Substance P and Vasoactive Intestinal Polypeptide in the Streptozotocin-Induced Diabetic Rat Retina

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Purpose. Little knowledge exists about how neurotransmitters behave in the diabetic retina. In this study, the authors measured the concentration of two neuropeptides, substance P and vasoactive intestinal polypeptide, in the streptozotocin-induced diabetic rat retina in a time-dependent manner.

Methods. The retinas of 1-, 3-, 5-, 8-, and 12-week diabetic rats were processed using a highly sensitive radioimmunoassay for both substance P and vasoactive intestinal polypeptide. Furthermore, the peptide-immunoreactivities were characterized by high-pressure liquid chromatography.

Results. Substance P and vasoactive intestinal polypeptide were found to be significantly reduced with a maximum decrease of 28.6% (±6.7) and 64.5% (±10.7) after 5 weeks, respectively. The peptide-immunoreactivities were found in a major peak coeluting with the synthetic peptides indicating that the quantitative values measured by radioimmunoassay represent the authentic peptides.

Conclusions. The reduction of substance P and vasoactive intestinal polypeptide is in clear contrast to the amino acid transmitters GABA and glycine, which have been shown to be elevated in this early stage of diabetic retinopathy. This finding is important for three reasons: First, the decrease may result in reduced excitability of inner retinal neurons, as both peptides are known to modulate the excitability of these neurons; second, the decrease may be the consequence of a depressing and/or damaging effect by excitotoxins; and third, it may help explain why neovascularizations do not occur in this animal model, although VEGF is massively upregulated, as substance P is a very potent vascular growth factor. (*Invest Ophthalmol Vis Sci.* 2001;42:1045–1050)

Diabetes mellitus (DM) is one of the most serious diseases in ophthalmology. It is associated with severe microvascular complications including loss of pericytes, capillary dilatation, capillary leakage, and thickening of the capillary basement membrane. The most serious complication is the occurrence of fibrovascularizations due to endothelial cell proliferation, and these neovascularizations both at the disc and elsewhere are the source of recurrent vitreous hemorrhages and may lead to traction retinal detachment. Beside these microvascular changes, at present, little is known about how neuronal elements behave in this disease entity in the retina. The present study concentrates on two neuropeptides, substance P (SP) and vasoactive intestinal polypeptide (VIP), and intends to evaluate the effect of DM on these peptides in the rat retina.

SP belongs to the family of tachykinin (TK) peptides that includes SP, neurokinin A (NKA), neurokinin A-related peptides (neuropeptide K and neuropeptide χ), and neurokinin B (NKB). Two distinct, structurally related genes encode for SP, NKA, and NKA-related peptides, which are the protein products of the preprotachykinin (PPT)-A gene, while NKB is derived from the PPT-B gene (for reviews see References 1 and 2). TK peptides have been identified in the mammalian central nervous system, where they are widely distributed and act as neuroactive substances.[1–3] In the mammalian retina, SP/TK-immunoreactivity (IR)[4–17] and TK mRNAs[18] have been localized to mainly amacrine cells, with their processes arborizing at three distinct levels in the inner plexiform layer (IPL). In addition, the presence of SP/TK-IR in ganglion cells of the rat and rabbit retina has been documented.[6,7,14] TK peptides are likely to act at specific receptor sites.[19] In the rat, rabbit, and bovine retina, specific high-affinity SP binding sites are mostly concentrated in the IPL,[19–21] yet recent immunohistochemical investigations have detected the tachykinin receptor neurokinin 1 (NK1; whose main ligand is SP[22]) in amacrine, interplexiform, displaced amacrine and perhaps some ganglion cells of the rat retina.[23]

VIP, a 28-amino acid peptide, is also widely distributed in the peripheral and central nervous system, where it is likely to act as neurotransmitter or neuromodulator.[24,25] The presence of VIP in the vertebrate retina has been well documented using radioimmunoassay,[26,27] immunohistochemistry[28–43] and in situ hybridization,[44] and high affinity binding sites have been detected in rat and rabbit retinas[45] as well as in bovine retinal membranes.[46] VIP-IR in mammalian retinas is localized to sparsely occurring amacrine cells having multistratified processes within the IPL.[31,36–39,41,43] In the rat and rabbit retinas, these cells also contain GABA and constitute a distinct subpopulation of GABAergic neurons.[47,48]

