Arginine Vasopressin- and Oxytocin-like Immunoreactive Neurons in the Hypothalamic Paraventricular and Supraoptic Nuclei of Streptozotocin-Induced Diabetic Rats*

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Summary. This study describes ultrastructural and morphometric changes in the arginine vasopressin (AVP)-like immunoreactive and oxytocin (OT)-like immunoreactive neurons in the hypothalamic paraventricular nuclei (PVN) and supraoptic nuclei (SON) of streptozotocin-induced diabetic rats at 1-12 months post-diabetes. At 1-6 months post diabetes, both AVP-immunoreactive and OT-immunoreactive neuronal somata were hypertrophied in the PVN and SON. These neuronal somata contained highly dilated rough endoplasmic reticulum in the cytoplasm. The reaction product for AVP as well as OT localization was dispersed throughout the cytoplasm and cell nucleus, but not within the nucleolus. Moreover, the reaction product appeared to be studded onto the ribosomes on dilated cisterns of the endoplasmic reticulum. At 9-12 months post-diabetes, both AVP-immunoreactive and OT-immunoreactive dendrites contained dilated endoplasmic reticulum, autophagic vacuoles, lipid bodies, microtubules, membranous bodies and occasionally swollen mitochondria. Labelled hypertrophied axonal profiles containing neurosecretory granules, autophagic vacuoles, membranous bodies and tubulovesicular elements were also observed in the neuropil. Morphometric study showed that both AVP-immunoreactive and OT-immunoreactive neuronal somata of the PVN and SON in the diabetic rats were markedly hypertrophied at all the time intervals examined. It is concluded that the morphometric changes observed represent hyperactivity of both AVP- and OT-immunoreactive neurons, while the concurrent ultrastructural changes observed at later stages may be indicative of degeneration.

The magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei in the hypothalamus of the rat and numerous other species including humans, are known to synthesize two types of neurohormones, arginine vasopressin (AVP) and oxytocin (OT), which are present in separate neuronal populations (SWAAB et al., 1975; VANDESANDE and DIERICKX, 1975; SILVERMAN and PICKARD, 1983). The AVP- and OT-producing magnocellular neurons in the PVN and SON have been reported to be activated by prolonged osmotic stimulation resulting in an increase in the synthesis of neurohormones (FRANCO-BOURLAND and FERNSTROM, 1981; MAJZOUB et al., 1983; BURBACH et al., 1984; VAN TOL et al., 1987). Diabetes mellitus has been shown to be associated with hyperosmotic dehydration (YOUNG, 1969; FERNSTROM et al., 1990). Recently, syntheses of AVP and OT in the hypothalamus have also been observed to increase in male rats 10 days after streptozotocin-induced diabetes (FERNSTROM et al., 1990). Earlier, LOESCH et al. (1988) have reported on the ultrastructural changes in the AVP- and OT-containing neuronal profiles of the hypothalamo-neurohypophysial system in male rats 8 weeks after streptozotocin-induced diabetes. However, their report did not provide any morphometric or morphological evidence for structural changes in long-term diabetes. With this in mind, a systematic investigation was carried out to examine the morphometric as well as morphological changes in AVP- and OT-like immunoreactive neurons in the PVN and SON of streptozotocin-induced diabetic rats at survival periods ranging from 1 to 12 months.

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MATERIALS AND METHODS

A total of 80 adult male Wistar rats (200-250 g) was used in the present study. The rats were divided into 5 groups, each consisting of eight streptozotocin-injected rats and eight controls (injected with normal saline). Four diabetic rats and four controls from each group were used for each neuropeptide analysis.

Diabetes was induced in aseptic conditions. Under light ether anaesthesia, each rat was given a intravenous injection of either streptozotocin (60 mg/kg body weight, freshly dissolved in 0.01 M citrate buffer, pH 4.5) or normal saline (0.9% sodium chloride), either one via the right external jugular vein, using a 1 ml syringe. Blood glucose was measured using a blood glucometer (Ames Glucometer II) before induction, 24 h after induction and just before sacrifice. Rats were considered diabetic when their blood glucose concentrations exceeded 400 mg/dl. The rats were maintained with access to water ad libitum and standard rat feed (Milne Feeds, Welshpool, W. Australia). Both diabetic and control rats were perfused at 1, 3, 6, 9 and 12 months post-diabetes. Before perfusion, each rat was anaesthetized with 1-1.5 ml of chloral hydrate (70 mg/ml). Tracheostomy was carried out and artificial respiration maintained with a Harvard rodent ventilator (Model 683). Five min before perfusion, 1000 units of heparin and 1 ml of 1% sodium nitrite per kg body weight were given by intracardiac injection. Each animal was then perfused through the left cardiac ventricle with 100 ml of Ringer’s solution (pH 7.4) followed by 500 ml of fresh fixative (4% formaldehyde + 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). The brains were removed immediately and immersed in the same fixative for 4 h at 4°C. These were then transferred into 0.1 M phosphate buffer with 20% sucrose and stored overnight at 4°C.

