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# STREPTOZOTOCIN-INDUCED DIABETES IS ASSOCIATED WITH CHANGES IN NGF LEVELS IN PANCREAS AND BRAIN

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## INTRODUCTION

Type 1 diabetes mellitus (DM) is a chronic metabolic sequel of pancreatic-β cell loss associated with various metabolic, neuronal, endocrine and immune alterations at cellular, tissue and organ levels (1, 6, 7, 39). Nerve growth factor (NGF) is a prototypic member of the neurotrophin family of proteins, known to promote growth, differentiation and function of peripheral sensory and sympathetic neurons (2, 28, 41). NGF is produced and released during embryonic and adult life by numerous cells, including pancreatic-β cells (26, 34, 36, 44,45). NGF has also been shown to promote the survival of pancreatic-β cells, to exerts beneficial effects on transplanted pancreatic islets (26, 32, 35), and to stimulates insulin secretion (44).

NGF is produced and stored in the central nervous system (CNS), is involved in neuroendocrine mechanisms and in promoting differentiation, survival and function of forebrain cholinergic neurons (FBCN). These brain cells play a crucial role in learning and memory abilities and degenerate in age-related neurological disorders and in Alzheimer's disease (21, 28, 29, 41). Animal and human studies have shown that reduction in NGF synthesis may contribute to DM-associated neuropathies (3-5, 22), such as skin ulcers (15, 16, 30), and vascular disorders (3, 14, 17, 38, 42). It has also been reported that brain-derived neurotrophic factor (BDNF), a neurotrophin belonging to the NGF family, decreases in some cardiometabolic diseases, such as coronary atherosclerosis and metabolic syndrome, suggesting their involvement not only in diabetic-related disorders, but also vascular physiopathology (4, 17, 20-22, 27). Recent pathological studies have suggested that diabetes is one risk factors for senile dementia of Alzheimer type (18, 19). However, the effect of diabetes on the brain itself and the relationship between diabetes and the presence of brain NGF is not clearly known. The present study was undertaken to investigate these aspects and, more specifically, to study the effect of streptozotocin(STZ)-induced Type 1 diabetes on NGF production and on expression of NGF receptors, low-affinity $p75^{NTR}$ and high-affinity receptor kinase A (TrkA) in the pancreas and brain.

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Abbreviations: NGF, Nerve Growth Factor; STZ, Streptozotocin; DM, Diabetes Mellitus; HI, Hippocampus; CNS, Central Nervous System; VEGF, Vascular Endothelial Growth Factor.

## MATERIAL AND METHODS

Sprague Dawley rats were raised in our animal house. For these studies we used 4 month-old rats weighing 200 g, maintained on a 12 h light/dark cycle and provided with food and water *ad libitum*.

*1. Induction of diabetes.*

Diabetes was induced in rats (n = 16) by a single intravenous injection of STZ, (Sigma St Louis, USA) at a dose of 60 mg/kg dissolved in a physiological solution (0.9% NaCl). The rats were allowed to drink a 10% dextrose solution overnight and were then placed back on standard lab conditions. Rats were considered diabetic and included in the study if they had a fasting plasma glucose level > 350 mg/dl. An equal number of rats (n = 16) received a physiological solution and served as controls.

For housing, care and experimental procedures we followed the guidelines indicated by Intramural Committee and Institutional Guidelines in accordance with National and International law (EEC council directive 86/609, OJ L 358, 1, 12 December 1987) and the NIH principles of laboratory animal care (NIH publication no. 85-23, revised 1985).

*2. Tissue Dissection and histological and immunoistochemical analysis.*

After 9 weeks, controls and STZ-treated rats were deeply euthanized with an overdose of Pentobarbital (Carlo Erba Reagenti, Italy). The pancreas and the brain were then quickly removed for biochemical, molecular, histological and immunoistochemical analysis.

The hippocampus (HI), frontal cortex, hypothalamus and pituitary gland tissues were dissected out using a mouse brain matrix (ASI Instruments, Inc. Co. USA) (see also the methodology described by Cuello (12)) and stored at -70 °C until used. Tissues were homogenized and centrifuged at 8500 rpm and the supernatant used for NGF determination.

For histological analysis, the pancreas of STZ-treated and untreated rats were fixed in 4% paraformaldheyde and sectioned at 20 μm thick and stained with 0.1% toluidine blue. Sections were then dehydrated, covered with cover glass and observed under a Zeiss Axiophot microscope for evaluate the structural aspect of the pancreas and, specifically, islets of Langherans.

