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Research paper

mRNA GPR162 changes are associated with decreased food intake in rat, and its human genetic variants with impairments in glucose homeostasis in two Swedish cohorts

Vanni Caruso [a,b,*,1], Smitha Sreedharan [a,1], Valeria P. Carlini [a,c], Josefin A. Jacobsson [a], Tatjana Haitina [a], Joanna Hammer [a], Olga Stephansson [a], Filip Crona [a], Wolfgang H. Sommer [d], Ulf Risérus [e], Lars Lannfelt [f], Claude Marcus [g], Markus Heilig [h], Susana R. de Barioglio [c], Robert Fredriksson [a], Helgi B. Schiöth [a]

[a] Department of Neuroscience, Unit of Functional Pharmacology, Uppsala University BMC, Uppsala SE 75124, Sweden
[b] Faculty of Pharmacy, University of Tasmania (UTAS), 7001 Hobart, Australia
[c] Departamento de Farmacología, Facultad de Ciencias Químicas, Haya de la Torre y Medina Allende, Ciudad Universitaria, Universidad Nacional de Córdoba, 5016 Córdoba, Argentina
[d] Department of Psychopharmacology, Central Institute of Mental Health, Mannheim, Germany
[e] Department of Public Health and Caring Sciences, Clinical Nutrition and Metabolism, Uppsala University, Uppsala, Sweden
[f] Department of Public Health and Caring Sciences, Geriatrics, Uppsala University, Uppsala, Sweden
[g] Department for Clinical Science, Intervention and Technology, Karolinska Institute, Division of Pediatrics, National Childhood Obesity Centre, Stockholm, Sweden
[h] Laboratory of Clinical and Translational Studies, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD, USA

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ABSTRACT

G protein–coupled receptors (GPCRs) are a class of integral membrane proteins mediating intercellular interactions of fundamental physiological importance for survival including regulation of food intake, blood pressure, and hormonal sensing signaling, among other roles. Homeostatic alterations in the physiological status of GPCRs are often associated with underlying causes of disease, and to date, several orphan GPCRs are still uncharacterized.

Findings from our previous study demonstrate that the *Rhodopsin* family protein GPR162 is widely expressed in GABAergic as well as other neurons within the mouse hippocampus, whereas extensive expression is observed in hypothalamus, amygdala, and ventral tegmental area, regions strictly interconnected and involved in the regulation of energy homeostasis and hedonic feeding.

In this study, we provide a further anatomical characterization of GPR162 in mouse brain via in situ hybridization as well as detailed mRNA expression in a panel of rat tissues complementing a specie-specific mapping of the receptor. We also provide an attempt to demonstrate a functional implication of GPR162 in food intake-related behavior via antisense knockdown studies. Furthermore, we performed human genetic studies in which for the first time, variants of the GPR162 gene were associated with impairments in glucose homeostasis.

1. Introduction

G protein–coupled receptors (GPCRs) are a class of integral membrane proteins mediating intercellular interactions through activation of GTP-binding proteins (G-proteins) (Fredriksson et al., 2003). Physiological implications of GPCRs include embryonic development, hormonal sensing signaling mediations, neuroendocrine regulation of food intake, as well as blood pressure (Almen et al., 2009; Lagerstrom and Schioth, 2008). Detrimental changes in the physiological status of GPCRs are often associated with underlying causes of disease (Lagerstrom and Schioth, 2008; Fredriksson et al., 2003), hence GPCRs constitute the target of one-third of all marketed drugs (Lagerstrom and Schioth, 2008).

Based on sequence similarity within their transmembrane regions, GPCRs are divided into five subfamilies, named Rhodopsin, Glutamate,

Abbreviations: ZI, Zona incerta; STN, Subthalamic nucleus; PSth, Parasubthalamic nucleus; BLA, Basolateral nucleus of the amygdala; LaDL, Lateral amygdaloid nucleus dorsolateral part; Den, Dorsal endopiriform claustrum; ACo, Anterior cortical amygdaloid area; SO, Supraoptic nucleus; LD, Laterodorsal thalamic nuclei; LH, Lateral hypothalamus; MH, Medial hebanula; MD, Mediodorsal thalamic nuclei; PV, Paraventricular thalamic nucleus; CM, Central medial thalamic nucleui; PAG, Periaqueductal gray; VTA, Ventral tegmental area; SN, Substantia nigra; SCN, Suprachaismatic nucleus; AHC, Anterior hypothalamic area central; ML, Medial mammilary nucleus lateral part; MM, Medial mammilary nucleus medial part; DG, Dendate gyrus; CA1, Field CA1 hippocampus; CA2, Field CA2 hippocampus and field CA3 hippocampus CA3; PVH, Paraventricular hypothalamus; CPu, Caudate putamen; DMH, Dorsomedial hypothalamus; VMH, Ventromedial hypothalamus; LHA, Lateral hypothalamic area; ARH, Arcuate hypothalamic nucleus.

