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Hypothalamic orexin, OX1, αMSH, NPY and MCRs expression in dopaminergic D2R knockout mice

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ABSTRACT

In 5-month-old male and female dopamine receptor 2 (D2R) knockout mice food intake per animal was unaltered while food per g BW was increased. We wished to evaluate the effect of D2R disruption on different components of energy balance and food intake regulation. We determined hypothalamic orexin precursor (PPO) expression, its receptor OX1, serum leptin levels, hypothalamic leptin receptor (OBR), circulating and pituitary α MSH levels, as well as central MC3 and MC4 receptors and NPY mRNA in wildtype and D2R knockout mice (KO).

Loss of D2R caused a marked increase in serum prolactin levels, to higher levels in females compared to male KO mice. On the other hand, it produced a female-specific increase in circulating αMSH, and hypothalamic αMSH content, while neurointermediate αMSH content was decreased in both sexes. No differences were found in hypothalamic NPY, MC3R or MC4R concentration. Hypothalamic PPO mRNA expression was significantly decreased only in female KOs, while OX1 mRNA was not different between genotypes. Serum leptin levels were also similar in both genotypes.

Our results show that in female and not in male mice disruption of the D2R produces two potentially anorexigenic events: an increase in serum and hypothalamic αMSH, and a decrease in hypothalamic orexin expression. Very high prolactin levels, which are orexigenic, probably counterbalance these effects, so that food intake is slightly altered. In males, on the other hand, hypothalamic PPO, and serum or hypothalamic αMSH are not modified, and increased prolactin levels may account for increased food intake per g BW. These results suggest a sexually dimorphic participation of the D2R in food intake regulation.

1. Introduction

Dopamine receptor D2 (D2R) knockout mice (KO) generate anterior pituitary lactotrope hyperplasia and have chronic hyperprolactinemia (Diaz-Torga et al., 2002; Cristina et al., 2005). These mice also present altered body growth, with reduction of pituitary GH, and serum IGF-I and IGF BP3 (Garcia-Tornadu et al., 2006). KO males have no differences in 24 h food intake, however when food intake is normalized to body weight there is actually a significant increase in food intake/gram of body weight, which indicates that food intake efficiency is greater in wildtype males (Diaz-Torga et al., 2002). In KO females there is a catch up in body weight after three months of age, and food intake has not been evaluated.

Many components of the neuroendocrine system act as metabolic regulators of energy balance and food intake (Cone, 1999, 2001). Neuropeptides interact with monoamines in the hypothalamus to control physiologic states such as hunger and satiety (Ramos et al., 2005). In particular, dopamine reduces food intake by acting in specific hypothalamic areas, through the dopamine receptors type 1 and 2 (Leibowitz, 1986; Terry et al., 1995) and different energy states (fasting, obesity, anorexia) modulate dopamine receptor expression (Fetissov et al., 2002; Sato et al., 2001). The anorectic dopamine action may be caused by transinaptic activation of orexin neurons (Fadel and Deutch, 2002).

The hypothalamic peptides orexins A and B are so named for their influence on food intake (de Lecea et al., 1998; Sakurai et al., 1998). An intracerebroventricular injection of orexin A does not merely affect eating behavior, but it also induces an increase in heart rate (Monda et al., 2005), blood pressure (Samson et al., 1999) and metabolic rate (Lubkin and Stricker-Krongrad, 1998). A role for the orexins in sleep regulation has also been demonstrated (Beuckmann and Yanagisawa, 2002), and deficiency in orexin neurotransmission results in the sleep disorder narcolepsy in mice,

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dogs, and humans (Taheri et al., 2002). Orexins act mainly at the orexin 1 (OX1) G protein-coupled receptor in the brain (Trivedi et al., 1998) (Silveyra et al., 2007).

On the other hand, the D2R negatively regulates α-melanocyte-stimulating hormone (αMSH), an anorexigenic peptide (Cote et al., 1986). αMSH is a 13 amino acid peptide produced by post translational processing of proopiomelanocortin (POMC) in the intermediate lobe of the pituitary. In addition to the skin-darkening effect in amphibians and other vertebrates, to which it owes its name, this peptide has anti-inflammatory and antimicrobial effects and probably contributes to innate immunity (Catania et al., 2000). It participates in the control of feeding behavior, reduces food consumption and stimulates catabolism acting at the melanocortin 3 (MC3R) and melanocortin 4 receptors (MC4R). αMSH is expressed primarily in the arcuate nucleus of the hypothalamus, and expressed and secreted to peripheral circulation by the intermediate lobe of the pituitary. Its synthesis and secretion in the pituitary is mainly controlled by the D2R (Chen et al., 1983). αMSH may modify the expression of the orexigenic peptides, orexin A and B in the hypothalamus (Lopez et al., 2007), and there is anatomical evidence that POMC neurons send projections to neurons within the region expressing orexins (Elias et al., 1998).

