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Sex-dependent regulation of hypothalamic neuropeptide Y-Y1 receptor gene expression in leptin treated obese (*ob*/*ob*) or lean mice.

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Abstract

Pharmacological and genetic studies have shown that the Y_1 receptor (Y_1R) for Neuropeptide Y (NPY) plays a crucial role in the control of feeding behavior under metabolic conditions of low leptin levels or leptin deficiency. In this study, we investigated the effect of leptin deficiency and leptin replacement on Y_1R gene expression in the hypothalamus of lean and obese *Y_1R/LacZ* transgenic mice (Tg*Y_1R/LacZ*) carrying the murine Y1R promoter linked to the *LacZ* gene that induces the expression of β-galactosidase. Two daily intraperitoneal injections with leptin (1 μg/g of body weight for one week) of male and female lean (Tg*Y_1R/LacZ*$^{+/+}$) and obese (Tg*Y_1R/LacZ*$^{ob/ob}$) mice induced a significant decrease of body weight in both sexes and genotypes. In males, leptin administration decreased β-galactosidase activity in the PVN and DMH of lean mice, and increased transgene expression in the same hypothalamic nuclei of obese mice. Sex-related differences were also observed in both genotypes, since leptin treatment failed to affect transgene expression in hypothalamus of lean and obese female mice. These results provide further evidence for a sexual dimorphism of the hypothalamic NPY-Y_1R-mediated pathway in response to changes in leptin circulating levels.

Keywords: NPY; Y_1 receptor; leptin; *ob*/*ob* mice; hypothalamus; sex.

1. Introduction

Leptin, an adipocyte-derived hormone, is a signal molecule that decreases food intake and reduces body weight, thus contributing to energy homeostasis (Campfield, 2000; Myers et al., 2008). Mice lacking leptin gene (ob) or leptin receptor genes (db) show obesity, hyperphagia, decreased energy expenditure, increased body fat deposition, hyperglycemia, hyperinsulinemia and hypothermia (Coleman, 1985; Kaiyala et al., 2015) and are considered a model for morbid obesity in humans (Wang et al., 2014).

Neuropeptide Y (NPY) is highly expressed in the central nervous system (CNS) and it potently stimulates feeding, reduces energy expenditure and induces obesity (Loh et al., 2015). Hypothalamic NPY-containing neurons express leptin receptors and are prime targets of leptin action (Sahu, 2003; Schwartz et al., 2000). Leptin negatively regulates NPY gene expression in the arcuate nucleus (ARC) (Morrison et al., 2005) and counteracts the orexigenic effect of NPY, whereas NPY opposes the anorectic effect of leptin (Kalra and Kalra, 2007; Schwartz et al., 1999). Increased hypothalamic NPY activity has been suggested in some forms of genetic obesity linked to defective leptin signaling, including the *ob*/*ob*, *db*/*db* mice and *fa*/*fa* Zucker rats (Kim et al., 2000; Roseberry et al., 2004), on the other hand genetic depletion of NPY attenuates obesity in *ob*/*ob* mice (Erickson et al., 1996).

The NPY Y1 receptor (Y1R) is expressed in the hypothalamic nuclei involved in the regulation of ingestive behavior and energy balance (Park and Ahima, 2014) and it plays a crucial role in the control of energy homeostasis (Bertocchi et al., 2011; Eva et al., 2006; Herzog, 2003; Pedrazzini, 2004). A role of hypothalamic Y1R in genetic obesity has also been suggested by the observation that depletion of *Npy1r* gene reduces hyperphagia and partially corrects the obese syndrome in *ob*/*ob* mice (Pralong et al., 2002). However, it still remains unclear whether changes in leptin levels affects the expression of hypothalamic Y1R that was found to be either decreased (Beck et al., 2001), or unchanged (Huang et al., 2003; Widdowson et al., 1997) in rodent models of obesity. These discrepancies possibly reflect the limitation of analyses done on the whole hypothalamus or

they may depend on the underlying cause of obesity, i.e lack of leptin in monogenetic obesity models or leptin resistance in diet-induced common obesity models (Spiegelman and Flier, 2001). With the use of transgenic mice harboring a *lacZ* reporter gene driven by the murine *Npy1r* gene promoter (Tg*Y_1R/LacZ* mice), we previously showed that changes of energy balance, during pregnancy, fasting, or leptin, glucose and high fat diet administration, modulate hypothalamic Y_1R gene expression at the transcriptional level in a nucleus-specific manner, suggesting that changes in *Y_1R/LacZ* transgene expression reflect altered Y1R steady state and signal transduction (Oberto et al., 2003; Zammaretti et al., 2001; Zammaretti et al., 2007). In this study we generated obese leptin-deficient (*ob*/*ob*) mice carrying the *Y_1R*/LacZ transgene (Tg*Y_1R/LacZ*$^{ob/ob}$ mice) to investigate the effect of genetic leptin deficiency and leptin replacement on the *Npy1r* gene transcription in four hypothalamic nuclei [ARC, paraventricular (PVN), dorsomedial (DMH) and ventromedial (VMH)] (Schwartz et al., 2000). In addition, since we previously reported that changes in energy balance differentially modulate Y1R gene expression in the hypothalamus of male and female mice (Zammaretti et al., 2007), both sexes were included in the study.

2. Results

2.1 Effect of leptin deficiency and leptin treatment on body weight

Two months-old male and female Tg*Y1R/LacZ*$^{ob/ob}$ mice were morbidly obese, with body weights 1.6- and 2.1-fold higher than lean Tg*Y1R/LacZ*$^{+/+}$ mice, respectively. Female obese Tg*Y1R/LacZ*$^{ob/ob}$ mice were significantly heavier and female lean Tg*Y1R/LacZ*$^{+/+}$ mice were significantly lighter compared to obese and lean males, respectively (Fig.1).

The treatment with murine recombinant leptin (1 μg/g body weight, twice a day for one week, significantly decreased body weight in both sexes and genotypes; however, a significantly greater response to leptin was observed in obese Tg*Y1R/LacZ*$^{ob/ob}$ as compared to lean Tg*Y1R/LacZ*$^{+/+}$ mice of both sexes. Moreover, leptin treatment induced a significantly greater weight loss in female than in male obese Tg*Y1R/LacZ*$^{ob/ob}$ mice (Fig. 1).

