Immunocytochemical localization of vasopressin in the human brain; its possible consequences for therapeutic strategies in aging and dementia

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Immunocytochemistry on human brain material

Immunocytochemistry is widely used in current research on the human brain, in order to selectively mark cells, enzymes, disease-related compounds (e.g. tangles, Pick bodies, tumor antigens) and neurotransmitters. This has proven to be possible even in conventionally fixed autopsy material. Immunocytochemistry is in fact often indispensable for this purpose, since changes in the brain during development, aging or disease generally take place in specific cell or transmitter systems that cannot be distinguished by conventional histological techniques. A number of immunocytochemical techniques are available at present which can be applied to the human brain, each with its own advantages and drawbacks with respect to sensitivity and specificity (for reviews see Swaab and Boer, 1980; Swaab, 1982a; Pool et al., 1983).

For each antigen and each brain area one has to determine the extent to which post-mortem changes, type and duration of fixation, and sectioning procedures affect the immunocytochemical staining. The post-mortem stability of the neurotransmitters acetylcholine, biogenic amines and amino acids is extremely low. Nevertheless, spectacular progress has recently been made in localizing these compounds in the rat brain. However, the fixation of these compounds in brain tissue requires such strict conditions of perfusion (Geffard et al., 1984; Seguala et al., 1984; Buijs et al., 1984), that

application to the human brain seems unlikely in the near future. These systems, however, can be marked in the human brain by immunocytochemical staining of the enzymes involved in the neurotransmitter synthesis, e.g. choline acetyl transferase, dopamine- β -hydroxylase and glutamate dehydrogenase, although such indirect procedures have obvious disadvantages. Peptides, on the other hand, are extremely stable compounds. Storage of unfixed brain tissue for 2 days at room temperature does not change the vasopressin content of the rat septum, one of its major sites of termination, and still allows a good immunocytochemical staining of cell bodies and fibers in the hypothalamus. Also in human brain material the stainability of vasopressin- or oxytocin-containing cells and fibers is not noticeably affected by a post-mortem storage for 2 days before the onset of immersion fixation. Nor does the duration of fixation seem to be critical for such compounds: even in human brains that had been kept in 4% formalin in our museum for 50 years, immunocytochemical staining of vasopressin cells could be obtained (although certainly not optimally!) (Swaab, 1982a).

In the present paper, examples will be given of immunocytochemical localization of neuropeptides in the human brain in order to investigate age-related and disease-related changes. Data will concern the neurohypophyseal hormones vasopressin (AVP) and oxytocin (OXT), and will serve to illustrate the necessity of selectively marking cells in

particular areas (viz., the paraventricular (PVN) and the supraoptic (SON) nucleus) by means of immunocytochemistry, since AVP-producing cells show changes during aging, whereas OXT-producing cells apparently do not. In addition, the use of AVP as an immunocytochemical marker allows for visualization of the suprachiasmatic nucleus (SCN), the hypothalamic "clock". This nucleus cannot be clearly delineated in conventionally stained human brain tissue but, by means of the peptide marker, it becomes easily distinguishable, and changes taking place during aging and dementia can be recognized.

Although the localization of neurohormones and neurotransmitters in the brain is important data in itself, the power of immunocytochemistry is best utilized by combining it with other techniques. In the present study immunocytochemistry was combined with morphometric techniques in an attempt to find cytomorphological correlates for functional changes of AVP cells with aging. Immunocytochemistry revealed differential changes in the AVP

system in the brain, both in man and in the rat. An attempt will be made to interpret these observations in relation with therapeutic strategies for age-related diseases.

