Glucose metabolism, diet composition, and the brain

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Chapter 6

Infusion of fluoxetine, a serotonin reuptake inhibitor, in the shell region of the nucleus accumbens increases blood glucose concentrations in rats

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Abstract

The brain is well known to regulate blood glucose, and the hypothalamus and hindbrain, in particular, have been studied extensively to understand the underlying mechanisms. Nuclei in these regions respond to alterations in blood glucose concentrations and can alter glucose liver output or glucose tissue uptake to maintain blood glucose concentrations within strict boundaries. Interestingly, several cortico-limbic regions also respond to alterations in glucose concentrations and have been shown to project to hypothalamic nuclei and glucoregulatory organs. For instance, electrical stimulation of the shell of the nucleus accumbens (sNAc) results in increased circulating concentrations of glucose and glucagon and activation of the lateral hypothalamic area (LHA). Whether this is caused by the simultaneous increase in serotonin release in the sNAc remains to be determined.

To study the effect of sNAc serotonin on systemic glucose metabolism, we implanted bilateral microdialysis probes in the sNAc of male Wistar rats and infused fluoxetine, a serotonin reuptake inhibitor, or vehicle after which blood glucose, endogenous glucose production (EGP) and glucoregulatory hormones were measured.

Fluoxetine in the sNAc for 1 hour significantly increased blood glucose concentrations without an effect on glucoregulatory hormones. This increase was accompanied by a higher EGP in the fluoxetine infused rats compared to the controls.

These data provide further evidence for a role of sNAc-serotonin in the regulation of glucose metabolism.

Introduction

The ongoing obesity epidemic, leading to an increased risk for disturbances in glucose metabolism, led to regain of scientific interest in the physiology of glucose homeostasis. In addition to glucoregulatory hormones, such as insulin and glucagon, which decrease and increase blood glucose concentrations respectively, the brain has been shown to play an important role in regulating glucose metabolism (Yi *et al*., 2010). The brain orchestrates glucose metabolism through innervation of glucoregulatory organs like liver, skeletal muscle and adipose tissue, that are involved in endogenous glucose production (EGP) and glucose uptake (Lam *et al*., 2009; Sandoval *et al*., 2009; Watts, 2014). Moreover, central innervation of β-cells in the pancreas have been described, providing a route via which the brain influences insulin and glucagon secretion (Bertrand *et al*., 1996; Tsutsumi *et al*., 2002). The classical view of glucose control by the hypothalamus and hindbrain has recently been expanded to include other brain areas (Diepenbroek *et al*., 2013a). For example, the nucleus accumbens (NAc), which is part of the reward circuitry controlling feeding behaviour, contains glucose sensing cells (Papp *et al*., 2007), and provides input to the lateral hypothalamic area (LHA) (Groenewegen *et al*., 1984), an area implicated in glucose regulation (Morgan *et al*., 2015; Yi *et al*., 2009). The NAc consists of a core (cNAc) and a shell (sNAc) region, which are functionally different, for instance, with regard to feeding behaviour, (Bassareo *et al*., 2015; Smith *et al*., 2015) and input and output areas (Groenewegen *et al*., 1999). We recently showed that electrically stimulating the sNAc using deep brain stimulation (DBS) increases plasma concentrations of glucose and glucagon and activated neurons in the LHA (Diepenbroek *et al*., 2013b), pointing to a role for the sNAc in control of glucose homeostasis. It remains, however, to be determined which neurotransmitter system in the sNAc underlies these changes.

The NAc receives dense input from different areas in the brain involved in energy metabolism, and is, for example, innervated by the serotonergic neurons from the raphe nuclei and periaqueductal grey (Steinbusch *et al*., 1981) forming synaptic contacts with cells in the sNAc particularly (Van Bockstaele *et al*., 1996). Central serotonin has been shown to control glucose metabolism (Zhou *et al*., 2007), and others have shown that DBS in the rat sNAc (and not in the cNAc) increases local serotonin and dopamine concentrations (Sesia *et al*., 2010).

