleus and progesterone/estradiol in rats in young and middle-aged rats in diestrus ($n = 6$; $R^2 = 0.647$; $P = 0.054$). C. Relationship between the magnitude of $5HT_{2A}$ immunoreactivity in the ventral half of the hypoglossal nucleus and progesterone/estradiol in young and middle-aged rats in estrus ($n = 6$; $R^2 = 0.603$; $P = 0.070$). D, diestrus; E, estrus; P, proestrus; Y, young; M, middle aged; O, old.