Activation of Vasopressinergic and Oxytocinergic Neurons During Aging in the Wistar Rat

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FLIERS, E. AND D. F. SWAAB. *Activation of vasopressinergic and oxytocinergic neurons during aging in the Wistar rat. PEPTIDES 4(2) 165-170, 1983.*—The activity of the hypothalamo-neurohypophyseal system (HNS) was determined in male Wistar rats from 3 to 32 months of age. Plasma levels of vasopressin (AVP) and oxytocin (OXT) were measured by means of a radioimmunoassay. In addition, the distribution of the Golgi apparatus marker enzyme thiamine-pyrophosphatase (TPP-ase) was measured as a parameter for neurosecretory activity in the hypothalamic supraoptic and paraventricular nuclei (SON and PVN). Plasma levels of radioimmunoassayable AVP were increased in the 32-month-old animals. Plasma levels of radioimmunoassayable OXT in 32-month-old animals did not differ from the levels found in the youngest group, but were higher than in 11-month-old animals. Neurosecretory activity in the SON was similar in 3- and 32-month-old animals, whereas in the PVN neurosecretory activity was increased in the 32-month-old animals. Urine excretion decreased between 6 and 11 months of age and remained on the same level until 32 months of age. In other words, instead of a loss of HNS function as has been suggested in the literature, an increased neurosecretory activity was observed in aged rats.

Aging	Vasopressin	Oxytocin	Neurosecretory activity	Supraoptic nucleus
Paraventricular nucleus		Thiamine pyrophosphatase	Golgi apparatus	

AGING of the central nervous system is generally considered to be accompanied by both degenerative changes and cell loss, resulting in a shrinkage of the brain [26]. The changes are, however, not found to the same extent throughout the brain, but are dependent on the area and the cell type studied [14,20].

The function of the hypothalamo-neurohypophyseal system (HNS) has been suggested to diminish during the course of aging. Human plasma levels of neurophysin decrease during aging [17], and vasopressin was claimed to improve performance on memory tests in 50-65 year-old men [16]. Furthermore, oxytocin (OXT) injections resulted in a prolongation of lifespan in rats [4].

A study of changes during aging in the "neurosecretory neurons" of the HNS has some unique advantages as compared with studying "conventional" neurons. Vasopressinergic and oxytocinergic neurons project via the hypothalamo-neurohypophyseal tract to the neural lobe of the pituitary, where they release vasopressin (AVP) and oxytocin (OXT) into the bloodstream [2]. The activity of these peptidergic neurons can be followed by assaying vasopressin and oxytocin in the peripheral circulation. In addition, vasopressin and oxytocin neurons project into a large number of brain areas [6, 7, 25]. In such regions they terminate by means of synapses that cannot be distinguished morphologically from conventional aminergic or aminoacid containing synapses [8].

The older literature unanimously states that the changes during aging in the rat HNS indicate a decrease in neurosec-

retory activity. The amount of neurosecretory material was reported to be reduced in the posterior lobe of rats older than 24 months, as revealed by Gomori staining [24]. Urinary excretion of vasopressin and plasma levels of this peptide were claimed to decline with advancing age in the rat, while the turnover of water would increase [31]. Recently, decreased amounts of vasopressin were observed in the hypothalamic and extrahypothalamic areas of old rats [11], while an impaired dehydration-induced response of the HNS in old rats was reported [28]. In addition, AVP content in the hypothalamus and plasma levels of AVP were reported to be decreased in aged Sprague-Dawley rats [33]. Also some of the behavioral deficits in old rats have been attributed to age-dependent changes in the secretion of AVP [9]. Furthermore, recent immunocytochemical studies revealed age-related changes in the neurosecretory cells, although the direction of the change in activity was not clear. A decreased staining intensity of magnocellular neurons was reported, especially in neurons containing oxytocin, the paraventricular nucleus (PVN) being more severely affected than the supraoptic nucleus (SON) [32]. Other investigators, however, reported an increase in the number of neurons that stained darkly for neurophysin in the SON, while the PVN remained unaltered [19].