We know from these studies that both peptides are expressed in the mammalian retina possibly participating in visual processing and they may underlie changes under diabetic metabolism conditions. Application of streptozotocin destroys the B-cells of the pancreas inducing type 1 DM. This drug is used in an animal model to induce DM and this diabetes model constitutes a widely accepted model to explore diabetes-associated pathologies, even peptidergic changes in several organs. We applied this animal model to evaluate diabetes-associated peptidergic changes in the rat retina. The objective of the present study was to characterize the effect of diabetes mellitus on SP and VIP and we report here significantly reduced peptide levels.

Materials and Methods

Animals

Sprague–Dawley rats (male albino, approximately 200 g) were used. The animals were housed in stainless steel cages and fed standard rat

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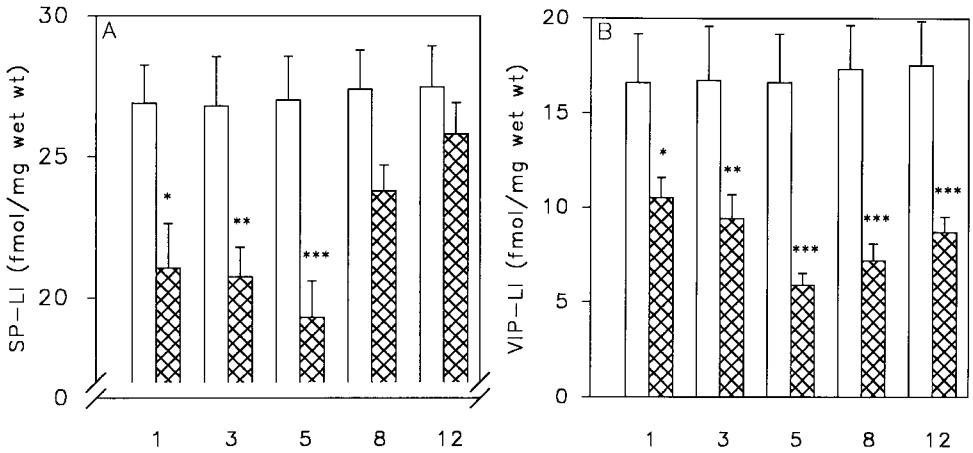

FIGURE 1. Concentration of SP (**A**) and VIP (**B**) in the retina in saline-injected control rats (*open bars*) and in streptozotocin-induced diabetic rats (*cross-hatched bars*). The retinas were gently removed after 1 week (controls, $n = 10$; diabetics, $n = 11$), 3 weeks (controls, $n = 10$; diabetics, $n = 12$), 5 weeks (controls, $n = 11$; diabetics $n = 13$), 8 weeks (controls, $n = 10$; diabetics, $n = 10$) and 12 weeks (controls, $n = 10$; diabetics, $n = 6$) after intraperitoneal administration of saline or streptozotocin and subjected to detection of the concentration by means of RIA. The data are expressed as means ± SEM. Statistical calculation was performed with the student's *t*-test and significance refers to saline-injected controls, i.e., $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

chow and tap water ad libitum. They were held in a room on a 12-hour light–dark cycle with an ambient temperature of 22° ± 1°C. All experimental and animal care procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Diabetes mellitus was induced by a single intraperitoneal injection of 65 mg/kg streptozotocin (Sigma, Vienna, Austria) and the animals were held without insulin treatment until sacrifice. Age-matched rats treated with saline were used as controls. Tail vein blood glucose was measured after 7 days and rats were considered hyperglycemic with a blood glucose reading of > 450 mg/dl. Animals with blood glucose < 450 mg/dl were excluded from the study. The animals were kept for 1 week, 3 weeks, 5 weeks, 8 weeks, and 12 weeks for radioimmunoassays and 12 weeks for high pressure-liquid chromatography. The animals were killed by an ether overdose and decapitated at the respective date. The eye was then enucleated, the animals retinas were dissected, the wet weight measured and the specimens were processed by the following methods:

Radioimmunoassay

The content of SP and VIP was determined in the retinas of saline-injected and streptozotocin-induced diabetic rats by means of radioimmunoassay (RIA) at various durations of the disease. Immediately after removal, the retinas were stored at -80°C and were kept frozen until the last tissue samples were collected from the 12-week time point. Each retinal sample was then homogenized in 0.6 ml of 2 M acetic acid, centrifuged (3500*g*, 10 minutes) and the supernatant subjected to analyzation of the SP- and VIP-immunoreactivities. 100 μl of the clear supernatant was used for the SP- and VIP-RIA. The SP- and VIP-RIA was performed with a specific antiserum (RD2 [gift from Leeman SE, Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, MA] and VIP2 [gift from Theodorsson E, Department of Clinical Chemistry, Karolinska Hospital, Stockholm, Sweden]). Incubation was performed for 48 hours without and a further 48 hours with the tracer added ([^{125}I]-Bolton Hunter-SP, [^{125}I] iodohistidyl-VIP; Amersham, Vienna, Austria). Separation of bound and free radioactivity was carried out with dextran-coated charcoal. Under these conditions, the detection limit of the assays was 0.1 to 0.2 fmol. The values were given as fmol/mg wet weight. Statistical calculations for comparison of control with diabetic data were done with the student's *t*-test.

High Pressure Liquid Chromatography

For determination of the chromatographic homogeneity of the SP- and VIP-like immunoreactivities (LI), tissue extracts of one retina were injected to a reversed phase high pressure liquid chromatography (HPLC) column (μBondapak Phenyl; Waters, Milford, MA) and eluted with 0.15 M 80% triethylammonium formate buffer (pH 2.5) and 20% acetonitrile at a flow rate of 1 ml/min. After 10 minutes, the acetonitrile concentration was increased linearly to 60% during the subsequent 40 minutes. Fractions (1.0 ml) were collected, lyophilized, reconstituted in assay buffer, and analyzed with the SP- and VIP-antibodies by means of RIA as described previously. The elution positions of the respective peptides were determined in separate runs with small amounts of synthetic peptides.

RESULTS

Animals

Diabetic rats exhibited typical characteristics of this experimental model, that is, marked hyperglycemia and glucosuria, retarded growth, scruffy fur, and loose stools. The mean blood glucose at various durations of diabetes mellitus is not specified because in certain cases it was higher than the detection limit of 600 mg/dl. Instead, another indicator of the diabetic metabolism condition should be mentioned, namely the body weight. The body weight of diabetic rats was significantly reduced at all stages of the disease compared with age-matched controls, the diabetic animals weighed 85.2% (± 4.3), 75.6% (± 5.4), 70.1% (± 5.1), 63.6% (± 7.7), and 58.8% (± 10.8) of control animals at the 1-week, 3-week, 5-week, 8-week, and 12-week time point, respectively.

Concentration of SP and VIP

The concentration of SP and VIP obtained by RIA varied between 26.8 (±1.75) and 27.5 (±1.46) fmol/mg wet weight for SP and between 16.6 (±2.56) and 17.5 (±2.35) fmol/mg wet weight for VIP in saline-injected control rats. There was no statistical significance observed between control values. Diabetic rats featured significantly reduced concentrations of both peptides depending on the time interval of determination and the impairment of the detection signal was more pronouncedly seen for VIP than for SP.

For SP (Fig. 1A), a 21.7% (±7.5) decrease was observed after 1 week, a 22.6% (±5.01) decrease after 3 weeks culminating in a 28.6% (±6.7) decrease after 5 weeks. At the 8-week and 12-week period the peptide levels returned to approximately normal values, that is, 86.7% (±3.9) and 93.9% (±4.3) of controls, respectively.

In contrast, the other peptide under investigation, VIP, was significantly reduced at all time points of the diabetic state (Fig. 1B). A 36.7% (±10.5) decrease was observed after 1 week, a 43.7% (±13.6) decrease after 3 weeks, reaching a maximal reduction of the concentration after 5 weeks, i.e., a 64.5% (±10.7) decrease. The peptide levels thereafter also rose again, as was found for SP, but did not return to control levels. After

