Unchanged Amounts of Vasopressin mRNA in the Supraoptic and Paraventricular Nucleus during Aging and in Alzheimer’s Disease

P. J. Lucassen, J. J. Van Heerikhuize, S. E. F. Guldenaar, C. W. Pool, M. A. Hofman and D. F. Swaab
Graduate School Neurosciences Amsterdam, Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam, The Netherlands.

Key words: In situ hybridization, vasopressin, aging, Alzheimer’s disease, supraoptic nucleus, paraventricular nucleus.

Abstract

The paraventricular (PVN) and supraoptic nucleus (SON) demonstrate a striking stability with respect to cell numbers during aging and Alzheimer’s disease (AD). Vasopressin (AVP) neurons even become activated during aging as judged from several parameters for neuronal activity, such as increased AVP plasma levels, enlarged nucleolus as well as cell size and an increased size of the Golgi apparatus in AVP-neurons. The activation possibly occurs as compensation for an age-related loss of AVP-receptors in the kidney. As a specific marker for AVP synthesis, we used quantitative in situ hybridization and estimated total amounts of AVP-mRNA in the entire SON and PVN of 14 control subjects and 14 AD patients that were matched for age, fixation time, postmortem delay and storage time of the tissue in paraffin. Following quantification, no differences were observed in total amounts of AVP-mRNA in the SON or PVN between young and old controls or between young and old AD patients, nor between the entire group of controls and AD patients. A significant negative correlation was found between the volume of the AVP-mRNA signal in the AD SON and age while the total amount of mRNA remained the same. This suggests a redistribution of cells or cell compartments in aging. A significant positive relation in both SON and PVN of AD patients was found between storage time of the paraffin-embedded tissue and the total amount of AVP-mRNA. A significant positive relation was present in the PVN, but not SON between pH of the cerebrospinal fluid, which is a marker for agonal state and the total amount of AVP mRNA. The present unchanged AVP-mRNA levels in SON and PVN confirm earlier observations on the stability of cell numbers in these nuclei in aging and AD. Although on the basis of other parameters, AVP-mRNA upregulation was expected, gradual, chronic stimulation over prolonged periods of time may, possibly, induce alternative mechanisms of regulation such as changes in translatability or in mRNA stability.

Neurosecretory neurons of the hypothalamic paraventricular (PVN) and supraoptic nucleus (SON) transcribe, translate and process vasopressin (AVP) and oxytocin (OXT) from high molecular weight precursor molecules (1, 2). One of the triggers inducing an upregulation of AVP gene expression in the rat is osmotic stress (3, 4) after which biologically active AVP is released from the posterior pituitary into the circulation, where it plays a role in water retention and vasconstriction (4, 5). In addition, AVP-neurons project to extrahypothalamic sites in the brain and spinal cord and are involved in central functions (6–9). Oxytocin (OXT) neurons are involved in lactation, parturition, eating behavior (10) and sexual behavior and send also projections to the neurohypophysis and several brain stem nuclei (11, 12). In contrast to the situation in rat, a clear subdivision in parvo- and magnocellular AVP or OXT neurons and a distribution of these two cell types in distinct subnuclei of the PVN is absent in human (10).

Earlier studies on human SON and PVN demonstrated a striking stability of these nuclei with aging and in Alzheimer’s disease (AD). No decreases have been observed in total cell numbers or in the AVP and OXT cell numbers (13–16), except for one single report (17). Activation of AVP neurons during aging has become apparent from several parameters, such as enhanced AVP plasma levels in elderly subjects, increases in AVP neuronal size, nucleolar size, Golgi apparatus size and an increase in the number of AVP-expressing neurons in the human PVN with normal aging (13, 15, 18–27), although others did not find increased basal AVP plasma levels in older subjects (28, 29). In AD, some of these age-related activity changes appear less pronounced or absent (15, 24).

In rats, marked strain differences in aging and age-related renal pathology are present, yielding inconsistency in the literature on AVP elevations in relation to age (30–34, 59). However, when discarding rat strains that were either not old enough or displayed pronounced nephropathology (33, 34, 37), AVP-neurons of the hypothalamo-neurohypophyseal system (HNS) were found to be metabolically highly active and to become extra activated with aging, possibly as the result of an age-related loss of AVP-receptors in the kidney (35–38). The combination of high neuronal metabolic activity and stability of cell numbers during aging
has been found also in other neuronal systems, and has led to
the concept that activated neurons may have a better chance to
survive the aging or Alzheimer process, paraphrased as ‘use it or
lose it’ (39).