To determine the effect of elevated extracellular serotonin concentrations in the sNAc on glucose metabolism, we bilaterally implanted rats with microdialysis probes aimed at the sNAc and subjected rats to 1 hour reverse microdialysis with the selective serotonin reuptake inhibitor (SSRI) fluoxetine, which has been shown to increase extracellular serotonin concentrations (Dailey *et al*., 1992; Rada *et al*., 1993). We assessed EGP and concentrations of blood glucose and the glucoregulatory hormones glucagon, insulin and corticosterone. This study shows for the first time that infusing a serotonin reuptake inhibitor in the sNAc increases blood glucose concentrations, partly explained by changes in EGP but not glucoregulatory hormones. These findings are in line with earlier findings that the sNAc plays a role in glucose metabolism (Diepenbroek *et al*., 2013b).

Material and Methods

Animals

Male Wistar rats (250-280g Harlan, Horst, the Netherlands) were individually housed in Plexiglas cages in a temperature (20±2°C), humidity (60±2%) and light controlled room with a 12/12h light-dark schedule (lights on at 7:00 h). All animals had *ad libitum* access to laboratory chow (Teklad Global 18% Protein Rodent Diet, Harlan, Horst, the Netherlands) and tap water prior to testing.

After arrival at the animal facilities, rats received one week for acclimatization during which they were adapted to handling. The experiment was approved by the Committee for Animal Experimentation of the Academic Medical Centre of the University of Amsterdam, the Netherlands.

Surgery

Rats were anaesthetized with an intraperitoneal injection of a mixture of 80 mg/kg Ketamine (Eurovet Animal Health, Bladel, the Netherlands), 8 mg/kg Rompun® (xylazine, Bayer Health Care, Mijdrecht, the Netherlands) and 0.1 mg/kg Atropine (Pharmachemie B.V., Haarlem, the Netherlands), after which a silicone catheter was implanted in the jugular vein, according to the method of Steffens (1969), for intravenous infusions. Another silicone catheter was implanted in the left carotid artery for blood sampling. After catheter implantations, rats were fixed in a stereotact (Kopf®, David Kopf instruments, Tujunga, California) and microdialysis probes (molecular weight cut-off 6 Kda, U-shaped tip 1.5 mm long, 0.7 mm wide, and 0.2 mm thick; constructed as reported in (Kalsbeek *et al*., 1996)) were bilaterally implanted in the sNAc (AP+1.44 mm, ML±3 mm, DV-7.3 mm, angle 17°). Catheters and microdialysis probes were fixed on the skull with dental cement. Rats received Carprofen (5 mg/kg BW, subcutaneous) during surgery and the first post-surgery day, and a recovery period of 14 days in which food and water intake and body weight was measured 5 times a week. Jugular vein catheters were flushed twice a week.

Fluoxetine concentration

Fluoxetine (fluoxetine hydrochloride, Sigma Aldrich, Germany) was dissolved in Ringer (73.5 Na^{2+}, 2 K, 1.13 Ca^{2+}, 77.8 Cl in mmol/500mL; Baxter, Utrecht, the Netherlands) to a concentration of 250 µM. We chose the maximum dose of 250 µM based on previous studies using *in vivo* reverse microdialysis of fluoxetine in the NAc in rats (Rada *et al*., 1993; Zangen *et al*., 1999). Also, we performed a pilot experiment including 3 groups infusing either vehicle (Ringer), fluoxetine 83 µM or fluoxetine 250 µM. The probes were correctly placed in 4 animals in the 83 µM fluoxetine group and 4 in the vehicle group. Plasma glucose and EGP were not different between the 83 µM dose fluoxetine group and the vehicle group. We therefore performed the experiment, described in this report, with the 250 µM fluoxetine concentration.

Glucose kinetics and reverse microdialysis

On the evening prior to the experiment, rats were connected to a multi-channel fluid infusion swivel (Instech Laboratories, PA, USA) to adapt. Food was restricted to 20 grams of chow to avoid differences in the nutritional state and basal blood glucose concentrations of the rats.

On experimental days, remaining food was removed at the beginning of the light period (8:00h in the morning), which was 2 hours before the experiment started. Subsequently, animals were connected to the blood-sampling catheter and microdialysis lines which were, via the multi-channel fluid infusion swivel, connected to an infusion pump (Harvard Apparatus, Holliston, Massachusetts, USA). The sampling catheter and cables were kept out of reach by means of a counterbalanced beam. This allowed the animals to move freely during the experiment and allowed all manipulations to be performed outside the cages without handling the animals.

