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Diabetes Increases the Expression of Hypothalamic Neuropeptides in a Spontaneous Model of Type I Diabetes, the Nonobese Diabetic (NOD) Mouse

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SUMMARY

1. Synthesis of oxytocin (OT) and arginine-vasopressin (AVP) is increased in induced models of Type I diabetes, such as the streptozotocin model. However, these parameters have not yet been evaluated in spontaneous models, such as the nonobese diabetic mouse (NOD). Therefore, we studied in the magnocellular cells of the paraventricular nucleus (PVN) of nondiabetic and diabetic 16-week-old female NOD mice and control C57Bl/6 mice, the immunocytochemistry of OT and AVP peptides and their mRNA expression, using nonisotopic in situ hybridization (ISH).

2. In nondiabetic and diabetic NOD female mice, the number of OT- and AVP-immunoreactive cells were similar to those of the controls, whereas immunoreaction intensity was significantly higher for both peptides in diabetic NOD as compared with nondiabetic NOD and control C57Bl/6 mice.

3. ISH analysis showed that the number of OT mRNA-containing cells was in the same range in the three groups, whereas higher number of AVP mRNA expressing cells was found in diabetic NOD mice. However, the intensity of hybridization signal was also higher for both OT and AVP mRNA in the diabetic group as compared with nondiabetic NOD and control mice.

4. Blood chemistry demonstrated that haematrocrit, total plasma proteins, urea, sodium, and potassium were within normal limits in diabetic mice. Thus, NOD mice were neither hypernatremic nor dehydrated.

5. We suggest that upregulation of OT and AVP reflects a high-stress condition in the NOD mice. Diabetes may affect neuropeptide-producing cells of the PVN, with the increased AVP and OT playing a deleterious role on the outcome of the disease.

KEY WORDS: diabetes mellitus; vasopressin; oxytocin; paraventricular nucleus; nonobese diabetic (NOD) mouse.

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INTRODUCTION

The neuropeptides arginine-vasopressin (AVP) and oxytocin (OT) are synthesized by magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nucleus of the hypothalamus. These peptides are present in two neuronal populations with distinct regional localization (De Wied *et al.*, 1993; Swaab *et al.*, 1975; Vandesande and Dierickx, 1975) and are involved in various physiological functions. Magnocellular-derived AVP, in addition to vasoconstrictor and antidiurectic actions, is implicated in hydrosaline balance, cardiovascular regulation, liver glycogenolysis, learning, and several behaviors (Dorner and Plagemann, 1994; Keppens and De Wulf, 1975). Although OT is generally considered a reproductive hormone, it also plays a role in osmoregulation, sodium balance, and secretion of pancreatic hormones (Blackburn *et al.*, 1992; Huang *et al.*, 1995; Widmaier *et al.*, 1991). Centrally, both peptides may function as peptidic neurotransmitters/neuromodulators. Several central nervous system areas contain AVP and OT immunoreactivity in projection fibers (Sofroniew, 1985; Whitnall *et al.*, 1985) and these neuropeptides are also released from dendrites, thereby affecting local as well as distant neurons (Morris and Pow, 1993; Van Tol *et al.*, 1987).

In experimentally induced Type I diabetes in rats such as the streptozotocin (STZ) model, increased synthesis of neurohypophysial AVP and OT was demonstrated at the ultrastructural level in the PVN and SON (Bestetti and Rossi, 1980; Lincoln *et al.*, 1989), leading to the suggestion that chronic dehydration, which developed during diabetes, upregulated AVP and OT expression. Shortly afterwards, it was shown that STZ diabetic rats presented increased AVP and OT peptide synthesis in hypothalamus and hypertrophy of AVP and OT-immunoreactive neurons (Dheen *et al.*, 1994; Fernstrom *et al.*, 1990). Moreover, STZ-induced diabetes caused, after 4 weeks, a significant increase in AVP and OT mRNA in the PVN and SON (Serino *et al.*, 1998). However, since STZ-induced diabetes is a pharmacological model that suddenly induces the clinical onset of the disease in mature nondiabetic-prone animals, we thought it of interest to investigate the central expression of AVP and OT at the peptide and mRNA levels in a spontaneous genetic model, such as the NOD mouse during progression from prediabetic to diabetic stages (Amrani *et al.*, 1988).

