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Effects of Water Deprivation on Neurokinin B Production by the Arginine-Vasopressin Neurons of Hypothalamic Paraventricular and Supraoptic Nuclei

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Abstract In order to clarify the role of neurokinin B (NKB), the dynamic changes in NKB expression and synthesis following water deprivation were examined in the arginine-vasopressin (AVP) neurons of hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei. In intact rats, NKB and AVP showed almost the same high level of immunohistochemical reactivity in the magnocellular neurons of the PVN and SON, as well as in the varicose fibers in the median eminence (ME). In contrast, NKB precursor peptide (NKBp) immunoreactivity in the SON and PVN were relatively weak. Five days after water deprivation, AVP and NKB immunoreactivity decreased drastically, while NKBp-immunoreactivity increased in both the PVN and SON magnocellular neurons. Reverse transcription-polymerase chain reaction analysis of control animals revealed high levels of AVP mRNA and substantial amounts of NKB mRNA in the SON. This was contrast to the relatively low levels of AVP mRNA and undetectable levels of NKB mRNA in the PVN. After five days of water deprivation, AVP mRNA in the PVN and NKB mRNA in both the PVN and the SON increased considerably.

These results indicate that synthesis and release of NKB, which colocalizes to AVP neurons, are enhanced by water deprivation in the same manner as AVP in the PVN and SON. Thus, NKB seems to be involved in the central control of body fluid levels. The results also suggest that the production rate of NKB under normal conditions in SON dominant.

Key words: Arginine-Vasopressin, Neurokinin B, Water deprivation, Hypothalamus

Introduction

Neurokinin B (NKB) is a member of the tachykinin peptide family[8]. It has been reported that NKB is mainly distributed in the central nervous system, where it is specifically located to the hypothalamus[12]. Also, intracerebroventricular injection[11)13)14)] as well as microinjection of the hypothalamic paraventricular nucleus (PVN)[4] with a specific NKB receptor (NK-3 receptor) agonist induces an increase in the level of plasma arginine-vasopressin (AVP). Therefore, NKB has been considered to take part in the central control of

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body fluid mediated by AVP release. In a previous double fluorescence immunohistochemical study, we demonstrated that almost all AVP neurons in the PVN and supraoptic nuclei (SON) contain NKB and possess NK-3 receptors on their cell bodies and dendrites[6]. However, the role of NKB in AVP neurons is unclear.

In order to clarify the significance of dynamic changes in NKB synthesis, we investigated the expression of NKB and AVP mRNA as well as examining immunohistochemical reactivities of NKB precursor (NKBp), NKB and AVP in the PVN and SON of rats subjected to water deprivation.

Materials and Methods

Animals and Tissue Preparation

Male Sprague-Dawley rats (250-300 g) were divided into two groups. In the water deprivation group, animals were deprived of water intake for five days, but were allowed free access to food. In the control group, animals were allowed free access to food and tap water. The animal experiments were approved by the Ethics Committee for Use of Experimental Animals at Saga Medical School. Animals of the experimental and control groups were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). For the immunohistochemical study, animals were fixed by transaortic perfusion of Zamboni's solution (2% paraformaldehyde, 15% saturated picric acid, 0.1 M phosphate buffer, pH 7.3), followed by perfusion with saline containing heparin (1 IU/ml). The brains containing the hypothalamus were dissected out and fixed by immersion in the same fixative for 24-48 h. Then, the brain blocks were immersed in 0.05 M phosphate buffer saline (PBS ; pH 7.3) containing 20% sucrose for 24 h, and cut into 20 μm thick sections using a freezing microtome.

For the reverse transcription-polymerase chain reaction (RT-PCR) method, animals were killed by decapitation. The brains containing the hypothalamus were removed, immediately immersed in ice cold PBS, and cut into 2 mm thick sections using a vibratome. Under a dissecting microscope, the PVN and the SON regions in the brain slices were identified, and tissue fragments (550 μm in diameter) containing the PVN and the SON were separatory punched out. The bilateral tissue fragments punched out from each nucleus were used as a sample per one animal.