2.2 Effect of leptin deficiency and leptin treatment on Y1R/LacZ transgene expression in the hypothalamus

Y1R/LacZ transgene expression was determined by computer-assisted quantitative analysis of histochemical β-galactosidase activity of PVN, ARC, VMH and DMH from male and female obese *Tg*Y$_1$R/LacZ$^{ob/ob}$ and lean *Tg*Y$_1$R/LacZ$^{+/+}$ mice. No significant changes of β-galactosidase activity were observed in the PVN, DMH, VMH and ARC of obese Tg*Y1R/LacZ*$^{ob/ob}$ mice, compared to lean Tg*Y1R/LacZ*$^{+/+}$ mice, independently of the sex (Fig. 2-4).

Conversely, genotype and sex-dependent differences in the effect of leptin-treatment on Y$_1$R/LacZ transgene expression were observed in different hypothalamic nuclei.

In male mice, leptin treatment significantly decreased β-galactosidase activity in the PVN (Fig. 2 and 4) and DMH (Fig. 3 and 4) of lean Tg*Y1R/LacZ*$^{+/+}$ male as compared to vehicle-treated mice. In contrast, the same treatment significantly increased β-galactosidase activity in the PVN and DMH of obese Tg*Y1R/LacZ*$^{ob/ob}$ mice (Fig. 2-4). Similar effects were observed in the VMH of leptin treated Tg*Y1R/LacZ*$^{+/+}$ and Tg*Y1R/LacZ*$^{ob/ob}$ male mice, respectively, although changes in β-

galactosidase activity were not significant in this nucleus (Fig. 4). Leptin treatment did not altered *Y_1R/LacZ* transgene expression in the ARC of male obese Tg*Y_1R/LacZ*$^{ob/ob}$ and lean Tg*Y_1R/LacZ*$^{+/+}$ mice (Fig. 4). Leptin treatment failed to modify Y_1R/LacZ expression in the PVN, DMH, VMH and ARC of both lean TgY_1R/LacZ$^{+/+}$ and obese TgY_1R/LacZ$^{ob/ob}$ female mice (Fig. 4).

Discussion

Previous evidences have shown that leptin action across its full biologic dose-response curve, which extends over a broad range, from lack of lepin signal in monogenetic obesity, to low levels during starvation, to high levels characteristic of common obesity, may differentially affect neuronal pathways controlling energy homeostasis (Bergonzelli et al., 2001; Spiegelman and Flier, 2001). In the basal state, the anabolic pathways (NPY/AgRP containing neurons) that stimulate food intake and decrease energy expenditure, are strongly inhibited in response to physiological concentrations of leptin, whereas catabolic pathways are stimulated to prevent excessive weight gain.

In turn, a decrease in energy balance, that is associated with low circulating leptin levels, activates anabolic pathways and inhibits catabolic pathways and this regulation is essential to vigorously defend against deficits of body fat (Morton et al., 2006).

In the present study we demonstrated that chronic leptin treatment induced opposite changes in the transcriptional activity of the Y_1R gene in the hypothalamus of lean and obese male mice, suggesting that the NPY-Y_1R pathway might differentially respond to the increase in the circulating levels of leptin, depending on the steady state of the hormone and/or on the activity of NPY neurons. The elevation of plasma leptin levels above the normal fed range, induced by repeated leptin administration to lean mice, decreased Y_1R gene expression in PVN and DMH. This observation suggests that leptin treatment inhibits the NPY anabolic pathway by decreasing Y_1R signal transduction in the hypothalamic nuclei involved in the regulation of feeding behavior. The leptin-induced inhibition of Y_1R gene expression seems to require a long-term administration since a three day treatment with 1μg/mg of leptin failed to affect *Y_1R/LacZ* transgene expression (Zammaretti et al., 2001).

Leptin replacement in *ob*/*ob* mice increases *Y_1R/LacZ* transgene expression in the PVN and the DMH. Previous studies demonstrated that *ob*/*ob* mice display increased spike activity of arcuate NPY/AgRP neurons (Takahashi and Cone, 2005) and over-express hypothalamic NPY mRNA

(Schwartz et al., 1996). Leptin administration to *ob*/*ob* mice (Schwartz et al., 1996; Sousa-Ferreira et al., 2011) or to fasted mice (Jequier, 2002) and rats (D'Souza A et al., 2014) attenuates these responses. Leptin deficiency results in hyperphagia that partially depends on a chronic stimulation of the Y_1R, as suggested by the observation that the ablation of Y_1R reduces hyperphagia and partially corrects the obese syndrome in *ob*/*ob* mice (Pralong et al., 2002). Present data suggest that the inhibition of an over-activated NPY pathway, induced by leptin treatment, might activate compensatory changes of Y_1R gene transcriptional activity.

Interestingly, in spite of the genotypic differences in the baseline body weight, we found no significant changes in Y1R gene expression in obese mice, as compared to lean mice. Several lines of evidence demonstrated that arcuate NPY neurons project to second-order downstream neurons in PVN or DMH, which also receive inputs from other brain regions, to regulate the overall balance between food intake and energy expenditure. For instance, neural projections from arcuate NPY neurons to oxytocin, TRH and cocaine- and amphetamine-regulated transcript (CART) positive neurons in the PVN and DMH have been identified (Broberger, 1999; Cyr et al., 2013; Kishi et al., 2005). In addition, recent studies suggest that both hypothalamic ARC and DMH NPYergic neurons directly control energy expenditure by modulating sympathetic output via the activation of Y1 receptors colocalised on the thyrosine hydroxylase producing neurons in the PVN (Loh et al., 2015). Therefore, we cannot exclude that there is a basal difference in the number of specific cell types that express Y1R gene in ob/ob mice, compared with lean mice, which could explain, at least in part, the differential changes in the LacZ transgene expression in response to leptin treatment observed in the two genotypes.