Other peptides related to food intake are leptin and neuropeptide Y (NPY). Leptin is an anorexigenic satiety factor, which governs energy balance through a negative feedback loop acting on hypothalamic centers in the brain through its receptor OBR (Ahima and Flier, 2000). There is extensive evidence demonstrating that the central melanocortin system is important in mediating the effects of leptin. First, leptin receptors are expressed on the majority of POMC neurons in the arcuate nucleus (Cheung et al., 1997) and the anorectic effects of exogenously administered leptin in rodents are partially reversed by treatment with a melanocortin receptor antagonist (Seeley et al., 1997; da Silva et al., 2004), indicating that the central melanocortin system is downstream of leptin receptor signaling and plays a key role in mediating the effects of this important anorexigenic hormone (Cowley et al., 2001).

NPY is one of the most abundant peptides of the hypothalamus (Kamiji and Inui, 2007). The major sites of neuronal expression of NPY in the hypothalamus are the arcuate nucleus and the dorso medial hypothalamus (DMH). It is a potent orexigenic neuropeptide, and centrally applied stimulates food intake. Prolactin may activate NPY gene expression in the DMH (Chen and Smith, 2004), and GH also increases NPY hypothalamic expression (Hurley et al., 2003). Finally, a relation of the dopaminergic system and NPY has been proposed, as dopaminergic agonists can decrease feeding behavior by an antagonistic action on hypothalamic NPY-containing neurons (Bina and Cincotta, 2000).

In view of the interdependence of the dopaminergic system with prolactin, αMSH, orexins, NPY and leptin, we sought to evaluate the effect of D2R disruption on food intake in both sexes in relation to hypothalamic orexin precursor (PPO) and OX1 expression, serum leptin levels, and expression of its hypothalamic receptor, OBR. Furthermore, we studied circulating, pituitary and hypothalamic α MSH levels, as well as central MC3 and MC4 receptors, and hypothalamic NPY mRNA levels.

2. Materials and methods

2.1. Animals

D2 dopamine receptor knockout mice, official strain designation B6.129S2-*Drd2*^{tm1low} by the Induced Mutant Resource at The Jackson Laboratory (Bar Harbor, ME), generated by targeted mutagenesis of the D2R gene in embryonic stem cells (Asa et al., 1999; Kelly et al., 1997) were used. The original F_2 hybrid strain (129S2/Sv × C57BL/6J) containing the mutated D2 receptor allele was backcrossed for ten generations to wildtype C57BL/6J mice. Mutant and wildtype mice were generally the product of heterozygote crossings, and in all cases sibling controls were used. Mice were separated by sex, housed in groups of 4 or 5 with mixed genotypes in an air-conditioned room with lights on at 07.00 and off at 19.00 h. They had free access to laboratory chow and tap water. Wildtype, heterozygous and knockout mice were identified by PCR of genomic DNA. As previously demonstrated (Diaz-Torga et al., 2002) body weight was 15% lower in male ($P < 0.05$) and not in female KO mice at six months of age. Weights of liver and inguinal/gonadal fat pads were similar in females of both genotypes, and significantly reduced in male KO compared with male wildtype mice (Diaz-Torga et al., 2002). All experimental procedures were reviewed and approved by the institutional animal care and use committee of either the Instituto de Biología y Medicina Experimental, Buenos Aires (Division of Animal Welfare, Office for Protection of Research Risks, NIH, A#5072-01).

2.2. Food intake

Five-month-old KO or wildtype male and female mice, and two-month-old female mice of both genotypes were weighed, individually caged and provided with a known amount of chow pellets (5% fat, 19% protein, and 5% fiber by weight; 3.4 kcal/g) in one corner of their cage. The mice and residual food were weighed at 1600 h on 5 consecutive d.

2.3. DNA extraction and PCR

DNA extraction and PCR used for genotyping was described in detail previously (Diaz-Torga et al., 2002)

2.4. Drugs

Unless specified, all chemicals were purchased from Sigma (St. Louis, MO).