Immunohistochemistry

For AVP, NKB and NKBp immunohistochemistry, the peroxidase-antiperoxidase (PAP) method was used. Free-floating sections were treated with 0.6% H_2O_2 and 0.4% Triton X-100 in PBS (for AVP) or 0.05 M tris buffer saline (TBS, pH 7.3) (for NKB and NKBp) for 15 min to block endogenous peroxidase activity. The sections were then preincubated with 2% normal swine serum for 90 min and subsequently incubated with rabbit anti-AVP (Chemicon, CA, U.S.A. ; diluted 1 : 2,000), anti-NKB (Marksteiner et. al.[10] ; diluted 1 : 500) or anti-NKBp (Marksteiner et. al.[10] ; diluted 1 : 1500) at 4°C for 48 h. Then, the sections were incubated with anti-rabbit swine IgG (DAKO, Glostrup, Denmark ; diluted 1 : 40) or, with rabbit-PAP (DAKO ; diluted 1 : 80) for 120 min each at room temperature. The sections were finally reacted with 0.02% diaminobenzidine tetrahydrochloride (Dotite, Kumamoto, Japan) in TBS, pH 7.6 containing 0.005% H_2O_2 for 15 min.

RT-PCR

Total cellular RNA was extracted from the rat brain tissues using ISOGEN (Nippon Gene,

Co., Toyama, Japan). The RNA samples were precipitated with ethanol, vacuum-dried, and resuspended in RNase-free water. Reverse transcription was carried in reactions containing 10 μg of total cellular RNA, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 0.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM of each dNTP (TaKaRa, Kyoto, Japan) and 200 units Super Script II RNase H-Reverse Transcriptase (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD, U.S.A.) in a final volume of 20 μl. After incubation at 42°C for 50 min, the samples were heated at 70°C for 15 min to terminate the reaction. Oligonucleotide primers were constructed from the cDNA sequences of rat AVP and NKB as described below.

The sequences of the AVP primers corresponded to bases 9-28 (sense strand) and dases 410-429 (antisense strand) of the vasopressin/neurophysin precursor cDNA sequence. The sequences of the NKB primer were 56-75 (sence strand) and 536-555 (antisense strand) of the NKBp sequence. The predicted sizes of the amplified AVP and NKB PCR products were 421 and 500 base pairs (bp), respectively.

Each transcription mixture was diluted 1 : 5 in RNase-free water and 2 μl aliquots were then transferred to fresh tubes for amplification. Each sample contained the upstream and downstream primers (0.2 μM each primer), which spanned the sequence for amplification, 200 μM of each dNTP (dATP, dCTP, dGTP, dTTP), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 0.01% (V/V) gelatin, and 2.5 units of ExTaq DNA polymerase (TaKaRa, Kyoto, Japan) in a final volume of 25 μl. The reaction mixture was amplified for 30 cycles in a Perkin Elmer Cetus thermal cycler (Norwalk, CT, U.S.A.). The amplification profiles consisted of denaturation for 1 min at 94°C; primer annealing for 30 s at 62°C, and extension for 1 min at 72°C.

Negative controls, consisting of reactions lacking the reverse transcription reaction products, were routinely included in PCR amplifications with both primer sets. In order to control for variations in mRNA levels between samples, GAPDH gene expression was used. No remarkable differences in levels of GAPDH mRNA were observed in all samples.

Results

In rats of the control group, AVP- and NKB-immunoreactivities were observed in magnocellular neurons and their associated varicose axon fibers in the SON and PVN, as previously reported[7] (Figs. 1A, C, 2A, C). A number of AVP- and NKB-immunoreactive varicosities or varicose fibers were observed in the internal zone of the ME (Fig. 3A, C). The number and distribution of these NKB-immunoreactive neuronal cell bodies and varicose fibers were comparable to those of AVP-immunoreactive neurons. In contrast to NKB, the cell bodies of magnocellular neurons in the SON displayed relatively weak NKBp-immunoreactivity, and only a few magnocellular neurons of the PVN were weakly immunoreactive for NKBp (Figs. 1E, 2E).

Five days after water deprivation, mean body weights of experimental rats were significantly reduced compared the weights of the rats before the start of the experiment and to the weight of control rats, indicating severe dehydration (Table 1).

In the water-deprived rats, AVP-immunoreactivity was extensively reduced in the PVN and the SON, and the magnocellular neurons showed only weak AVP-immunoreactivity in their cell bodies. Some of the immunoreactive varicose axon fibers in the nuclei and ME also displayed weak AVP-immunoreactivity (Figs.