The aim of the present study was to reinvestigate the suggested decline in basal functional activity of the rat HNS during aging, using a combination of techniques. Plasma levels of AVP and OXT were measured by means of radioimmunoassay (RIA). As a measure for neurosecretory

FIG. 1. Body weight as a function of age. Bars represent the mean $\pm$ standard error of the mean (SEM) for each age group (n represents the number of animals in each group). Note the decline in weight after 11 months of age. *Significantly ($p < 0.05$) higher than 3 month group, **significantly ($p < 0.05$) higher than any other group.

activity the distribution of the Golgi apparatus was determined in the SON and PVN. This organelle is involved in packaging neurosecretory granules and can be labelled by the enzyme thiamine-pyrophosphatase (TPP-ase). The distribution of this marker increases with enhancement of neurosecretory activity, e.g., during water deprivation, pregnancy, parturition, lactation, castration and during the oestrous [15,29].

METHOD

Animals

Adult male Wistar rats (CPB:WU, 5 groups of different ages, $n=98$) were obtained from TNO-CPB (Zeist, The Netherlands). The youngest (3-month-old) animals were delivered directly from TNO-CPB, whereas the older animals (6–32-month-old) were kindly donated by Dr. H. Rigter (Organon, Oss, The Netherlands), who had used them as untreated controls in short-lasting behavioral experiments. They were housed in groups of no more than 5, under constant temperature and humidity, and received tap water and food (Hope Farms, Woerden, The Netherlands) ad lib. Lights were on between 7 a.m. and 7 p.m.

General Procedures

The 24-hr urine excretion was measured in metabolism cages, during which period the animals received tap water ad lib, but no food. This was because only the old animals often spoiled large amounts of food in the cages, which disturbed

FIG. 2. Brain wet weight as a function of age. Bars represent the mean $\pm$ SEM for each age group (n represents the number of animals in each group). Note the gradual increase in weight until 11 months of age. *Significantly ($p < 0.05$) higher than 3 month group; **significantly ($p < 0.05$) higher than 3 and 6 month group.

the urine measurements. The animals were weighed and decapitated between 09:00 and 11:30 a.m., after which the brains were dissected immediately and weighed. One animal which, at 26 months of age, was found to have a pituitary tumor, was excluded from further study. Blood was collected from the trunk in chilled and heparinized plastic tubes and immediately processed for RIA. All assays were set up in duplicate. Details on the extraction procedure and RIA for AVP and OXT have been described elsewhere [10]. The AVP and OXT-RIA had a negligible cross-reaction of 0.01% with OXT and AVP, respectively. The inter-assay coefficients of variation of extracted samples in our assay procedure were 13.9% for AVP and 8.6% for OXT. The intra-assay coefficients of variation were 15.7% and 10.2%, respectively. The lower limit of detection was 1.5 pg/ml for AVP and 11.1 pg/ml for OXT. If the values were below the limit of the assay, the detection limit was used as an actual value.

Histochemistry

Animals used for this part of the study were selected at random. Hypothalamic areas were excised, fixed in 4% glyoxal in cacodylate buffered sucrose (4°C) for 24 hr, and washed in cacodylate buffered sucrose. After a washing period of 2×24 hr with constant agitation the tissue was frozen in liquid Freon-12 at -80°C . Cryostat sections (16 μm) of the SON and the PVN were used for the staining of the Golgi apparatus marker enzyme TPP-ase, which was quantified as described before [15,29]. In brief, the distribution of TPP-ase deposits in the SON and the posterior magnocellular part (cf., [30]) of the PVN was determined by means of a Zeiss Hennig ocular, combined with an oil immersion (100 $\times$) objective. The distribution of the enzyme deposits was expressed as the percentage of the 100 points of the Hennig

FIG. 3. Urine excretion as a function of age. Bars represent the mean $\pm$ SEM for each age group (n represents the number of animals in each group). Note the decrease between 6 and 11 months of age. *Significantly ($p < 0.05$) lower than 3 and 6 month group.

ocular hitting the Golgi apparatus in each SON or PVN section. In each SON 16 sections were counted and in each PVN 8 sections were counted per animal.

