

Apoptosis of vasopressinergic hypothalamic neurons in chronic diabetes mellitus

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The hyperosmolality associated with diabetes mellitus triggers an increase in neuronal activity and vasopressin production within magnocellular neurosecretory cells (MNCs) of the hypothalamic supraoptic nucleus (SON). In this study, we examined the effect of chronic diabetes on the function and survival of these neurons. After 6 months, but not 6 weeks, of streptozotocin (STZ)-induced diabetes, we observed an increase in the appearance of small hyperchromatic neurons and a decrease in SON neuronal density. A subpopulation of neurons within the SON at this time point demonstrated positive staining for cleaved caspase-3 and TUNEL, two markers of apoptosis. In addition, the number of vasopressin-positive neurons was decreased. Markers for apoptosis did not colocalize with vasopressin immunopositivity; this was probably due to a diabetes-induced degenerative process causing downregulation of vasopressin expression or depletion of neuropeptide. Although the phenotypes of the apoptotic neurons were not identified, other SON neurons including oxytocin-producing neurons are unlikely to be affected by chronic hyperglycemia. Microglial hypertrophy and condensation were also observed in the 6-month diabetic SON. Although upregulation of vasopressin production in response to acute hyperosmolality is adaptive, prolonged overstimulation of vasopressin-producing neurons in chronic diabetes results in neurodegeneration and apoptosis.

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Introduction

The diabetic state results in an increase in plasma vasopressin levels primarily as a result of hyperglycemia-induced hyperosmolality. This effect is observed in humans with diabetes mellitus and in experimental models of diabetes (Bankir et al., 2001; Brooks et al., 1989; Van Itallie and Fernstrom, 1982). While elevated

vasopressin levels may be adaptive in the short term, there is evidence suggesting that prolonged elevated levels of vasopressin can cause chronic renal hyperfiltration, albuminuria, and hypertrophy, and ultimately contribute to diabetic nephropathy and renal failure (Ahloulay et al., 1999; Bardoux et al., 1999).

Vasopressin is synthesized in magnocellular neurosecretory cells (MNCs) within the supraoptic nucleus (SON) of the hypothalamus and released from the neurohypophysis in response to elevated plasma osmotic pressure. In diabetes, there is an increased demand on these neurons to produce enough vasopressin to maintain euvolemia and minimize fluid shifts between intracellular and extracellular environments.

Experimental evidence suggests that chronic overactivation of these neurons may have adverse effects on their survival. Ultrastructural studies first demonstrated that in streptozotocin (STZ)-induced diabetes there is an age-dependent progressive degeneration of SON neurons including the appearance of abnormal somata, dendrites, axonal profiles, and cytoplasmic vacuoles (Dheen et al., 1994). This same study reported a significant increase in mean cross-sectional area and cross-sectional nuclear area of diabetic SON neurons compared to controls (Dheen et al., 1994). Nuclear and somal hypertrophy is suggestive of increased mRNA and protein synthesis, which reflects the increased demand for and production of vasopressin in diabetic MNCs (Crespo et al., 1990). While the diabetic SON is indeed characterized by hypertrophic neurons, Luo et al. (2002) recently observed that in addition, shrunken and hyperchromatic neurons are also present.

Changes in the transcription of several genes in diabetic SON neurons have been examined. After 4 months of STZ-induced diabetes, vasopressin levels in SON neurons remain elevated compared to controls, but there is less upregulation compared to earlier time points (Luo et al., 2002), suggesting that in chronic diabetes, a functional insufficiency of vasopressin production may develop. The expression of voltage-gated sodium channels Nav1.2 and Nav1.6, which support the bursts of action potentials that release vasopressin, is upregulated in diabetic SON neurons (Klein et al., 2002). Likewise, the glutamate receptor NMDAR and neuronal nitric oxide synthase (nNOS), which plays a role in the modulation of secretion of vasopressin, are also upregulated in diabetic SON neurons (Kadowaki et al., 1994; Luo et al., 2002;

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Serino et al., 1998). In these neurons, NMDAR and nNOS overactivation may be excitotoxic and result in degeneration or apoptosis (Brecht et al., 2001). Following STZ-induced diabetes, neuronal apoptosis has in fact been demonstrated in both hippocampal neurons (Li et al., 2002) and retinal ganglion cells (Barber et al., 1998; Zeng et al., 2000).

In the present study, we asked whether chronic STZ-induced diabetes leads to apoptotic cell death in vasopressinergic neurons of the SON. We report here that in chronic STZ-induced diabetes, there is increased expression of activated caspase-3 and markers of DNA degradation within SON neurons compared to age-matched normoglycemic controls. These changes were evident after 6 months of diabetes, but not after 6 weeks of diabetes. We propose that long-term neuronal overstimulation in chronic diabetes induces a heterogeneous response in the SON: some neurons degenerate or undergo apoptosis while others continue to produce and secrete vasopressin.

Materials and methods

Induction of diabetes

Adult male Sprague–Dawley rats (225–250g) were injected with streptozotocin (STZ, 60 mg/kg ip, Sigma, St. Louis, MO). Animals were housed in a 12 h light–dark cycle with free access to water and food. Plasma glucose (Encore Glucometer, Miles Inc., Elkhart, IN) was measured at 6 weeks and 6 months postinjection and compared to control rats injected with saline. In total, 18 animals were used: five diabetic and four control rats at 6 weeks, and five diabetic and four control rats at 6 months. All experimental manipulations were carried out in accordance with National Institutes of Health guidelines for the care and use of laboratory animals, and all animal protocols were approved by the Yale University Institutional Animal Care and Use Committee.