Neurohypophyseal peptides; hormones and transmitters

AVP and OXT neurons project as the classical neurosecretory neurons from the SON and PVN to the neurohypophysis, where AVP and OXT are released into the bloodstream (Bargmann, 1949). In the periphery, these peptides are involved in diuresis, lactation and labor. In addition (Fig. 1), AVP is produced by the SCN (Swaab et al., 1975) and by a number of other nuclei, i.e. the bed nucleus of the stria terminalis, amygdala, dorsomedial nucleus of the hypothalamus, and locus ceruleus (Van Leeuwen and Caffé, 1983; Caffé and Van Leeuwen, 1983). AVP- and OXT-containing fibers project to a large number of brain areas, where they

Fig. 1. Vasopressin (AVP) pathways and possible sites of termination (—<) in the rat brain projected to the sagittal plane. The pathways of the PVN (squares) are indicated by pecked-dotted lines (—·—·—) and pathways of the SCN (triangles) are indicated by dotted lines (.....). The AVP cell groups found only after colchicine treatment are indicated by large black dots, while the pathways of the stria terminalis (BST) are indicated by pecked lines (— · — · —). A question mark indicates that the source of the AVP innervation in this area is still unknown. Abbreviations: A, amygdala; DBB, diagonal band of Broca; DMH, dorsomedial nucleus of the hypothalamus; DVC, dorsal vagal complex; LC, locus ceruleus; LH, lateral habenula; LS, lateral septum; ME, median eminence; OVLT, organum vasculosum of the lamina terminalis; PVG, periventricular grey; PVS, periventricular nucleus; RD, dorsal raphe nucleus; VHi, ventral hippocampus; VTA, ventral tegmental area. (from Buijs et al., 1983 by courtesy)

terminate by means of synapses that cannot be distinguished morphologically from cholinergic, aminergic or amino acid containing terminals (Buijs and Swaab, 1979). These extrahypothalamic projections are presumably the anatomical basis for the hypothesized central functions of AVP and OXT, such as the regulation of temperature, osmotic pressure, circulation, and memory processes (De Wied, 1983; Schmid et al., 1984; Swaab, 1982b). Many of these functions appear to be disturbed during aging, a condition during which a decreased release of neurohypophyseal hormones into the periphery has also been reported (e.g. Legros, 1975). Because of the effects of neurohypophyseal hormones on memory consolidation, the memory disorders in the elderly and the presumed deficiency of neurohypophyseal function in this condition, Legros et al. (1978) studied the influence of vasopressin administered in men aged 50–65 and reported a positive effect. In order to see whether or not a decreased production of vasopressin would actually take place in the course of aging, we have in the first place measured cell size of immunocytochemically identified AVP and OXT cells by means of a digitizer (MOP system), as a parameter for peptide production in the PVN and SON (see Fliers et al., 1985a).

The SON and PVN in aging and dementia

The size of AVP cell profiles in the dorsolateral SON and the PVN showed an initial small decrease from childhood until the sixth decade. After this age a significant increase was observed in the size of AVP cell profiles, with the highest values being found in the age group of 80–100 years (Swaab et al., 1984; Fliers et al., 1985a). In brains from patients with SDAT, this morphometric parameter was within the range of their respective control age group (Fig. 2). OXT cell size did not change significantly, either with age or in SDAT patients. Assuming that the well-known relation between neurosecretory cell size and peptide synthesizing activity (cf. Fliers et al., 1985a) also holds for the *aging*

Fig. 2. Life-span changes in the size of the AVP cell profile. Note the decrease in cell profile until 60 years of age and the rise in this parameter for cell activity beyond this age (ANOVA; $p < 0.03$ during normal aging; ●). ○, SDAT patients, n , number of subjects investigated in each group. The asterisk indicates a significant ($p < 0.05$) rise as compared with the 40–60-y group (Student Newman-Keuls multiple range (SNK) test).

brain, the observed increase in AVP cell sizes after 60 years of age may imply an increased activity of these cells. This interpretation is strengthened by nucleolar measurements which show similar results (Hoogendijk et al., 1985). Our morphometric observations are in approximate agreement with the decreased blood levels of neurophysins reported between 50 and 60 years of age (Legros, 1975). In addition, the activation of AVP cells which is found at older ages appears to be reflected in the increased peripheral levels of this peptide (Frolkis et al., 1982; Kirkland et al., 1984). Similarly Legros et al. (1980) have reported a secondary increase in immunoreactive neurophysin blood levels after the age of 70.