* Corresponding author at: Department of Neuroscience, Unit of Functional Pharmacology, Biomedical Center, Box 593, SE 75124 Uppsala, Sweden.
E-mail address: vanni.caruso@neuro.uu.se (V. Caruso).
[1] These authors contributed equally to this work.

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Adhesion, Frizzled/Taste 2, and Secretin, of which Rhodopsin are the largest component consisting of more than 670 human GPCRs including the olfactory receptors (Gloriam et al., 2007). Recent phylogenetic analyses revealed nine new members of the Rhodopsin family including GPR162 (Gloriam et al., 2005; Sreedharan et al., 2011), whose function and physiological implication remain unknown.

In our previous study, we used a custom-made polyclonal antibody to chart the expression of GPR162 in the mouse brain (Caruso et al., 2014a). For the first time, our results provided direct evidence of high GPR162 expression in the limbic system, and specifically, we reported that GPR162 is widely expressed in GABAergic as well as other neurons particularly in areas related to energy homeostasis and hedonic feeding such as hypothalamus, amygdala, and ventral tegmental, which are brain regions extensively interconnected with each other and involved in the regulation of food intake via reward mechanisms (Ahn and Phillips, 2002; Carlini et al., 2004).

Here, we provide a further anatomical characterization of GPR162 in mouse brain via in situ hybridization studies as well as an attempt to describe its functional implication in food intake through antisense knockdown studies. Additionally, we performed human genetic studies investigating the possible association between genetic variants of GPR162 with BMI, insulin, and glucose levels as well as insulin resistance in two Swedish cohorts.

2. Materials and methods

All animal work was approved by the Uppsala Ethics Committee and followed the guidelines of the European Communities Council Directive (86/609/EEC).

2.1. Animals studies

2.1.1. Quantitative real-time PCR (qRT-PCR)

Three Wistar male rats, 6 months old, were used to complete the mRNA GPR162 tissue panel. Animal handling, tissue isolation, and cDNA synthesis were performed as previously described (Lagerstrom et al., 2007). After dissection, tissues were preserved in the RNA later solution (Ambion, Austin, TX, USA) and stored at −80 °C. Primers for housekeeping genes (rGAPDH, Cyclo, and RPL19) and GPR162 were designed using Beacon Primer Design 4.0 software (Premier Biosoft, USA). Primer design is provided in Supplemental Table 1. The amplification was carried out in a total volume of 20 μl consisting of cDNA equivalent to 25 ng RNA, 0.25 μM of forward and reverse primer, 20 μM Tris–HCl (pH: 8.4), 50 μM KCl, 4 μM $MgCl_2$, 0.2 μM dNTP, SYBR Green (1:50 000), and 0.02 U/μl Taq DNA polymerase (Invitrogen, Sweden). PCR reactions were prepared in duplicates and heated to 95 °C for 3 min followed by 50 cycles of denaturation at 95 °C for 15 s, at optimal annealing temperature for 15 s, and extension at 72 °C for 30 s. Negative and positive controls as well as a melting curve were included in each run to validate the method. RT-PCR raw data were analyzed using the MyiQ software v. 1.04 (Bio-Rad Laboratories AB, Sundbyberg, Sweden). Primer efficiencies were evaluated using LinRegPCR program and relative expression levels using the GeNorm software (Vandesompele et al., 2002). Normalized expression value of gDNA (genomic DNA) was set to 100% and the relative expression values were displayed as change in expression related to the gDNA.

2.1.2. In situ hybridization

Three male C57BL/6 J mice, 10 weeks old, were anesthetized with an i.p. injection of ketamine (7 μg/kg bw) and medetomidine hydrochloride (70 μg/kg bw) and transcardially perfused with saline followed 4% PFA/PBS. Brains were removed and postfixed in 4% PFA/PBS at 4 °C overnight, embedded in 4% agarose, and sectioned (70 μm) on a Vibratome (Leica, Sweden).

The antisense probe for mouse GPR162 was generated from the EST clone 5042682 (Invitrogen, Sweden). Plasmid preparation was performed using the JETSTAR Plasmid Midi Kit (Genomed, USA). Plasmid was linearized with BamHI (Fermentas, Sweden) and used as a template for antisense digoxigenin (DIG)-labeled probe synthesis and T7 polymerase was used for probe synthesis. Synthesis of cDNA probes and in situ hybridization were performed as previously described (Lagerstrom et al., 2007; Kalnina et al., 2013).