The NOD mouse represents a rare spontaneous model of Type I diabetes, whose clinical and pathophysiological features make it a suitable model for the human disease (Bach, 1994; Huang *et al.*, 1995). Clinical onset of the disease is preceded by a latent period during which T-cell infiltration of the pancreas (insulitis) develops. Then, insulitis leads to extensive β-cell destruction, lack of insulin secretion, and hyperglycemia. In our colony of NOD mice, hyperglycemia appears in females from 12 weeks of age onwards and 80% of them become diabetic by 6 months of age (Bach, 1994; Homo-Delarche, 1997). At 16 weeks of age, 60% of the mice are diabetic and 40% nondiabetic, although animals in the latter group may develop the disease at later time periods. The incidence of diabetes in males is lower than in females. Diabetes in NOD mice is characterized by polydipsia, glycosuria, reduced body weight, hyperglycemia, and ketoacidosis. Without insulin treatment diabetic NOD mice died after 4–8 weeks. (Amrani *et al.*, 1988; Bach, 1994; Homo-Delarche, 1997; Saravia-Fernandez *et al.*, 1996).

Our objective therefore, was to characterize the expression of hypothalamic neuropeptides in diabetic NOD mice. Our investigations included: (1) analysis of OT and AVP peptide by immunocytochemistry; (2) determination of the OT and AVP mRNA using *in situ* hybridization. These parameters were compared in the hypothalamus from female nondiabetic and diabetic NOD mice and C57Bl/6 mice taken as controls.

MATERIALS AND METHODS

Animals

NOD originally provided by Clea Japan (Tokyo, Japan) and C57BI/6 mice were bred and kept at the facilities of Hopital Necker, Paris, under pathogen-free conditions. Mice were fed standard pellets and water *ad libitum* and maintained at $22 \pm 1^{\circ}$C on a 12/12 h light–dark cycle, according to the norms stipulated by the European Community. From 10 weeks of age onwards, animals were tested weekly for glycosuria. After a positive test was obtained, animals were bled by retroorbital puncture and glucose levels in blood measured first by Haemoglucotest and then by Refoflux (Boehringer-Mannheim). Animals showing blood glucose >11 mM were considered diabetic. In our colony, hyperglycemia appears in NOD females from 12 weeks of age onwards and 80% become diabetic at 6 months (Amrani *et al.*, 1988; Bach, 1994; Homo-Delarche, 1997). In our study, blood glucose levels were 6.8 ± 0.5, 29.2 ± 2.2, and 7.05 ± 0.4 mmol/l in nondiabetic NOD, diabetic NOD, and C57Bl/6 controls, respectively. Mice were ether anesthetized and perfused intracardially with 0.9% NaCl. Brains were removed, frozen in dry ice over Tissue-Tek (Sakura, USA) and kept at -80°C until used. Coronal sections (10–15 μm) were obtained in a cryostat and placed in gelatin-coated glass slides.

Blood collected at the time of killing was used for determination of haematocrit and plasma levels of urea, total protein and electrolytes, using routine clinical laboratory facilities at Hopital Necker. Laboratory data from diabetic NOD mice showed that haematocrit ($43.9 \pm 0.8\%$), plasma sodium and potassium (119.6 ± 14.1 and 6.5 ± 0.8 mmol/l, respectively), total plasma proteins (53.6 ± 1.0 g/l), and urea (6.3 ± 0.4 mmol/l) were within normal limits (Homo-Delarche, 1997; Jaboby and Fox, 1984).