To study glucose kinetics, [6.6-$^2H_2$] glucose was used as a tracer (>99% enriched; Cambridge Isotope Laboratories, Cambridge, USA). Five minutes prior to infusion of the stable isotope tracer, a blood sample was drawn to assess background isotopic enrichment. At 10:00 h a primed (3000 µL/ h in 5 min (=250 µL)) followed by a continuous [6.6-$^2H_2$] glucose (>99% enriched; Cambridge Isotope Laboratories, Cambridge, USA) (500 µL/h) infusion was started using the infusion pump. After 90 minutes of equilibration time, three blood samples (180 µL) were drawn to determine blood glucose concentrations and isotopic enrichment, to calculate EGP. Thereafter, microdialysis of Ringer was switched to either Ringer (as vehicle (control)) or fluoxetine (3 µL/min) and blood samples were drawn at t=5, 10, 15, 20, 30, 60 min. After 1 hour of either Ringer or fluoxetine infusion, all animals were switched back to Ringer and final blood samples were drawn at t=65, 90, 120 min. At the end of the experiment (t=120), animals were anaesthetized with a CO_2/O_2 mixture (6:4) followed by 100% CO_2 and killed by decapitation. Brains were then rapidly removed, frozen on dry ice and stored at -80°C.

Analytical methods

Blood glucose concentrations were measured directly during the experiment, using a glucose monitor device (Freestyle Freedom Lite, Abbott, Hoofddorp, the Netherlands). Blood samples were immediately chilled on ice in Eppendorf tubes with 5 µL heparin: saline (10x) solution and centrifuged (15 min, 3000 rpm). Plasma samples were stored at -20°C until further analysis. Plasma concentrations of insulin, glucagon and corticosterone were measured in duplo using radioimmunoassay kits (Millipore, St Charles, MO, USA and Biochemicals, Costa Mesa, CA, respectively). The amount of sample-, standards-, label-, antibody and precipitating reagent, described in the manufacture's protocol, were divided by four. Radioimmunoreactivity was measured by use of a gamma counter (Wizard 2 PerkinElmer, Waltham, Massachusetts). The intra assay variation-coefficient of the immunoassays was <10%. Plasma [6.6-$^2H_2$] glucose enrichment was measured by gas

chromatography-mass spectrometry (GCMS) (Ackermans *et al*., 2001), EGP was calculated using Steele equations (Steele, 1959).

Histology and immunocytochemistry

Brain tissue was cut on a cryostat in 35 µm sections. All sections were collected on gelatin coated slides and fixed for 10 min in 4% paraformaldehyde at room temperature. For verification of probe placement, every 2 slides were Nissl-stained after fixation and examined with a microscope to determine precise location of the probes. Probe placement was considered misplaced when microdialysis probe tips were observed outside the sNAc according to the delineation of Paxinos and Watson (2007). Probes were drawn on rat brain atlas prints by 3 separate researchers blind for the conditions. Data of 25% of all animals undergoing surgery were excluded from the analysis due to misplaced probes**.**

Statistics

All data are presented as means±SEM. We evaluated the kinetics of concentrations of systemic glucose, glucagon, corticosterone, insulin and EGP flux during sNAc reverse microdialysis by the increments of the concentrations with regard to their baseline values (t=0). Statistical analysis was performed using a repeated-measure analysis of variance (rmANOVA) (SPSS Inc, Chicago, USA) to test for effects of *Time, Stimulation* and *Time*Stimulation* interaction. When an overall *Time, Treatment* or *interaction* effect was detected, a *LSD fisher* was used to test for group differences. A difference was considered significant when $p<0.05$.

Results

Histology

Verification of microdialysis probe placements showed correctly placed probes in $n=6$ for the vehicle group and $n=7$ for the fluoxetine group in the sNAc (Figure 1). Unilateral correctly placed probes were also included in the dataset when the probe was fully placed in the sNAc.

Baseline metabolic parameters

Basal concentrations of blood glucose, insulin, glucagon and corticosterone were not significantly different between infusion groups. In line, EGP did not differ between groups at baseline (Table 1).

