High glucose induces hypogonadotropic hypogonadism by interfering with GPR54 signaling in the preoptic area of hypothalamus

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Introduction

Male hypogonadism is defined as a gonadal failure due to alterations of the hypothalamus-pituitary-gonadal axis and is relatively frequent in middle-aged and elderly subjects.
Low circulating levels of testosterone and gonadotropins are associated with age-dependent comorbidities such as hypertension, type II diabetes mellitus, and metabolic syndrome (MetS), a clustering of metabolic and cardiovascular risk factors often associated to male hypogonadotropic hypogonadism (HH).
Although MetS and HH are correlated to insulin resistance and increased waist circumference, the pathogenetic mechanisms linking the two conditions remain to be elucidated.

Aims

In the present study we investigated the mechanisms involved in MetS-induced hypothalamic alterations potentially influencing the gonadotropin-releasing hormone (GnRH) neuron functions

Methods

The HFD-induced MetS rabbits, obtained as previously described (1), developed all MetS features and HH (1-4)and showed a reduced GnRH positivity in the hypothalamus area, where GnRH neurons express kisspeptin/KiSS-1 receptor (GPR54). To evaluate the role played by HFD-induced glucose dysregulation and by systemic pro-inflammatory status on hypothalamic function, two subgroups of HFD rabbits were treated with the farnesoid-X receptor agonist obeticholic acid (OCA) or a monoclonal anti-TNFα (Infliximab), respectively. In addition, *in vitro* studies were performed to evaluate the effects of high glucose concentrations on neuroblasts GnRH-secreting human FNCB4 (5), which expresses the system kisspeptin/KiSS-1R. The RNA expression and protein quantification were evaluated by RT-PCR and immunohistochemistry analysis, respectively.

Table 1. Metabolic hormonal parameters of RD and HFD rabbits at 12 weeks

* p< 0.05, ** p< 0.01, *** p< 0.001 vs. RD

	RD (n=30)[a]	HFD (n=18)
Blood glucose (g/L)	1.22 ± 0.04	1.92± 0.09***
OGTT (iAUC)	151.82 ± 7.35	215.71 ± 10.32***
Cholesterol (mg/dL)	45.33 ± 6.09	1588.24 ± 119.68***
Triglycerides (mg/dL)	93.54 ± 6.87	241.54 ± 43.30***
MAP (mmHg)	92.61 ± 3.42	142.20 ± 5.71*
VAT weight (g)	36.21 ± 1.91	42.85 ± 1.86*
Testosterone (nM)	5.12 ± 0.67	1.23 ± 0.26***
17β-estradiol (pM)	168.50 ± 16.58	274.55 ± 28.73**
LH (ng/mL)	0.63 ± 0.16	0.17 ± 0.08**
FSH (ng/mL)	8.33 ± 1.04	4.05 ± 0.61**
Seminal vesicles (mg)	737.19 ± 66.86	461.90 ± 35.79**

Results

MetS and hormones plasma levels

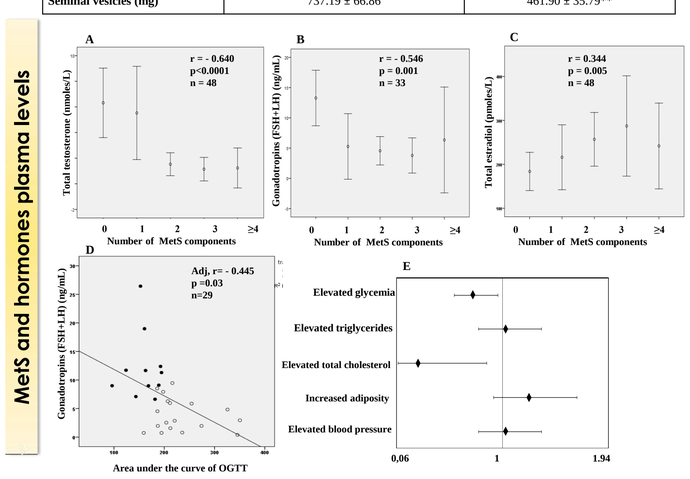

Figure 1. Relationship between the number of MetS components and testosterone (**A**), estradiol (**B**) or gonadotropins (**C**), and the incremental AUC during OGTT and gonadotropin plasma levels (**D**) univariate Spearman's regression (**E**) r: relative coefficient of correlation; p: level of significance; AUC: area under the curve; OGTT: oral glucose tolerance test.

