# Effect of Sugars on mRNA Expression of 11β-HSD1in the Hypothalamus of Rats after 24 hour Exposure

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## Abstract

*Fructose can quickly increase the 11β-HSD1 expression in both the liver and adipose tissue of rats. The intra cellular glucocorticoids regeneratedby11β-HSD1 increase the risk of obesity development. However, how sugars affect the expression of hypothalamic 11β-HSD1 is not clear. In the current paper, we treated Sprague Dawley rats with 15% of different sugar solutions including glucose, sucrose, high fructose corn syrup (HFCS) and fructose. After 24 hour exposure, we sampled portions of the par ventricular hypothalamic nucleus (PVN), ventromedial hypothalamus (VMH) and lateral hypothalamus (LH). We then measured mRNA expression of 11β-HSD1 in these three regions of hypothalamus using quantitative PCR techniques. Results showed that although sugars have no significant effect on 11β-HSD1 expression in the PVN, VMH or LH compared to control, HFCS did increase 11β-HSD1 expression compared with glucose and sucrose group in both the PVN and VMH.*

**Keywords:** micro-dissection, sugar, PVN, VMH, LH, 11β-HSD1

## *1. Introduction*

Glucocorticoid dysregulation has been implicated in several forms of obesity. For example, elevated circulating glucocorticoids are found in patients with Cushing's disease(Bista & Beck, 2013). By contrast, bilateral adrenalectomy of genetically- or dietary-obese rats leads to body weight loss and reduced fat appetite. Corticosterone replacement can restore these conditions(Castonguay, Dallman, & Stern, 1984). Liver and adipose tissue intracellular glucocorticoid concentrations are elevated in obese rats and humans (Chapman, Holmes, & Seckl, 2013; Masuzaki et al., 2001; Rask et al., 2001), leading to the speculation that the enzyme 11 β -HSD1 plays an important role in the control of body weight. 11β-HSD1 is an enzyme that catalyzes the inter-conversion of active and inactive glucocorticoids (e.g. cortisol to cortisone in humans and corticosterone to hydroxycorticosterone in rats). The reaction direction of 11β-HSD1 varies by cell types. 11β-HSD1 mainly acts as an oxoreductase to facilitate active glucocorticoid generation in intact cells (Bujalska, Walker, Hewison, & Stewart, 2002; Jamieson, Chapman, Edwards, & Seckl, 1995).

Glucocorticoids may affect behavioral and cognitive functions via targeting specific brain areas(Fietta, Fietta, & Delsante, 2009; Green, Wilkinson, & Woods, 1992). 11β-HSD1 isalso expressed in the hypothalamus of both rats (Moisan, SECKL, & EDWARDS, 1990)and humans(Bisschop et al., 2013). However, the expression pattern of specific neuropeptides is dependent on strain (Schlussman et al., 2011). 11β-HSD1 coexists with corticotropin-releasing hormone (CRH), OXT and AVP in the PVN of humans(Bisschop et al., 2013).Intraventricular glucocorticoid injection increases the body weight gain in adrenalectomized rats (Green et al., 1992) and 11β-HSD1 deficiency increases glycolysis and energy substrate (lactate) in the brain (Verma et al., 2014), indicating that hypothalamic 11β-HSD1 may play a critical role in the control of energy homeostasis. The fructose hypothesis (that high intake of HFCS or fructose has caused the current obesity epidemic)has been hotly discussed for years(Bray & Popkin, 2013; Chiavaroli, Ha, de Souza, Kendall, & Sievenpiper, 2014; Forshee et al., 2007; Nakagawa, Tuttle, Short, & Johnson, 2005; Stanhope, 2012). Our lab showed that fructose decreased the expression of 11β-HSD1 both in liver and visceral adipose after 24 h exposure(London & Castonguay, 2011) indicating the possible role of fructose in the development of obesity. Recently we also found that different sugars affect expression of the hypothalamic neuropeptides in different ways(Colley & Castonguay, 2015).

However, how sugars affect the expression of hypothalamic 11β-HSD1 is not known yet. Different hypothalamic regions have differing effects on feeding behaviors(Schwartz, Woods, Porte, Seeley, & Baskin, 2000). For example, bilateral PVN or VMH ablations increase food intake and lead to obesity whereas bilateral LH area ablations results in decreased intake(Stellar, 1954; Weingarten, Chang, & McDonald, 1985). The work summarized in this paper begins to chronicle our findings as we set out to determine how sugars affect the expression of 11β-HSD1 in different hypothalamic regions.