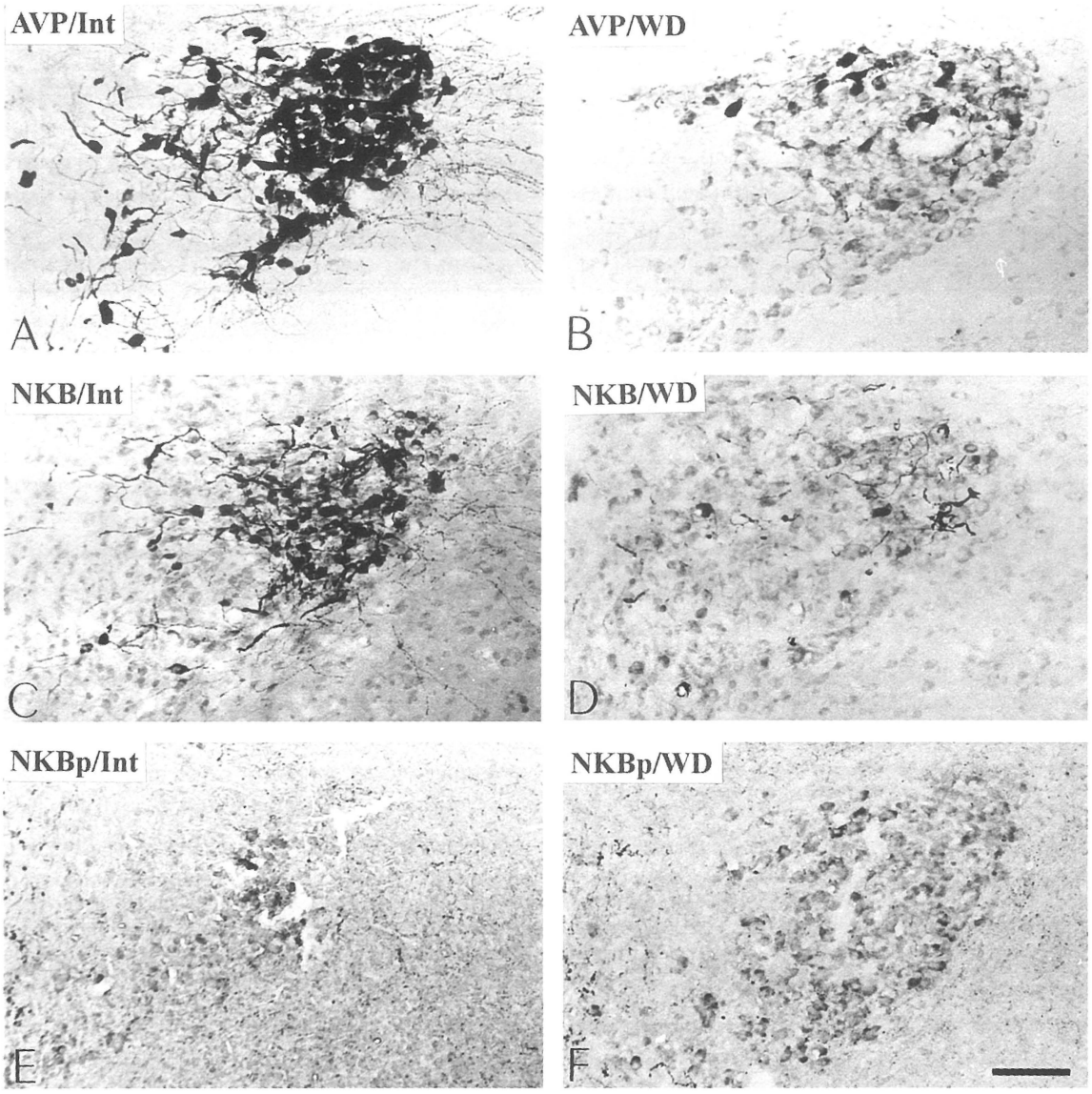

Fig. 1 Effects of water deprivation on arginine-vasopressin (AVP), neurokinin B (NKB) and the neurokinin B precursor (NKBp) in the hypothalamic paraventricular nucleus. In intact rats (Int), AVP (A)- and NKB (C)-immunoreactivities were observed in magnocellular neurons and their varicose axon fibers. Only a few weak NKBp (E)-immunoreactive magnocellular neurons were found (E). In water deprived rats (WD), AVP (B)- and NKB (D)-immunoreactivities were extensively reduced. NKBp (F)-immunoreactivity was increased, and distinctive immunoreaction was observed in the almost all cell bodies of magnocellular neurons after five days of water deprivation. Bar=100 μm