2.5. Immunoblotting

Anterior hypothalami from 6-month-old male and female mice of both genotypes were homogenized individually in ice-cold buffer containing 60 mM Tris–HCl, 1 mM EDTA (pH 6.8) and a mix of protease inhibitors (phenyl-methyl-sulphonile, TPCK, TAME, ZPCK and TLCK) in a handheld microtissue homogenizer. The homogenate was then centrifuged at 700*g* for 10 min at 4 °C. 1.5 mg of initial tissue weight were diluted in sample buffer (62.5 mM Tris–HCl, 2% w/v SDS, 10% w/v glycerol, 0.1% w/v bromophenol blue, 50 mM dithiotreitol, pH 6.8). Samples were heated for 5 min at 95 °C and stored at −20 °C until used in Western blot analysis. Samples were subjected to SDS–polyacrylamide gel electrophoresis (6% w/v for OBR and 12% w/v for MC4, MC3 and actin, acrylamide-bis acrylamide gels were used). Gels were blotted onto a nitrocellulose membrane (Bio-Rad, Buenos Aires, Argentina) and probed with the corresponding primary antibody followed by a secondary antibody conjugated with horseradish peroxidase. MC4, MC3, actin and OBR polyclonal antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Immunoreactive proteins were detected by enhanced chemiluminescence (ECL, Amersharm). Bands were quantitated using the ImageQuant software.

The calculated molecular masses of the OBR receptor, the MC4R, MC3R, and actin were 123, 45, 37 and 43 kDa, respectively.

2.6. Blood samples

Blood samples were obtained by decapitation. Blood sampling was performed in 4–6-month-old male and female mice of both genotypes. Sera were kept at −20 °C until RIAs were performed.

2.7. Radioimmunoassays

Leptin was determined using antimouse leptin (#AFP3011199) and recombinant mouse leptin (AFP 341C) for standard and iodinated hormone, respectively (both reagents provided and distributed by the Hormone Distribution Program of the NIDDK and Dr. A.F. Parlow). The intra- and inter-assay coefficients of variation were 8.3% and 10.2%, respectively.

Prolactin was measured by RIA using kits provided by the NIDDK. Results are expressed in terms of mouse prolactin RP3. Intra- and inter-assay coefficients of variation were 7.2% and 12.8%, respectively.

2.8. MSH

Serum αMSH was measured using a commercial kit (αMSH EIA kit, Phoenix Pharmaceuticals, Inc., Belmont, CA), minimum detectable concentration was 0.5 pg. Sera was extracted using 0.1 M acetic acid.

Pituitary αMSH was measured by RIA using an anti-αMSH developed in rabbit (1:10000 dilution, Sigma, St. Louis, MO), and αMSH (Sigma) for standards and iodinated hormone. Neurointermediate lobe was extracted and homogenized in 0.25 M acetic acid, and centrifuged 5 min at 800*g*. Supernatants were diluted in saline phosphate buffer with 1% w/v bovine seroalbumin. Hypothalami were homogenized in 0.25 M acetic acid, and centrifuged 5 min at 800*g*; supernatant was transferred, and the precipitate was homogenized in 0.25 M acetic acid, and centrifuged 5 min at 800*g*; the second supernatant was pooled with the first one, and used for RIA after appropriate dilution with SPB BSA. Intra and inter-assay coefficients of variation were 7.8% and 9.5%, respectively. Minimum detectable concentration was 0.05 ng.

2.9. Immunohistochemistry

Immunohistochemistry was performed as previously described using the avidin–biotin peroxidase method (Cristina et al., 2007). Primary antibody was anti-αMSH developed in rabbit (1:1000 dilution, Sigma, St. Louis, MO), and the secondary antibody anti-rabbit HRP conjugated (Vectors Labs, Burlingame, CA). Three or four animals were used for each experimental group, and a minimum of 4–5 pituitary sections obtained at different levels of each gland were used for quantitation. αMSH positive cells were expressed as a percentage of total nucleated cells in the section at 100×. Nucleated cells were evidenced by counterstaining with hemotoxylin.

2.10. Tissue extraction for real-time PCR

The brains were rapidly removed and placed on ice for dissection. An area limited anteriorly by the cephalic fissure of the optic chiasm, laterally by the hypothalamic fissures, posteriorly by the fissure caudal to the mammilary bodies, and in depth by the subthalamic sulcus was excised. All tissue samples were immediately homogenized in TRIzol reagent (Invitrogen, Buenos Aires, Argentina) and kept at −70 °C until used. Levels of expression of mRNAs for PPO and OX1, were determined by quantitative RT-PCR.

2.11. Total RNA preparation and cDNA synthesis

Total RNA was isolated from tissue homogenates by use of the TRIzol reagent method. The RNA concentration was determined on the basis of absorbance at 260 nm, and its purity was evaluated by the ratio of absorbance at 260/280 nm (>1.8). RNAs were kept frozen at −70 °C until analyzed.

After digestion of genomic DNA by treatment with deoxyribonuclease I (Ambion, Austin, TX), first-strand cDNA was synthesized from 1 μg of total RNA in the presence of 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.6), 75 mM KCl, 0.5 mM deoxy-NTPs, 1 mM DTT, 1 U/μl RnaseOUT (Invitrogen), 0.5 μg oligo$(dT)_{15}$ primer (Biodynamics, Buenos Aires, Argentina), and 20 U of MMLV reverse transcriptase (Epicentre, Madison, WI). To validate successful deoxyribonuclease I treatment, the reverse transcriptase was omitted in control reactions. The absence of PCR-amplified DNA fragments in these samples indicated the isolation of RNA free of genomic DNA.

