MUTANT VASOPRESSIN PRECURSORS IN THE HUMAN HYPOTHALAMUS: EVIDENCE FOR NEURONAL SOMATIC MUTATIONS IN MAN

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Abstract—We report here the expression of mutant proteins displaying the +1 reading frame of the vasopressin and oxytocin precursors in magnocellular neurons of the human hypothalamus. Our data demonstrate a high frequency of frameshift mutations in these neurons and thus provide the first evidence of somatic mutations in neurons of the human brain.

The results imply that other neuronal populations and specific genes may also undergo similar mutational events with possible consequences for neuronal functioning and pathology.

Key words: genomic instability, frameshift mutation, supraoptic nucleus, paraventricular nucleus, neurohypophysics, oxytocin.

The neuropeptides vasopressin and oxytocin are synthesized in the magnocellular neurons of the supraoptic (SON) and paraventricular (PVN) nuclei, axonally transported to the neurohypophysis and released into the blood circulation.²³ In the periphery, vasopressin is involved in the regulation of water metabolism, whereas oxytocin plays a role in lactation and labour.^{3,16} Vasopressin and oxytocin neurons of the human PVN also project into the brain⁷ and may influence central processes such as sexual behaviour. In addition, vasopressin is synthesized in parvocellular neurons of the suprachiasmatic nucleus (SCN), which is considered to be the regulator of hormonal and behavioural circadian and circannual rhythms.¹¹ The nonapeptide vasopressin is derived from a precursor protein consisting of a signal peptide, vasopressin, neurophysin II and a glycoprotein. The oxytocin precursor includes a signal peptide, oxytocin and neurophysin I. During axonal transport within secretory granules the precursor protein is cleaved enzymatically, yielding the individual peptides.¹⁰

Recent studies revealed that magnocellular neurons are subject to genomic alterations affecting the expression of vasopressin and oxytocin genes. We have previously reported the occurrence of +1 frameshift mutations at high frequency in vasopressin transcripts of the rat. Magnocellular neurons display-

ing this frameshift increase in number with age both in homozygous (di/di) Brattleboro and wild-type rats. The mutations consist predominantly of a di-nucleotide deletion (Δ GA) occurring at two separate GAGAG motifs.^{5,24,26} Since the hypothalamic neurons of the rat do not undergo cell division after fetal day 15,¹ these mutations point to a yet unknown post-mitotic mechanism of mutagenesis. Additionally, the magnocellular hypothalamic neurons express altered vasopressin precursors that appear to be derived by somatic recombination between the vasopressin and oxytocin genes.¹⁴ These hybrid precursors are most likely non-functional, thereby revealing a DNA recombination event that suggests that magnocellular neurons are prone to genomic instability.

It is evident that genomic instability may be associated with carcinogenesis,¹³ genetic disease¹⁸ and aging²⁸ in organs other than the brain. However, nothing is known about the occurrence of spontaneous mutations in specific genes of the human brain and their effect on neuronal functioning.

EXPERIMENTAL PROCEDURES

Human brain tissue

Brains from six control subjects (three males and three females, 30–82 years of age) who had not suffered from a primary neurological or psychiatric disorder were obtained at autopsy. The brains were weighed and fixed in 10% formalin at room temperature. Details on age, *post mortem* delay, fixation time and clinical diagnoses of the subjects are given in Table 1. The hypothalamic area was dissected, dehydrated in graded ethanol and embedded in paraffin. Serial 6- μ m transverse sections were cut on a Leitz microtome, numbered and mounted on chrome-alum-coated

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Abbreviations: di/di, homozygous Brattleboro rat; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus.

Table 1. Number of human hypothalamic neurons expressing mutant vasopressin and oxytocin precursors and clinical and pathological data of subjects studied

Pat. no.	Sex (f/m)	Age (years)	BW (g)	PM (h)	Fix (days)	Clinico-pathological data	Vasopressin + 1 mutants		Oxytocin + 1 mutants	
							Cell number	Total SON + PVN	Cell number	Total SON + PVN
90090	m	30	1325	4.55	ND	Fallot's tetralogy, arrhythmia, hypoxia	5	250	0	0
9182	f	36	1348	< 72	90	faecal peritonitis, ulcera duodeni/pylori	4	200	0	0
89411	f	49	1260	< 24	31	lung carcinoma, metastases, cytostatic, leucopenia, septic shock	1	50	0	0
9326	m	50	1646	< 12	51	ureter carcinoma, metastases, hypovolaemic shock by sepsis, lung oedema, bronchopneumonia	16	800	2	200
9341	f	73	1344	< 12	34	lung emphysema, dyspnea, bronchopneumonia, hypertension, hypercapnia	9	450	1	100
9075	m	82	1268	5.20	28	ureter carcinoma, metastases, respiratory insufficiency, hydronephrosis	3	150	0	0

Abbreviations: Pat. no., patient number; BW, brain weight; PM, post mortem delay; Fix, fixation time; f, female; m, male; ND, not determined.

slides. Every 50th section was stained with Thionine in order to locate the SON and PVN before immunocytochemical staining.

