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Running title: Effects of TBT on mice leptin-NPY system

Key words: TBT; NPY; food intake; arcuate nucleus; paraventricular nucleus; dorsomedial nucleus; C57BL/6 mice

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ABSTRACT

Tributyltin (TBT) is a pesticide, used in antifouling paints, toxic for aquatic invertebrates. In vertebrates, TBT may act as obesogen inducing adipogenetic genes transcription for adipocites differentiation. In a previous study we demonstrated that acute administration of TBT induces c-fos expression in arcuate nucleus, therefore, in the present study we tested the hypothesis that adult exposure to TBT may alter part of the nervous pathways controlling animal food intake. In particular, we investigated the expression of NPYimmunoreactivity. This neuropeptide forms neural circuits dedicated to food assumption and its action is mediated by Y1 receptors that are widely expressed in the hypothalamic nuclei responsible for the regulation of food intake and energy homeostasis. To this purpose, TBT was orally administered at a dose of 0.025 mg/Kg/day/body weight to adult animals (male and female C57BL/6□Y1-LacZ transgenic mice) for 4 weeks.

No differences were found in body weight and fat deposition, but we observed significant increase of feed efficiency in TBT-treated male mice and a significant decrease of circulating leptin in both sexes. Computerized quantitative analysis of NPY immunoreactivity and Y1-related beta-galactosidase activity, demonstrated a statistically significant reduction of NPY and Y1 transgene expression in the hypothalamic circuit controlling food intake of treated-male mice in comparison to controls. In conclusion, present results indicate that adult exposure to TBT is profoundly interfering with the nervous circuits involved in the stimulation of food intake.

INTRODUCTION

Obesity is defined as excessive deposition of body fat, and it is caused by complex interactions between genetic, behavioral and environmental factors. In humans, the most common causes are overeating high caloric fatty diets combined with a sedentary lifestyle and a genetic predisposition for the disease (Newbold, et al., 2007). Adipose tissue is not just a passive lipid repository, it can, in fact, act as an endocrine organ producing several factors including a peptide hormone (called leptin or Ob protein) which is responsible to communicate energy reserve information from adipocites to other body's organs including the central nervous system (Myers, et al., 2009).

Leptin is produced by the Ob-gene that is expressed in all adipose cells in proportion to fat accumulated, and it has the highest expression in the subcutaneous fat (Montague, et al., 1998). Leptin serves to communicate the levels of energy storage, to induce the state of satiety and to suppress food intake by its action on neuroendocrine systems (Ahima, et al., 2000). These actions are therefore finalized to increase energy expenditure (Bates, et al., 2003, Friedman and Halaas, 1998). Circulating leptin can pass across the blood-brain-barrier (Banks and Kastin, 1996) and leptin binds, in the brain, to Ob-receptors (Ob-R) expressed in the hypothalamus, mainly in arcuate (ARC), paraventricular (PVN), dorsomedial (DMH) hypothalamic nuclei, and lateral hypothalamic area (LHA) (Mercer, et al., 1996). Part of Ob-R-expressing neurons of LHA are inhibiting elements, therefore, through these neurons leptin may modulate the mesolimbic dopamine reward system to suppress feeding behavior (Leinninger, et al., 2009). These data indicate that the hypothalamus is a key site for leptin action, although other brain regions may be also involved in direct or indirect manner. In the hypothalamic circuits, leptin at the same time reduces food intake [by its inhibition of neuropeptide Y (NPY) system] and increases energy expenditure [by stimulating the α-melanocyte-stimulating hormone (α-MSH) system] (Myers, et al., 2009, Robertson, et al., 2008). NPY is an orexigenic peptide, made of 36 aminoacids, representing the most abundant peptide of the hypothalamus (for a recent review see Nguyen, et al., 2011). In the circuit controlling food intake NPY is synthesized by neurons, located within the ARC, projecting to other hypothalamic nuclei of the circuit: PVN, ventromedial nucleus

(VMH) and DMH [for a review see (Valassi, et al., 2008)]. Acute central administration of NPY induces increase of food intake (Stanley and Leibowitz, 1985), whereas chronic administration produces hyperfagia, decrease of thermogenesis and obesity (Stanley, et al., 1986). The action of NPY is mediated by several receptors, among them: Y1 and Y5 receptors are mainly related to the food control (Beck, 2006). In addition to its role to regulate feeding behavior, Y1-R is involved in several NPY-induced responses, such as anxiolytic effects and stress responses (Bertocchi, et al., 2011, Longo, et al., 2015), ethanol drinking behavior, activation of neuroendocrine axis (for a review see Eva, et al., 2006). The Y5-R subtype may have some role in the control of nesting behavior, i.e. during lactation with effects on litter growth rates (Ladyman and Woodside, 2009). Thus, the central nervous system plays a fundamental role in food intake regulation integrating neural and endocrine factors. According to this point of view, obesity is not only dependent by peripheral change of energy homeostasis, but can also be considered a "neuroendocrine disease" depending on alteration of the axis leptin/hypothalamic circuits controlling food intake (Austin and Marks, 2009).

Several studies demonstrated that so-called endocrine disrupting chemicals (EDCs) may alter several neural circuits and behaviors (for reviews see Frye, et al., 2012, Panzica, et al., 2011, Panzica, et al., 2007). In particular, it has been predicted the existence of EDCs that inappropriately regulate lipid metabolism and adipogenesis to promote obesity, defined as obesogens (Grun and Blumberg, 2006), and now classified among the more inclusive category of metabolic disruptors (Heindel, et al., 2015). For example, organotins, in particular tributyltin (TBT), largely used as antifouling agents in paints and in many industrial processes, can promote obesity by activating peroxisome proliferator-activated receptor γ (PPARγ) and retinoid X receptor (RXRα, RXRβ, and RXRγ), that induces in vitro differentiation of pre-adipocites to adipocites, while *in vivo* can lead to an increase of body fat storage (for a recent review see Grun, 2014). The wide distribution and the high persistence of TBT in fresh and salt water, have raised concern about its adverse effects to the animal (in particular marine invertebrates and fishes, Leung, et al., 2006, Lima, et al., 2015) and human health (Rantakokko, et al., 2014, Rantakokko, et al., 2013).

Data collected by several experimental studies clearly indicate a role of TBT on

obesity development in vertebrates (Grun, et al., 2006) probably through its action to predispose multipotent stem cells to become adipocytes (Kirchner, et al., 2010). However, currently, the effects of this compound on the central nervous system have received less attention (Decherf and Demeneix, 2011). *In vitro* studies demonstrated that TBT may induce the expression of c-fos (Matsuoka and Igisu, 1996), apoptosis (Nakatsu, et al., 2008, Nakatsu, et al., 2006, Nakatsu, et al., 2007), and inhibit dopamine biosynthesis (Lee, et al., 2006) in PC12 cell cultures. In primary cultures of dissociated neurons, TBT increased the Ca^{2+} -activated K^{+} current induced by NMDA application (Kanemoto, et al., 2002), is involved in cell death by glutamate excitotoxicity (Nakatsu, et al., 2006), and it has different effects on dissociated cells from different brain regions (Mitra, et al., 2014).

With respect to the *in* vivo studies, it has been demonstrated that TBT may cross the placenta (Kimura, et al., 2005) and the blood-brain barrier (Hara, et al., 1994), passing into the maternal milk and accumulating in pups' tissues, including the brain (Cooke, et al., 2008). Therefore, the central nervous system is a potential target for its action, at least for exposition during pregnancy or lactation. The exposure to TBT during the gestational period induces hypothyroidism in the progeny, whereas the acute treatment of pregnant females in the same period induces a dose-dependent increase of T_3-independent TRH transcription levels (Decherf, et al., 2010). TBT induces oxidative damages in various region of the adult rat brain, including cerebellum, hippocampus, hypothalamus, and striatum (Mitra, et al., 2015). In an earlier study we demonstrated that TBT acute administration induces, in short time, the expression of c-fos in ARC (Bo, et al., 2011) a key hypothalamic nucleus for the control of food intake (Myers, Munzberg, Leinninger and Leshan, 2009). More recently, He and coworkers (He, et al., 2014) demonstrated alterations in the mRNA content of hypothalamic peptides [NPY, pro-opiomelanocortin (POMC)] in adult male and female rats exposed to TBT for 8 weeks. In the present study, we investigated if the reported adverse effects of TBT on some physiological parameters may also affect brain NPY/Y1-R circuits involved in feeding control (PVN, ARC, DMH and VMH). As in previous studies in rodents (Cooke, et al., 2004, Tryphonas, et al., 2004), the doses of TBT selected for this study was based on the NOEL (No Observed Effect Level) dose corresponding to 0.025µg/g body weight /day (EFSA, 2004).

MATERIALS AND METHODS

Chemicals

Tributyltin chloride (TBT) in stock solution (96%) was obtained from Sigma-Aldrich, Europe (Aldrich Catalogue, T50202).

The primary antibody anti-NPY (a generous gift from H. Vaudry, Rouen, France) was raised in rabbit against synthetic porcine NPY; its production and the results of the assays performed to control its specificity and cross-reactivity with other neuropeptides have been previously described (Pelletier, et al., 1984, Pelletier, et al., 1984).

The Enzyme-Linked Immunoabsorbent Assay (ELISA) kit to detect leptin levels in mice's blood was purchased from SpiBio (INALCO, Milano, Italy). All other chemicals were obtained from commercial sources.

Tributyltin (96%) liquid was diluted in olive oil (Sigma-Aldrich, Europe) to obtain a final amount of TBT corresponding to the selected doses of 0.025mg/kg of body weight in a final volume of 30 µl that was delivered to each animal. The dosing solutions were prepared fresh weekly and varied with the current weight of the mice.

Animals and treatment

Animal care and handling throughout the experimental procedures were in accordance with the European Union Council Directive of 24 November 1986 (86/609/EEC) and the Italian Ministero della Sanità and the ethical Committee of the University of Torino approved the procedures reported in the present study.

Adult (3 months old) male and female C57BL/6 mice, from our laboratory, were housed at 22 ± 1°C under a photoperiod of 12 h light each day, with water and food *ad libitum*. Mice belong to a transgenic strain generated in our laboratory (Oberto, et al., 1998), carrying the 1.3 Kb sequence of the mouse Y1-R promoter fused with the coding region of the Escherichia Coli *LacZ* gene. Analysis of Y1-R/LacZ transgene activity by histochemical staining of β-galactosidase with X-gal demonstrated that this construct contains sufficient information to replicate the expression pattern of the endogenous Y1-R gene in a CNS-restricted and developmental stage-specific manner. This transgenic model has been previously employed in our laboratory to quantitatively evaluate in vivo

changes in transgene expression (Ferrara, et al., 2001, Martini, et al., 2011, Oberto, et al., 2003, Oberto, et al., 2000, Oberto, et al., 2001, Oberto, et al., 1998, Zammaretti, et al., 2007, Zammaretti, et al., 2001).

First experiment

A preliminary experiment was performed with 48 C57BL/6-Y1-R/LacZ male mice in order to choose the correct dose. According to some previous studies made on rats (Cooke, et al., 2004, Tryphonas, et al., 2004) we tried a range of 0.025, 0.25, or 2.5mg of tributyltin (TBT) per Kg of body weight per day. Males were randomly divided in the four experimental groups, and the experiment should originally cover 4 weeks (see below for the description of the TBT administration). However, the mice of the 2.5 and 0.25 groups died in a few days before the end of the first week (see Table 1). The dose of 0.025mg/Kg of body weight (classified as NOAEL dose in the literature) was therefore selected for the second experiment.