1B, 2B, 3B). Similarly, NKB-immunoreactivity in the magnocellular neurons and associated axon fibers was reduced to a level that was equal to that of AVP-immunoreactivity in the PVN, SON and ME of water deprived rats (Figs. 1D, 2D, 3D). NKBp-immunoreactivity was increased, and distinctive immunoreactive patterns were observed in almost all the cell

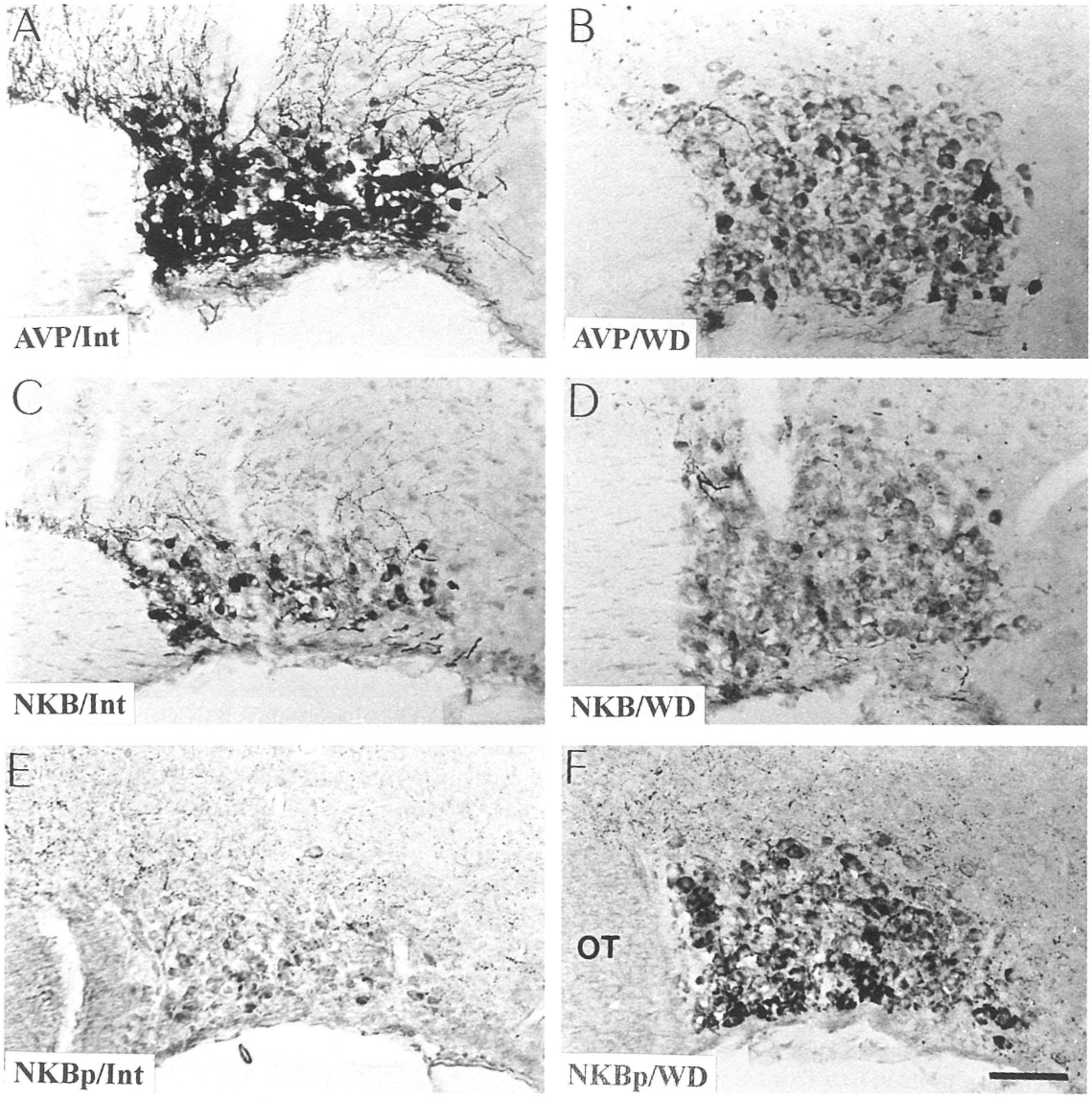

Fig. 2 Effects of water deprivation on arginine-vasopressin (AVP), neurokinin B (NKB) and neurokinin B precursor (NKBp) in the supraoptic nucleus. In intact rats (Int), a number of AVP (A)- and NKB (C)-immunoreactivities were observed in magnocellular neurons and their varicose axon fibers. Relatively weak NKBp (E)-immunoreactivities were observed in cell bodies of magnocellular neurons. In water deprived rats (WD), AVP (B)- and NKB (D)-immunoreactivities were extensively reduced. NKBp (F)-immunoreactivity was increased, and distinctive immunoreactivity was observed in the almost all cell bodies of magnocellular neurons after five days of water deprivation. Bar=100 μm