So far, the parameters for increased neuronal activity in AVP
neurons were only based upon general measures for metabolic
activity. Therefore, we set out to ascertain whether AVP gene
expression increases in the SON and PVN during aging or in
AD. To that end, we estimated total amounts of AVP-mRNA in
these nuclei of control subjects and AD patients of different ages
using previously developed quantitative in situ hybridization
(ISH) procedures (40).

Results
Labeled AVP-probe was detected as a strong signal on film of
which the distribution was in accordance with the location of the
SON and PVN in thionine-stained sections (Fig. 1). The system-
atric samples taken throughout the SON and PVN resulted in
complete profiles since no signal was present in the first or last
sampled section of a nucleus. Comparison of the total amounts
of AVP-mRNA, or volume, as a measure for AVP-mRNA based
on the hybridization signal on film, only revealed a significant
difference between the young and old age group in volume of the
SON (P = 0.03). In the control or AD group in either nucleus,
no additional significant differences were observed (Fig. 2a and
b; Fig. 3a and b); P-values are depicted in Table 2. In addition,
no difference was found between the control and AD group in
total amount of AVP-mRNA either in the SON (P = 0.61) or
PVN (P = 0.71).

Exclusion of the two Parkinsonian patients had no influence
on the results (SON: P = 0.66, PVN: P = 0.77). Moreover, no
significant differences were present in volume between young
and old controls in both nuclei, nor in AD patients (Table 2). Also
for this parameter, no difference was present between controls
and AD patients (SON: P = 0.15, PVN: P = 0.25). No interference
was expected since no significant change in the number of AVP-
expressing neurons was found in the PVN in Parkinsonian
patients (41).

Multiple regression revealed that within the control group, no
significant correlations were present for age or any of the other
variables including postmortem delay (Table 2). The significant
difference present between the control and Alzheimer group (P =
0.006) in the postmortem delay, has, consequently, not influenced
our data. Within the AD group, significant positive relations were
found between total amounts of the SON ((F1,12) = 7.13; P =
0.02) and PVN (F1,12) = 8.6; P = 0.01) and storage time as well
as between volume of the two nuclei and storage time (SON:
F (1,11) = 6.5; P = 0.01, PVN: F (1,12) = 8.3; P = 0.01). For the total
amounts in the PVN, a positive relation with pH of the CSF was
observed as well (F1,11) = 6.9; P = 0.01). In addition, a significant
negative relation was observed between volume of the SON and
age (F (1,12) = 5.7; P = 0.03) (Table 2; Fig. 3). No significant
differences were found for PMD or fixation time in AD.

It is interesting to note that patient # 91107, who was mentioned
in the clinical records to be severely dehydrated (Table 1), yielded
high total amounts of AVP-mRNA. P-values obtained after
excluding this datapoint from the analysis are depicted in Table 2
and marked by **. For storage time, even more significant values
for the total amounts were obtained in the AD group whereas
the significant differences for the volume of the PVN and SON
have disappeared (Table 2). A negative relation was now found
with pH for both total amounts and volume of the SON and
volume of the PVN, whereas the total amounts in the PVN in
AD was not significantly different anymore.

Discussion
As shown before, quantitative ISH allows for the estimation of the
total amount of mRNA in an entire hypothalamic nucleus
while preserving the anatomical localization. It thus enables an
assessment of the relative differences in the level of gene expression
between individuals as well as between groups of controls and
diseased patients (40, 42, 43).

The use of human tissue implicated that variability might be
expected on the basis of duration of fixation, PMD or storage
time of the tissue (40, 42–46). In addition, tissue-PH or pH of
the CSF, a measure for agonal state that is independent of post
mortem delay (47, 48) was recently shown to contribute to the
variability in mRNA (49, 50). We attempted, therefore, to match
the control subjects and AD patients for all these factors. Since
data on the CSF-PH of a number of controls was lacking,
matching for this parameter was only possible in the AD group.
As expected on the basis of the clinical data, the highest total
amounts were found in AD patient # 91107, who was severely
dehydrated several days before his death (Table 1). Together with
the increased amounts of CRH-mRNA found in the PVN in
Alzheimer and depressed patients (43), this shows that the used
quantitative ISH procedure for formalin-fixed, paraffin-embedded
tissue, which is based on the integration of mRNA values
throughout an entire anatomical structure, is indeed able to detect
clinically relevant increases in mRNA. Although the observation
on patient # 91107 has still to be confirmed for a larger number
of dehydrated patients, it agrees with data of others who related
levels of AVP-mRNA to antemortem volume status (51).