Second experiment

Twenty four C57BL/6-Y1-R/LacZ mice with adequate weight gain and without clinical signs were divided randomly into four experimental groups and individually housed: six males (M) and six females (F) were the control groups treated with vehicle only (OIL), six males and six females received daily 0.025mg/kg of body weight per day of TBT. The vehicle or the TBT solution was administered through a pipette with a not stressful procedure (Palanza, et al., 2002). In brief, to reduce stress in animals, they were manipulated for a period of ten days before starting the experiment, and then mice were picked up by the skin between the shoulders and held upright. A micropipette tip was placed into the mouth with the pipette tip gently touching the roof of the mouth, and the oil (final volume 30µl) was ejected from the pipette. Mice readily consume oil. The treatment was prolonged for 4 weeks.

Feed efficiency and body weight

Animals were fed with a standard diet 4RF21 GLP certificate (Mucedola, Italy) containing 2.668 Kcal/g of metabolizable energy with 21.7 % as protein, 0.4% as fat and 66.5% as carbohydrate.

Body weight was recorded daily, before the oral administration of TBT, with an electronic precision balance (Mod. Kern-440, capacity 500g, and accuracy 1mg). Then, we calculated a mean body weight for each week of treatment for each animal. To eliminate differences due to variability between animals randomly assigned to different groups, we expressed the body weight gain as percentage of the initial body weight.

Food consumption (grams per mouse) was determined twice a week on the 3rd and 7th day of each week, at 4.00 PM. The amount of food consumed per cage during these time intervals was estimated by subtracting the residual food recovered from each cage from the total amount presented. The daily food intake was calculated dividing the amount of food consumed during the week by seven. After the measurement, the mice received fresh quantities of their food (Zammaretti, et al., 2007).

Energy intake was obtained by multiplying daily food intake by the caloric value of the chow (2.668 Kcal/g), and daily feed efficiency was expressed as body weight (g)/Kcal eaten (Heine, et al., 2000, Michel, et al., 2003). In order to compensate for the differences in the initial body weight in the different groups, we calculated also the changes of the feed efficiency from the beginning of the experiment (conventionally put equal to 1). These data were analyzed by two-way analysis of variance (ANOVA) for repeated measures with a mixed design using the treatment and the sex as independent factors and the variations of feed efficiency for each of the 4 weeks as repeated factor.

Tissue sampling and histological examination

Adult mice were sacrificed 4 weeks after the beginning of the treatment**.** Mice were deeply anesthetized with 2,2,2-Tribromoethanol (Sigma-Aldrich, Europe). Before the sacrifice we collected the blood from the retro orbital vein. Animals were then perfused through the left ventricle with a saline solution (NaCl 9 g/l) until vessels were completely blood free, followed by 4% paraformaldehyde in 0.1 M saline-phosphate buffer (PBS), pH 7.3–7.4. After perfusion, brain, liver, kidneys, gonads, and fat were dissected. Brains were post-fixed for 24 hours in the same fixative at 4°C, rinsed several time in PBS, placed overnight at 4°C in PBS containing 30% sucrose solution, frozen on powdered dry ice and stored at -80°C.

Liver, kidney and gonads were weighed and stored with the same procedure utilized for the brain, for further analyses. In order to analyze the hepatic tissue integrity, livers were cut with a cryostat at 10µm of thickness, collected on glass slides and then processed for hematoxylin/eosin staining.

Hormone assay

Blood samples, collected in EDTA-treated tubes were centrifuged at 2000 rpm for 10 min to separate the plasma that was stored frozen at -80°C. Circulating leptin was evaluated in plasma samples by using a commercial EIA kit anti-mouse leptin (SpiBio, INALCO, Milano, Italy). Briefly, 100µl of diluted samples (1:2) were dispensed in duplicate into wells of the microtiter plates for ELISA, and the plates were incubated for 1hr at room temperature, followed by washing three times with EIA-buffer. Biotin-conjugated anti-leptin antibody was diluted to 1:10 in buffer containing bovine serum albumin 1 mg/ml, and 100µl of the diluted antibody solution was added to each well. The plates were incubated for 1hr at room temperature and washed thoroughly with EIA-buffer. Streptavidin-HRP complex was added (100µl) to each well and incubated for 30 min at room temperature. After washing, the substrate solution (100µl) was added to each well, and the plate was incubated for exactly 10 min at room temperature in the dark. To stop the reaction of color development, which is dependent on the amount of leptin in each well, 100µl of blocking solution were added. The intensity of color that developed was measured at 490 nm with a microplate reader within 5 min from the stop of the reaction. Leptin concentrations in diluted plasma samples were calculated by the standard curve after subtracting the small value of nonspecific color development with a EIA buffer blank.

Adipose tissue evaluation

After brain dissection, two representative pads of White Adipose Tissue (WAT) were manually dissected and weighted: adipose subcutaneous and gonadal fat pad (Cinti, 2011).

The fat pads were post-fixed in paraformaldeyde solution for 24 hours at 4°C, cryoprotected with sucrose solution (30% in saline-phosphate buffer 0.1M), frozen in liquid isopentane at –35°C, and stored at –80°C for future analysis.

NPY immunohystochemistry

Brains were cut in the coronal plane with a cryostat at 25µm of thickness, collected in multiwell dishes for free-floating procedure with a cryoprotectant solution (Watson, et al., 1986) and kept at -20°C. One series of sections (every fourth section, i.e. one section every 100 µm) was stained for NPY immunohistochemistry. Brain sections were always stained in groups containing males and females treated and non-treated, so that between assays variance could not cause systematic group differences.

The procedure was previously tested and optimized in the same mouse strain (Eva, et al., 2008, Oberto, et al., 2003). Briefly, after overnight washing in PBS, sections were exposed to Triton X-100 (0.2% in PBS) and then treated for blocking endogenous peroxidase activity [methanol/hydrogen peroxide for 20 minutes (Streefkerk, 1972)], incubated with normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30 minutes. Then, they were incubated overnight at room temperature with a rabbit polyclonal antibody against synthetic porcine NPY (a generous gift of H. Vaudry, Rouen, France) diluted 1:6,000 in PBS, pH 7.3-7.4, containing 0.2% Triton X-100, followed by a biotinylated anti-rat IgG (Vector, Peterborough, UK, cat. #: VC-BA-9400-MC15), diluted 1:250 with PBS-Triton. Sections were then processed for 1 hour in Avidin-Biotin-Peroxidase Complex (Vector, Peterborough, UK) and rinsed in PBS. The peroxidase activity was visualized with a solution containing 0.15 mg/ml 3,3'-diamino-benzidine (DAB, Sigma-Aldrich, Europe) and 0.025% hydrogen peroxide in 0.05 M Tris-HCl buffer pH 7.6. Sections were collected on chromalum-coated slides, air-dried, washed in xylene, and coverslipped with Entellan (Merck, Milano, Italy).

The specificity of anti-NPY antibody has been previously reported (Pelletier, et al., 1984, Pelletier, et al., 1984). This antibody was largely used for immunohistochemical detection of NPY immunoreactive system in a wide range of vertebrate species, including rodents and humans (Aste, et al., 1991, Danger, et al., 1991, Danger, et al., 1990, Pelletier, et al., 1984, Pelletier, et al., 1984, Perroteau, et al., 1988). In addition, we have

performed the following controls in our material: a) the primary antibody was omitted or replaced with an equivalent concentration of normal serum (negative controls); b) the secondary antibody was omitted. In these conditions, cells and fibers were totally unstained.

A second series of brain sections was Nissl-stained with toluidine blue for anatomical orientation. All structures were identified using the stereotaxis atlas of the mouse brain (Paxinos and Franklin, 2001).

β-galactosidase histochemistry

Y1/LacZ expression was determined by β-galactosidase staining on a third series of brain coronal sections, as previously described (Oberto, et al., 2003, Oberto, et al., 1998, Zammaretti, et al., 2007, Zammaretti, et al., 2001). Briefly, sections were incubated overnight at 37°C in a solution containing 1mg/ml X-gal, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$, 0.01% Triton-X 100 in PBS. Slices were then washed in deionized water for 5min, counterstained with nuclear fast red, coverslipped with Entellan (Merck, Milano, Italy) and analyzed. The transgene is expressed in the neuronal cell body and is typically detected as a juxtanuclear blue dot (Oberto, et. al, 1998).

Quantitative analysis

1. NPY-immunoreactivity quantification

For quantification we selected four nuclei involved in circuits controlling food intake and showing various degrees of NPY immunoreactivity: ARC, VMH, DMH, and PVN The density of NPY-immunoreactive structures was measured on three consecutive sections per nucleus per animal (ARC, VMH, DMH: bregma –1.46mm, -1.58mm –1.70mm; PVN: bregma –0.70mm, -0.82mm, -0.94mm, mouse brain atlas, Paxinos and Franklin, 2001). All sections were observed with a Olympus BH2 microscope and selected fields were then acquired by a Leica DFC320 video camera connected to a Power PC G4 Macintosh. The staining density of NPY-immunoreactive structures was measured in selected nuclei with Image J 1.38 software (Wayne Rasband, NIH,,

Bethesda, Maryland, USA) by calculating in binary transformations of the images (threshold function) the fractional area (percentages of pixels) covered by immunoreactive structures in predetermined fields (area of interest, AOI) as described in previous studies (Oberto, et al., 2003, Pierman, et al., 2008, Plumari, et al., 2002). The AOI selected for each nucleus was a box of fixed size and shape, selected to cover immunoreactive material only within the boundaries of each nucleus (140,000 μm^2 for VMH and DMH; 110,000 μm^2 for Arc; and 200,000 μm^2 for PVN).

The results for each nucleus were analyzed by two-way analysis of variance (ANOVA) for repeated measures with a mixed design using the treatment and the sex as independent factors and the antero-posterior neuroanatomical levels as repeated factor. When preliminary analyses revealed no significant effects of anterior-posterior levels, the levels were collapsed and the average number (calculated using the average values from two sections) was analyzed by a two-way ANOVA (treatment and sex as independent factors).

2. β-Galactosidase histochemistry quantification

Quantification of Y1R/LacZ transgene expression was performed by computer-assisted analysis of β-galactosidase histochemical stained coronal brain sections (25μm) (Oberto, et al., 2003, Zammaretti, et al., 2007, Zammaretti, et al., 2001). Three standardized sections of comparable levels of the PVN, ARC, VMH and DMH were examined. Selected sections were placed on Nikon microscope and analyzed with Image J software. Using a manual threshold method, dots were selected. The AOI selected for each nucleus was the same box of fixed size and shape used for NPY-ir quantification. For each animal and nucleus, the cumulative number of dots (corresponding to the number of cell expressing the transgene) and the cumulative areas of the analyzed sections were considered to obtain the density expression of the transgene expressed as dots per μm2.

Statistical analysis

Quantitative data were examined with SPSS statistic software (SPSS inc., Chicago, USA) by analysis of variance (two-way ANOVA), where sex and treatment were considered

independent variables. When appropriated, we performed a multivariate test (Bonferroni) to compare groups. Differences between groups were considered significant for values of $p \leq 0.05$

RESULTS

According to some previous studies on rats (Cooke, et al., 2004, Tryphonas, et al., 2004) we tried a range of 0.25-2.5mg of tributyltin (TBT) per Kg of body weight, however, the mice died in a few days (Table 1). The only dose compatible with long treatment was the lowest one, classified as NOAEL dose in the literature (0.025mg/Kg of body weight). Even in this small amount, TBT was highly aggressive by oral administration, in fact, many animals developed dermatitis and hair loss.

