Attenuated Dopaminergic Tone in the Paraventricular Nucleus

Contributing to Sympatho-Excitation in Rats with Type 2 Diabetes

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Running title: Central nervous system regulation of sympatho-excitation in diabetes

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ABSTRACT

The study was conducted to investigate the role for dopamine in the centrally mediated sympatho-excitatory response in rats with type 2 diabetes (T2D). T2D was induced by a combination of high-fat diet (HFD) and low-dose streptozotocin (STZ). HFD/STZ treatment for 12-14 weeks, resulted in significant increase in the number of FosB positive cells in the paraventricular nucleus (PVN) and rostral ventrolateral medulla (RVLM). In anesthetized rats, administration of exogenous dopamine (dopamine hydrochloride, 20 mM) in the PVN, but not in the RVLM, elicited decreases in renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) in control rats and but not in the T2D rats. Blocking the endogenous dopamine with dopamine D₁/D₅ receptor antagonist SCH39166 (2 mM) in the PVN and RVLM, resulted in increases in RSNA, MAP and heart rate (HR) in both control and T2D rats. These responses were significantly attenuated in T2D rats compared to control rats (PVN - ΔRSNA: 21 ± 10 vs. 44 ± 2 %; ΔMAP: 7 ± 3 vs. 19 ± 6 mmHg, ΔHR: 17 ± 5 vs. 32 ± 4 bpm, P < 0.05). There were no significant increases in response to dopamine D₂/D₃ receptor antagonist raclopride application in the PVN and RVLM of both control and T2D rats. Furthermore, there were decreased dopamine D₁ receptor and D₂ receptor expressions in the PVN of T2D rats. Taken together these data suggest that reduced endogenous dopaminergic tone within the PVN may contribute to the sympatho-excitation in T2D.

Keywords: central nervous system; dopaminergic; sympathetic nerve activity
INTRODUCTION

Type 2 diabetes (T2D) is known to be closely linked with insulin resistance and elevated sympathetic activation (11, 23). The elevated activation of the sympathetic nervous system contributes to the onset and maintenance of cardiovascular complications such as hypertension, cardiac arrhythmias and atherosclerosis in T2D (41). The evidence for an increased activation of the sympathetic nervous system in diabetes is the observation of an increased heart rate (HR) and blood pressure (BP), diminished HR variability, baroreceptor dysfunction, and finally high concentrations of catecholamine in the plasma and urine (16, 18). The underlying mechanisms linking diabetes with sympathetic activation are complex and not yet clearly understood. Previous studies suggest that the central nervous system is critical in regulating sympathetic activation and thus contributing to the altered neuro-humoral drive during diabetes (1, 39, 42, 53).

Dopamine is involved in the regulation of a broad range of biological functions, including locomotor activity, cognition, food intake and hormone secretion (38). Dopamine signal is mediated by at least five distinct G-protein coupled receptor subtypes, classified as D1-like (D1 and D5) and D2-like (D2, D3 and D4) receptors (26). The D1 receptor is the most widespread dopamine receptor in the brain which has been found in the striatum, olfactory tubercle, limbic system, hypothalamus and thalamus (5). The D2 receptor is also abundant in the mammalian brain. It has been detected in many areas, including the paraventricular nucleus (PVN) of the hypothalamus (26, 40) and the dorsal vagal complex of the human medulla (12). Dopamine neurons are found in the periventricular, the anterior, medial and lateral parvocellular regions of the PVN (49). Tyrosine hydroxylase (TH)- and dopamine-beta-hydroxylase (DBH)-like immunoreactive terminals were found distributed throughout the rat hypothalamus and were
abundant in all parts of the PVN (9). Dopamine innervation of the PVN may be responsible the 
euroendocrine, behavior response and sympathetic regulation (21, 24, 54).

A number of investigations have attempted to elucidate the role of dopamine in the 
pathogenesis and treatment of high BP (26, 30). The D₁ receptor of smooth muscle cells induces 
vasodilatation in the vasculature in the systemic circulation, whereas in the kidney they modulate 
sodium excretion, induce natriuresis, diuresis, and improve renal blood flow and glomerular 
filtration in renal tubules (30). The D₂ receptors are located in the sympathetic endings and their 
stimulation reduces norepinephrine release and thus induces vasodilatation, decreasing HR and 
BP (26). In the central nervous system, dopaminergic effects on the cardiovascular system 
include inhibitory or excitatory actions. Low dose of dopamine increases renal sympathetic nerve 
activity (RSNA), and a high dose of dopamine decreases RSNA suggesting that dopamine may 
mediate some of the central sympatho-inhibitory effects upon hyperactivation of the 
dopaminergic system (54).