The following day, 40 μm coronal sections through the hypothalamus were cut on an Oxford vibratome and collected in incubation-wells containing phos-
phate buffered saline (PBS, pH 7.4). The sections were divided serially into two groups and separately processed for light microscopy or electron microscopy. They were processed for immunocytochemistry using the free floating technique with avidin/biotin peroxidase immunostaining (Hsu et al., 1981). Initially, the free sections were rinsed in PBS and incubated in 4% normal goat serum (NGS) for 1 h at room temperature. The sections were washed in 3 changes of PBS (10 min each) and subsequently incubated for 40 h at 4°C with either rabbit AVP antiserum (Peninsula Corp., California) at a 1:1000 final dilution in 1% NGS or rabbit OT antiserum (Incstar Corp., Minnesota) at a 1:500 final dilution in 1% NGS. Subsequent antibody detection was carried out using the vectastain ABC-Kit (PK-4001, Vector Lab., Burlingame, California) against rabbit IgG with 3,3'-diaminobenzidine as a peroxidase substrate. The PBS for light microscopic preparations contained 0.1% Triton X-100, whereas that for electron microscopic preparations contained no Triton X-100. Sections for light microscopy were mounted on gelatinized glass slides, air-dried, dehydrated in a graded series of ethanol and coverslipped with Permount. For electron microscopy, the free sections were osmicated for 15-30 min at 4°C and dehydrated in a graded series of ethanol and flat-embedded in an Araldite mixture. Areas containing the PVN and SON were trimmed out from the embedded-sections and remounted on Araldite filled capsules for ultrathin sectioning. Ultrathin sections were cut with a Reichert OMU4 ultramicrotome, stained with lead citrate only and viewed in a JEM 1200CX or Philips 400T electron microscope. Immunostaining was abolished by incubating the sections in 1% NGS without the antiserum or by pre-incubation of the diluted antiserum with 200 μg/ml of either synthetic AVP or synthetic OT.

Morphometric study

Light microscopic sections containing PVN and SON were viewed and analysed separately under an invert-
ed microscope (Olympus IMT-2) which was linked to Jandel Video Analysis System (JAVA Software, Version-1.20). One hundred nucleated AVP-like immunoreactive AVP-IR and OT-like immunoreactive OT-IR neurons from both the controls and diabetic rats of each group (i.e. 1, 3, 6, 9 and 12 months) were chosen randomly. Random sampling of sections was achieved by using every third section (40 um each) from 4 animals. Their neurons were outlined and the cross-sectional areas were measured. Mean values were taken for all the measurements. The data obtained were expressed as Mean ± Standard Deviation (n=100) and then subjected to statistical analysis using Student’s ‘t’-test. The frequency distributions for the cross-sectional areas of the AVP and OT neurons in the PVN and SON of both control and diabetic rats were calculated.

RESULTS

The rats developed diabetes mellitus with a blood glucose level exceeding 400 μg/dL within 24 h after streptozotocin administration, and remained diabetic until the time of sacrifice. Most of the diabetic rats developed cataracts after 6 months.

Qualitative analysis

Control animals

The structural features of the AVP-immunoreactive and OT-immunoreactive neurons in the PVN and SON were in general agreement with those described by other workers (LECLERC and PELLETIER, 1974; KRISCH, 1980; PIEKUT, 1983; SILVERMAN et al., 1983; LOESCH, 1985; LOESCH et al., 1988). Both AVP- and OT-immunoreactive magnocellular neurons (Figs. 1, 2) were observed in the PVN and SON. Intense staining was detected in the cell nucleoplasm and the cytoplasm of cell bodies and dendritic processes of the neurons. However, the nucleolus appeared to be unlabelled (Figs. 1, 2). Moreover, light staining was observed in the perinuclear zone that contained Golgi saccules. In labelled dendrites, the reaction product was closely associated with the parallel arrays of neurotubules. Most of the labelled dendrites were postsynaptic to unlabelled axon terminals containing small spherical agranular vesicles. Very few labelled axon profiles were observed.
1-6 months post-diabetes
At this stage, both AVP-immunoreactive and OT-immunoreactive neuronal somata were hypertrophied in the PVN and SON (Figs. 3, 4). These neuronal somata showed numerous vacuoles which had possibly developed from distended cisterns of rough endoplasmic reticulum. Most of the cytoplasmic organelles such as the Golgi saccules, lysosomes and mitochondria in the vacuolated somata appeared normal (Fig. 4). The reaction product was dispersed throughout the cytoplasm and cell nucleus, but not within the nucleolus. In the cytoplasm, the perinuclear zone that contained Golgi saccules was lightly stained. Moreover, the reaction product appeared to be studded onto the ribosomes of the dilated endoplasmic reticulum. It was difficult to identify the reaction product in electron-dense lysosomal droplets. Intense staining was observed in the cell nucleus of some immuno-positive neurons. In the neuropil, both AVP- and OT-immunoreactive vacuolated dendrites were also observed.