Results presented in this paper do not address the molecular mechanisms through which leptin modifies Y_1R gene transcription (Higuchi et al., 2005). However, since leptin was found to regulate NPY gene transcription, and NPY regulates leptin receptor gene expression through a Y_1R dependent mechanism (Di Yorio et al., 2014), a direct effect of leptin on NPY and Y_1R transcriptional pathway cannot be excluded (Dhillon and Belsham, 2011).

In the present study we also demonstrated that, although leptin treatment induces a greater weight loss in females, it fails to affect Y1R gene expression in the hypothalamus of lean and obese female mice. These results suggest that Y_1R is differentially sensitive to leptin treatment in males and females. A sexual dimorphism of the expression of NPY and Y_1R receptors has been described in Tg*Y1R/LacZ* mice (Bo et al., 2016; Martini et al., 2011). In addition, ablation of NPY in obese *ob*/*ob* mice induces a decrease on body weight that is significantly more pronounced in female than in male mice (Erickson et al., 1996; Naveilhan et al., 2002). Finally, Y_1R knockout female, but not male, mice develop late onset obesity (Kushi et al., 1998; Lin et al., 2006). Results from the present study suggest that these differences might be related to a different sensitivity of the NPY-Y_1R pathway to the anorectic effect of leptin. Accordingly, we have previously reported that the consumption of high fat diet increases body weight and modulates Y_1R gene expression in the VMH and DMH of Tg*Y1R/LacZ* male but not in female mice (Zammaretti et al., 2007).
In conclusion, in the present study we provide further insights on the regulation of NPY-Y_1R pathway by leptin that might contribute to adaptive responses to weight loss in males.

Experimental Procedure

4.1 Experimental Animals

Y1R/LacZ FVB mice (Tg*Y1R/LacZ*) from transgenic line 62 of our breeding colony were used in this study (Oberto et al., 1998). Heterozygous *ob*/+ C57BL/6-6J mice were purchased from Charles River Laboratories (Lecco, Italy). To obtain genetically leptin-deficient Tg*Y1R/LacZ* mice, heterozygous *ob*/+ mice were crossed with homozygous Tg*Y1R/LacZ* mice. Double heterozygous mice were crossed again to obtain mice homozygous for the *Y1R/LacZ* transgene and bearing the *ob*/*ob*, *ob*/+ or +/+ genotypes. Tg$Y_1R/LacZ^{ob/+}$ mice were used for breeding to expand the mouse line. Genetically obese Tg$Y_1R/LacZ^{ob/ob}$ and lean Tg$Y_1R/LacZ^{+/+}$ mice were used from F10 for the experiments. Offspring were genotyped by PCR followed by restriction enzyme digestion (for *ob*

mutation) or by Southern blot hybridization (for *Y_1R/LacZ*) (Chehab et al., 1996; Oberto et al., 1998).

Mice were individually housed in standard cages and maintained at constant temperature (21± 2°C) in a 12:12 hr light/dark cycle. Food and water were provided *ad libitum*. All experiments were conducted in accordance with the European Community Council Directive of 24 November 1986 86/609/EEC and 6106/10/EU and approved by the University of Turin Ethical Committee for animal research and by the Italian Ministry of Health (License No. 180/2006-B).

Two months-old obese (Tg*Y_1R/LacZ$^{ob/ob}$*) male and female mice or lean (Tg*Y_1R/LacZ$^{+/+}$*) male and random cycling female mice were used in this study. Mice were treated for one week with two daily intraperitoneal injections (8:00 a.m. and 8:00 p.m) of 1 μg/g murine recombinant leptin (Sigma-Aldrich, Milano, IT) or saline (Ahima et al., 1999; Zammaretti et al., 2001). All mice were weighted at 9:00 am immediately before (D1) and at the end (D7) of the treatment and killed by cervical dislocation at the end of D7. Brains were quickly removed, placed in 10% embedding medium (Bio-optica, Milano, Italy) in PBS, frozen on crushed dry ice, and stored at -80 °C until assayed.

Y_1R/LacZ expression was determined by histochemical staining of β-galactosidase activity in mice brain coronal sections, as previously described (Zammaretti et al., 2001). Briefly, frozen brains were cut on a cryostat at -20°C and 25 μm-thick sections were collected on clean slides starting from a level corresponding to the end of the anterior commissure. Sections were dehydrated with acetone-chloroform (1:1), air dried and shortly fixed in 2.5 % glutaraldehyde in PBS (each step for 5 minutes on ice), and incubated overnight at 37° C in a solution containing 1 mg/ml of X-gal, 5mM potassium ferrocyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% Triton X-100 in PBS. After washing in water, sections were counterstained with nuclear fast red, dried and coverslipped with DPX mounting medium (Fluka, Buchs, Switzerland).

4.2 Quantitation of transgene expression as determined by β-galactosidase activity

Three standardized sections of comparable levels per animal were analyzed for each of these nuclei: PVN (around bregma -0.70/-0.82 mm), rostral ARC (around bregma -1.34 mm), rostral VMH (around bregma -1.22 mm) and rostral DMH (around bregma -1.46 mm) (Paxinos and Franklin, 2001). Quantification of the *Y_1R/LacZ* transgene expression was made by computer assisted morphometric analysis (Image J, Rasband, 2008), as previously described (Oberto et al., 2003; Zammaretti et al., 2001; Zammaretti et al., 2007). Briefly, the region of interest was identified through a built-in green filter, whereas a red filter was employed to increase the contrast of beta-galactosidase blue dots. The transgene is expressed in the neuronal cell body and is typically detected as a juxtanuclear blue dot (Oberto et al., 1998).

In our conditions, the size, in square pixels, of the dots ranges from 4 to 25 square pixels. Therefore we set the Image J particle analyzer to remove from calculations all the objects less than 4 square pixels. For each animal and nucleus, the cumulative number of dots and the cumulative areas of the analyzed sections were considered to obtain the density of expression of the transgene as dots per μm^2.

