

# A refined high carbohydrate diet is associated with changes in the serotonin pathway and visceral obesity

PAOLA A. SPADARO<sup>1</sup>, HELEN L. NAUG<sup>1,2</sup>, EUGENE F. DU TOIT<sup>1,2</sup>, DANIEL DONNER<sup>1</sup>  
AND NATALIE J. COLSON<sup>1,2\*</sup>

<sup>1</sup>School of Medical Science, Griffith University Gold Coast Campus, Griffith University, Qld, Australia, 4222

<sup>2</sup>Menzies Health Institute of Queensland School of Medical Science, Griffith University Gold Coast Campus, Griffith University, Qld, Australia, 4222

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## Summary

Consumption of palatable foods high in refined carbohydrate has been implicated as a contributing factor to the epidemic levels of obesity. Such foods may disrupt appetite regulation in the hypothalamus through alterations in hunger and satiety signalling. This investigation examined whether a palatable high refined carbohydrate (HRC) diet with the potential to induce obesity was linked to modulation of serotonin and dopamine signalling within the hypothalamus of rats. Male Wistar rats were allowed *ad libitum* access to either a palatable refined carbohydrate enriched (HRC) diet or standard chow (SC). Visceral fat percentage was used as a measure of the animals' weight gain during the trial. Real-time PCR was applied to determine any variation in levels of expression of the serotonin (*Slc6A4* or *Sert*) and dopamine transporter (*Slc6A3* or *Dat*) genes. After 29 weeks, the HRC group showed a significant increase in visceral fat percentage accompanied by increased expression of *Sert*. Higher levels of circulating triglycerides were also seen. This investigation determined that a refined high carbohydrate diet is associated with visceral obesity, increased circulating lipids in the blood and distorted serotonergic signalling, which possibly alters satiety and hunger signals.

## 1. Introduction

Current lifestyle and consumption of highly refined carbohydrate dense foods have changed the evolutionary programming of food intake in humans, possibly through disruption of appetite regulation and satiety (King, 2013). Early investigations have indicated the serotonergic and dopaminergic systems within the hypothalamus as key regions for energy control in mammals (Erlanson-Albertsson, 2005). The hypothalamus connects higher cortical centres, such as the reward and motivation related limbic system with peripheral signals, influencing food intake and energy expenditure (Suzuki *et al.*, 2012; Stice *et al.*, 2013). There is evidence that the hypothalamic energy control response and reward pathways in the brain appear to be functionally linked (Kempadoo *et al.*, 2013).

Although neural signalling modifications in the hypothalamus in response to dietary changes have been explored (Erlanson-Albertsson, 2005; Berthoud *et al.*, 2012; Murray *et al.*, 2014), there are limited studies exploring the specific effects of a palatable refined carbohydrate diet on mRNA levels.

The dopaminergic circuit has long been associated with food intake (Quarta *et al.*, 2014). Dopamine appears to function as an inhibitor of food consumption and hyperphagia by establishing control over the number and duration of meals (Meguid *et al.*, 2000). High carbohydrate content diets with protein restriction have an effect on dopamine efflux in the striatum of rats associated with coping response (Yeghiayan *et al.*, 2004). A major regulator of dopamine in the brain is the sodium dependent dopamine transporter (*Dat* or *Slc6A3*) and its modification due to high fat diet exposure has also been linked with obesity (Vucetic *et al.*, 2012).

Serotonin is known as a major regulator of feeding (Lam *et al.*, 2010). Although there is growing support for the function of serotonin in central and peripheral control of food intake and energy balance regulation,

\* Corresponding author: Natalie J. Colson, Menzies Health Institute of Queensland School of Medical Science, Griffith University Gold Coast Campus, Griffith University, Qld, Australia, 4222. Tel: +61 7 55529075. Fax: +61 7 55528908. E-mail: n.colson@griffith.edu.au

conserved from mammals to fish, the specific neuronal mechanisms underlying the serotonergic pathway are still emerging (Johnston & Glanville, 1994; Donovan & Tecott, 2013). Serotonergic signalling is further controlled by the serotonin transporter (*Sert* or *Slc6A4*), which traffics serotonin from the synaptic cleft to the pre-synaptic membrane (Zhou *et al.*, 1996). Agents that block the re-uptake of serotonin, such as fenfluramine and selective serotonin reuptake inhibitors, elicit powerful anorectic effects (Garfield & Heisler, 2009), suggesting a role for *Sert* in feeding behaviour.