A similar activation of AVP production and release was found in aged Wistar rats (Fliers and Swaab, 1983). Immunocytochemical staining of AVP binding sites in the rat kidney showed that the activation of AVP cells in the aged rat is likely to be a compensatory mechanism for a decrease in AVP binding sites in both the distal convoluted and the collecting ducts of the kidney (Ravid et al., 1985b). Similar changes have been proposed to occur in the human kidney in senescence (Legros et al., 1980).

The suprachiasmatic nucleus: changes in rhythmical processes during aging

The suprachiasmatic nucleus is considered to be an endogenous "clock" of the mammalian brain, which coordinates hormonal and behavioral circadian rhythms. Age-related changes in biological circadian rhythms with aging have been reported in man as well as in other species. The increase in time spent in wakefulness during the night, and in daytime naps can be characterized as a disruption of the circadian sleep/wake rhythm in senescence. Since this phenomenon is even more prominent in patients with senile dementia of the Alzheimer type (SDAT) than in age-matched controls (Prinz et al., 1982), the study of this nucleus in relation to age and SDAT seems to be of particular interest. However, the very existence of this nucleus in the human brain was questioned until recently, because of difficulties visualizing it by means of conventional histological staining techniques (see Lydic et al., 1980). Immunocytochemical staining with antibodies against AVP has turned out to be a good marker for the human SCN (Dierickx and Vandesande, 1977), similar to what had been found earlier in the

rat (Swaab et al., 1975). This fact has enabled us to apply morphometric techniques to the investigation of the human SCN in order to follow age-related changes in volume and cell number of this nucleus.

A marked decrease in SCN volume, AVP cell number and total SCN cell number was found in 80–100 year-old patients as compared with the younger age groups (Swaab et al., 1984, 1985). The SCN changes in SDAT were even more pronounced than those observed during normal aging (Fig. 3). From animal experiments it appears that SCN cell number is important for its pacemaker properties (Van den Pol and Powley, 1979; Pickard and Turek, 1983). The decrease observed in SCN volume and cell number in senescence and, even more pronounced, in SDAT, suggests therefore, a causal relationship between age-related changes in the SCN and disturbances of circadian rhythmicity, such as sleep/wake patterns. A changed temporal organization of sleep, in combination with quantitative changes in various sleep parameters, e.g. reduction in the proportion of REM sleep, have also been found in aged Wistar and in Brown-Norway rats (Van Gool and Mirmiran, 1983), that may serve therefore in this respect as a model for the study of the age-related sleep changes in the human.

Fig. 3. Suprachiasmatic nucleus AVP cell number. Note the decrease in cell number in the oldest age group (81–100 years) and the even stronger decrease in the demented patients (DEM) in spite of their relatively young mean age (76 years). The effect of age on AVP cell number was significant (ANOVA; $p = 0.026$). The decrease in the oldest age group was significant as compared with the 0–20 and 40–80-y old group (SNK; $p < 0.05$).

Some problems of a vasopressin substitution therapy

From the cell profile measurements in the SON and PVN and from the literature the conclusion can hardly be avoided that vasopressin "substitution therapy" in elderly and demented patients has in most cases probably been given to patients in whom neurohypophyseal function was not deficient at all, but in fact quite adequately activated due to a decreased renal sensitivity for AVP. This would partially explain the inconsistent results of this therapy (see Jolles, 1983).