9-12 months post-diabetes
At this stage, vacuolated and necrotic dendrites as well as axon profiles were observed in both PVN and SON. Both AVP- and OT-immunoreactive dendrites appeared to be hypertrophied, and contained dilated endoplasmic reticulum, autophagic vacuoles, lipid bodies, microtubules, membranous bodies and occasionally swollen mitochondria (Figs. 5, 6). The cytoplasm of these profiles was intensely stained by the reaction product, but the vacuoles present were not labelled. Some of the hypertrophied AVP-immunoreactive neuronal profiles contained large vacuoles together with labelled strands of endoplasmic reticulum (Fig. 7). In these neuronal profiles, the dilated strands of endoplasmic reticulum appeared to have anastomosed with one another to form large vacuoles. Other labelled hypertrophied neuronal profiles containing neurosecretory granules, autophagic vacuoles and membranous bodies were also observed in the neuropil (Fig. 8).

Morphometric analysis
Cross-sectional cell areas of AVP-immunoreactive and OT-immunoreactive neurons in the PVN and SON of streptozotocin-induced diabetic rats as well as controls were measured at survival periods of 1, 3, 6,
and 12 months. The results of this morphometric study show that both AVP-immunoreactive and OT-immunoreactive neurons of the PVN and SON in the diabetic rats were markedly hypertrophied at all the time intervals studied (Figs. 9 a-d). The frequency distributions for the cross-sectional cell areas of the AVP-immunoreactive and OT-immunoreactive neurons in the PVN and SON of both control and diabetic rats are illustrated in Figures 10 and 11. From the histograms, it is evident that, at all the time intervals studied, the majority of the AVP-immunoreactive neurons in the PVN and SON of diabetic rats were hypertrophied. OT-immunoreactive neurons of the PVN and SON in diabetic rats were also hypertrophied, but the number of hypertrophied neurons was less in comparison with AVP-immunoreactive neurons in the diabetic rats.

**DISCUSSION**

The present morphometric investigations revealed that both AVP-immunoreactive and OT-immunoreactive neurons of the diabetic rats were markedly hypertrophied at all the time intervals studied in comparison with the age-matched controls. The cellular hypertrophy probably reflects increased protein synthesis as well as greater electrical activity in the neurons (Sokol and Valtin, 1965). Moreover, both AVP- and OT-immunoreactive neurons in the diabetic rats showed vacuoles of various sizes which possibly were derived from the dilated endoplasmic reticulum. This distension has been attributed to the hypersecretion of neurohormones, although extreme cases of vacuolation may indicate exhaustion (Kalimo, 1975). Dellmann (1973) reported in his review article that the appearance of vacuoles and tubulovesicular elements indicates hyperactivity of the hypothalamo-
neurohypophyseal complex. These findings are more plausible in view of the observed increase in hypothalamic levels of AVP and OT in male rats 10 days after streptozotocin-induced diabetes (FERNSTROM et al., 1990).