Previously, our laboratory has carried out a series of experiments documenting the central 
mechanisms involved in sympathetic abnormalities contributing to the altered neuro-humoral 
drive during streptozotocin (STZ)-induced type 1 diabetes (36, 55, 56). Evidence indicates that 
the PVN of the hypothalamus is involved in the blunted renal sympatho-inhibition in response to 
acute volume expansion in diabetic rat. We also demonstrated significant increases in the neural 
activity in the PVN of rats with diabetes (17). These data suggest that the neurons in the PVN are 
activated and this may contribute to the autonomic dysfunction observed during diabetes. In 
T2D, reduced dopaminergic tone in hypothalamic neural circuits have been observed by the 
others (38). Reduced dopaminergic neurotransmission in the suprachiasmatic nucleus of obese 
animals appears to drive norepinephrine and neuropeptide Y mediated transmissions in other
nuclei to induce the obesity syndrome. Treatment with D₂ receptor agonists can reverse the metabolic syndrome in these animals (38). Despite this evidence, the central mechanisms by which dopamine contributes to altered neuro-humoral drive during T2D remain unclear. In the present study, we hypothesized that reduced dopaminergic activity in the PVN neurons may contribute to the enhanced sympatho-excitation commonly observed in T2D.

**MATERIALS AND METHODS**

**Induction of type 2 diabetes**

This study was approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee, and conforms to the guidelines for the care and use of laboratory animals of the National Institutes of Health and the American Physiological Society. Male Sprague-Dawley rats (150-180 g, Sasco, Omaha, NE) were maintained in vivarium with a 12-h light/12-h dark cycle and placed on the high fat diet (HFD, Harlan, Indianapolis, IN), in which 42% of calories are from fat. After 4 weeks of HFD feeding, the rats were injected once with low-dose STZ (30 mg/kg, i.p) to induce partial insulin deficiency. These rats were then placed on the HFD for another 8 weeks. The “normal diet” fed rats were used as non-diabetic controls.

Postprandial plasma glucose, body weight, and food consumption were monitored weekly. The levels of plasma insulin and leptin were measured by immunoassay (ALPCO, Salem, NH), while plasma triglyceride level was measured by triglyceride quantification kit (BioVision, Milpitas, CA). Rats were fasted for 4 h before glucose tolerance tests. Oral glucose load was administered at 2 g/kg of body weight. Glucose levels were measured from tail bleeds with a glucometer at specified time points after glucose administration.
**Urinary Norepinephrine Excretion Measurements**

Urinary norepinephrine excretion was measured as an index of overall sympathetic activity. Six to eight weeks after surgery, rats from all groups were placed in metabolic cages and urine was collected for 24 hrs. The 50 ml collecting tubes contained mineral oil to prevent evaporation losses. After collection, urine was centrifuged and transferred to eppendorf tubes containing 1N HCl to prevent auto-oxidation of catecholamines, and then stored at -80ºC.

Urinary norepinephrine concentration of thawed samples was measured using a commercially available ELISA kit (Labor Diagnostika Nord, Nordhorn, Germany), following the manufacturer’s instructions. The limit of detection of the assay is 1.5 ng/ml norepinephrine in urine. Urinary excretion of norepinephrine was calculated using urine flow rate.

**FosB and dopamine receptor immunohistochemistry**

The rats were anesthetized with pentobarbital (65 mg/kg) and perfused transcardially with 150 ml of heparinized saline followed by 250 ml of 4 % paraformaldehyde in 0.1 M sodium phosphate buffer. The brain was removed and postfixed at 4°C for 4 h in 4 % paraformaldehyde solution and then placed in 30 % sucrose. The brain was blocked in the coronal plane and sections 30 µm in thickness were cut with a cryostat.

One set of floating sections was rinsed 4 times in 0.1 M phosphate buffered saline (PBS) and then incubated in 0.3 % hydrogen peroxide for 30 min at room temperature. After rinsing, sections were incubated in PBS containing 3 % normal horse serum and 0.25 % Triton X-100 for 2 h. Sections were then incubated with a polyclonal rabbit anti-FosB antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) for 48 h at 4°C. Sections were rinsed and incubated with
biotinylated goat anti-rabbit IgG for 2 h at room temperature (1:100), then incubated with avidin-biotin complex (1:200; Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 1 h, rinsed in PBS for 30 min. Immunostaining was visualized by incubation in diaminobenzidine kit (DAB kit, Vector Laboratories, Burlingame, CA). Finally, they were mounted on gelatin-coated glass slides, dried and cover-slipped.

The presence of FosB positive cells in discrete regions of the PVN, supraoptic nucleus (SON), media preoptic nucleus (MnPO), subfornical organ (SFO) and rostral ventrolateral medulla (RVLM) was examined with a Leica microscope under bright field from all four groups of rats. Images were captured with a Qimaging digital camera (Qimaging, Surrey, Canada). The staining was evaluated by counting the number of nuclei positively stained for FosB by ImageJ software (National Institutes of Health, Bethesda, MD). Three adjacent sections were considered to represent one coronal level. The average number of cells in the sections (PVN: 1.80 ± 0.1 mm; SON: 1.80 ± 0.1 mm; MnPO: 0.4 ± 0.1 mm; SFO: 0.9 ± 0.1 mm, RVLM: 12.0 ± 0.1 mm posterior to bregma) was taken to represent the number of cells unilaterally. An independent observer blind to the two experimental groups counted cells within the identified boundaries of the PVN, SON, MnPO, SFO and RVLM according to the atlas of Pellegrino LJ (37).