Neuronal morphology

All rats were anaesthetized with ketamine/xylazine (80/5 mg/kg ip) and then underwent intracardiac perfusion with 0.01 M PBS followed by a 4% solution of cold buffered paraformaldehyde. Brains were removed, postfixed, and cryoprotected in 30% sucrose in 1 M phosphate buffer solution (PBS), and coronal cryosections (10 μ m) of cortex containing the SON were cut. Sections were stained with Cresyl Violet (Sigma). Neuronal area and diameter were determined by outlining individual neurons whose nuclei were visible in the plane of section using IPLab v3.0 Image Processing software (Scanalytics, Fairfax, VA). Counts of neurons within the SON were performed by arbitrarily selecting an area within the SON and averaging multiple counts ($n = 6$) of neurons from the 6-week and 6-month control and diabetic animals.

Immunocytochemistry

Sections were incubated in blocking solution (5% normal goat serum and 1% BSA in PBS) containing 0.1% Triton X-100 and 0.02% sodium azide at room temperature for 30 min, then incubated with antibodies to either vasopressin (1:1000, Oncogene Research Products, San Diego, CA), cleaved caspase-3 (1:50, Cell

Signaling Technology, Beverly, MA), glial fibrillary acidic protein (GFAP) (1:50, Chemicon, Temecula, CA), or OX-42 (CD11b/c) (1:50, BD Biosciences, Franklin Lakes, NJ) overnight at 4°C. Sections reacted with the caspase-3 antibody were washed in PBS and incubated with biotinylated goat anti-rabbit serum (1:1000, Sigma) in blocking solution for 3 h, then washed in PBS, and incubated in ExtrAvidin-HRP (1:1000, Sigma) in blocking solution for 3 h. These sections were then washed again in PBS and exposed to heavy metal enhanced 3,3'-diaminobenzidine-4HCl in 1 \times peroxide substrate buffer (Pierce, Rockford, IL) for 7 min, washed in PBS, and mounted with Aqua-Polymount (Polysciences, Warrington, PA). Alternatively, for double labeling experiments utilizing fluorescent labels, goat anti-rabbit IgG-Cy3 (1:2000, Amersham, Piscataway, NJ) and goat anti-mouse IgG-Cy2 (1:1000, Molecular Probes, Eugene, OR) secondary antibodies were used. Vasopressin signal was detected using rhodamine epifluorescence illumination (emission wavelength: 570–620 nm) and GFAP and OX-42 signals were detected using fluorescein epifluorescence illumination (emission wavelength: 516–565 nm).

For detection of terminal d-UTP nick end labeling (TUNEL) of genomic DNA, 10- μ m coronal sections of SON that had been previously reacted with vasopressin antibody were incubated sequentially in: PBS, 3% H₂O₂ in methanol for 10 min, PBS, 0.1% Triton X-100 in freshly prepared 0.1% sodium citrate for 15 min, PBS, and a mix of 100 ml label solution (containing fluorescein-dUTP) and 400 ml enzyme solution (containing terminal deoxynucleotidyl transferase, TdT; In Situ Cell Death Detection Kit, Roche, Indianapolis, IN), at room temperature for 3 h.

Following vasopressin and TUNEL localization, sections were reacted with the nuclear stain Hoechst 33342 (10 μ g/ml, Molecular Probes) for 10 min, washed with PBS, and mounted with Aqua-Polymount. TUNEL-positive neurons were detected using fluorescein epifluorescence illumination. Hoechst 33342 signal was observed using ultraviolet (UV) fluorescence illumination. To evaluate colocalization of staining, rhodamine-, fluorescein-, and UV-filtered images were merged using Adobe Photoshop v5.5.

Data analysis

Quantitative microdensitometry was performed with a Nikon Eclipse E800 light microscope (20X objective) using IPLab software (Scanalytics). Signal intensities were determined by outlining individual neurons, and IPLab integrated densitometry functions were used to calculate mean signal intensities for the selected areas. Only neurons with distinct borders and visible nuclei in the plane of section were counted. Immunopositive neurons were defined as a signal greater than 2 \times background levels and specifically localized within the margins of the neuronal plasma membrane. Immunopositive neurons were counted as a fraction of total neurons in the SON. TUNEL-positive neurons were also compared to counts of vasopressin-positive neurons in the SON. Data from neuron-counting procedures were analyzed using one-way ANOVA with post hoc multiple comparison analysis. Microglial morphology was quantified by comparing the maximum microglial cell diameter including dendritic processes in control and diabetic animals (Kreutzberg, 1996) and analyzed using a nonpaired *t* test. An alpha level of 0.05 was used as a threshold for statistical significance. All data are presented as mean \pm SE.

Results

Morphological changes occur in diabetic MNCs

All animals injected with streptozotocin (STZ) developed hyperglycemia (>250 µg/dl) compared to control animals injected with saline. After either 6 weeks or 6 months, tissue sections of supraoptic nucleus (SON) from control and diabetic animals were stained with Cresyl Violet (Nissl) to examine changes in cellular morphology. In control animals at 6 weeks and 6 months, there was relatively little variation in cell size, with the majority of neurons in the 10–15-µm range (Figs. 1A, A', C, and C'). In contrast, after 6 weeks of diabetes, there was a change in size distribution of magnocellular neurosecretory cells (MNCs) such that a greater proportion of neurons were larger (15–25 µm) (Figs. 1B and B', arrows). After 6 months of diabetes, an even greater heterogeneity of SON size was observed: there was a similar trend toward larger neurons, but in addition, there was an increased proportion of smaller neurons (<10 µm) (Figs. 1D and D'), which were not seen in controls or the 6-week diabetic SON. The larger neurons showed nuclear distention, cytoplasmic swelling, and vacuolization

(arrows), while the smaller neurons tended to be hyperchromatic (arrowheads).

Counts of neurons revealed no significant difference between 6-week and 6-month control SONs, or between 6-week diabetic and 6-month control SONs. In the 6-month diabetic animals, neuronal density was significantly decreased by 34.5% compared to 6-month controls (Fig. 1E, *P < 0.05).