Neuronal hypertrophy and distension of the endoplasmic reticulum have been reported in the hypothalamic magnocellular neurons of osmotically stimulated rats (HILLARP, 1949; ZAMBRANO and DEROBERTIS, 1966; TALANTI, 1971; KALIMO and RINNE, 1972; KRISCH, 1974). Measurements of the biosynthetic activity of the AVP- and OT-synthesizing neurons by in vivo incorporation of (35S) cysteine into AVP and OT molecules resulted in an increase in AVP and OT biosynthesis after osmotic dehydration (FRANCO-BOURLAND and FERNSTROM, 1981). Moreover, the increased biosynthetic activity has been shown to be accompanied by an increase in the AVP mRNA (BURBACH et al., 1984; 1986) and OT mRNA (VAN TOL et al., 1987) levels of the PVN and SON, indicating that the gene expression of AVP and OT is altered by osmotic stimulus. Experimentally induced diabetic rats have been shown to exhibit serum hyperosmolality (FERNSTROM et al., 1990), hyperosmotic dehydration (YOUNG, 1969) as well as neuronal changes in the hypothalamic neurohypophyseal system (LOESCH et al., 1988). Since all the diabetic rats in the present study displayed polyuria, the activation of AVP-immunoreactive and OT-immunoreactive neurons in the PVN and SON could be the result of osmotic stress caused by hyperglycaemia. However, AVP- and OT-mRNA levels in the hypothalamic magnocellular neurons of streptozotocin-induced diabetic rats have not yet been ascertained to determine whether the gene expression of AVP and OT is altered by hyperosmotic dehydration associated with hyperglycaemia.

It ought to be mentioned that the frequency of hypertrophied OT-immunoreactive neurons encountered in the present study in the PVN and SON of the diabetic rats was less than that of AVP-immunoreactive neurons. This implies that AVP-immunoreactive neurons could be more sensitive to osmotic stress resulting from hyperglycemia than OT-immunoreactive neurons. The exact mechanism by which hyper-

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**Fig. 7.** An extensively vacuolated AVP-immunoreactive neuronal profile in the SON showing unlabelled vacuoles and labelled strands of endoplasmic reticulum (arrows). In this profile, the reticulum strands appear to be anastomosed with one another to form a large vacuole. 9 months post-diabetes. ×8,700

**Fig. 8.** An AVP-immunoreactive hypertrophied neuronal profile showing neurosecretory granules, autophagic vacuoles, membranous bodies and tubulovesicular elements in the PVN. 9 months post-diabetes. ×6,100
glycaemia causes the morphological changes observed in the present study is obscure, however. Recently it has been reported that in severe insulin deficiency, glucose cannot permeate the osmoreceptor in the hypothalamus, leading to an osmotic gradient between the intracellular and extracellular fluids, and thus becomes an effective osmotic stimulus for vasopressin release (YOKES and ROBERTSON, 1985). This view implies that the osmoreceptor is dependent on insulin for glucose transport. Although it is believed that the cells of the central nervous system do not require insulin for the transport and metabolism of glucose, recent studies have shown high-affinity receptors for circulating insulin in the circumventricular organs and areas of the anterior hypothalamus (VAN HOUTON et al., 1979; CORP et al., 1986) where the osmoreceptor is thought to reside. More recently, it has been reported that AVP and OT increase

Fig. 9 a-d. Histograms showing the mean cross-sectional area of the AVP- and OT-immunoreactive neurons in the PVN and SON of control and diabetic rats at different time intervals (1, 3, 6, 9, 12 months). Each value is mean ± S.D. Control vs Diabetes: * P<0.01; ** P<0.001.
Fig. 10. Size-frequency distribution (cross-sectional area in μm²) of the AVP- and OT-immunoreactive neurons in the PVN of control and diabetic rats at 1, 3, 6, 9 and 12 months post-diabetes.
Fig. 11. Size-frequency distribution (cross-sectional area in \( \mu \text{m}^2 \)) of the AVP- and OT-immunoreactive neurons in the SON of control and diabetic rats at 1, 3, 6, 9 and 12 months post-diabetes.
insulin amounts from the pancreatic islet cells of the mouse by activating specific receptors (Gao and Henquin, 1993). This suggests the interesting possibility that the neurohormones (both AVP and OT) and pancreatic insulin may exist as a complex feedback loop. Hence it is postulated that hypoinsulinemia could have caused the hyperglycaemia and hyperosmolality which could then be perceived by the hypothalamic osmoreceptors resulting in the hyperactivity of the AVP- and OT-immunoreactive neurons, which could feedback to stimulate insulin release in the pancreas. Since the pancreatic ß cells were destroyed in the streptozotocin-induced diabetic rats (Rakieten et al., 1963; Junod et al., 1967; Rerup, 1970), there could be damage to such a feedback loop, resulting in metabolic dysfunction and thereby contributing to the degenerative changes observed in the present study 6 months after the onset of diabetes.

It is concluded that, although the morphometric changes observed represent the hyperactivity of both AVP- and OT-immunoreactive neurons, the ultrastructural changes observed at later stages may be indicative of degeneration. Moreover, it is suggested that the hyperosmotic stress resulting from chronic dehydration in the diabetic rats may be responsible for some of the ultrastructural changes observed in the present study.

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