HAL
open science

Association of TMEM18-variants with BMI and waist circumference in children and correlation of mRNA expression in the PFC with body weight in rats

Helgi B Schiöth, Mathias Helge Rask-Andersen, Josefin A. Jacobsson, George Moschonis, Rohit A Chavan, Md Noman Abu Sikder, Elin Allzén, Johan Alsiö, George P Chrousos, Yannis Manios, et al.

To cite this version:

Helgi B Schiöth, Mathias Helge Rask-Andersen, Josefin A. Jacobsson, George Moschonis, Rohit A Chavan, et al.. Association of TMEM18-variants with BMI and waist circumference in children and correlation of mRNA expression in the PFC with body weight in rats. European Journal of Human Genetics, 2011, 10.1038/ejhg.2011.176 . hal-00683156

HAL Id: hal-00683156

https://hal.science/hal-00683156v1

Submitted on 28 Mar 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Association of TMEM18-variants with BMI and waist circumference in children and correlation of mRNA expression in the PFC with body weight in rats

Mathias H Rask-Andersen[1], Josefin A. Jacobsson[1], George Moschonis[2], Rohit A Chavan[1], Md Abu Noman Sikder[1], Elin Allzén[1], Johan Alsiö[1], George P. Chrousos[3], Yannis Manios[2], Robert Fredriksson[1], and Helgi B. Schiöth[1§]

[1]Department of Neuroscience, Functional Pharmacology, Uppsala University, BMC, Uppsala SE 75124, Sweden.

[2]Department of Nutrition & Dietetics, Harokopio University of Athens, Athens, Greece

[3]First Department of Pediatrics, Athens University Medical School, Aghia Sophia Children's Hospital, Thivon and Levadias, 115 27, Goudi, Athens, Greece

[§] Corresponding Author: Helgi.Schioth@neuro.uu.se

Running title: TMEM18-variants and BMI in children

Keywords: Obesity, Tmem18, FTO

Abstract

Genome Wide Association (GWA) studies have shown a strong association of single nucleotide polymorphisms in the near vicinity of the TMEM18 gene. The effects of the TMEM18 associated variants are more readily observed in children. TMEM18 encodes a 3TM protein which locates to the nuclear membrane. The functional context of TMEM18 and the effects of its associated variants are as of yet undetermined. To further explore the effects of near TMEM18 variants, we have genotyped two TMEM18 associated SNPs, rs6548238 and rs4854344, in a cohort of 2352 Greek children (Healthy Growth Study). Included in this study is data on anthropomorphic traits body weight, BMI z-score and waist circumference. Also included is dietary energy and macronutrient intake as measured via 24-h recall interviews. Major alleles of rs6548238 and rs4854344 were significantly associated with an increased risk of obesity (OR=1.489 (1.161 – 1.910) and 1.494 (1.165 – 1.917) respectively) and positively correlated to body weight (p=0.017, p=0.010) and waist circumference (p=0.003, p=0.003). An association to energy and macronutrient intake was not observed in this cohort. We also correlated food intake and body weight in a food choice model in rats, to Tmem18 expression in central regions involved in feeding behavior. We observed a strong positive correlation between TMEM18 expression and body weight in the prefrontal cortex (PFC) (r= 0.5694, p=0.0003) indicating a potential role for TMEM18 in higher functions related to feeding involving the PFC.

41 **Introduction**

42 Genome wide association (GWA) studies have led to the identification of several loci in the
43 human genome containing genetic variants conferring an increased risk of developing
44 overweight and obesity[1-2]. One such gene to be associated with a higher BMI, the fat mass
45 and obesity associated gene (FTO), was identified by Frayling et al. in 2007[3]. FTO has
46 since been shown to be involved in regulation of feeding behavior via homeostatic
47 hypothalamic pathways[4-6], and its obesity associated variants to confer gain-of-function by
48 increasing gene transcription[7]. The most recent meta analyses of GWA-studies on obesity,
49 which included genetic information from 249,796 individuals, identified variants at a total
50 of 32 genetic loci affecting the development of BMI[2]. Single nucleotide polymorphisms
51 (SNPs) in the FTO gene, followed by SNPs in the proximity of TMEM18 and MC4R, have
52 consistently given the strongest associations to obesity in large scale GWA-studies.
53 TMEM18 associated SNPs were first found to be associated with a higher BMI in 2009 by
54 the GIANT consortium[1], and confirmed the same year by an independent group[8].

55

56 Results from child cohorts have shown stronger effects of near TMEM18 SNPs to obesity,
57 compared to adults. Zhao et al found near-TMEM18 SNPs to confer the strongest effect on
58 pediatric BMI out of 25 studied obesity associated variants in a cohort of 6078 children of
59 European descent[9]. A similar effect was observed in a cohort of 2042 children and
60 adolescents from four European countries when the effects of 17 variants were studied. The
61 strongest effect-size for BMI and sum of skin folds observed in this study was for the near-
62 TMEM18 SNP rs6548238[10]. The rs6548238 variant also had a moderate effect on waist
63 circumference and height. However, no correction was made for multiple testing in this