2.1.3. Knockdown studies

Sixteen Wistar male rats, 2 months of age, weighing 260–290 g were maintained under controlled temperature at 21 ± 1 °C and a light/dark cycle (12 h light, 12 h dark) with food and water *ad libitum*. Rats were anesthetized with 55 mg/kg ketamine HCl (Kensol König, Argentina) and 11 mg/kg xylazine (Kensol König, Argentina) and placed in the stereotaxic apparatus for intracerebroventricular cannulation (ICV) using brain infusion kit (Alzet Corp, Palo Alto, USA). Brain coordinates were −3.6 mm (A), 2.0 mm (L), 2.8 mm (V) from bregma. Cannula was connected to an osmotic minipump (Alzet Corp, Palo Alto, USA) and oligonucleotide solution was delivered (1 μl/h) during following 7 days from the surgery (Sommer et al., 2000). Infusions were practiced between 10:00 am and 12:00 am and toxicity tests were performed on brain sections from rats administered with GPR162 antisense (AS) oligodeoxynucleotide (ODN), and rats receiving GPR162 mismatch (mm) ODN. No effect with regards to inflammation, necrosis, and basic tissue structure was observed (Supplemental Fig. 1).

2.1.4. Antisense oligonucleotides design and infusion

Rat AS and mm ODN for GPR162 (rGPR162AS and rGPR162) were resuspended together in artificial cerebrospinal fluid (ACSF: NaCl 130 mM, KCl 2.8 mM, $MgCl_2$ 1 mM, NaH_2PO_4 0.05 mM, $NaHCO_3$ 20 mM, and $CaCl_2$ 1.2 mM) in equimolar quantities to give a final concentration of 1.7 nmol/μl. Oligonucleotides sequences are provided in Supplemental Table 2.

2.1.5. Food intake and body weight

Food intake of each animal was recorded for 4 days before the surgery in order to establish a mean individual baseline of food intake. Body weight of each animal was recorded for 5 consecutive days before surgery in order to establish a mean baseline of body weight for each animal. Data were analyzed using two-way ANOVA followed by the Bonferroni's post-hoc test.

2.2. Human studies-analysis of genetic association to obesity and related traits

2.2.1. Obese children and adolescents

Cohort of children and adolescents includes 551 severely obese children (286 girls and 265 boys) enrolled at National Childhood Obesity Centre at Karolinska University Hospital, Huddinge, Sweden (Table 1). This study was approved by the Regional Committee of Ethics, Stockholm (Sundberg et al., 2008).

2.2.2. Adult men

The Uppsala Longitudinal Study of Adult Men (ULSAM) is a population-based cohort (n = 2322) initiated in 1970 in which men born between 1920 and 1924 and residing in Uppsala, Sweden, were

Table 1
Descriptive characteristics of the two cohorts.

Characteristics	Obese	Normal weight*
N (boys/girls)	551 (265/286)	1152
Age (years)	12.7 ± 3.2	49.6 ± 0.6
Body weight (kg)	91.2 ± 29.5	77.4 ± 10.0
Length (cm)	158.0 ± 17.0	176.5 ± 5.8
BMI z-score	3.5 ± 0.6	1.1 ± 0.8

Values are geometric means ± SD.
* Men in ULSAM cohort.

invited to participate in a health survey (Table 1) (Hedstrand, 1975). Design and selection criteria for the cohort have been described previously (Ingelsson et al., 2005). All participants gave their written informed consent, and the study was approved by the Ethics Committee of Uppsala University, Faculty of Medicine (Ingelsson et al., 2005).

2.2.3. Phenotype characterization

Among adult men (50 years old), height and body weight were recorded and BMI was calculated as body weight divided by height squared (kg/m^2), whereas in children BMI z-score was calculated relative to the International Obesity Task Force (IOTF) definitions (Cole et al., 2000). Blood glucose, serum insulin concentrations during an intravenous glucose tolerance test (IVGTT) were measured and HOMA-IR (as an index of insulin resistance) were determined as previously described (Zethelius et al., 2008). The level of serum insulin was analyzed from blood samples drawn after 12 h overnight fasting (Table 2).

2.3. Genotyping

DNA was available for 1152 men obtained from examination at 70 years of age and for 551 children and adolescents (Table 1). Four SNPs were genotyped in these two cohorts (Table 2): one located approximately 1300 bp downstream of GPR162 (rs1045261), one in intron 2 (rs874627), one in intron 4 (rs2071081), and one non-synonymous in exon 5 (rs11612427). Genotyping in the ULSAM cohort was carried out at the SNP technology platform at Uppsala University (http://www.genotyping.SE/) using the Illumina Golden Gate Assay (Fan et al., 2003). The genotype call rate in the samples was 96.8%. Genotyping in the children and adolescents was carried out with predesigned Taqman single-nucleotide polymorphism genotyping assays (Applied Biosystems, Foster City, USA) and an ABI7900 genetic analyzer with SDS 2.2 software at the Uppsala Genome Center (http://www.genpat.uu.se/node462). The genotype call rate was 90.6%. Test for deviation from Hardy–Weinberg equilibrium was performed using the Pearson's χ^2 test (1 d.f.) and none of the SNPs did deviate from Hardy–Weinberg equilibrium in any of the studied groups.

