Short communication

Morphometric analysis of vasopressin and vasoactive intestinal polypeptide neurons in the human suprachiasmatic nucleus: influence of microwave treatment

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Accepted 25 June 1996

Abstract

The vasopressin (VP) and vasoactive intestinal polypeptide (VIP)-expressing neurons in the human suprachiasmatic nucleus (SCN) were morphometrically determined with or without microwave (MW) treatment. Both an enlarged volume and an increased number of neurons were found in the VP and VIP subnucleus of the SCN following MW treatment. The staining of VIP neurons by MW treatment improved much more than that of the VP neurons. Without MW treatment the VP- and VIP-expressing neurons were localized mainly in the central part of the subnuclei, whereas by increasing the sensitivity of the staining by MW treatment more neurons became visible, particularly in the peripheral areas of the subnuclei.

Keywords: Vasopressin; Vasoactive intestinal polypeptide; Suprachiasmatic nucleus; Microwave; Antigen retrieval; Morphometry

The suprachiasmatic nucleus (SCN) generates and coordinates circadian rhythms, such as sleep/wakefulness, body temperature, hormonal and behavioural rhythms [9]. Our previous studies indicate that the number of vasopressin (VP) and vasoactive intestinal polypeptide (VIP)-expressing neurons in the SCN is related to sexual orientation and gender, and that they show circadian and seasonal fluctuations [3,4,13,15,18]. Furthermore, a dramatic decrease in the number of VP and a less obvious one in VIP neurons was found in the SCN in Alzheimer's disease [14,18]. The number of peptide-expressing neurons is a functional measure that is dependent on the sensitivity of the immunocytochemical procedure. Formalin-fixation and paraffin-embedding, being routinely carried out in our group for practical reasons, may cause masking of some antigens as has been shown also in the human hypothalamus [6]. Antigen retrieval by microwave (MW) treatment can bypass such masking effects [6,10] and make the staining much more sensitive. In order to determine the influence of MW treatment on the immunocytochemistry of VP and VIP-expressing neurons of the human SCN, numbers of these peptidergic neurons were determined morphometrically with and without MW treatment.

After autopsy, for which special permission had been obtained, brains of nine patients without primary neurological or psychiatric disease were examined. General neuropathology was performed by Dr. W. Kamphorst, Prof. F. Stam or Dr. D. Troost. Each group consisted of six subjects (Table 1). In three patients alternating sections were treated with MW, or not. Four series of adjacent 6 μ m paraffin sections, two for VIP with and without MW and two for VP with and without MW, were taken at 25- μ m intervals throughout the complete SCN region of three subjects. In addition, two series of adjacent sections, one for VIP and one for VP, were taken, either for MW or without MW, from the other three subjects in each group. The immunocytochemical procedure without MW has been used and described extensively before [11–14]. For MW treatment, the hypothalamic sections were mounted in an aquadest solution on microwave-resistant AAS-coated glass slides (Kreatech, batch no. cg9556b). The sections were dried overnight at 37°C, deparaffinized and rehydrated. The sections were heated by a microwave-oven at 700 W at 90°C for 10 min (for details see [6]). The sections were incubated with anti-VIP (Viper, 18/8/86) 1:1000 or anti-vasopressin (Truus, 18/9/85) 1:1000, followed by the PAP method. The number of AVP- or VIP-expressing neurons in the SCN was estimated by multiplying the numerical cell density (as determined by the deconvolution

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technique) by the volume of each cell population in the SCN (for details see [14,15,17,18]). Differences among the groups were statistically evaluated using the two-tailed Mann–Whitney *U*-test. A 5% level of significance was used in all statistical tests. Throughout this study values are expressed as mean $\pm$ S.E.M..

The age, brain weight, postmortem delay, fixation time and time of death of the groups did not differ (Table 2). The VIP-expressing neurons were mainly present in the ventrolateral and centromedial part of the SCN, while the VP-expressing neurons were mainly distributed in the dorsal and centromedial portion of the SCN, which is in agreement with previous studies [7,8,12,14,15,17,18]. The intensity of the immunocytochemical staining was much stronger for both peptides in the MW-treated sections than for those that had not been treated with MW (Fig. 1).