The presently observed positive relation between AVP-mRNA
and storage time agrees with Guldenaar & Swaab (42) on OXT-
mRNA, but contrasts with Raadsheer et al. (43), who found a
negative relationship in control subjects. This relation was, how-
ever, found for CRH-mRNA and for storage times in considerably
older tissue with a larger age range and was not present in AD
or depressed patients. Apparently, time- and tissue-dependent
effects of storage time may be expected which illustrates the
necessity to match for this factor in human ISH studies. Our
observation on the positive relationship between mRNA and pH
agrees with that of others (49) for aldolase C, β-tubulin, prepro-
encephalin and preprotachykinin. Although only in our AD
group sufficient pH values were recorded to perform statistical
analysis, the data from Kingsbury et al. (49), based on both
control and Parkinsonian tissue, suggest that the influence of pH
on mRNA in pathological brain samples is similar to that in
control material. The present results underline the importance
of pH of the CSF and storage time as matching factors for ISH on
human brain (42–45, 47–50, 52).

On the basis of previous research, one would expect the
activational changes in AVP in the course of aging (see introduc-
tion) to be most pronounced in the SON, since the vast majority
of cells in this nucleus produce AVP and the AVP cell numbers
outnumber those in the PVN (53). Moreover, it was shown in
rat that increases in AVP and AVP-mRNA upon osmotic stimula-

Fig. 1. Photomicrograph depicting AVP-mRNA signal on film in a supraoptic (SON) and a paraventricular nucleus (PVN). a) Control subject, age 85, and b) an Alzheimer patient, age 87. Bar represents 1 mm. Note the stronger signal in the SON as compared with the PVN.

Identification is more pronounced in the SON than in the PVN (3, 4, 54, 55). It should be noted though, that although different quantitation methods were used, a 25 day period of 2% salt loading in rats resulted only in a 1.85-fold increase in AVP-mRNA levels in the SON and a 1.6-fold increase in the PVN (55). In view of the variation in the present human data, it may be difficult to detect increases of such a relatively small magnitude. Also, others have shown increases in SON AVP-mRNA content after dehydration in young and middle aged, but not in old rats. Fos induction, however, did take place in all 3 age groups to a similar extent, suggesting, in spite of the activational response in all age groups, an inability to increase the amount of AVP-mRNA available for translation in old animals (59). Whether similar mechanisms exist in human is, however, unknown.
No significant age-related changes in AVP-mRNA were found in the present study with both control subjects and AD patients showing comparable total amounts of AVP-mRNA in SON and PVN with mRNA values being in the SON than in the PVN (Figs. 1 and 2, Table 2). The significant negative relationship in the AD SON with age as found for volume, but not for total amounts, suggests that the same amount of mRNA is concentrated in a smaller volume. This can be interpreted as changes in the distribution either of the AVP-mRNA within the neuron itself, e.g. due to the accumulation of lipofuscin (56), or as an increased packaging of AVP-mRNA expressing cells within the SON. Because density measures of AVP-mRNA alone would have been influenced by these factors and so provided inconsistent results, these findings stress again the importance of measuring the integrated signal over an entire nucleus (40). In Fisher 344 rats, Flood & Coleman (57) have reported a decrease in SON volume in old age as measured on the basis of dendritic branching patterns, that coincided with increases in neuronal density. Since they did not observe any age-related changes in neuronal number, this resembles our findings in the aging human SON. For another theoretical possibility, i.e. a decrease in glia cell number in the SON, no evidence is present.