Body weight and Feed Efficiency (FE)

No differences were observed in the percentage of body weight gain (Fig. 1A). On the contrary, we observed a reduction in food consumption at the end of the treatment both in TBT males and TBT females (Fig. 1B,C). The two-way ANOVA for repeated measures, being sex and treatment the independent variables and weekly food consumption the repeated measure, showed effects of sex ($F_{(1,1)}$=20.482 and $p \leq 0.001$) and of treatment ($F_{(1,1)}$=4.386 and $p \leq 0.05$) on food consumption, but no effects of interaction between sex and treatment ($F_{(1,1)}$=0.136). The Bonferroni's test revealed significant differences only in males at the end of treatment ($p<0.05$), whereas in females the difference was not significant ($p=0.11$).

Results for feed efficiency (Body weight/Kcal) were statistically analyzed by two-way ANOVA for repeated measures (with sex and treatment as independent variables and feed efficiency as repeated measure) demonstrating a global effects of sex ($F_{(1)}$=7.530 and $p \leq 0.05$) and treatment ($F_{(1)}$=11,248 and $p \leq 0.01$). The multiple comparisons between groups displayed that, in the week before treatments no differences were observed in feed efficiency between animals; while at the end of the experimental period in both TBT-exposed males and females there was an increase in feed efficiency compared to controls (respectively $p \leq 0.05$ for males and $p \leq 0.01$ for females) (Fig. 2A, B).

Due to the fact that, at day 0, the weight of TBT-treated females was different from that of Oil-treated females, we have also analyzed the changes of the feed efficiency in comparison to the values of the feed efficiency for each group at the beginning of the experiment (conventionally put equal to 1). In this case, the two-way ANOVA for repeated measures (with sex and treatment as independent variables and percentual variations of feed efficiency as repeated measures) demonstrated effects of the interactions among repeated measures and sex ($F_{(1,3)}$=3.584 and $p<0.05$) as well as among repeated measures and treatment ($F_{(1,3)}$=5,967 and $p\leq0.01$). The multiple comparisons between groups displayed that no significant differences were present in the variations of feed efficiency for weeks 1 to 3. At week 4 we observed an increase for both sexes, but it was significant only in TBT-exposed males compared to controls ($p\leq0.05$), whereas for control females it was not significant ($p=0.1$) (Fig. 2C, D).

Blood leptin concentration

Exposure to TBT induced a reduction of blood circulating leptin measured with the EIA technique in both sexes. Two-ways ANOVA (sex and treatment as independent variables) showed a statistically significant effect of treatment ($F_{(1)}$=39.346 and $p\leq0.001$) on blood leptin levels, but no effects of sex ($F_{(1)}$=0.388) or of the interation between sex and treatment $F_{(1,1)}$=0.654). Multiple comparisons analysis confirmed the significant reduction of blood leptin levels in treated males ($p\leq0.001$) and females ($p\leq0.01$) compared to controls (Fig. 3B).

Liver

The volume of the liver was not altered in TBT-treated mice. This was confirmed by two-way ANOVA (sex and treatment as independent variables, and liver weight as dependent variable) showing a statistically significant effect of sex on liver weight ($F_{(1,1)}$=13.821 and $p\leq0.01$), but no effects of treatment ($F_{(1,1)}$=0.018). Multiple comparison analysis between groups confirmed the gender differences in control ($p\leq0.05$) animals, whereas the large variations in treated females prevented to have a significant difference with treated males (Fig. 3A).

On the contrary, histological inspection of hematoxylin-eosin stained sections of liver

showed impairment in tissues of treated animals. In fact, in sections from TBT-treated animals we observed severe congestion of the tissue and prominent cytoplasmic hepatocyte degeneration while the cytoplasm seems to be empty (Fig.4).

Adipose tissue

A first qualitative analysis displayed no relevant differences of considered fat pads, except for treated females that showed a reduction of the perigonadic fat deposition. This was confirmed by statistical analysis. In fact, the two-way ANOVA (sex and treatment as independent variables and fat weight (g) as dependent variable), showed no effects of sex ($F_{(1)}$=3.862) and of treatment ($F_{(1)}$=0.282) on subcutaneous white fat. In a similar way, we observed no effects of sex ($F_{(1)}$=1.219) and of treatment ($F_{(1,1)}$=2.813) for the perigonadic fat, but the interaction between sex and treatment had some effects ($F_{(1,1)}$=8.034 and $p \leq 0.05$). In fact, multiple comparison analysis showed statistically significant reduction of perigonadic fat in TBT-exposed females ($p \leq 0.01$).

NPY immunoreactivity

Immunostaining for NPY in control males reported a hypothalamic distribution of positive elements similar to those already reported in several previous contributions. In particular, due to the lack of pretreatment with colchicine, we have only rarely observed positive cell bodies, whereas a large amount of positive fibers was observed along the entire hypothalamus (Fig. 5). These fibers were particularly dense within the PVN and the ARC nuclei, but they were also abundant within the suprachiasmatic, supraoptic and DMH nuclei. Other regions have less dense innervations, as for example the VMH. Qualitative inspection of the stained sections revealed obvious differences among treated and control male mice. We have therefore quantitatively analized NPY-immunoreactivity (NPY-ir) within those nuclei that are involved in the food intake controlling circuit: PVN (Fig. 6), DMH, ARC and VMH.

For all nuclei, the preliminary two-way ANOVA for repeated measures has not evidenced a statistically significant effect for the anterior-posterior levels; we have therefore repeated the two-way ANOVA (treatment and sex were the independent factors) considering the average value of fractional area covered by immunoreactive

structures.

The quantitative analysis of stained sections indicated a decrease of NPY-ir in all the considered nuclei of TBT-treated males (Fig.7). The two-way ANOVA reported significant effects of the interaction between sex and treatment in DMH ($F_{(1,1)}$=8.661 and $p \leq 0.05$) and VMH ($F_{(1,1)}$=9.602 and $p \leq 0.01$). In PVN and ARC the p-values for the interaction were very close to significance ($p<0.07$), therefore we performed a multiple comparison analysis between groups for all nuclei. These tests demonstrated a significant sex differences (i.e. males showing a higher NPY-immunoreactivity than females) in OIL groups (PVN, ARC and DMH, $p<0.05$, VMH $p<0.01$), this difference is abolished by the treatment for all the nuclei. In particular, in PVN, ARC, and DMH we observed a significant reduction of NPY-ir in treated males compared to controls ($p \leq 0.05$), while no significant effects were detected for females. On the contrary, in the VMH TBT-treated females have a significant increase of NPY-ir compared to control females ($p \leq 0.05$), while no significant differences were found for males.

β-galactosidase histochemistry

In our specimens, the distribution of β-galactosidase activity (reflecting the activation of Y1-R promoter) in the hypothalamus was comparable to earlier description (Oberto, et al., 1998, Zammaretti, Panzica and Eva, 2007, Zammaretti, et al., 2001), in particular, we observed a high density of positive elements in the PVN, VMH and medial preoptic area (MPOA). Other regions as DMH or ARC showed a less intense positivity. Overall, the amount of positive elements and their size was smaller if compared to previous works in which we have not perfused the animals (Oberto, et al., 2003), but was very similar to our study performed in perfused animals (Martini, et al., 2011). In part of these nuclei we observed an obvious decrease of the histochemical staining in TBT-treated animals (Fig. 8). The subsequent quantification partly confirmed the visual inspection (Fig. 9).

In PVN and VMH the two-way analysis of variance showed significant effects of sex (respectively $F_{(1)}$=14.548 and $p \leq 0.01$; $F_{(1)}$=6.672 and $p \leq 0.05$) and treatment (respectively $F_{(1)}$=17.933 and $p \leq 0.001$; $F_{(1)}$=17.768 and $p \leq 0.001$). In VMH we observed a significant effect of the interaction between the two variables ($F_{(1,1)}$=5.414 and $p \leq 0.05$). In both PVN and VMH of control group, multiple comparisons showed differences in Y1R-transgene

expression between males and females, having females a higher number of positive elements ($p \leq 0.01$). The TBT treatment induced a significant decrease in both nuclei of females ($p \leq 0.01$, and $p < 0.001$ respectively). Also in male PVN and VMH nuclei we observed a reduction of Y1R-transgene expression, but the p-value indicated a tendency to significance ($p = 0.06$). In ARC the ANOVA showed the effect of sex ($F_{(1)} = 20.388$ and $p \leq 0.001$) and treatment ($F_{(1)} = 6.876$ and $p \leq 0.05$), but no effect was found for the interaction between the two variables ($F_{(1,1)} = 0.129$). In DMH there was only a significant effect of treatment ($F_{(1)} = 9.207$ and $p \leq 0.05$), while nor sex ($F_{(1)} = 0.020$) neither the interaction sex-treatment ($F_{(1,1)} = 0.065$) were significant. Subsequent multiple comparison analysis, showed a significant dimorphism (where females have a higher expression of Y1R-transgene in comparison to males) in ARC ($p \leq 0.01$), but not in DMH. Moreover TBT–treated female showed a reduction in Y1R transgene expression in both nuclei; in ARC was close to significant ($p = 0.07$), while in DMH this reduction was statistically significant ($p \leq 0.05$).

DISCUSSION

Present data suggest that, in adult C57BL/6 mice, the oral administration of 0.025µg/g/day of TBT for a period of four weeks is able to induce profound alterations of the leptin-NPY-Y1 receptor axis, even if the peripheral obesogenic effect is not strongly evident. In this work, we used the NOAEL dose, instead of higher doses that were applied to rats in other laboratories (Cooke, et al., 2008, Cooke, et al., 2004, et al., 2004), because, in our hands, these doses (2.5µg/g/day or 0.25µg/g/day) were highly toxic for our mice provoking animals' death in a short time (see Table 1).

In the present experiment, 4-weeks of TBT treatment in adult mice did not increase the body weight (Fig. 1A). However, both males and females showed a decrease of food intake at the end of treatment (significant only in males, see Fig. 1B.C). We have therefore calculated the feed efficiency, as well as its relative changes during the treatment (Fig. 2). In both cases, at the end of the treatment we observed an increase of feed efficiency and of its relative changes in both TBT-treated male and female mice. This increase is always significant in males, whereas the relative changes were not significant in females, probably due to the differences in body weight and food

consumption for the two female groups. These results mean that, at the end of the experiment the TBT-treated male mice had the same weight while consuming less food, with a similar tendency also for females.

Previous studies demonstrated that alterations in food intake and feed efficiency can lead to differences in body composition, providing a partial explanation for the obesity observed in some mouse model (Djazayery, et al., 1979). Although the susceptibility of the FVB mouse strain to obesity is not well studied, it was previously reported that FVB mice fed with a high fat diet become obese and show an increase in circulating leptin correlated with body weight (Frederich, et al., 1995, Martin, et al., 2006). In addition, 3 week-old males, of our transgenic strain (*Y1R/LacZ*), exposed for 8 weeks to a moderate-high fat diet showed a significant increase of body weight associated with a decrease of Y1R gene expression in the DMH and VMH in (Zammaretti, et al., 2007). However, in the present experiment, performed in older animals (3 month-old), we have not observed any significant increase of fat mass in TBT-exposed mice. This seems in contrast to studies demonstrating that TBT stimulates adipocytes differentiation *in vitro* (Kanayama, et al., 2005) and increases adipose mass *in vivo* (Grun, et al., 2006). In particular, *in utero* exposure to TBT (with i.p. doses 2 or 20 times higher than the one we used) may increase body fat mass (and not body weight), especially the fat located around the male gonadal ducts (Grun, et al., 2006). It is possible that the low dosis of TBT orally administered to adult mice in our experiment was not sufficient, or the period was too short to induce fat deposition, even if at the end of the treatment our TBT-treated animals showed a higher feed efficiency.