64 study. Results from this group also showed an association of rs6548238 and rs7561317
65 with obesity in a cohort of 1027 children from the Stockholm area but no association to
66 anthropomorphic traits was detected in this study[11]. Results from adult cohorts have been
67 more inconsistent. A study on 4923 adults from northern Sweden was unable to detect an
68 association of rs6548238 to obesity, type 2 diabetes or measures of body composition and
69 adipose distribution[12]. Another study on a cohort of 6013 Chinese type 2 diabetes patients,
70 1087 healthy adolescents and 605 healthy adults, was also unable to detect an association of
71 the rs7561317 genotype to type 2 diabetes, waist circumference and waist-to-hip ratio.
72 There was a trend towards an association to BMI but this did not reach statistical
73 significance [13]. In a Japanese adult cohort of 1129 obese and 1736 normal weight controls,
74 the TMEM18 associated SNPs: rs2867125, rs6548238, rs4854344 and rs7561317 were all
75 observed to be associated to obesity [14]. The near-TMEM18 SNP rs7561317 was also tested
76 for association to phenotypes of metabolic disorder in this study, due to its previous
77 association to type II diabetes. BMI, blood pressure, fasting plasma glucose, triglycerides,
78 total cholesterol and HDL cholesterol were compared in obese and controls but no
79 association to rs7561317 genotype was observed.
80
81 TMEM18 is a highly conserved transmembrane protein containing three membrane-
82 spanning regions containing a nuclear localization signal, as well as a coiled coil domain, at
83 the C-terminal [11]. It most likely localizes to the nuclear membrane after translation and was
84 discovered to be involved in the migration of neural stem cells and neural progenitor cells
85 towards glioma cells as part of the downstream signaling pathway of chemokine receptors
86 [15]. Expression profiling in rat and mouse showed TMEM18 to be expressed in all tissues

87 tested. It is also abundantly expressed in the brain and in central regions including those
88 involved in food intake. However, in contrast to, for example: FTO, NPY, POMC, AgRP or
89 MC4R; TMEM18 has not been found to be regulated in brain regions regulating food
90 intake. Our earlier studies found no regulation of TMEM18 to take place in the
91 hypothalamus or brain stem during food deprivation or consumption of a palatable high fat
92 diet [11], this was in contrast to canonical feeding regulatory peptides such as POMC, NPY,
93 MC4R and AgRP[16], as well as FTO [4-5]. The anatomical, molecular and functional basis of
94 how TMEM18 may be involved in regulating BMI is thus obscure.
95
96 In the present study, we genotyped two TMEM18 SNPs previously associated with BMI:
97 rs6548238 and rs4854344, in a cohort of 2352 Greek schoolchildren containing
98 anthropomorphic data on body weight, BMI and waist circumferences as well as dietary
99 intake data, such as energy and macronutrient intake as determined by 24-h recall
100 interviews. Food preference and body weight in a rodent food choice model [17] were also
101 correlated to expression levels of Tmem18 in several tissues involved in different aspects of
102 feeding, such as components of the mesolimbic and mesocortical pathways: the amygdala,
103 nucleus accumbens and hippocampus, as well as the prefrontal cortex, which is involved in
104 behavioral inhibition and executive function.

105 **Materials and methods**

106

107 *Subjects and phenotype characterization*

108 The cohort of Greek children comprised 2658 schoolchildren, attending the 5th and 6th
109 grades of primary schools (table 1). This cohort was part of the "Healthy Growth Study", a

110 large scale cross-sectional epidemiological study initiated in May 2007 as described
111 previously [17-18]. An extended letter explaining the aims of the current study and a consent
112 form were provided to each parent who had a child in one of the primary schools
113 participating in the study. Those parents who agreed to participate in the study gave their
114 informed consent by signing the consent form, and provided their contact details. Body
115 weight and height were measured in all study participants using standard procedures and
116 equipment. Body weight was measured to the nearest 10 g and height was measured to the
117 nearest 0.1cm in standing position. BMI z-score was calculated relative to the International
118 Obesity Task Force (IOTF) definitions [19]. Subjects were categorized as normal-weight and
119 obese using IOTF BMI cut-off values equivalent to adult BMI of ≤24 and ≥30,
120 respectively. Waist circumference was measured to the nearest 0.1 cm with the use of a
121 non-elastic tape (Hoechstmass, Germany) around the trunk, at the level of umbilicus
122 midway between the lower rib margin and the iliac crest, and with the subject at a standing
123 position. Dietary intake data were obtained by trained dieticians and nutritionists via
124 morning interviews with the children at school-site for two consecutive weekdays and one
125 weekend day, using the 24-hour recall technique. All study participants were asked to
126 describe the type and amount of foods, as well as all beverages consumed during the
127 previous day, provided that it was a usual day according to the participant's perception. To
128 improve the accuracy of food descriptions, standard household measures (cups,
129 tablespoons, etc.) and food models were used to define amounts when appropriate. At the
130 end of each interview the interviewers, who were dieticians rigorously trained to minimize
131 interviewer effect, reviewed the collected food intake data with the respondent in order to
132 clarify entries, servings and possible forgotten foods. The ratio of reported energy intake

133 and predicted basal metabolic rate was used to asses underreporting of calorie intake. Basal
134 metabolic rate was estimated according to Schofield equations [20], taking into account age,
135 sex, and body weight and with cut-off limits developed by Goldberg et al. [21].
136
137 Food intake data were analyzed using the Nutritionist V diet analysis software (version 2.1,
138 1999, First Databank, San Bruno, CA), which was extensively amended to include
139 traditional Greek recipes, as described in Food Composition Tables of Greek Cooked Foods
140 and Dishes [22]. Furthermore, the database was updated with nutritional information of
141 processed foods provided by independent research institutes, food companies and fast-food
142 chains. DNA for genotyping was available for 2 352 subjects (1 311 girls and 1 064 boys).
143 Approval of the consent procedure, and to conduct the study, was granted by the Greek
144 Ministry of National Education and the Ethical Committee of Harokopio, University of
145 Athens.
146
147 *Genotyping and linkage disequilibrium analysis*
148 The genotyping of *TMEM18* SNPs was carried out with pre-designed Taqman single-
149 nucleotide polymorphism genotyping assays (Applied Biosystems, Foster City, USA) and
150 an ABI7900 genetic analyzer with SDS 2.2 software at the Uppsala Genome Center
151 (http://www.genpat.uu.se/node462). The genotype call rate was 99.6%. Test for deviation
152 from Hardy-Weinberg equilibrium was performed using the Pearson´s χ^2-test (1 d.f) and
153 none of the SNPs did deviate from Hardy Weinberg equilibrium ($p>0.4$). Haploview [23] was
154 used for linkage disequilibrium (LD) measurements according to confidence intervals by
155 Gabriel et al [24] as well as graphical representation of the LD structure indicated as r^2. The