2.12. Quantitative real-time PCR

Sense and antisense oligonucleotide primers were designed on the basis of the published cDNA of PPO, OX1, and cyclophilin sequences by use of the PrimerExpress software (Applied Biosystems, Foster City, CA). Oligonucleotides were obtained from Invitrogen. The sequences of the primers were as follows: PPO sense GCCTCAG ACTCCTTGGGTATTTG, PPO antisense GGCAATCCGGAGAGATGGT; OX1 sense CAGAGAGCGTTGTAAACCGTC, OX1 antisense AGCCAAG CTCTGATAGGGTG; cyclophilin sense GTGGCAAGATCGAAGTGG, cyclophilin antisense TAAAAATCAGGCCTGTGG; NPY sense GATGCT AGGTAACAAGCGAATG, NPY antisense TCAGCCAGAATGCCCAAAC.

Quantitative measurements of PPO, OX1 and cyclophilin cDNA were performed by kinetic PCR using SYBR Green I as fluorescent dye (Invitrogen). The PCR reaction consisted of 100 ng cDNA, 0.4 μM primers, 10 mM Tris–HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM deoxy-NTPs, and 1.25 U *Taq* polymerase (Invitrogen) in a final volume of 25 μl. After denaturation at 95 °C for 5 min, the cDNA products were amplified with 40 cycles, each cycle consisting of denaturation at 95 °C for 15 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s. The accumulating DNA products were monitored by the ABI 7500 sequence detection system (Applied Biosystems), and data were stored continuously during the reaction. The results were validated on the basis of the quality of dissociation curves generated at the end of the PCR runs by ramping the temperature of the samples from 60 to 95 °C, while continuously collecting fluorescence data. Product purity was confirmed by agarose gel electrophoresis. Each sample was analyzed in triplicate along with specific standards and no template controls to monitor contaminating DNA.

The calculations of the initial mRNA copy numbers in each sample were made according to the cycle threshold (C_T) method. The C_T for each sample was calculated at a fluorescence threshold (R_n) using the ABI 7500 sequence detection system software with an automatic baseline setting. For all designed primer sets, linearity of real-time RT-PCR signaling was determined with wide-range serial dilutions of reference cDNA that covered the amount of target mRNA expected in the experimental samples, and clear linear correlations were found between the amount of cDNA and the C_T for the duration of at least 40 real-time -PCR rounds.

The relative PPO, NPY and OX1 gene expression were normalized to that of the cyclophilin housekeeping gene by use of the standard curve method, as described by the manufacturer (User Bulletin no. 2). Results are expressed as arbitrary units (AU) for comparison among samples. AU is defined as the expression level relative to a sample of wildtype mice (calibrator sample).

2.13. Statistical analyses

Results are expressed as means + SE.

Realtime PCR results were analyzed by one way ANOVA for the effect of genotype. Western blot and RIA data were analyzed by two-way analysis of variance (ANOVA) for the effects of sex and genotype. If F of interaction was found significant, individual means were compared by Tukey's honest significant difference or Fisher's protected least significant difference tests, if it was not significant, groups of means were analyzed by the same tests. $P < 0.05$ was considered significant.

3. Results

3.1. Food intake in wildtype and knockout mice

Food intake per mouse per day was not modified by the genotype in male or female mice at two or five months of age (Fig. 1A). When food intake was normalized to body weight there was a significant increase in food intake/gram of body weight in KO mice of both sexes at 5 months (Fig. 1B, $P = 0.045$) and not in 2-month-old females.

3.2. Serum PRL and in wildtype and knockout mice

As expected, prolactin levels were higher in KO mice than in wildtype littermates of both sexes at 5 months of age. Furthermore, prolactin was 3-fold higher in female KOs compared with male KOs and female 2-month-old KOs (P interaction = 0.0003; KO female vs. KO male $P = 0.00012$, and vs. 2-month-old KO females $P = 0.00012$, Fig. 1C).

3.3. Serum αMSH levels, and pituitary and hypothalamic αMSH content

Disruption of D2Rs caused a female-specific increase in circulating αMSH (female KO vs. wildtype $P = 0.035$, Fig. 2A).