Immunocytochemistry

OT and AVP immunocytochemistry was carried out following previously published procedures (Saravia *et al.*, 1999; Saravia-Fernandez *et al.*, 1996). After inhibition of endogenous peroxidase activity with H_2O_2 and blocking with 10% goat serum, slices were incubated overnight at 4°C with a 1/500 dilution of the polyclonal rabbit anti-OT antibody (Peninsula, CA, USA) or a 1/1000 dilution of rabbit polyclonal anti-AVP serum (a generous gift from Dr Ruud Buijs, Netherlands Institute for Brain Research, Amsterdam, The Netherlands; Buijs *et al.*, 1978) prepared in PBS containing 0.3% Triton × 100 and 1% goat serum. After extensive washings in PBS, slices were incubated with a 1:200 dilution of the second antibody (antirabbit

goat serum, Vector Labs., Burlingame. CA, USA) in PBS containing 0.3% Triton × 100 and 1% goat serum for 1 h at room temperature. Afterwards, slices were washed again in PBS and incubated in 10 μl/ml biotin–avidin solution (Vectastain ABC Elite, Vector) for 45 min at room temperature. The last step consisted of washing in PBS and addition of 0.25% 3,3′-diaminobenzidine (DAB, Sigma, St Louis, MO, USA) dissolved in 0.1 M Tris pH 7.4 containing 0.01% H_2O_2 for 6–8 min to develop the reaction. Finally, slices were mounted in Permount and coverslipped. Substitution of the primary antibody by nonimmune serum completely blocked staining of the magnocellular cells.

In Situ Hybridization

Nonisotopic *in situ* hybridization was used to measure mRNA levels, following EMBO (European Molecular Biology Organization) procedures (Landry and Roche, 1994). The 30 mer OT probe 5′-CTC-GGA-GAA-GGC-AGA-CTC-AGG-GTC-GCA-GGC-3′ corresponded to a portion of exon C of the OT gene (Morris *et al.*, 1994). The 48 mer AVP probe 5′-GTA-GTA-GAC-CCG-GGG-CTT-GGC-AGA-ATC-CAC-GGA-CTC-TTG-TGT-CCC-AGC-CAG-3′ was complementary to the glycoprotein coding region of the AVP gene (Ivell and Richter, 1984). Probes were labeled with digoxigenin (Boehringer) at the 3′ end using the enzyme terminal transferase (GIBCO, Maryland, USA) (Saravia-Fernandez *et al.*, 1996). Slices placed on glass slides were immersed in 4% paraformaldehyde for fixation during 6 min at 4°C and then washed with PBS. Prehybridization was carried out in the presence of 4 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate), 1 × Denhardt (0.02% Ficoll, 0.02% PVP, 0.02% bovine serum albumin) during 1 h at 42°C. Hybridization was carried out overnight at 42°C in hybridization buffer consisting of 50% formamide, 600 mM NaCl, 80 mM Tris-HCl pH 7.5, 4 mM EDTA, 0.05% sodium pyrophosphate, 0.05% tetrasodium pyrophosphate, 0.2% *N*-lauryl-sarcosine, 10 mM dithiothreitol) containing 10 nM of the labeled probe. Afterwards, slices were washed twice in 2 × SSC for 30 min, once in 1 × SSC and once in 0.5 × SSC at 42°C. This step was followed by two additional washes in 0.1 × SSC for 30 min at room temperature.