In addition, one might wonder, whether or not administration of an analog that has little or no intrinsic antidiuretic effect, such as DGAVP, is advisable in conditions where what the kidney actually requires is extra AVP, in order to compensate

for the loss of renal binding capacity. There is, in addition, a possibility that such an analog competes with AVP for the kidney vasopressin receptor, and, consequently, inhibits the anti-diuretic action of endogenous AVP. Such an effect seems to occur with DGAVP in *in vitro* experiments (Ravid et al., 1985a). The crucial *in vivo* experiments to detect such a possible antagonistic effect of DGAVP have still to be performed.

Another problem encountered in "substitution" therapy with vasopressin is that the various vasopressinergic systems in the brain do not seem to change in a similar way in aging and dementia. Thus, the SON and PVN are activated, while, in contrast, SCN cells degenerated to a large extent. Cells of the SON, PVN and SCN project to different areas in the brain (Buijs et al., 1978, 1983; Mason et al., 1984; Sawchenko and Swanson, 1982; De Vries and Buijs, 1983). The possibility of a differential alteration of vasopressinergic innervation according to its site of origin is supported by our recent observations in the 34-month old rat which reveal that the extrahypothalamic sites of AVP fiber terminations show no overall changes with age. Certain areas show a decrease in the AVP density of immunocytochemically stained fiber networks, while other sites seem to remain unaltered (Fliers et al., 1985b). Such differential changes in the vasopressinergic innervation make it very difficult to substitute to normal vasopressin levels in an area without interfering with the normal vasopressin levels in other areas.

Apart from these aspects, one cannot realistically expect to mimic the complex and naturally occurring spatial-temporal fluctuation of the transmitter activity by global administration of a chemical substance. Such considerations may even apply to the example "par excellence" of a successful substitution therapy, viz. L-dopa treatment in Parkinson's disease. The death of nigro-striatal and other nearby neurons constitutes a major part of parkinsonian pathology. The L-dopa therapy of this disease has therefore often been presented as an example of replacement of the disappearing neurons by their natural transmitter, dopamine. The remaining neurons

compensate in the beginning stages of the disease effectively for the loss of the rest of the dopaminergic neurons by means of presynaptic overactivity and postsynaptic supersensitivity (Hornykiewicz, 1982). L-Dopa is able to cross the blood-brain barrier and is efficiently converted into the neurotransmitter dopamine by the enzyme dopamine decarboxylase that is present in redundant amounts. Notwithstanding the impressive initial therapeutic effect of L-dopa (Hornykiewicz, 1982) including even at times mental symptoms (Drachman and Stahl, 1975), 15% of parkinsonian patients do not respond to this therapy (Jankovic, 1982) and in those patients in which the therapy is effective, long-term treatment eventually results in loss of therapeutic efficacy (Riklan et al., 1976; Yahr, 1978). The benefits of L-dopa decline with time, most probably because the progression of the pathology causes further degeneration of the aminergic synaptic terminals, thus leading to a loss of capacity to store and release the transmitters (Marsden and Jenner, 1981; Jankovic, 1982). Consequently, the initial effect does not seem to result from replacement of the lost substantia nigra neurons by their transmitter, but rather from a temporary aid to the still remaining neurons. For peptides, however, neither the compensatory pre- and postsynaptic mechanisms nor the efficient reversal of an administered precursor into the neurotransmitter and storage of this compound in the synaptic terminals have been demonstrated. These considerations urge us to be reserved about the potentialities of neurotransmitter "replacement" therapy in the case of neuropeptides. Certainly this does not rule out the possibility of other than neurotransmitter effects of exogenously administered peptides during aging. For instance, peptides of the proopiomelanocortin family might act as a "trophic factor" in aging (Landfield et al., 1981). This possibility remains to be investigated with respect to AVP. The Accurel device, which allows a continuous long-term release of peptides (Boer and Kruisbrink, 1984) could prove to be an important tool in this type of study in research on aging.