4.3 Data analysis

A four-way analysis of variance (ANOVA) for repeated measures (sex, genotype, and treatment as independent variables, D1 and D7 as repeated measures) was used to compare mean body weight. This analysis was followed by a three-way ANOVA for repeated measures (sex and treatment as independent variables, D1 and D7 weights as repeated measures) independently performed on obese Tg*Y_1R/LacZ*$^{ob/ob}$ or lean Tg*Y_1R/LacZ*$^{+/+}$ mice.

Quantitative analysis of transgene expression was examined using four-way ANOVA for repeated measures (sex, genotype, and treatment as independent variables, different nuclei values as repeated measures). This analysis was followed by a three-way ANOVA for repeated measures (genotype, and treatment as independent variables, different nuclei values as repeated measures) independently performed on males and females. A two-way factorial ANOVA was then performed on each nucleus of male obese Tg*Y_1R/LacZ*$^{ob/ob}$ or lean Tg*Y_1R/LacZ*$^{+/+}$ mice.

All data are expressed as means ± S.E.M and the level of statistical significance was set at $p<0.05$ for all comparisons. When the results of the ANOVA were significant, the appropriate contrasts were analysed by Fisher's PLDS test.

Conflict of interest

The authors report no conflict of interest.

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Figure Legends

Fig.1

Leptin treatment decreases body weight of lean Tg*Y1R*/*LacZ*$^{+/+}$ and obese Tg*Y1R*/*LacZ*$^{ob/ob}$ male and female mice. Body weight of lean Tg*Y1R*/*LacZ*$^{+/+}$ (lean) and obese Tg*Y1R*/*LacZ*$^{ob/ob}$ (obese) male and female mice before (day 1, D1) and at the end (day 7, D7) of treatment with murine recombinant leptin (1 μg/g, twice a day). Data are the mean ±SEM; n= 9-11 (males) and 8-12 (females) from three litters. Three-way analysis of variance for repeated measures revealed a significant effect of genotype [$F(1,36)= 555$, $p<0.001$], day of treatment [$F(1,36)= 340$, $p<0.001$], genotype-sex interaction [$F(1,36)= 25.8$, $p<0.001$], genotype-day of treatment interaction [$F(1,36)= 111$, $p<0.001$] and genotype-sex-day of treatment interaction [$F(1,36)= 4.94$, $p=0.033$]. # $P<0.01$ versus D1; ** $P<0.01$ versus males paired for genotype and day of treatment; § $P<0.01$ versus lean males and females at D1, respectively.

Fig.2

Coronal sections illustrating β-galactosidase activity in the paraventricular nucleus (PVN) of male Tg*Y$_1$R*/*LacZ* mice from different experimental groups.

+/+, lean Tg*Y$_1$R*/*LacZ*$^{+/+}$ mice treated twice a day for one week with vehicle; **+/+ (+Lep)**, lean Tg*Y$_1$R*/*LacZ*$^{+/+}$ mice treated twice a day for one week with 1 μg/g of murine recombinant leptin; **ob**, obese Tg*Y$_1$R*/*LacZ*$^{ob/ob}$ mice treated twice a day for one week with vehicle; **ob (+Lep)**, obese Tg*Y$_1$R*/*LacZ*$^{ob/ob}$ mice treated twice a day for one week with 1 μg/g of murine recombinant leptin. Scale bar= 100 μm.

Pictures were digitized using a red filter to enhance the histochemical staining.

Fig. 3

Coronal sections illustrating β-galactosidase activity in the dorsomedial nucleus (DMH) of male Tg*Y$_1$R*/*LacZ* mice from different experimental groups.

+/+, lean Tg$Y_1R/LacZ^{+/+}$ mice treated twice a day for one week with vehicle; **+/+ (+Lep)**, lean Tg$Y_1R/LacZ^{+/+}$ mice treated twice a day for one week with 1 μg/g of murine recombinant leptin; **ob**, obese Tg$Y_1R/LacZ^{ob/ob}$ mice treated twice a day for one week with vehicle; **ob (+Lep)**, obese Tg$Y_1R/LacZ^{ob/ob}$ mice treated twice a day for one week with 1 μg/g of murine recombinant leptin. Scale bar= 200 μm. Pictures were digitized using a red filter to enhance the histochemical staining.

Fig. 4

Effect of leptin treatment on β-galactosidase activity in the paraventricular (**PVN**), dorsomedial (**DMH**),ventromedial (**VMH**) and arcuate (**ARC**) hypothalamic nuclei of lean and obese male and female mice.

+/+, lean Tg$Y_1R/LacZ^{+/+}$ mice treated twice a day for one week with vehicle; **+/+ (+Lep)**, lean Tg$Y_1R/LacZ^{+/+}$ mice treated twice a day for one week with 1 μg/g of murine recombinant leptin; **ob**, obese Tg$Y_1R/LacZ^{ob/ob}$ mice treated twice a day for one week with vehicle; **ob (+Lep)**, obese Tg$Y_1R/LacZ^{ob/ob}$ mice treated twice a day for one week with 1 μg/g of murine recombinant leptin. Data are expressed as density of blue dots and are the mean ± SEM; n= 9-14 (males) and 7-12 (females) from three litters.

Four-way ANOVA for repeated measures to determine significant effects of sex, genotype and treatment on different nuclei (repeated measures), and their interactions, showed significant effects of sex [$F(1,74)= 4.99$, $P=0.028$], nuclei [$F(3,222)= 870$, $P<0.001$] and for sex x genotype ($F(1,74)=6.84$, $P=0.011$), sex x genotype x treatment ($F(1,74)=7.18$, $P=0.009$), nuclei x sex ($F(3,222)=4.93$, $P=0.002$), nuclei x sex x genotype x treatment ($F(3,222)=3.15$, $P=0.026$) interactions. Sex-dependent differences were analyzed by three-way ANOVA for repeated measures independently performed on males and females that showed significant effects of genotype [$F(1,41)= 8.37$, $P=0.006$], nuclei [$F(3,123)= 578,8$, $P<0.001$] and genotype x treatment [$F(1,41)= 10.69$, $P=0.002$) and nuclei x genotype x treatment interactions [$F(1,123)= 5.98$, $P<0.001$) only in males. A two-way factorial ANOVA performed on each nucleus in males showed significant effects

of genotype [DMH: F(1,41)=13.68, P<0.001; VMH: F(1,41)=14.06, P<0.001] and of treatment x genotype interaction [PVN: F(1,41)= 13.86, P<0.001, DMH: F(1,41)=13.07, P<0.001]. *: p<0.05 versus Tg$Y_1R/LacZ^{+/+}$ male mice, **: p<0.05 versus Tg$Y_1R/LacZ^{ob/ob}$ male mice by Fisher's test.