bodies of magnocellular neurons in both the SON and the PVN after five days of water deprivation (Figs. 1F, 2F).

AVP and NKB mRNA contents were compared by RT-PCR analysis of total RNA extracted from punched out tissues containing the SON and PVN. In control rats, AVP mRNA was detected both in the PVN and the

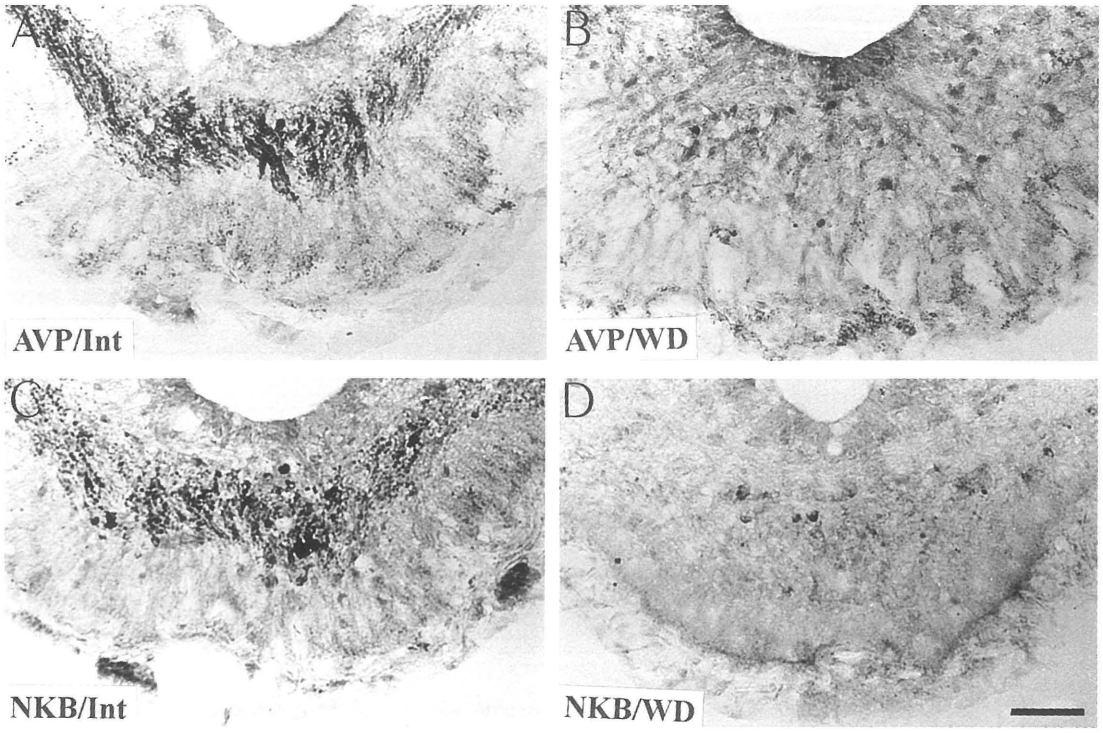

Fig. 3 Effects of Water deprivation on arginine-vasopressin (AVP) and neurokinin B (NKB) in the median eminence. In intact rats (Int), a number of AVP (A)- and NKB (C)-immunoreactive varicosities or varicose fibers were observed in internal zone. In water deprived rats (WD), AVP (B)- and NKB (D)-immunoreactivities were extensively reduced. Bar=100 μm

Table 1 Change in body weight following water deprivation for five days (W. D. 5) in the rats.