156 LD pattern was generated using HapMap data version 2, release 21 and CEU as analysis
157 panel.
158
159 *Statistical analyses*
160 Association with obesity was analyzed with logistic regression and odds ratio (OR) with a
161 95% confidence interval (CI). Associations between genotypes and phenotypes were
162 analyzed with linear regression, assuming an additive model. Quantitative skewed variables
163 were normalized by transformation before analysis. The models were adjusted for age,
164 gender, BMI and length when needed. Statistical analyses were performed with PLINK
165 (http://pngu.mgh.harvard.edu/purcell/plink/) [25]. Multiple testing was adjusted with the False
166 Discovery Rate (FDR) according to the procedure of Benjamini and Hochberg [26] and a p-
167 value < 0.005 was considered statistically significant.
168
169 *Power calculations*
170 For the case/control analyses, power calculations were carried out with the CaTS power
171 calculator (http://www.sph.umich.edu/csg/abecasis/CaTS/index.html) [27]. We had 80 %
172 power to detect association with obesity with a relative risk of 1.3.
173
174 *Food choice model*
175 Thirty-six outbred male Wistar rats (Scanbur BK AB, Sollentuna, Sweden) were housed in
176 standard macrolon cages at constant temperature (22±1ºC) and humidity (50±5%). Rats
177 were 10 weeks old at the start of the study and housed individually for 8 days during the
178 food preference paradigm. Water and standard food chow (R36, Lactamin, Lidköping,

179 Sweden) was supplied *ad libitum* during the entire experiment except for the 5-day food
180 preference test, during which the animals had free access to three diets: a palatable high-fat
181 diet and two reference diets with high amounts of either casein (high-protein diet) or maize
182 starch (high-carbohydrate diet). Compositions of the different diets are described in Alsiö et
183 al 2009. The food was provided in bowls and weighed each day to measure the ingested
184 amount. This model allowed the determination of the following outcomes: body weight at
185 endpoint, total food intake during palatable diet presentation, high-fat diet preference (high-
186 fat diet consumption divided by total intake), consumption of standard chow. Subsequent to
187 the 5-day food choice, the animals were kept on standard chow for 12 days; this 'washout'
188 period was inserted to allow effects of the food-choice diets on gene expression to subside.
189 Animals were then killed by decapitation. All samples were collected immediately
190 following decapitation. Dissection of the brain is described by Alsiö et al 2009 [28]. Samples
191 then immersed in RNAlater (applied biosystems/Ambion, Austin, TX, USA) and stored at
192 room temperature for 2h before being stored at -20°C until preparation. All animal
193 procedures were approved by the Uppsala Animal Ethical Committee (ID: C 285/5) and
194 followed the guidelines of Swedish legislation on animal experimentation (Animal Welfare
195 Act SFS1998:56) and European Union legislation (Convention ETS123 and Directive
196 86/609/EEC).
197
198 *Generation of cDNA*
199 Tissues were homogenized by sonification (Branson sonifier B 15) and RNA was purified
200 from the samples using the TRIzol method (Sigma-Aldrich, Sweden) [29]. Samples were then
201 treated with Dnase I (Roche Diagnostics, Scandinavia) to remove residual DNA

202 contamination. Presence of residual DNA contamination after Dnase I treatment was
203 checked for using PCR and products were run on agarose gel. Complementary DNA
204 (cDNA) was then generated using reverse MLV reverse transcriptase (Invitrogen, Sweden)
205 according to the manufacturer's specifications.
206
207 *qRT-PCR*
208 PCR reactions were run in a total volume of 20ul using Taq polymerase kits (Biotools,
209 Sweden). Each reaction was performed in duplicate according to the manufacturers
210 specifications and contained 75mM Tris/HCL, 50mM KCl and 20mM $(NH_{4)2}SO_{4,}$ 4mM
211 $MgCl_2$, 0.25mM dNTPs, 1:20 DMSO, 20mU/ul Taq polymerase, 50mM forward and
212 reverse primer and 1:4 SYBR-green (Invitrogen, Sweden). Reactions were run on iCycler
213 temperature cyclers and fluorescence was measured using MyiQ single color real time PCR
214 detection system. Data were analyzed using iQ5 software (BioRad, Sweden). Primer
215 temperatures were optimized for specificity using temperature gradients and analyzing
216 melting curves of the PCR-products.
217
218 *Statistical analysis:*
219 Gene expression was normalized using the geometric mean of the most stable housekeeping
220 genes (M<1.5), in accordance with the geNorm-method as previously published by
221 Vandesompele et al. [30]. Statistical analysis was performed in Prism v 5.02 for Windows
222 (GraphPad Software, San Diego California USA, www.graphpad.com). The non-parametric
223 test for correlation (Spearman's test, Prism v. 5.02) was used to test for correlation between
224 mRNA expression and model parameters: body weight at the end of the food preference