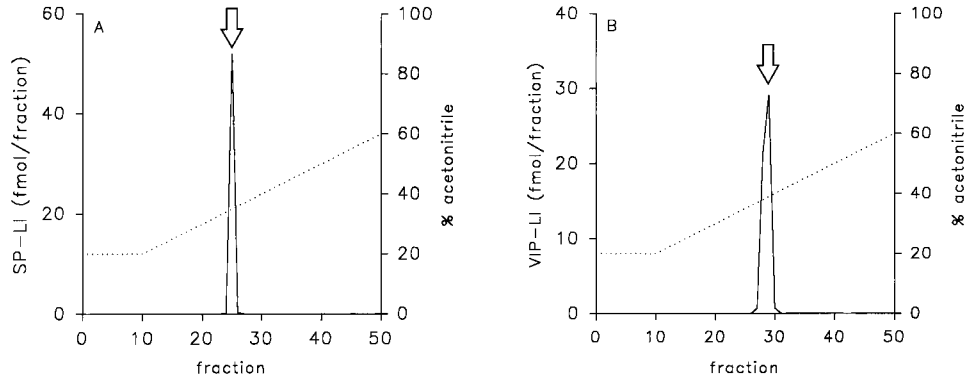

FIGURE 2. Analysis of SP- (**A**) and VIP- (**B**) like immunoreactivities by HPLC. Then, 100 μl of retinal extracts were injected into a reversed phase column (μBondapak Phenyl; Waters) and eluted with 0.15 M 80% triethylammoniumformate buffer (pH = 2.5). The *dotted line* represents the gradient profile (percent acetonitrile, *right ordinate*). One ml/min fractions were collected, lyophilized, reconstituted in assay buffer, and immunoreactivities were determined by RIA. The elution positions of synthetic SP and VIP are indicated by the *arrow*. Thus, both peptides are found in a major peak coeluting with the synthetic peptides indicating that the quantitative data measured by RIA represents the authentic SP and VIP, respectively.

8 weeks, the concentration of VIP in the diabetic rat retina was 41.6% (±12.1) of controls and after 12 weeks, 49.7% (±9.2).

High Pressure Liquid Chromatography

The peptide-LIs measured by means of RIA were further characterized by gradient high pressure liquid chromatography. The results are illustrated in Figures 2A and 2B. Thus, the immunoreactivities of both SP and VIP are found in only one peak coeluting with synthetic SP and VIP, respectively. This indicates that the quantitative values of SP and VIP measured by RIA represent the authentic peptides.

DISCUSSION

In the central nervous system, most neuropeptides have been shown to fulfill the criteria of neurotransmitters; that is, expression in certain specialized cells, presence and wide distribution, release after depolarization-induced stimulation, presence of a receptor or at least binding sites, and functional effects. In the retina, SP is one of the best characterized peptides and has been qualified according to these criteria. This study briefly aimed to descriptively evaluate DM-associated SP-ergic changes in the rat retina. It must be emphasized that we anticipated our study to reveal a nearly complete picture about diabetes-associated SP-ergic changes in this rat model excluding functional experiments. For this purpose, we examined the expression of the SP-precursor PPT-A by Northern blot analysis at the same time points as were carried out for the RIA experiments, but we found no effect of streptozotocin-induced DM. Thus neither an upregulation nor downregulation was observed at the mRNA level (data not shown). Then, we also examined the expression of the NK1 receptor by Northern blot analysis; again, neither an upregulation nor downregulation at the mRNA level was observed at 1, 3, 5, 8, and 12 weeks after streptozotocin-injection (data not shown). Finally, the authors also conducted experiments on the release of this peptide by an in vitro perfusion system comparing the release of SP from the retina of 12-week diabetic rats versus controls. Neuropeptides are known to be stored in large dense core vesicles, and the release is an exocytotic process mediated via opening of voltage sensitive Ca^{2+}-channels. In the hypothalamus, we found the L-type dihydropyridine-sensitive channel to be the one triggering neuropeptide release.[49] Using the same method in the rat retina, neither a basal nor an evoked release by electrical field stimulation could be detected, even when high concentrations of K^+ were added to the perfusion medium. This was surprising as other authors found an increased release at least for SP in the bovine retina with K^+.[21] One possible explanation for this may be the low concentration of the peptides in the rat retina. On the other hand, the diffusion of K^+ into the inner nuclear layer and of the peptides from amacrine cells after stimulation may be the limiting step. An altered release under diabetic metabolism conditions would have been indicative for pathologies of the exocytotic apparatus. However, our experiments show that the rat retina is not the appropriate tissue to study this exocytotic process by in vitro perfusion, even when pooled retinas are used.