	0 day	5days
control	226.7±7.2 g	267.0±7.8 g*
W. D. 5	223.3±8.1 g	159.3±9.8 g*,**

Values are the means±S.E. of the values obtained from measurements in 6 to 12 rats.
*, $P<0.05$ compared with 0 days value, **, $P<0.05$ compared with control 5 days.
Change of body weight was analyzed by using student's t-test.

SON, and the AVP mRNA content of the SON was much higher than that of the PVN (Fig. 4A, C). NKB mRNA was only detected in the SON and was visibly absent from the PVN (Fig. 4E, G). Five days after water deprivation, AVP mRNA content in the PVN was markedly increased (Fig. 4B). NKB mRNA content also increased strongly in the SON and was detectable in the PVN (Fig. 4F, H).

Discussion

The present study shows that changes in NKB immunoreactivity in the AVP neurons of the PVN and SON match similar changes in the AVP-immunoreactivity of these neurons following water deprivation. Five days after water deprivation, AVP-immunoreactivity was markedly reduced in the cell bodies and varicose axon fibers of magnocellular AVP neurons in the PVN, while AVP mRNA content increased in the PVN. These results are in agreement with previous reports which indicated that AVP content in AVP neurons decreases

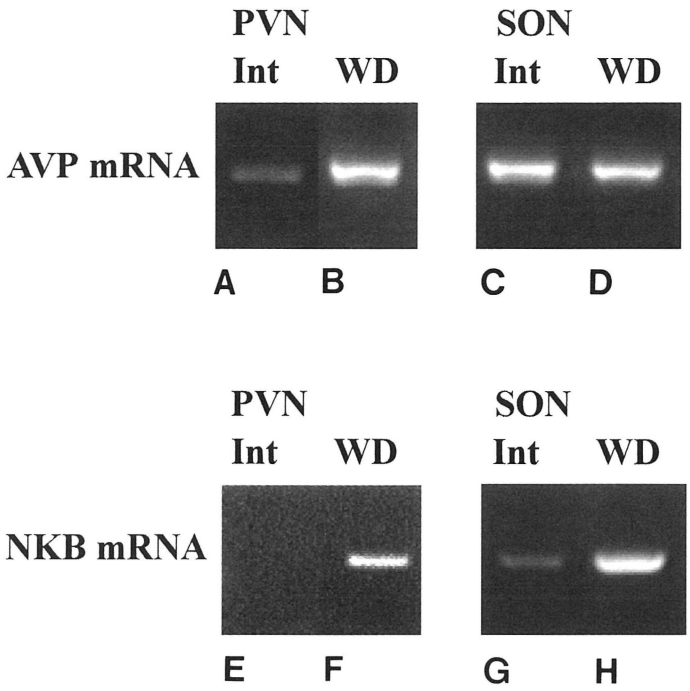

Fig. 4 Effects of water deprivation on arginine-vasopressin (AVP) and neurokinin B (NKB) mRNA in the supraoptic (SON) and hypothalamic paraventricular nuclei (PVN). In intact rats (Int), AVP mRNA was detected both in the SON (C) and the PVN (A). NKB mRNA was detected in the SON (G), but not in the PVN (E). In water deprived rats (WD), AVP mRNA content in the PVN (B) markedly increased to a higher level than that of the SON (D), which failed to show any remarkable change. NKB mRNA content also increased extensively in the SON (H) and became detectable in the PVN (F).

in rats deprived of water for three days[5)7)] and that, AVP mRNA content increases after 2.5 or 3 days of water deprivation[7)9)]. On the other hand, after a shorter period (48 h) of water deprivation, both AVP-immunoreactivity and AVP mRNA content were shown to increase in the PVN and SON[1)]. Many studies have reported that plasma AVP concentration is significantly increased by water deprivation[1)4)9)]. Taken together with these results, the present findings suggest that both production and release of AVP are enhanced by water deprivation but that the release of AVP is far superior to the rate of AVP production, resulting in an overall drop in AVP storage in AVP neurons. This notion is also supported by our observation that water deprivation caused a remarkable reduction in the AVP-immunoreactivity of the varicose axon fibers descending through the ME to the posterior hypophysis.