Statistics

Plasma AVP and OXT levels. Since the observed distribution of values of radioimmunoassayable AVP and OXT showed too much deviation from a normal distribution, non-parametric Kruskal-Wallis one-way analysis of variance (including correction for ties) was used to assess significance of differences. If p -values were less than 0.05, individual groups were compared using the Mann-Whitney U test (0.05 level of significance).

Weight, urine excretion, neurosecretory activity. The distribution of individual values over the various age groups was tested by means of analysis of variance. If the p -values were below 0.05, the Newman-Keuls method was used to test differences between pairs of means (0.05 level of significance).

RESULTS

Body Weight, Brain Weight and Urine Excretion

Body weight increased significantly ($p < 0.05$) from 3 months up to 11 months, after which a decrease was observed until 32 months of age (Fig. 1). Brain weight continuously increased ($p < 0.05$) up to 11 months, whereas differences between 11, 26 and 32-month-old animals were not significant (see Fig. 2). Urine excretion (total 24 hr volume $\pm$ standard error of the mean (SEM) decreased from 28.4 ± 2.4 ml at 3 months to 17.7 ± 2.3 ml at 32 months, the

FIG. 4. Plasma levels of radioimmunoassayable vasopressin (AVP) and oxytocin (OXT) in 3 different age groups. Bars indicate the median, n represents the number of animals in each group. Note the increased AVP levels in the oldest age group. OXT levels are lowest in the 11 month-old animals. *Significantly higher than 3 and 11 month group ($p < 0.03$ and $p < 0.02$, respectively; **significantly lower than 3 and 32 month group ($p < 0.02$ and $p < 0.01$, respectively).

differences between successive age groups being only significant ($p < 0.05$) between 6 and 11 months (Fig. 3).

Plasma Levels of AVP and OXT

At 32 months of age, the levels of radioimmunoassayable AVP (range: 1.5–59.5 pg/ml) were significantly higher than those at 3 ($p < 0.03$) and 11 ($p < 0.02$) months (range: 1.5–41.7 pg/ml and 1.5–13.3 pg/ml, respectively), while the latter groups did not differ from each other (Fig. 4). Also the levels of radioimmunoassayable OXT were highest in the oldest age group (range: 11.1–108.8 pg/ml), while the lowest values were measured in the 11-month group (range: 11.1–31.6 pg/ml). The values for 3 months (range: 11.1–100.9 pg/ml) and for 32 months did not differ significantly from one another, but the 32-month value was significantly ($p < 0.01$) higher than the 11-month value.

Neurosecretory Activity

Five age groups were studied for the determination of the distribution of TPP-ase. Lead sulphide deposits could be discerned as brown, perinuclear bands in the PVN and SON neurons of all animals. The TPP-ase staining of the Golgi apparatus could easily be distinguished from the staining in nucleoli and blood vessels which was not counted. The SON neurons in the various age groups did not show any striking

FIG. 5. Thiamine pyrophosphatase staining of the Golgi apparatus in the paraventricular nucleus. n: nucleolus; b: blood vessel. $\times 900$ (a) at 3 months of age; (b) at 32 months of age. Note the increase in the distribution of the Golgi apparatus marker enzyme and in staining intensity in the older age group.

difference in the size of the Golgi apparatus. In the PVN of most 32-month-old animals, however, the staining was more intense and mostly covered all of the perikaryon, in contrast to the perinuclear localization observed in the younger age-groups (Fig. 5).

Quantitative determination of the distribution of the enzyme deposits (Fig. 6) in the PVN revealed a non-significant trend to decline up to 26 months of age. In the PVN the percentage of positive hits in 32-month-old animals was significantly ($p < 0.05$) higher than in all the younger age groups, which did not differ significantly from each other. Regarding the SON, 32-month-old animals did not differ significantly from 3- and 6-month old animals. However, the mean percentage of hits in 11- and 26-month-old animals was significantly lower than in 32-month-old animals ($p < 0.05$).