For immunohistochemistry, STZ-treated and control rats were deeply anaesthetized with Pentobarbital and perfused through the ascending aorta with paraformaldehyde 4% in 0.1M PBS via aorta with 0.1M PBS. The brain was then removed and post-fixed for 24 hrs with the same fixative, cryoprotected in 0.1M PBS with 20% sucrose. Coronal brain sections, 20 μm in thickness, were then cut on a cryostat at the temperature of -18 ± 2 °C. After collection, brain sections were coded and immunostained. Free-floating sections were incubated overnight at 4 °C with a monoclonal antibody against the low-affinity NGF receptor ($p75^{NTR}$) the first antibody. After a brief rinse, sections were incubated with biotinylated anti-mouse or rabbit IgG, washed again, and incubated with an avidin-biotin-horseradish peroxidase (HRP) complex using the Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, USA), following the manufacturer's instructions. Negative controls were performed by replacing the primary antibody with 10% of normal goat serum. Negative controls did not reveal specific immunoreactivity.

For quantitative analysis, immunostained cells were observed under a x-40 objective frame, with a Zeiss Axiophot microscope equipped with an image analysis program (IAS 2000; Delta Sistemi, Rome, Italy) connected to a computer. The number of immunoreactive cells present in 8 different fields of each immunostained section (n = 10) were counted and compared. To exclude the possibility of measuring cell fragments or group of cells, only the $p75^{NTR}$-immunopositive cells ranging from 14 to 28 μm of diameter were examined.

*3. NGF assay.*

The brain tissues and pancreas were homogenized with ultrasonication in extraction buffer (tris-acetate 20 mM, pH7.5, NaCl 150 mM, EDTA 1 mM, EGTA 1 mM, Sodium-Pyrophosphate 2.5 mM,

Ortovanadate 1 mM, β-Glycerolphosphate 1 mM, NaF 100 mM, PMSF 1 mM, Leupeptin 1 μg/ml) and centrifuged at 4 °C for 10 min, 13000 rpm and supernatants were recovered (EDTA, Ethylenediamine-Tetraacetic acid; EGTA, Ethyleneglycol-Tetraacetic acid; PMSF, Phenylmethylsulfonyl Fluoride). The tissue concentration of NGF was measured by a highly sensitive and specific two-site enzyme immunoassay ELISA kit "NGF Emaxtm ImmunoAssay System number G7631" by Promega, (Madison, WI, USA), following the instructions provided by the manufacturer.

*4. Western blotting analysis.*

$p75^{NTR}$, TrkA, and VEGF were evaluated in the pancreas and brain of STZ-treated and control rats, using western blot analysis. Tissues were homogenized in the sample buffer (0.01 M TRIS-HCl buffer pH 7.6, containing 0.25 M sucrose, 0.1 M NaCl, 1 mM EDTA, and 1 mM PMSF) and centrifuged at 8,000 g for 10 min at 4 °C. The supernatants were then used for western blotting. Briefly, samples (30 μg total protein) were dissolved with loading buffer (0.1 M TRIS-HCl buffer (pH 6.8) containing 0.2 M DTT, 4% SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 12,5% SDS-PAGE, and electrophoretically transferred to PVDF for 3 h. The membranes were incubated for 40 minutes at room temperature with blocking buffer (5% non-fat dry milk, 10 mM TRIS pH 7.5, 100 mM NaCl, 0.1% Tween 20) and were washed three times for 10 minutes each at room temperature in TTBS (10 mM TRIS pH 7.5, 100 mM NaCl, 0.1% Tween 20). This was followed by an incubation overnight at 4 °C with monoclonal mouse anti-VEGF 1:1000 (Santa Cruz, CA, USA); with polyclonal rabbit anti-TrkA 1:1000 (Santa Cruz, CA, USA); and with $p75^{NTR}$ 1: 2000. Membranes were also washed three times for 10 minutes each at room temperature in TTBS and incubated for 1 h with horseradish peroxydase-conjugated anti-rabbit IgG or anti-mouse IgG (Cell Signaling, USA) as the secondary antibody. The blots were developed with ECL (Amersham Bioscience) as the chromophore. The optical density of β-actin bands was used as an internal control for difference in sample loading.

*5. Statistical analysis.*

Data were obtained by means of ANOVA using the SuperANOVA package for Macintosh (Abacus Concepts Inc., CA, USA), considering the treatments with STZ as variable. The difference between groups was determined by Tukey-Kramer comparison; a $p < 0.05$ was considered statistically significant.