In contrast with the lack of effect on the weight of fat tissue we detected significant effects on circulating levels of leptin (Fig. 3B). In fact, according to our results on fat distribution, no significant variations of the leptin levels should occur, whereas, we observed a significant decrease of the hormone. The leptin fall in TBT-treated adult mice could be related to the putative xenoandrogenic action of TBT (Grote, et al., 2004), in fact, estrogens increase leptin production, while androgens act in the opposite way decreasing leptin levels (for a review see Mayes and Watson, 2004). The reduction of leptin synthesis could be also mediated by TBT-dependent activation of PPARγ, a nuclear receptors that play important roles in lipid homeostasis and adipogenesis. In fact,

PPARγ activation is known to be responsible of inhibition of genes that induce lipolysis in adipocytes of white adipose tissue (Evans, et al., 2004, Ferré, 2004) and TBT was shown to disrupt normal development and homeostatic controls over adipogenesis and energy balance, resulting obesity through its action on PPARγ and RXR (Grun, et al., 2006).

The liver, which is one of the targets of the toxicant action of TBT, even if not macroscopically altered (the volume was not significantly different in control or TBT-treated mice) shows histological signals of hepatocitic degeneration, it seems therefore that the supposed no effect dose has already a deleterious effect on this important organ (Figs 3A-4).

As reported in the introduction, the food intake is regulated by cerebral neuroendocrine circuits, mainly located in the hypothalamus, and leptin communicates the levels of energy storage, inducing the state of satiety while suppressing food intake by its action on these neuroendocrine circuits (Ahima, et al., 2009). However, reports on the action of TBT on hypothalamic circuits are very rare and limited to studies on neurotransmitters' expression and metabolisms in homogenates of discrete regions (Konno, et al., 2001, Tsunoda, et al., 2004), or in cell cultures (He, et al., 2014, Mizuhashi, et al., 2000, Nakatsu, et al., 2010, Nakatsu, et al., 2009). Only recently, we demonstrated a short-term action of TBT in vivo, by inducing a significant increase of c-fos expression in ARC elements after acute exposure to TBT (Bo, et al., 2011).

In the present experiment we studied changes induced by TBT in the expression of a neuropeptide strictly related to the control of food intake, the NPY. In adult TBT-treated males we observed a significant reduction of NPY expression in some of the investigated hypothalamic nuclei (Figs 6-7). On the basis of the levels of circulating leptin in TBT-treated animals and of the well known relationships among leptin and NPY (Myers, et al., 2009, Robertson, et al., 2008), the leptin decrease should induce a parallel increase of NPY-ir in ARC and, consequently, in its main targets: PVN, DMH, and VMH. On the contrary, we observed, a significant reduction of NPY-immunoreactivity in male ARC, PVN, and DMH, but not in females. In the VMH, a nucleus implicated both in the control of energy metabolism and in the control of lordosis behavior (for reviews see Asarian and Geary, 2013, Flanagan-Cato, 2011), we observed a reverse effect: no decrease in males

and a significant increase in TBT-treated females. Thus, there is a sex dimorphism in the sensitivity of NPY-based neural circuits controlling food intake to TBT. This is probably in agreement with other studies that indicate sex differences in body fat distribution as the result of differences in sex hormones between males and females (for reviews see Lovejoy and Sainsbury, 2009, Shi and Clegg, 2009).

The analysis of Y1-R transgene expression (Figs 8-9) revealed the presence of a sex dimorphism in PVN, VMH and ARC. In general, TBT-treated females show a significant decrease of the transgene expression (only a tendency for ARC) thus determining the disappearance of the dimorphism when compared to control males. In all these nuclei the exposure to TBT induced a significant decrease of Y1-R transgene expression in females, whereas in males we observed a strong reduction, but this has not reached statistical significance (p values 0.06-0.07) and it is probably due to the large variations in the transgene expression in males. As previously demonstrated in the same transgenic strain (Oberto, et al., 2003), lower levels of NPY-ir should induce an increase in Y1-R transgene expression. On the contrary, in the present experiment we observed a decrease of both NPY-ir and Y1-R transgene expression. Therefore, we have here a sex-oriented defect in the fine-tuning among up- and down-regulation of NPY and its main receptor. Previous studies have suggested that gonadal hormones may play a role in the expression of Y1-R during the estrous cycle (Martini, et al., 2011) or in its regulation after exposure to different diets (Zammaretti, et al., 2007). In addition, in this study we observed a sex dimorphism in Y1-R expression, having females more β-galactosidase positivity than males, thus the TBT is mainly acting masculinizing the Y1-R expression. It is therefore possible that the decrease of Y1R expression in TBT-treated animals may depend on one hand by a putative xenoandrogenic effect of TBT or, on the other hand, by the inhibition of aromatase activity mediated by TBT (Cooke, 2002, Heidrich, et al., 2001, Li, et al., 2015).

In conclusion, on the basis of the present findings, we can assume that TBT, in addition to its effects on peripheral fat tissue, has some effects on neural circuits, particularly directed to alter the neuroendocrine relationships among circulating leptin levels and the hypothalamic circuits controlling food intake, and these effects seems to be

sexually differentiated. Further studies should investigate if other parts of the food intake controlling system, as for example the α-MSH or the orexin circuits, may be affected by TBT exposure.

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Disclosures

The authors declare no conflict of interest

Authors' contribution

E.B., C.E., S.G. and G.C.P. designed and supervised the experiments; E.B., A.F., M.M. and D.S. performed the experiments; E.B., A.F., S.G. and G.C.P. performed the statistical analysis; E.B., S.G. and G.C.P. prepared the figures for publication; E.B., C.E., S.G. and G.C.P. wrote the first draft of the manuscript; all authors read and approved the manuscript.

LEGENDS TO THE FIGURES

Figure 1. A- Histograms represent body weight gain (expressed as percentage) during the treatment. No statistically significant differences were found between groups. **B-C** - The lines represents variations of the amount (in grams) of food intake during the treatment in control and treated males (**B**) and females (**C**). * $p<0.05$ (Bonferroni's test).

Figure 2. - Changes of feed efficiency (expressed as index of body weight/Kcal introduced) calculated during the period of treatment in control and TBT-treated males (**A**) and females (**B**). Feed efficiency is significantly increased at the end of treatment in TBT mice: * $p<0.05$, ** $p<0.01$ (Bonferroni's test). **C, D** - Variations of feed efficiency in comparison to the values at the beginning of the experiment (conventionally put equal to 1). TBT-treated males are significantly different in the last week of treatment, whereas the females show only a tendency during the same week, * $p<0.05$ (Bonferroni's test).

Figure 3- A - Histograms represent the liver's weight at the day of sacrifice. No statistically significant differences were found between treated and control groups, a sex difference is observed among control groups. The significant differences (ANOVA followed by the Bonferroni's test at a level of $P<0.05$) are denoted by **a** or **b**. **B** - Histogram representing the concentration of circulating leptin (detected with ELISA method). In both TBT-treated males and TBT-treated females we observe a significant drop of leptin levels in the blood. The significant differences (ANOVA followed by the Bonferroni's test at a level of $P<0.05$) are denoted by **a** or **b**.

Figure 4. Microphotographs of hematoxylin-eosin stained sections of liver from control (**A**) and TBT-treated (**B**) males showing profound alterations of the parenchima. Bar represents 100 microns

Figure 5. Microphotographs illustrating the immunohistochemical staining for NPY in the nuclei that have been considered in the present study. Microphotograps are taken at the same enlargement. Bar represents 500 microns. **A-** rostral section containing the paraventricular nucleus (PVN). **B-** More caudal section containing the dorsomedial (DMH), ventromedial (VMH) and arcuate (ARC) nuclei. * Third ventricle.

Figure 6. Sections of the paraventricular nucleus from different experimental groups immunostained for NPY. Microphotograps are taken at the same enlargement. Bar represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated male; F OIL - oil-treated female; F TBT - TBT-treated female. * Third ventricle.

Figure 7. Histograms representing NPY-ir in hypothalamus (expressed as percentage of area covered by stained elements). The multiple comparison analysis between groups for all nuclei demonstrated: in PVN, ARC, and DMH, a significant reduction of NPY-ir in treated males compared to controls), while no significant effects were detected for females. The significant differences (ANOVA followed by the Bonferroni test at a level of $P<0.05$) are denoted by **a** or **b**.

Figure 8. Histochemical detection of beta-galactosidase activity (expression of Y1R-LacZ transgene) in sections of the paraventricular nucleus from different experimental groups. Microphotograps are taken at the same enlargement. Bar represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated male; F OIL - oil-treated female; F TBT - TBT-treated female. * Third ventricle.

Figure 9. Histograms representing Y1R-transgene expression in hypothalamus (expressed as dots/µm2). In females the treatment significantly reduced Y1R expression in PVN and VMH. Also in males, TBT reduced Y1R-transgene expression in both nuclei but the statistical p-value is only close to significance. The significant differences (ANOVA followed by the Bonferroni test at a level of $P<0.05$) are denoted by **a** or **b,** ^ indicates a close to significant difference ($P<0.07$).

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Adult exposure to tributyltin affects hypothalamic neuropeptide Y, Y1 receptor distribution, and circulating leptin in mice.

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Running title: Effects of TBT on mice leptin-NPY system

Key words: TBT; NPY; food intake; arcuate nucleus; paraventricular nucleus; dorsomedial nucleus; C57BL/6 mice

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ABSTRACT

Tributyltin (TBT) is a pesticide, used in antifouling paints, toxic for aquatic invertebrates. In vertebrates, TBT may act as obesogen inducing adipogenetic genes transcription for adipocites differentiation. In a previous study we demonstrated that acute administration of TBT induces c-fos expression in arcuate nucleus, therefore, in the present study we tested the hypothesis that adult exposure to TBT may alter part of the nervous pathways controlling animal food intake. In particular, we investigated the expression of NPYimmunoreactivity. This neuropeptide forms neural circuits dedicated to food assumption and its action is mediated by Y1 receptors that are widely expressed in the hypothalamic nuclei responsible for the regulation of food intake and energy homeostasis. To this purpose, TBT was orally administered at a dose of 0.025 mg/Kg/day/body weight to adult animals (male and female C57BL/6 Y1-LacZ transgenic mice) for 4 weeks.

No differences were found in body weight and fat deposition, but we observed significant increase of feed efficiency in TBT-treated male mice and a significant decrease of circulating leptin in both sexes. Computerized quantitative analysis of NPY immunoreactivity and Y1-related beta-galactosidase activity, demonstrated a statistically significant reduction of NPY and Y1 transgene expression in the hypothalamic circuit controlling food intake of treated-male mice in comparison to controls. In conclusion, present results indicate that adult exposure to TBT is profoundly interfering with the nervous circuits involved in the stimulation of food intake.

INTRODUCTION

Obesity is defined as excessive deposition of body fat, and it is caused by complex interactions between genetic, behavioral and environmental factors. In humans, the most common causes are overeating high caloric fatty diets combined with a sedentary lifestyle and a genetic predisposition for the disease (Newbold, et al., 2007). Adipose tissue is not just a passive lipid repository, it can, in fact, act as an endocrine organ producing several factors including a peptide hormone (called leptin or Ob protein) which is responsible to communicate energy reserve information from adipocites to other body's organs including the central nervous system (Myers, et al., 2009).