Gene expression in rabbit hypothalamus

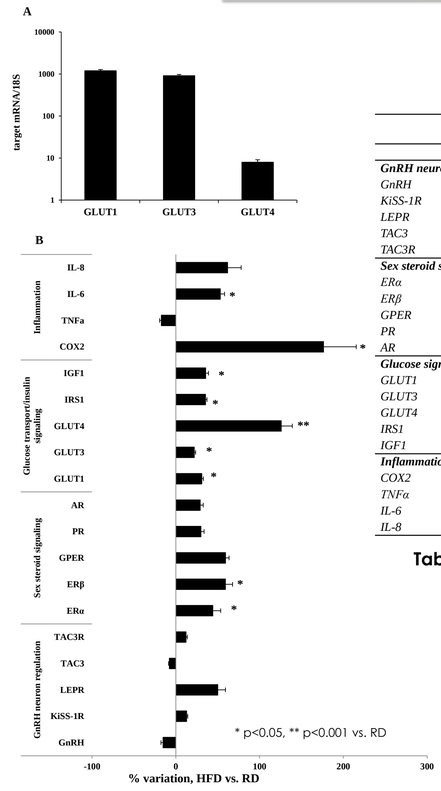

Figure 2 A: Relative expression of GLUT 1, GLUT3 and GLUT4 mRNA in hypothalamus from RD (regular diet) rabbits. **B**: HFD-induced variations of hypothalamic genes, compared to RD.

	GnRH mRNA			gonadotropins		
	r	p value	n	r	p value	n
GnRH neuron regulation						
GnRH	/	/		-0,067	0.768	17
KiSS-1R	**0.324**	**0.042**	40	-0.036	0.899	15
LEPR	0.243	0.116	43	-0.159	0.542	17
TAC3	**0.597**	**<0.0001**	43	-0.032	0.903	17
TAC3R	**0.490**	**0.001**	44	-0.043	0.870	17
Sex steroid signaling						17
ERα	**0.550**	**<0.0001**	44	-0.272	0.290	17
ERβ	0.303	0.051	42	-0.367	0.148	17
GPER	0.018	0.909	44	-0.397	0.114	17
PR	0.167	0.279	44	-0.298	0.246	17
AR	**0.584**	**<0.0001**	44	-0.183	0.483	17
Glucose signaling						
GLUT1	**-0.345**	**0.024**	43	**-0.538**	**0.026**	17
GLUT3	-0.031	0.845	43	**-0.536**	**0.027**	17
GLUT4	-0.195	0.209	43	**-0.756**	**0.001**	16
IRS1	-0.029	0.850	44	-0.345	0.175	17
IGF1	-0.237	0.121	44	-0.085	0.747	17
Inflammation						
COX2	0.017	0.913	44	-0.430	0.085	17
TNFα	0.083	0.595	43	-0.244	0.345	17
IL-6	0.024	0.876	44	**-0.535**	**0.027**	17
IL-8	0.083	0.596	43	-0.362	0.154	17

Table 2. Association of GnRH mRNA or gonadotropins (LH plus FSH) plasma levels with genes in rabbit hypothalamus.