Pituitary αMSH content was similar in female and male wildtype mice, and loss of D2R caused a decrease in both sexes, probably indicating reduced storage (effect of genotype:$P = 0.0012$;

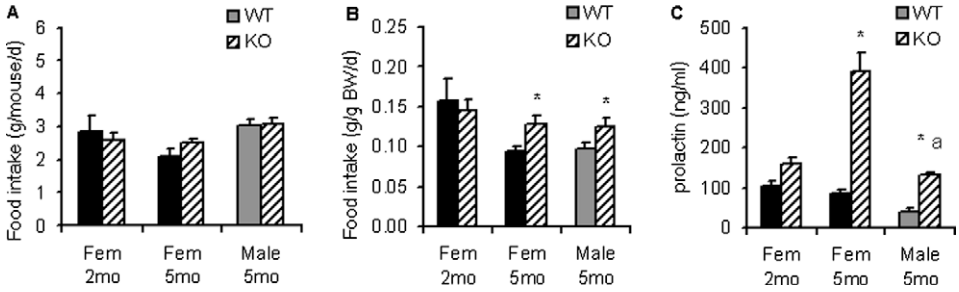

Fig. 1. (A) Food intake (g/mouse/d), and (B) g/g BW/d, in adult female and male mice of both genotypes at 5 months of age (fem 5mo, male 5mo) and in female 2-month-old mice (fem 2mo). KO: knockout, WT: wildtype. *$P < 0.05$ vs. respective WT, $N = 6$ per group. (C) Serum prolactin levels in adult female and male mice of both genotypes (fem 5mo, male 5mo) and in female 2-month-old mice (fem 2mo). KO: knockout, WT: wildtype. *$P < 0.05$ vs. respective WT; (a) $P < 0.05$ vs. KO females. $N = 22$, 20, 23 and 25.

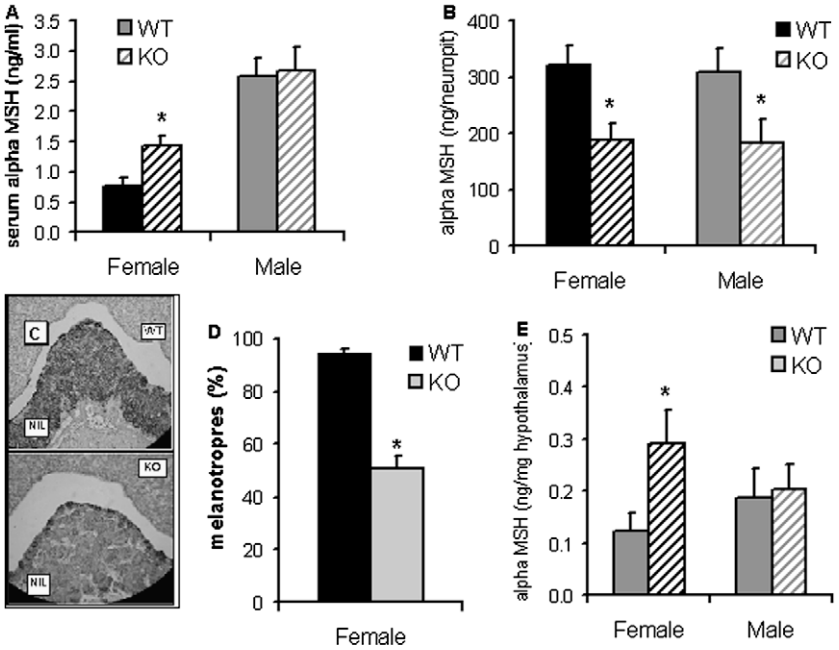

Fig. 2. (A) Serum αMSH concentration in adult female and male mice of both genotypes. KO: knockout, WT: wildtype. *$P < 0.05$ vs. respective WT. $N = 7$, 6, 5 and 5; (B) pituitary αMSH content (ng/neurointermediate lobe) in adult female and male mice of both genotypes. *$P < 0.05$ vs. respective WT. $N = 15$, 13, 16 and 14; (C) representative immunohistochemistry of neurointermediate lobes (NIL) of WT and KO female mice, stained with αMSH antibody, and DAB reaction. (D) Percentage of αMSH immunoreactive cells in relation to total cells in the intermediate pituitary lobe of female mice, *$P < 0.05$ vs. WT $N = 4$, 3; (E) hypothalamic αMSH content (ng/mg hypothalamus) in adult female and male mice of both genotypes, *$P < 0.05$ vs. respective WT. $N = 15$, 13, 14 and 13.

Fig. 2B). This was confirmed by immunohistochemistry which revealed a decrease in the percentage of αMSH cells in the intermediate lobe (Fig. 2C and D). On the other hand, hypothalamic αMSH content was increased in KO females compared to wildtype females ($P = 0.027$; Fig. 2E).

3.4. Hypothalamic MC3 and MC4 receptors

Even though circulating and hypothalamic αMSH was increased in female knockout mice, hypothalamic MC3 and MC4 receptors were not different between genotypes or sexes (Fig. 3A and B).