Although the present data confirm the stability of the SON and PVN in both aging and AD, they do not indicate an upregulation of the AVP production in these conditions. This could, in principle, be explained by an impaired transcriptional upregulation in aging or in AD. However, this possibility seems highly unlikely because previous observations, based on various independent parameters, suggested rather an activation of AVP production in these conditions (13, 15, 18, 20–27, 35, 36, 58, 60). The present absence of AVP-mRNA upregulation (Figs. 2 and 3), as is e.g. induced following osmotic stimulation in the rat (54, 61), points, however, to an alternative possibility that detectable increases in AVP-mRNA take place only after acute osmotic stimulation, inducing depletion of the AVP-stores in the hypothalamus, and not so much after chronic stimulation over several years. Chronic osmotic stimulation may rather give rise to an increased translatability or an elevated stability of the AVP-mRNA, e.g. due to an increased poly(A) tail length, as has been described following osmotic stimulation in rat (62–66).

On the basis of related gene products, it has been proposed that enhanced mRNA stability or changes in translatability may be just as effective in the control of protein synthesis as changing transcription rate (61, 64, 66–68). Such an enhanced mRNA stability or changes in translatability as a basis for increased neurosecretion would agree with the age-related changes in Golgi apparatus size, the number of AVP-expressing neurons and AVP blood levels (15, 18, 21, 23, 24, 69). Furthermore, an other theoretical possibility is regulation of AVP synthesis and release at the level of the AVP stores in the neurohypophysis that has been suggested in rat studies (64, 70). Also, measures for mRNA-splicing and turnover such as e.g. by measuring heteronuclear RNA using intron-probes have been suggested to reflect activational changes in these areas rather accurately (71, 72). However, little is known about these mechanisms and the possible influences of PMD and pH in this respect in human brain.

In conclusion, no age-related differences were observed in the total amounts of AVP-mRNA in the SON and PVN in control subjects or in AD patients. Significant positive relations were present between total amounts of RNA and pH of the CSF in the AD PVN and with storage time, for both total amounts and volume, in the SON and PVN of AD patients. Since matching was performed for these and other variables, these factors have, however, not contributed to the absence of differences with age. The unchanged AVP-mRNA level in the SON and PVN during aging confirms earlier observations on the stability of these nuclei. The lack of an AVP-mRNA upregulation during aging as could have been expected on the basis of other parameters in human, points to possible changes in translatability or mRNA stability during aging.

Materials and methods

Human tissue

Brains of 13 Alzheimer patients (5 males and 8 females) and 13 controls (7 males and 6 females) who died from different causes, were obtained...
### Table 1. Clinicopathological Data of the Human Brain Material Used.