225 paradigm, total amount of food consumed, preference for the high fat diet and the amount
226 of chow consumed during the food preference paradigm.
227
228 **Results**
229
230 *TMEM18 associated SNPs rs4854344 and rs6548238 are associated to obesity, BMI and*
231 *waist circumference but not dietary intake*
232 Cohort characteristics are displayed in table 1. Rs6548238 and rs4854344 are located about
233 30kb downstream of TMEM18 and in almost absolute linkage disequilibrium with each
234 other, r^2=0.98 (supplementary Figure 1). Association to obesity was determined by
235 comparing normal weight subjects (BMI<25) with obese (BMI>30). Analysis showed
236 major alleles of rs6548238 and rs4854344 to be significantly associated with an increased
237 risk of obesity (rs6548238: 1.489 (1.161-1.910), p=0.002; rs4854344: 1.494 (1.165-1.917),
238 p=0.001) (table 3) which is in the line with previous results on a cohort of 1027 children
239 and adolescents from the Stockholm area [11]. By linear regression analysis we also observed
240 higher BMI (rs6548238: p=0.006, rs4854344: p=0.005) and waist circumference
241 (rs6548238: p=0.003, rs4854344: p=0.003) for carriers of the major allele of rs6548238 and
242 rs4854344 and trends for higher body weight (rs6548238: p=0.017, rs4854344: p=0.010)
243 (table 3). The two SNPs were not associated with the average dietary energy intake
244 (Kcal/day). Similarly, daily intake of fiber and cholesterol, as well as percentages of energy
245 intake derived from total fat and protein, were independent on TMEM18 genotypes in this
246 cohort (table 3). Underreporting of calorie intake was assessed for 22.6% of the entire
247 cohort and 12.1 % of the cohort for which genotype data was available. However, no effect

248 on the outcome was observed when we removed subjects assessed to be underreporting
249 from the analysis. This indicates that the lack of an association between food intake and
250 TMEM18-related SNPs observed in our cohort was likely not caused by the underreporting
251 of calorie intake by the participants.
252
253 *Expression of prefrontal cortex Tmem18 correlates to body weight in rats*
254 The rodent food choice paradigm was previously employed to identify associations
255 between hypothalamic gene expression levels and food intake as well as body weight of
256 rats [28]. We did observe expression of Tmem18 in all the tissues studied. However, we
257 observed no correlation for Tmem18 expression with high fat diet preference, amount of
258 chow, amount of total food ingested or body weight in the hypothalamus, nucleus
259 accumbens, amygdala or hippocampus. We did, however, observe a strong correlation
260 between Tmem18 expression and body weight in the rat prefrontal cortex (r = 0.5694,
261 p=0.0003) (figure 1). We also investigated the expression of several proteins known to be
262 involved in cortical signaling (supplementary Table 1). Body weight was significantly
263 correlated to expression of GABA receptor subunit α-3 (Gabra3) (p=0.0052), as well as 5-
264 HT_{2A} receptor (p=0.049), alpha-1B adrenergic receptor (0.0168) and GABA B receptor 2
265 (p=0.0426) (supplementary Figure 1).
266
267 **Discussion**
268
269 Here we show that the major alleles of the near-TMEM18 SNPs: rs6548238 and
270 rs4854344, were significantly associated with an increased risk to develop obesity in the

271 studied cohort of 2352 Greek children. These results are in line with previous results on a
272 cohort of children from the Stockholm area [11] as well as with the results from Zhao et al.
273 who observed strong associations of three TMEM18 associated SNPs to obesity in a cohort
274 of 6078 children of European descent [9]. We also observed associations of rs6548238 and
275 rs4854344 to anthropomorphic traits: waist circumference, body weight and BMI z-score
276 which were not detected in the Stockholm cohort. We did however not observe any
277 association to dietary energy and macronutrient intake (Table 3).
278
279 Studies on adult cohorts have yielded more inconsistent results indicating that the effects of
280 TMEM18-associated variants may be more pronounced in early childhood and
281 adolescence. This is supported a study by Elks et al. who observed obesity risk-alleles
282 identified through GWA studies, including TMEM18 associated SNPs, to confer an effect
283 on weight gain also in early childhood and adolescence. This effect was observed as early
284 as in the first weeks after birth [31]. An association study for BMI-increasing alleles and birth
285 weight reported a slight effect of rs6548238 on birth weight, but not when multiple testing
286 was accounted for [32].
287
288 Rs6548238 and rs4854344 are located about 30kb downstream of TMEM18. Haplotype
289 analysis showed rs6548238 and rs4854344 to be in almost complete linkage disequilibrium.
290 Both SNPs are located in a haplotype block which does not encompass the TMEM18 gene.
291 It is thus unlikely that rs6548238 and rs4854344 are linked to causative SNPs inside the
292 TMEM18 gene. They may however have an effect on the transcription of TMEM18 by

293 affecting the binding of transcription factors or co-regulators of transcription. At this stage,
294 the mechanisms behind the effect of these variants are, as of yet, undetermined.
295
296 We present for the first time an association of body weight in rats with gene expression of
297 Tmem18. This association was neuroanatomically localized to the PFC. The PFC has been
298 proposed to play an important role in integrating behavioral cues from multiple sources in
299 the brain (reviewed by Miller and Cohen in 2001 [33]). It receives information from various
300 sources, among these the hippocampus, hypothalamus, mesolimbic circuit, motor cortex
301 and sensory cortices. In the context of feeding behavior, a model for the prefrontal cortex in
302 prioritization of long term goals by inhibiting behavioral cues from the hypothalamus,
303 brainstem and mesolimbic pathway, i.e. hunger and craving, has been suggested. This has
304 been termed "top-down" control of-, or inhibition of-, behavior which denotes higher
305 cognitive function inhibiting behavioral cues from the basal ganglia, brain stem and
306 hypothalamus. Other aspects of prefrontal cortex function includes what has been described
307 as impulse control and working memory. A functional role for TMEM18 in the prefrontal
308 cortex is, as of yet, unclear. Earlier reports of involvement of TMEM18s in neural
309 migration may be a valuable lead and point to a role in the development of the prefrontal
310 cortex, or in dynamic plasticity related to memory formation and learning. Learning
311 dependant cortical remodeling has been reported in the primate PFC in response to afferent
312 dopamine signaling [34] and synaptic plasticity in the PFC has been shown to be modulated
313 by monoaminergic signaling from PFC afferents [35].
314