In the present study, NKB mRNA content of the PVN and SON as well as the NKBp-immunoreactivity of the magnocellular neurons increased remarkably after water deprivation.

These results indicate that NKB synthesis is positively regulated by water deprivation. On the other hand, the NKB-immunoreactivity of the cell bodies of magnocellular PVN and SON neurons, as well as their varicose axon fibers in the ME decreased in the same way as AVP-immunoreactivity. Although a possibility that lower regulation of the conversion from NKBp to NKB cause the decrease of the NKB-immunoreactivity can not be completely ruled out, it seems likely that the synthesis of the NKB peptide is largely compensated by the loss of the peptide from the cell bodies during water deprivation as described for AVP. If this were the case, these findings would indicate that the release rate of NKB from the cells is higher than the conversion rate of NKBp to NKB, and that this conversion rate is somewhat slower than the synthesis rate of NKBp, which would result in accumulation of NKBp in the cell bodies of the PVN and SON magnocellular neurons.

In the control animals, NKB mRNA expression and NKBp-immunoreactivity of the PVN neurons were far lower than those of the SON neurons, in which substantial amounts of NKB mRNA and NKBp peptide were detected. Nevertheless, the strength of NKB immunoreactivity of PVN neurons was similar to that of SON neurons. These results indicate that the synthesis of NKBp as well as the release of NKB from PVN neurons is much slower than those of SON neurons under normal conditions, and that NKB is stored in the PVN. Thus, under normal conditions, it is conceivable that SON neurons mainly work to produce and release NKB and that, under conditions of water deprivation, both SON and PVN neurons work only for NKB release. In relation to this, it is interesting that the expression of AVP mRNA was also lower in the PVN than in the SON.

At present, we have no valid explanation for the functional role of the concomitant release of NKB and AVP. It is possible that NKB is neurosecreted into the vascular system at the posterior pituitary and that it acts on the kidney, since NK-3 receptors have been found in the kidney[2]. NK-3 receptors have also been found in the AVP/NKB neurons in the PVN and the SON[3], and microinjection of senktide, a NK-3 receptor specific agonist, into the PVN induced an increase in plasma AVP[6]. Therefore, it is also possible that NKB release regulates the neurosecretion of AVP via NK-3 autoreceptors.

In conclusion, the present study has shown that the synthesis and release of NKB, which colocalizes to AVP neurons in the PVN and the SON, are positively regulated in the same manner as AVP by water deprivation. This suggests that NKB synthesis in AVP neurons takes part in the central control mechanisms governing body fluid levels. It was also demonstrated that NKB and AVP syntheses of SON neurons are superior to those of PVN neurons in normal animals, suggesting that these two distinct AVP secreting neuron groups play different roles in fluid control.

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視床下部室傍核および視索上核のアルギニン-バソプレシン神経におけるニューロキニンB産生に対する絶水の影響

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ニューロキニンB（NKB）の役割を調べる目的で，視床下部室傍核および視索上核のアルギニン-バソプレシン（AVP）神経におけるNKB発現および産生に対する絶水の影響を調べた．

正常ラットでは，視床下部室傍核の大細胞領域，視索上核および正中隆起において，多くの強いAVP免疫陽性反応およびNKB免疫陽性反応が観察された．それに対して，NKB前駆体ペプチド（NKBp）免疫陽性反応は，室傍核および視索上核において，弱い反応が観察された．

5日間絶水したラットでは，視床下部室傍核の大細胞領域および視索上核において，多くのAVP免疫陽性細胞とNKB免疫陽性細胞の陽性反応が共に減弱した．それに対して，NKBp免疫陽性反応は，室傍核および視索上核において，共に強い反応が観察された．

RT-PCRによる実験では，正常ラットの視索上核で，AVP mRNAおよびNKB mRNAが検出されたが，室傍核ではAVP mRNAのみが検出され，NKB mRNAは検出されなかった．

5日間絶水したラットでは，視索上核のNKB mRNAおよび室傍核のAVP mRNAが増加し，室傍核のNKB mRNAが検出できるようになった．

以上の結果から，視床下部室傍核および視索上核のAVP神経に共存するNKBの産生と放出は，AVPと同様に絶水によって増加することが明らかとなった．そのことから，NKBは中枢性の体液調節に関与する可能性が示唆された．また，正常時においてNKBの産生は，視索上核が優位であることが示唆された．