This investigation focused on the neural processes that govern the tendency to overeat palatable carbohydrates beyond homeostatic needs. Here we examine whether such diets are associated with changes in hypothalamic expression of *Sert* and *Dat* and lipid metabolisms in rats.

## 2. Methods

### (i) Animal training and tissue collection

A total of 49 ten-week old male Wistar rats were housed in the Griffith University Animal Facility in a room maintained at 23 °C with a 12-hour day–night cycle. All studies were performed in accordance with the guidelines of the Animal Ethics Committee of Griffith University (Permit No: MSC/08/10/AEC), which is accredited by the Queensland Government, Department of Primary Industries and Fisheries under the guidelines of The Animal Care and Protection Act 2001, Section 757. The animals were randomly assigned to one of two diet groups for a 29-week feeding period. Following a model developed by du Toit *et al.* (2008), the two diet groups had *ad libitum* access to food and water. The high refined carbohydrate (HRC) diet was made by combining 3.2 kg of rat chow soaked in water with 3 kg of sweetened condensed milk and 0.56 kg of raw sugar. The resulting macronutrient composition was 82% carbohydrate, 11% protein and 7% fat; while the standard chow (SC) diet consisted of 73% carbohydrate, 18% protein and 9% fat. Energy intake was measured by daily food consumption documented by weighing the food left in the cage when doing the daily food replacement. Knowing the weight of food consumed and the macronutrient content of the diets, we were able to calculate the daily caloric intake of the SC and HRC rats. All rats were weighed before being euthanized by lethal injection of anaesthesia at the age of 39 weeks. Each rat brain was rapidly removed from the skull and placed on a flat surface on ice with the ventral surface uppermost. The hypothalamus was quickly dissected from the fresh brain with the aid of a standard rat brain atlas (Paxinos *et al.*, 1980), stored in RNAlater (Qiagen, Hilden, Germany) and snap frozen at –80 °C until required. Total visceral fat tissue was

collected to determine the percentage of visceral fat of each individual to be used as an indicator of obesity.

### (ii) DNA–RNA extraction and real-time PCR

RNA extractions were performed using Qiagen AllPrep DNA/RNA kits (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Final concentrations and purity were measured in ng/μl using Nanodrop (Thermo Scientific, Waltham, MA, USA) and Lonza Flashgel (Basel, Switzerland). The RNA (2 μg) was immediately reverse transcribed using the Advanced Avian RT First Strand Synthesis Kit (Sigma, St. Louis, MO, USA) as per the manufacturer's instructions. Real-time PCR (rtPCR) was used to determine any variation in levels of target gene expression applying the  $2^{-\Delta\Delta C_t}$  method (Livak & Schmittgen, 2001). The primers used in this investigation (Table 1) were selected from the scientific literature (Jijun *et al.*, 2010).

All rtPCR runs were performed on the Corbett RotorGene 3000. Six genes of known expression levels in the rat brain (Bonfeld *et al.*, 2008), were examined using the geNorm program (available at: <https://genorm.cmgg.be/>) to determine the most stably expressed reference gene to be used for analysis. Consequently, *Actin B* was the reference gene selected to establish the delta cycle threshold values ( $\Delta C_t$ ) following the method published by Vandesompele *et al.* (2002).