2.4. Statistical analysis

Associations between genotypes and phenotypes were analyzed in each cohort with linear regression, assuming an additive model. Quantitative skewed variables were normalized by transformation before the analysis. For the ULSAM cohort, covariates such as BMI, height, and age, were tested for dependence on the response variables and included in the model if significant. Among the children and adolescents, the models were adjusted for age, gender, BMI, and height when needed. Statistical analyses were performed with PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007; Bax et al., 2006). Due to the number of SNPs and phenotypes tested, the significant levels in the different cohorts were adjusted with the False Discovery Rate (FDR) according to the procedure of Benjamini and Hochberg (Benjamini and Hochberg, 1995) and a p-value ≤0.004 was considered statistically significant. Haploview (Barrett et al., 2005) was used for linkage disequilibrium (LD) measurements according to confidence intervals by Gabriel et al. (2002) as well as graphical representation of the LD structure indicated as r^2. Data for LD analysis was obtained from HapMap phase 3 per November 17, 2015 (http://www.hapmap.org). Fixation values (Fst) were calculated for the CEU population vs the entire HapMap database using PLINK 1.09b3 (Purcell et al., 2007).

3. Results

3.1. mRNA GPR162 is abundant in the CNS and negligible in peripheral tissues

qRt-PCR was performed on a rat panel tissue (Fig. 1). Overall, GPR162 expression was high in the CNS whose highest levels were observed in the cerebellum (844 ± 20) and in the hindbrain (726 ± 90); conspicuous expression was also detected in brain cross-section VII (650 ± 26) and cross-section VI (630 ± 29). Similar levels were found in the brain stem (426 ± 10), hypothalamus (406 ± 13), spinal cord (333 ± 48), and pituitary (228 ± 65). Moderate to low expression was detected in the eye (44 ± 14) and almost negligible expression with values ranging from 2 to 15 was detected in all peripheral tissues examined.

3.2. Significant expression of GPR162 in regions related to hedonic feeding

In situ hybridization was performed on coronal sections of the mouse brain and revealed consistency with our qRT-PCR results. *In situ* hybridization findings revealed a widespread differential expression of Gpr162 mRNA in the gray matter but not in the white matter (Fig. 2). Higher GPR162 expression was detected in the amygdala, with dense expression in the basal amygdaloid nucleus (BLA) and anterior cortical amygdaloid area (ACo). High expression was found in the hippocampus, particularly in the granular layer of the dentate gyrus (GrDG) and in the pyramidal cell layer (Py); substantial expression

Table 2
Association study of GPR162 SNPs with obesity phenotypes.

				Obese children		Adult men	
SNP	Location	MAF	Phenotype	Beta	P	Beta	P
rs1045261	Downstream	0.14	BMI	−0.045	0.495	0.061	0.270
T > C			Glucose	−0.012	0.907	−0.016	0.043
			Insulin	−0.260	0.010	−0.072	0.013
			HOMA	−0.248	0.017	−0.165	0.006
rs874627	Intron 2	0.40	BMI	0.002	0.966	0.009	0.808
G > T			Glucose	0.009	0.904	−0.001	0.795
			Insulin	−0.165	0.024	0.009	0.648
			HOMA	−0.158	0.035	0.018	0.664
rs2071081	Intron 4	0.25	BMI	0.052	0.379	−0.016	0.720
A > C			Glucose	−0.021	0.822	−0.002	0.662
			Insulin	−0.287	0.001*	−0.050	0.030
			HOMA	−0.261	0.004*	−0.100	0.040
rs11612427	Exon 5	0.29	BMI	0.039	0.474	0.026	0.526
G > A			Glucose	0.029	0.740	−0.002	0.680
			Insulin	0.002	0.980	0.036	0.103
			HOMA	0.001	0.999	0.066	0.152

Beta indicates transformed beta-values. P indicates p-values adjusted for significant covariates.
* p-value ≤0.004 was considered statistically significant.