Cleaved caspase-3 expression is upregulated in the chronically diabetic SON

Cleaved caspase-3 is the activated form of caspase-3, a critical effector of apoptosis (Cohen, 1997). Immunocytochemistry using an antibody directed against cleaved caspase-3 revealed low levels of caspase-3 immunoreactivity in neurons from control animals at 6 weeks and 6 months, and from diabetic animals at 6 weeks (Figs. 2A–C). Six-month diabetic animals demonstrated a large upregulation of cleaved caspase-3 expression, which was visible in the cytoplasmic compartment of the neurons (Fig. 2D). SON neurons (28%) in the 6-month diabetic group were immunopositive compared to approximately 5% in the other groups (Fig. 2E, *P <

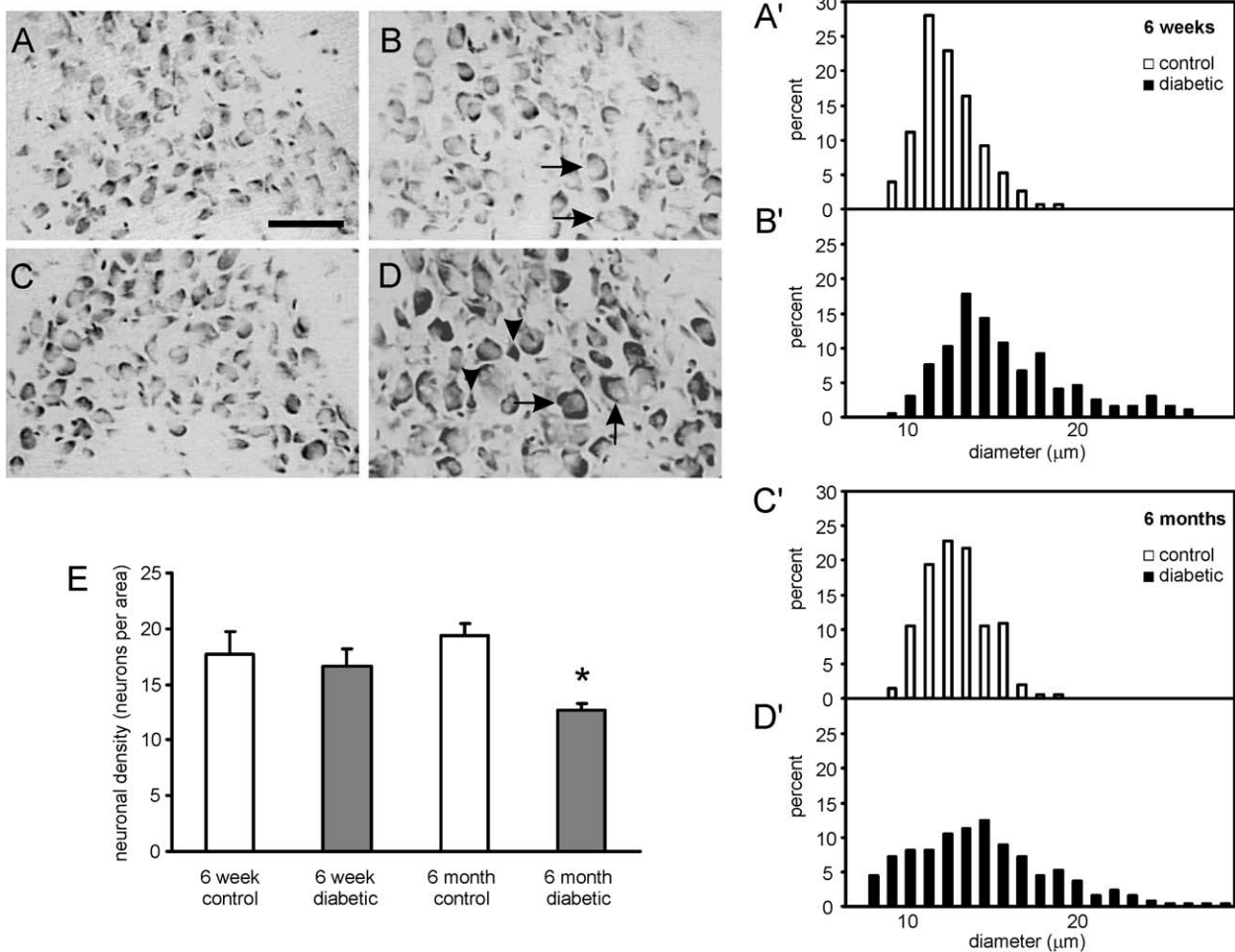


Fig. 1. Morphological changes of diabetic neurons. Cresyl violet staining of sections of supraoptic nucleus from 6-week control (A) and diabetic (B), and 6-month control (C), and diabetic (D) animals. Arrows in B and D indicate hypertrophic neurons; arrowheads in D indicate shrunken, hyperchromatic neurons. Size frequency histograms corresponding to A–D are shown in A'–D'. Neuronal density is quantified in E. Scale bar in A = 50 µm. Data are plotted as mean ± SE, *P < 0.05.

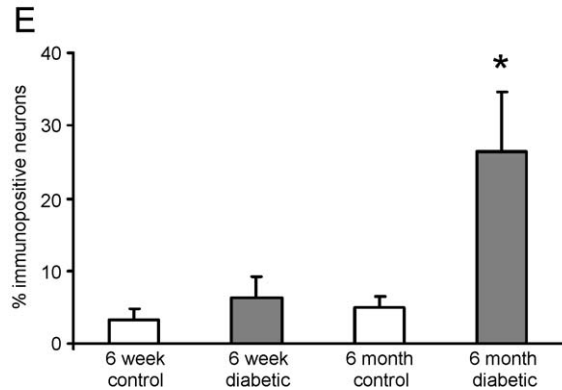
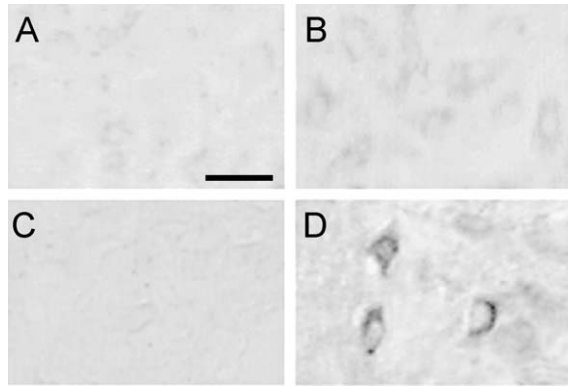


Fig. 2. Cleaved caspase-3 immunoreactivity in sections of supraoptic nucleus from 6-week control (A) and diabetic (B), and 6-month control (C), and diabetic (D) animals. Images were converted to gray scale and identically digitally contrast-enhanced to qualitatively illustrate differences in immunostaining. Quantitative microdensitometry of unenhanced images was used to obtain data shown in E. Neurons defined as immunopositive demonstrated signal greater than $2\times$ background levels with staining specifically localized within the margins of the plasma membrane. Scale bar in A = 50 μm . Data are plotted as mean \pm SE, $*P < 0.05$.