## *2. Materials & Methods*

### 2.1 Animal Treatment

Forty experimental adult male Sprague-Dawley (CD strain) rats (Charles River Laboratories, Wilmington, MA) weighing 300 grams on average were used. All animals were individually housed under a 12h light/dark cycle in a temperature controlled room (22 ±1°C). All rats were fed a nutritionally complete low fat diet [Rodent diet 7012] prepared by Harlan Teklad (Bethlehem, PA) containing 3.41 kcal/g of diet, of which 2.14 kcals were derived from carbohydrate per gram of chow. All animals were given free access to water throughout the experiment. Rats were randomly assigned to one of five weight-matched groups (eight rats per group). One group of rats had ad libitum chow and water and served as the control group. Rats assigned to the other groups had ad libitum access to the chow and water and to one of four solutions: a 15% weight/volume (w/v) fructose (Tate & Lyle, Decatur IL) solution, a 15% (w/v) glucose (Sigma Aldrich, St Louis MO) solution, a 15% (total solute per volume) high fructose corn syrup (HFCS) (IsoSweet® 5500, 55% fructose–41% glucose, 77% solids, Tate & Lyle, Decatur IL) or a 15% (w/v) sucrose (Domino Foods, Baltimore MD) solution. All sugar solutions were prepared 24 h in advance and stored at 4 °C until use. The rats were maintained with free access to their respective diets for 24 h before sacrifice. All rats were killed by slow replacement of air with pure $CO_2$in a specialized chamber followed by rapid decapitation and exsanguination. This method has been approved for use by the Panel on Euthanasia of the American Veterinary Medical Association as well as the UM IACUC. All procedures described herein are in compliance with the University of Maryland's ACUC guidelines. At the time of sacrifice, the brains were dissected, snap frozen in isopentane/dry ice and then stored at -80 °C until use.

### 2.2 Brain Slicing

The frozen brains were embedded using M1 embedding matrix (Lipshaw, Pittsburgh, PA). The IEC Minot Custom Microtome (Damon/IEC Division) was used for brain cutting. The blade and antiroll plate was pretreated with RNaseZap® to remove any possibility of RNase contamination and cleaned with paper towel presoaked in DEPC-treated water. The brain was slice at -10°C.The thickness of each slice was110 µM. The slices were carefully transferred to pre-cleaned slides (Fisher Scientific, Pittsburgh, PA) and then stored at -80 °C until sampled.

### 2.3 Hypothalamic Dissection

Hypothalamic dissection was carried out within one month after brain slicing. Because sliced samples after standard staining can lead to RNA loss by 10% in as little as 30 minutes(Clément-Ziza, Munnich, Lyonnet, Jaubert, & Besmond, 2008), ethanol dehydration was used in our experiment. Preliminary test showed that cresyl violet staining did not help much in distinguishing hypothalamic structures due to the thickness of our samples. As a result we employed three steps of ethanol dehydration without staining, i.e. 95% ethanol, 30 sec, 100% ethanol, 1min and 100% ethanol, 1min. Before the slices were completely dried out, PVN, VMH and LH were sampled using a sterile 23 G X 1"hypodermic needle (B-D Precision Glide, Franklin Lakes. NJ) under a light microscope. 6-7 brain slices were used for PVN dissection. PVN sampling was initiated approximately at -1.30 mm behind Bregma (Paxinos & Watson, The Rat Brain in Stereotaxic Coordinates, Second Edition).VMH and LH samples were dissected starting at around -2.12 mm behind Bregma. VMH samples were approximately 1.5mm thick while LH samples were approximately 3 mm thick. Captured tissues were carefully transferred into cold 1.5ml eppendorf tubes. 350 µl RLT lysis buffer (containing10 µl β-ME per 1 ml Buffer RLT) was added into each tube followed by 30 sec vortexing to facilitate cell breakage and RNA release. RLT lysis buffer is strong enough to inactivate RNase according to the manufacturer's protocol. The tubes were stored in the -80°C freezer until RNA extraction.

### 2.4 RNA extraction and cDNA synthesis

Tissue lysates were thawed in a 37°C water bath until all the salts were dissolved. The lysates of VMH and LH were centrifuged (Eppendorf centrifuge model 5424) at full speed for 3min to remove cell debris, as per the manufacturer's instructions (Qiagen RNeasy micro kit). DNase I was used to remove any DNA which may affect the downstream applications. RNA quality was estimated using a Nano Drop 2000 spectrophotometer based on A260/A280 values. cDNA synthesis was carried out using iScript™ cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocol. The cDNA products were stored at -20°C until used.

### 2.5 Quantitative Real Time PCR

The PCR reaction with iQ SYBR Green Super mix was carried out in two replicates using a CFX96 Bio-Rad system. β-action was used as the reference gene. The program used for all PCR reactions was 95°C for 3min and 40cycles of 95°C for 15sec, annealing temperature (Ta) for 30sec (Table 1) and 68°C for 30sec.