Hypothalamic GLUT4 expression

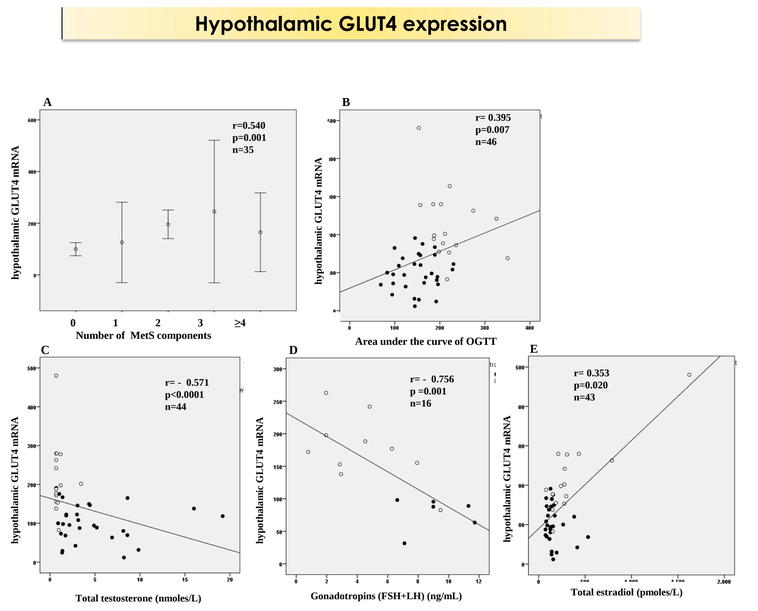

Figure 3 A-C: Relationship between hypothalamic GLUT4 mRNA expression and the number of MetS components (**A**), incremental AUC of glucose during OGTT (**B**), testosterone (**C**), gonadotropins (**D**) or estradiol (**E**), as derived from univariate Spearman's regression analysis in n samples. r: relative coefficient of correlation; p: level of significance; AUC: area under the curve; OGTT: oral glucose tolerance test

Immunohistochemical analysis of GLUT4 and inflammation markers

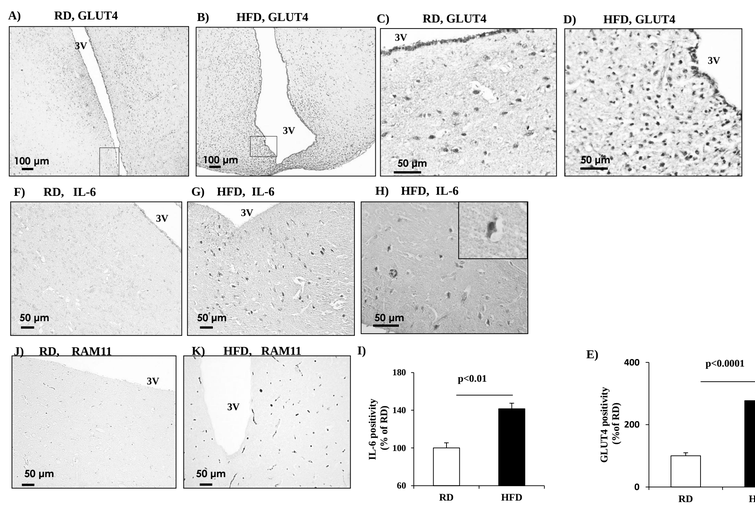

Figure 4. Representative images of coronal sections of rabbit hypothalamus, including the region lining the 3rd ventricle (3V) in RD (A, C, F, J) or HFD (B, D, G, H, K) rabbits. **A-B**: immuno-detection of GLUT4 (magnification x4). **C-D**: higher magnification of box in A and B panels (magnification x20), respectively. **F-H**: representative images of IL-6 immunopositivity in RD (**F**) or HFD (**G**) rabbits; (magnification x10). **H**: Higher-magnification images (x20) show macrophage-like IL-6 positive cells in blood vessels. **J-K**: immunopositivity of the macrophagic marker RAM11 (magnification x10) in RD (**J**) and HFD (**K**) rabbit hypothalamus. **F, J**: quantification of GLUT4 and IL-6 immunopositivity, respectively.

Immuno analysis of microglial and astroglia morphology

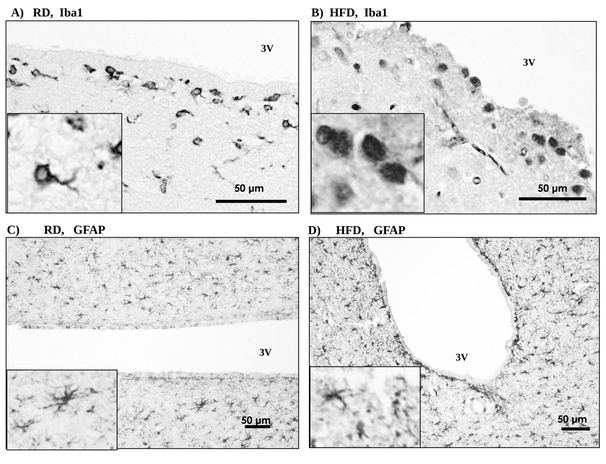

Figure 5. Representative images of coronal sections of rabbit hypothalamus, including the region lining the 3rd ventricle (3V) in RD (**A-C**) or HFD (**B-D**) rabbits. Immuno-detection of the microglial marker Iba1 (ionized calcium-binding adapter molecule 1) in hypothalamic microglial cells from RD rabbits (ramified morphology, **panel A**) and from HFD-rabbits ("ameboid" morphology, **panel B**). Immuno-detection of the astrocyte marker GFAP (Glial Fibrillary Acidic Protein) in RD (**C**) and HFD (**D**) rabbit hypothalamus. GFAP-positive cells are similarly detected in both RD and HFD rabbits (magnification x10).