315 In this context, it is of interest that we observe concomitant correlation in the rat prefrontal
316 cortex of GABA receptor transcripts Gabra3, Gabbr2, and monoamine receptor transcripts
317 5Ht2a and Adrb1, with body weight at the end of the feeding paradigm. The α_{1A} adrenergic
318 receptor and the 5-HT_{2A}-receptor both act as excitatory G protein-coupled receptors
319 coupling to the $G\alpha_q$ signaling cascade and elicits phospholipase C-mediated Ca^+ release [36].
320 Postsynaptically, they act to stimulate GABA-release from cortical interneurons and also
321 glutamate release from pyramidal neurons [37]. Stimulation of α_1-adrenergic receptors in the
322 rat prefrontal cortex has also been shown to increase local release of 5-HT [36]. The GABA-
323 receptor is a ligand gated ion channel commonly composed of five subunits: two α-, two β-,
324 and one γ-subunit. Each subunit also has several subtypes which creates a large number of
325 combinations making up the GABA-receptor isoform diversity. The subunit composition of
326 the receptor in turn affects ligand affinity and can also affect its electrophysiological
327 properties [38]. The α3 GABA receptor-subunit is frequently a component of the $\alpha_3\beta_2\gamma_2$
328 GABA-receptor subtype, which is expressed in the cerebral cortex and in monoaminergic
329 neurons of the basal forebrain. Knock out studies have shown this subunit to be involved in
330 sensorimotor signaling and a hyperdopaminergic phenotype which has been suggested to be
331 related to schizophrenia [39]. The correlation of the expression of these genes in the prefrontal
332 cortex of rats with high body weight indicates prefrontal cortex serotonergic and adrenergic
333 signaling to be affected in these animals with concurrent effects on monoaminergic receptor
334 expression and subsequent effects on GABA receptor gene transcription.
335

336 In summary we show that SNPs in TMEM18 are associated with obesity and body weight,
337 as well as anthropomorphic measures of adiposity: waist circumference, in a cohort of 2352
338 Greek children. No association was found to behavioral phenotypes: dietary energy and
339 macronutrient intake as measured by 24-h recall interviews. It should be mentioned that
340 every evaluation of dietary intake is limited, and the limitations of 24-h recalls in capturing
341 habitual intake at the individual level are well known, because of the large day-to-day
342 variation in intake that exists and possiable difficulties in remembering what has actually
343 been ingested. However, for the purpose of this study, three 24-h recalls on a reasonably
344 large group, with no obvious bias, was deemed appropriate to study a plausible association
345 with energy intake and TMEM18. However, replication of this with other estimates of
346 energy intake would be valuable. Surprisingly, we found that expression of TMEM18 in the
347 rat prefrontal cortex is correlated to body weight in our animal model. This is the first
348 functional data showing a link between expression of TMEM18 and phenotype.
349 Concomitant regulation of monoaminergic- and GABA-ergic receptor transcripts could
350 prompt further studies for a role on TMEM18 in synaptic plasticity.
351
352
353 **Acknowledgements**
354 The study was supported by the Swedish Research Council, Brain Research Foundation,
355 Novo Nordisk, Tore Nilsons foundation and Åhlens foundation. R.F. was supported by the
356 Göran Gustafsson foundation. The SNP genotyping was performed by the SNP Technology
357 Platform, Uppsala, Sweden (http://www.genotyping.se) with support from Uppsala
358 University and the Knut and Alice Wallenberg foundation and at the Uppsala Genome

Centre. We thank the Healthy Growth Study Group for their contribution in this study. The Healthy Growth Study Group consists of (1) Harokopio University Research Team/Department of Nutrition and Dietetics: Yannis Manios (Coordinator), George Moschonis (Project manager), Katerina P Skenderi, Evangelia Grammatikaki, Odysseas Androutsos, Sofia Tanagra, Alexandra Koumpitski, Paraskevi-Eirini Siatitsa, Anastasia Vandorou, Aikaterini-Efstathia Kyriakou, Vasiliki Dede, Maria Kantilafti, Aliki-Eleni Farmaki, Aikaterini Siopi, Sofia Micheli, Louiza Damianidi, Panagiota Margiola, Despoina Gakni, Vasiliki Iatridi, Christina Mavrogianni, Kelaidi Michailidou, Aggeliki Giannopoulou, Efstathoula Argyri, Konstantina Maragkopoulou, Maria Spyridonos, Eirini Tsikalaki, Panagiotis Kliasios, Anthi Naoumi, Konstantinos Koutsikas, Katerina Kondaki, Epistimi Aggelou, Zoi Krommyda, Charitini Aga, Manolis Birbilis, Ioanna Kosteria, Amalia Zlatintsi, Elpida Voutsadaki, Eleni-Zouboulia Papadopoulou, Zoi Papazi, Maria Papadogiorgakaki, Fanouria Chlouveraki, Maria Lyberi, Nora Karatsikaki-Vlami, Eva Dionysopoulou and Efstratia Daskalou (2) Aristotle University of Thessaloniki/School of Physical Education and Sports Sciences: Vassilis Mougios, Anatoli Petridou, Konstantinos Papaioannou, Georgios Tsalis, Ananis Karagkiozidis, Konstantinos Bougioukas, Afroditi Sakellaropoulou and Georgia Skouli. (3) University of Athens/ Medical School: George P Chrousos, Maria Drakopoulou and Evangelia Charmandari.

Conflict of interest.

The authors declare no conflict of interest.

382 **References:**

383 1. Willer CJ, Speliotes EK, Loos RJ *et al*: Six new loci associated with body mass
384 index highlight a neuronal influence on body weight regulation. *Nat Genet* 2009;
385 **41:** 25-34.
386
387 2. Speliotes EK, Willer CJ, Berndt SI *et al*: Association analyses of 249,796
388 individuals reveal 18 new loci associated with body mass index. *Nat Genet* 2010;
389 **42:** 937-948.
390
391 3. Frayling TM, Timpson NJ, Weedon MN *et al*: A common variant in the FTO gene
392 is associated with body mass index and predisposes to childhood and adult obesity.
393 *Science* 2007; **316:** 889-894.
394
395 4. Fredriksson R, Hagglund M, Olszewski PK *et al*: The obesity gene, FTO, is of
396 ancient origin, up-regulated during food deprivation and expressed in neurons of
397 feeding-related nuclei of the brain. *Endocrinology* 2008; **149:** 2062-2071.
398
399 5. Olszewski PK, Fredriksson R, Olszewska AM *et al*: Hypothalamic FTO is
400 associated with the regulation of energy intake not feeding reward. *BMC Neurosci*
401 2009; **10:** 129.
402
403 6. Church C, Moir L, McMurray F *et al*: Overexpression of Fto leads to increased food
404 intake and results in obesity. *Nat Genet* 2010; **42:** 1086-1092.