### 2.6 Data Analysis

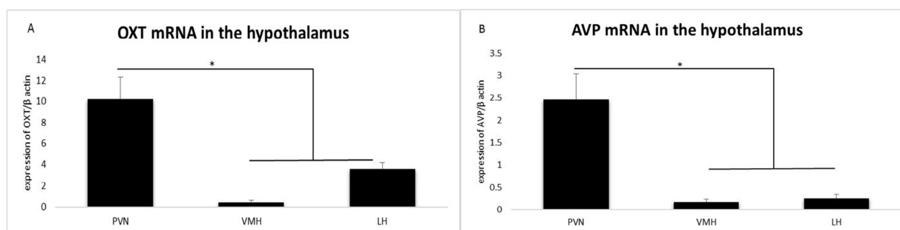
Mean values from β action were used as reference. Data were evaluated using Dixon's Q test, and outliers were removed prior to subsequent analyses. Student's t tests were used to evaluate $2^{-\Delta Ct}$ values so as to determine significance of differences between groups using JMP Pro 10.0.2.

## *3. Results*

### 3.1 Expression of PVN markers

OXT and AVP were mainly expressed in PVN. Expression of these hormones was significantly higher in the PVN than in the VMH or the LH ($p<0.05$; Figure 2).

**Figure 1 Expression of PVN markers. (A-B) Expression of OXT and AVP mRNA in the PVN is significantly higher than that in VMH and LH.**

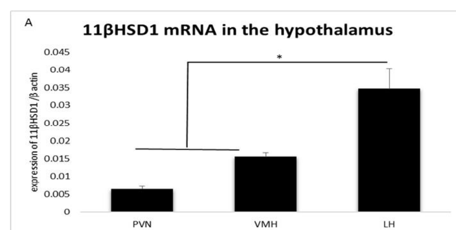



*: P<0.05. PVN: Paraventricular hypothalamic nucleus, VMH: Ventromedial hypothalamus, LH: Lateral hypothalamus, OXT: oxytocin, AVP: arginine vasopressin

### 3.2 Distribution of 11β-HSD1 mRNA in the hypothalamus

11β-HSD1 was abundantly expressed in the hypothalamus. Specifically, 11β-HSD1 was mostly expressed in the LH. The remaining two hypothalamic regions (PVN and VMH) also produced 11β-HSD1 (Figure2).

**Figure 2: Expression of 11β-HSD1 mRNA in hypothalamus. 11β-HSD1 was mostly expressed in the LH. PVN and VMH also expressed 11β-HSD1 mRNA.**



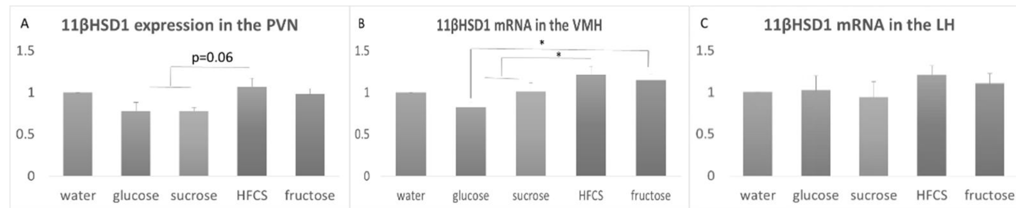

*: P<0.05, PVN: Paraventricular hypothalamic nucleus, VMH: Ventromedial hypothalamus, LH: Lateral hypothalamus, 11β-HSD1: 11 β-hydroxysteroid dehydrogenase type 1

### 3.3 Expression of hypothalamic 11β-HSD1 in rats fed with different sugars

Sugars had no significant effect on 11β-HSD1 mRNA expression in PVN, VMH or LH compared with water group (Figure3A-C).However, HFCS increased 11β-HSD1 mRNA expression in the PVN compared with glucose or sucrose ($p=0.06$; refer to Figure3A).

Similarly, HFCS increased 11β-HSD1 mRNA expression in the VMH compared with glucose or sucrose. Fructose also increased 11β-HSD1 mRNA expression in the VMH compared with glucose only ($p<0.05$; refer to Figure3B).

**Figure 3: Different sugars affect the expression of 11β-HSD1in the PVN, VMH and LH. (A)Sugars did not significantly change 11β-HSD1 mRNA expression in the PVN compared with water group. HFCS increased 11β-HSD1 mRNA expression in the PVN compared with glucose or sucrose ($p=0.06$). (B) Similarly, Sugars did not significantly change 11β-HSD1 mRNA expression in VMH compared with water group. HFCS significantly increased 11β-HSD1 mRNA expression in the VMH compared with glucose or sucrose ($p<0.05$). (C)All sugars failed significantly change 11β-HSD1 mRNA expression in theLH compared with water group.**




*: $P<0.05$, PVN: Paraventricular hypothalamic nucleus, VMH: Ventromedial hypothalamus, LH: Lateral hypothalamus, 11β-HSD1: 11 β-hydroxysteroid dehydrogenase type 1