Expression of KiSS-1R (GPR54)

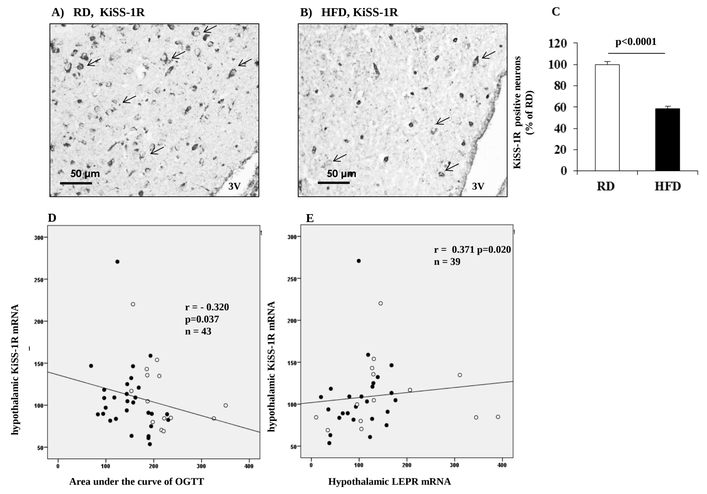

Figure 6. Representative images (magnification x20) of KiSS-1R (GPR54) immunodetection in coronal sections of rabbit hypothalamus, including the region lining the 3rd ventricle (3V), from a RD (**A**) or HFD (**B**) rabbits. Reduced amount of positive neurons (arrows) are detectable in HFD rabbits (**B**), while several groups of KiSS-1R positive neurons (arrows) are visible in RD hypothalamus (**A**). **C**: quantification of KISS-1R immunopositivity. **D-E**: Relationship between hypothalamic KiSS-1R mRNA expression and the incremental AUC of glucose during OGTT (**D**), or hypothalamic LEPR (leptin receptor) mRNA expression (**E**), as derived from univariate Spearman's regression analysis in n samples. r: relative coefficient of correlation; p: level of significance; AUC: area under the curve; OGTT: oral glucose tolerance test.

Effects of chronic treatment with OCA o infliximab

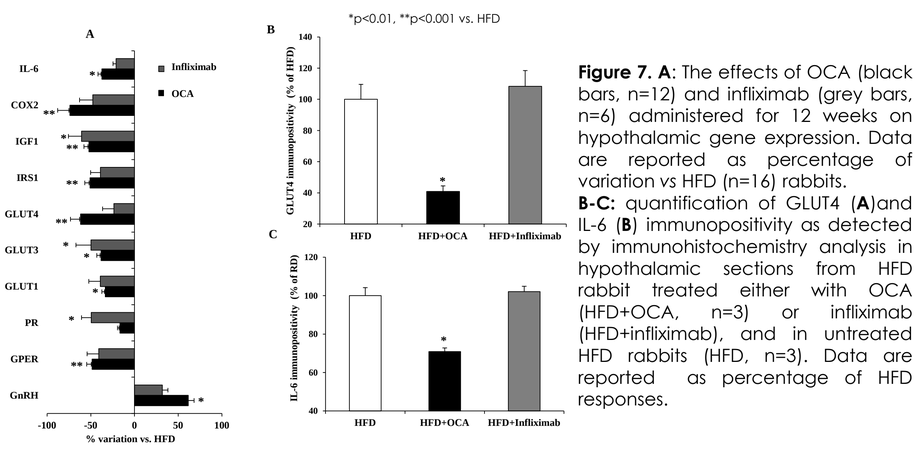

Figure 7. A: The effects of OCA (black bars, n=12) and infliximab (grey bars, n=6) administered for 12 weeks on hypothalamic gene expression. Data are reported as percentage of variation vs HFD (n=16) rabbits.
B-C: quantification of GLUT4 (**A**)and IL-6 (**B**) immunopositivity as detected by immunohistochemistry analysis in hypothalamic sections from HFD rabbit treated either with OCA (HFD+OCA, n=3) or infliximab (HFD+infliximab), and in untreated HFD rabbits (HFD, n=3). Data are reported as percentage of HFD responses.