Antisera

To determine whether frameshift mutations are present in human vasopressin and oxytocin transcripts, antibodies were raised in rabbits against synthetic peptides predicted by the amino acid sequence that would result from a +1 frameshift mutation (antisera huva +1 and humox +1, respectively). The synthetic peptides corresponded to nucleotides 2217–2246 of the vasopressin gene (amino acid sequence: GARALRARPA) and to nucleotides 1173–1205 of the oxytocin gene (amino acid sequence: LKLDGSQH-PRS). The nucleotide numbering is according to Sausville *et al.*¹⁹ The frameshifted vasopressin precursors detected by the huva +1 antiserum are derived by +1 frameshift mutations which are present downstream of nucleotide position 1817, since a stop codon will be created in the +1 reading frame at this site. This results in mutant vasopressin precursors of which the N-terminus, encoding the vasopressin peptide, contains the wild-type amino acid sequence. The +1 reading frame of both the vasopressin and oxytocin precursor eliminates the translational stop codon at the C-terminus.

Immunocytochemistry

Sections were taken at regular 300- μ m intervals throughout the SON and the PVN and stained with the huva +1 antiserum. Sections taken at 600- μ m intervals were stained with the humox +1 antisera. As a positive control two consecutive sections from each subject were stained with antisera recognizing vasopressin neurons (Boris-Y-2, directed against human glycoprotein_{22–39})⁸ and oxytocin neurons (O-1-V, 4-4'-75).²⁹ Immunocytochemical staining was performed by the following protocol: sections were hydrated and incubated for 1 h at room temperature and overnight at 4°C with huva +1 (1:500 dilution), humox +1 (1:500 dilution), Boris-Y-2 (1:2000 dilution) or O-1-V purified with vasopressin (1:500 dilution) in Tris-buffered saline (0.05 M Tris/0.5 M NaCl, pH 7.6) containing 0.5% Triton X-100. In addition, the huva +1 antiserum was solid-phase preadsorbed with its antigen as a control for specificity. All the subsequent incubations were performed at room temperature for 1 h. The sections were washed in Tris-buffered saline (0.05 M Tris/0.9% NaCl, pH 7.6) and incubated with goat-anti-rabbit immunoglobulin G serum (Betsie 1:100 dilution) in Tris-buffered saline–Triton X-100. After washing in Tris-buffered saline, sections were incubated with peroxidase–antiperoxidase (1:1000 dilution) in Tris-buffered saline–Triton X-100. Finally, the sections were washed in 0.05 M Tris–HCl, pH 7.6, and stained with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M Tris, pH 7.6, containing 0.01% H₂O₂ and 0.2% nickel ammonium sulphate at room temperature for 10 min. After rinsing in distilled water the sections were dehydrated via ethanol and mounted in Entellan (Merck).

Estimation of the total number of magnocellular neurons expressing mutant precursors

In order to estimate the total numbers of immunoreactive neurons per hypothalamus, the sum of the counts for the individual sections was multiplied by the sample periodicity (i.e. 50 for vasopressin +1 and 100 for oxytocin +1). Only cells containing a nucleolus were counted. Nucleoli were considered to be hard particles which, during sectioning, were pushed either completely into or out of the section; thus, no correction factor for split nucleoli was needed.^{12,15}

RESULTS

Hypothalami of three male and three female individuals aged 30–82 years (Table 1) were studied for the expression of both wild-type and +1 frameshifted

Fig. 1. Consecutive sections of subject no. 89411 stained for vasopressin and oxytocin neurons. The immunoreactive staining is representative for all human subjects studied. (A) Dorsolateral SON stained with Boris-Y-2 antiserum directed against the glycoprotein₂₂₋₃₉ moiety of the vasopressin precursor. (B) Dorsolateral SON stained for oxytocin peptide using the O-1-V antiserum purified with vasopressin. Note that the oxytocin cell population is clearly separated from the vasopressin cell population. Asterisks, capillaries present in consecutive sections; OC, optic chiasm. Scale bar = 100 μ m.