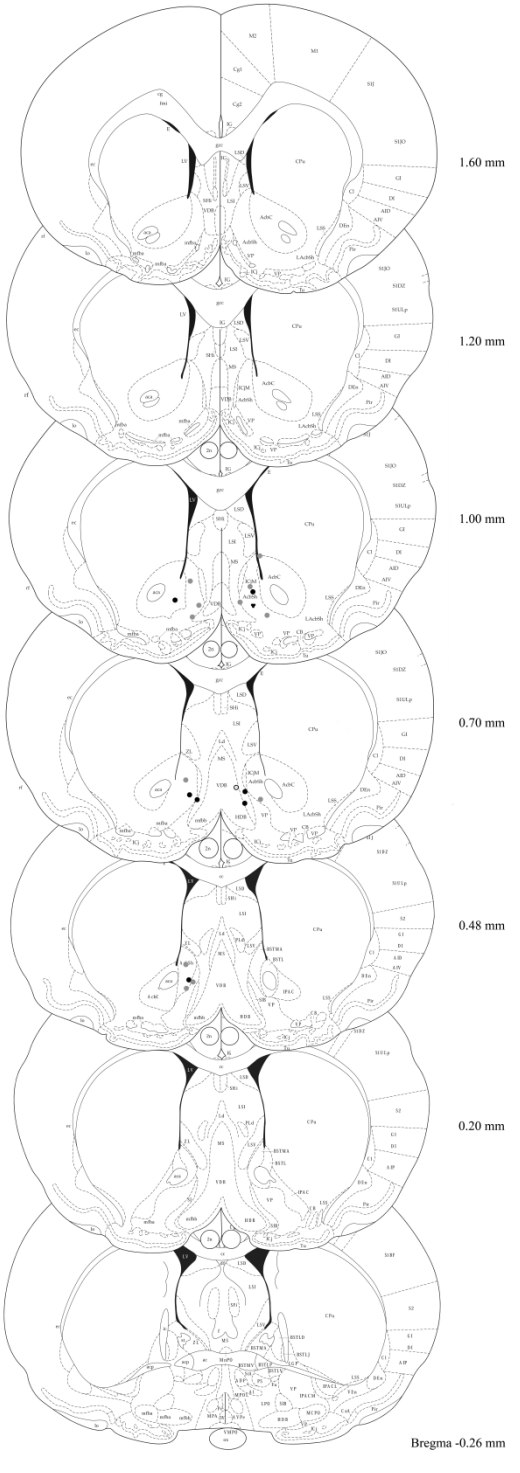

Figure 1. Localisation of the end of the microdialysis probes in the brains of rats infused with vehicle (black dots) or fluoxetine (250 μM, grey dots). The dots represent the end of the probe, and thus the area of infusion extends 1.5mm above this point (which is the length of the probe). Images are modified from Paxinos and Watson (1998).

Table 1. Body weight, basal endogenous glucose production (EGP) and basal concentrations of blood glucose, plasma glucagon, corticosterone and insulin in the vehicle (n=6) and fluoxetine (n=7) infused rats. Data are means±SEM. No significant differences detected using Student's t-test.

	vehicle	fluoxetine
body weight (g)	315.2 ± 5.7	314.2 ± 2.8
EGP (µmol/kg.min)	58.3 ± 3.7	52.4 ± 2.6
glucose (mmol/L)	5.0 ± 0.1	4.7 ± 0.2
glucagon (ng/L)	85.4 ± 8.2	82.8 ± 6.0
corticosterone (nmol/mL)	13.9 ± 9.4	10.3 ± 5.9
insulin (ng/mL)	2.0 ± 0.2	1.7 ± 0.2

Abbreviation: EGP, endogenous glucose production

Fluoxetine effects on blood glucose and EGP

rmANOVA of blood glucose concentrations showed a significant effect of *Time* (p<0.001) and of *Treatment* (p=0.025) but no interaction effect between *Time* and *Treatment* (p=0.1). *Post hoc* analysis further showed that blood glucose concentrations were significantly higher during fluoxetine infusion compared to vehicle infusion between t=5 and t=15 (Figure 2A). In addition, overall rmANOVA of EGP data did not show an effect of *Time* (p=0.27), interaction (*Time***Treatment*: p=0.23) or *Treatment* (p=0.20) (Figure 2B). When analysing the time curves separately for vehicle and fluoxetine infused rats, EGP in the vehicle group decreased over time (p<0.01), while EGP did not change in the fluoxetine infused rats (p= 0.70) (Figure 2B).

