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Effects of aging on stress-related responses of serotonergic neurons in the dorsal raphe nucleus of male rats

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ABSTRACT

Responses to various stressors in the brain change with age. However, little is known about the neural mechanisms underlying age-dependent changes in stress responses. It is known that serotonin, a stress-related transmitter, is closely related with the regulation of stress responses in the brain and that serotonergic function is modulated by various factors, including estrogen, in both sexes. In the present study, to elucidate the effects of aging on stress responses in serotonergic neurons, we examined the expression levels of tryptophan hydroxylase (TPH; a marker of serotonergic neurons) in the dorsal, ventral and lateral parts of the dorsal raphe nucleus (DRN) in young and old intact male rats. In young males, repeated restraint stress significantly increased the number of TPH-positive cells in all subdivisions of the DRN. In contrast, the stress-induced increase in TPH expression was only observed in the ventral part of the DRN in old males. Pretreatment with an estrogen receptor β antagonist had no effect on the number of TPH-positive cells in the dorsal and lateral DRN in young stressed males, whereas the antagonist decreased the number of TPH-positive cells in all DRN subdivisions in old stressed males. Our results suggest that the effects of repeated stress exposure on the expression of TPH in serotonergic neurons in the DRN change with age and that estrogenic effects via estrogen receptor β on TPH expression in stressed old males differ from those in young males.

1. Introduction

Aging changes physiological and pathological states in humans and animals, including rodents. Responses to various stressors in the brain also change with age. Under basal conditions, the function of the hypothalamic-pituitary-adrenocortical (HPA) axis, as measured by adrenocorticotrophic hormones and cortisol/corticosterone secretions, seems to be unchanged or somewhat hyperactive in aged humans and rodents compared with younger adults (Cizza et al., 1995a, b; Lamberts et al., 1997). However, stress-induced reactions of the HPA axis in the aged are greater than those in younger adults, and tend to have long-lasting influences (Bazhanova et al., 2000; Cizza et al., 1995a; Sapolsky et al., 1986), raising the possibility of an age-dependent dysfunction of the negative feedback regulation in stress responses including HPA axis. In addition, the diurnal variation of adrenocorticotropin and cortisol secretion found in young adults flattens gradually with age (Rehman and Masson, 2001). These findings strongly suggest hyperactivation or impairment of adequate responses to stressors and increases in stress vulnerability in old age.

Stress responses are mediated by various neurotransmitters and neuropeptides, including serotonin, which is closely related with the regulation of physiological and psychological functions, such as anxiety and stress responses in the brain (Fuller, 1992; Handa and Weiser, 2014). Serotonergic neurons directly project to corticotropin-releasing factor-containing neurons in the paraventricular hypothalamic nucleus and control HPA function (Fuller, 1992; Weidenfeld et al., 2002). In the dorsal raphe nucleus (DRN), which is a major region of serotonin production in the midbrain and is composed of several subdivisions (Donner and Handa, 2009; Shikanai et al., 2012), innocuous stressors enhance neuronal activation in young male rats (Hale et al., 2008; Shikanai et al., 2012). In addition, the administration of a serotonin 1A receptor antagonist decreases the acute restraint stress-induced corticosterone response and neuronal activation in the paraventricular hypothalamic nucleus in adult rats (Goel et al., 2014). This evidence

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indicates the importance of the serotonergic system in stress responses in young rodents. The function of the serotonergic system changes with age (Miura et al., 2002; Rehman and Masson, 2001), and it has been proposed that such dysfunction of the serotonergic system is heavily involved with mood disorders including depression (Donner and Handa, 2009; Swaab et al., 2005). Therefore, it is highly possible that age-dependent changes in the serotonergic system contribute to the vulnerability to stress exposure in old age.

It has been reported that the serotonergic system is closely linked to estrogen (Barth et al., 2015; Donner and Handa, 2009). Estrogen modulates serotonergic function by the regulation of tryptophan hydroxylase (TPH) and the expression of serotonin transporters and receptors (Barth et al., 2015; Goel et al., 2014; Pecins-Thompson et al., 1996). It is well known that stress exposure increases estrogen levels in female rat brain (Liu et al., 2011), and estrogen in the brain is closely involved with the regulation of stress responses (Handa and Weiser, 2014). Estrogen exerts its action via the estrogen receptors (ER)-α and ER-β. It has been reported that ER-β mediates estrogenic effects in stress responses in young rodents (Liu et al., 2011; Lund et al., 2006), and ER-β is abundantly distributed in the DRN (Shughrue et al., 1997; Yamaguchi and Yuri, 2012), suggesting the possibility that regulatory function of serotonergic system under stress condition is partly due to an ER-β-mediated estrogenic effect.

In the present study, to elucidate the effects of aging on repeated stress-induced changes in the expression of serotonergic neurons in the DRN of male rats, we examined the expression levels of TPH, the rate-limiting enzyme in serotonin synthesis, as a marker of serotonergic neurons, in the dorsal, ventral and lateral parts of the DRN in young and old male rats. In addition, to reveal whether the role of endogenous estrogen via ER-β in serotonergic responses under stress condition change with age, we examined the effect of the ER-β blockade on TPH expression in the DRN in young and old male stressed rats. Furthermore, we examined the effects of stress experience in young adulthood on stress-related TPH expression in aged male rats. Finally, we tried to reveal whether stress-responsive TPH expression in old males differs from those in old females.