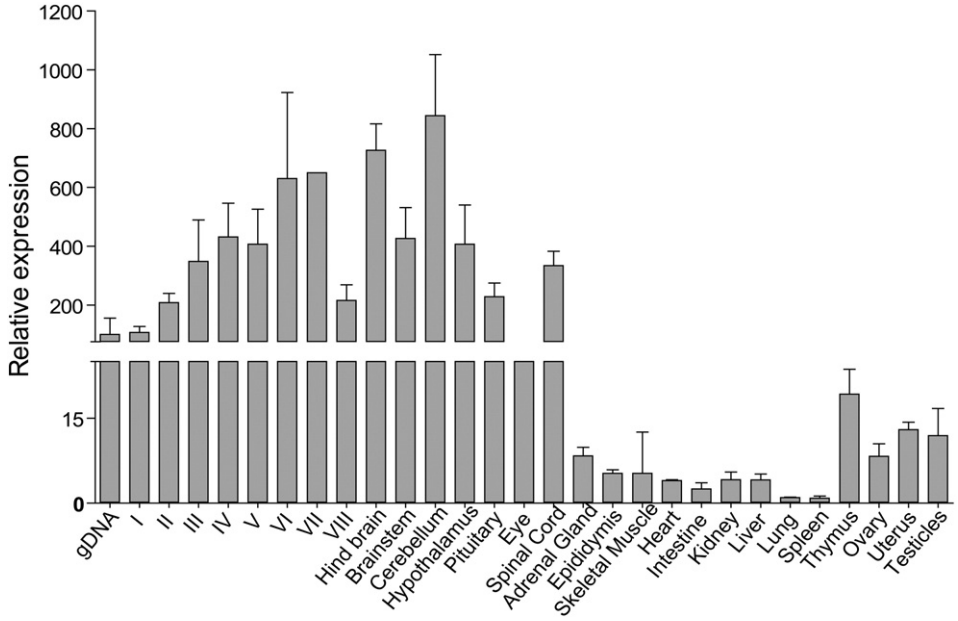

Fig. 1. GPR162 mRNA expression in rat. Results are shown as relative expression to rat genomic DNA. In rat panel, abbreviations I–VIII indicate brain cross-sections adapted from Lagerström et al. (2007).

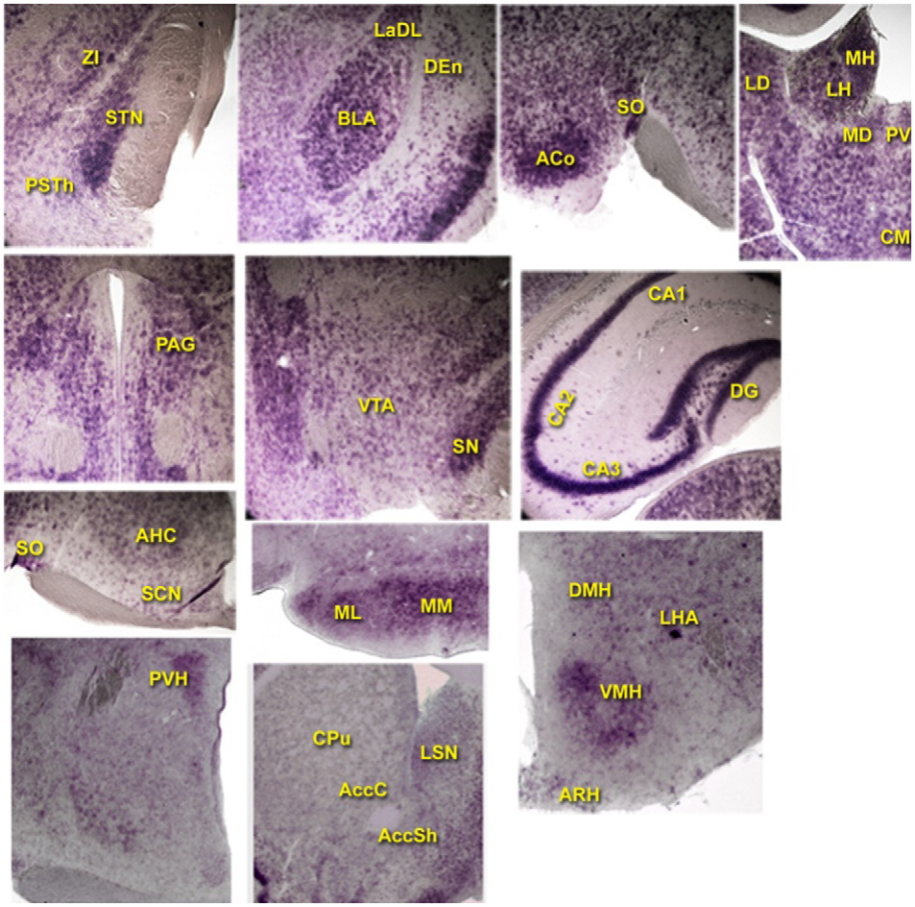

Fig. 2. Floating *in situ* hybridization experiment in coronal sections of the mouse brain. Floating *in situ* hybridization using 300 ng of digoxigenin labeled mouse GPR162 antisense probe. Abbreviations: ZI, zona incerta; STN, subthalamic nucleus; PSth, parasubthalamic nucleus; BLA, basolateral nucleus of the amygdala; LaDL, lateral amygdaloid nucleus dorsolateral part; Den, dorsal endopiriform claustrum; ACo, anterior cortical amygdaloid area; SO, supraoptic nucleus; LD, laterodorsal thalamic nuclei; LH, lateral hypothalamus; MH, medial hebanula; MD, mediodorsal thalamic nuclei; PV, paraventricular thalamic nucleus; CM, central medial thalamic nucleui; PAG, periaqueductal gray; VTA, ventral tegmental area; SN, substantia nigra, SCN, suprachaismatic nucleus; AHC, anterior hypothalamic area central; ML, medial mammilary nucleus lateral part; MM, medial mammilary nucleus medial part; DG, dendate gyrus; CA1, field CA1 hippocampus; CA2, field CA2 hippocampus; and CA3, field CA3 hippocampus; PVH, paraventricular hypothalamus; CPu, caudate putamen; DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; LHA, lateral hypothalamic area; and ARH, arcuate hypothalamic nucleus.

was detected in various nuclei of the thalamus and substantia nigra as well as periaqueductal gray (PAG) and supraoptic nucleus. Abundant expression was seen in the hypothalamus, in particular, the ventromedial (VMH), paraventricular (PVH), and lateral hypothalamus (LH). Moderate to high expression was found in the ventral tegmental area (VTA) and caudate putamen and suprachiasmatic nucleus (SCN) (Fig. 2).