The last wash was followed by an incubation in 2% sheep serum prepared in 0.1% Triton × 100 in PBS for 30 min at room temperature and a second incubation (overnight) at 4°C with an alkaline phosphatase conjugated antidigoxigenin antibody (1:5000; Boehringer) prepared in buffer A containing 1 M NaCl, 2 mM $MgCl_2$ in 0.1 M Tris pH 7.5. The last step was followed by new washes lasting 10 min each at room temperature with the following buffers: Buffer A (3 washes), Buffer B (1 M NaCl, 4.4 mM $MgCl_2$ in 0.1 M Tris pH 9.5, 1 wash), and Buffer C (0.1 M ClNa, 4.4 mM $MgCl_2$ in 0.1 M Tris pH 9.5, 1 wash). Development of the reaction was carried out in the dark at room temperature by exposing the sections with the alkaline phosphatase substrates NTB (nitroblue tetrazolium) and BCIP (bromochloride-indolphosphate; GIBCO) made in Buffer C for 2–3 h. The specificity of the hybridization was determined by (1) competition of the labeled probe with a 20-fold excess of unlabeled probe and (2) replacing the antidigoxigenin antibody by nonreactive serum. Under these conditions, signals for both OT and AVP were reduced to background levels.

Computerized Image Analysis

The number of cells expressing peptide or mRNA and the intensity of immunocytochemical and *in situ* hybridization reactions within the magnocellular division of the PVN (Paxinos and Watson, 1982) were analyzed by computerized image analysis (Optimas Bioscan Program; Ferrini *et al.*, 1995; Saravia *et al.*, 1999). For this purpose, densitometry methods previously validated for quantitation of AVP and other hypothalamic neuropeptides were followed (Bestetti *et al.*, 1990; Saravia *et al.*, 1999; Sladek and Olschowka, 1994). Digited images of tissue sections were displayed on the video screen under identical lighting conditions and the program was made to calculate the average optical density of the pixels contained within the boundaries of the outlined area by using a 256 unit gray level scale. The densitometric reading of a comparable area outside the PVN but devoid of AVP or OT immunostaining was considered as background and subtracted from the staining readings of magnocellular cells. Results were expressed as the inverse log of gray intensity (ILIGV), which is proportional to the unweighted average optical density (Saravia *et al.*, 1999). This parameter is related to the relative amount of immunoreactive peptide or mRNA signal. Using the Optimas program we were also able to determine the number of OT and AVP peptide and mRNA-containing cells in the magnocellular PVN (Bestetti *et al.*, 1990; Saravia *et al.*, 1999; Sladek and Olschowka, 1994).

Statistical Analysis

For statistical analysis, reaction intensity and number of cells expressing OT or AVP mRNA and peptide were compared between the groups of control and NOD mice. For each mice, data from at least six sections obtained at the PVN level were pooled for the individual animal. Results are presented as mean $\pm$ SEM of average readings in 6 mice per group. To compare multiple group means, we used one way ANOVA followed by Bonferroni's "post hoc" test. Statistical significance was established at $p < 0.05$.

RESULTS

OT and AVP Immunocytochemistry in the PVN Nucleus From C57Bl/6 and NOD Mice

Immunocytochemistry revealed an intense and specific labelling for OT in magnocellular cells arranged anteroventromedially in the PVN, in agreement with previous reports (Armstrong, 1985). Meanwhile, parvocellular cells showed a much lower level of expression, as reported by Whitnall *et al.* (1985). The number of OT-positive cells in the magnocellular division (Fig. 1A), was similar regardless of the group of 16-week-old mice: controls C57Bl/6, nondiabetic and diabetic NOD females. However, OT immunoreaction intensity (ILIGV, Fig. 1B) in diabetic NOD mice was significantly higher as compared with nondiabetic NOD and C57Bl/6 control mice ($p < 0.001$ in both cases).