3.5. Hypothalamic PPO, OX1 and NPY mRNA expression

Hypothalamic PPO, OX1 and NPY mRNA content were assessed by real-time PCR. Loss of D2R caused a sex specific decrease in PPO mRNA expression in females (P interaction 0.0095, wildtype female vs. KO female: $P = 0.00029$; Fig. 4A). On the other hand, hypothalamic OX1 and NPY expression were similar between genotypes (Fig. 4B and C).

3.6. Serum leptin levels and hypothalamic OBR expression in wildtype and D2R knockout mice

In non fasted mice, serum leptin levels were not different between genotypes (Fig. 5A). In 12 h-fasted animals higher levels were found in females, but there was no effect of genotype (P interaction = 0.18, effect of sex $P = 0.0033$, effect of genotype $P = 0.43$; Fig. 5B).

Hypothalamic OBR protein expression was not significantly different between genotypes or sexes (Fig. 5C).

4. Discussion

Dopamine regulates hunger and satiety by acting in specific hypothalamic areas. The effects of dopamine on food intake have

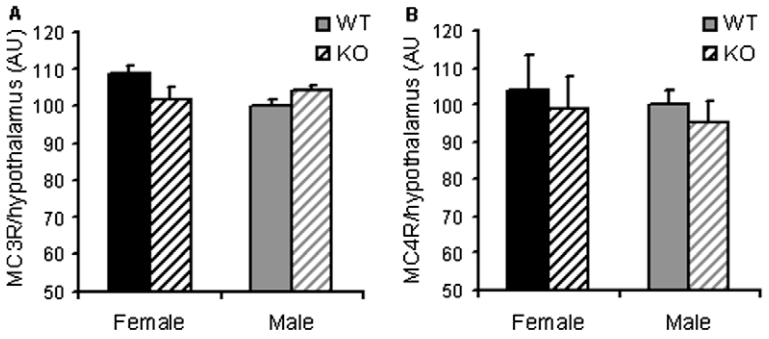

Fig. 3. (A) Hypothalamic MC3 receptor (MC3R) content (arbitrary units (AU)/hypothalamus) in adult female and male mice of both genotypes. KO: knockout, WT: wildtype. $N = 6, 6, 15$ and 16. (B) Hypothalamic MC4 receptor (MC4R) content (arbitrary units (AU)/hypothalamus) in adult female and male mice of both genotypes. $N = 9, 7, 18$ and 19.

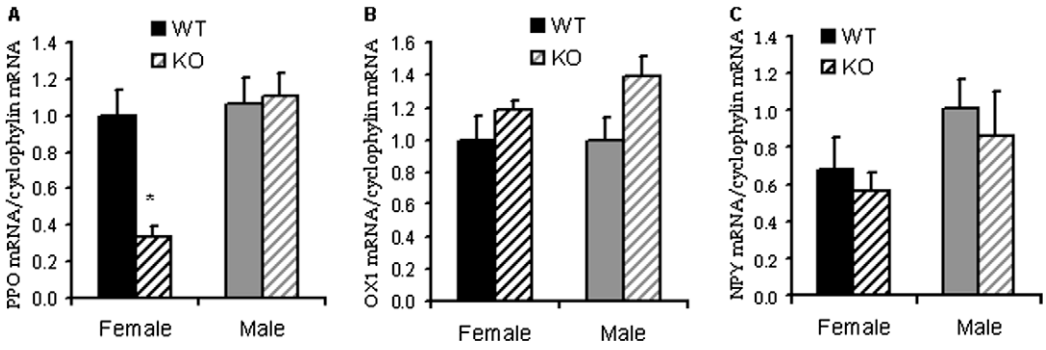

Fig. 4. (A) Orexin precursor (PPO), (B) OX1 and (C) NPY mRNA concentration normalized to cyclophylin mRNA of the sample in adult female and male mice of both genotypes. KO: knockout, WT: wildtype. $^{*}P < 0.05$ vs. respective WT. $N = 10, 10, 7$ and 7 for PPO, 7, 8, 8 and 7 for OX1 and 5, 7, 6, and 6 for NPY, respectively.