3.3. GPR162 knockdown is associated with reduced food intake

GPR162 knockdown rats were generated by ICV infusion (7 days) of GPR162 AS ODN. During this period, rats received food *ad libitum*. The baseline food intake for both group remained similar (7.56 ± 0.52 mg) whereas baseline body weight varied slightly between the two groups (control: 282 ± 11.6 g, knockdown: 300 ± 9 g). Food consumption and body weights were monitored every day during the treatment period. Data for food intake and body weight collected during the first 2 days of experiment were not included in the statistical analysis to avoid initial aberrant behavior after the surgery. The persistent effect of GPR162 AS ODN to knockdown the expression of GPR162 was associated with the significant reduction in food intake ($p = 0.003$) compared to the control group. The reduction in food intake was consistent during the last 3 days of the treatment period ($p \leq 0.01$; Fig. 3A). A strong trend toward a reduction in body weight was observed after GPR162 knockdown (Fig. 3B).

3.4. Analysis of genetic association to obesity and related traits

Four SNPs (rs1045261, rs874627, rs2071081, and rs11612427), one located downstream and three located in GPR162, were genotyped in two Swedish cohorts. Descriptive characteristics of the two cohorts are presented in Table 1. Pairwise linkage disequilibrium (LD) tests calculated based on the HapMap data between the SNPs genotyped show that, according to the LD structure, these are not located in the same haplotype blocks (Fig. 4). For these SNPs, we studied the effect on BMI, serum insulin levels, glucose levels, and insulin resistance in both cohorts (Table 2). Among obese children and adolescents, we observed the effect on serum insulin levels and insulin resistance, measured as HOMA, for one SNP located in intron 4 (rs2071081). Carriers of the minor allele of this variant had decreased serum insulin levels and insulin resistance compared to non-carriers. Trends for decreased serum insulin levels and decreased insulin resistance among carriers of the minor allele were also observed for the variant located downstream (rs1045261) and for the one located in intron 2 (rs874627). The effects on insulin levels and insulin resistance were also observed among normal-weight men for rs1045261 and rs2071081, although no statistical significance was reached. No effect on glucose levels or BMI was observed for any of the variants.

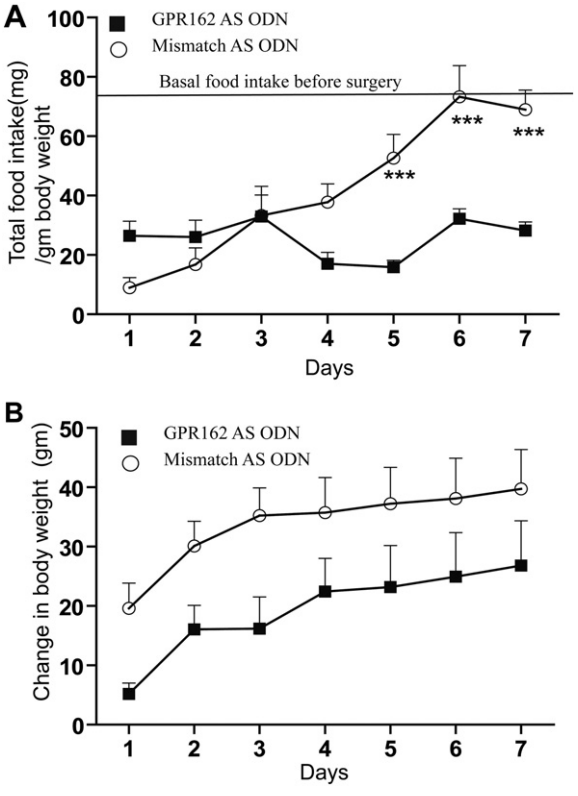

Fig. 3. Effects of GPR162 AS ODN on food intake and body weight. Percentage of food intake/gram of body weight (A) and change in body weight compared to baseline (B) was measured for 7 consecutive days after the surgical implantation of the osmotic pump in rats receiving GPR162 AS ODN ($n = 8$) versus the rats receiving mismatch AS ODN ($n = 8$). Values are means ± SEM. Data were analyzed using two-way ANOVA followed by the Bonferroni's post-hoc test. Significant differences between the groups: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

4. Discussion

Previous work from our laboratory has provided an anatomical characterization of GPR162 protein in mouse brain demonstrating that GPR162 is widely expressed in GABAergic as well as other neurons within the mouse hippocampus, whereas extensive expression is observed in areas related to energy homeostasis and hedonic feeding such as hypothalamus, amygdala, and ventral tegmental area, regions known to be involved in the regulation of palatable food consumption (Caruso et al., 2014a). In this study, we provide further anatomical characterization of GPR162 mRNA expression in mouse brain via *in situ* hybridization as well as detailed mRNA expression in a panel of rat tissues complementing a species-specific mapping of the receptor. Furthermore, we performed human genetic studies in which, for the first time, variants of the GPR162 gene were associated with impairments in insulin levels and resistance.