405

406 7. Berulava T, Horsthemke B: The obesity-associated SNPs in intron 1 of the FTO
407 gene affect primary transcript levels. *Eur J Hum Genet* 2010; **18:** 1054-1056.
408

409 8. Thorleifsson G, Walters GB, Gudbjartsson DF *et al*: Genome-wide association
410 yields new sequence variants at seven loci that associate with measures of obesity.
411 *Nat Genet* 2009; **41:** 18-24.
412

413 9. Zhao J, Bradfield JP, Li M *et al*: The role of obesity-associated loci identified in
414 genome-wide association studies in the determination of pediatric BMI. *Obesity*
415 *(Silver Spring)* 2009; **17:** 2254-2257.
416

417 10. den Hoed M, Ekelund U, Brage S *et al*: Genetic susceptibility to obesity and related
418 traits in childhood and adolescence: influence of loci identified by genome-wide
419 association studies. *Diabetes* 2010; **59:** 2980-2988.
420

421 11. Almen MS, Jacobsson JA, Shaik JH *et al*: The obesity gene, TMEM18, is of ancient
422 origin, found in majority of neuronal cells in all major brain regions and associated
423 with obesity in severely obese children. *BMC Med Genet* 2010; **11:** 58.
424

425 12. Renstrom F, Payne F, Nordstrom A *et al*: Replication and extension of genome-
426 wide association study results for obesity in 4923 adults from northern Sweden.
427 *Hum Mol Genet* 2009; **18:** 1489-1496.

428

429 13. Ng MC, Tam CH, So WY *et al*: Implication of genetic variants near NEGR1,
430 SEC16B, TMEM18, ETV5/DGKG, GNPDA2, LIN7C/BDNF, MTCH2,
431 BCDIN3D/FAIM2, SH2B1, FTO, MC4R, and KCTD15 with obesity and type 2
432 diabetes in 7705 Chinese. *J Clin Endocrinol Metab* 2010; **95:** 2418-2425.
433

434 14. Hotta K, Nakamura M, Nakamura T *et al*: Association between obesity and
435 polymorphisms in SEC16B, TMEM18, GNPDA2, BDNF, FAIM2 and MC4R in a
436 Japanese population. *J Hum Genet* 2009; **54:** 727-731.
437

438 15. Jurvansuu J, Zhao Y, Leung DS *et al*: Transmembrane protein 18 enhances the
439 tropism of neural stem cells for glioma cells. *Cancer Res* 2008; **68:** 4614-4622.
440

441 16. Lindblom J, Haitina T, Fredriksson R, Schioth HB: Differential regulation of
442 nuclear receptors, neuropeptides and peptide hormones in the hypothalamus and
443 pituitary of food restricted rats. *Brain Res Mol Brain Res* 2005; **133:** 37-46.
444

445 17. Moschonis G, Tanagra S, Vandorou A *et al*: Social, economic and demographic
446 correlates of overweight and obesity in primary-school children: preliminary data
447 from the Healthy Growth Study. *Public Health Nutr* 2010; **13:** 1693-1700.
448

449 18. Jacobsson JA, Rask-Andersen M, Riserus U *et al*: Genetic variants near the
450 MGAT1 gene are associated with body weight, BMI and fatty acid metabolism
451 among adults and children. *Int J Obes (Lond)* 2011.
452
453 19. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH: Establishing a standard definition for
454 child overweight and obesity worldwide: international survey. *BMJ (Clinical*
455 *research ed* 2000; **320:** 1240-1243.
456
457 20. Schofield WN: Predicting basal metabolic rate, new standards and review of
458 previous work. *Hum Nutr Clin Nutr* 1985; **39 Suppl 1:** 5-41.
459
460 21. Goldberg GR, Black AE, Jebb SA *et al*: Critical evaluation of energy intake data
461 using fundamental principles of energy physiology: 1. Derivation of cut-off limits to
462 identify under-recording. *Eur J Clin Nutr* 1991; **45:** 569-581.
463
464 22. Trichopoulou A: *Composition tables of foods and Greek dishes. School of*
465 *Medicine: Department of Hygiene and Epidemiology*. Athens, Greece, 2004.
466
467 23. Barrett JC, Fry B, Maller J, Daly MJ: Haploview: analysis and visualization of LD
468 and haplotype maps. *Bioinformatics* 2005; **21:** 263-265.
469
470 24. Gabriel SB, Schaffner SF, Nguyen H *et al*: The structure of haplotype blocks in the
471 human genome. *Science* 2002; **296:** 2225-2229.