Other two groups of sections were incubated with 10 % normal donkey serum in PBS for 1 h at room temperature and then incubated with primary antibody against D1 receptor (anti-rabbit, 1:200, Santa Cruz), D2 receptor (anti-goat, 1:200, Santa Cruz) or a neuronal marker microtubule-associated protein 2 (MAP2, anti-mouse, 1:200, Abcam, Cambridge, MA) overnight at 4°C. After washing with PBS, the sections were incubated with Cy3-conjugated donkey anti-rabbit/anti-goat secondary antibody (1:200) and Cy2-conjugated donkey anti-mouse secondary antibody (1:400, Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. After
washing with PBS and drying, the sections were cover-slipped with fluoromounting-G (SouthernBiotech, Birmingham, AL). Distribution of dopamine receptor immunofluorescence within the PVN was viewed using an Olympus fluorescence microscope (Japan) equipped with a digital camera (Qimaging, Canada). The images were taken under same conditions (exposure time and resolution) in both groups and not adjusted prior to quantification. ImageJ software (National Institutes of Health) was used to identify the total intensity of positive staining with Cy3. Three alternate sections (1.80 ± 0.1 mm posterior to bregma) representing the PVN were analyzed in this way, and then the mean data was calculated. Although the images showed some overlap of D1/D2 receptor with the neuronal marker MAP2, still there is the lack of phenotypic characterization of the D1 and D2 positive cells (i.e. RVLM/spinally projecting).

**General surgery for recording of renal sympathetic nerve activity and arterial pressure**

Rats were anesthetized with α-chloralose (70 mg/kg, i.p) and urethane (0.75 g/kg, i.p). The femoral vein was cannulated for administration of supplemental anesthesia and 0.9 % saline. The femoral artery was cannulated for recording mean arterial pressure (MAP) and HR. The left renal nerve was isolated and cut. The central end of the nerve was used to record the electrical signal with the PowerLab (ADInstruments, Colorado Spring, CO) to monitor RSNA as described before (14, 56). Basal nerve activity was determined at the beginning of the experiment, and background noise was determined by nerve activity recorded at the end of the experiment (after the rat was euthanized). The nerve activity during the experiment was calculated by subtracting the background noise from the recorded value. The changes in integration and frequency of the nerve discharge were expressed as a percentage from basal value. Responses of MAP and HR were expressed as the absolute difference between the basal value and the value after each dose.
of a drug.

Microinjection of dopamine, D<sub>1</sub>/D<sub>5</sub> antagonist and D<sub>2</sub>/D<sub>3</sub> antagonist into the PVN

Rats were placed in a stereotaxic apparatus. An incision was made on the midline of the scalp to expose bregma. The coordinates of the right PVN with reference to bregma were calculated as being, 1.5 mm posterior, 0.4 mm lateral, and 7.8 mm ventral to the dura (14). Thirty-45 mins after the surgery, a needle (0.2 mm OD) that was connected to a microsyringe (0.5 μl) was lowered into the PVN. Stable baseline parameters were monitored for 10-20 minutes before each microinjection. At the completion of the experiment, monastral blue dye (2 % Chicago blue, 30 nl) was injected into the brain for histological verification (Figure 7). As a comparison, microinjection was also performed in the RVLM within the brain stem. The coordinates of the right RVLM with reference to bregma were calculated as being, 12.5 mm caudal to the bregma, 2.1 mm lateral to the midline and 9.2 mm ventral to the surface of cerebellum (52). The RVLM was chemically identified by a transient pressor response (at least 20 mmHg) to injection of l-glutamate (50 mM in 100 nl).

Experiment 1: In a set of six control rats and six T2D rats, dopamine hydrochloride (Tocris, Ellisville, MO) was microinjected (20 mM in 100 nl) into the PVN and RVLM. The responses in RSNA, MAP and HR over the 30 min were recorded.

Experiment 2: In another set of control (n = 8) and T2D rats (n = 8), RSNA, MAP and HR were measured while D<sub>1</sub>/D<sub>5</sub> antagonist SCH39166 (Tocris, Ellisville, MO) or D<sub>2</sub>/D<sub>3</sub> antagonist raclopride (Tocris, Ellisville, MO) was microinjected into the PVN and RVLM (2 mM in 100 nl), respectively. The changes in RSNA, MAP and HR over the 30 min were recorded.
Micropunch of PVN for Western blot measurements

In a separate group of control (n = 6) and T2D (n = 6) rats, the brains were removed and frozen on dry ice after the sacrifice of the rats with overdose of pentobarbital (150 mg/kg, i.p.). The location for the punching of the PVN sections (between 1.60 and 2.20 mm posterior to bregma) was determined relative to the fornix and the optic tract as shown in Figure 7. Six frozen serial coronal sections (100 μm each) of the PVN were cut with a cryostat according to a stereotaxic atlas of the rat brain by Pellegrino LJ (37) and bilaterally punched with an 18-gauge needle using the Palkovits and Brownstein technique (31), such that there were 12 total punches per brain. The punches for each brain were combined and placed in 100 μl of protein extraction buffer (10 mM Tris, 1 mM EDTA, 1 % SDS, 0.1 % Triton-X-100, and 1 mM phenylmethylsulfonyl fluoride) to extract the protein. As a comparison, 10 serial sections of RVLM (100 μm/section, 1.5 to 2.5 mm rostral to the obex) were also cut and bilaterally punched (total 20 punches) with an 18-gauge needle using the Palkovits and Brownstein technique (31), then processed for protein extraction.