* p< 0.05, ** p< 0.01, *** p< 0.001 vs. HFD

	HFD+ OCA (n=13)	HFD + infliximab (n=7)
Blood glucose (g/L)	1.38 ± 0.09***	1.98 ± 0.21
OGTT (iAUC)	174.42 ± 10.10*	198.00 ± 17.50
Cholesterol (mg/dL)	1309.54 ± 182.35	1302.57 ± 71.77
Triglycerides (mg/dL)	199.46 ± 58.70	200.14 ± 49.05
MAP (mmHg)	138.11 ± 5.24	143.93 ± 9.92
VAT weight (g)	11.85 ± 1.82***	25.31 ± 5.03**
Testosterone (nM)	0.76 ± 0.04	0.90 ± 0.21
17β-estradiol (pM)	119.17 ± 10.15**	139.37 ± 14.61***
LH (ng/mL)	0.14 ± 0.06	0.11± 0.03
FSH (ng/mL)	3.14 ± 0.99	2.42 ± 0.58
Seminal vesicles (mg)	447.50± 48.69	415.00 ± 50.58

Table 3. Effects of 12 weeks treatments with OCA or Infliximab on metabolic /hormonal parameters in HFD rabbits.

High-glucose effects in FNCB4 cells

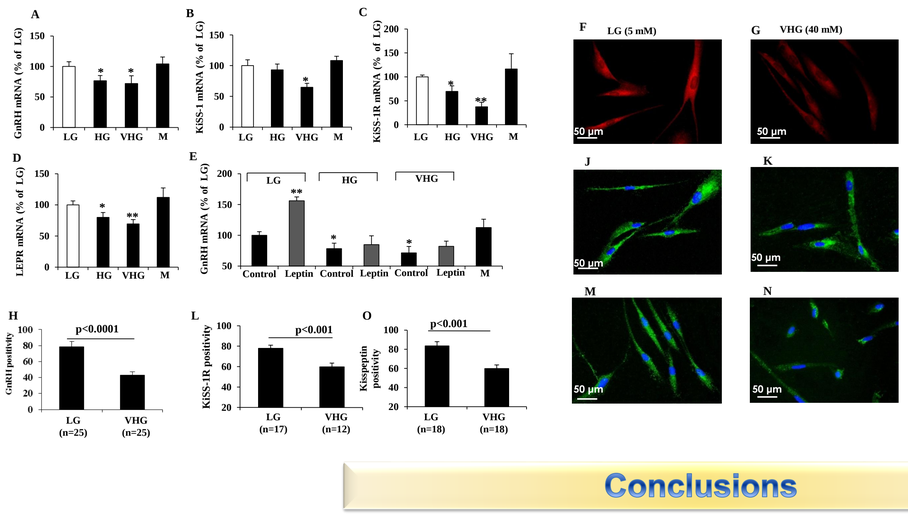

Figure 8. A-D: Quantitative RT-PCR analysis of expression of GnRH (**A**), KiSS-1 (**B**), KiSS-1R (**C**) and LEPR (**D**) in FNCB4 cells exposed to low (LG, 5 mM), high (HG, 22 mM), and very high (VHG, 40 mM) glucose concentration or mannitol (M, 22 mM) for 24 hours. **E**: Effect of leptin (1nM, 24 hours) on GnRH mRNA expression after exposure to different glucose concentrations (LG, HG, VHG) or mannitol (M). **F-O**: Immuno-localization of GnRH (**F-G**), KiSS-1R (**J-K**) and kisspeptin (**M-N**) proteins in FNCB4 cells exposed to LG (**F,J, M**) or VHG (**G, K, N**). Dual labeling with the nuclear staining DAPI (blue color) and anti-KiSS-1R (green color; J and K), or anti-kisspeptin (green color, M and N) antibodies is also shown (l magnification x20). **H,L,O:** quantification of GnRH, KiSS-1R and kisspeptin immunopositivity. n= number of cells analyzed.

Conclusions

In this study, we add new informations with regard to the mechanisms linking MetS development and HH, first demonstrating that HFD-associated glucose intolerance and hypothalamic inflammation was associated to an impairment of GnRH network. Our results suggest that HFD-induced glucose dysregulation negatively affects the GnRH neuron function, most likely through an inflammatory injury at the hypothalamic level.
The metabolic derangements, mainly ascribed to glucose intolerance and hallmarked by GLUT4 upregulation, may activate pro-inflammatory pathways within the hypothalamus, thus compromising a key brain area involved in the control of reproduction.

References
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