vasopressin and oxytocin precursor proteins. Mutant precursors were detected by antisera directed against synthetic peptides predicted by the +1 reading frame of vasopressin and oxytocin cDNAs as outlined in the methodology section. Figure 1A and B shows specific immunoreactive staining for peptides derived from wild-type vasopressin and oxytocin genes. All six human hypothalami displayed intense vasopressin and oxytocin peptide expression in the SON and PVN and their localization was in agreement with the anatomical distribution of vasopressin and oxytocin cells described by Dierickx and Vandesande.⁴

In addition, clearly immunoreactive solitary magnocellular neurons were found in all subjects using the huva + 1 antiserum, which is directed against the vasopressin + 1 reading frame (Fig. 2A–C, Table 1). These mutant solitary neurons were present both in the dorsolateral and dorsomedial SON and in the PVN. Furthermore, vasopressin + 1-immunoreactive fibres were detected in these nuclei (Fig. 2B, C). No immunoreactive cells were observed with the huva + 1 antiserum after solid-phase absorption with the vasopressin + 1 synthetic peptide.

The highest numbers of immunoreactive neurons were found in subjects nos 9326 and 9341, i.e. 16 and nine positive cells respectively corresponding to a total number of 800 and 450 neurons expressing mutant vasopressin precursors in the entire hypothalamus (Table 1). No explanation can be given for

the individual differences on the basis of the clinical records. The distribution of huva + 1-immunoreactive cells in the SON ($\pm 75\%$) and the PVN ($\pm 25\%$) as determined for subject nos 9326 and 9341 (data not shown) is in agreement with the total number of vasopressin neurons in these two nuclei (Table 2). Using the humox + 1 antiserum, directed against the oxytocin + 1 reading frame, low numbers of heavily stained magnocellular neurons were detected in the PVN of subject nos 9326 and 9341 (Fig. 2D, Table 1). No cells expressing mutant oxytocin precursors were found in the SON, which is in agreement with the small number of oxytocin neurons in this nucleus (Table 2). The number of neurons expressing mutant vasopressin and oxytocin precursors in the six individuals did not show a relationship with age.

DISCUSSION

The immunocytochemical identification of mutant vasopressin and oxytocin precursors in magnocellular neurons of the human hypothalamus suggests that +1 frameshift mutations exist in vasopressin and oxytocin transcripts. These results provide the first evidence of somatic mutations in neurons of the human brain. From the total number of magnocellular vasopressin neurons in the hypothalamus, which is approximately 183,000 cells (Table 2), the

six individuals studied display 50–800 mutant vasopressin neurons. Assuming that the mutant vasopressin cells of the human hypothalamus are heterozygous, as has been shown for vasopressin neurons expressing mutant vasopressin precursors in the *di/di* rat,²⁴ the number of mutant vasopressin neurons would correspond to a +1 frameshift mutation frequency of the vasopressin gene of the order of 10^{-4} – 10^{-3} . If compared to the overall spontaneous mutation frequency in somatic cells as determined in human lymphocytes,^{2,27} which is of the order of 10^{-6} – 10^{-4} , the mutation frequency in the vasopressin locus is considerably higher. The occurrence of such mutations is remarkable, since mutagenesis is generally associated with meiosis and mitosis.²¹ Since the magnocellular neurons of the hypothalamus represent a post-mitotic cell population, as yet unidentified mutational mechanisms seem to be operative in this human neuronal cell type. In the rat, a post-mitotic mechanism is involved in mutagenesis, as shown by the linear increase in the number of mutant vasopressin neurons with age.^{5,24,26} The frameshift mutations are most likely introduced at the level of the gene. The high levels of frameshifted transcripts³ and precursors^{5,24,26} in a small number of solitary neurons and the heterozygous phenotype of these reverted cells²⁴ argue against a form of RNA “editing”. Whether similar mutational events

underlie the presence of mutant vasopressin and oxytocin precursors in the human hypothalamus remains to be determined.

The absence of an age-related increase in the number of neurons expressing human vasopressin in the +1 reading frame does not yet exclude such a process (as reported in rats^{5,24,26}) at the earlier stages of life. In the homozygous Brattleboro rat, we indeed did not find any further increase in the number of revertant neurons at the age of approximately 100 weeks.²⁶ It would therefore be of interest to assess the number of these cells present in the hypothalami of human subjects younger than 30 years.