Western blot measurement of D₁ receptor and D₂ receptor protein

The total protein concentration from the extracted protein described above was measured with a bicinchoninic acid assay kit (Pierce, Rockford, IL). Samples were adjusted to contain the same concentration of total protein, and then equal volumes of 2 X 4% SDS sample buffer were added. The protein samples were loaded onto a SDS PAGE gel and subjected to electrophoresis. The fractionated proteins on the gel were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was probed with primary antibody [rabbit anti-D₁
receptor, goat anti-D<sub>2</sub> receptor (1:500, Santa Cruz, CA), or rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH 1:2,000, Santa Cruz, CA)] overnight, and then probed with secondary antibody (peroxidase conjugated anti-rabbit or goat IgG, 1:5,000, Pierce, IL). An enhanced chemiluminescence substrate (Pierce, IL) was applied to the membrane, followed by an exposure within an UVP system (UVP BioImaging, Upland, CA) for visualization. Kodak 1D software (Kodak, NY) was used to quantify the signal. The expression of protein was calculated as the ratio of intensity of the D<sub>1</sub> receptor and D<sub>2</sub> receptor respectively, relative to the intensity of GAPDH band.

**Statistical analysis**

Data are presented as means±SE. The data were subjected to two-way ANOVA followed by comparison for individual group differences with the Newman-Keuls test. Statistical significance was indicated by a value of $P < 0.05$.

**RESULTS**

**General data**

T2D was induced by a combination of both HFD and injection of low-dose STZ. Table 1 illustrates the general characteristics of control and T2D rats used in these experiments. After 12-14 weeks of the treatments (HFD and STZ injection), the body weight and weight of the retroperitoneal fat pad were significantly higher in T2D rats. The T2D rats also show increased plasma triglyceride and decreased brown adipose tissue. The fasting blood glucose, 2 h plasma glucose level, and plasma leptin were significant higher in T2D rats than those in control rats. Blood glucose tolerance (Fig. 1) and the insulin sensitivity index (Table 1) were significantly
decreased in the T2D rats compared with that in control rats. These data indicated that HFD and low dose STZ induced hyperglycemia, hyperleptinemia, hyperlipidemia, and insulin resistance (lower insulin sensitivity index and blood glucose tolerance) in T2D rats.

Meanwhile, the basal RSNA and 24 h urinary norepinephrine levels were significantly increased in T2D rats, suggesting that there was an increased overall sympathetic tone in the T2D rats (Table 1). However, there were no significant differences in basal MAP and HR between control and T2D rats.

**Increased FosB positive cells in rats with T2D**

Significant increases in the numbers of FosB positive cells in the PVN and other nuclei (SON and SFO) of the brain were observed after 12-14 weeks HFD/STZ treatment. The number of FosB positive cells was significantly increased in the PVN (207 ± 31 vs. 139 ± 22, \( P < 0.05 \)), SON (32 ± 5 vs. 19 ± 4, \( P < 0.05 \)), SFO (114 ± 10 vs. 67 ± 15, \( P < 0.05 \)) and RVLM (50 ± 10 vs. 23 ± 9, \( P < 0.05 \)) of T2D rats compared to the control rats (Fig. 2). In T2D rats, there was no significant alteration of FosB positive cells in the MnPO (29 ± 5 vs. 23 ± 5, \( P > 0.05 \)) and the adjacent areas of the PVN T2D rats. Increased FosB may indicate chronic neuronal activation within the hypothalamus in T2D rats (50, 57). Figure 2A shows the representative images of FosB staining from the PVN, SON, MnPO and SFO.

**Decreased responses to microinjection of dopamine into the PVN in T2D rats**

Administration of dopamine hydrochloride (20 mM), in the PVN elicited decreases in RSNA and MAP in control rats but barely showed any change in the T2D rats. Microinjection of dopamine elicited decreases in RSNA and MAP, reaching -17 ± 4 % (0.51 ± 0.12 μv.s) and -10 ±
2 mmHg, in control rats. The RSNA and MAP responses were significantly attenuated in T2D rats compared to the control rats, reaching -7 ± 3 % (0.28 ± 0.11 μv.s) and -3 ± 2 mmHg, respectively \((P < 0.05)\) (Fig. 3). Microinjection of dopamine did not change the HR in both control and T2D groups. As a comparison, administration of dopamine hydrochloride (20 mM), in the RVLM elicited no decreases in RSNA, MAP and HR in both groups (Fig. 3).

**Decreased responses to microinjection of dopamine antagonists into the PVN in T2D rats**

Administration of dopamine D1/D5 antagonist SCH39166 (2 mM), in the PVN and RVLM elicited increases in RSNA, MAP and HR in both groups (Fig. 4A and 4B). Microinjection of SCH39166 elicited significant increases in RSNA, MAP and HR, reaching 44 ± 2 % (1.32 ± 0.06 μv.s), 19 ± 6 mmHg and 32 ± 4 bpm in the PVN in control rats. The RSNA, MAP and HR responses were significantly attenuated in T2D rats compared to the control rats, reaching 21 ± 10 % (0.84 ± 0.38 μv.s), 7 ± 3 mmHg and 17 ± 5 bpm in the PVN \((P < 0.05)\). Microinjection of SCH39166 also elicited significant increases in RSNA, MAP and HR in the RVLM [reaching 30 ± 8 % (0.91 ± 0.24 μv.s), 14 ± 3 mmHg and 29 ± 5 bpm] in control rats. In the RVLM, the responses were also significantly blunted in T2D rats compared to the control rats [RSNA 17 ± 3 % (0.68 ± 0.11 μv.s), MAP 7 ± 4 mmHg, and HR 14 ± 4 bpm, \(P < 0.05\)] (Fig. 4B).