## RESULTS

*1. Body weight and serum glucose.*

Consistent with previous studies, STZ-injected rats lost weight and/or did not gain as much weight as control animals. The serum glucose concentration of STZ-treated rats was significantly higher compared to non-diabetic rats (data not shown).

*2. Effect of STZ on the islet of Langerhans.*

Figures 1A, B show histological sections of the pancreas of non diabetic rats (A) compared to those of diabetic rats (B). The size of IL and the number of cells in the pancreas of STZ – treated rats are markedly reduced.

*3. Effect of STZ on NGF levels and NGF receptor expression in pancreas.*

Figure 1C reports that NGF amount in the pancreas of diabetic rats is significantly reduced compared to controls, 2584.1 ± 150.6 *vs.* 4125.2 ± 215.4 pg/gr/tissue. As reported in Figure 1D, western blotting analysis indicates a moderate down-regulation of TrkA protein and an increase of $p75^{NTR}$.

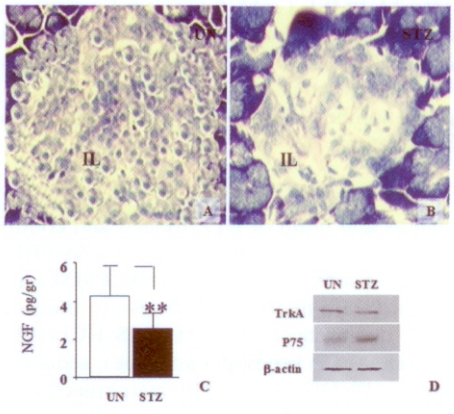


Fig. 1A-D. – *Histological preparations of pancreas of untreated (AN) and STZ-treated (B) rats showing the reduction of islets of Langerhans (IL) and the decreased cells in the pancreas of diabetic rats. Toluidine blue stained, magnification X-120.*

The pancreas of STZ-treated rats is characterized by a significant decrease of NGF (C), a moderate decrease of TrkA and up-regulation of the low-affinity NGF receptor p75NTR (D) compared to untreated pancreas (UN).

*4. Effect of STZ on brain NGF level.*

We next measured the concentration of NGF in the CNS. As reported in Figure 2, the levels of NGF in the HI (**$p < 0.001$) and pituitary gland (*$p < 0.05$) undergo a significant increase in diabetic rats. In the cortex and hypothalamus, DM also causes a moderate increase of NGF levels, though this increase does not reach statistical significance.

*5. Effect of STZ on NGF receptor expression in the brain.*

As reported in Figure 3, western blotting analysis in the brain of STZ-treated rats compared to controls indicates that the expression TrkA is reduced in hypothalamus ($p < 0.05$) and pituitary gland, while in the HI and cortex no differences were found.

*6. Immunochemistry of $p75^{NTR}$.*

To further characterize the role of NGF in the CNS of diabetic rats, the expression of $p75^{NTR}$ in two brain regions was also investigate. As reported in Figure 4 qualita-

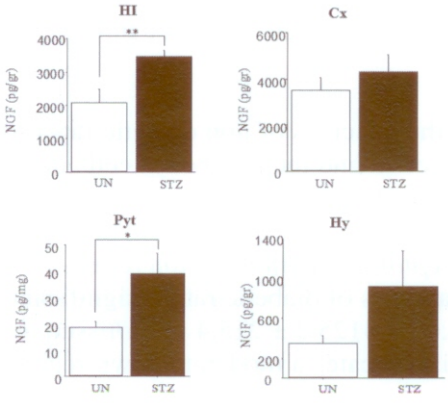


Fig. 2 – *Level of NGF in the hippocampus (HI), cortex (Cx), pituitary (Pyt) and hypothalamus (Hy) of untreated (UN) and STZ-treated rats (STZ).*

Level of NGF in the HI, cortex, pituitary and hypothalamus of untreated (UN) and STZ-treated rats. Note marked increase of NGF in the HI (**$p < 0.001$) and pituitary (*$p < 0.05$) and to a lesser extent also in the hypothalamus of diabetic rats compared to controls.