REFERENCES

Ahima, R.S., Bjorbaek, C., Osei, S., Flier, J.S., 1999. Regulation of Neuronal and Glial Proteins by Leptin: Implications for Brain Development 1. Endocrinology. 140, 2755-2762.

Beck, B., Richy, S., Dimitrov, T., Stricker-Krongrad, A., 2001. Opposite regulation of hypothalamic orexin and neuropeptide Y receptors and peptide expressions in obese Zucker rats. Biochem Biophys Res Commun. 286, 518-23.

Bergonzelli, G.E., Pralong, F.P., Glauser, M., Cavadas, C., Grouzmann, E., Gaillard, R.C., 2001. Interplay between galanin and leptin in the hypothalamic control of feeding via corticotropin-releasing hormone and neuropeptide Y. Diabetes. 50, 2666-72.

Bertocchi, I., Oberto, A., Longo, A., Mele, P., Sabetta, M., Bartolomucci, A., Palanza, P., Sprengel, R., Eva, C., 2011. Regulatory functions of limbic Y1 receptors in body weight and anxiety uncovered by conditional knockout and maternal care. Proc Natl Acad Sci U S A. 108, 19395-400.

Bo, E., Farinetti, A., Marraudino, M., Sterchele, D., Eva, C., Gotti, S., Panzica, G.C., 2016. Adult exposure to tributyltin affects hypothalamic neuropeptide Y, Y1 receptor distribution, and circulating leptin in mice. Andrology, 2016, doi: 10.1111/andr.12222

Broberger, C., 1999. Hypothalamic cocaine-and amphetamine-regulated transcript (CART) neurons: histochemical relationship to thyrotropin-releasing hormone, melanin-concentrating hormone, orexin/hypocretin and neuropeptide Y. Brain research. 848, 101-113.

Campfield, L.A., 2000. Central mechanisms responsible for the actions of OB protein (leptin) on food intake, metabolism and body energy storage. Front Horm Res. 26, 12-20.

Chehab, F.F., Lim, M.E., Lu, R., 1996. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. Nat Genet. 12, 318-20.

Coleman, D.L., 1985. Increased metabolic efficiency in obese mutant mice. Int J Obes. 9 Suppl 2, 69-73.

Cyr, N.E., Toorie, A.M., Steger, J.S., Sochat, M.M., Hyner, S., Perello, M., Stuart, R., Nillni, E.A., 2013. Mechanisms by which the orexigen NPY regulates anorexigenic α-MSH and TRH. American Journal of Physiology-Endocrinology and Metabolism. 304, E640-E650.

D'Souza A, M., Asadi, A., Johnson, J.D., Covey, S.D., Kieffer, T.J., 2014. Leptin deficiency in rats results in hyperinsulinemia and impaired glucose homeostasis. Endocrinology. 155, 1268-79.

Dhillon, S.S., Belsham, D.D., 2011. Leptin differentially regulates NPY secretion in hypothalamic cell lines through distinct intracellular signal transduction pathways. Regul Pept. 167, 192-200.

Di Yorio, M.P., Bilbao, M.G., Faletti, A.G., 2014. Neuropeptide Y regulates the leptin receptors in rat hypothalamic and pituitary explant cultures. Regul Pept. 188, 13-20.

Erickson, J.C., Hollopeter, G., Palmiter, R.D., 1996. Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. Science. 274, 1704-7.

Eva, C., Serra, M., Mele, P., Panzica, G.C., Oberto, A., 2006. Physiology and gene regulation of the brain NPY Y1 receptor. Frontiers in Neuroendocrinology. 27, 308-339.

Herzog, H., 2003. Neuropeptide Y and energy homeostasis: insights from Y receptor knockout models. Eur J Pharmacol. 480, 21-9.