Therefore, the only clear effect of streptozotocin-induced DM was found at the protein level. Diabetes induced alterations in neuropeptide content have been reported in various organs—including the gastrointestinal tract, skin, urogenital-tract, blood vessels, peripheral nerve, spinal cord, and brain—and the described changes do not follow a predictable pattern. Thus, levels of particular peptides have been reported to increase, decrease or remain unchanged depending on the target tissue innervation. In the eye for example, elevated levels of certain neuropeptides, that is, SP, calcitonin gene-related peptide,[50] and VIP[51] have been found in the rat iris of streptozotocin-induced DM and these changes have been held responsible for reported clinical deficits in pupillary diameter regulation, but no effects were found in the cornea.[50] As the time point of investigation is also crucial, we decided to evaluate streptozotocin-induced SP-ergic changes in the rat retina in a time-dependent manner. In this study, SP was found to be transiently significantly reduced in the diabetic rat retina. This decrease is not unique for this peptide as another peptide, VIP, was also found to be reduced. This is in clear contrast to certain other transmitters such as GABA and glycine, which have been found to be elevated in this early stage of the disease.[52] As the expression of SP at the mRNA level was not decreased, posttranscriptional modifications must lead to this result. The other main finding of our study concerns the chromatographic separation and purification. There is evidence that both peptides are present as free peptides within the diabetic rat retina, at least the antibodies used in the RIA experiments only recognized the respective peptides. Similar results for SP have already been obtained by other authors in the bovine retina.[21]

The role of both peptides in the retinal physiology is not clear but an involvement in visual processing has been insinu-

ated by many authors. This is supported by the finding that there exist high affinity binding sites for radioactively labeled SP in the human retina[19–21] and the most important study published by Casini et al. demonstrates the localization of the NK1 receptor in the rat retina.[23] Thus NK1-IR processes were found to be densely distributed across the IPL, and cells expressing the SP receptor are predominantly GABA and TH-IR amacrine cells, displaced amacrine, interplexiform, and some ganglion cells. From pharmacological and physiological investigations, there is evidence of excitatory actions of SP in nonmammalian and mammalian retinas. For instance, exogenously applied SP depolarizes amacrine cells that are likely to be GABAergic in the rabbit retina,[53] and it elicits the release of dopamine from amacrine cells in the rat retina.[54] Furthermore, direct and long-lasting effects have been reported of SP in modulating the excitability of ganglion cells in the rabbit retina[53] and SP has also a long-lasting excitatory effect on most ganglion cells in the mudpuppy and fish retina.[55,56] These investigations are in agreement with the receptor studies of Casini et al. and with the report of SP-IR synaptic input from amacrine cells to amacrine and ganglion cell bodies and processes in the guinea pig retina.[57] Taken together, it is suggested that SP has an excitatory and long-lasting influence on multiple retinal cell populations in the inner retina. For VIP, on the other hand, high-affinity binding sites also have been shown in the rat retina,[45] but an exact localization of the receptor has not been characterized. On the physiological basis, VIP has been shown to potentiate the GABA-induced chloride currents at $GABA_A$ receptors in isolated bipolar and ganglion cells of the rat retina[58] and to significantly increase the maintained activity of both ON- and OFF-center ganglion cells of the rabbit retina,[59] suggesting that VIP-containing amacrine cells exert modulatory roles on the flow of visual information through the retina. Our results provide evidence of profound SP- and also VIP-ergic changes in the diabetic rat retina and furthermore support the observation that neurotransmitters change even before clinical abnormalities are visible. The significance of the reduced peptide levels for the retinal physiology in diabetics remains to be examined. As both peptides are generally thought to act modulatorily on inner retinal neurons, the reduction of the peptides may result in reduced excitability of these neurons, thus impairing the efficacy of synaptic transmission in the retina. To confirm this, it would also require studies on the release of the peptides under diabetic metabolism conditions and as we were not able to investigate this, further studies are needed to clearly establish the significance of our results.