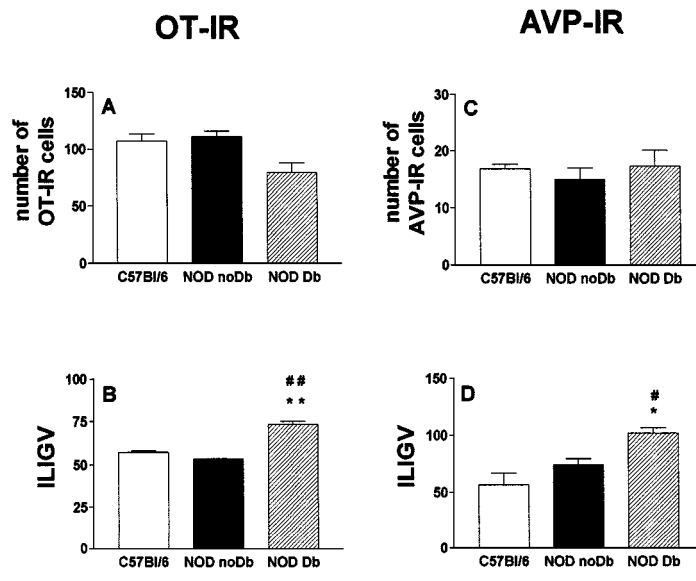

Fig. 1. Immunoreactive oxytocin (OT-IR) and vasopressin (AVP-IR) in the paraventricular nucleus of control C57Bl/6 strain (white columns), nondiabetic (dark columns) and diabetic NOD mice (stippled columns) at 16 weeks of age. Data correspond to the number of OT-IR (A) and AVP-IR (C) cells, and reaction intensity per cell (ILIGV, inverse log of gray intensity) for OT (B) and AVP (D). Significance: $^{*}p < 0.05$ and $^{**}p < 0.001$ vs. nondiabetic NOD; $^{\#}p < 0.01$ and $^{\#\#}p < 0.001$ vs. C57BI/6. (ANOVA followed by Bonferroni's post hoc test).

AVP-immunoreactive neurons were found mainly in the lateral magnocellular portion of the PVN, separated from the anteroventrally placed OT-immunoreactive neurons as already described by Armstrong (1985). Similar to findings with OT, the number of AVP-expressing cells in nondiabetic and diabetic NOD mice was similar to those of controls (Fig. 1C). However, the intensity of the immunochemical reaction (ILIGV, Fig. 1D) was significantly higher in diabetic NOD mice as compared with nondiabetic NOD and control groups ($p < 0.05$ and $p < 0.01$, respectively).

OT and AVP mRNA Levels in the PVN From C57Bl/6 and NOD Mice

In situ hybridization for OT and AVP mRNA revealed that regional segregation of messengers followed the pattern of peptide-containing cells described earlier, that is, OT-producing cells located anteroventromedially while the AVP cell population was found in the lateral magnocellular portion.

OT mRNA analysis was carried out in the three groups of 16-week-old mice, that is C57Bl/6 (control), nondiabetic, and diabetic NOD mice. The number of cells expressing mRNA as well as reaction intensity (ILIGV) was assessed as for immunocytochemistry. Figure 2A shows that the number of cells expressing OT mRNA were similar in control C57Bl/6 and NOD mice regardless of the glycemic state. However,