0.05). In general, the level of immunostaining of neurons in the 6-week diabetic group (Fig. 2B) was greater compared to the controls (Figs. 2A and C), but many of these moderately stained neurons

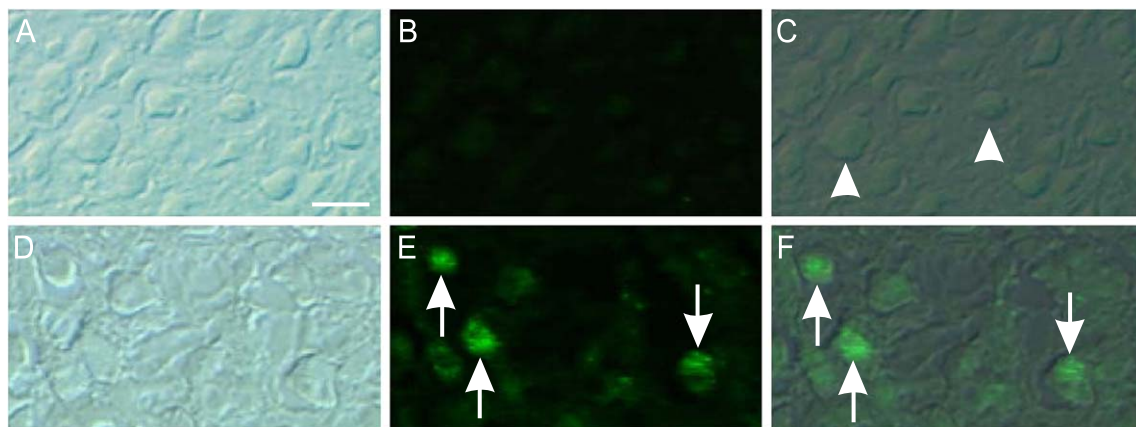


Fig. 3. TUNEL assay for apoptosis-related DNA fragmentation. Phase-contrast images of 6-month control (A) and diabetic (D) supraoptic nucleus are shown to identify neuronal boundaries. TUNEL staining is shown for 6-month control (B) and diabetic (E) sections. C is an overlay of A and B, and F is an overlay of D and E. Positive TUNEL staining (E, arrows) occurs within neuronal boundaries (F, arrows), and is not detected in control SON neurons (C, arrow heads). Scale bar in A = 50 μm . Data are plotted as mean \pm SE, $*P < 0.05$.

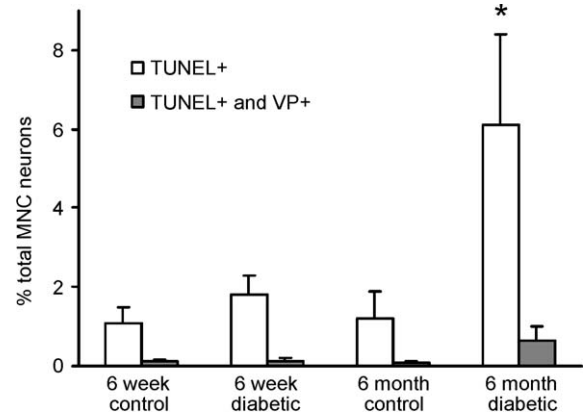


Fig. 4. Quantification of TUNEL-positive neurons within the supraoptic nucleus of 6-week and 6-month control and diabetic animals (white bars). TUNEL-positive neurons demonstrated signal greater than $2\times$ background levels. The percentage of neurons that were both TUNEL-positive and vasopressin-immunopositive is shown (gray bars). Data are plotted as mean \pm SE, $*P < 0.05$.

(Fig. 2B) were not above the threshold for inclusion in the immunopositive group.

DNA fragmentation occurs in the chronically diabetic SON

Having demonstrated an upregulation of caspase-3 in the 6-month diabetic SON, we sought to confirm these findings using an additional marker of apoptosis. The TUNEL assay was used to identify DNA strand breaks which occur in apoptotic neurons as a result of cleavage of genomic DNA. At 6 months, TUNEL-positive neurons were not seen in control MNCs (Fig. 3B), but were clearly visible in neurons in the diabetic SON (Fig. 3E, arrows). Figs. 3A and D are phase-contrasted images of the cells shown in B and E. Figs. 3C and F are overlays of A and B, and D and E, respectively. TUNEL-positive neurons were detected extremely infrequently in the 6-week control, 6-week diabetic, and 6-month control SON, and were significantly increased in 6-month diabetic SON (Fig. 4, $*P < 0.05$).

Table 1
Quantification of SON neuronal phenotype

	VP+	VP–	Total
6-week control	20.0 ± 5.5 (29.3%)	48.3 ± 3.5 (70.7%)	68.3 ± 5.5 (100.0%)
6-week diabetic	24.3 ± 3.3 (40.1%*)	36.3 ± 2.6* (59.9%*)	60.7 ± 3.8 (100.0%)
6-month control	20.0 ± 2.0 (29.9%)	46.6 ± 5.5 (70.1%)	66.7 ± 5.4 (100.0%)
6-month diabetic	14.0 ± 0.8* (27.5%)	37.0 ± 1.5* (72.5%)	51.0 ± 2.2* (100.0%)

Data are shown as mean ± SE.