### (iii) Biochemical analyses

We also examined any potential differences in levels of serum triglycerides, glucose and insulin, and determined the homeostatic model assessment of insulin resistance (HOMA-IR) prior to sacrifice. Animals examined were fasted for 5 hours before blood to be used in the biochemical analyses was collected. Serum triglycerides and glucose levels were determined in fresh whole blood using a Blood-Lipid and Glucose Analyser (L.D.X. Cholestech Analyser, Alere, Australia). For serum analysis, the remaining blood samples were placed in serum separation tubes (BD Vacutainer tubes), centrifuged and the serum stored at –80 °C for later analysis. Levels of insulin (ALPCO Immunoassays, Salem, NH, USA) were determined in 96-well enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions. The HOMA index was used to assess insulin resistance in the HRC and SC animals using fasting blood glucose and insulin levels and applying the standard formula:  $[\text{fasting insulin } (\mu\text{IU/ml}) \times \text{fasting glucose (mmol/l)}] / 22.5$ .

### (iv) Animal training and tissue collection

Data for gene expression and biochemical parameters were analysed by independent *t* tests using SPSS

Table 1. *Oligo sequences for measurement of gene expression.*

Gene	5'–3'	Sequence – real-time PCR
<i>Slc6a3</i>	Forward	GCGGAGATGAGGAATGAAGATGTG
	Reverse	AGGAAGAAGATGATGGCAAAGAAC
<i>Slc6a4</i>	Forward	TTGGGTTTGGAGTGCTGATTGC
	Reverse	AGAAGACGACGAAGCCAGAGG
<i>ActB</i>	Forward	TGTCACCAACTGGGACGATA
	Reverse	GGGGTGTGAAGGTCTCAA

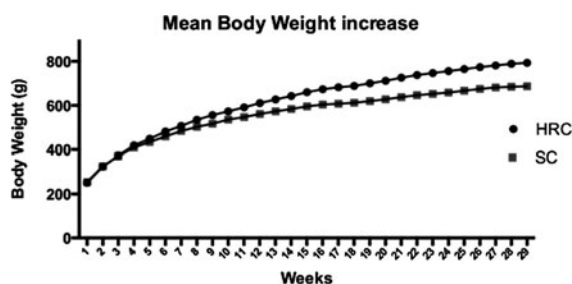


Fig. 1. Differences in visceral fat percentage of rats on the high refined carbohydrate and standard chow (control) diets at sacrifice. HRC: high refined carbohydrate; SC: standard chow.

Version 17. The differences were considered to be significant at  $p < 0.05$ .

### 3. Results

We initially examined whether *ad libitum* access to the palatable refined carbohydrate diet induced obesity in the rats according to a well-established model (du Toit *et al.*, 2008). Differences in mean body weights were seen from week 6 of *ad libitum* access to the SC or HRC diet, and increased weekly (Fig. 1).

After 29 weeks the HRC group ( $n = 18$ ) displayed a marked increase in energy intake at  $570 \pm 23$  kJ/day compared to the SC ( $n = 19$ ) diet group that consumed only  $371 \pm 18$  kJ/day. At the time of sacrifice, there was a significant increase in visceral fat for the HRC group (mean visceral fat percentage, HRC = 8.0%, SC = 6.541%;  $p = 0.018$ ,  $t = 2.479$ ,  $df = 35$ ; Fig. 2). These data indicated the development of visceral obesity within the HRC group of animals.

We then examined triglycerides, glucose and insulin levels and calculated HOMA-IR in a subset of the animals (SC = 10, HRC = 10 for triglycerides and glucose; SC = 5, HRC = 5 for insulin and HOMA-IR; Table 2).

Here we detected a significant difference between SC and HRC rats for triglycerides measurements, while there was no evidence of insulin resistance or development of diabetes. This is consistent with previous findings linking elevated circulating triglycerides levels with increase in body weight and obesity (Bocarsly *et al.*, 2010).