The consequences of SP- and VIP-ergic reductions in the diabetic rat retina are only speculative as no functional studies were carried out in this study. However, reduced amplitudes in oscillatory potentials (OPs) observed in the electroretinogram (ERG) characterize major electrophysiological abnormalities in diabetes. These changes are commonly seen in the early stage of the disease even before the onset of retinopathy, indicating a particular susceptibility of OPs to the altered metabolic conditions induced by diabetes. The exact pathogenesis of the reduced OP amplitudes has still not been fully elucidated, but they either arise as a postsynaptic response to glutamate-releasing neurons,[60,61] or in a feedback circuit mediated by amacrine cells.[62] Considering the origin of OPs in the inner retina, Ishikawa et al. were the first to suggest changes in the metabolism of certain amino acid transmitters in this portion as being responsible for the reduction of OPs in diabetes as they found an elevated retinal content of GABA and glycine, which are known depressants of OPs.[52] This study also proved that the earlier OPs are more susceptible to the effect of diabetes. Concerning our results of reduced peptide levels in this early stage of diabetes, a possible relationship can be suggested between reduced early OPs and reduced SP and VIP. According to the excitotoxin theory that makes excitatory amino acids responsible for reduced OP amplitudes, our results may reflect a depressing and/or damaging effect on certain amacrine transmitters by excitotoxins. As the decrease of VIP was found to be more pronounced than that of SP in the diabetic rat retina, it might be speculated that VIPergic systems are more vulnerable. The reincrease of SP argues against a toxic effect of both streptozotocin and excitotoxins for the decrease, this decrease is rather a depressing effect. Thus, this depressing effect on SP seems to be overcome by prolonged diabetes mellitus. However, there is an imbalance in retinal neurotransmitters at least in this early stage of retinopathy.

Another important fact worth discussing concerns the biological activities of SP. Although streptozotocin-diabetic rats exhibit some retinal vascular abnormalities,[63–65] a typical proliferative diabetic retinopathy, similar to that which develops in humans, does not develop in these rats. Recent studies made IGF-1 and FGFs responsible for the ischemia-induced proliferative response, the most modern studies mainly dealt with VEGF as the proangiogenic agent (for review, see Reference 66). Interestingly, these neovascularizations do not occur in this diabetes model, although both VEGF and its receptors are upregulated in the retinas.[67] The reason for this is not clear. On the one hand, the animals may perish before the occurrence of neovascularizations in the course of the diabetic metabolism conditions; on the other hand, intrinsic antiangiogenic factors that counteract angiogenic stimuli may be highly active in the rat retina, although direct evidence for such a hypothesis is lacking. Alternatively, proliferative diabetic retinopathy might not only be the product of these factors alone, but also including other currently unrecognized factors. One of these factors may be hepatocyte growth factor (HGF), which has been found to be elevated in the vitreous of patients with proliferative diabetic retinopathy.[68] Another factor constitutes SP, which has the same endothelial cell migratory effect at similar dosages as has bFGF,[69] and SP induces vasoproliferation in vivo, that is, has a vascular growth factor potential.[70] Both effects are mediated by nitric oxide.[71] The reduction of SP, as has been found in this study, may support the idea of a deficiency of proangiogenic stimuli and may thus provide further insight into the fact that neovascularizations do not occur, although VEGF and its receptors are upregulated. The damaging effect of laser photocoagulation onto peptide-expressing cells with reduction of SP as the consequence, would be in agreement with the beneficial effect of this therapy regime. Thus, regression of neovascularizations subsequent to laser photocoagulation would also include this peptide. This is no more than a hypothesis, but it constitutes a novel aspect that should, however, encourage further investigations all the more because there are potent even nonpeptide antagonists available for this peptide. As a first step to evaluate whether SP may be involved in the pathogenesis of proliferative diabetic retinopathy, the authors are currently collecting vitreous samples of patients with proliferative retinal diseases and intend to find out whether certain neuropeptides are present in these specimens.

In conclusion, we found significant changes of SP in the streptozotocin-induced diabetic rat retina. The concentration of this peptide is decreased in a time-dependent manner, and this is the result of posttransciptional modifications as the expression was found not to be altered. There is also no influence of streptozotocin-induced DM on the expression of the NK-1 receptor. The decrease of SP is in clear contrast to certain amino acid transmitters in this diabetes model and furthermore, is not unique for SP, as VIP also was found to be reduced.

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