Decreased Number of Vasopressin Immunoreactive Neurons in the Medial Amygdala and Locus Coeruleus of the Aged Rat

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Vasopressin	Immunocytochemistry	Aging	Cell number	Bed nucleus of the stria terminalis	Medial
amygdala	Locus coeruleus	Subcoeruleus			

IN addition to the classical neurosecretory vasopressin (VP) system, i.e. the supraoptic and paraventricular nucleus (SON and PVN, respectively), several other VP-containing cell groups and their projections in the brain of rat, monkey, and human have been described (3,4,13,27). The VP fiber density in several extrahypothalamic brain regions was strongly diminished in the senescent rat (e.g., in the medial amygdala (MA), locus coeruleus (LC), vertical limb of diagonal band, basal nucleus of Meynert, lateral habenular nucleus, substantia nigra, and lateral septum (12). The VP innervation of several of these regions appeared to be sexually dimorphic and depend on circulating levels of plasma testosterone. These sexually dimorphic VP systems comprise cells of the bed nucleus of the stria terminalis (BST) (20) and MA and their projection areas (9,27). The sexually dimorphic VP innervation of the senescent rat brain was in some extrahypothalamic regions restored by testosterone supplementation (14) showing the causality of the correlation between sex hormone levels and vasopressin innervation.

The present study investigated whether the cells of origin of the sexually dimorphic VP fibers also show age-related changes in vasopressin immunoreactivity related to decreasing plasma testosterone levels.

METHOD

Male Brown-Norway rats (BN/BiRij), 3, 19, and 33 ($n = 18$) months of age, were used (TNO/IVEG, Rijswijk, The Netherlands). After decapitation, the brains were immediately removed

from the skull and trunk blood was collected for measuring plasma testosterone levels by means of radioimmunoassay. The brains were cut into 3 mm thick transverse slices and, using a razor blade, each slice was further divided into left and right sections. One hemisphere of each animal, at random left or right, was used for the present study. The chosen hemisphere was subsequently immersed in Ringer medium for 6 h postmortem and processed for immunocytochemical (ICC) staining as described Van Zwieten et al. (30). Standard specificity controls (i.e., pre-immune serum), VP-adsorbed antivasopressin, and Brattleboro rat brain were included, but these showed no aspecificity. An additional step of pretreating the sections with methanol was introduced to prevent pseudoperoxidase staining of erythrocytes. This procedure has no negative effect on the final diaminobenzidine precipitation (25).

The VP cell number was determined by counting the number of VP-immunoreactive cell profiles in all sections through the brain regions of interest. The brain regions studied included the BST, MA, LC, and subcoeruleus (SubC).

Testosterone assays were performed as described by Ravid et al. (23). Since the previous study had shown that plasma levels of total and free testosterone are positively correlated ($\rho = 0.99$; $p < 0.001$), only total testosterone levels were determined in the present study.

STATISTICS

One-way analysis of variance (ANOVA) was applied for statistical evaluation of possible age-related differences in the cell

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number of each brain region. When a significant difference was found, the student Newman-Keuls Test was used to identify the two groups which were significantly different from each other. Variations in cell number per brain region between the right and left hemisphere were also tested by one-way ANOVA. Spearman's rank correlation was used to study the relation between vasopressin-immunoreactive cell number of the various brain regions and plasma testosterone levels. The level of significance was $p < 0.05$ for all tests.

RESULTS

As shown in Table 1, plasma testosterone levels were significantly decreased in the old and middle-aged group as compared to the young group. Furthermore, no significant differences in cell number were observed between the left and right hemispheres in any of the four brain regions tested.

A significant decrease in cell number was observed between the young and the old group, in the MA, and the LC which correlated with the decrease in plasma testosterone levels. No significant age-related change in cell number was found in the BST and SubC. VP cell number and plasma testosterone levels were not significantly correlated in these brain regions.

DISCUSSION

The present study showed that all previously described hypothalamic and extrahypothalamic VP immunoreactive cell bodies and fibers are made visible without pretreatment with colchicine. The post-mortem procedure used induces not only a substantial increase in VP content of the SCN (30) but can also be a successful alternative for colchicine treatment in experimental designs in which old rats are involved. In addition, it shows that in material in which a postmortem interval is inevitable (e.g., human material), this interval is not always detrimental and might even be advantageous when immunocytochemical stainings are performed.

A decrease in the number of VP immunoreactive cells was found in the MA of the old rats which is in agreement with the

earlier observed decrease in their site of termination (i.e., in the lateral septum and hippocampus) (3,12). The MA is involved in male rat mating behavior (18) and the disappearance of VP immunoreactivity in the projection regions of the MA following gonadectomy (9) is concomitant with the reduction in sexual behavior of the gonadectomized rat (8). Our finding that there is a significant correlation between decreasing plasma testosterone levels and the number of VP-expressing cells in the MA suggests that the decrease in testosterone levels, VP-expression in neurons in the MA, and fibers in their projection sites is directly related to the changes in sexual behavior. Supplementation of testosterone restores VP innervation in affected regions (14). However, because several studies have shown a lack of effect of testosterone supplementation on the decline in sexual behavior of the aging male rat (5,16,17), VP expression of MA neurons cannot be the only factor determining the changes in sexual behavior during aging.