In rat, qRT-PCR results show that GPR162 expression is abundant within the CNS and negligible in peripheral tissues, with some exceptions including reproductive organs, such as ovaries, uterus, and testicles, in agreement with previous findings (Fig. 1) (Gloriam et al., 2005). Furthermore, we investigated mRNA GPR162 expression via *in situ* hybridization providing a detailed distribution of the receptor in the brain of mouse (Fig. 2). Our results demonstrated that mRNA GPR162 expression levels are in agreement with results obtained in the rat panel revealing a similar mRNA expression pattern between species. Likewise, *in situ* hybridization findings of the present study are in agreement with GPR162 protein expression levels of our previous investigations (Sreedharan et al., 2011; Caruso et al., 2014a).

The hypothalamus integrates neural and peripheral hormonal signals generated by the pancreas, liver, intestinal tract, and adipose tissue for the regulation of food intake (Klok et al., 2007). Hypothalamic lesions studies have revealed alterations in feeding circuitry (Brobeck et al., 1943), and in our study, extensive GPR162 mRNA transcription levels were found in critical hypothalamic nuclei integrating central and peripheral information involved in appetite regulation such as ARC, LH, DMH, and VMH nucleus (Yeo and Heisler, 2012; Schwartz et al., 2000). Similarly, high expression was observed in physiological relevant areas implicated in motivation for food and reward (Pecina et al., 2003; Mietlicki-Baase et al., 2013) such as nucleus accumbens and amygdala. Taken together, our results suggest that GPR162 gene could possibly play a role in the complex mechanisms of feeding circuitry (Ahn and Phillips, 2002; Carlini et al., 2004), and to further support this hypothesis, we examined the relationship between alteration in hypothalamic GPR162 expression and food intake (Fig. 3). In agreement, ICV infusion of GPR162 antisense oligodeoxynucleotide resulted in a significant decrease in food intake compared to control group, suggesting a plausible implication of the gene in the "hunger center"