*P < 0.05; significant difference from corresponding control group.

TUNEL and vasopressin are not colocalized in the chronically diabetic SON

It is well established that vasopressin is upregulated in diabetic MNCs, and it has been hypothesized that neuronal overactivation due to chronic hyperosmotic stimulation may lead to neuronal degeneration and apoptosis. Table 1 shows quantification of neuronal phenotype in control and diabetic animals after 6 weeks and 6 months of diabetes. These measurements indicate that (1) the percent of vasopressin-positive neurons is increased in 6-week diabetic animals; (2) the number of vasopressin-negative neurons is decreased in 6-week and 6-month diabetic animals; (3) the number of vasopressin-positive neurons is decreased in 6-month diabetic animals, and (4) the number of total SON neurons is decreased in 6-month animals. We then attempted to correlate vasopressin

immunoreactivity with TUNEL staining in 6-month diabetic animals. Vasopressin-immunopositive neurons are clearly visible in Fig. 5A (arrowheads). TUNEL-positive neurons can be seen in Fig. 5A (arrowheads). TUNEL-positive neurons can be seen in Fig.

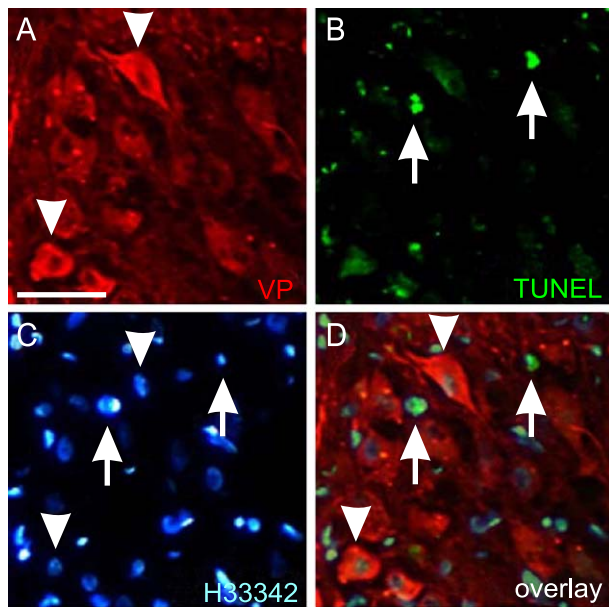


Fig. 5. Vasopressin (VP), TUNEL, and Hoechst 33342 (H33342) images from 6-month diabetic supraoptic nucleus. Vasopressin-immunopositive neurons are clearly visible in A (arrowheads). TUNEL-positive cells are visible in B (arrows). Hoechst 33342 nuclear stain is shown in C. Arrowheads in C showing low intensity nuclear staining correspond to vasopressin-immunopositive neurons in A. Arrows in C showing pyknotic, condensed, and hyperchromatic nuclei correspond to TUNEL-positive cells in B. Overlay of A, B, and C is shown in D; arrowheads correspond to vasopressin-immunopositive neurons, arrows correspond to TUNEL-positive neurons. Scale bar in A = 50 μm.

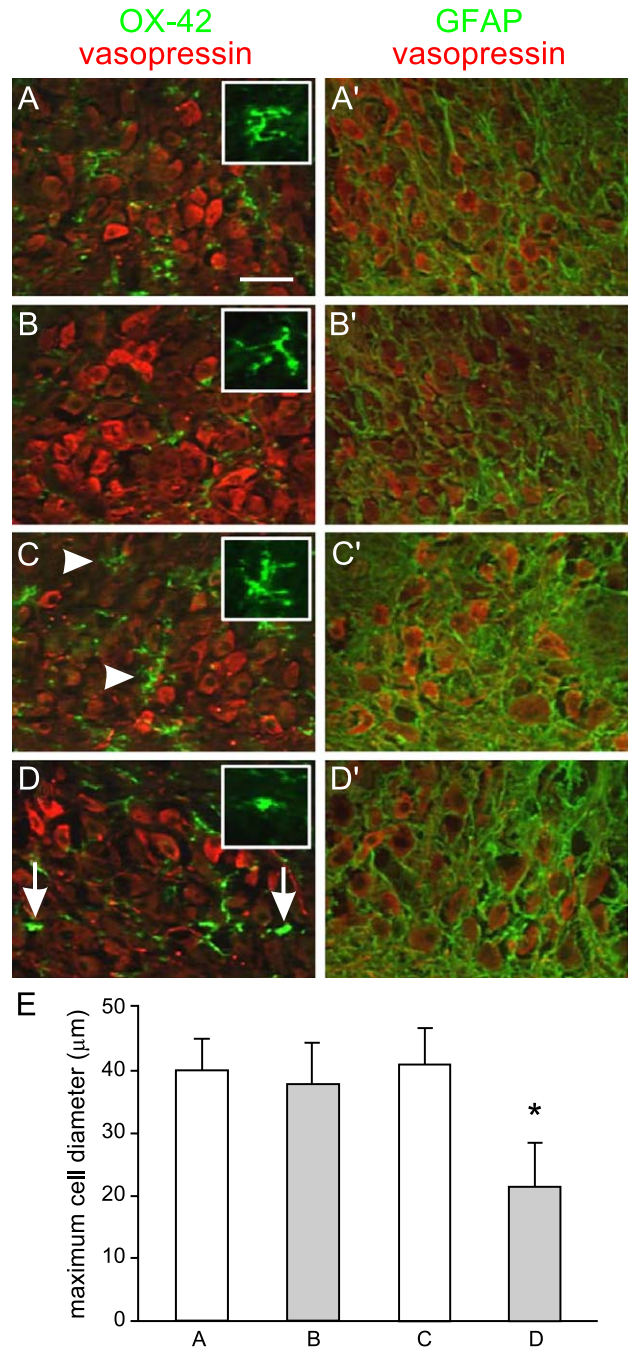


Fig. 6. Glial response to chronic diabetes. Sections of supraoptic nucleus were stained with vasopressin (red) and either OX-42 (green, left panels, A–D) or GFAP (green, right panels, A'–D'). A and A' are from 6-week controls; B and B' are from 6-week diabetics; C and C' are from 6-month controls; and D and D' are from 6-month diabetics. Arrowheads in C show normal branched microglial morphology. Arrows in D show hypertrophic and condensed microglia. The histogram in E is a quantification of changes in microglial morphology. Six-month diabetic animals demonstrate reduced maximum cell diameter suggesting microglial activation. Data are plotted as mean ± SE, *P < 0.05. Scale bar in A = 50 μm.