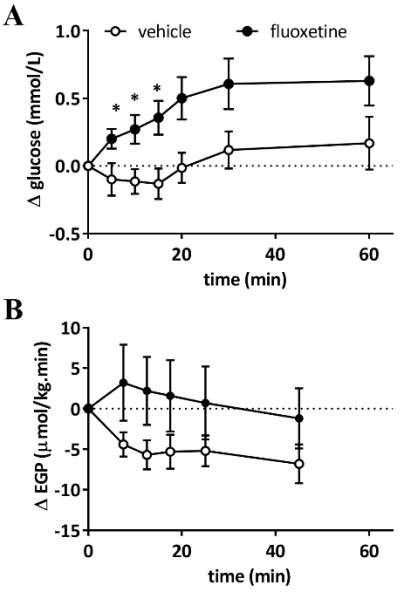

Figure 2. Blood glucose concentrations were significantly higher during 1 hour of sNAc fluoxetine infusion (250 µM) (black circles: n=7) compared to vehicle infusion (open circles; n=6) (A). Endogenous glucose production (EGP) in vehicle infused rats (n=6, open circles) decreased significantly over the course of 1 hour infusion whereas EGP in fluoxetine infused rats (n=7, black circles) did not (B). Concentrations are expressed as the difference from the respective t=0 values. Absolute t=0 values are displayed in Table 1. All data are presented as mean±SEM. *p<0.05.

Fluoxetine effects on glucoregulatory hormones

There was no effect of fluoxetine on plasma concentrations of insulin, glucagon and corticosterone when comparing rats infused with vehicle to rats infused with fluoxetine (Figure 3A, B and C).

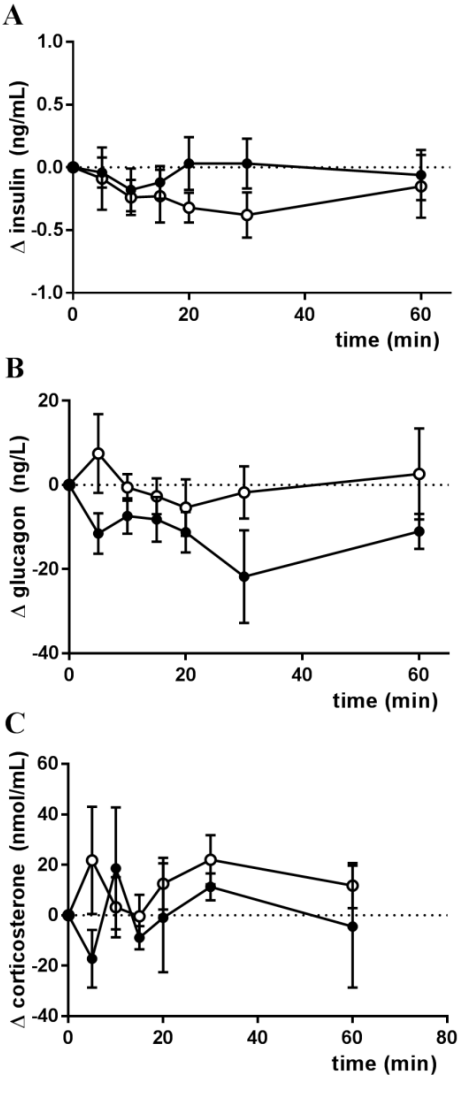

Figure 3. (A) Plasma insulin (B) plasma glucagon, and (C) plasma corticosterone concentrations during 60 minutes of vehicle (n=6, white circles) or fluoxetine (250 µM) infusion in the sNAc (n=7, black circles). Concentrations are expressed as the difference from the respective t=0 values. Absolute t=0 values are displayed in Table 1. All data are presented as mean±SEM. $^{*}p<0.05$.

Discussion

We here show that local infusion of a serotonin reuptake inhibitor in the sNAc of rats increases blood glucose concentrations without alterations in glucoregulatory hormones. These findings provide further evidence that the sNAc is implicated in peripheral glucose metabolism. The increase of blood glucose is in line with our earlier report on the effects of stimulating the sNAc using DBS (Diepenbroek *et al*., 2013b).