The volume of the VP subnucleus in the SCN was 2.4 times larger in the MW-treated sections than in the untreated ones ($P < 0.01$). The number of VP-expressing neurons increased by 76% following MW treatment. However, the difference was on the borderline of significance ($P = 0.055$, Table 2). There was an extremely strong and significant 8-fold increase in the volume of the VIP subnucleus, whereas the number of VIP-expressing neurons in the SCN increased 4-fold after the MW treatment. The increased volume of the VIP subnucleus was due to both a larger cross-sectional area and a significantly longer rostral-caudal axis in the MW-treated sections (Table 2). A similar pattern was found in the three subjects of whom the hypothalamic tissue had been treated either with MW or without MW, that is, the volume of the VP subnucleus was 2.5 times larger and the number of VP-expressing neurons increased by 70% after MW treatment.

Although the SCN can be identified in the human hypothalamus by immunocytochemistry [2,7,8,12,14] its

boundaries are often difficult to discern, especially the rostral and caudal ones. In addition, at some developmental stages quite a high proportion of the SCNs did not stain for VIP [15]. In fact, a high variability of SCN staining between individuals has been found in all studies dealing with the human brain [7,8,12,14,15,18]. In addition to the biological and pathological factors that may affect the peptide content of the SCN and thus the staining [3,4,14,15,17,18], the sensitivity of the immunocytochemical staining will also influence the number of cells stained. Indeed, a much more sensitive immunocytochemical staining was obtained following MW pretreatment. Not only were more immunoreactive neurons visible, but the volumes of the VP- and VIP-expressing subnuclei in the SCN were also strongly increased. An influence of, for example, postmortem delay, fixation time or time of death of the different subjects on the MW-induced changes of the SCN can be excluded because a similar pattern of change was found in three subjects of whom SCN sections had been treated either with MW or without MW.

The main findings of the present study were (i) that the number of peptide-expressing neurons in the SCN increased dramatically by increasing the sensitivity of the method by MW treatment and (ii) that the two cell populations were affected by MW treatment in a different way: VIP neurons were affected much stronger than VP neurons. In addition, (iii) the immunoreactive neurons that were visible without MW were localized mainly in the central area of the VP- and VIP-subnuclei. In contrast, neurons that became only visible after increasing the sensitivity by MW treatment were mainly localized in the peripheral area of the subnuclei (Fig. 2). The increased sensitivity following MW treatment went, therefore, together with an expansion of the size of the subnucleus and a tendency to a decreased cell density.

Table 1
Brain material

Patient no.	Sex	Age (years)	Brain weight (g)	PMD (h)	Fix. (days)	Time of death	Month of death	Diagnosis
<i>With MW</i>								
94019	M	20	1490	8	83	08:30	Jan.	Pancytopenia
81020 *	F	50	1210	7	40	07:00	Jan.	M. Kahler, renal insufficiency
93047	M	70	1454	4	33	15:20	Feb.	Urinary tract infection
93041 *	F	73	1344	8	34	09:10	Feb.	Septic shock, pneumonia
94191	M	78	1441	8	24	12:15	July	Arrhythmia cordis
82175 *	M	85	1400	16	44	16:50	July	Chronic myelocytic leukemia
<i>Without MW</i>								
87110	M	23	1310	13	11	11:00	March	Encephalitis in brainstem
81020 *	F	50	1210	7	40	07:00	Jan.	M. Kahler, renal insufficiency
84049	M	63	1420	32	35	01:00	March	Myocardial infarction; cardiac failure
93041 *	F	73	1344	8	34	09:10	Feb.	Septic shock, pneumonia
81064	M	83	1280	22	42	07:00	March	Divericulitis
82175 *	M	85	1400	16	44	16:50	July	Chronic myelocytic leukemia

PMD, postmortem delay; fix, fixation time; MW, microwave; M, male; F, female.

* Brain material of patients was used both for MW and without MW treatment.