472

473 25. Purcell S, Neale B, Todd-Brown K *et al*: PLINK: a tool set for whole-genome
474 association and population-based linkage analyses. *American journal of human*
475 *genetics* 2007; **81:** 559-575.
476

477 26. Benjamini Y, Hochberg Y: Controlling the False Discovery Rate - a Practical and
478 Powerful Approach to Multiple Testing. *J Roy Stat Soc B Met* 1995; **57:** 289-300.
479

480 27. Skol AD, Scott LJ, Abecasis GR, Boehnke M: Joint analysis is more efficient than
481 replication-based analysis for two-stage genome-wide association studies. *Nat*
482 *Genet* 2006; **38:** 209-213.
483

484 28. Alsio J, Roman E, Olszewski PK *et al*: Inverse association of high-fat diet
485 preference and anxiety-like behavior: a putative role for urocortin 2. *Genes Brain*
486 *Behav* 2009; **8:** 193-202.
487

488 29. Rio DC, Ares M, Jr., Hannon GJ, Nilsen TW: Purification of RNA using TRIzol
489 (TRI reagent). *Cold Spring Harbor protocols*; **2010:** pdb prot5439.
490

491 30. Vandesompele J, De Preter K, Pattyn F *et al*: Accurate normalization of real-time
492 quantitative RT-PCR data by geometric averaging of multiple internal control
493 genes. *Genome biology* 2002; **3:** RESEARCH0034.
494

495 31. Elks CE, Loos RJ, Sharp SJ *et al*: Genetic markers of adult obesity risk are
496 associated with greater early infancy weight gain and growth. *PLoS Med* 2010; **7:**
497 e1000284.
498
499 32. Kilpelainen TO, den Hoed M, Ong KK *et al*: Obesity-susceptibility loci have a
500 limited influence on birth weight: a meta-analysis of up to 28,219 individuals. *Am J*
501 *Clin Nutr* 2011.
502
503 33. Miller EK, Cohen JD: An integrative theory of prefrontal cortex function. *Annu Rev*
504 *Neurosci* 2001; **24:** 167-202.
505
506 34. Kuboshima-Amemori S, Sawaguchi T: Plasticity of the primate prefrontal cortex.
507 *Neuroscientist* 2007; **13:** 229-240.
508
509 35. Goto Y, Yang CR, Otani S: Functional and dysfunctional synaptic plasticity in
510 prefrontal cortex: roles in psychiatric disorders. *Biol Psychiatry* 2010; **67:** 199-207.
511
512 36. Amargos-Bosch M, Adell A, Bortolozzi A, Artigas F: Stimulation of alpha1-
513 adrenoceptors in the rat medial prefrontal cortex increases the local in vivo 5-
514 hydroxytryptamine release: reversal by antipsychotic drugs. *J Neurochem* 2003; **87:**
515 831-842.
516

517 37. Andrade R: Serotonergic regulation of neuronal excitability in the prefrontal cortex.
518 *Neuropharmacology* 2011.
519
520 38. Keramidas A, Harrison NL: The activation mechanism of alpha1beta2gamma2S and
521 alpha3beta3gamma2S GABAA receptors. *J Gen Physiol* 2010; **135:** 59-75.
522
523 39. Yee BK, Keist R, von Boehmer L *et al*: A schizophrenia-related sensorimotor
524 deficit links alpha 3-containing GABAA receptors to a dopamine hyperfunction.
525 *Proc Natl Acad Sci U S A* 2005; **102:** 17154-17159.
526
527

528 **Figure Legends:**

529 Figure 1: a) Slopes for best-fit curves ± 95% confidence interval, generated from linear
530 regression analysis of mRNA expression of TMEM18 in regions of the brain in rats (n=36)
531 as measured by quantitative reverse transcriptase PCR, against body weight in grams.
532 Linear regression was performed in Prism v5.02 (GraphPad Software, San Diego California
533 USA, www.graphpad.com). b) Relative expression of TMEM18 mRNA in the rat
534 prefrontal cortex, as measured by quantitative reverse transcriptase PCR, plotted against
535 body weight in grams. Results from linear regression analysis of TMEM18 mRNA-
536 expression against body weight at the end of the food preference paradigm shows a
537 significant correlation between body weight and expression of TMEM18. *** denotes $p <$
538 0.001.

539

Figure 1. Correlation of TMEM18 expression and body weight in rat brain regions associated with feeding behavior.