DISCUSSION

The observations concerning the decreased human plasma neurophysin levels between 51 and 60 years of age [17] and the various animal experimental observations suggesting degenerative changes of the rat HNS during aging (see introductory paragraphs) indicated that the rat might be

a useful animal model to study the mechanisms underlying the hypothesized decline of HNS function in man. However, up till now most observations on the HNS itself have been based upon methods that do not allow any conclusions to be drawn about the nature of the changes during aging (i.e., inhibition vs. activation). Therefore we determined changes in the distribution of the Golgi apparatus, which has been proved to be a measure for neurosecretory activity. The observation of an inverse relationship between the quantity of intraneuronal lipofuscin accumulation and the size of the Golgi apparatus in the aged rhesus monkey [1] made this parameter, in addition, of special interest to gerontology.

The fact that the cells of the PVN appeared to be activated at 32 months of age was a surprise in view of the earlier reports in the literature, suggesting decreased neurosecretory activity. Although there is no neuron loss in the SON and PVN of senescent male Sprague-Dawley rats [21], data on cell numbers in the SON and PVN of aged Wistar rats do not exist. However, the activation of the HNS is not to be considered a compensatory mechanism for cell loss, in view of the enhanced plasma levels of AVP in the 32 month old animals.

In the present study the changes in intracellular distribution of the Golgi apparatus in the SON and PVN were related

FIG. 6A. Percentage of thiamine pyrophosphatase-positive hits in the supraoptic nucleus of rats from 3 to 32 months of age. Bars indicate the mean for each age group, vertical lines indicate the SEM (n represents the number of animals in each group). *Significantly ($p < 0.05$) higher than 11 and 26 month group.

FIG. 6B. Percentage of thiamine pyrophosphatase-positive hits in the paraventricular nucleus of rats from 3 to 32 months of age. Bars indicate the mean for each age group, vertical lines indicate the SEM (n represents the number of animals in each group). The oldest age group shows an increase in the distribution of the Golgi apparatus enzyme. *Significantly ($p < 0.05$) higher than any other age group.

to changes in plasma levels of radioimmunoassayable AVP and OXT. The increased neurosecretory activity in the PVN at 32 months proved to be concomitant with a clearcut increase in the plasma level of radioimmunoassayable AVP. Data appearing during the preparation of this manuscript, showing a rise in the plasma level of vasopressin during aging, both in humans and the rat [12], support the finding of an activation of the HNS during aging. However, the particular strain of rats was not mentioned. These findings contrast with earlier bioassay data of other investigators [31], who reported a fall in the plasma level of AVP in 23–30 month-old male Wistar rats. In male Fischer 344 rats, basal plasma levels of AVP were reported to be similar in 3- and 30-month-old animals [28]. Some of the discrepancies may be explained by strain differences and by the fact that no generally accepted definition of "the aged rat" exists in the literature. If the 50% survival age is used as a definition, 30 months of age should be considered as "old" for most laboratory rat strains [5]. However, since the rate of aging is highly dependent on dietary and other environmental factors [18], even 30-month-old rats of the same strain are not necessarily comparable with respect to age-related variables. Our "aged" animals had been fed ad lib and had been housed in groups of approximately 5 animals, which may be considered to be "environmental enrichment" in comparison with the (standard) single housed condition (e.g. [13]). The differences in AVP plasma levels between our 11- and 32 month-old animals cannot be explained by differences in environmental factors, since all animals older than 3 months had been obtained from Organon (Oss) and had been exposed to the same conditions.

It is not clear how the enhanced HNS activity is caused. It

might be secondary to changes in the target organ for vasopressin, the kidney. Impairment of the urinary concentrating ability of the kidney and a decrease in the vasopressin dependent cAMP generation has been reported in 24-month-old Fisher 344 rats [3]. We were unable to confirm earlier statements on the occurrence of diabetes insipidus in the old rat [31], as we found no differences in urine excretion between 11 and 32 months of age. Still, our animals may have developed a decreased kidney sensitivity to vasopressin, maintaining the urinary excretion at the same level as the 11-month old animals by higher AVP levels. However, it is unexpected in this condition that the PVN shows the most pronounced activation. In young animals, osmotic stress results in increased activity in the SON as well as in the PVN, the SON reacting earlier and stronger than the PVN [15]. A possible explanation for the increased neurosecretory activity mainly in the PVN may be differential age-related alterations in the pattern of afferent innervation of the SON and the PVN. An age-related decline in the pattern of noradrenergic innervation of magnocellular elements in the SON of the rat was reported [27]. The PVN, however, was not taken into consideration.