<table>
<thead>
<tr>
<th>Controls</th>
<th>Pat.#</th>
<th>Age</th>
<th>Sex</th>
<th>PMD</th>
<th>BrWt</th>
<th>Fix</th>
<th>pH</th>
<th>StT</th>
<th>Clinicopathological data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Morbid adipositas, dystrofica myotonica, Curschman-Steinert syndrome</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Foetal and benzodiazepine intoxication, heart failure</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Carcinoma of the bladder, metastases, shock due to sepsis, peritonitis, lung oedema</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Traffic accident</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Renal insufficiency, a. basillaris trombosis, haemorrhage in pons</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sudden cardiac death, arteriosclerosis of coronary, brain and kidney arteries, Morbus Crohn</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mamma tumor, chemotherapy</td>
</tr>
<tr>
<td>Mean</td>
<td>53.0</td>
<td>&lt;11.9</td>
<td>1383</td>
<td>30.0</td>
<td>–</td>
<td>43.7</td>
<td></td>
<td>Sudden death</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>10.2</td>
<td>7.8</td>
<td>203.5</td>
<td>14.9</td>
<td>–</td>
<td>25.4</td>
<td></td>
<td>Heart failure</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>0.19</td>
<td>0.66</td>
<td>0.15</td>
<td>0.50</td>
<td>–</td>
<td>0.58</td>
<td></td>
<td>Bronchus carcinoma, pulmonary embolus, cardiac failure</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hydronephrosis, ureter carcinoma with metastases, respiratory insufficiency</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Myocardial infarctions, cardiac failure, pulmonary embolus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Parkinson’s disease, atherosclerosis, status lacunaris</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Myocardial infarction, emphysema, pneumonia, ischaemia cerebri</td>
</tr>
<tr>
<td>Mean</td>
<td>80.7</td>
<td>8.0</td>
<td>1099</td>
<td>23.2</td>
<td>6.81</td>
<td>42.0</td>
<td></td>
<td>Presenile AD, many amorphic plaques, cachexia</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>8.2</td>
<td>5.7</td>
<td>144.8</td>
<td>8.6</td>
<td>0.66</td>
<td>19.6</td>
<td></td>
<td>Presenile AD, uremia, pneumonia</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>0.10</td>
<td>0.71</td>
<td>0.13</td>
<td>0.37</td>
<td>0.10</td>
<td>0.47</td>
<td></td>
<td>AD</td>
<td></td>
</tr>
<tr>
<td>Alzheimer patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AD, cachexia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Presenile AD, sudden death</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AD, epilepsy, dehydration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AD; changes most pronounced paretically and occipitally, aspiration pneumonia</td>
</tr>
<tr>
<td>Mean</td>
<td>53.7</td>
<td>4.3*</td>
<td>1207</td>
<td>31.3</td>
<td>6.51</td>
<td>43.7</td>
<td></td>
<td>AD, pneumonia, aorta insufficiency, sinus sagitai thrombosis and cerebral infarctions</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>8.8</td>
<td>1.2</td>
<td>229.6</td>
<td>3.7</td>
<td>0.43</td>
<td>18.9</td>
<td></td>
<td>AD, depression, epilepsy</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>0.16</td>
<td>0.28</td>
<td>0.19</td>
<td>0.12</td>
<td>0.07</td>
<td>0.43</td>
<td></td>
<td>Parkinson’s disease with outspoken AD changes, dehydration, pneumonia</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AD, congophilic angiopathy, cachexia, septic shock</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AD, diabetes mellitus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AD, cachexia, some Lewy bodies in the substantia nigra</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AD, decompensatio cordis</td>
</tr>
<tr>
<td>Mean</td>
<td>84.7</td>
<td>4.0*</td>
<td>1046</td>
<td>35.6</td>
<td>6.60</td>
<td>42.0</td>
<td></td>
<td></td>
<td>AD</td>
</tr>
<tr>
<td>SD</td>
<td>10.5</td>
<td>1.06</td>
<td>142.3</td>
<td>9.8</td>
<td>0.12</td>
<td>21.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>0.12</td>
<td>0.27</td>
<td>0.14</td>
<td>0.18</td>
<td>0.02</td>
<td>0.52</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations used: Pat.#: patient number; PMD: post mortem delay (hours and tenths of hours); Fix: fixation time (in days); pH: pH of the CSF; StT: Storage time of the tissue (in months); m: male; f: female; nr: not recorded; SD: standard deviation; CV: coefficient of variation. AD: Alzheimer’s disease.*: indicates significantly different from the control group (see text for details).

from the Netherlands Brain Bank. For reasons of matching (see below), also two Parkinson’s patients were included, one without AD changes in the control group and one with outspoken AD changes in the AD group. The AD patients had been clinically assessed as having ‘probable AD’ by excluding other possible causes of dementia by history, physical examination and laboratory tests (73). All AD and control cases were neuropathologically confirmed by Dr W. Kamphorst (Free University, Amsterdam).

Dr D. Troost (Academic Medical Centre, Amsterdam) or Dr F. C. Stam (Netherlands Brain Bank) on the basis of the presence or absence of large numbers of plaques and tangles in hippocampal and neocortical areas. Patient data are presented in Table 1.

Hypothalami were dissected and fixed in 4% formaldehyde at room temperature for approximately 30 days (see Table 1 for details), subsequently dehydrated in alcohol, cleared in xylene and embedded in
Table 2. Scheme Depicting P-Values for Differences and Correlations between Total Amounts of AVP-mRNA in the SON and PVN in Aging or AD and Several Matching Variables.