## *4. Discussion*

Two methodological issues encouraged us to develop the alternative method described above. First, brain slices after standard staining can lose as much as 10% of its RNA within 30 minutes (Clément-Ziza et al., 2008).Here we have demonstrated that the micro-dissection of brain slices can be performed under a microscope without staining(Benicky et al., 2011). Additionally, this method allowed us the use of thick slices for dissection/sampling and that way minimized steps that could have affected the structure or intracellular molecules for downstream analysis. Secondly, immunohistochemical methods have been used to identify peptides within a known anatomical region such as the PVN(Cho, Lee, Park, Hong, & Ryu, 2007; Fuller, Clements, & Funder, 1985). However, this technique is both costly and labor-intensive. As an alternative, we have micro dissected and sampled regions of the PVN and verified their accuracy by amplifying RNA that is known to be localized only in the PVN. Our findings confirmed that we have been able to sample the PVN, as both OXT and AVP messages were not measured to any extent in samples of the VMH or LH.It should be noted that a similar validation was not performed on VMH and LH samples. When compared with the PVN, these two regions are much larger and anatomically distinct, making it unnecessary to further examine if they were accurately sampled.

Adipose tissue 11β-HSD1 has been studied for its possible roles in obesity development (Masuzaki et al., 2003; Morton et al., 2001; Morton et al., 2004), whereas the brain 11β-HSD1 is rarely reported. Early reports showed that 11β-HSD1 was expressed in the forebrain (hypothalamus, hippocampus, and cortex) of Wistar rats(Moisan et al., 1990). One group recently investigated the distribution of 11β-HSD1 in the human brain, and reported that 11β-HSD1 was also abundantly detected in the human hypothalamus(Bisschop et al., 2013). However, the expression pattern of specific neuropeptides can vary by strain(Schlussman et al., 2011). The hypothalamus is the food intake control center, and plays a pivotal role in energy balance. Here we determined where the 11β-HSD1 mRNA was distributed in three hypothalamic regions using quantitative PCR in the Sprague Dawley rats. We found that 11β-HSD1 was most expressed in the LH, but it is also abundantly expressed in the other two hypothalamic regions – PVN and VMH. This result is consistent with the previous report on Wistar rats that 11β-HSD1 was highly expressed in the preoptic area and also posterior arcuate nucleus (ARC) (not included in the current study)(Moisan et al., 1990). The previous report failed to clearly detect 11β-HSD1 expression in other hypothalamic areas. This is probably because in situ hybridization is less sensitive than the quantitative PCR technique. Bisschop et al. reported that 11β-HSD1 was found to be coexpressed with CRH, OXT and AVP in the PVN(Bisschop et al., 2013). Similarly, we found 11β-HSD1 was detected in the PVN, where CRH, OXT and AVP were highly expressed. Our observations make it plausible that hypothalamic 11β-HSD1 in the PVNparticipates in the hypothalamic-pituitary-adrenal axis and/or other biological metabolism, e.g. food intake control. Centralglucocorticoids play an important role in energy homeostasis (Fietta et al., 2009; Green et al., 1992). We have previously shown that sugars can affect the expression of several neuropeptides in the hypothalamus.

Here we further showed that sugars also increased the hypothalamic expression of 11β-HSD1 in both PVN and VMH. Interestingly, when we combined the PVN, VMH and LH data together, we failed to detect any significant differences. Therefore, we believe the effect of sugars on the hypothalamic glucocorticoids is probably mainly in the PVN and VMH areas. It has been argued that the health issues caused by HFCS consumption are mainly attributable to its fructose content. In fact, HFCS is more preferred to simple glucose and fructose mixtures because of the existence of some polycose (Ackroff & Sclafani, 2011). Fructose can facilitate glucose absorption and intake of fructose in excess of glucose can lead to malabsorption (Rumessen, 1992). In the current research, we found that HFCS is more effective than fructose in increasing11β-HSD1 expression in the VMH specifically.


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**Table 1 Primer Sets**

| **Primer Name** | **sequences (5' to 3')** | **Ta (℃)** |
|---|---|---|
| β actin sense | TGTCACCAACTGGGACGATA | 60 |
| β actin antisense | GGGGTGTTGAAGGTCTCAAA | |
| 11β-HSD1 sense | GTGTCTCGCTGCCTTGAAC | 55 |
| 11β-HSD1 antisense | AGTGGTCTGTGTGATGTGATTG | |
| OXY sense | ACCCTGAGTCTGCCTTCT | 54 |
| OXY antisense | ATGGGGAATGAAGGAAGCG | |
| AVP sense | ACCTCTGCCTGCTACTTC | 53 |
| AVP antisense | ACACTGTCTCAGCTCCAT | |