Leptin is produced by the Ob-gene that is expressed in all adipose cells in proportion to fat accumulated, and it has the highest expression in the subcutaneous fat (Montague, et al., 1998). Leptin serves to communicate the levels of energy storage, to induce the state of satiety and to suppress food intake by its action on neuroendocrine systems (Ahima, et al., 2000). These actions are therefore finalized to increase energy expenditure (Bates, et al., 2003, Friedman and Halaas, 1998). Circulating leptin can pass across the blood-brain-barrier (Banks and Kastin, 1996) and leptin binds, in the brain, to Ob-receptors (Ob-R) expressed in the hypothalamus, mainly in arcuate (ARC), paraventricular (PVN), dorsomedial (DMH) hypothalamic nuclei, and lateral hypothalamic area (LHA) (Mercer, et al., 1996). Part of Ob-R-expressing neurons of LHA are inhibiting elements, therefore, through these neurons leptin may modulate the mesolimbic dopamine reward system to suppress feeding behavior (Leinninger, et al., 2009). These data indicate that the hypothalamus is a key site for leptin action, although other brain regions may be also involved in direct or indirect manner. In the hypothalamic circuits, leptin at the same time reduces food intake [by its inhibition of neuropeptide Y (NPY) system] and increases energy expenditure [by stimulating the α-melanocyte-stimulating hormone (α-MSH) system] (Myers, et al., 2009, Robertson, et al., 2008). NPY is an orexigenic peptide, made of 36 aminoacids, representing the most abundant peptide of the hypothalamus (for a recent review see Nguyen, et al., 2011). In the circuit controlling food intake NPY is synthesized by neurons, located within the ARC, projecting to other hypothalamic nuclei of the circuit: PVN, ventromedial nucleus

(VMH) and DMH [for a review see (Valassi, et al., 2008)]. Acute central administration of NPY induces increase of food intake (Stanley and Leibowitz, 1985), whereas chronic administration produces hyperfagia, decrease of thermogenesis and obesity (Stanley, et al., 1986). The action of NPY is mediated by several receptors, among them: Y1 and Y5 receptors are mainly related to the food control (Beck, 2006). In addition to its role to regulate feeding behavior, Y1-R is involved in several NPY-induced responses, such as anxiolytic effects and stress responses (Bertocchi, et al., 2011, Longo, et al., 2015), ethanol drinking behavior, activation of neuroendocrine axis (for a review see Eva, et al., 2006). The Y5-R subtype may have some role in the control of nesting behavior, i.e. during lactation with effects on litter growth rates (Ladyman and Woodside, 2009). Thus, the central nervous system plays a fundamental role in food intake regulation integrating neural and endocrine factors. According to this point of view, obesity is not only dependent by peripheral change of energy homeostasis, but can also be considered a "neuroendocrine disease" depending on alteration of the axis leptin/hypothalamic circuits controlling food intake (Austin and Marks, 2009).

Several studies demonstrated that so-called endocrine disrupting chemicals (EDCs) may alter several neural circuits and behaviors (for reviews see Frye, et al., 2012, Panzica, et al., 2011, Panzica, et al., 2007). In particular, it has been predicted the existence of EDCs that inappropriately regulate lipid metabolism and adipogenesis to promote obesity, defined as obesogens (Grun and Blumberg, 2006), and now classified among the more inclusive category of metabolic disruptors (Heindel, et al., 2015). For example, organotins, in particular tributyltin (TBT), largely used as antifouling agents in paints and in many industrial processes, can promote obesity by activating peroxisome proliferator-activated receptor γ (PPARγ) and retinoid X receptor (RXRα, RXRβ, and RXRγ), that induces in vitro differentiation of pre-adipocites to adipocites, while *in vivo* can lead to an increase of body fat storage (for a recent review see Grun, 2014). The wide distribution and the high persistence of TBT in fresh and salt water, have raised concern about its adverse effects to the animal (in particular marine invertebrates and fishes, Leung, et al., 2006, Lima, et al., 2015) and human health (Rantakokko, et al., 2014, Rantakokko, et al., 2013).

Data collected by several experimental studies clearly indicate a role of TBT on

obesity development in vertebrates (Grun, et al., 2006) probably through its action to predispose multipotent stem cells to become adipocytes (Kirchner, et al., 2010). However, currently, the effects of this compound on the central nervous system have received less attention (Decherf and Demeneix, 2011). *In vitro* studies demonstrated that TBT may induce the expression of c-fos (Matsuoka and Igisu, 1996), apoptosis (Nakatsu, et al., 2008, Nakatsu, et al., 2006, Nakatsu, et al., 2007), and inhibit dopamine biosynthesis (Lee, et al., 2006) in PC12 cell cultures. In primary cultures of dissociated neurons, TBT increased the Ca^{2+} -activated K^{+} current induced by NMDA application (Kanemoto, et al., 2002), is involved in cell death by glutamate excitotoxicity (Nakatsu, et al., 2006), and it has different effects on dissociated cells from different brain regions (Mitra, et al., 2014).

With respect to the *in* vivo studies, it has been demonstrated that TBT may cross the placenta (Kimura, et al., 2005) and the blood-brain barrier (Hara, et al., 1994), passing into the maternal milk and accumulating in pups' tissues, including the brain (Cooke, et al., 2008). Therefore, the central nervous system is a potential target for its action, at least for exposition during pregnancy or lactation. The exposure to TBT during the gestational period induces hypothyroidism in the progeny, whereas the acute treatment of pregnant females in the same period induces a dose-dependent increase of T_3-independent TRH transcription levels (Decherf, et al., 2010). TBT induces oxidative damages in various region of the adult rat brain, including cerebellum, hippocampus, hypothalamus, and striatum (Mitra, et al., 2015). In an earlier study we demonstrated that TBT acute administration induces, in short time, the expression of c-fos in ARC (Bo, et al., 2011) a key hypothalamic nucleus for the control of food intake (Myers, Munzberg, Leinninger and Leshan, 2009). More recently, He and coworkers (He, et al., 2014) demonstrated alterations in the mRNA content of hypothalamic peptides [NPY, pro-opiomelanocortin (POMC)] in adult male and female rats exposed to TBT for 8 weeks. In the present study, we investigated if the reported adverse effects of TBT on some physiological parameters may also affect brain NPY/Y1-R circuits involved in feeding control (PVN, ARC, DMH and VMH). As in previous studies in rodents (Cooke, et al., 2004, Tryphonas, et al., 2004), the doses of TBT selected for this study was based on the NOEL (No Observed Effect Level) dose corresponding to 0.025µg/g body weight /day (EFSA, 2004).

MATERIALS AND METHODS

Chemicals

Tributyltin chloride (TBT) in stock solution (96%) was obtained from Sigma-Aldrich, Europe (Aldrich Catalogue, T50202).

The primary antibody anti-NPY (a generous gift from H. Vaudry, Rouen, France) was raised in rabbit against synthetic porcine NPY; its production and the results of the assays performed to control its specificity and cross-reactivity with other neuropeptides have been previously described (Pelletier, et al., 1984, Pelletier, et al., 1984).

The Enzyme-Linked Immunoabsorbent Assay (ELISA) kit to detect leptin levels in mice's blood was purchased from SpiBio (INALCO, Milano, Italy). All other chemicals were obtained from commercial sources.

Tributyltin (96%) liquid was diluted in olive oil (Sigma-Aldrich, Europe) to obtain a final amount of TBT corresponding to the selected doses of 0.025mg/kg of body weight in a final volume of 30 µl that was delivered to each animal. The dosing solutions were prepared fresh weekly and varied with the current weight of the mice.

Animals and treatment

Animal care and handling throughout the experimental procedures were in accordance with the European Union Council Directive of 24 November 1986 (86/609/EEC) and the Italian Ministero della Sanità and the ethical Committee of the University of Torino approved the procedures reported in the present study.

Adult (3 months old) male and female C57BL/6 mice, from our laboratory, were housed at 22 ± 1°C under a photoperiod of 12 h light each day, with water and food *ad libitum*. Mice belong to a transgenic strain generated in our laboratory (Oberto, et al., 1998), carrying the 1.3 Kb sequence of the mouse Y1-R promoter fused with the coding region of the Escherichia Coli *LacZ* gene. Analysis of Y1-R/LacZ transgene activity by histochemical staining of β-galactosidase with X-gal demonstrated that this construct contains sufficient information to replicate the expression pattern of the endogenous Y1-R gene in a CNS-restricted and developmental stage-specific manner. This transgenic model has been previously employed in our laboratory to quantitatively evaluate in vivo

changes in transgene expression (Ferrara, et al., 2001, Martini, et al., 2011, Oberto, et al., 2003, Oberto, et al., 2000, Oberto, et al., 2001, Oberto, et al., 1998, Zammaretti, et al., 2007, Zammaretti, et al., 2001).

First experiment

A preliminary experiment was performed with 48 C57BL/6-Y1-R/LacZ male mice in order to choose the correct dose. According to some previous studies made on rats (Cooke, et al., 2004, Tryphonas, et al., 2004) we tried a range of 0.025, 0.25, or 2.5mg of tributyltin (TBT) per Kg of body weight per day. Males were randomly divided in the four experimental groups, and the experiment should originally cover 4 weeks (see below for the description of the TBT administration). However, the mice of the 2.5 and 0.25 groups died in a few days before the end of the first week (see Table 1). The dose of 0.025mg/Kg of body weight (classified as NOAEL dose in the literature) was therefore selected for the second experiment.

Second experiment

Twenty four C57BL/6-Y1-R/LacZ mice with adequate weight gain and without clinical signs were divided randomly into four experimental groups and individually housed: six males (M) and six females (F) were the control groups treated with vehicle only (OIL), six males and six females received daily 0.025mg/kg of body weight per day of TBT. The vehicle or the TBT solution was administered through a pipette with a not stressful procedure (Palanza, et al., 2002). In brief, to reduce stress in animals, they were manipulated for a period of ten days before starting the experiment, and then mice were picked up by the skin between the shoulders and held upright. A micropipette tip was placed into the mouth with the pipette tip gently touching the roof of the mouth, and the oil (final volume 30µl) was ejected from the pipette. Mice readily consume oil. The treatment was prolonged for 4 weeks.

Feed efficiency and body weight

Animals were fed with a standard diet 4RF21 GLP certificate (Mucedola, Italy) containing 2.668 Kcal/g of metabolizable energy with 21.7 % as protein, 0.4% as fat and 66.5% as carbohydrate.

Body weight was recorded daily, before the oral administration of TBT, with an electronic precision balance (Mod. Kern-440, capacity 500g, and accuracy 1mg). Then, we calculated a mean body weight for each week of treatment for each animal. To eliminate differences due to variability between animals randomly assigned to different groups, we expressed the body weight gain as percentage of the initial body weight.

Food consumption (grams per mouse) was determined twice a week on the 3rd and 7th day of each week, at 4.00 PM. The amount of food consumed per cage during these time intervals was estimated by subtracting the residual food recovered from each cage from the total amount presented. The daily food intake was calculated dividing the amount of food consumed during the week by seven. After the measurement, the mice received fresh quantities of their food (Zammaretti, et al., 2007).

Energy intake was obtained by multiplying daily food intake by the caloric value of the chow (2.668 Kcal/g), and daily feed efficiency was expressed as body weight (g)/Kcal eaten (Heine, et al., 2000, Michel, et al., 2003). In order to compensate for the differences in the initial body weight in the different groups, we calculated also the changes of the feed efficiency from the beginning of the experiment (conventionally put equal to 1). These data were analyzed by two-way analysis of variance (ANOVA) for repeated measures with a mixed design using the treatment and the sex as independent factors and the variations of feed efficiency for each of the 4 weeks as repeated factor.

Tissue sampling and histological examination

Adult mice were sacrificed 4 weeks after the beginning of the treatment**.** Mice were deeply anesthetized with 2,2,2-Tribromoethanol (Sigma-Aldrich, Europe). Before the sacrifice we collected the blood from the retro orbital vein. Animals were then perfused through the left ventricle with a saline solution (NaCl 9 g/l) until vessels were completely blood free, followed by 4% paraformaldehyde in 0.1 M saline-phosphate buffer (PBS), pH 7.3–7.4. After perfusion, brain, liver, kidneys, gonads, and fat were dissected. Brains were post-fixed for 24 hours in the same fixative at 4°C, rinsed several time in PBS, placed overnight at 4°C in PBS containing 30% sucrose solution, frozen on powdered dry ice and stored at -80°C.