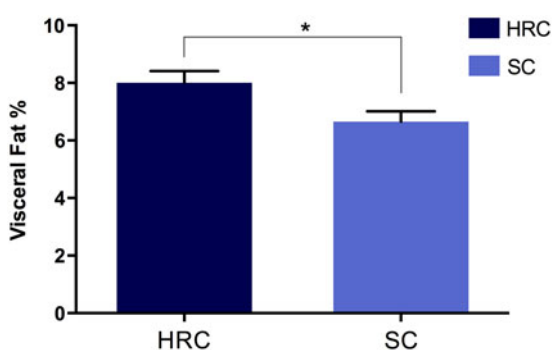


Fig. 2. Differences in visceral fat development between rats on the high refined carbohydrate and standard chow diets at 39 weeks of age. \*Statistically significant difference. HRC: high refined carbohydrate; SC: standard chow.

Next, we investigated whether the visceral obesity present in the HRC group was associated with alterations in serotonin and dopamine signalling within the rat hypothalamus by measuring the mRNA levels of both neurotransmitter transporters. Our results showed that expression of *Sert* was significantly increased in the HRC diet group compared to the SC group ( $p = 0.032$ ,  $t = 2.227$ ,  $df = 35$ ; Fig. 3). Conversely, there was no difference in expression levels of *Dat* ( $p = 0.720$ ,  $t = 0.620$ ,  $df = 34$ ). These results implied the involvement of the serotonergic but not dopaminergic pathway in the development of visceral obesity.

### 4. Discussion

This study has examined the effect of a palatable HRC diet on food consumptive behaviour and the expression of two important genes involved in hypothalamic control of feeding in rodents. We verified that increased levels of *Sert* within the rat hypothalamus, as a consequence of carbohydrate overconsumption, leads to aberrant eating behaviour. Development of visceral obesity and higher levels of circulating triglycerides follow this response.

Here we found that the palatable refined carbohydrate diet altered consumptive behaviour that led to distorted lipid metabolism, with no evidence of insulin resistance or diabetes. The role of triglycerides and lipid

Table 2. Biometric comparisons of standard chow and high refined carbohydrate-fed rats.

Biochemical parameter	SC	HRC	p-value
Triglycerides (mmol/l)	1.28 ± 0.13	1.69 ± 0.14	0.046
Glucose (mmol/l)	9.78 ± 0.29	9.23 ± 0.55	0.404
Insulin (ng/ml)	6.61 ± 0.18	6.79 ± 0.10	0.411
HOMA-IR	62.99 ± 1.94	61.11 ± 3.27	0.635

HRC: high refined carbohydrate; SC: standard chow.

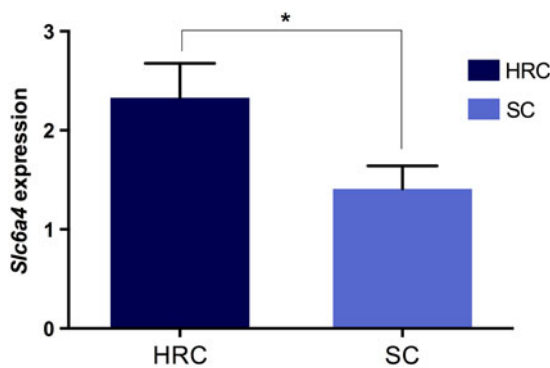


Fig. 3. Differences in *Slc6A4* (*Sert*) gene expression between rats on the high refined carbohydrate and standard chow diets at 39 weeks of age. \*Statistically significant difference. HRC: high refined carbohydrate; SC: standard chow.

metabolism is only now emerging and its influence in consumptive behaviour is yet to be clarified. Recent investigations nonetheless have indicated the presence of triglycerides in the mesolimbic brain region as a regulator of the reward pathway (Cansell *et al.*, 2014). In high saturated fat diets, increased circulation of triglycerides has also been shown to alter the hypothalamus circuitries and induce overeating in rodents (Barson *et al.*, 2012). Given that triglyceride excess has been associated with heart disease (Harchaoui *et al.*, 2009), our results are consistent with a link between carbohydrate overconsumption and development of coronary disease risk factors.