2. Materials and methods

2.1. Animals

Young male (7 weeks, n = 12), old male (20 months, n = 16) and old female (20 months, n = 8) Wistar/ST rats (body weight, 250–270 g, 560–615 g and 340–360 g, respectively) were used. All rats were purchased from SLC (Shizuoka, Japan) at the age of 4 weeks and were maintained in our animal facilities under a controlled light and temperature environment (14:10 h light:dark cycle, lights on at 6:00 a.m., 23 °C), with food and water provided *ad libitum*. Both male and female rats were gonadally intact when they were used in all experiments, because we attempted to examine the effects of stress exposure under normal physiological conditions and the role of endogenous estrogen under stress condition. Old female rats were checked for their oestrus cycle stage by the vaginal smear method for 10 days before the start of the experiment, and showed constant diestrus. Our experimental procedures were reviewed and approved by Kochi University, and all rats were treated in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by Kochi University.

2.2. Experimental design

Table 1 summarizes the experimental groups used in this study (n = 4 in each group). All rats were assigned to each experimental group at the age of 4 weeks (n = 8 in each group). To analyze the data from rats with no obvious abnormality such as cancer or abnormal behavior, the number of rats was eventually culled to 4 in each group. The rats of the same experimental group were housed in each cage (two rats per cage).

Table 1
Summary of experimental groups.

Group	7-week		20-month		Sex
	RS	Treatment	RS	Treatment	
1	(−)	Vehicle			Male
2	(+)	Vehicle			Male
3	(+)	PHTPP			Male
4	(−)	(−)	(−)	Vehicle	Male
5	(−)	(−)	(+)	Vehicle	Male
6	(−)	(−)	(+)	PHTPP	Male
7	(+)	(−)	(+)	Vehicle	Male
8	(−)	(−)	(−)	Vehicle	Female
9	(−)	(−)	(+)	Vehicle	Female

RS, restraint stress.

In the young male group, rats were used in the experiments at the age of 7 weeks, and there were three subgroups: vehicle-treated non-stressed young males (Group 1), vehicle-treated stressed young males (Group 2) and ER-β antagonist-treated stressed young males (Group 3). In the old male group, rats were used in the experiments at the age of 20 months, except for one subgroup (Group 7), and there were four subgroups: vehicle-treated non-stressed old males (Group 4), vehicle-treated stressed old males (Group 5), ER-β antagonist-treated stressed old males (Group 6) and vehicle-treated stressed old males that were exposed to repeated restraint stress in young adulthood (Group 7). In the old female group, rats were used in the experiments at the age of 20 months, and there were two subgroups: vehicle-treated non-stressed old females (Group 8) and vehicle-treated stressed old females (Group 9).

2.3. ER-β antagonist administration

A selective ER-β antagonist, 4-[2-phenyl-5,7-*bis*(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP; Tocris Bioscience, Minneapolis, MN, USA), was dissolved in dimethyl sulfoxide (1.0 mg/0.2 ml). PHTPP or vehicle was administered intraperitoneally in a volume of 0.2 ml/kg body weight at 60 min before restraint stress in 5 consecutive exposures. The dose of PHTPP was determined based on previous studies showing the effects of PHTPP administration on nitric oxide production in the paraventricular hypothalamic nucleus (Grassi et al., 2013) and food intake (Santollo et al., 2010). To minimize the number of rats used in the experiments, all rats in the groups other than the PHTPP-treated groups were administered vehicle intraperitoneally.

2.4. Restraint stress

At the age of 7 weeks (young groups) or 20 months (old groups), rats in the stressed groups were exposed to repeated restraint stress. Stress exposure was performed according to our previous report (Yamaguchi et al., 2010) with slight modifications. They were retained in an acrylic rodent restrainer (KN-325, Natsume, Tokyo, Japan; type C-4 [63-mm diameter × 216 mm] for young males and old females; type C-5 [89-mm diameter × 228 mm] for old males) for 1 h per day. The duration of stress exposure was decided based on previous studies showing the HPA responses to acute restraint stress (Figueiredo et al., 2002; Liu et al., 2011) and our previous study (Yamaguchi et al., 2010). It has been reported that repeated

exposure to a homotypic stressor generally results in habituation of HPA response, and that daily exposure to 2-hr restraint stress for 1–4 weeks produces habituation of corticosterone response (Melia et al., 1994; Natelson et al., 1988). Moreover, the exposure to chronic unpredictable stress at unpredictable times of day seems to enhance various stress responses including HPA reactions (Munhoz et al., 2006; Radley and Sawchenko, 2015; Ulrich-Lai and Herman, 2009). Therefore, the stress exposure was performed for 5 consecutive days at a different time of a day (15:00–16:00 on the first and third days; 10:00–11:00 on the other days). For only one old male group (Group 7), which was planned to experience stress exposure in both young adulthood and older age, the rats were exposed to restraint stress for 12 consecutive days, starting at the age of 7 weeks, and then they were returned to their home cages and maintained in the same way as the other old groups until the next stress exposure at the age of 20 months. In contrast, rats in the non-stressed groups were kept undisturbed in their home cages without food and water for 1 h during the experimental periods.

Stressed rats were sacrificed immediately after the last stress exposure. Rats in the non-stressed groups were sacrificed after removal from their home cages without any handling. All rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After collecting blood samples by cardiac puncture, the rats were perfused through the left cardiac ventricle with 100 ml of 0.1 M phosphate-buffered saline (pH 7.4), followed by 500 ml of ice-cold fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and were post-fixed overnight in the same fixative. After cryoprotection in 20% sucrose in 0.1 M phosphate buffer, coronal frozen sections (30-μm thickness) were cut on a cryostat.