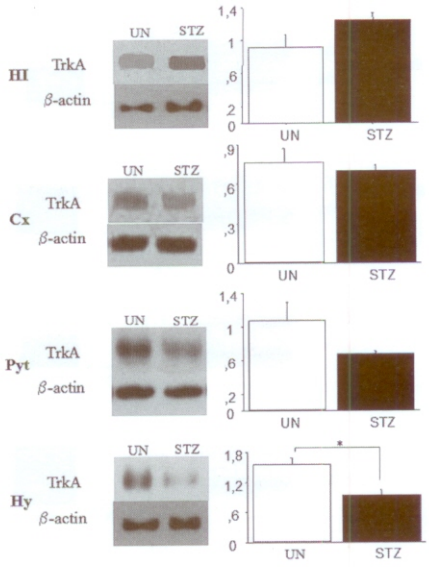


Fig. 3 – *Western blot analysis of TrkA protein presence in the hippocampus (HI), cortex, (Cx) pituitary (Pyt) and hypothalamus (Hy) of untreated and STZ-treated rats.*

These studies revealed that STZ-treatment cause a decrease of TrkA protein only in the hypothalamus ($p < 0.05$).

tive and quantitative immunohistochemical analyses revealed that the expression of this receptor in septal neurons of STZ-treated rats increase significantly (**$p < 0.01$), while in neurons of nucleus basalis, which project to the cortex, no differences between STZ and control rats were observed.

*7. Effect of STZ on brain VEGF protein.*

As reported in Figure 5, STZ treatment induces in rats a marked reduction of VEGF in the pancreas and in the CNS, particularly in the hippocampus and pituitary, suggesting that DM alters the expression of molecules involved in the functional activity of vascular tissue. Whether this effect is linked to NGF synthesis and release is not known.

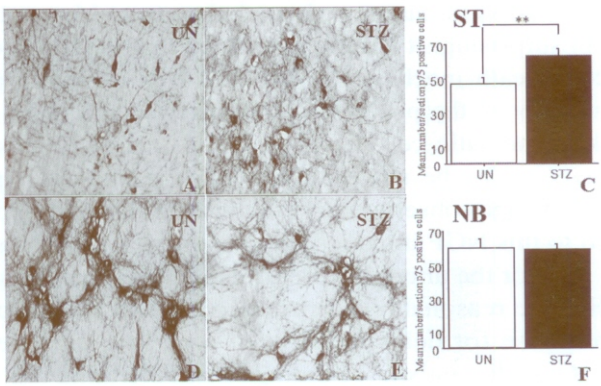


Fig. 4A-F – *The STZ treatment causes an increase in the number of p75NTR-positive neurons of the ($p < 0.01$) in the septum receiving trophic support from the NGF produced in the HI (A-C) and no differences in the number of p75NTR-positive neurons localized in nucleus basalis receiving trophic support from the cortex (D-F).*

Magnification, A, B, D, E: X-120.

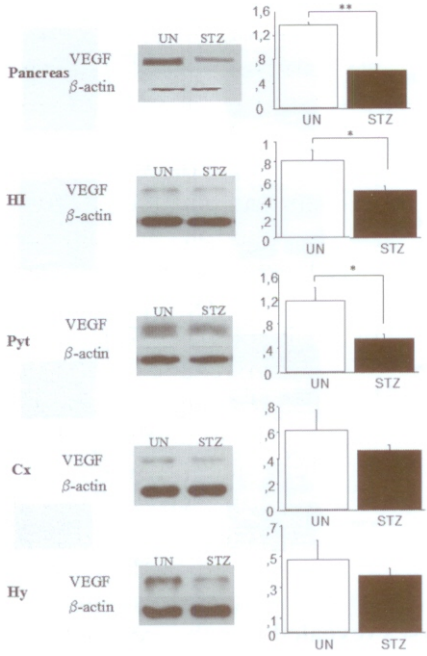


Fig. 5 – *Western blotting analysis showed that STZ treatment causes a marked reduction of VEGF expression in the pancreas, hippocampus (HI), pituitary (Pyt) gland and, to a lesser extent, also in the cortex (Cx) compared to untreated rats. No changes were found in the hypothalamus.*

## DISCUSSION

A number of recent studies have demonstrated that NGF has survival and trophic effects on pancreatic-β cells in vitro and in vivo (26, 34-36), as well as stimulation of insulin secretion (44, 45). The present study demonstrates that STZ treatment causes a dramatic loss of pancreatic-β cells in rats which is accompanied by a significant decrease of NGF pancreatic level. The present study also indicated that STZ-induced DM is associated with (i) elevated NGF levels in the HI and of pituitary gland and, to a lesser extent, in the cortex and hypothalamus, and (ii) down-regulation of NGF-receptor in forebrain cholinergic neurons.