A recent report, using micropunches of the MA followed by RIA, revealed no change in the VP concentration in the MA during aging (15) but did show a significant correlation between VP neurons in the MA and plasma testosterone levels. The seeming discrepancy with the present study regarding the VP content may be due to the higher spatial resolution of immunocytochemistry as compared to radioimmunoassay.

The present observation of decreasing numbers of VP immunoreactive cells in the LC during aging is in line with observations by Dorsa and Bottemiller (11). Using radioimmunoassay these authors showed a decrease of VP content in the LC of 26-month-old Long-Evans rats. In addition, the rat LC shows an age-related decline in total cell number (26) and the number of melanin-containing noradrenergic neurons in the human LC decreases by 30% in aging (7). Caffé and co-workers (2) showed the co-existence of noradrenaline (NA) and VP in LC neurons and using antibodies against dopamine-beta-hydroxylase (DBH), Luque and co-workers showed a sexual dimorphism of DBH-stained neurons in the rat LC (19). Because the LC provides efferents to almost every brain region, changes at the cellular level in the VP system of this nucleus may affect processes it is involved in (e.g., level of

TABLE 1
PLASMA TESTOSTERONE LEVELS AND VASOPRESSIN CELL NUMBERS IN FOUR BRAIN REGIONS OF YOUNG, MIDDLE-AGED, AND OLD BROWN-NORWAY RATS

Group	Age (months)	Cell Numbers Per Brain Region								Testosterone (pg/ml)
		BST	(n)	MA	(n)	LC	(n)	SubC	(n)	
Young	3	77.8 + 8.6	5	319.3 + 33.2	6	630.4 + 67.7	5	210.2 + 39.4	5	21.3 + 4.6
Middle-aged	19	71.2 + 32.8	6	274.4 + 28.4	5	492.8 + 91.8	5	143.8 + 67.0	5	4.6 + 0.8
Old	33	59.5 + 21.6	6	184.4 + 70.4	5	341.2 + 33.1	5	133.6 + 39.4	5	2.8 + 0.3
Statistics										
Left/right difference		$p = 0.665$		$p = 0.229$		$p = 0.079$		$p = 0.142$		
Correlation with testosterone		$\rho = 0.31$		$\rho = 0.80$		$\rho = 0.63$		$\rho = 0.55$		
Age-related differences		$p = 0.247$		$p < 0.001^*$		$p < 0.002^*$		$p = 0.051$		
		$p = 0.494$		$p < 0.028^*$		$p < 0.036^*$		$p = 0.309$		
				Y <> O		Y <> O				

Data are given as Means $\pm$ SEM; n represents number of animals.

BST: bed nucleus of the stria terminalis; MA: medial amygdala; LC: locus coeruleus; SubC: subcoeruleus.

* statistical significant difference ($p < 0.05$).

Y <> O: young compared to old.

FIG. 1. Vasopressin immunoreactive cell bodies (arrows) in the bed nucleus of the stria terminalis (A), the medial amygdala (B), the locus coeruleus (C), and subcoeruleus (C: short arrows) of a young rat, in vibratome sections (50 μ m) stained with antivasopressin (Truus 18-9-85, 1:1000). Bar represents 500 μ m.

vigilance, emotion, memory retrieval, and sleep-wake cycles (24). Furthermore, in several neurodegenerative diseases, the LC is severely compromised, e.g., in Alzheimer's disease a neuron loss of up to 87% can be detected, while in Parkinson's disease this neuron loss can reach 94% (7). Degeneration of this nucleus may, in addition, cause depression in neurodegenerative diseases (6,7). Our results provide further evidence for age-related changes in a specific neurotransmitter system in the LC of the senescent rat brain.

Sexually dimorphic extra-hypothalamic brain regions known to receive VP fibers originating from the BST, i.e. lateral septum, lateral habenula show a decrease in VP fiber density during aging (12), whereas VP fiber density in these regions is significantly increased following testosterone supplementation (14).

Considering the fact that VP neurons in the BST of rat brain possess receptors for gonadal hormones (1) and that a substantial age-related decrease in testosterone was found, our observation that there is no significant decrease in VP-positive cell number in the BST is remarkable. Recently, in a study Dobie and co-workers (10) showed an age-related decline in VP-mRNA in the BST of Fisher 344 rats. Not only the number of labeled cells had decreased but also the number of grains per cell (viz. the amount of mRNA). Testosterone supplementation could reverse this age-related decline in VP gene expression in the BST of castrated 24-month-old Fisher 344 male rats (10). An explanation for the apparent discrepancy may be the strain difference or that both immunocytochemistry and in situ hybridization only show the

presence of a compound without providing information on its turnover.

A general point of consideration in this study is that cell numbers as reported in the present and in similar studies (29) have to be regarded as relative and not absolute numbers. In vibratome sections, VP cells under the surface of the section are optimally stained, whereas cells in the center of the sections are not reached by the antibodies (21).

This report shows that age-related changes in VP innervation regions coincide with age-related changes in the VP expression of the neurons of origin (i.e., the MA and LC but not in the BST). Furthermore, the age-related change in the VP neurons of certain brain regions is related to the decreased plasma testosterone levels in a similar way as found earlier for vasopressin terminals. Whether these parameters are indeed causally related should be studied by testosterone substitution experiments (14).

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