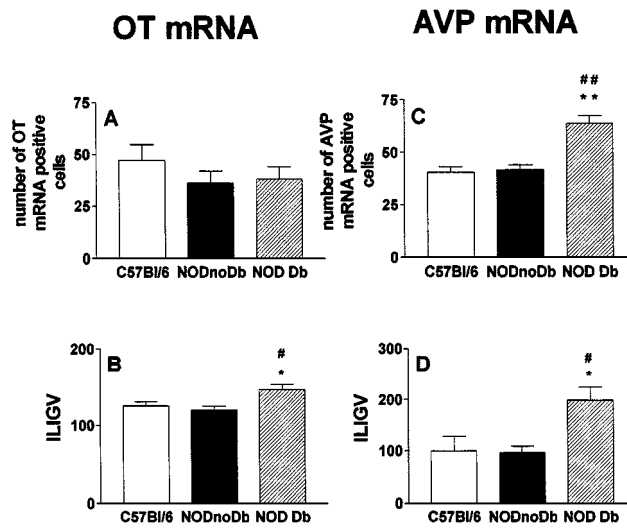

Fig. 2. OT mRNA and AVP mRNA in the paraventricular nucleus of control C57Bl/6 strain (white columns), nondiabetic NOD (dark columns) and NOD mice (stippled columns). Data correspond to the number of OT-mRNA (A) and AVP-mRNA (C) containing cells, and reaction intensity per cell for OT mRNA (B) and AVP mRNA (D). Significance: $^{\star}p < 0.05$ and $^{\star\star}p < 0.001$ vs. nondiabetic NOD; $^{\#}p < 0.01$ and $^{\#\#}p < 0.001$ vs. C57Bl/6 (ANOVA followed by Bonferroni's post hoc test).

mRNA signal intesity (ILIGV, Fig. 2B) was significantly higher for NOD diabetic as compared with control C57Bl/6 and nondiabetic mice ($p < 0.05$ in both cases).

Regarding the number of cells expressing AVP mRNA and the reaction intensity, both parameters were significantly increased in the diabetic group (Fig. 2C and 2D) compared with control C57Bl/6 and nondiabetic groups. Statistical analysis demonstrated that the NOD diabetic group was higher at the $p < 0.001$ level for AVP mRNA positive cells and $p < 0.05$ for mRNA staining intensity against the other two groups. The photomicrographs of Figs. 3 and 4 present the increased OT and AVP mRNA labeling of the diabetic NOD hypothalamus as compared with nondiabetic NOD and C57Bl/6 mice.

DISCUSSION

In the spontaneous NOD model of type I diabetes, overt disease was associated with a significant upregulation of immunoreactivity for OT and AVP in the magnocellular portion of the PVN. More specifically, we demonstrated that the number of OT and AVP immunoreactive cells were similar in C57Bl/6 control mice and nondiabetic and diabetic NOD mice. However, diabetic NOD mice at 16 weeks of age exhibited a significant increase of OT and AVP immunoreaction intensity as compared with the other two groups. These data suggest that in a spontaneous model of

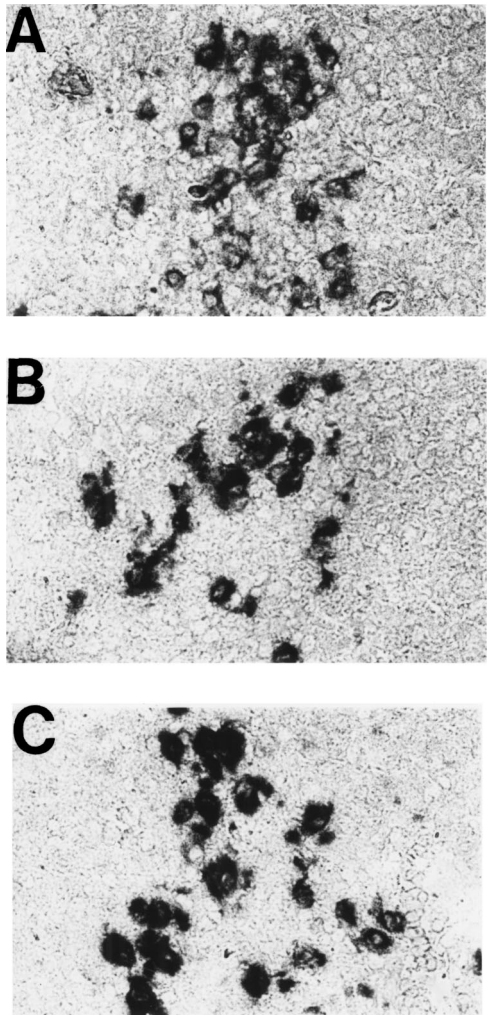

Fig. 3. Photomicrographs corresponding to non-isotopic *in situ* hybridization for OT mRNA in the PVN from a control C57Bl/6 mouse (A) a nondiabetic NOD mouse (B) and a diabetic NOD mouse (C). Magnification: 400×.