Administration of dopamine D2/D3 antagonist raclopride (2 mM) in the PVN and RVLM did not elicit significant increases in RSNA [11 ± 3 % (0.33 ± 0.09 μv.s) vs. 4 ± 5 % (0.16 ± 0.20 μv.s) in the PVN, 6 ± 3 % vs. 5 ± 6% in the RVLM, \(P > 0.05\)], MAP (7 ± 2 mmHg vs. 4 ± 2 mmHg in the PVN, 3 ± 3 mmHg vs. 6 ± 4 mmHg in the RVLM, \(P > 0.05\)) and HR (10 ± 4 bpm vs. 6 ±1 bpm in the PVN, 11 ± 2 bpm vs. 6 ± 3 bpm in the RVLM, \(P > 0.05\)) in both control and
T2D groups (Fig. 4B).

**Decreased dopamine receptor protein expression in the PVN and RVLM in T2D rats**

Western blotting analysis showed 74 kDa bands representing D$_1$ receptor and 51 kDa bands representing D$_2$ receptor in the PVN and RVLM of control and T2D rats. T2D rats had significantly lower protein level of D$_1$ receptor (ratio of intensity: 0.9 ± 0.2 vs. 1.9 ± 0.2, $P < 0.05$) and D$_2$ receptor (ratio of intensity: 0.4 ± 0.1 vs. 0.8 ± 0.1, $P < 0.05$) in the PVN (Fig. 5). In the RVLM, both D$_1$ receptor and D$_2$ receptor protein expression were not significantly changed in the T2D compared to the control rats (ratio of intensity: D$_1$ receptor 0.8 ± 0.2 vs. 0.6 ± 0.1; D$_2$ receptor 1.6 ± 0.3 vs. 2.3 ± 0.3, $P > 0.05$).

As an in situ confirmation of the alteration in D$_1$ receptors and D$_2$ receptors within the PVN, the immunofluorescence for D$_1$ receptors and D$_2$ receptors were found to be decreased in the PVN from rats with T2D compared with control rats (Fig. 6). Both D$_1$ receptor and D$_2$ receptor immunofluorescent signals were co-localized with neuronal marker MAP2 within the PVN. There were no significant differences of MAP2 and DAPI immunofluorescence between the groups.

**DISCUSSION**

In the present study, we have found significant increases in the numbers of FosB positive cells in the PVN of HFD/STZ induced T2D rats. Second, exogenous dopamine administration in the PVN elicited decreases in RSNA and MAP in control group and not in the T2D rats. Third, blocking the endogenous actions of dopamine with D1/D5 receptor antagonist in the PVN resulted in increases in RSNA, MAP and HR in both control and T2D rats. These responses were
significantly attenuated in T2D rats compared to control rats. Fourth, there were no significant increases in responses to dopamine receptor D2/D3 antagonist application in the PVN of both control and T2D rats. Consistent with these observations we also found decreased D1 receptor and D2 receptor expression in the PVN of T2D rats. Taken together these results show that reduced endogenous dopaminergic tone within the PVN may contribute to the sympatho-excitation in T2D.

There are a number of different animal models that are used for studying T2D (46). Rats fed HFD nutrition develop obesity, hyperinsulinemia, and insulin resistance but not frank hyperglycemia (45, 47). In our studies, T2D has been induced by HFD combined with single low dose injection of STZ. Our study shows that HFD and low dose STZ induces hyperglycemia, hyperleptinemia, hyperlipidemia, and insulin resistance (lower insulin sensitivity index and blood glucose tolerance). Clinical T2D is characterized by a progressive increase of the insulin resistance and is followed by inadequate compensatory insulin secretion from pancreatic β cells. In this T2D rat model, HFD initiates the insulin resistance, which is one of the key characteristics of the T2D patients. Low dose STZ induces a mild impairment of insulin release, one feature of the late stage of the human T2D, which closely mimics the natural history of the disease events of human T2D (from insulin resistance to β cell dysfunction) (19, 22, 29, 33). Elevated 24 h urinary level of norepinephrine and increased basal integrated RSNA, observed in this study (Table 1) are indications of increased overall sympatho-excitation in T2D rats. It has been recognized that this model of T2D would produce a spectrum of dysfunction (a range of blood glucose: 12.5-17.5 mM mild hyperglycemia, ~80 %; > 17.5 mM severe hyperglycemia, ~20 %). We took rats that had mild hyperglycemic (blood glucose: 12.5-17.5 mM) to get a fairly consistent model of T2D.
FosB has been extensively used as an indicator of synaptic activation in the central nervous system (10, 15), although FosB activation may not distinguish between excitatory and inhibitory neuronal activation. The present study identified and mapped central structures that demonstrated long-term neuronal activation (12-14 weeks after HFD/STZ induction) in rats using immunohistochemical detection of FosB. The results showed an increased number of FosB positive cells in the PVN, SON, SFO and RVLM in rats with T2D. This suggests that activation of neurons including autonomic and neuroendocrine neurons in the hypothalamus and brain stem may play a major role in the processes leading to sympathetic hyperactivity in rats with T2D. This is consistent with the observation that global sympatho-excitation is higher in T2D condition, confirmed in our studies by elevated levels of catecholamine (24 h urinary excretion of norepinephrine).