2.5. Immunohistochemistry

Free-floating sections were pretreated with 1% H_2O_2 solution for 20 min and then with 3% normal rabbit serum in 0.1 M PBS for 1 h at room temperature to eliminate non-specific antibody binding. The sections were incubated with sheep anti-TPH polyclonal antibody (1:5000; AB1541; Millipore, Temecula, CA, USA) overnight at 4 °C. After the incubation and washes, the sections were incubated with biotinylated rabbit anti-sheep IgG (1:400; Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature, and then treated with avidin-biotin complex-labeled peroxidase (1:250; Vector Laboratories, Burlingame, CA, USA) for 45 min at room temperature. Finally, the sections were reacted with 0.03% 3,3'-diaminobenzidine and 0.03% H_2O_2 in 0.05 M Tris-buffered saline (pH 7.2).

2.6. Measurement of plasma testosterone

Blood samples were centrifuged (3000× *g*, 10 min) and plasma samples were stored at −80 °C for a hormone assay. Plasma testosterone concentrations were measured by enzyme immunoassay using the ELISA kit (ADI-900-065; Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacture's methods. The intra-assay coefficient of variation was 6.4%.

2.7. Data analysis and statistics

The classification of subdivisions was according to the atlas of Paxinos and Watson (2005). The expression of TPH-positive cells was observed, and photomicrographs were captured with an all-in-one microscope system (BZ-9000, Keyence, Osaka, Japan).

To quantify the expression of TPH in the three DRN subdivisions (the dorsal part, the ventral part and the lateral part), the TPH-positive cells were counted within a grid (200 × 200 μm) placed in each section. Cell counting was performed using seven sections of each subdivisions of the DRN for each rat, and the approximate anterior-posterior level from bregma of each section is shown as follows: −7.20, −7.32, −7.44, −7.56, −7.68, −7.80, −7.92 mm (Paxinos and Watson, 2005). Cell counting was performed manually by an observer who was blind to the experimental conditions. The data were analyzed by two-way or one-way analysis of variance (ANOVA) followed by *post hoc* analysis with the Bonferroni method. A difference was considered to be significant if the *p* value was less than 0.05.

3. Results

3.1. Plasma testosterone level

As shown in Fig. 1, two-way ANOVA indicated a significant main effect of age [$F(1,12) = 14.013$, $p < 0.05$], but no significant main effect of stress exposure and interaction between age and stress exposure on testosterone concentration. The rats in the young groups exhibited similar levels of plasma testosterone to those in young adult males at the age of 8–12 weeks previously reported (Bourke and Neigh, 2011; Wu and Gore, 2010; Wu et al., 2009), indicating that the rats in the young groups used in this study attained young adult levels of testosterone. In old males, plasma testosterone level was similar to those in male rats at the age of 20 or 24 months previously reported (Madeira et al., 2000; Wu et al., 2009).

3.2. Effects of aging on restraint stress-induced TPH expression in male rats

To reveal the effect of restraint stress on TPH expression and the effect of aging on stress-related changes in TPH expression in the DRN, we examined the number of TPH-positive cells in the dorsal, ventral and lateral parts of the DRN of non-stressed and stressed young and old male rats. In the dorsal part, two-way ANOVA indicated significant main effects of age [$F(1,12) = 5.21$, $p < 0.05$] and stress exposure [$F(1,12) = 23.57$, $p < 0.05$] and a significant interaction between age and stress exposure [$F(1,12) = 5.79$, $p < 0.05$] on the number of TPH-positive cells (Figs. 2 and 3A). In the ventral part, there were significant effects of age [$F(1,12) = 10.64$, $p < 0.05$], stress exposure [$F(1,12) = 44.17$, $p < 0.05$] and interaction between age and stress exposure [$F(1,12) = 6.07$, $p < 0.05$] on the number of TPH-positive cells (Figs. 2 and 3B). In the lateral part, there was a significant effect of stress exposure [$F(1,12) = 9.547$, $p < 0.05$] and a significant interaction between age and stress

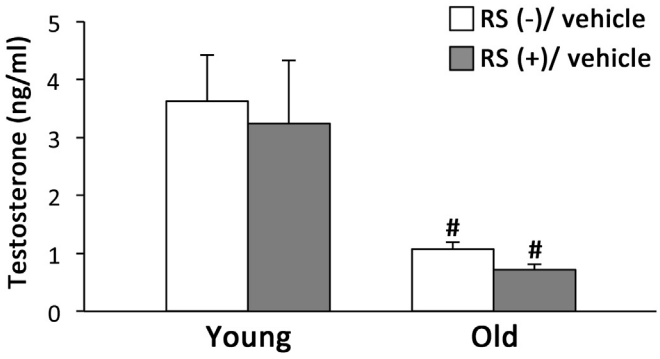

Fig. 1. Plasma testosterone levels in young and old male rats. The white bars indicate vehicle-treated non-stressed [RS (−)] male rats, and the gray bars indicate vehicle-treated stressed [RS (+)] male rats. The data represent mean ± SEM. #Significantly different ($p < 0.05$) from young rats of the same stress condition.