5B (arrows). Hoechst 33342 staining of nuclei is shown in Fig. 5C. The vasopressin-immunopositive neurons had normal-appearing nuclei (Fig. 5C, arrowheads), while the TUNEL-positive neurons showed condensed, pyknotic, and irregular nuclei (Fig. 5C, arrows). Overlay of Figs. 5A–C, as seen in Fig. 5D, demonstrated that after 6 months of diabetes, vasopressin-positive neurons do not exhibit overlap with TUNEL-positive neurons. This result was quantified in Fig. 4. The apoptotic neurons appeared to comprise a separate population from the presumably functional vasopressin-producing neurons.

Microglial activity is enhanced in the diabetic SON

Because neuronal density in the 6-month diabetic SON was decreased (Fig. 1E), we examined the possibility that microgliosis or astrogliosis occurs in conjunction with neuronal degeneration and apoptosis. Vasopressin and either OX-42 (a marker for microglia) or GFAP (a marker for astrocytes) were detected simultaneously in tissue slices containing the SON using fluorescence microscopy. At 6 weeks, the SON from both control and diabetic animals showed similar staining for OX-42 (Figs. 6A and B) and GFAP (Figs. 6A' and B'). At 6 months, GFAP staining in both the control and diabetic SON was modestly increased compared to 6 weeks, and GFAP staining in control and diabetic SON appeared similar at this time point (Figs. 6A', B', C', and D). In contrast, OX-42 staining in the 6-month diabetic SON showed hypertrophic and condensed microglia (Fig. 6D, arrows) which differed from the normal branched dendritic pattern seen in controls (Fig. 6C, arrowheads). The maximum diameter of microglial cells including their dendritic processes was quantified (Fig. 6E). Six-month diabetic animals demonstrated truncated processes and reduced maximum cell diameter suggesting microglial activation in these animals.

Discussion

The hyperosmolality associated with diabetes mellitus triggers both structural and metabolic changes within magnocellular neurosecretory cells (MNCs) of the hypothalamic supraoptic nucleus (SON). These changes occur, at least in part, as a result of neuronal activation and the demand for increased production and secretion of vasopressin. It is well established that chronic neuronal overactivation can lead, by a variety of mechanisms including nNOS activation and progressive intracellular calcium accumulation, to neuronal degeneration and apoptosis (Brecht et al., 2001; Dawson et al., 1991). In the present study, we asked the question of whether apoptosis occurs among vasopressin-producing neurons after prolonged diabetes. We utilized morphometric and immunocytochemical criteria to examine diabetic SON neurons.

Although we did not label SON neurons that produce and secrete oxytocin, it is unlikely that these neurons are affected by experimental diabetes. Our use of male animals also limited gender effects on oxytocin secretion. Assuming that oxytocin neurons do not become overactivated or undergo apoptosis as a result of chronic diabetes, then our estimates of vasopressin neuron loss in diabetes are, if anything, an underestimate of the actual percentage of vasopressin neurons lost in chronic diabetes.

Our principal findings are that after 6 months of diabetes: (1) small hyperchromatic neurons with condensed and irregular somal and nuclear profiles are present in addition to hypertrophic neurons;

(2) neuronal density is significantly decreased in the SON; (3) cleaved caspase-3 immunoreactivity is upregulated in a subpopulation of SON neurons; (4) DNA fragmentation is detectable in some SON neurons; (5) the number of vasopressin-positive neurons is decreased; (6) TUNEL-positive neurons do not colocalize with vasopressin-producing neurons; and (7) microglial hypertrophy and condensation are evident. Identification of neuronal apoptosis in the SON after chronic diabetes is an important finding, as tight regulation of plasma osmolality is a critical physiologic requisite, without which dysregulation of fluid homeostasis will occur.

Analysis of neuronal area and diameter by Cresyl Violet staining provided the first suggestion of neuronal degeneration and apoptosis in this study. The 6-month diabetic SON contained a unique population of neurons that were <10 μm in diameter and appeared hyperchromatic. Neuronal density was also decreased in the 6-month diabetic SON. We reasoned that cell death by an apoptotic mechanism might account for these changes, and assayed for several hallmarks of apoptosis to test this hypothesis.

Caspase-3 is an important regulator of apoptosis, and its proteolytically cleaved form is known to be upregulated in neurons undergoing apoptosis (Kermer et al., 1999; Srinivasan et al., 1998). In our study, cleaved caspase-3 immunoreactivity was strongly upregulated in 6-month diabetic SON neurons (Fig. 2). The interval over which cleaved caspase-3 yields an immunopositive signal is thought to span the entire active process of neuronal degeneration, and may thus include much of the time course of apoptosis (Brecht et al., 2001). Therefore, the percent of immunopositive neurons reported in Fig. 2E may not be an accurate measure of the incidence of apoptosis, and almost certainly represents an overestimate of the rate of neuronal death at any given time point. Morphological assessment of neurons expressing cleaved caspase-3 might be helpful in determining how far along the apoptotic process individual neurons are.