Non-pharmacological therapies

Recently some non-pharmacological therapies seem to bear promise in research on aging. The changes in sleep/wake patterns found in aged rats are opposite in various respects to those induced by an enriched environment in young ones, e.g. in respect to the increased amount of REM sleep found in the latter condition (Mirmiran et al., 1982). In order to determine whether or not such a response to the environment still exists at an advanced age, 25-month old rats were recently placed in "enriched environment" cages. These large cages are outfitted daily with a new arrangement of objects like ladders, boxes and tubes. The sleep pattern of such old but "enriched" rats came to resemble in certain respects that of young "standard" rats, in particular the age-related REM sleep reduction was no longer present after enrichment rearing (Van Gool and Mirmiran, 1984). This non-pharmacological "therapeutic" effect of the environment on rhythmical brain processes, however, was not accompanied by improvements in performance on a radial maze task, contrary to what has been reported for young enriched rats. More behavioral studies are clearly indicated; plasticity in senescence is also being studied at a cytomorphological level in various brain areas.

The mechanism of action of the enriched environment "therapy" on the aging brain is not known but it does have in common with food restriction a decrease in body weight. This may point to a common mechanism, e.g. to increased motoric activity or alternatively to decreased rates of O_2 consumption (Harman, 1983). Feeding of rats only on alternate days turned out to be a highly effective method for increasing life-span by 40–80%. In addition, the loss of dopamine receptors in the aging brain was substantially retarded (Levin et al., 1981; Goodrich et al., 1982). The actual mechanism of such dietary induced changes is poorly understood.

Another non-pharmacological strategy that might be effective in aging animals is brain transplantation (Gage et al., 1983). This approach may circumvent the theoretical drawbacks of neuro-

transmitter suppletion, as mentioned above, since the transplanted neurons are actually incorporated into the neuronal network of the host. Aged rats show a decline in, among other things, motor coordination, and this is believed to be correlated with impairment of the neurotransmitter dopamine. Suspensions of dissociated cells, prepared from the substantia nigra of rat embryos and subsequently grafted in the caudate putamen of aged rats, produced extensive new dopaminergic connections in the neostriatum, resulting in a significant improvement in motor coordination (Gage et al., 1983). Improved "memory" has already been reported following transplantation of noradrenergic neurons into aged rats (Collier et al., 1984), but this claim should still be substantiated, especially from the behavioral point of view. The transplantation technique has not yet been applied to the AVP system in aging, but it seems to be an attractive approach, since AVP neurons can be successfully transplanted (Gash et al., 1983). Moreover, recently, the fetal SCN was grafted into SCN-lesioned adult rats, which resulted in a restoration of circadian periodicity (Drucker-Colin et al., 1983). This might prove to be an effective procedure for restoring disturbed circadian processes in the aged rats as well. Although brain grafting in human beings obviously raises both practical problems and ethical questions, such techniques have already been applied to Parkinson patients (Backlund, 1984). However, for the time being, trials of relatively non-specific stimulation of the brain by means of environmental and dietary manipulation would be more advisable in the clinical situation.

Summary and conclusions

In conclusion, ¹²⁵I-immunocytochemistry in combination with morphometric techniques has revealed that the AVP neurons in both the SON and PVN are activated in normal senescence and, to the same degree, in SDAT patients. On the other hand, in the SCN (which was immunocytochemically visualized using AVP as a marker) a marked reduction

in cell number was observed in senescence, while an even stronger decrease was found in SDAT. Since, depending on the area studied, AVP neurons can either be activated or have largely degenerated and since AVP acts within the brain as a transmitter, little benefit may probably be expected from a therapy aiming at the substitution of the transmitter function of AVP by the administration of exogenous AVP during aging or dementia. Of course this does not rule out beneficial effects as a result of other, e.g. metabolic or trophic effects of neuropeptides or their analogs.

Recently, non-pharmacological treatments (i.e. activation by means of environmental manipulations, dietary changes, or brain transplantation techniques) appear to carry a good potential in aging research, as judged from the partial restoration of, e.g. sleep-wake patterns, dopamine receptors and motor performance in senescent rats.

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