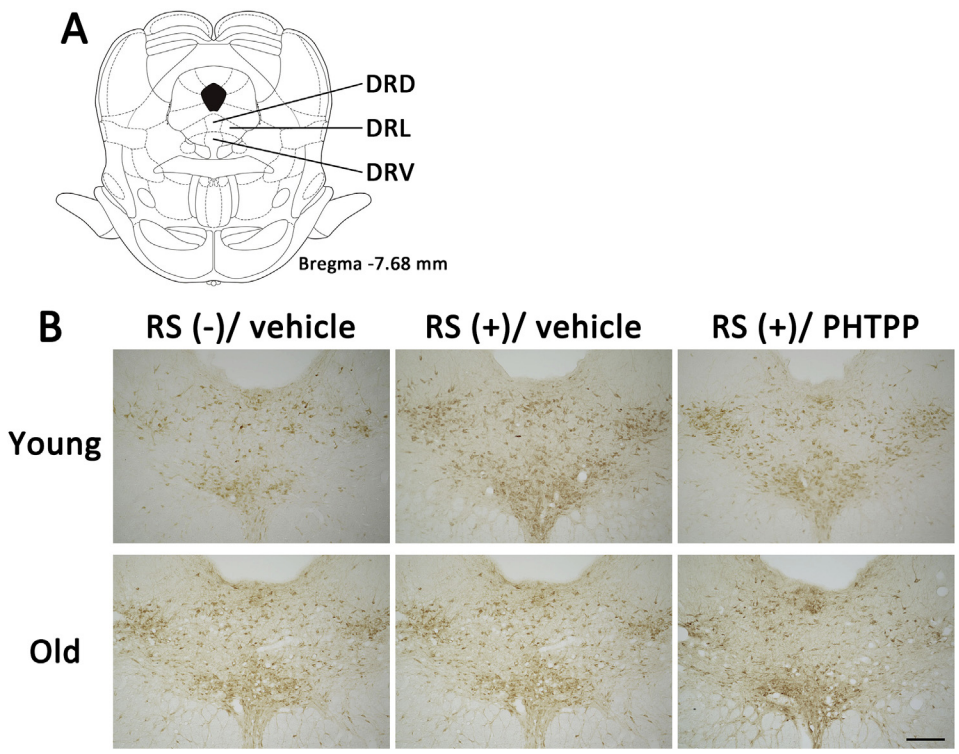

Fig. 2. TPH-positive cells in the dorsal raphe nucleus (DRN) in male rats. (A) Schematic illustration of a coronal DRN section, modified from the rat brain atlas of Paxinos and Watson (27). (B) Representative photomicrographs show TPH-positive cells in the DRN of young and old male rats. Scale bar = 200 μm. DRD, dorsal part of the DRN; DRL, lateral part of the DRN; DRV, ventral part of the DRN.

exposure [$F(1,12) = 5.145$, $p < 0.05$] on the number of TPH-positive cells, but the main effect of age was not significant (Figs. 2 and 3C). *Post hoc* analyses revealed that the number of TPH-positive cells was increased significantly in stressed young males compared to non-stressed young males in all DRN subdivisions. On the other hand, in old males, increases in the number of TPH-positive cells in stressed males were not detected in the dorsal and lateral parts of the DRN. In addition, the expression levels of TPH in all subdivisions in non-stressed old males were significantly higher than those in non-stressed young males.

3.3. Effects of PHTPP, an ER-β antagonist, on restraint stress-induced TPH expression in male rats

To reveal the role of endogenous estrogen via ER-β on restraint stress-induced TPH expression, we examined the number of TPH-positive cells in young and old male stressed rats pretreated with vehicle or PHTPP. Two-way ANOVA indicated a significant main effect of PHTPP treatment in the dorsal DRN [$F(1,12) = 7.10$, $p < 0.05$] and a significant interaction between age and PHTPP treatment [$F(1,12) = 5.21$, $p < 0.05$] on the number of TPH-positive cells (Figs. 2 and 4A), but no significant main effect of age. In the ventral part, there was a significant effect of PHTPP treatment [$F(1,12) = 68.95$, $p < 0.01$] and a significant interaction between age and PHTPP treatment [$F(1,12) = 7.80$, $p < 0.05$] on the number of TPH-positive cells (Figs. 2 and 4B). In the lateral part, significant effects of age [$F(1,12) = 8.65$, $p < 0.05$] and PHTPP treatment [$F(1,12) = 9.75$, $p < 0.05$] were found on the number of TPH-positive cells (Figs. 2 and 4C). *Post hoc* analyses revealed that pretreatment with PHTPP affected the number of TPH-positive cells only in the ventral DRN in young stressed males, whereas the ER-β antagonist significantly decreased the number of TPH-positive cells in all DRN subdivisions in old stressed males.

3.4. Effects of stress exposure in young adulthood on stress-induced TPH expression in aged male rats

To reveal the effect of stress experience in young adulthood on the stress response in old age in male rats, we examined the number of TPH-positive cells in old male rats with or without stress exposure at the age of 7–8 weeks. One-way ANOVA indicated a significant effect of stress in young adulthood on the number of TPH-positive cells in the dorsal [$F(2,9) = 14.44$, $p < 0.05$] (Fig. 5A) and ventral [$F(2,9) = 4.84$, $p < 0.05$] parts of the DRN (Fig. 5B). Stress exposure in young adulthood significantly increased TPH expression only in the dorsal part of the DRN in old stressed males.