Type I diabetes, overt diabetes was associated with increased peptide expression per cell and corroborate, in part, with those obtained in STZ-rats and show that levels of neuropeptides are upregulated in the hypothalamus from diabetic rodents.

To elucidate if the observed changes were accompanied by increased synthesis, mRNA levels were quantified by *in situ* hybridization using rat oligonucleotide probes showing full cross-reaction with their respective mouse mRNAs. In this case, the number of cells containing OT mRNA was similar in control C57Bl/6, nondiabetic, and diabetic NOD mice. However, diabetic NOD mice exhibited a significant increase in OT mRNA expression per cell. Therefore, efficient translation of this synthesized OT mRNA could explain the high immunoreaction intensity for OT peptide found in the group of diabetic NOD mice. For AVP, both the number of mRNA-expressing cells and reaction intensity were upregulated in diabetic NOD

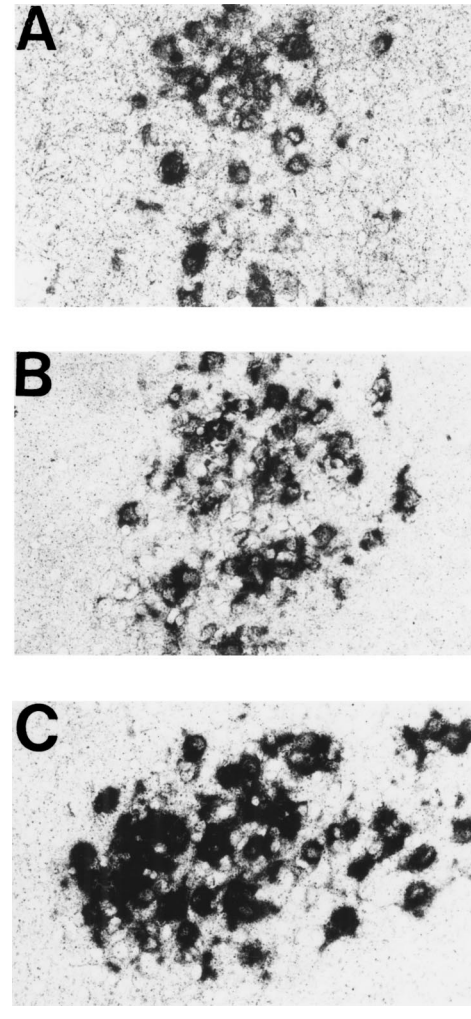

Fig. 4. Photomicrographs corresponding to non-isotopic *in situ* hybridization for AVP mRNA in the PVN from a control C57Bl/6 mouse (A) a nondiabetic NOD mouse (B) and a diabetic NOD mouse (C) Magnification: 400×.

mice. This finding suggests that the diabetic state was a stronger stimulus for AVP than for OT gene expression, as more AVP mRNA-expressing magnocellular cells were present in diabetic NOD mice than in controls and the nondiabetic group, whereas cell counting for OT mRNA was similar in the three groups. However, not all vasopressinergic cells translated AVP mRNA into peptide, because the number of AVP peptide-containing cells were similar in the three groups of mice. Moreover, a rapid release with depletion of AVP from these additional cells would also explain this discrepancy.

Our data obtained in a spontaneous model of Type I diabetes support literature reports of increased synthesis of AVP and OT peptide and mRNA, which occur in the PVN of STZ-diabetic rats (Dheen *et al.*, 1994; Fernstrom *et al.*, 1990; Lincoln *et al.*, 1989; Serino *et al.*, 1998). Regarding the mechanism(s) leading to changes in AVP

and OT mRNA and peptide levels in NOD mice, we first excluded an effect of age, by comparing age-matched control C57Bl/6 and NOD mice. Instead, the aging process was shown to enhance synthesis and release of AVP from the PVN (Hatzinger *et al.*, 2000). Second, osmotic stimulation powerfully stimulate the magnocellular neurons, which subsequently increase synthesis and release of OT and AVP into the circulation (Burbach *et al.*, 1984; Franco-Bourland and Fernstrom, 1981; Majzoub *et al.*, 1983; Van Tol *et al.* 1987). Diabetic NOD mice are characterized by polyuria and polydypsia. We checked that these mice were neither hyperosmotic nor dehydrated by measuring haematocrit values and concentration of proteins, urea, and Na^+ and K^+, which remained within normal limits.