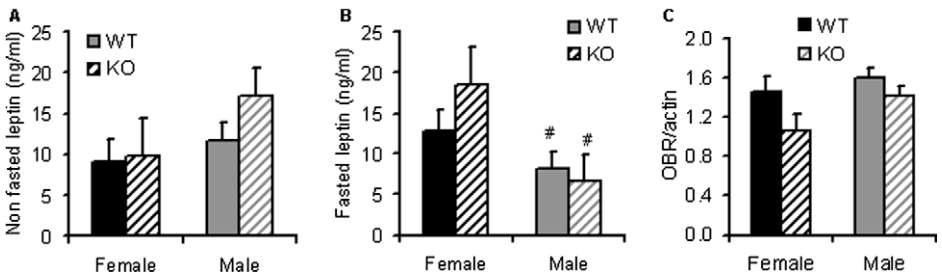

Fig. 5. (A) Serum leptin levels in non fasted male and female mice of both genotypes, KO: knockout, WT: wildtype. $N = 20, 17, 22, 14$. (B) Serum leptin levels in fasted adult female and male mice of both genotypes. $^{\#}P < 0.05$ vs. respective female. $N = 15, 13, 16$ and 14. (C) OBR expression in adult female and male mice of both genotypes. KO: knockout, WT: wildtype.

yielded conflicting results in the literature due to the different actions of dopamine on various hypothalamic nuclei, the involvement of multiple receptors, and different responses in food intake when administered systemically or locally into the hypothalamus (Ramos et al., 2005). Systemic treatment with D1/D2 agonists decrease food intake (Leibowitz, 1986; Terry et al., 1995), whereas it has also been described that selective D2R agonists may increase food intake (Hillebrand et al., 2002).

We have previously described that in the D2R knockout male mouse food intake per mouse is similar to that of the wildtype male, but food intake per unit of body weight is increased (Diaz-Torga et al., 2002). In the present work we found similar results in female 5-month-old mice but not in two-month-old KO mice. We show that different neuroendocrine signals might compensate food intake in males and females lacking D2Rs. First, in 5-month-old female KO mice chronic hyperprolactinemia is 3-fold greater than in male KO mice. It has been shown that prolactin stimulates food intake and fat deposition in female rats (Sauve and Woodside, 1996; Naef and Woodside, 2007), and that prolactin receptor-deficient mice have a progressive reduction in body weight, with females affected to a greater degree than males (Freemark et al., 2001). Injections of prolactin into the paraventricular nucleus increase food intake (Sauve and Woodside, 2000), which indicates that, in addition to its postulated actions on peripheral sites such as the pancreas and adipose tissue, prolactin affects hypothalamic orexigenic and anorexigenic systems that regulate appetite. It is therefore plausible that chronic increased prolactin concentration in KO females is mechanistically related to the increased food intake. To this respect, we also demonstrated that in 2-month-old female knockout mice food intake was not increased, in correlation with only a moderate increase in serum prolactin. But even though D2R knockout mice eat more they gain less, and this may be explained by the effect of elevated serum αMSH levels which increase energy expenditure (Fan et al., 2000) and fat loss (Haynes et al., 1999; Kastin et al., 1975), and may counterbalances the hyperphagic effect of prolactin. Long-term hyperprolactinemia is often accompanied by weight gain in humans. Sustained elevation of prolactin, caused by either antipsychotic drugs or prolactinomas, leads to increased body weight, which can be ameliorated by normalization of serum prolactin (Ben-Jonathan et al., 2006).

As mentioned, serum αMSH was increased in female KO mice, probable reflecting decreased storage in the intermediate lobe due to the lack of D2R inhibition on melanotropes (Cote et al., 1986). But, even though storage was decreased in both sexes, in males this was not reflected in increased αMSH levels. Different metabolic clearance or secretion patterns between sexes may be involved (Fadel and Deutch, 2002), and higher basal αMSH observed in wildtype males may be related to the sexual differences in the activity of periventricular-hypophysial dopamine neurons terminating in the intermediate lobe, with a stronger hypothalamic dopaminergic control in females (Manzanares et al., 1992). Furthermore, hypothalamic αMSH content was increased in female and not in male KOs.

On the other hand, the hypothalamic expression of the orexin precursor was decreased in female and not in male knockout mice compared to their wildtype littermates. OX1 mRNA expression was similar between genotypes, indicating that orexin did not modify its own receptor. The decrease in PPO mRNA indicates that D2R is involved directly or indirectly in regulating the expression of orexins. To this regard it has been described that dopamine regulates orexin neurons. The dopamine agonist apomorphine increased Fos expression in orexin neurons, and combined D1 antagonist and D2 antagonist pretreatment blocked apomorphine-induced activation of orexin neurons (Bubser et al., 2005). Furthermore, patients with schizophrenia treated with haloperidol have lower cerebrospinal fluid orexin A levels than unmedicated patients (Dalal et al., 2003). Therefore, disruption of D2Rs may induce a decrease in orexin expression. But, as orexin expression was unmodified in male KOs, additional factors may be involved. *In situ* hybridization histochemistry revealed that neurons in the lateral hypothalamus and adjacent perifornical area rarely express mRNAs encoding dopamine receptors (Bubser et al., 2005), suggesting that orexin cells are trans-synaptically activated by dopamine agonists.