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Age regr.</th>
<th>Storage t.</th>
<th>Storage t.*</th>
<th>pH</th>
<th>pH*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SON Total amounts</td>
<td>0.56</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Volume</td>
<td>0.40</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>PVN Total amounts</td>
<td>0.84</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Volume</td>
<td>0.40</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Alzheimer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SON Total amounts</td>
<td>0.17</td>
<td>ns</td>
<td>+0.02*</td>
<td>+0.0003*</td>
<td>ns</td>
<td>−0.0003*</td>
</tr>
<tr>
<td>Volume</td>
<td>0.06</td>
<td>−0.03*</td>
<td>F(1,12)=5.7</td>
<td>F(1,10)=29.4</td>
<td>ns</td>
<td>F(1,10)=23.6</td>
</tr>
<tr>
<td>PVN Total amounts</td>
<td>0.40</td>
<td>ns</td>
<td>+0.01*</td>
<td>+0.0002*</td>
<td>+0.01*</td>
<td>F(1,10)=39.5</td>
</tr>
<tr>
<td>Volume</td>
<td>0.17</td>
<td>ns</td>
<td>F(1,12)=8.6</td>
<td>F(1,10)=30.6</td>
<td>F(2,11)=6.9</td>
<td>−0.002*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations used: Total Amounts: of AVP-mRNA signal (in arbitrary units); Volume: occupied by the AVP-mRNA signal (arbitrary units); SON: supraoptic nucleus; PVN: paraventricular nucleus; Age: P-values of the differences between the young and old group (Table 1) after a two-tailed Mann-Whitney U-test; Age regr.: P-value of the correlation with age as obtained after multiple regression analysis; Storage t: P-value of the correlation with storage time of the tissue; pH: P-value of the correlation with pH of the CSF; F: F-value with degrees of freedom; *: indicates the presence of a significant relation; + or − indicates whether the significant relationship was positive or negative; **: indicates significance of the same parameter after excluding dehydrated patient 91107; ns: not significant.

Fig. 3. Graphs showing Volume of the AVP-mRNA signal on film (see text for details) with age for both the control group (open symbols) and Alzheimer patients (solid symbols) in: a) SON (circles). Line depicts the significant negative relation with age that is present in the Alzheimer group in this nucleus (P = 0.03; F(1,12) = 5.7). b) PVN (squares). ▶ AD; ○ control; ▼ AD; □ control.

paraffin wax (Histowax, melting point 56–58 °C, Histo-Lab, Sweden) after which serial 6 μm sections were cut. The PVN and SON were localized by thionine staining and by using serial AVP-immunostained sections to check the boundaries of both nuclei. Tissue sections were mounted throughout the SON and PVN with a section-to-section interval of 300 μm since in a previous study, less frequent sampling strategies turned out to influence the accuracy of the estimation of the total amounts of AVP-mRNA (40). On average 25 ± 6.4 (mean ± SD) sections per patient, including both SON and PVN, were mounted under RNAase-free conditions on 2% amino-alkyl-silane coated glass slides (74), dried for 48 h at 38 °C and kept at room temperature until hybridization.

Earlier studies suggested possible influences of several factors such as post mortem delay (PMD), duration of fixation, storage time of the dehydrated and embedded paraffin tissue as well as tissue pH on mRNA conservation and detection in brain tissue (40, 42–45, 49, 50). Therefore, control subjects and AD patients were matched as much as possible for these variables and for age in such a way that mean, standard deviation (SD) and coefficient of variation (CV) values of the patient groups were comparable (Table 1). However, for post mortem delay, a significant difference between the control and AD group could not be avoided (P = 0.006; Table 1) due to availability constraints of the tissue. This has, however, not influenced our data (see results section). The border between the young and old groups was arbitrarily set at 65 years of age, but correlations with age were performed over the entire age range.

In situ hybridization (ISH)

Patient material was randomly devided over 6 hybridization assays of approximately 120 sections each. Quantitative ISH was performed as described earlier (40) with minor adaptations. Briefly, all sections were deparaffinized together and rehydrated to phosphate buffered saline (PBS)(pH 7.6), treated with 0.2 N HCl for 20 min and deproteinized with 10 μg Proteinase K (PK) (Sigma) per ml PK buffer (10 mM Tris, 2 mM CaCl2) at 37°C for 15 min. Sections were subsequently rinsed in glycine buffer (2 mg/ml PBS), washed in PBS twice and dehydrated and delipidated as described earlier (40) after which they were dried in a vacuum dessicator for 3–4 h and kept at room temperature for 3–9 days until hybridization commenced.

The AVP probe (hvp3) consisted of an oligomer of 48 nucleotides complementary to bases 411–458 of the preprovasopressin precursor (75).

It was prepared and checked for specificity as described earlier including Northern blot, melting curve analysis and hybridization with sense probe (76, 77). No homologies with other sequences were furthermore found after a BLAST search (J. UdodeHaes and S. E. F. Guelden, unpublished results). The probe was 3’ labeled in 5 tripartite reactions using terminal
doxynucleotidyl transferase (Boehringer Mannheim) and [\textsuperscript{32}P]-dATP (>1400 Ci/mmol, ICN Biomedicals Benelux) as described (40) to a specific activity of 5136 Ci/mmol probe and was recovered by ethanol precipitation.