Higuchi, H., Hasegawa, A., Yamaguchi, T., 2005. Transcriptional regulation of neuronal genes and its effect on neural functions: transcriptional regulation of neuropeptide Y gene by leptin and its effect on feeding. J Pharmacol Sci. 98, 225-31.
Huang, X.-F., Han, M., Storlien, L.H., 2003. The level of NPY receptor mRNA expression in diet-induced obese and resistant mice. Molecular Brain Research. 115, 21-28.
Jequier, E., 2002. Leptin signaling, adiposity, and energy balance. Ann N Y Acad Sci. 967, 379-88.
Kaiyala, K.J., Ogimoto, K., Nelson, J.T., Schwartz, M.W., Morton, G.J., 2015. Leptin signaling is required for adaptive changes in food intake, but not energy expenditure, in response to different thermal conditions. PLoS One. 10, e0119391.
Kalra, S.P., Kalra, P.S., 2007. To subjugate NPY is to improve the quality of life and live longer. Peptides. 28, 413-8.
Kim, E.M., O'Hare, E., Grace, M.K., Welch, C.C., Billington, C.J., Levine, A.S., 2000. ARC POMC mRNA and PVN alpha-MSH are lower in obese relative to lean zucker rats. Brain Res. 862, 11-6.
Kishi, T., Aschkenasi, C.J., Choi, B.J., Lopez, M.E., Lee, C.E., Liu, H., Hollenberg, A.N., Friedman, J.M., Elmquist, J.K., 2005. Neuropeptide Y Y1 receptor mRNA in rodent brain: Distribution and colocalization with melanocortin-4 receptor. Journal of Comparative Neurology. 482, 217-243.
Kushi, A., Sasai, H., Koizumi, H., Takeda, N., Yokoyama, M., Nakamura, M., 1998. Obesity and mild hyperinsulinemia found in neuropeptide Y-Y1 receptor- deficient mice. Proceedings of the National Academy of Sciences of the United States of America. 95, 15659-15664.
Lin, E.J., Sainsbury, A., Lee, N.J., Boey, D., Couzens, M., Enriquez, R., Slack, K., Bland, R., During, M.J., Herzog, H., 2006. Combined deletion of Y1, Y2, and Y4 receptors prevents hypothalamic neuropeptide Y overexpression-induced hyperinsulinemia despite persistence of hyperphagia and obesity. Endocrinology. 147, 5094-101.
Loh, K., Herzog, H., Shi, Y.C., 2015. Regulation of energy homeostasis by the NPY system. Trends Endocrinol Metab. 26, 125-35.
Martini, M., Sica, M., Gotti, S., Eva, C., Panzica, G.C., 2011. Effects of estrous cycle and sex on the expression of neuropeptide Y Y1 receptor in discrete hypothalamic and limbic nuclei of transgenic mice. Peptides. 32, 1330-4.
Morrison, C.D., Morton, G.J., Niswender, K.D., Gelling, R.W., Schwartz, M.W., 2005. Leptin inhibits hypothalamic Npy and Agrp gene expression via a mechanism that requires phosphatidylinositol 3-OH-kinase signaling. Am J Physiol Endocrinol Metab. 289, E1051-7.
Morton, G.J., Cummings, D.E., Baskin, D.G., Barsh, G.S., Schwartz, M.W., 2006. Central nervous system control of food intake and body weight. Nature. 443, 289-95.
Myers, M.G., Cowley, M.A., Munzberg, H., 2008. Mechanisms of leptin action and leptin resistance. Annu Rev Physiol. 70, 537-56.
Naveilhan, P., Svensson, L., Nystrom, S., Ekstrand, A.J., Ernfors, P., 2002. Attenuation of hypercholesterolemia and hyperglycemia in ob/ob mice by NPY Y2 receptor ablation. Peptides. 23, 1087-91.
Oberto, A., Tolosano, E., Brusa, R., Altruda, F., Panzica, G., Eva, C., 1998. The murine Y1 receptor 5' upstream sequence directs cell-specific and developmentally regulated LacZ expression in transgenic mice CNS. European Journal of Neuroscience. 10, 3257-68.
Oberto, A., Mele, P., Zammaretti, F., Panzica, G.C., Eva, C., 2003. Evidence of Altered Neuropeptide Y Content and Neuropeptide Y1 Receptor Gene Expression in the Hypothalamus of Pregnant Transgenic Mice. Endocrinology. 144, 4826-4830.
Park, H.K., Ahima, R.S., 2014. Physiology of leptin: energy homeostasis, neuroendocrine function and metabolism. Metabolism. 64, 24-34.
Paxinos, G., Franklin, K.B.J., 2001. The Mouse Brain in Stereotaxic Coordinates. Vol., Academi Press, San Diego.

Pedrazzini, T., 2004. Importance of NPY Y1 receptor-mediated pathways: assessment using NPY Y1 receptor knockouts. Neuropeptides. 38, 267-75.
Pralong, F.P., Gonzales, C., Voirol, M.J., Palmiter, R.D., Brunner, H.R., Gaillard, R.C., Seydoux, J., Pedrazzini, T., 2002. The neuropeptide Y Y1 receptor regulates leptin-mediated control of energy homeostasis and reproductive functions. FASEB Journal. 16, 712-714.
Rasband, W.S., 2008. Image J. Bethesda, MD: US National Institutes of Health; 1997–2007. World Wide Web (http://rsb. info. nih. gov/ij/).
Roseberry, A.G., Liu, H., Jackson, A.C., Cai, X., Friedman, J.M., 2004. Neuropeptide Y-mediated inhibition of proopiomelanocortin neurons in the arcuate nucleus shows enhanced desensitization in ob/ob mice. Neuron. 41, 711-22.
Sahu, A., 2003. Leptin signaling in the hypothalamus: emphasis on energy homeostasis and leptin resistance. Front Neuroendocrinol. 24, 225-53.
Schwartz, M.W., Baskin, D.G., Bukowski, T.R., Kuijper, J.L., Foster, D., Lasser, G., Prunkard, D.E., Porte, D., Jr., Woods, S.C., Seeley, R.J., Weigle, D.S., 1996. Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. Diabetes. 45, 531-5.
Schwartz, M.W., Baskin, D.G., Kaiyala, K.J., Woods, S.C., 1999. Model for the regulation of energy balance and adiposity by the central nervous system. Am J Clin Nutr. 69, 584-96.
Schwartz, M.W., Woods, S.C., Porte, D., Jr., Seeley, R.J., Baskin, D.G., 2000. Central nervous system control of food intake. Nature. 404, 661-671.
Sousa-Ferreira, L., Garrido, M., Nascimento-Ferreira, I., Nobrega, C., Santos-Carvalho, A., Alvaro, A.R., Rosmaninho-Salgado, J., Kaster, M., Kugler, S., de Almeida, L.P., Cavadas, C., 2011. Moderate long-term modulation of neuropeptide Y in hypothalamic arcuate nucleus induces energy balance alterations in adult rats. PLoS One. 6, e22333.
Spiegelman, B.M., Flier, J.S., 2001. Obesity and the regulation of energy balance. Cell. 104, 531-543.
Takahashi, K.A., Cone, R.D., 2005. Fasting induces a large, leptin-dependent increase in the intrinsic action potential frequency of orexigenic arcuate nucleus neuropeptide Y/Agouti-related protein neurons. Endocrinology. 146, 1043-7.
Wang, B., Chandrasekera, P.C., Pippin, J.J., 2014. Leptin- and leptin receptor-deficient rodent models: relevance for human type 2 diabetes. Curr Diabetes Rev. 10, 131-45.
Widdowson, P.S., Upton, R., Henderson, L., Buckingham, R., Wilson, S., Williams, G., 1997. Reciprocal regional changes in brain NPY receptor density during dietary restriction and dietary-induced obesity in the rat. Brain Res. 774, 1-10.
Zammaretti, F., Panzica, G.C., Eva, C., 2001. Fasting, leptin treatment and glucose administration differentially regulate Y1 receptor gene expression in the hypothalamus of transgenic mice. Endocrinology. 142, 3774-3782.
Zammaretti, F., Panzica, G., Eva, C., 2007. Sex-dependent regulation of hypothalamic neuropeptide Y-Y1 receptor gene expression in moderate/high fat, high-energy diet-fed mice. J Physiol. 583, 445-54.