The TUNEL assay is a useful marker for apoptosis-related genomic DNA fragmentation (Gavrieli et al., 1992; Migheli et al., 1995) and was used in conjunction with caspase-3 immunoreactivity in this study as a second marker of apoptosis. DNA fragmentation results from oxidative DNA damage and is a final irreversible step of the apoptotic process (Brecht et al., 2001). Neurons only transiently show positive TUNEL staining and therefore TUNEL positivity may be a more accurate reflection of the incidence of neuronal death compared to caspase-3 immunoreactivity (Duan et al., 2003). Detection of TUNEL positivity, however, is challenging. Whereas immunocytochemical procedures benefit from signal-boosting amplification steps such as the biotin–avidin–HRP process, the TUNEL reaction is a one-step procedure. In our study, it was necessary to increase the gain (identically for diabetic and control tissue slices) on the microscopic images to detect and compare TUNEL-positive and TUNEL-negative neurons (Fig. 3). It is possible, as a result of this manipulation, that our false-positive rate was increased, and that the percentages of TUNEL-positive neurons shown in Fig. 4 are in fact an overestimate. To more accurately confirm TUNEL positivity, we used Hoechst 33342 nuclear staining as a means of colocalization with the TUNEL signal (Fig. 5). TUNEL-positive neurons showed pyknotic and irregular nuclei which are characteristic of apoptotic cell death (Fig. 5C, arrows).

In addition to apoptosis, the decreased neuronal density observed in the chronically diabetic SON could also be due to gliosis and reflect a relative dilution of neurons by proliferating astrocytes and microglia. Perineuronal astrocytes can modulate hormone

release by altering their processes. In rats subjected to dehydration (and consequent hyperosmolality), a gross retraction of astrocytic processes has been reported in the SON, which is reversible with hydration (Hawrylak et al., 1998). Detailed ultrastructural analysis of this phenomenon reveals that in dehydrated rats neurovascular astrocytic contacts expand while neuroglial contacts decrease in size (Miyata and Hatton, 2002; Miyata et al., 2001). The increase in terminal-capillary contacts provides a route for increased neuropeptide release.

Like the dehydrated state, chronic diabetes subjects the SON to a hyperosmotic environment and triggers an increase in vasopressin production and release. Immunocytochemical experiments have shown astrocyte retraction and condensation in the diabetic SON (Luo et al., 2002). In contrast to this response to dehydration, astrocytes can also hypertrophy following neuronal injury and degeneration. GFAP immunoreactivity in the chronically diabetic SON may therefore reflect multiple (and possibly opposing) factors, and in this study, the pattern of GFAP staining in diabetic SON was not distinguishable from age-matched control SON.

Previous work has shown that microglia within the SON hypertrophy and shorten their processes in response to diabetes (Luo et al., 2002). In the present study, this effect was clearly evident after 6 months of diabetes. Microgliosis and microglial activation can occur in response to a variety of stimuli including neuronal ischemia, inflammation, infection, neoplasia, and neurodegeneration (Kreutzberg, 1996). Neurotoxic overactivation and apoptosis were the likely triggers for microglial proliferation, condensation, and truncation of processes as seen in Fig. 6.

Several important questions are raised by this study. First, why were the apoptotic changes not seen at 6 weeks? Because there is no time point at which acute diabetes is delineated from chronic diabetes, it is not possible to conclude that the onset of neuronal apoptosis corresponds to chronic diabetes. Instead, the detection of apoptosis after 6 months of diabetes is indicative of a dynamic process of accumulating neuronal injury which is likely to be present at lower levels, in this case, largely indistinguishable from controls, at the 6-week time point.

Second, as shown in Fig. 4, the rate of vasopressin and TUNEL overlap is very low in the diabetic SON. A possible explanation for this is that overstimulated neurons reach a degeneration threshold, where normal function (vasopressin production) is attenuated and the irreversible steps of apoptosis (positive TUNEL signal) begin. The few neurons that demonstrated positive labeling for both vasopressin and TUNEL may have been transitioning between functioning and degenerating states. The molecular changes that account for this transition may result from alterations in intracellular calcium homeostasis, secondary to neuronal overstimulation. Elevated intracellular calcium levels are known to exist within many diabetic tissues (Levy et al., 1994). Diabetic hippocampal neurons, for example, undergo a slow progressive dysregulation of calcium homeostasis, which at first alters their synaptic function (Biessels et al., 2002; Kamal et al., 1999), and can eventually lead to apoptosis (Li et al., 2002).

Diabetes remains a chronic progressive disease with significant vascular and neurologic complications (Biessels et al., 1994; Donnelly et al., 2000; McCall, 1992). While chronically upregulated circulating levels of vasopressin can cause glomerular damage within the kidney, we have shown here that chronically overstimulated vasopressin-producing neurons within the SON undergo apoptosis. While upregulation of vasopressin is adaptive in an acute hyperosmotic state, our results suggest that chronic

hyperosmolality may result in a pathologic sequence of neurotoxic events which ultimately lead to neuronal loss.