No solitary cells immunoreactive for the mutant vasopressin precursor were found in the SCN, which comprises 4000–12,000 parvocellular vasopressin neurons in the age group up to 80 years.²² An absence of frameshifted vasopressin precursors was also observed in the SCN of the *di/di* and wild-type rats,^{5,24} which suggests that mutations are only present in vasopressin transcripts of magnocellular neurons. This may be explained by the highly active metabolic state of these latter cells. In the *di/di* rat, the mutation rate is decreased following inhibition of the activity of the vasopressin neurons, indicating that the mechanism of mutagenesis is affected by cellular activity.⁵

The lower mutation frequency of oxytocin neurons may be due to the absence of GAGAG motifs in the

Fig. 2. Representative immunocytochemical staining of solitary neurons of the human hypothalamus expressing mutant vasopressin (vasopressin +1, A–C) and oxytocin (oxytocin +1, D) precursors displaying the +1 reading frame. (A–C) Solitary neurons of the supraoptic nucleus stained with huva + 1 antiserum directed against vasopressin + 1 precursors. The arrowheads in B and C indicate fibres immunoreactive for vasopressin + 1 precursors. (D) Solitary neuron of the paraventricular nucleus stained with humox + 1 antiserum directed against oxytocin + 1 precursors. Note that intense immunoreactivity in all positive cells is exclusively present in the cytoplasm. Scale bar = 25 μ m.

Table 2. Total number of magnocellular neurons expressing vasopressin and oxytocin in the human supraoptic and paraventricular nuclei

	Vasopressin cell number	Oxytocin cell number	Vasopressin + oxytocin cell number
SON	138,906	18,054	156,960
PVN	44,000	53,200	97,200
SON + PVN	182,906	71,254	254,160

For the PVN, based upon Purba *et al.*,¹⁷ for the dorsolateral part of the SON on Purba (unpublished observations; SON, 49,240 vasopressin and 5460 oxytocin cells) and for oxytocin with the presumption that 90% of the cells of the central part of the dorsolateral SON contain vasopressin and 10% oxytocin.⁶ The exact numbers of vasopressin and oxytocin neurons in the medial part (dorsomedial and ventromedial) of the SON have never been determined. Based upon Morton,¹⁵ 69.7% of the SON is occupied by the dorsolateral part. Consequently, the medial part of the nucleus would contain 23,780 neurons. Since Dierickx and Vandesande⁴ report that 85% of the medial neurons contain vasopressin, the medial SON would contain 20,213 vasopressin and 3567 oxytocin neurons. Consequently, the entire SON contains 69,453 vasopressin and 9027 oxytocin neurons. Since the counting was only done on one side of the SON, the total number in the table has been multiplied by two. The accessory nuclei were not included in these countings. Since the numbers were partially obtained by presumption, no S.E.M. is given; however, in Purba *et al.*,¹⁷ S.E.M. values are given for vasopressin and oxytocin cell numbers in the PVN.

oxytocin nucleotide sequence. These motifs were shown earlier to be hotspots of frameshift mutations at two sites in vasopressin transcripts of the di/di rat.⁵ In contrast to the oxytocin gene, a GAGAG motif is present at positions 2086–2090¹⁹ in the human vasopressin gene. The low number of mutant-expressing oxytocin neurons as compared to vasopressin neurons in the human hypothalamus is in agreement with observations in wild-type rats in which no expression of +1 frameshifted oxytocin precursors could be detected (Evans *et al.*, unpublished results).

The detection in the human hypothalamus of several fibres immunoreactive for the mutant vasopressin precursor (Fig. 2B, C) indicates that the mutant product is axonally transported. Whether the mutant vasopressin precursor is subject to processing and the vasopressin peptide, located at the unaltered N-terminal region of the mutant precursor, is enzymatically cleaved cannot be concluded from these observations. There is a possibility that abnormal proteins impair cellular functions and activities as illustrated by the absence of immunoreactivity for several peptides (e.g., angiotensin II, 7B2) in magnocellular neurons of the di/di rat lacking the normal vasopressin precursor. Angiotensin II and 7B2 immunoreactivity reappears in solitary neurons reverted to the wild-type phenotype by +1 frameshift mutations.^{9,25} Apparently, the mutant vasopressin precursor of the di/di rat, which is arrested in the endoplasmic reticulum,²⁰ affects the expression of other proteins. The consequences of the expression of frameshifted vasopressin precursors in the human hypothalamus remains to be investigated.

The observation of +1 frameshift mutations in vasopressin neurons in the human brain and the presence of GAGAG motifs in many genes may imply that other neuronal genes also display higher somatic mutation frequencies than thought before. This may provide novel mechanisms of pathology in neurodegenerative diseases and aging.

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