In the HFD/STZ-induced diabetic rats, we did not see an increase in the baseline blood pressure and heart rate. Blood pressure is a consequence of a number of factors including RSNA mediated changes in reno-vasoconstriction. There may have been some differential changes in sympathetic outflow such that there may be vasodilation in other vascular beds. Such differential outflow has been demonstrated previously in other studies (34). The lack of an increase in blood pressure with a concomitant increase in RSNA in diabetic animals maybe attributed to a number of possible contributing factors including but not limited to: 1) decreased cardiac output due to a dysfunction in the myocardium; 2) differential sympathetic outflow; 3) hypovolemia due to osmotic diuresis; 4) impairment of sympathetic innervation of heart and blood vessels (2).

There is accumulating evidence for a neurotransmitter-like role for dopamine in the regulation of sympathetic activation (7, 40). The PVN of the hypothalamus is an important site for autonomic and endocrine homeostasis. Dopamine neurons have been found in various areas
of PVN, including dorsal parvocellular PVN, medial and periventricular subdivisions of the PVN (13, 48), areas where preautonomic neurons reside. The PVN-mediated inhibitory response in RSNA can be mimicked by dopamine and the effect can be reversed by application of the D<sub>1</sub> receptor antagonists SCH23390 (54). In our study, exogenous administration of dopamine within the PVN caused inhibitory effect of RSNA and BP. On the contrary, D<sub>1</sub> receptor antagonist injections, not D<sub>2</sub> receptor antagonist, in the PVN significantly increased RSNA, BP and HR. These responses were significantly attenuated in T2D rats. These results suggest that central dopamine (within the PVN) may exert an inhibitory influence on the sympathetic activity in descending pathways via D<sub>1</sub> receptor. This combined with the data showing decreased expression of dopamine receptors in the PVN, it is possible that any disturbance of dopaminergic regulation within the PVN would change the tonic inhibitory influence on the overall sympathetic nervous outflow, leading to the sympatho-excitation commonly observed in T2D.

Our study showed that application of dopamine receptor D<sub>1</sub>/D<sub>5</sub> antagonist specifically within the PVN resulted in increases of RSNA, MAP and HR in both control and T2D rats. However, there were no significant increases in responses to dopamine receptor D<sub>2</sub>/D<sub>3</sub> antagonist application in the PVN of both control and T2D rats. This would indicate that the dopaminergic sympatho-inhibitory effect in the PVN may not be mediated by D<sub>2</sub> receptor because they were unaffected by blocking the D<sub>2</sub> receptor with raclopride. There is a previous study (54) which indicated that stimulation of the PVN in the presence of intrathecally administered D<sub>2</sub> receptor antagonist does not prevent the sympatho-excitatory response in renal nerve activity. However, the inhibitory responses on renal nerve activity after PVN stimulation can be blocked by intrathecal application of D<sub>1</sub> receptor antagonist (54). This would indicate dopamine via D<sub>1</sub> receptor might mediate some of the PVN mediated inhibitory effects. Thus, dopamine may have
a mixed response on sympathetic nerve activity and cardiovascular regulation depending on receptor subtype activation.

Our results also showed differential sympathetic and BP responses to dopamine administration in the PVN and RVLM. Exogenous administration of dopamine within the PVN caused inhibitory effect on RSNA and MAP but not from the RVLM. This would suggest a tonic inhibitory dopaminergic influence on the sympathetic outflow dictated from the PVN. It is recognized that the RVLM is also critical for maintaining basal sympathetic vasomotor tone and is an essential component of many sympathetic reflexes (4). Dopaminergic pathway has shown to be involved in RVLM C1 neuron mediated cardiovascular and autonomic function (3, 43). In our study, application of dopamine receptor D1/D5 antagonist to the RVLM resulted in increases of RSNA, MAP and HR. This would support the notion that dopaminergic pathway within the RVLM is also involved in the regulation of sympathetic activity. Furthermore, this dopaminergic regulation by RVLM is also blunted in the diabetic condition.