Third, it is possible that diabetes constituted a powerful stressor acting on the PVN, a nucleus playing a critical role in stress reactivity. It is worth noting here that NOD mice are animals hypersensitive to stress, particularly females, presenting, as adults, strong corticosterone response to various stimuli whereas basal levels of corticosterone are normal. The hypersensitivity of the HPA axis of the NOD mouse is observed early in life (Amrani *et al.*, 1988; Bach, 1994; Homo-Delarche, 1997; Johansson *et al.*, 1991) and is accompanied by transient periods of hyperinsulinemia observed at birth and after weaning. It is known that hyperinsulinism occurring during a crucial period of brain development may adversely affect hypothalamic control centers (Dorner and Plagemann, 1994). Thus, early high insulin titers could have a priming effect for late stress effects at the time of clinical diabetes. Stress not only activates parvocellular cells of the PVN containing AVP and corticotrophin-releasing hormone but also the magnocellular AVP system (Angulo *et al.*, 1991), which subsequently releases stored peptide into the hypothalamic-pituitary portal system (Antony, 1993). Hyperactivity of the CNS-pituitary-adrenal axis is observed in experimental diabetes in rats (De Nicola *et al.*, 1991). Increased sensitivity to stress due to the metabolic derangement of diabetes would also cause a cascade of neuroendocrine responses, leading to increased synthesis of AVP and OT in the hypothalamus. AVP is an accepted modulator of the stress response to a large variety of stimuli, while support for a role of the PVN OT system in the response to stress has been recently described (Gibbs, 1986; Nishioka *et al.*, 1998). In this regard, it is worth mentioning that elevation of plasma AVP is a characteristic feature of both Type I and Type II diabetic patients (Zerbe *et al.*, 1979). This AVP elevation also occurs in animal models of diabetes mellitus, whether experimental or genetically determined (Young, 1969). Since the responsible stimulus for this AVP increase does not appear to be hyperglycemia per se (Bardoux *et al.*, 1999; Zerbe *et al.*, 1979), the possibility of the hyperresponse to metabolic stress should be seriously considered.

If increased synthesis of hypothalamic neuropeptides is coupled to increased secretion, excess AVP and OT may cause additional side effects (Zhi-Young and Henkin, 1993) including paracrine pancreatic effects (Amico *et al.*, 1988; Johansson *et al.*, 1991; Zhi-Young and Henkin, 1993). OT has been shown to exert glucoregulatory functions, leading to hyperglycemia mediated by increases in pancreatic secretion of glucagon (Widmaier *et al.*, 1991). In this regard, a hyperglucagonemia has been observed in diabetic NOD mice (Ohneda *et al.*, 1984; Pelegri *et al.*, 1999; Yibchok-anun and Hsu, 1998). Magnocellular-derived AVP could act peripherally by increasing liver glycogenolysis with secondary hyperglycemia (Keppens and De

Wulf, 1975), and also by releasing ACTH from pituitary corticotrophs (Antony, 1993). ACTH would then stimulate adrenal corticosteroid secretion, further aggravating hyperglycemia. Finally, AVP was shown to contribute to hyperfiltration, albuminuria, and renal hypertrophy in diabetes mellitus, therefore contributing to the worsening of the disease at the kidney level (Bardoux *et al.*, 1999). Clinical and animal studies will be required in the near future to deeply understand the abnormalities of brain function in insulin-dependent diabetes mellitus.

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