The observed activation of the PVN in the aged rat may have consequences for central functions in which AVP and OXT are supposed to be involved, as the central and peripheral release of neurohypophyseal hormones seem to be coupled under various conditions [22]. In a preliminary experiment Tj. B. Van Wimersma Greidanus found in a single sample of pooled cerebrospinal fluid (CSF) of 3 aged rats (24 months of age) an AVP level of approximately 65 pg/ml, which is about three times higher than the level found in 3-month-old rats (personal communication). In this context it

is of interest to note that aged animals of the same strain showed remarkably few deficits on memory tests [23].

Future research will be directed towards the question if there are any consequences of the observed activation of the PVN for the extrahypothalamic peptidergic pathways. Furthermore, we shall focus on the human HNS in order to establish whether the changes related to aging in the human and rat HNS are paralleled.

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REFERENCES

- Barden, H. Relationship of Golgi thiaminepyrophosphatase and lysosomal acid phosphatase to neuromelanin and lipofuscin in cerebral neurons of the aging Rhesus monkey. *J Neuropathol Exp Neurol* 29: 225-240, 1970.
- Bargmann, W. Über die neurosekretorische Verknüpfung von Hypothalamus und Neurohypophyse. *Z Zellforsch Mikrosk Anat* 34: 610-634, 1949.
- Beck, N. and B. P. Yu. Effect of aging on urinary concentrating mechanism and vasopressin-dependent cAMP in rats. *Am J Physiol* 243: (Renal Fluid Electrolyte Physiol 12), F121-F125, 1982.
- Bodanszky, M. and S. L. Engel. Oxytocin and the life-span of male rats. *Nature* 210: 751, 1966.
- Burek, J. D. and C. F. Hollander. Experimental gerontology. In: *The Laboratory Rat vol 2, Research Applications*, edited by H. J. Baker, J. R. Lindsay and S. H. Weisbroth. New York: Academic Press, 1980, pp. 149-159.
- Buijs, R. M., D. F. Swaab, J. Dogterom and F. W. Van Leeuwen. Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. *Cell Tissue Res* 186: 423-433, 1978.
- Buijs, R. M. Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. Pathways to the limbic system, medulla oblongata and spinal cord. *Cell Tissue Res* 192: 423-435, 1978.
- Buijs, R. M. and D. F. Swaab. Immunoelectronmicroscopical demonstration of vasopressin and oxytocin synapses in the rat limbic system. *Cell Tissue Res* 204: 355-365, 1979.
- Cooper, R. L., M. C. McNamara and W. G. Thompson. Vasopressin and conditioned flavor aversion in aged rats. *Neurobiol Aging* 1: 53-57, 1980.
- Dogterom, J., Tj. B. Van Wimersma Greidanus and D. F. Swaab. Evidence for the release of vasopressin and oxytocin into cerebrospinal fluid: measurements in plasma and CSF of intact and hypophysectomized rats. *Neuroendocrinology* 24: 108-118, 1977.
- Dorsa, D. M. and L. Bottemiller. Age-related changes of vasopressin content of microdissected areas of the rat brain. *Brain Res* 242: 151-156, 1982.
- Frolkis, V. V., S. F. Golovchenko, V. I. Medved and R. A. Frolkis. Vasopressin and cardiovascular system in aging. *Gerontology* 28: 290-302, 1982.
- Greer, E. R., M. C. Diamond and J. M. W. Tang. Effects of age and enrichment on certain brain dimensions in Brattleboro rats deficient in vasopressin. *Exp Neurol* 75: 11-22, 1982.
- Hsu, H. K. and M. T. Peng. Hypothalamic neuron number of old female rats. *Gerontology* 24: 434-440, 1978.