3.5. Sex differences in restraint stress-induced TPH expression in aged rats

To reveal whether the effect of stress exposure on TPH expression in old males differs from those in old females, we examined the number of TPH-positive cells in male and female old rats. Two-way ANOVA indicated a significant main effect of sex [$F(1,12) = 10.83$, $p < 0.05$] and a significant interaction between sex and stress exposure [$F(1,12) = 10.83$, $p < 0.05$] on the number of TPH-positive cells in the dorsal DRN (Fig. 6A). In the ventral part, there were significant effects of sex [$F(1,12) = 25.49$, $p < 0.05$] and a significant interaction between sex and stress exposure [$F(1,12) = 50.07$, $p < 0.05$] on the number of TPH-positive cells (Fig. 6B). In the lateral part, no significant main effects or significant interaction was found (Fig. 6C). *Post hoc* analyses revealed that the number of TPH-positive cells decreased significantly in stressed old females compared to non-stressed old females in the ventral part, but not in the dorsal and lateral parts.

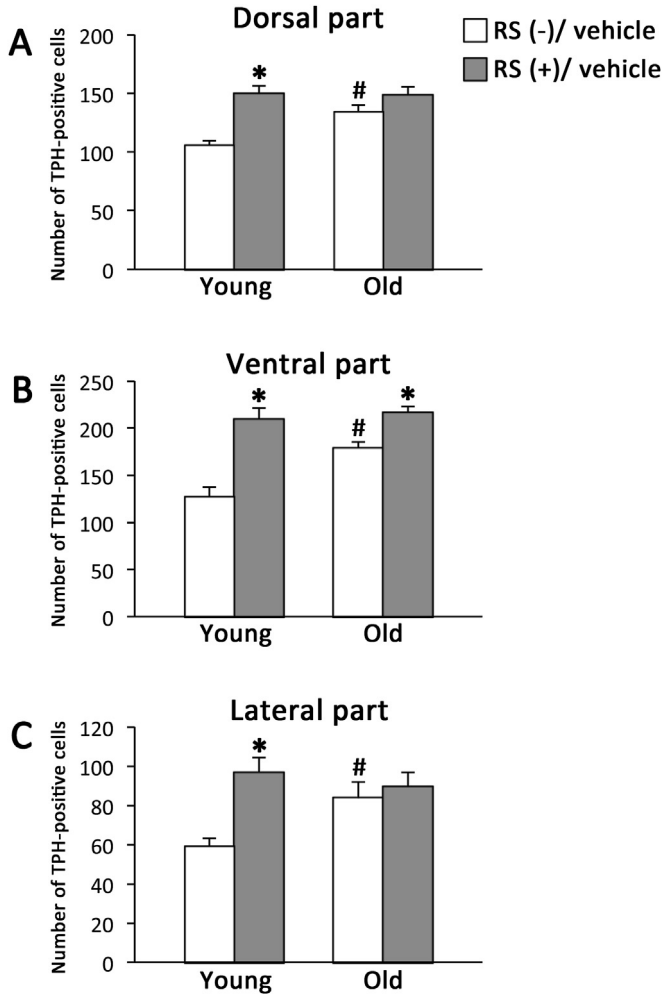

Fig. 3. Effects of aging on restraint stress-induced TPH expression in male rats. Quantification of the expression of tryptophan hydroxylase (TPH)-positive cells in the dorsal part (A), ventral part (B) and lateral part (C) of the dorsal raphe nucleus in young and old male rats. The white bars indicate vehicle-treated non-stressed [RS (−)] male rats, and the gray bars indicate vehicle-treated stressed [RS (+)] male rats. The data represent mean ± SEM. *Significantly different ($p < 0.05$) from vehicle-treated non-stressed rats of the same age group; #significantly different ($p < 0.05$) from young rats of the same stress condition.

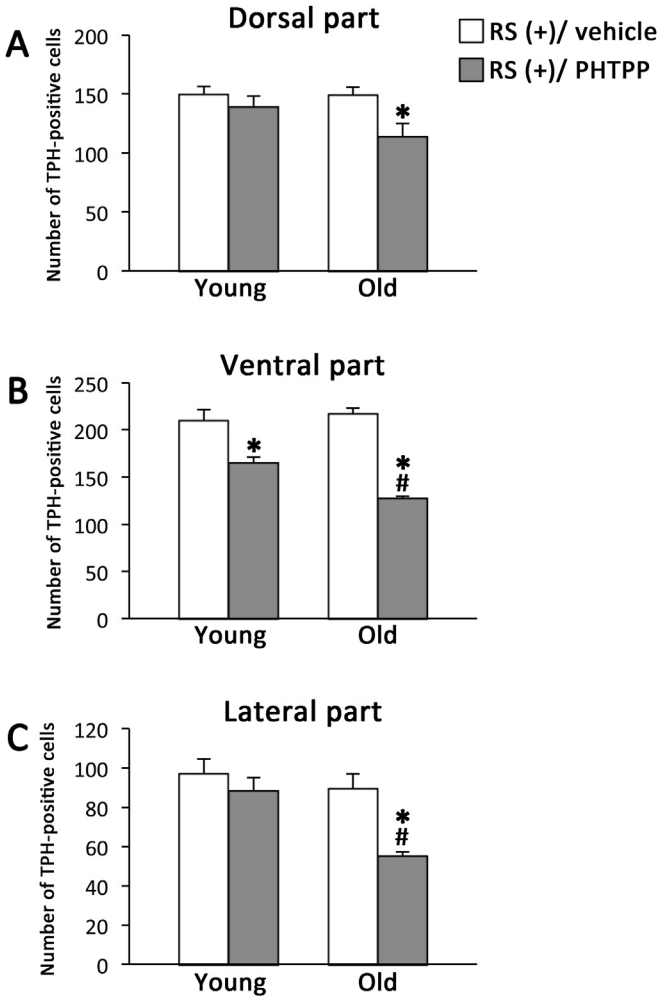

Fig. 4. Effects of PHTPP, an ER-β antagonist, on restraint stress-induced TPH expression in male rats. Quantification of tryptophan hydroxylase (TPH)-positive cells in the dorsal part (A), ventral part (B) and lateral part (C) of the dorsal raphe nucleus in young and old male rats. The white bars indicate vehicle-treated stressed [RS (+)] male rats, and the gray bars indicate ER-β antagonist-treated stressed [RS (+)] male rats. The data represent mean ± SEM. *Significantly different ($p < 0.05$) from vehicle-treated rats of the same age group; #significantly different ($p < 0.05$) from young rats of the same treatment group. The data on vehicle-treated stressed male rats (both young and old) were cited from Fig. 3.