The functional significance of such a differential pattern of NGF/NGF receptor expression in pancreas, hypothalamus and hypophysis of diabetic rats is at present not surprising. It has been reported that the receptor $p75^{NTR}$ is involved in apoptotic cells death mechanism (10), and it is therefore possible that the increase of this receptor in the pancreas of diabetic rats might be linked to mechanism involved in the pancreatic cell death observed in DM. It has also been reported that NGF is involved in the modulation of the hypothalamic-pituitary-adrenal axis (2, 28) and the changes observed in hypothalamus and hypophysis in diabetic rats may be related to this NGF impaired neuroendocrine mechanisms.

As for the altered expression of NGF-receptors in the septum and of NGF in the HI, one reasonable interpretation is that their increase may represent an adaptive response for delaying or even reversing damaged forebrain cholinergic neurons induced by diabetes. Findings demonstrating that intracerebroventricular adminis-

tration of STZ causes a whole spectrum of biochemical and behavioral, Alzheimer-type alterations associated to altered gene expression of neurotrophins, including NGF (18, 19), and that diabetes can be one of the risk factor for dementia support this hypothesis. Findings showing that diabetes can be implicated in neurodegeneration in cerebral arteries (24, 27) and in the modulation of NMDA receptor activity in the CNS (13) seem to be in line with this hypothesis. NGF is one important growth factor involved in brain development and plasticity and is a good candidate for mediating survival and plasticity of brain neurons in adults (28, 41). Because brain NGF responsive neurons are involved in age-related disorders, one reasonable hypothesis is that diabetes can have negative effects also on learning and memory abilities. Yet, a question remains unanswered: STZ-induced DM or STZ itself, or both, are responsible for these particular brain changes.

Of note, NGF can stimulate VEGF synthesis endothelial cell migration in vivo and in vitro (14, 17, 20, 27, 37, 38, 43), and in tissues of cardiovascular-related disorders (8, 9, 31). This suggests that a cross-talk between NGF and VEGF may also operate in the brain (27; also see 19), and that a low presence of NGF in the brain can contribute to the development of neurodegeneration and vascular deficits observed in DM (33). The hypothesis that changes in the synthesis and release of NGF can have a pathogenic relevance for disturbed trophism in DM is suggested by evidence that: (a) the serum and skin concentration of NGF in patients with DM are decreased when compared to healthy subjects (4); (b) pancreas β-cells produce and are receptive to the action of NGF (40); (c) NGF can promote pancreatic cell survival and function (26, 32, 35); (d) peripheral neuropathies occurring in diabetic subjects can be prevented or reversed by exogenous administration of NGF (4, 5); (e) NGF promotes recover of skin and ischemic limb healing (11, 25, 43); (f) topical application of NGF to cutaneous wounds accelerate wound healing in rats (23, 30); (g) NGF stimulates VEGF-A production by human endothelial cells incubated in a high-glucose medium and confer resistance against high-glucose (37). The observation that diabetes causes decrease of VEGF protein amount not only in the pancreas, but also in the HI and pituitary gland is in line with the hypothesis that diabetes have negative effects in the brain and changes in NGF and NGF-receptor expression. Whether cells these changes are functionally linked to NGF remains to be investigated.

Collectively, these findings point to a role of NGF in a number cell deficits induced by diabetes. However, whether NGF can exert a similar protective role on damaged brain cell induced by DM needs to be further investigated.

## SUMMARY

Type 1 diabetes mellitus (DM), a "classical" result of a pancreatic-β cell damage, is associated with various metabolic, neuronal, endocrine and immune alterations at cellular, tissue and organ levels. Nerve growth factor (NGF) is one of the most extensively studied neurotrophic factors, which is produced and released by numer-

ous cells including the pancreatic β cells. NGF plays an important role during brain development and may be able to delay or even reverse damaged forebrain cholinergic neurons that undergo degeneration in aged animals and in Alzheimer's disease (AD). Recent reports indicate that experimentally induced DM in rodents can cause brain biochemical and molecular alterations similar to those observed in sporadic AD. Given the importance of NGF in the pathophysiology of brain cholinergic neurons, we looked for NGF changes in the pancreas and brain of diabetic rats. The aim of this study was, therefore, to investigate the effect of streptozotocin-induced DM on NGF and NGF receptor expression in pancreas and brain. The results showed that DM is associated with altered NGF, NGF-receptor expression in both pancreas and brain.

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