Liver, kidney and gonads were weighed and stored with the same procedure utilized for the brain, for further analyses. In order to analyze the hepatic tissue integrity, livers were cut with a cryostat at 10µm of thickness, collected on glass slides and then processed for hematoxylin/eosin staining.

Hormone assay

Blood samples, collected in EDTA-treated tubes were centrifuged at 2000 rpm for 10 min to separate the plasma that was stored frozen at -80°C. Circulating leptin was evaluated in plasma samples by using a commercial EIA kit anti-mouse leptin (SpiBio, INALCO, Milano, Italy). Briefly, 100µl of diluted samples (1:2) were dispensed in duplicate into wells of the microtiter plates for ELISA, and the plates were incubated for 1hr at room temperature, followed by washing three times with EIA-buffer. Biotin-conjugated anti-leptin antibody was diluted to 1:10 in buffer containing bovine serum albumin 1 mg/ml, and 100µl of the diluted antibody solution was added to each well. The plates were incubated for 1hr at room temperature and washed thoroughly with EIA-buffer. Streptavidin-HRP complex was added (100µl) to each well and incubated for 30 min at room temperature. After washing, the substrate solution (100µl) was added to each well, and the plate was incubated for exactly 10 min at room temperature in the dark. To stop the reaction of color development, which is dependent on the amount of leptin in each well, 100µl of blocking solution were added. The intensity of color that developed was measured at 490 nm with a microplate reader within 5 min from the stop of the reaction. Leptin concentrations in diluted plasma samples were calculated by the standard curve after subtracting the small value of nonspecific color development with a EIA buffer blank.

Adipose tissue evaluation

After brain dissection, two representative pads of White Adipose Tissue (WAT) were manually dissected and weighted: adipose subcutaneous and gonadal fat pad (Cinti, 2011).

The fat pads were post-fixed in paraformaldeyde solution for 24 hours at 4°C, cryoprotected with sucrose solution (30% in saline-phosphate buffer 0.1M), frozen in liquid isopentane at –35°C, and stored at –80°C for future analysis.

NPY immunohystochemistry

Brains were cut in the coronal plane with a cryostat at 25µm of thickness, collected in multiwell dishes for free-floating procedure with a cryoprotectant solution (Watson, et al., 1986) and kept at -20°C. One series of sections (every fourth section, i.e. one section every 100 µm) was stained for NPY immunohistochemistry. Brain sections were always stained in groups containing males and females treated and non-treated, so that between assays variance could not cause systematic group differences.

The procedure was previously tested and optimized in the same mouse strain (Eva, et al., 2008, Oberto, et al., 2003). Briefly, after overnight washing in PBS, sections were exposed to Triton X-100 (0.2% in PBS) and then treated for blocking endogenous peroxidase activity [methanol/hydrogen peroxide for 20 minutes (Streefkerk, 1972)], incubated with normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30 minutes. Then, they were incubated overnight at room temperature with a rabbit polyclonal antibody against synthetic porcine NPY (a generous gift of H. Vaudry, Rouen, France) diluted 1:6,000 in PBS, pH 7.3-7.4, containing 0.2% Triton X-100, followed by a biotinylated anti-rat IgG (Vector, Peterborough, UK, cat. #: VC-BA-9400-MC15), diluted 1:250 with PBS-Triton. Sections were then processed for 1 hour in Avidin-Biotin-Peroxidase Complex (Vector, Peterborough, UK) and rinsed in PBS. The peroxidase activity was visualized with a solution containing 0.15 mg/ml 3,3'-diamino-benzidine (DAB, Sigma-Aldrich, Europe) and 0.025% hydrogen peroxide in 0.05 M Tris-HCl buffer pH 7.6. Sections were collected on chromalum-coated slides, air-dried, washed in xylene, and coverslipped with Entellan (Merck, Milano, Italy).

The specificity of anti-NPY antibody has been previously reported (Pelletier, et al., 1984, Pelletier, et al., 1984). This antibody was largely used for immunohistochemical detection of NPY immunoreactive system in a wide range of vertebrate species, including rodents and humans (Aste, et al., 1991, Danger, et al., 1991, Danger, et al., 1990, Pelletier, et al., 1984, Pelletier, et al., 1984, Perroteau, et al., 1988). In addition, we have

performed the following controls in our material: a) the primary antibody was omitted or replaced with an equivalent concentration of normal serum (negative controls); b) the secondary antibody was omitted. In these conditions, cells and fibers were totally unstained.

A second series of brain sections was Nissl-stained with toluidine blue for anatomical orientation. All structures were identified using the stereotaxis atlas of the mouse brain (Paxinos and Franklin, 2001).

β-galactosidase histochemistry

Y1/LacZ expression was determined by β-galactosidase staining on a third series of brain coronal sections, as previously described (Oberto, et al., 2003, Oberto, et al., 1998, Zammaretti, et al., 2007, Zammaretti, et al., 2001). Briefly, sections were incubated overnight at 37°C in a solution containing 1mg/ml X-gal, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$, 0.01% Triton-X 100 in PBS. Slices were then washed in deionized water for 5min, counterstained with nuclear fast red, coverslipped with Entellan (Merck, Milano, Italy) and analyzed. The transgene is expressed in the neuronal cell body and is typically detected as a juxtanuclear blue dot (Oberto, et. al, 1998).

Quantitative analysis

1. NPY-immunoreactivity quantification

For quantification we selected four nuclei involved in circuits controlling food intake and showing various degrees of NPY immunoreactivity: ARC, VMH, DMH, and PVN The density of NPY-immunoreactive structures was measured on three consecutive sections per nucleus per animal (ARC, VMH, DMH: bregma –1.46mm, -1.58mm –1.70mm; PVN: bregma –0.70mm, -0.82mm, -0.94mm, mouse brain atlas, Paxinos and Franklin, 2001). All sections were observed with a Olympus BH2 microscope and selected fields were then acquired by a Leica DFC320 video camera connected to a Power PC G4 Macintosh. The staining density of NPY-immunoreactive structures was measured in selected nuclei with Image J 1.38 software (Wayne Rasband, NIH,,

Bethesda, Maryland, USA) by calculating in binary transformations of the images (threshold function) the fractional area (percentages of pixels) covered by immunoreactive structures in predetermined fields (area of interest, AOI) as described in previous studies (Oberto, et al., 2003, Pierman, et al., 2008, Plumari, et al., 2002). The AOI selected for each nucleus was a box of fixed size and shape, selected to cover immunoreactive material only within the boundaries of each nucleus (140,000 μm^2 for VMH and DMH; 110,000 μm^2 for Arc; and 200,000 μm^2 for PVN).

The results for each nucleus were analyzed by two-way analysis of variance (ANOVA) for repeated measures with a mixed design using the treatment and the sex as independent factors and the antero-posterior neuroanatomical levels as repeated factor. When preliminary analyses revealed no significant effects of anterior-posterior levels, the levels were collapsed and the average number (calculated using the average values from two sections) was analyzed by a two-way ANOVA (treatment and sex as independent factors).

2. β-Galactosidase histochemistry quantification

Quantification of Y1R/LacZ transgene expression was performed by computer-assisted analysis of β-galactosidase histochemical stained coronal brain sections (25µm) (Oberto, et al., 2003, Zammaretti, et al., 2007, Zammaretti, et al., 2001). Three standardized sections of comparable levels of the PVN, ARC, VMH and DMH were examined. Selected sections were placed on Nikon microscope and analyzed with Image J software. Using a manual threshold method, dots were selected. The AOI selected for each nucleus was the same box of fixed size and shape used for NPY-ir quantification. For each animal and nucleus, the cumulative number of dots (corresponding to the number of cell expressing the transgene) and the cumulative areas of the analyzed sections were considered to obtain the density expression of the transgene expressed as dots per µm2.

Statistical analysis

Quantitative data were examined with SPSS statistic software (SPSS inc., Chicago, USA) by analysis of variance (two-way ANOVA), where sex and treatment were considered

independent variables. When appropriated, we performed a multivariate test (Bonferroni) to compare groups. Differences between groups were considered significant for values of $p \leq 0.05$

RESULTS

According to some previous studies on rats (Cooke, et al., 2004, Tryphonas, et al., 2004) we tried a range of 0.25-2.5mg of tributyltin (TBT) per Kg of body weight, however, the mice died in a few days (Table 1). The only dose compatible with long treatment was the lowest one, classified as NOAEL dose in the literature (0.025mg/Kg of body weight). Even in this small amount, TBT was highly aggressive by oral administration, in fact, many animals developed dermatitis and hair loss.

Body weight and Feed Efficiency (FE)

No differences were observed in the percentage of body weight gain (Fig. 1A). On the contrary, we observed a reduction in food consumption at the end of the treatment both in TBT males and TBT females (Fig. 1B,C). The two-way ANOVA for repeated measures, being sex and treatment the independent variables and weekly food consumption the repeated measure, showed effects of sex ($F_{(1,1)}$=20.482 and p≤0.001) and of treatment ($F_{(1,1)}$=4.386 and p≤0.05) on food consumption, but no effects of interaction between sex and treatment ($F_{(1,1)}$=0.136). The Bonferroni's test revealed significant differences only in males at the end of treatment (p<0.05), whereas in females the difference was not significant (p=0.11).

Results for feed efficiency (Body weight/Kcal) were statistically analyzed by two-way ANOVA for repeated measures (with sex and treatment as independent variables and feed efficiency as repeated measure) demonstrating a global effects of sex ($F_{(1)}$=7.530 and p≤0.05) and treatment ($F_{(1)}$=11,248 and p≤0.01). The multiple comparisons between groups displayed that, in the week before treatments no differences were observed in feed efficiency between animals; while at the end of the experimental period in both TBT-exposed males and females there was an increase in feed efficiency compared to controls (respectively p≤0.05 for males and p≤0.01 for females) (Fig. 2A, B).

Due to the fact that, at day 0, the weight of TBT-treated females was different from that of Oil-treated females, we have also analyzed the changes of the feed efficiency in comparison to the values of the feed efficiency for each group at the beginning of the experiment (conventionally put equal to 1). In this case, the two-way ANOVA for repeated measures (with sex and treatment as independent variables and percentual variations of feed efficiency as repeated measures) demonstrated effects of the interactions among repeated measures and sex ($F_{(1,3)}$=3.584 and $p<0.05$) as well as among repeated measures and treatment ($F_{(1,3)}$=5,967 and $p\leq0.01$). The multiple comparisons between groups displayed that no significant differences were present in the variations of feed efficiency for weeks 1 to 3. At week 4 we observed an increase for both sexes, but it was significant only in TBT-exposed males compared to controls ($p\leq0.05$), whereas for control females it was not significant ($p=0.1$) (Fig. 2C, D).

Blood leptin concentration

Exposure to TBT induced a reduction of blood circulating leptin measured with the EIA technique in both sexes. Two-ways ANOVA (sex and treatment as independent variables) showed a statistically significant effect of treatment ($F_{(1)}$=39.346 and $p\leq0.001$) on blood leptin levels, but no effects of sex ($F_{(1)}$=0.388) or of the interation between sex and treatment $F_{(1,1)}$=0.654). Multiple comparisons analysis confirmed the significant reduction of blood leptin levels in treated males ($p\leq0.001$) and females ($p\leq0.01$) compared to controls (Fig. 3B).