4. Discussion

With age, stress responses in the brain change gradually. Dysfunctions in appropriate responses to stressors and stress vulnerability in the aged can be a major risk factor associated with various stress-related diseases such as depression and cardiovascular diseases. In the present study, we investigated age-dependent changes in stress responses in the serotonergic system in the DRN of male rats.

Many studies have shown age-related changes in stress responses in humans and rodents, although age-dependent effects on the basal activity of HPA axis remain somewhat controversial (unchanged or overactivation) (Cizza et al., 1995a). Acute and repeated restraint stress-induced neuronal activation indicated by Fos expression is enhanced with aging in several stress-related brain regions including the DRN (Shoji and Mizoguchi, 2010), raising the possibility that the function of the serotonergic system under stress conditions changes with age. In fact, several studies have reported the effect of aging on stress responses in the serotonergic system as measured by concentrations of serotonin and its metabolites in rodents, although the findings are not consistent. Most of these studies found age-dependent changes in the concentrations of serotonin and its metabolites after stress exposure compared to those in young rodents (Algeri et al., 1982; Gilad et al., 1993; Lorens et al., 1990; Miura et al., 2002), but these findings show both increases and decreases in serotonergic responses to stressors in the aged. One reason for these differences is the methodological differences in stress exposure, such as the kind of stress, duration of exposure and brain regions from which the samples were taken. In the present study, we revealed that the effects of repeated restraint stress on the expression of TPH in neurons in the DRN differ between young and old male rats. We used vehicle-treated rats as control groups to omit vehicle-effects by dimethyl sulfoxide, although there is a possibility that repeated injections may affect differently in young and old rats or in males and females. In young male rats, repeated restraint stress increased the number of TPH-positive cells in all DRN subdivisions, whereas such stress-induced increases in TPH expression were not observed in old male rats, except in the ventral part of the DRN.

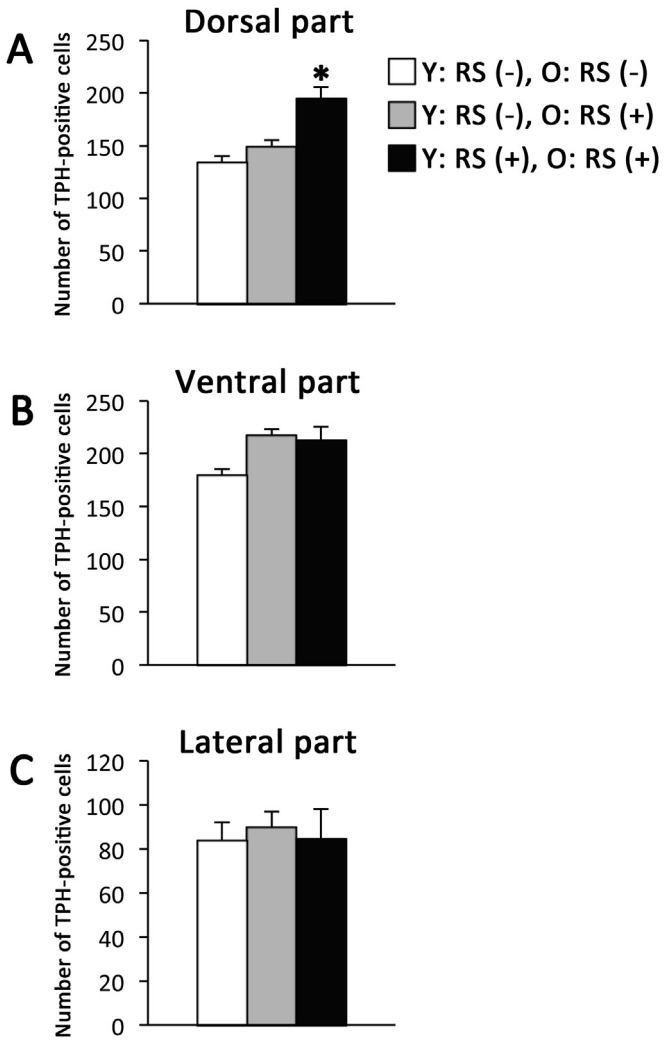

Fig. 5. Effects of stress experience in young adulthood on stress-induced TPH expression in old male rats. Quantification of the expression of tryptophan hydroxylase (TPH)-positive cells in the dorsal part (A), ventral part (B) and lateral part (C) of the dorsal raphe nucleus in old male rats. The white bars indicate vehicle-treated non-stressed [RS (−)] old male rats, the gray bars indicate vehicle-treated stressed [RS (+)] old male rats that were not exposed to restraint stress in young adulthood, and the black bars indicate vehicle-treated stressed [RS (+)] old male rats that were exposed to restraint stress in young adulthood. The data represent mean ± SEM. *Significantly different ($p < 0.05$) from vehicle-treated non-stressed rats. The data on vehicle-treated non-stressed (white bar) and vehicle-treated stressed (gray bar) male rats were cited from Fig. 3. O, old age; Y, young adulthood.