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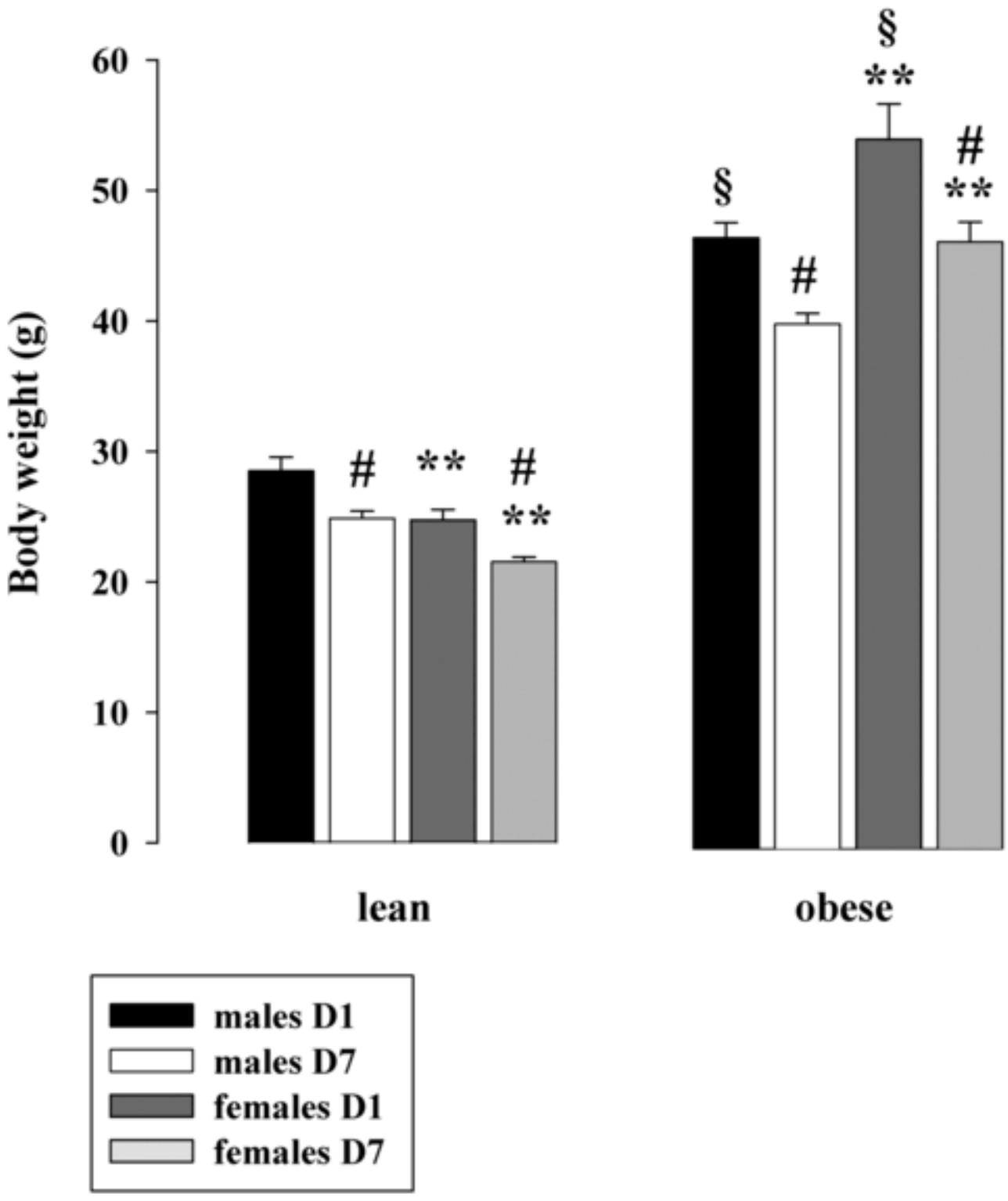