Further, labeled probe was diluted in hybridization buffer (HB) containing 50% de-ionized formamide (Merek), 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 x Denhardt's, 500 μg/ml yeast tRNA, 10% dextran sulphate and 50 mM DTT to an earlier established saturation concentration of 1 pmol/ml HB (J UdodeHaes and SEF Guldenaar, unpublished results). Hybridizations were performed on 6 consecutive days. 65 μl of reconstituted probe in HB was added to every section that was subsequently covered with a sterile glass coverslip, placed in a box humidified with a 2 x standard saline citrate (SSC)(1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7)/50% formamide solution and hybridized at 42°C for 15 h. The following day, coverslips were removed in 2 x SSC at 45°C and subsequently washed in 1 x SSC for 20 min at 45°C, 3 x 30 min in 0.1 x SSC at 45°C and 2 x 30 min in 0.1 x SSC at room temperature. Sections were then dehydrated in 70% ethanol and 100% ethanol, each for 5 min and dried in a vacuum desiccator for 2 h. Autoradiographic detection was performed by exposure to β-max hyperfilm (Amersham, UK) for 71 h.

Quantitative analysis

In order to quantify the ISH signal on film, densitometry of film autoradiograms was applied as described in detail before (40) using a Kontron AT IBAS image analysis system (Kontron, Germany) with a magnification setting that resulted in a resolution of 1 pixel representing 8.1 μm. In brief, a grey value transformation curve was constructed on the basis of radioactive standards. Images including SON or PVN were loaded into the IBAS followed by an automated structure extracting and thresholding procedure that yielded a mask coinciding exactly with the signal on film. Since both the number of cells expressing the gene may change or the expression in individual cells may alter, density as well as area of the signal covered by the mask were measured after a manual outline of the SON or PVN. For background correction, the density measured in a standardized outline per film that was devoid of any mask, was subtracted from every following density measurement in that film.

After multiplication of the area of the mask with the background corrected, mean density values for each structure in every section, a profile of integrated density values through an entire nucleus was obtained. The total area under the curve through these profiles, was an estimate for the total amount of radioactive label and thus for the total amount of AVP-mRNA present in the SON or PVN (total amounts). Likewise, of the area values on film, the total area under the curve yielded an estimate for the volume of the nucleus based on that part of the SON that is occupied by AVP-mRNA (volume).

Statistics

A two tailed Mann-Whitney test was used for comparison between the young and old control and AD groups. Multiple regression was applied to determine possible relationships between total amounts and volumes of the AVP-mRNA signal in both nuclei and age of the patients as well as matching variables like PMD, fixation time, storage time and pH of the cerebrospinal fluid (CSF). Statistical significance was set at P<0.05.

Acknowledgements

We thank the Netherlands Brain Bank (Coordinator: Dr R. Ravid) for the provision of human brain material and Mr A. Holtrop, Mr M. Kooreman and Dr E. J. van Zwieten for assistance with selecting and matching the patient data. Furthermore, we are indebted to Dr G. Mengod (Department of Neurochemistry, CTD/CSIC, Barcelona, Spain) and Dr J. M. Palacios (Laboratory Almirall, Barcelona, Spain) for their generous gift of the hyp3 probe. We also thank Dr A. Salehi for his assistance with the statistics, Mr G. van der Meulen for photography and Ms W. Verweij for correcting English. This work was supported by the Netherlands Organization for Scientific Research (NWO, Department of Medical Sciences: grant no. 900-552-083 to P. J. L.), the Dutch Ministry of Welfare, Public Health and Culture (Proj.Nr. 94-13/94.007) and the Innovation Fund of the Royal Dutch Academy of Arts and Sciences (both to S. E. F. G.).

Accepted 23 December 1996

References

20. Hoogenrijk JE, Fliers E, Swaab DF, Verwer RWH. Activation of vasopressin neurons in the human supraoptic and paraventricular
304 Vasopressin mRNA in aging and Alzheimer’s disease


64 Carter DA and Murphy D. Rapid changes in poly(A) tail length of...