Our results support the findings of Park *et al.* (1999) who detected increased *Sert* binding in the rats fed a palatable HRC diet within the hypothalamus and midbrain regions associated with obesity. Concordantly, high BMI in adult humans has also been associated with increased *Sert* binding in the anterior cingulate cortex and thalamus/hypothalamus (Koskela *et al.*, 2008), regions that are already known to be linked to the perception of sweet and fat flavour in humans (Grabenhorst *et al.*, 2010; Fernstrom *et al.*, 2012).

While early investigations indicated that serotonin acts as a satiety signal (Lawton *et al.*, 1995), new

research shows that carbohydrate consumption increases serotonin levels in the hypothalamus (Ventura *et al.*, 2014). This effect was not seen with the consumption of protein or fats (Rouch *et al.*, 1999). Consistent with our findings, numerous animal studies have shown that palatable food promotes altered eating behaviour (Erlanson-Albertsson, 2005; Avena *et al.*, 2008; Avena & Bocarsly, 2012; Alσιο *et al.*, 2012). Palatability stimulates excess consumption in rodents (Alσιο *et al.*, 2009), with sweetness being a clear driver for pleasure-linked eating responses (Lenoir *et al.*, 2007). Added to this, the hypothalamus regulates energy homeostasis by sensing and integrating changes in glucose levels (Song *et al.*, 2001), projecting directly to the nucleus accumbens, which influences the hedonic and motivational aspects of food (Stice *et al.*, 2013).

Furthermore, chronic ingesting of sugar has been shown to blunt activity of the oxytocin system, a pathway that mediates satiety in the hypothalamus (Mitra *et al.*, 2010). Previous investigations have identified links between oxytocin and serotonin pathways by which administration of serotonin or fenfluramine, a serotonin agonist, induces increased levels of oxytocin (Jorgensen *et al.*, 2003; Lee *et al.*, 2003). While the interaction between the two neuromodulators has additionally been confirmed in humans (Marazziti *et al.*, 2012), experimental work on non-human primates suggested a role for *Sert* in mediating this association (Emiliano *et al.*, 2007). Additionally, a recent publication has implied oxytocin as a regulator of serotonin signalling in the human brain associated with anxiety (Mottolese *et al.*, 2014) and previous data show that oxytocin infusion regulates serotonin release via activation of oxytocin receptors found in serotonergic neurons (Yoshida *et al.*, 2009). These precedents suggest that chronic consumption of a sugar-laden diet could potentially modulate the oxytocin system via *Sert*. Further investigations are merited as they may help to elucidate common pathways with consumptive behaviour and provide new perspectives to develop novel strategies to treat obesity.

The mechanisms resulting in the upregulation of *Sert* remain elusive. The complexity of *Sert* expression and function is not yet fully understood (Daws & Gould, 2011). Nonetheless, given that *in vitro* studies have shown that long-term exposure to high glucose concentrations further increases the activity of *Sert* (Goncalves *et al.*, 2008), it is possible that the consumption of refined carbohydrate over an extended time determined the patterns of expression of *Sert* seen in our investigation. Recent findings showed a correlation between increased serotonin signalling and obesity (Crane *et al.*, 2015), while earlier investigations suggested that decreased *Sert* protects against rising glucose levels in female rats (Homberg *et al.*, 2010). Growing evidence in support of a link between serotonin

trafficking, food intake and obesity warrants further research to identify the underlying molecular circuitry of these associations. A limitation of our study was that we were unable to confirm the specific influence of palatability on altered eating behaviour and serotonergic signalling as a group of rats fed with a non-palatable HRC diet was not included in the study.

## 5. Conclusion

We have demonstrated that a palatable HRC diet causes changes in the serotonergic pathway driving abnormal eating behaviour, visceral obesity and altered lipid metabolism in rats. To clarify the relationship between food quality, palatability, the level of consumption and *Sert* patterns of expression, future investigations could include a second treatment group fed with a non-palatable HRC diet, along with palatable HRC and SC diet groups.

Our results contribute to the growing body of knowledge implicating the serotonergic system in the hypothalamic control of energy intake and may assist in the understanding of neurophysiological mechanisms underlying the obesity epidemic.

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## Declaration of interest

None.

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