The number of VP- and VIP-expressing neurons in the SCN is a good indicator of the functional activity of the neurons as was shown, e.g. by the circadian fluctuations in the human SCN, revealing more VP-expressing neurons in the SCN during the day than during the night [3,4]. This measure of activity is consistent with circadian fluctuations in VP content and mRNA in the rat and hamster SCN [1,5,16] and VP release in vitro [11]. The observed staining patterns suggest thus that the centrally localized neurons

that contain more peptide and are already visible without MW treatment are the more active neurons, whereas the peripherally localized neurons that contain less peptide, and which become only visible following MW treatment, are the less active ones. Whether this is a general phenomenon, that holds for other hypothalamic nuclei as well, i.e. that there is a gradient in neuronal activity depending on the position of neurons in the nucleus from central to the periphery, remains to be studied.

Fig. 1. Vasoactive intestinal polypeptide (VIP) and vasopressin (VP)-expressing neurons in frontal sections ($6 \mu\text{m}$) of the human suprachiasmatic nucleus (SCN). A,B: microwave-treated sections. A: VIP-expressing neurons located in the ventrolateral part of the SCN. B: VP-expressing neurons distributed in the dorsal and centromedial part of the SCN. 3V: the third ventricle. Bar = $100 \mu\text{m}$. C–F: Comparison between nonmicrowave-treated sections (C,E) and microwave-treated sections (D,F). C,D: VIP-expressing neurons. E,F: VP-expressing neurons. Note the stronger intensity of the staining and the increased number of immunoreactive neurons in microwave-treated sections. Bar = $25 \mu\text{m}$.

Table 2

Morphometry of the VIP and VP neuron populations in the human suprachiasmatic nucleus (mean $\pm$ S.E.M.)

	With MW	Without MW	P values
Age (years)	62.7 $\pm$ 10	62.8 $\pm$ 10	0.936
Brain weight (g)	1390 $\pm$ 41	1327 $\pm$ 32	0.171
Postmortem (h)	9 $\pm$ 2	16 $\pm$ 4	0.120
Fixation (days)	43 $\pm$ 8	34 $\pm$ 5	0.936
Cell number ($\times 10^3$)			
VP population	11.683 $\pm$ 7.2	6.625 $\pm$ 1.0	0.055
VIP population	6.439 $\pm$ 1.1	1.584 $\pm$ 0.1	0.004
Cell population volume (mm ³)			
VP population	0.61 $\pm$ 0.09	0.25 $\pm$ 0.03	0.010
VIP population	0.34 $\pm$ 0.08	0.04 $\pm$ 0.01	0.004
Cell density ($\times 10^3/\text{mm}^3$)			
VP population	20.17 $\pm$ 3.52	26.85 $\pm$ 0.6	0.055
VIP population	23.95 $\pm$ 4.97	54.43 $\pm$ 18	0.055
Rostro-caudal axis (mm)			
VP population	2.36 $\pm$ 0.41	1.63 $\pm$ 0.20	0.200
VIP population	3.03 $\pm$ 0.64	1.44 $\pm$ 0.22	0.045
Maximum cross-sectional area (mm ²)			
VP population	0.75 $\pm$ 0.16	0.42 $\pm$ 0.07	0.109
VIP population	0.31 $\pm$ 0.07	0.04 $\pm$ 0.01	0.004

Abbreviations: MW, microwave treatment; VP, vasopressin; VIP, vasoactive intestinal polypeptide; P values, two-tailed Mann-Whitney U-test.

Fig. 2. Drawing of the distribution of VIP- and VP-expressing neurons in the SCN on the basis of four adjacent sections. Note that without microwave treatment VIP- (Δ) and VP- ($\bullet$) expressing neurons are mainly distributed in the 'central' areas of the subnucleus whereas after microwave treatment there are more VIP (Δ) and VP ($\circ$) neurons in the 'peripheral' areas of the subnucleus. Note that enlarged volume of the SCN after microwave treatment. III: the third ventricle. OC: the optic chiasma.

Acknowledgements

We thank B. Fisser, H. Stoffels, G. van der Meulen, and O. Pach for their help. Brain material was provided by the Netherlands Brain Bank (coordinator Dr. R. Ravid).

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