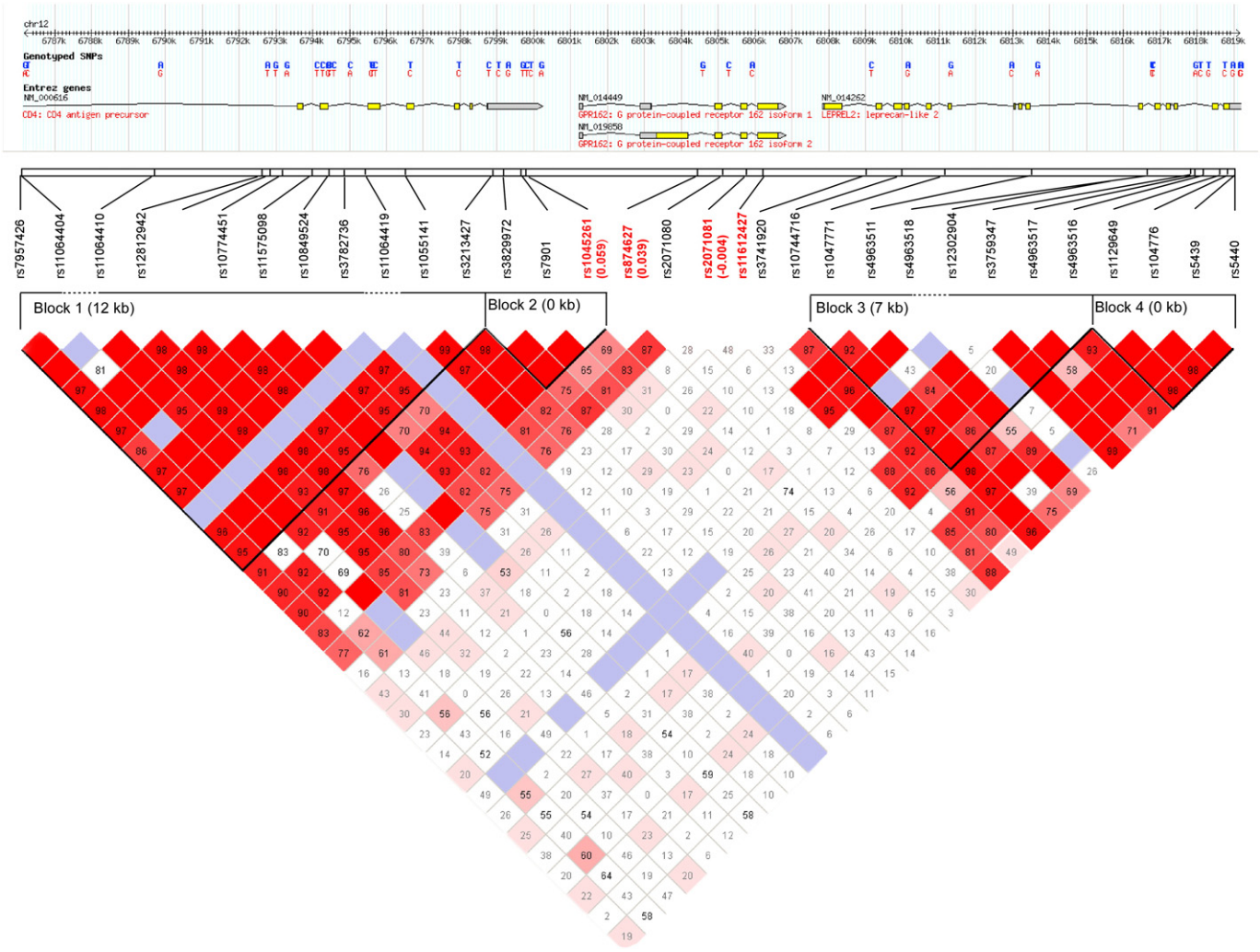

Fig. 4. Schematic structure of *GPR162* and the LD pattern based on data from HapMap Phase 3. The diamonds represent the pairwise LD expressed as r^2 defined by confidence interval according to Gabriel et al. (2002) (black $r^2 > 0.8$, dark gray 0.8–0.5, moderate gray 0.6–0.4, light gray 0.4–0.2, and white $r^2 < 0.2$). The SNPs in this paper are marked in red, with FST values in parenthesis. For rs11612427, no FST value could be obtained due to large amount of missing data. Fst values were calculated for the CEU population using PLINK 1.09b3 (Purcell et al., 2007). The haplotype analysis was performed using Haploview 4.2 (Barrett et al., 2005).

(Stellar, 1994; Zhang et al., 2011). Central control of feeding and energy expenditure is highly complex and it involves a plethora of regulatory pathways; however, results from the present study strengthen our previous findings highlighting the impact of GPR162 in food intake (Caruso et al., 2014a). Our findings demonstrated that high GPR162 expression is found in hypothalamic nuclei such as the ARC containing two opposing neuronal population, the appetite-suppressing proopiomelanocortin (POMC), the orexigenic neuropeptide Y (NPY), and agouti-related peptide (AgRP)-neuropeptide-expressing neurons (Cowley et al., 2001). Leptin and insulin act on POMC and AgRP-NPY neurons promoting respectively energy expenditure (van den Top et al., 2004) and regulating whole body glucose metabolism (Bruning et al., 2000). Mechanisms by which POMC neurons coordinate and integrate circulating leptin and insulin signaling are of high interest in current research (Dodd et al., 2015; Billes et al., 2012; Loh et al., 2011), and further studies investigating the association of GPR162 expression and the melanocortin system, together with a more extended repertoire of animal behavioral models, should shed further light on the complex mechanisms regulating energy homeostasis. Interestingly, a recent study reported that the expression of GPR162 was altered in several organs of diabetic rats including heart, kidney, and brain, compared to control group (Ruiz-Hernandez et al., 2015). Authors of this study concluded that GPR162 could be involved in the development of diabetes complications highlighting a possible link between alterations of GPR162 gene expression and the occurrence of impairments in glucose metabolism. In line with these recent findings, when we genotyped four SNPs of GPR162 gene among obese as well as normal-weight subjects of different age, our human genetic results revealed that variant rs2071081 in intron 4 is associated with impairments in insulin levels as well as insulin resistance measured as HOMA in children (Table 2). Although not significant, a similar trend was observed for the variant rs1045261 in adult men (insulin, $p = 0.013$; HOMA, $p = 0.006$). The association between SNPs of GPR162 gene and impairments in glucose metabolism requires further clinical investigations as the functional metabolic implication of the GPR162 gene in the complex mechanisms of energy homeostasis might be contingent on the cellular context (Bermak et al., 2001) and in response to environmental factors such as hormones and sensorial stimuli (Ritter and Hall, 2009). Taken together, in light of our previous (Sreedharan et al., 2011; Caruso et al., 2014a) and current findings in rodents as well as human genetic association studies, our results provide a significant anatomical characterization of GPR162 gene and a plausible functional implication in the mechanisms of feeding circuitry. Furthermore, neuroanatomical interactions between GPR162 and melanocortin system within hypothalamic circuits and mesocorticolimbic centers

could promote the design of multifunctional compounds for therapeutic purposes (Caruso et al., 2014b). Novel drugs containing hybridized structures able to independently interact with their respective receptors may potentially result in a greater therapeutic effect for the control of food intake in human.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2016.01.044.

Disclosure statement

The authors have nothing to disclose.

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