The higher blood glucose concentrations in the fluoxetine infused rats may be explained by a higher EGP, as EGP decreased in the vehicle but not in the fluoxetine infused rats. In addition to these modest differences in EGP, increased systemic glucose concentrations may be explained by a direct effect of serotonin availability in the sNAc on peripheral glucose uptake. For example, Sudo *et al.* (1991) showed that peripheral glucose uptake is under hypothalamic control, and the hypothalamus receives dense projections from the sNAc (Sano & Yokoi, 2007). Furthermore, an increase in blood glucose concentrations might also result from an arousal/stress response increasing plasma corticosterone concentrations, eventually altering EGP. However, plasma corticosterone concentrations were not different between groups, and so far there is no evidence that central administration of fluoxetine increases plasma corticosterone concentrations directly. In addition, neither plasma concentrations of insulin nor glucagon differed between both groups. The lack of altered insulin concentrations suggests that the increase of blood glucose concentrations in fluoxetine infused rats was not sufficient to induce insulin secretion. Alternatively, portal insulin might have been higher but this was not measured. However, the latter is not expected since an increase in portal insulin would reduce EGP.

Next to an increase in blood glucose concentrations, DBS of the sNAc increased systemic glucagon concentrations (Diepenbroek *et al*., 2013b). In contrast, we did not observe an increase in systemic glucagon concentrations during fluoxetine-sNAc dialysis. This suggests that serotonin is not the only neurotransmitter involved in the DBS-sNAc effect on glucose metabolism. (Sesia *et al*., 2010) also reported an increase in dopamine concentrations after sNAc-DBS, which thus could be involved in the DBS-sNAc induced changes in glucose metabolism as well.

Fluoxetine has been described as a specific ligand for the serotonin transporter, and serotonin has been shown to increase after infusion of comparable fluoxetine concentrations (Rada *et al*., 1993). Of note, serotonin also has some affinity, albeit very low for the noradrenaline (NA) and dopamine (DA) transporter (van Harten, 1993). However, the binding of fluoxetine to serotonin transporter has been shown to be 10 to 100 times higher compared to its binding to NA or DA transporters respectively (Frazer, 1997; Koch *et al*., 2002). Therefore the effects that we observe after fluoxetine microdialysis are likely to be the result of fluoxetine binding to the serotonin transporter and thus a specific serotonin effect.

The pathways underlying the effect of fluoxetine in the sNAc on glucose metabolism remain speculative at this point. Besides an increase in extracellular serotonin

concentrations in the NAc after systemic fluoxetine administration several studies have shown that fluoxetine or serotonin infusion in the posterior medial NAc dose dependently decreased acetylcholine (ACh) outflow by cholinergic interneurons (Guan & McBride, 1988; Kirby & Lucki, 1997), (Rada *et al*., 1993). ACh activates GABAergic medium spiny neurons via muscarinic receptors (Chau *et al*., 2011; Rada *et al*., 2006) and the inhibitory action of serotonin on sNAc cholinergic interneurons might therefore reduce stimulation of the GABAergic projections to the LHA. This hypothalamic disinhibition might result in higher plasma glucose concentrations. In line with this proposed pathway, it was previously shown that the LHA is neurally connected to the liver, and that a GABA antagonist administered to the LHA increased blood glucose concentrations (Yi *et al*., 2009). Moreover, we earlier showed that the sNAc DBS-induced increase in glucose concentrations was accompanied by activation of the LHA (Diepenbroek *et al*., 2013b).

Finally, verification of probe placements revealed that when the probe was placed in the septum blood glucose concentrations and EGP curves during fluoxetine infusion closely resembled those of vehicle infused rats (data not shown). This observation indicates that it is specifically the sNAc which is involved in the observed metabolic changes.

In summary, we here demonstrate that locally infusing a serotonin reuptake inhibitor in the sNAc of rats leads to an increase in blood glucose concentrations without overall changes in glucoregulatory hormones. The effect on glucose might be explained by a combined effect of a higher EGP and lower glucose uptake. These data establish a role for the sNAc in systemic glucose metabolism and suggest that serotonin might be an important player mediating these effects. These findings provide novel insights in the complex mechanisms of central regulation of glucose metabolism.