Several neuropeptides may mediate this transinaptic effect. In particular, the αMSH receptor, MC4R, is highly expressed in the lateral hypothalamic area (Kishi et al., 2003), and there is anatomical evidence that POMC neurons send projections to neurons within the region expressing orexins (Elias et al., 1998). Besides, a functional link between αMSH and orexin has been stated. *Pomc* knockout mice (which lack αMSH) have increased orexin expression in the lateral hypothalamic area, which is not reversed with corticosterone replacement (Lopez et al., 2007). Central administration of αMSH in these mice restored orexin expression to wildtype levels showing that αMSH may inhibit the expression of orexin in this model. Therefore, increased serum or hypothalamic αMSH, which is evidenced in female and not in male KOs may participate in decreased hypothalamic orexin expression. It remains to be discerned if hypothalamic neurons can sense circulating αMSH, and if increased αMSH content in the hypothalamus reflects peripheral levels or central D2R regulation. To this regard it has been demonstrated that some areas of the hypothalamus, such as the arcuate nucleus, lack a blood brain barrier (Peruzzo et al., 2000), and thus can be in contact with circulating factors. Even though MC3 and MC4 have been linked to food intake regulation, we did not find modifications in their expression in D2R KO mice, suggesting that the increase in αMSH did not increase the expression of its hypothalamic receptors.

Furthermore, high prolactin levels in KO mice may participate in decreasing orexin expression, as described in animal models of hyperprolactinemia (Garcia et al., 2003). Therefore, the sex difference in orexin decrease may also be related to higher prolactin levels found in KO females compared to KO males, as well as to increased αMSH. To this regard it could be envisaged that prolactin counterbalances its hyperphagic effect by increasing αMSH and decreasing orexin.

We also studied serum leptin and its hypothalamic receptor OBR. Leptin is considered one of the main peripheral anorectic signals that influences food intake and body weight. It is secreted by adipocytes and acts primarily at hypothalamic nuclei to relay information regarding the level of energy available as lipids in the adipose tissue. After its binding to OBR in the hypothalamus, leptin stimulates a specific signaling cascade that results in the inhibition of several orexigenic neuropeptides (NPY, melanin-concentrating hormone (MCH), orexins and AgRP), and stimulation of several anorexigenic peptides (αMSH, CART and CRH) (Hillebrand et al., 2002; Sahu, 2003). We did not find any significant differences in serum leptin or its hypothalamic receptor. Sex differences (females had higher serum leptin levels than males) were only evidenced when mice were fasted, and this effect was not modified by the genotype.

No differences were found in hypothalamic NPY levels. This could be the result of the complex regulation of the neuropeptide. D2R KO mice have increased prolactin levels, and also decreased GH and IGF I output (Diaz-Torga et al., 2002). While increased prolactin and decreased dopaminergic action would favor increased NPY expression (Chen and Smith, 2004; Bina and Cincotta, 2000), reduced GH would have an opposite effect (Hurley et al., 2003).

Finally it is pertinent to mention that we previously described that D2R KO mice present alterations in specific central components of movement: initiation of locomotion, time spent in motion, and horizontal distance traveled (Luukkaa et al., 2001). This could also partly explain the decreased food efficiency evidenced in KO

mice. Nevertheless, we also described that they have a relatively good locomotion function probably due to adaptations of the other dopamine receptors in the CNS (Kelly et al., 1998), and so the changes in neuropeptide expression and hormone levels are probably mandatory in altered food intake.

The mechanism of energy homeostasis control and body weight is a complex physiological system that involves an intricate network of signals (peptides, neurotransmitters, hormones, glucose) and receptors, tending to regulate the assimilation, storage and use of energy from nutrients at an optimum level. In humans, reduction in D2R is associated with addictive behavior, towards food or drugs, and individuals with low numbers of D2R may be more vulnerable to addictive behaviors including compulsive food intake. But, the involvement of dopamine in pathological eating and obesity is poorly understood. The evidence linking mutations of the D2R gene and obesity syndromes in humans is limited, and in general, loss of function mutations associate with overweight (Pijl, 2003; Fetissov and Meguid, 2009). In contrast, we show that complete loss of D2Rs does not cause obesity and associated neurochemical changes; but only an increase in food intake per g BW. Sexual differences in the expression of neuropeptides related to food intake are found. In females there is an increase in serum and hypothalamic αMSH and a decrease hypothalamic orexin expression, two anorexigenic events. These events may compensate the hyperphagic effect of the very high prolactin levels, and therefore food intake is only increased when related to BW. In males, levels of prolactin are only moderately increased, and changes in PPO or αMSH are not evidenced. These results reveal a participation of multiple factors in D2R mediation of food intake.

Conflict of interest statement

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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