SCH39166, has been shown to be a potent, specific, D1 receptor antagonist with several features which are advantageous over its predecessor such as SCH23390 (51). SCH39166 has shown more selective for binding to D1 receptors than SCH23390 with high affinity, saturable, and specific (51). SCH23390 can inhibit G protein-coupled inward rectifier K+ (GIRK) channels currents causing depolarization of neurons (20). As a consequence, blockade of GIRK channel in neurons may decrease spontaneous action potential formation and increase the release of excitatory neurotransmitters. However, there is no report about the similar effects in SCH39166 to date. Since SCH39166 is an analogue of SCH23390, we cannot exclude the possibility that SCH39166 has the similar GIRK inhibitory effect contributing to the sympatho-excitation. This remains to be explored further in the future.
Altered neurohormonal drive plays an important pathophysiological role in diabetes (32, 44). In our previous study, we have found an enhanced excitatory effect through an angiotensin II-superoxide mechanism (35) and attenuated inhibitory effect through a nitric oxide mechanism (56) contributing to sympatho-excitation in type 1 diabetes. In T2D, insulin resistance leads to activation of neurohormones, and this in turn leads to further insulin resistance (25). Insulin resistance of muscle and adipose tissue hampers glucose uptake in the tissues. As the brain appears to be a key regulator of energy metabolism, this may cause alterations of some key candidate neurotransmitters in relevant brain areas, such as PVN. Reduced dopaminergic neurotransmission in hypothalamic nuclei has been implicated in the pathogenesis of T2D (38). For example, selective destruction of dopaminergic neurons in the area of the suprachiasmatic nucleus severely impairs insulin sensitivity and promotes body fat accretion. Diminished dopaminergic tone in the suprachiasmatic area may affect neural circuits in these hypothalamic nuclei to impact metabolism and associated with markedly enhanced noradrenergic transmission in these same hypothalamic nuclei. The results from our study showed an attenuated dopaminergic inhibitory influence on the RSNA and MAP, suggesting that the impaired dopaminergic tone within the PVN may lead to the elevated sympatho-excitation, commonly observed in T2D.

Other evidence has indicated that insulin resistance may impair dopamine function in the central nervous system (27). In one study, 12 weeks HFD resulted in peripheral insulin resistance and oxidative stress. These changes were accompanied by impaired dopamine function in the dorsal striatum (27). Specifically, in vivo electrochemical measures of dopamine neuronal function revealed decreased potassium-evoked dopamine release, which correlated with the degree of insulin resistance. In our T2D rats, HFD/STZ induction resulted in impaired glucose
tolerance and peripheral hyperglycemia, hyperleptinemia, hyperlipidemia, and insulin resistance. It would be interesting for future studies to directly measure the effects of insulin, leptin and high lipid on the function of dopamine neurons in the PVN. HFD feeding also increases markers of oxidative stress in multiple brain regions (6, 28). Oxidative damage decreases dopamine transporter expression and dopamine metabolite levels (8). The contribution of oxidative stress to the observed deficits in dopamine function cannot be ruled out and remain a likely possibility.

In conclusion, we postulate that the reduced dopaminergic activity within the PVN of HFD/STZ-induced T2D rats leads to an altered renal sympatho-inhibition in diabetes. This altered dopaminergic mechanism/s within the PVN may contribute to the increased renal sympathetic neural activity observed in T2D. These results provide a potential target for the treatment of enhanced sympatho-activation commonly observed in T2D.

ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS

Figure 1. Plasma glucose during intraperitoneal glucose tolerance test in control and type 2 diabetic (T2D) rats. Data are means ± SE. *P < 0.05 vs. control rats.

Figure 2. A. Representative images of FosB staining in the paraventricular nucleus (PVN) (-1.8 mm from bregma), supraoptic nucleus (SON) (-1.8 mm from bregma), media preoptic nucleus (MnPO) (-0.4 mm from bregma), subfornical organ (SFO) (-1.1 mm from bregma) and rostral ventrolateral medullar (RVLM) (-12.0 mm from bregma) from one rat/group (control and T2D). Bar = 50 μm. B. Number of FosB positive cells in the PVN, SON, MnPO, SFO and RVLM in the two groups of rats. Data are mean ± SE. *P < 0.05 compared to control.

Figure 3. The mean data of changes in renal sympathetic nerve activity (RSNA), mean arterial pressure (MAP) and heart rate (HR) after microinjections of dopamine hydrochloride (20 mM) into the PVN and RVLM in control and T2D rats. *P < 0.05 vs. control group.

Figure 4: RSNA, MAP and HR responses to dopamine antagonists microinjected into the PVN and RVLM. A. Segments of original recordings from individual rats from each experimental group showing responses of HR, MAP, integrated RSNA (int.RSNA) and RSNA to D1/D5 antagonist SCH39166 microinjected into the PVN. B. The mean data of changes in RSNA, MAP and HR after microinjections of dopamine antagonists (D1/D5 antagonist SCH39166 and D2/D3 antagonist raclopride into the PVN and RVLM in control and T2D rats. *P < 0.05 vs. control group. Ant: antagonist.
Figure 5. A: Example of visualized bands of dopamine receptor (D₁ receptor and D₂ receptor) and GAPDH in the PVN and RVLM in control and T2D rats. B. Mean data of band densities normalized by GAPDH. *P < 0.05 versus control group.

Figure 6. A. Immunofluorescent photomicrographs from the sections of the PVN region (bregma -1.8 mm) stained for D₁ receptor (red), microtubule associated protein 2 (MAP2 in green) and 4',6-diamidino-2-phenylindole (DAPI in blue) in a control and a T2D rat, bar = 100 μm. B. Immunofluorescent photomicrographs from the sections of the PVN region (bregma -1.8 mm) stained for D₂ receptor (red), MAP2 (green) and DAPI (blue) in a control and a T2D rat, bar = 100 μm. C. Mean data of D₁ receptor and D₂ receptor, MAP2 and DAPI immunofluorescent signal in the PVN in control and T2D rats. M: magnocellular; D: dorsal cap; vP: ventrolateral parvocellular. *P < 0.05 versus control group.