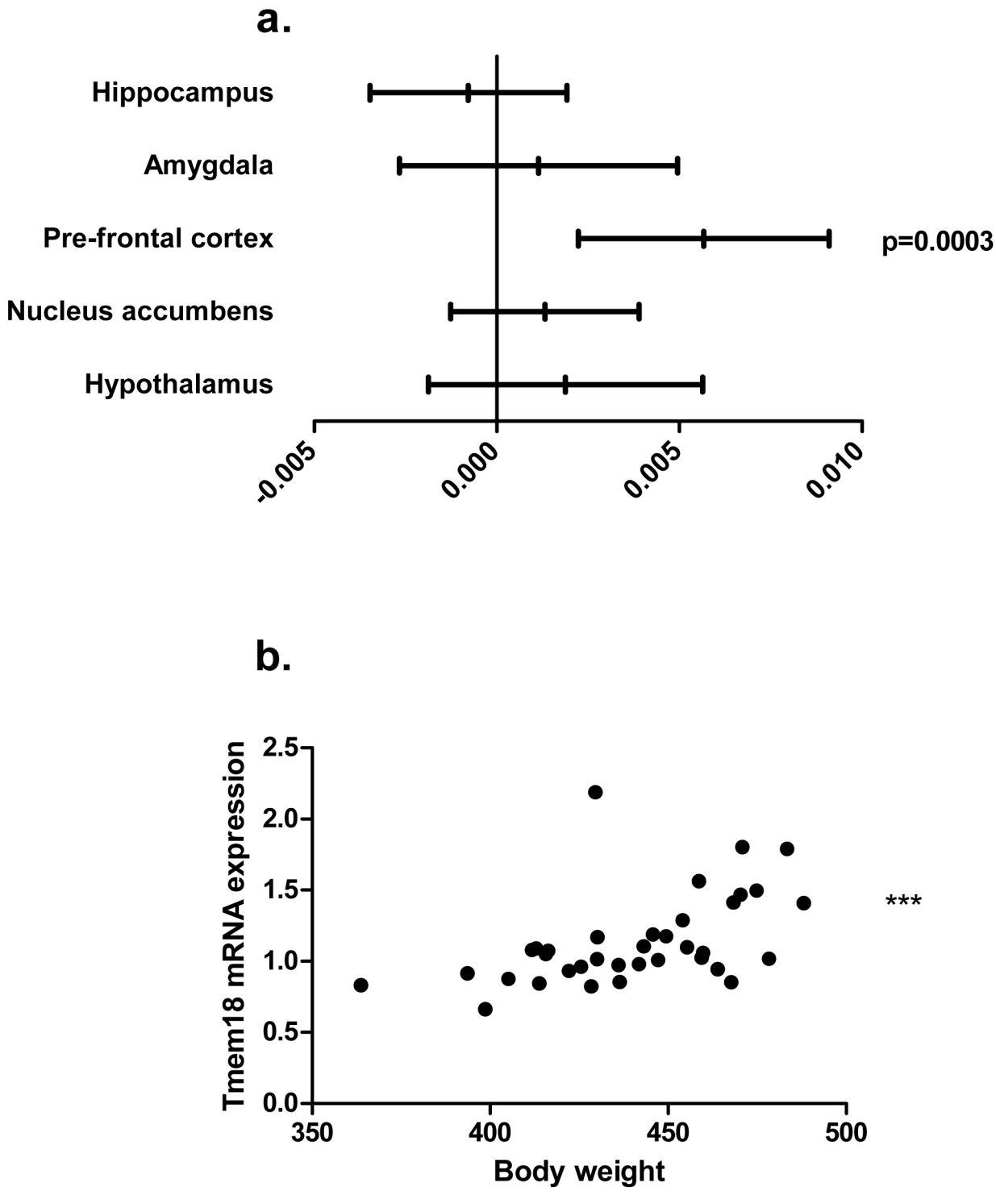

Table 1: Descriptive characteristics of the cohort of 2 352 Greek children, stratified according to weight-status

Characteristics	All	Normal-weight	Obese
N	2352	2080	272
Age (years)	11.2 ± 0.7	11.2 ± 0.7	11.1 ± 0.6
Body weight (kg)	45.3 ± 11.1	38.7 ± 6.3	64.5 ± 9.5
Height (cm)	148.7 ± 7.8	147.3 ± 7.7	152.3 ± 7.4
BMI z-score	0.9 ± 1.2	-0.01 ± 0.8	2.8 ± 0.3
BMI	20.3 ± 3.8	20.3 ± 3.8	27.6 ± 2.5
Average dietary energy intake (Kcal/day)	1791.6 ± 553.7	1801.5 ± 553.8	1715.9 ± 547.6
Fiber (g)	13.7 ± 7.5	13.8 ± 7.6	12.8 ± 6.5
Cholesterol (mg)	230.9 ± 127.7	284.1 ± 138.2	226.1 ± 120.8
% total energy from			
Fat	40.9 ± 7.3	40.9 ± 7.2	40.8 ± 7.9
Carbohydrates	45.6 ± 8.5	45.7 ± 8.4	45.2 ± 9.2
Protein	15.6 ± 3.5	15.6 ± 3.4	16.1 ± 3.7

Values are geometric means ± SD.

Table 2: Genotype distribution and odds ratio of two SNPs downstream *TMEM18* between obese and normal-weight Greek children.

		N	Genotype, n (%)			MAF, %	OR (95% CI)	P	HWE
			G1	G2	G3				
rs6548238	Normal weight	2073	82 (4.0)	689 (33.2)	1302 (62.8)	20.6			
A > G	Obese	270	9 (3.3)	62 (23.0)	199 (73.7)	14.7	1.489 (1.161-1.910)	0.002	0.461
rs4854344	Normal weight	2072	83 (4.0)	686 (33.1)	1303 (62.9)	20.5			
G > T	Obese	271	9 (3.3)	62 (22.9)	200 (73.8)	14.7	1.494 (1.165-1.917)	0.001	0.591

Data are number of subjects in each group and number of subjects for each genotype (G) (% in each group). Minor allele frequency (MAF) for each group is given in percentage. Odds ratio (OR) with a 95 % confidence interval (CI) was calculated assuming an additive model. Association with obesity was determined comparing subjects with normal weight (BMI < 25 kg/m^2) and obesity (BMI ≥ 30 kg/m^2). HWE indicate p-values for deviation from Hardy-Weinberg Equilibrium, excluded if p<=0.001.

Table 3: Association between two SNPs downstream TMEM18 and body weight, BMI z-score, waist circumference and dietary energy intake in 2 352 Greek children.

		Beta	95% CI	P_{Add}
rs6548238	Body weight (kg)	0.063	0.011 - 0.115	0.017
	BMI z-score	0.097	0.026 - 0.169	0.005
	Waist circumference	0.111	0.039 - 0.182	0.003
	Average dietary energy intake (Kcal/day)	-0.034	-0.036 - 0.106	0.343
	Fiber (g)	-0.007	-0.064 - 0.078	0.845
	Cholesterol (mg)	0.063	-0.008 - 0.135	0.081
	% total energy from			
	Fat	-0.239	-0.047 - 0.096	0.513
	Carbohydrates	0.034	-0.106 - 0.036	0.342
	Protein	-0.039	-0.033 - 0.112	0.289
rs4854344	Body weight (kg)	0.068	0.016 - 0.120	0.01
	BMI z-score	0.102	0.031 - 0.173	0.005
	Waist circumference	0.110	0.039 - 0.182	0.003
	Average dietary energy intake (Kcal/day)	-0.038	-0.03 - 0.109	0.291
	Fiber (g)	-0.003	-0.068 - 0.075	0.926
	Cholesterol (mg)	0.065	-0.006 - 0.136	0.071
	% total energy from			
	Fat	-0.029	-0.042 - 0.101	0.422
	Carbohydrates	0.041	-0.112 - 0.030	0.261
	Protein	-0.042	-0.030 - 0.114	0.260

Beta indicates transformed beta-values. P indicates p-values adjusted for significant covariates.