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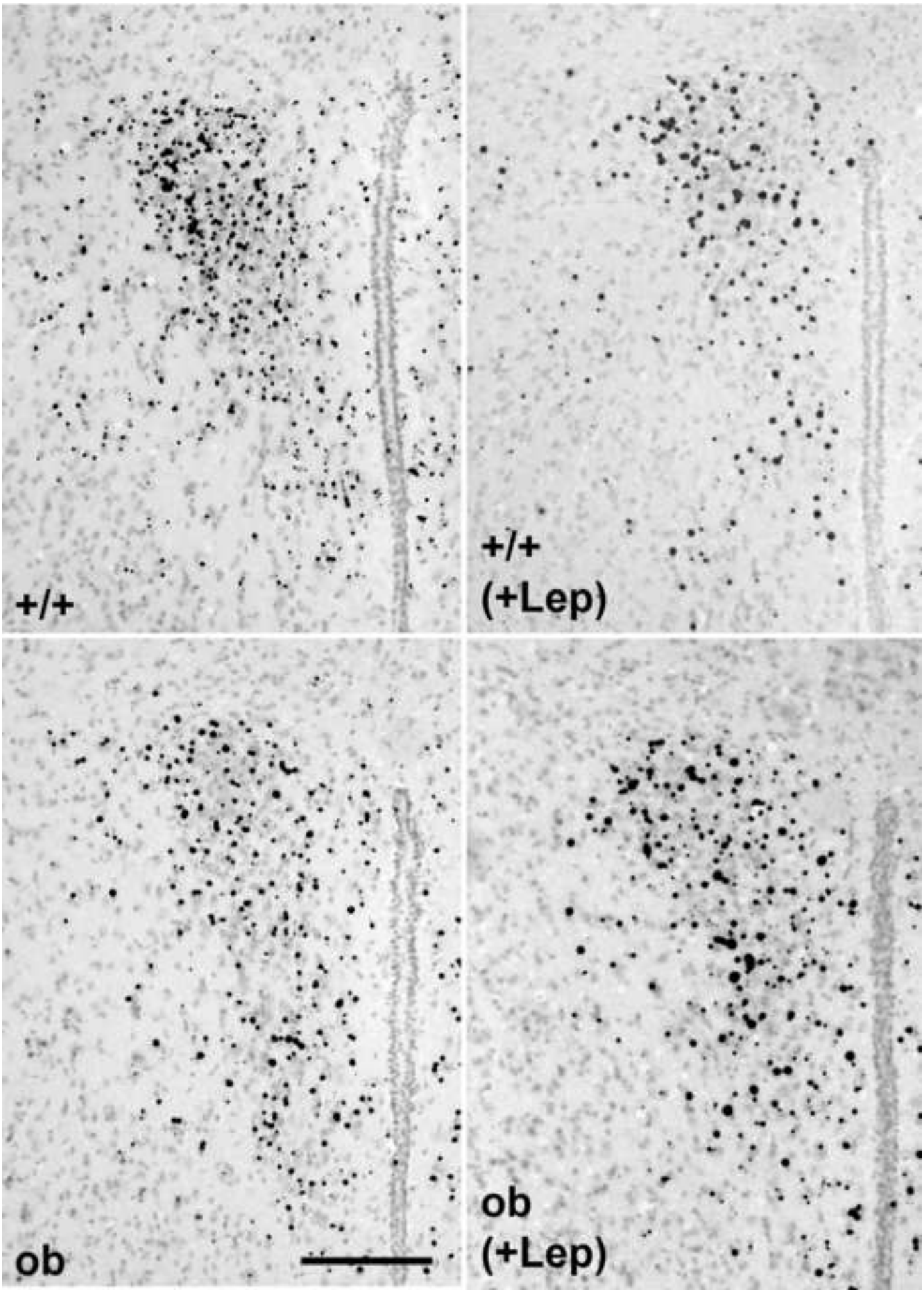

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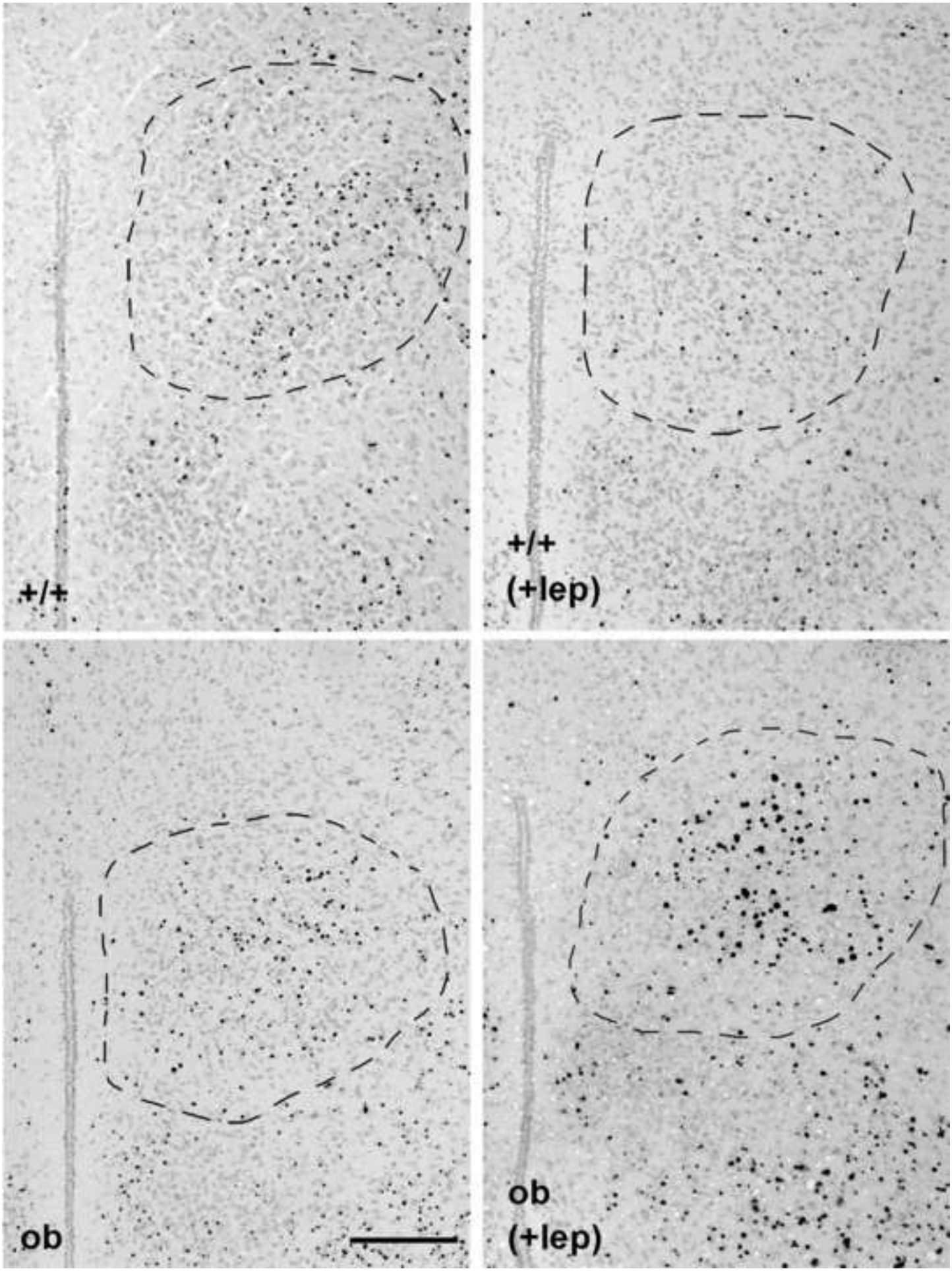

Figure 4

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