- Jongkind, J. F. and D. F. Swaab. The distribution of thiamine diphosphatephosphohydrolase in the neurosecretory nuclei of the rat following osmotic stress. *Histochemistry* 11: 319-324, 1967.
- Legros, J. J., P. Gilot, X. Seron, J. Claessens, A. Adam, J. M. Moeglen, A. Audibert and P. Berchier. Influence of vasopressin on learning and memory. *Lancet* 1: 41-42, 1978.
- Legros, J. J. The neurohypophyseal peptides: biosynthesis, biological role and prospects of use in neuropsychiatric therapy. *Triangle* 18: 17-30, 1979.
- Levin, P., J. K. Janda, J. A. Joseph, D. K. Ingram and G. S. Roth. Dietary restriction retards the age-associated loss of rat striatal dopaminergic receptors. *Science* 214: 561-562, 1981.
- McNeill, T. H., C. J. Clayton and J. R. Sladek. Immunocytochemical characteristics of hypothalamic magnocellular neurons in the aged rat. Abstract No. 47, Proc. 31st Annual Meeting of the Histochemical Society. New Orleans, 1980.
- Peng, M. T. and L. R. Lee. Regional differences of neuron loss of rat brain in old age. *Gerontology* 25: 205-211, 1979.
- Peng, M. T. and H. K. Hsu. No neuron loss from hypothalamic nuclei of old male rats. *Gerontology* 28: 19-22, 1982.
- Pittman, G. J., T. J. Malkinson, W. L. Veale and K. Lederis. Central release of arginine vasopressin (AVP) in rabbit brain. *Soc Neurosci Abstr* 7: 162.10, 1981.
- Rigter, H., J. L. Martinez and J. C. Crabbe. Forgetting and other behavioral manifestations of aging. In: *The Psychobiology of Aging: Problems and Perspectives*, edited by D. Stein. New York: Elsevier North Holland, 1980, pp. 161-175.
- Rodeck, H., K. Lederis and H. Heller. The hypothalamo-neurohypophyseal system in old rats. *J Endocrinol* 21: 225-228, 1960.
- Rossor, M. N., L. L. Iversen, C. Q. Mountjoy, M. Roth, J. Hawthorn, V. Y. Ang and J. S. Jenkins. Arginine vasopressin and choline acetyltransferase in brains of patients with Alzheimer type senile dementia. *Lancet* 9: 1367-1368, 1980.
- Sabel, B. A. and D. G. Stein. Extensive loss of subcortical neurons in the aging rat brain. *Exp Neurol* 73: 507-516, 1981.
- Sladek, J. R., H. Khachaturian, G. E. Hoffman and J. Scholer. Aging of central endocrine neurons and their aminergic afferents. *Peptides* 1: Suppl 1, 141-157, 1980.
- Sladek, C. D., T. H. McNeill, C. M. Gregg, M. L. Blair and R. B. Raggs. Vasopressin and renin response to dehydration in aged rats. *Neurobiol Aging* 2: 293-302, 1981.
- Swaab, D. F. and J. F. Jongkind. The hypothalamic neurosecretory activity during the oestrous cycle, pregnancy, parturition, lactation and persistent oestrus and after gonadectomy in the rat. *Neuroendocrinology* 6: 133-145, 1970.
- Swanson, L. W., P. E. Sawchenko, A. Berot, B. K. Hartman, K. B. Helle and D. E. Vanorden. An immunohistochemical study of the organization of catecholaminergic cells and terminal fields in the paraventricular and supraoptic nuclei of the hypothalamus. *J Comp Neurol* 196: 271-285, 1982.
- Turkington, M. R. and A. V. Everitt. The neurohypophysis and aging with special reference to the antidiuretic hormone. In: *Hypothalamus, Pituitary and Aging*, edited by A. V. Everitt and J. A. Burgess. Springfield, IL: C. C. Thomas, 1976, pp. 123-136.
- Watkins, W. B. and V. J. Choy. The impact of aging on neuronal morphology in the rat hypothalamo-neurohypophyseal system: An immunohistochemical study. *Peptides* 1: Suppl 1, 239-245, 1980.
- Zbuzek, V. K. and W. Wu. Age-related vasopressin changes in rat plasma and the hypothalamo-neurohypophyseal system. *Exp Gerontol* 17: 133-138, 1982.