Liver

The volume of the liver was not altered in TBT-treated mice. This was confirmed by two-way ANOVA (sex and treatment as independent variables, and liver weight as dependent variable) showing a statistically significant effect of sex on liver weight ($F_{(1,1)}$=13.821 and $p\leq0.01$), but no effects of treatment ($F_{(1,1)}$=0.018). Multiple comparison analysis between groups confirmed the gender differences in control ($p\leq0.05$) animals, whereas the large variations in treated females prevented to have a significant difference with treated males (Fig. 3A).

On the contrary, histological inspection of hematoxylin-eosin stained sections of liver

showed impairment in tissues of treated animals. In fact, in sections from TBT-treated animals we observed severe congestion of the tissue and prominent cytoplasmic hepatocyte degeneration while the cytoplasm seems to be empty (Fig.4).

Adipose tissue

A first qualitative analysis displayed no relevant differences of considered fat pads, except for treated females that showed a reduction of the perigonadic fat deposition. This was confirmed by statistical analysis. In fact, the two-way ANOVA (sex and treatment as independent variables and fat weight (g) as dependent variable), showed no effects of sex ($F_{(1)}$=3.862) and of treatment ($F_{(1)}$=0.282) on subcutaneous white fat. In a similar way, we observed no effects of sex ($F_{(1)}$=1.219) and of treatment ($F_{(1,1)}$=2.813) for the perigonadic fat, but the interaction between sex and treatment had some effects ($F_{(1,1)}$=8.034 and $p \leq 0.05$). In fact, multiple comparison analysis showed statistically significant reduction of perigonadic fat in TBT-exposed females ($p \leq 0.01$).

NPY immunoreactivity

Immunostaining for NPY in control males reported a hypothalamic distribution of positive elements similar to those already reported in several previous contributions. In particular, due to the lack of pretreatment with colchicine, we have only rarely observed positive cell bodies, whereas a large amount of positive fibers was observed along the entire hypothalamus (Fig. 5). These fibers were particularly dense within the PVN and the ARC nuclei, but they were also abundant within the suprachiasmatic, supraoptic and DMH nuclei. Other regions have less dense innervations, as for example the VMH. Qualitative inspection of the stained sections revealed obvious differences among treated and control male mice. We have therefore quantitatively analized NPY-immunoreactivity (NPY-ir) within those nuclei that are involved in the food intake controlling circuit: PVN (Fig. 6), DMH, ARC and VMH.

For all nuclei, the preliminary two-way ANOVA for repeated measures has not evidenced a statistically significant effect for the anterior-posterior levels; we have therefore repeated the two-way ANOVA (treatment and sex were the independent factors) considering the average value of fractional area covered by immunoreactive

structures.

The quantitative analysis of stained sections indicated a decrease of NPY-ir in all the considered nuclei of TBT-treated males (Fig.7). The two-way ANOVA reported significant effects of the interaction between sex and treatment in DMH ($F_{(1,1)}$=8.661 and p≤0.05) and VMH ($F_{(1,1)}$=9.602 and p≤0.01). In PVN and ARC the p-values for the interaction were very close to significance (p<0.07), therefore we performed a multiple comparison analysis between groups for all nuclei. These tests demonstrated a significant sex differences (i.e. males showing a higher NPY-immunoreactivity than females) in OIL groups (PVN, ARC and DMH, p<0.05, VMH p<0.01), this difference is abolished by the treatment for all the nuclei. In particular, in PVN, ARC, and DMH we observed a significant reduction of NPY-ir in treated males compared to controls (p≤0.05), while no significant effects were detected for females. On the contrary, in the VMH TBT-treated females have a significant increase of NPY-ir compared to control females (p≤0.05), while no significant differences were found for males.

β-galactosidase histochemistry

In our specimens, the distribution of β-galactosidase activity (reflecting the activation of Y1-R promoter) in the hypothalamus was comparable to earlier description (Oberto, et al., 1998, Zammaretti, Panzica and Eva, 2007, Zammaretti, et al., 2001), in particular, we observed a high density of positive elements in the PVN, VMH and medial preoptic area (MPOA). Other regions as DMH or ARC showed a less intense positivity. Overall, the amount of positive elements and their size was smaller if compared to previous works in which we have not perfused the animals (Oberto, et al., 2003), but was very similar to our study performed in perfused animals (Martini, et al., 2011). In part of these nuclei we observed an obvious decrease of the histochemical staining in TBT-treated animals (Fig. 8). The subsequent quantification partly confirmed the visual inspection (Fig. 9).

In PVN and VMH the two-way analysis of variance showed significant effects of sex (respectively $F_{(1)}$=14.548 and p≤0.01; $F_{(1)}$=6.672 and p≤0.05) and treatment (respectively $F_{(1)}$=17.933 and p≤0.001; $F_{(1)}$=17.768 and p≤0.001). In VMH we observed a significant effect of the interaction between the two variables ($F_{(1,1)}$=5.414 and p≤0.05). In both PVN and VMH of control group, multiple comparisons showed differences in Y1R-transgene

expression between males and females, having females a higher number of positive elements ($p \leq 0.01$). The TBT treatment induced a significant decrease in both nuclei of females ($p \leq 0.01$, and $p < 0.001$ respectively). Also in male PVN and VMH nuclei we observed a reduction of Y1R-transgene expression, but the p-value indicated a tendency to significance ($p = 0.06$). In ARC the ANOVA showed the effect of sex ($F_{(1)} = 20.388$ and $p \leq 0.001$) and treatment ($F_{(1)} = 6.876$ and $p \leq 0.05$), but no effect was found for the interaction between the two variables ($F_{(1,1)} = 0.129$). In DMH there was only a significant effect of treatment ($F_{(1)} = 9.207$ and $p \leq 0.05$), while nor sex ($F_{(1)} = 0.020$) neither the interaction sex-treatment ($F_{(1,1)} = 0.065$) were significant. Subsequent multiple comparison analysis, showed a significant dimorphism (where females have a higher expression of Y1R-transgene in comparison to males) in ARC ($p \leq 0.01$), but not in DMH. Moreover TBT–treated female showed a reduction in Y1R transgene expression in both nuclei; in ARC was close to significant ($p = 0.07$), while in DMH this reduction was statistically significant ($p \leq 0.05$).

DISCUSSION

Present data suggest that, in adult C57BL/6 mice, the oral administration of 0.025µg/g/day of TBT for a period of four weeks is able to induce profound alterations of the leptin-NPY-Y1 receptor axis, even if the peripheral obesogenic effect is not strongly evident. In this work, we used the NOAEL dose, instead of higher doses that were applied to rats in other laboratories (Cooke, et al., 2008, Cooke, et al., 2004, et al., 2004), because, in our hands, these doses (2.5µg/g/day or 0.25µg/g/day) were highly toxic for our mice provoking animals' death in a short time (see Table 1).

In the present experiment, 4-weeks of TBT treatment in adult mice did not increase the body weight (Fig. 1A). However, both males and females showed a decrease of food intake at the end of treatment (significant only in males, see Fig. 1B.C). We have therefore calculated the feed efficiency, as well as its relative changes during the treatment (Fig. 2). In both cases, at the end of the treatment we observed an increase of feed efficiency and of its relative changes in both TBT-treated male and female mice. This increase is always significant in males, whereas the relative changes were not significant in females, probably due to the differences in body weight and food

consumption for the two female groups. These results mean that, at the end of the experiment the TBT-treated male mice had the same weight while consuming less food, with a similar tendency also for females.

Previous studies demonstrated that alterations in food intake and feed efficiency can lead to differences in body composition, providing a partial explanation for the obesity observed in some mouse model (Djazayery, et al., 1979). Although the susceptibility of the FVB mouse strain to obesity is not well studied, it was previously reported that FVB mice fed with a high fat diet become obese and show an increase in circulating leptin correlated with body weight (Frederich, et al., 1995, Martin, et al., 2006). In addition, 3 week-old males, of our transgenic strain (*Y1R/LacZ*), exposed for 8 weeks to a moderate-high fat diet showed a significant increase of body weight associated with a decrease of Y1R gene expression in the DMH and VMH in (Zammaretti, et al., 2007). However, in the present experiment, performed in older animals (3 month-old), we have not observed any significant increase of fat mass in TBT-exposed mice. This seems in contrast to studies demonstrating that TBT stimulates adipocytes differentiation *in vitro* (Kanayama, et al., 2005) and increases adipose mass *in vivo* (Grun, et al., 2006). In particular, *in utero* exposure to TBT (with i.p. doses 2 or 20 times higher than the one we used) may increase body fat mass (and not body weight), especially the fat located around the male gonadal ducts (Grun, et al., 2006). It is possible that the low dosis of TBT orally administered to adult mice in our experiment was not sufficient, or the period was too short to induce fat deposition, even if at the end of the treatment our TBT-treated animals showed a higher feed efficiency.

In contrast with the lack of effect on the weight of fat tissue we detected significant effects on circulating levels of leptin (Fig. 3B). In fact, according to our results on fat distribution, no significant variations of the leptin levels should occur, whereas, we observed a significant decrease of the hormone. The leptin fall in TBT-treated adult mice could be related to the putative xenoandrogenic action of TBT (Grote, et al., 2004), in fact, estrogens increase leptin production, while androgens act in the opposite way decreasing leptin levels (for a review see Mayes and Watson, 2004). The reduction of leptin synthesis could be also mediated by TBT-dependent activation of PPARγ, a nuclear receptors that play important roles in lipid homeostasis and adipogenesis. In fact,

PPARγ activation is known to be responsible of inhibition of genes that induce lipolysis in adipocytes of white adipose tissue (Evans, et al., 2004, Ferré, 2004) and TBT was shown to disrupt normal development and homeostatic controls over adipogenesis and energy balance, resulting obesity through its action on PPARγ and RXR (Grun, et al., 2006).

The liver, which is one of the targets of the toxicant action of TBT, even if not macroscopically altered (the volume was not significantly different in control or TBT-treated mice) shows histological signals of hepatocitic degeneration, it seems therefore that the supposed no effect dose has already a deleterious effect on this important organ (Figs 3A-4).

As reported in the introduction, the food intake is regulated by cerebral neuroendocrine circuits, mainly located in the hypothalamus, and leptin communicates the levels of energy storage, inducing the state of satiety while suppressing food intake by its action on these neuroendocrine circuits (Ahima, et al., 2009). However, reports on the action of TBT on hypothalamic circuits are very rare and limited to studies on neurotransmitters' expression and metabolisms in homogenates of discrete regions (Konno, et al., 2001, Tsunoda, et al., 2004), or in cell cultures (He, et al., 2014, Mizuhashi, et al., 2000, Nakatsu, et al., 2010, Nakatsu, et al., 2009). Only recently, we demonstrated a short-term action of TBT in vivo, by inducing a significant increase of c-fos expression in ARC elements after acute exposure to TBT (Bo, et al., 2011).

In the present experiment we studied changes induced by TBT in the expression of a neuropeptide strictly related to the control of food intake, the NPY. In adult TBT-treated males we observed a significant reduction of NPY expression in some of the investigated hypothalamic nuclei (Figs 6-7). On the basis of the levels of circulating leptin in TBT-treated animals and of the well known relationships among leptin and NPY (Myers, et al., 2009, Robertson, et al., 2008), the leptin decrease should induce a parallel increase of NPY-ir in ARC and, consequently, in its main targets: PVN, DMH, and VMH. On the contrary, we observed, a significant reduction of NPY-immunoreactivity in male ARC, PVN, and DMH, but not in females. In the VMH, a nucleus implicated both in the control of energy metabolism and in the control of lordosis behavior (for reviews see Asarian and Geary, 2013, Flanagan-Cato, 2011), we observed a reverse effect: no decrease in males

and a significant increase in TBT-treated females. Thus, there is a sex dimorphism in the sensitivity of NPY-based neural circuits controlling food intake to TBT. This is probably in agreement with other studies that indicate sex differences in body fat distribution as the result of differences in sex hormones between males and females (for reviews see Lovejoy and Sainsbury, 2009, Shi and Clegg, 2009).