Acknowledgments

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References

- Ahloulay, M., Schmitt, F., Dechaux, M., Bankir, L., 1999. Vasopressin and urinary concentrating activity in diabetes mellitus. *Diabetes Metab.* 25 (3), 213–222.
- Bankir, L., Bardoux, P., Ahloulay, M., 2001. Vasopressin and diabetes mellitus. *Nephron* 87 (1), 8–18.
- Barber, A.J., Lieth, E., Khin, S.A., Antonetti, D.A., Buchanan, A.G., Gardner, T.W., 1998. Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. *J. Clin. Invest.* 102 (4), 783–791.
- Bardoux, P., Martin, H., Ahloulay, M., Schmitt, F., Boubry, N., Trinh-Trang-Tan, M.M., Bankir, L., 1999. Vasopressin contributes to hyperfiltration, albuminuria, and renal hypertrophy in diabetes mellitus: study in vasopressin-deficient Brattleboro rats. *Proc. Natl. Acad. Sci. U. S. A.* 96 (18), 10397–10402.
- Biessels, G.J., Kappelle, A.C., Bravenboer, B., Erkelens, D.W., Gispen, W.H., 1994. Cerebral function in diabetes mellitus. *Diabetologia* 37 (7), 643–650.
- Biessels, G.J., van der Heide, L.P., Kamal, A., Bleys, R.L., Gispen, W.H., 2002. Ageing and diabetes: implications for brain function. *Eur. J. Pharmacol.* 441 (1–2), 1–14.
- Brecht, S., Gelderblom, M., Srinivasan, A., Mielke, K., Dityateva, G., Herdegen, T., 2001. Caspase-3 activation and DNA fragmentation in primary hippocampal neurons following glutamate excitotoxicity. *Mol. Brain Res.* 94 (1–2), 25–34.
- Brooks, D.P., Nutting, D.F., Crofton, J.T., Share, L., 1989. Vasopressin in rats with genetic and streptozocin-induced diabetes. *Diabetes* 38 (1), 54–57.
- Cohen, G.M., 1997. Caspases: the executioners of apoptosis. *Biochem. J.* 326 (Part 1), 1–16.
- Crespo, D., Ramos, J., Gonzalez, C., Fernandez-Viadero, C., 1990. The supraoptic nucleus: a morphological and quantitative study in control and hypophysectomized rats. *J. Anat.* 169, 115–123.
- Dawson, V.L., Dawson, T.M., London, E.D., Bredt, D.S., Snyder, S.H., 1991. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. U. S. A.* 88 (14), 6368–6371.
- Dheen, S.T., Tay, S.S., Wong, W.C., 1994. Ultrastructural changes in the hypothalamic supraoptic nucleus of the streptozotocin-induced diabetic rat. *J. Anat.* 184 (Part 3), 615–623.
- Donnelly, R., Emslie-Smith, A.M., Gardner, I.D., Morris, A.D., 2000. ABC of arterial and venous disease: vascular complications of diabetes. *Br. Med. J.* 320 (7241), 1062–1066.
- Duan, W.R., Garner, D.S., Williams, S.D., Funckes-Shippy, C.L., Spath, I.S., Blomme, E.A., 2003. Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. *J. Pathol.* 199 (2), 221–228.
- Gavrieli, Y., Sherman, Y., Ben-Sasson, S.A., 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119 (3), 493–501.
- Hawrylak, N., Fleming, J.C., Salm, A.K., 1998. Dehydration and rehydration selectively and reversibly alter glial fibrillary acidic protein immu-

- noreactivity in the rat supraoptic nucleus and subjacent glial limitans. *Glia* 22 (3), 260–271.
- Kadowaki, K., Kishimoto, J., Leng, G., Emson, P.C., 1994. Up-regulation of nitric oxide synthase (NOS) gene expression together with NOS activity in the rat hypothalamo-hypophysial system after chronic salt loading: evidence of a neuromodulatory role of nitric oxide in arginine vasopressin and oxytocin secretion. *Endocrinology* 134 (3), 1011–1017.
- Kamal, A., Biessels, G.J., Urban, I.J., Gispen, W.H., 1999. Hippocampal synaptic plasticity in streptozotocin-diabetic rats: impairment of long-term potentiation and facilitation of long-term depression. *Neuroscience* 90 (3), 737–745.
- Kermer, P., Klocker, N., Labes, M., Thomsen, S., Srinivasan, A., Bahr, M., 1999. Activation of caspase-3 in axotomized rat retinal ganglion cells in vivo. *FEBS Lett.* 453 (3), 361–364.
- Klein, J.P., Craner, M.J., Cummins, T.R., Black, J.A., Waxman, S.G., 2002. Sodium channel expression in hypothalamic osmosensitive neurons in experimental diabetes. *NeuroReport* 13 (11), 1481–1484.
- Kreutzberg, G.W., 1996. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* 19 (8), 312–318.
- Levy, J., Gavin III, J.R., Sowers, J.R., 1994. Diabetes mellitus: a disease of abnormal cellular calcium metabolism? *Am. J. Med.* 96 (3), 260–273.
- Li, Z.G., Zhang, W., Grunberger, G., Sima, A.A., 2002. Hippocampal neuronal apoptosis in type 1 diabetes. *Brain Res.* 946 (2), 221–231.
- Luo, Y., Kaur, C., Ling, E.A., 2002. Neuronal and glial response in the rat hypothalamus–neurohypophysis complex with streptozotocin-induced diabetes. *Brain Res.* 925 (1), 42–54.
- McCall, A.L., 1992. The impact of diabetes on the CNS. *Diabetes* 41 (5), 557–570.
- Migheli, A., Attanasio, A., Schiffer, D., 1995. Ultrastructural detection of DNA strand breaks in apoptotic neural cells by in situ end-labelling techniques. *J. Pathol.* 176 (1), 27–35.
- Miyata, S., Hatton, G.I., 2002. Activity-related, dynamic neuron–glial interactions in the hypothalamo-neurohypophysial system. *Microsc. Res. Tech.* 56 (2), 143–157.
- Miyata, S., Takamatsu, H., Maekawa, S., Matsumoto, N., Watanabe, K., Kiyohara, T., Hatton, G.I., 2001. Plasticity of neurohypophysial terminals with increased hormonal release during dehydration: ultrastructural and biochemical analyses. *J. Comp. Neurol.* 434 (4), 413–427.
- Serino, R., Ueta, Y., Tokunaga, M., Hara, Y., Nomura, M., Kabashima, N., Shibuya, I., Hattori, Y., Yamashita, H., 1998. Upregulation of hypothalamic nitric oxide synthase gene expression in streptozotocin-induced diabetic rats. *Diabetologia* 41 (6), 640–648.
- Srinivasan, A., Roth, K.A., Sayers, R.O., Shindler, K.S., Wong, A.M., Fritz, L.C., Tomaselli, K.J., 1998. In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. *Cell Death Differ.* 5 (12), 1004–1016.
- Van Itallie, C.M., Fernstrom, J.D., 1982. Osmolal effects on vasopressin secretion in the streptozotocin-diabetic rat. *Am. J. Physiol.* 242 (6), E411–E417.
- Zeng, X.X., Ng, Y.K., Ling, E.A., 2000. Neuronal and microglial response in the retina of streptozotocin-induced diabetic rats. *Vis. Neurosci.* 17 (3), 463–471.