Figure 7. a-c Schematic representations of serial sections from the rostral (-1.4) to the caudal (-2.1) extent of the region of the PVN. The distance (in mm) posterior to bregma is shown for each section. Filled circle represents the site of termination of an injection that is considered to be within the PVN region in control group; "+" represents that in T2D group. d. Histological photo showing the injection site (arrow) in the PVN of one rat. AH, anterior hypothalamic nucleus; f, fornix; 3V, third ventricle; OX, optic tract; SO, supraoptic nucleus.
Table 1: Characteristics of control and T2D rats in the experiments

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 15)</th>
<th>T2D (n = 16)</th>
<th>Fasting plasma glucose (mM)</th>
<th>Control (n = 15)</th>
<th>T2D (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>423 ± 21</td>
<td>485 ± 22*</td>
<td>5.3 ± 0.6</td>
<td>13.4 ± 2.2*</td>
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<tr>
<td>Retroperitoneal fat pad (g)</td>
<td>5.2 ± 0.4</td>
<td>7.7 ± 2.1*</td>
<td>2h plasma glucose (mM)</td>
<td>14.3 ± 2.7</td>
<td>18.9 ± 3.9</td>
</tr>
<tr>
<td>Epydidimal fat pad (g)</td>
<td>8.6 ± 1.6</td>
<td>9.8 ± 1.5</td>
<td>2h plasma insulin (mU/L)</td>
<td>14.6 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Brown adipose tissues (g)</td>
<td>1.1 ± 0.3</td>
<td>0.3 ± 0.1*</td>
<td>3.6 ± 0.8*</td>
<td>482 ± 52*</td>
<td></td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>1.8 ± 0.3</td>
<td>3.6 ± 0.4*</td>
<td>Insulin sensitivity Index</td>
<td>-4.0 ± 0.2</td>
<td>-5.3 ± 0.3</td>
</tr>
<tr>
<td>Plasma triglyceride (mM)</td>
<td>1.1 ± 0.3</td>
<td>3.6 ± 0.4*</td>
<td>Plasma leptin (ng/ml)</td>
<td>328 ± 37</td>
<td>482 ± 52*</td>
</tr>
<tr>
<td>Basal MAP (mmHg)</td>
<td>94 ± 7</td>
<td>100 ± 5</td>
<td>Basal heart rate (bpm)</td>
<td>362 ± 23</td>
<td>372 ± 36</td>
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<tr>
<td>Urine NE (μg/ml)</td>
<td>12.9 ± 2.9</td>
<td>20.9 ± 2.5*</td>
<td>Basal int.RSNA (µV.s)</td>
<td>3.3 ± 0.3</td>
<td>4.3 ± 0.3*</td>
</tr>
<tr>
<td>24h urine volume (ml)</td>
<td>15 ± 3</td>
<td>33 ± 5*</td>
<td>24h urine NE (μg)</td>
<td>194 ± 54</td>
<td>689 ± 79*</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SE; * indicates $P < 0.05$ vs. control rats. MAP: mean arterial pressure; NE: norepinephrine; Int. bpm: beats/min; RSNA: integrate renal sympathetic nerve activity.
Figure 1

![Graph showing blood glucose levels over time for control and T2D groups. The graph indicates higher blood glucose levels in the T2D group compared to the control group at various time points, marked with asterisks.](image-url)
Figure 2

A. PVN  SON  MnPO  SFO  RVLM

Control

T2D
Figure 2

B.

![Bar chart showing the number of FOSB positive cells in different brain areas. The chart compares Control (n=5) and T2D (n=6) groups.](chart.png)
Figure 4

A. Control

- HR (bpm)
- MAP (mmHg)
- Int.RSNA (uv.s)
- RSNA (uv)

SCH39166 2 mM

T2D

- HR (bpm)
- MAP (mmHg)
- Int.RSNA (uv.s)
- RSNA (uv)

SCH39166
Figure 5

A. Control T2D

PVN

D1DR 74 kDa

GAPDH 37 kDa

RVLM

D1DR 74 kDa

GAPDH 37 kDa

D2DR 51 kDa

GAPDH 37 kDa

B. D1 receptor

PVN

Control (n=6) T2D (n=6)

Ratio of Intensity

0 0.4 0.8 1.2 1.6 2.0 2.4

PVN RVLM

D1 receptor

B. D2 receptor

PVN

Control (n=6) T2D (n=6)

Ratio of Intensity

0 0.5 1.0 1.5 2.0 2.5 3.0

PVN RVLM

* indicates a significant difference.
Figure 6

B. D2 receptor | MAP2 | DAPI | Merge

Control

T2D
C.

![Figure 6](image)

**Figure 6**

- Control (n=4) vs. T2D (n=4)

**Relative Intensity**

- D1 receptor
- D2 receptor
- MAP2
- DAPI
Figure 7

a.

b.

c.

d.