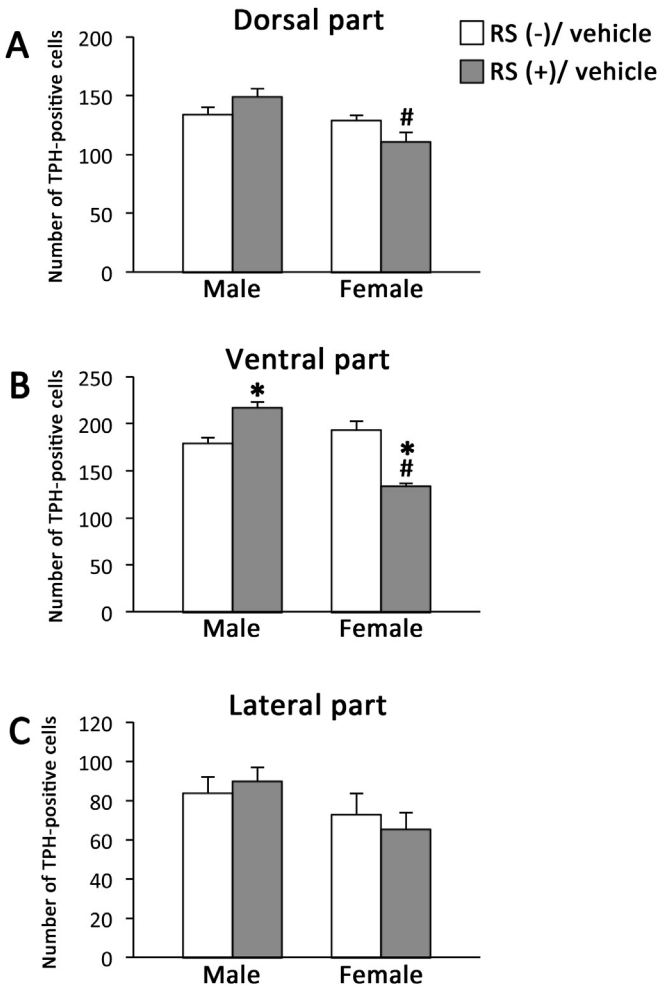

Fig. 6. Sex differences in stress-induced TPH expression in old rats. Quantification of tryptophan hydroxylase (TPH)-positive cells in the dorsal part (A), ventral part (B) and lateral part (C) of the dorsal raphe nucleus in male and female old male rats. The white bars indicate vehicle-treated non-stressed [RS (−)] rats, and the gray bars indicate vehicle-treated stressed [RS (+)] rats. The data represent mean ± SEM. *Significantly different ($p < 0.05$) from vehicle-treated non-stressed rats of the same sex; #significantly different ($p < 0.05$) from young rats of the same stress condition. The data on male rats (both stressed and non-stressed) were cited from Fig. 3.

These findings suggest that aging influences responses to repeated restraint stress in serotonergic neurons in the DRN of male rats. Our present data also showed that basal levels of TPH expression in all subdivisions differed between young and old male rats: the number of TPH-positive cells in non-stressed old males was larger than that in non-stressed young males. Suzuki et al. (2013) reported that the TPH expression level in the DRN in male middle-aged mice is similar to that in young adult males. Even there is a species difference, these might reflect an age-dependent change in TPH expression in the DRN occurring after middle age. Moreover, it has been reported that expression pattern of *tph2* mRNA in the DRN is dependent on daily fluctuations of glucocorticoids (Malek et al., 2007). The age-related increases in basal levels of TPH expression shown in our study may be, in part, due to attenuation of glucocorticoid negative feedback (Cizza et al., 1995a; Mizoguchi et al., 2009). Taken together, our data suggest the possibility that aging increased the basal level of TPH expression in the DRN in males, resulting in plateau effects in old males; therefore, stress exposure in old age failed to increase TPH expression in contrast to young adulthood.

The DRN contains topographically-organized subpopulations of serotonergic neurons and each subdivision has unique patterns of afferent/efferent pathways (Paul and Lowry, 2013), suggesting that respective subdivision are different functionally. The dorsal and ventral parts of the DRN have projections to stress- and anxiety-related regions such as the frontal cortex, the bed nucleus of the stria terminalis and the amygdala (Hale and Lowry, 2011; Muzerelle et al., 2016). Our result showing the age-dependent change in the effect of repeated restraint stress on TPH expression in the dorsal part raises a possibility that stress responses in brain regions mediated by serotonergic neurons in this subdivision may change with age. On the other hand, the lateral DRN subdivision contains a high density of GABAergic neurons (Day et al., 2004), and seems to be involved in not only sympathetic inhibition (Shikanai et al., 2012) but also inhibitory control to TPH expression in the ventral part of the DRN (Hale and Lowry, 2011). Our results demonstrated

that the stress-induced increase in TPH expression in the lateral part in young males disappeared with age, while the stress-induced increase in TPH expression was found in the ventral part, but not in the dorsal part, in old males. These differences might partly be due to age-dependent changes in an inhibitory control by the lateral part of the DRN.

Estrogen regulates the functions of the serotonergic system (Barth et al., 2015; Donner and Handa, 2009). In the DRN, it has been reported that ER-β mRNA and protein are more abundantly expressed compared to ER-α (Mitra et al., 2003; Shughrue et al., 1997). ER-β is localized primarily in serotonin-containing neurons in the DRN (Nomura et al., 2005; Vanderhorst et al., 2005). Under non-stressed conditions, ER-β is involved with TPH expression in the DRN and the level of serotonin content in several brain regions in young and middle-aged male mice (Donner and Handa, 2009; Imwalle et al., 2005; Nomura et al., 2005; Suzuki et al., 2013), and the administration of estrogen or ER-β agonist affects the expression of *tph2* mRNA and serotonin transporter mRNA in the DRN in rats (Donner and Handa, 2009; Hiroi et al., 2006; McQueen et al., 1999), indicating estrogenic effects via ER-β on the regulation of serotonergic system in the DRN. In this study, to reveal the role of endogenous estrogen via ER-β in serotonergic responses under stress condition in males change with age, we examined whether the ER-β blockade can affect TPH expression in the DRN under stress conditions in young and old male rats. Our results showed that the effect of ER-β blockade on TPH expression under stress condition intensifies with age in male rats. Our previous study showing the age-dependent change in ER-β mRNA expression in specific brain regions of male rats revealed that the expression level in the DRN did not change with age (Yamaguchi and Yuri, 2012). Therefore, the difference in the effect of ER-β blockade between young and old stressed males found in this study seems to be not due to age-dependent changes in ER-β mRNA expression. Instead, a possible reason for our result is a difference in the level of endogenous estrogen content in the DRN. Estrogen in males is mainly derived from testosterone by aromatization in the periphery and brain (Naftolin, 1994; Vermeulen et al., 2002), and the actions of testosterone to serotonin neurotransmission in the DRN seem to be mediated by its conversion by aromatase to estradiol in male adult rats (McQueen et al., 1999; Summer and Fink, 1998). Testosterone concentrations gradually decrease with age in males (Lamberts et al., 1997; Wu et al., 2009) and we also showed low testosterone level in old males compared to young males, raising the possibility of an age-dependent decrease in endogenous estrogen converted from testosterone in the DRN. The increased effect of ER-β blockade in old males in this study may be due to an age-dependent loss in endogenous estrogen and therefore a decrease in a competitive estrogenic effect in old males. Further studies are needed to reveal the age-related changes in the aromatase activity and the local level of estrogen in the DRN.

In the present study, we examined the effect of stress experience in young adulthood on the stress response in serotonergic neurons in old age. Our results showed that stress exposure in young adulthood significantly increased TPH expression only in the dorsal part of the DRN. As mentioned above, in this subdivision, stress exposure in only old age had no effect on the TPH expression in old male rats, although stress exposure in young adulthood seemed to elicit a stress response in serotonergic neurons in old age. The HPA axis in neonatal animals shows lower reactivity, and matures during puberty (Romeo, 2010). Adverse experiences in early life such as neonatal maternal separation cause long-lasting behavioral and psychological effects, and the effects of neonatal experience can remain detectable in the aged (Akers et al., 2008; Arborelius and Eklund, 2007). Neonatal and pubertal stress experiences have no small effect on the development and maturation of the HPA axis, and cause organizational effects resulting in changes in emotional function and the stress response (Cordero et al., 2013; Romeo, 2010, 2003). Our findings showed the long-term influence of stress experience in young adulthood on stress responses in old age. Taken together, stress experience not only in the neonatal and pubertal periods but also in young adulthood after completed pubertal maturation of the HPA axis can exert long-lasting effects on the serotonergic system related to the stress response and can change the stress response in old age. At the present time it is unclear whether the effect of stress exposure in young adulthood on stress response in serotonergic neurons in old age is due to a constitutive overexpression of TPH or sensitization to stress exposure caused by stress experience in young adulthood. Further studies are needed to clarify the long-lasting effect of stress experience in young adulthood on stress responses in old age.

It is well known that there is a sex difference in stress responses in young adulthood (Goel et al., 2014; Lund et al., 2006; Swaab et al., 2005). As for old age, it has been reported that intact male and female mice show similar responses in the HPA activity to predator-odor stressor (Harris and Saltzman, 2013), although it remains unknown whether other stress responses including serotonergic system in males are similar to those in females in old age. Therefore, in this study, we compared the effect of repeated restraint stress on TPH expression in the DRN of male and female old rats. Our results in old rats indicated that basal expression levels of TPH-positive neurons in all subdivisions did not differ between male and female non-stressed rats. Moreover, we demonstrated that repeated restraint stress had no effect on the number of TPH-positive cells in the dorsal and lateral parts of the DRN in both old males and females. Interestingly, in only the ventral part, restraint stress decreased TPH expression in old females, in stark contrast to old males, in which TPH expression was increased by stress exposure. Neurons in the ventral parts of the DRN innervate various brain regions related to the regulation of stress responses such as the central and basolateral subnuclei of the amygdala, bed nucleus of the stria terminalis, and locus coeruleus (Hale and Lowry, 2011; Muzerelle et al., 2016; Paul and Lowry, 2013; Peyron et al., 1998). The difference in responses of serotonergic neurons in this subdivision to stress exposure between sexes might contribute to a sex difference in stress responses in old age.

In conclusion, our findings suggest that the responses to repeated restraint stress in serotonergic neurons change with age in male rats. The responses seem to be regulated by ER-β in the specific subdivisions of the DRN in young adulthood, and regulation via ER-β also changes with age. In addition, the present data showed stress responses in serotonergic neurons in old males differ from those in old females.

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