The analysis of Y1-R transgene expression (Figs 8-9) revealed the presence of a sex dimorphism in PVN, VMH and ARC. In general, TBT-treated females show a significant decrease of the transgene expression (only a tendency for ARC) thus determining the disappearance of the dimorphism when compared to control males. In all these nuclei the exposure to TBT induced a significant decrease of Y1-R transgene expression in females, whereas in males we observed a strong reduction, but this has not reached statistical significance (p values 0.06-0.07) and it is probably due to the large variations in the transgene expression in males. As previously demonstrated in the same transgenic strain (Oberto, et al., 2003), lower levels of NPY-ir should induce an increase in Y1-R transgene expression. On the contrary, in the present experiment we observed a decrease of both NPY-ir and Y1-R transgene expression. Therefore, we have here a sex-oriented defect in the fine-tuning among up- and down-regulation of NPY and its main receptor. Previous studies have suggested that gonadal hormones may play a role in the expression of Y1-R during the estrous cycle (Martini, et al., 2011) or in its regulation after exposure to different diets (Zammaretti, et al., 2007). In addition, in this study we observed a sex dimorphism in Y1-R expression, having females more β-galactosidase positivity than males, thus the TBT is mainly acting masculinizing the Y1-R expression. It is therefore possible that the decrease of Y1R expression in TBT-treated animals may depend on one hand by a putative xenoandrogenic effect of TBT or, on the other hand, by the inhibition of aromatase activity mediated by TBT (Cooke, 2002, Heidrich, et al., 2001, Li, et al., 2015).

In conclusion, on the basis of the present findings, we can assume that TBT, in addition to its effects on peripheral fat tissue, has some effects on neural circuits, particularly directed to alter the neuroendocrine relationships among circulating leptin levels and the hypothalamic circuits controlling food intake, and these effects seems to be

sexually differentiated. Further studies should investigate if other parts of the food intake controlling system, as for example the α-MSH or the orexin circuits, may be affected by TBT exposure.

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Disclosures

The authors declare no conflict of interest

Authors' contribution

E.B., C.E., S.G. and G.C.P. designed and supervised the experiments; E.B., A.F., M.M. and D.S. performed the experiments; E.B., A.F., S.G. and G.C.P. performed the statistical analysis; E.B., S.G. and G.C.P. prepared the figures for publication; E.B., C.E., S.G. and G.C.P. wrote the first draft of the manuscript; all authors read and approved the manuscript.

LEGENDS TO THE FIGURES

Figure 1. A- Histograms represent body weight gain (expressed as percentage) during the treatment. No statistically significant differences were found between groups. **B-C** - The lines represents variations of the amount (in grams) of food intake during the treatment in control and treated males (**B**) and females (**C**). * $p<0.05$ (Bonferroni's test).

Figure 2. - Changes of feed efficiency (expressed as index of body weight/Kcal introduced) calculated during the period of treatment in control and TBT-treated males (**A**) and females (**B**). Feed efficiency is significantly increased at the end of treatment in TBT mice: * $p<0.05$, ** $p<0.01$ (Bonferroni's test). **C, D** - Variations of feed efficiency in comparison to the values at the beginning of the experiment (conventionally put equal to 1). TBT-treated males are significantly different in the last week of treatment, whereas the females show only a tendency during the same week, * $p<0.05$ (Bonferroni's test).

Figure 3- A - Histograms represent the liver's weight at the day of sacrifice. No statistically significant differences were found between treated and control groups, a sex difference is observed among control groups. The significant differences (ANOVA followed by the Bonferroni's test at a level of $P<0.05$) are denoted by **a** or **b**. **B** - Histogram representing the concentration of circulating leptin (detected with ELISA method). In both TBT-treated males and TBT-treated females we observe a significant drop of leptin levels in the blood. The significant differences (ANOVA followed by the Bonferroni's test at a level of $P<0.05$) are denoted by **a** or **b**.

Figure 4. Microphotographs of hematoxylin-eosin stained sections of liver from control (**A**) and TBT-treated (**B**) males showing profound alterations of the parenchima. Bar represents 100 microns

Figure 5. Microphotographs illustrating the immunohistochemical staining for NPY in the nuclei that have been considered in the present study. Microphotograps are taken at the same enlargement. Bar represents 500 microns. **A-** rostral section containing the paraventricular nucleus (PVN). **B-** More caudal section containing the dorsomedial (DMH), ventromedial (VMH) and arcuate (ARC) nuclei. * Third ventricle.

Figure 6. Sections of the paraventricular nucleus from different experimental groups immunostained for NPY. Microphotograps are taken at the same enlargement. Bar represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated male; F OIL - oil-treated female; F TBT - TBT-treated female. * Third ventricle.

Figure 7. Histograms representing NPY-ir in hypothalamus (expressed as percentage of area covered by stained elements). The multiple comparison analysis between groups for all nuclei demonstrated: in PVN, ARC, and DMH, a significant reduction of NPY-ir in treated males compared to controls), while no significant effects were detected for females. The significant differences (ANOVA followed by the Bonferroni test at a level of $P<0.05$) are denoted by **a** or **b**.

Figure 8. Histochemical detection of beta-galactosidase activity (expression of Y1R-LacZ transgene) in sections of the paraventricular nucleus from different experimental groups. Microphotograps are taken at the same enlargement. Bar represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated male; F OIL - oil-treated female; F TBT - TBT-treated female. * Third ventricle.

Figure 9. Histograms representing Y1R-transgene expression in hypothalamus (expressed as dots/µm2). In females the treatment significantly reduced Y1R expression in PVN and VMH. Also in males, TBT reduced Y1R-transgene expression in both nuclei but the statistical p-value is only close to significance. The significant differences (ANOVA followed by the Bonferroni test at a level of $P<0.05$) are denoted by **a** or **b,** ^ indicates a close to significant difference ($P<0.07$).

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Treatment	Day 0	Day 3	Day 4	Day 5
OIL	12	12	12	12
2.5 mg/Kg	12	7	0	0
0.25 mg/Kg	12	12	5	0
0.025 mg/Kg	12	12	12	12

Table 1 – Number of male mice surviving at the treatment with TBT at different doses after 3, 4, 5 days of treatment.

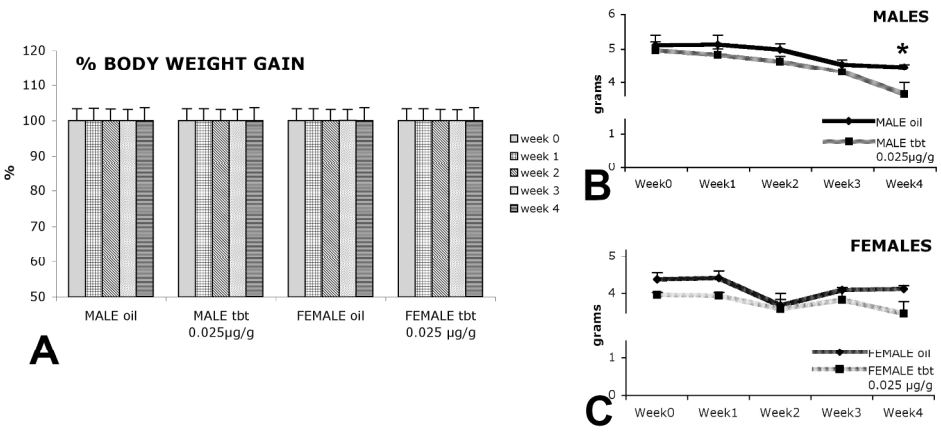

Figure 1. A- Histograms represent body weight gain (expressed as percentage) during the treatment. No statistically significant differences were found between groups. B-C - The lines represents variations of the amount (in grams) of food intake during the treatment in control and treated males (B) and females (C). * $p<0.05$ (Bonferroni's test).
253x117mm (300 x 300 DPI)

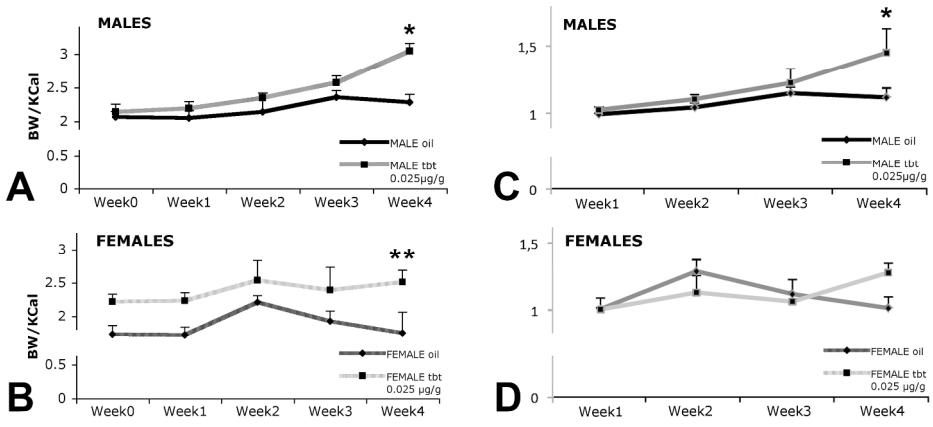

Figure 2. - Changes of feed efficiency (expressed as index of body weight/Kcal introduced) calculated during the period of treatment in control and TBT-treated males (A) and females (B). Feed efficiency is significantly increased at the end of treatment in TBT mice: * $p<0.05$, ** $p<0.01$ (Bonferroni's test). C, D - Variations of feed efficiency in comparison to the values at the beginning of the experiment (conventionally put equal to 1). TBT-treated males are significantly different in the last week of treatment, whereas the females show only a tendency during the same week, * $p<0.05$ (Bonferroni's test).
265x122mm (300 x 300 DPI)

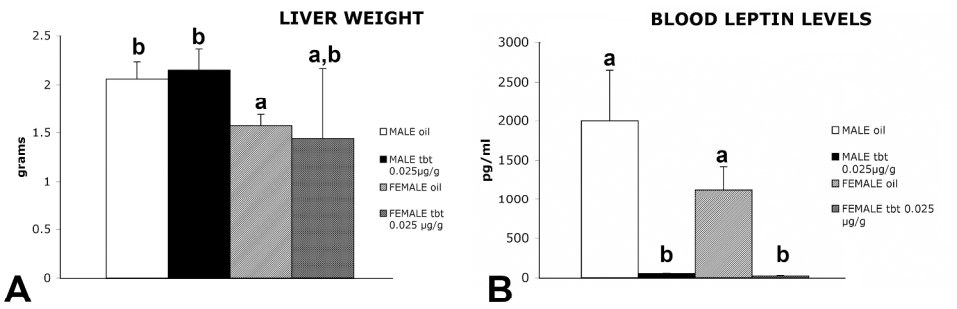

Figure 3- A - Histograms represent the liver's weight at the day of sacrifice. No statistically significant differences were found between treated and control groups, a sex difference is observed among control groups. The significant differences (ANOVA followed by the Bonferroni's test at a level of $P<0.05$) are denoted by a or b. B - Histogram representing the concentration of circulating leptin (detected with ELISA method). In both TBT-treated males and TBT-treated females we observe a significant drop of leptin levels in the blood. The significant differences (ANOVA followed by the Bonferroni's test at a level of $P<0.05$) are denoted by a or b.
279x140mm (300 x 300 DPI)

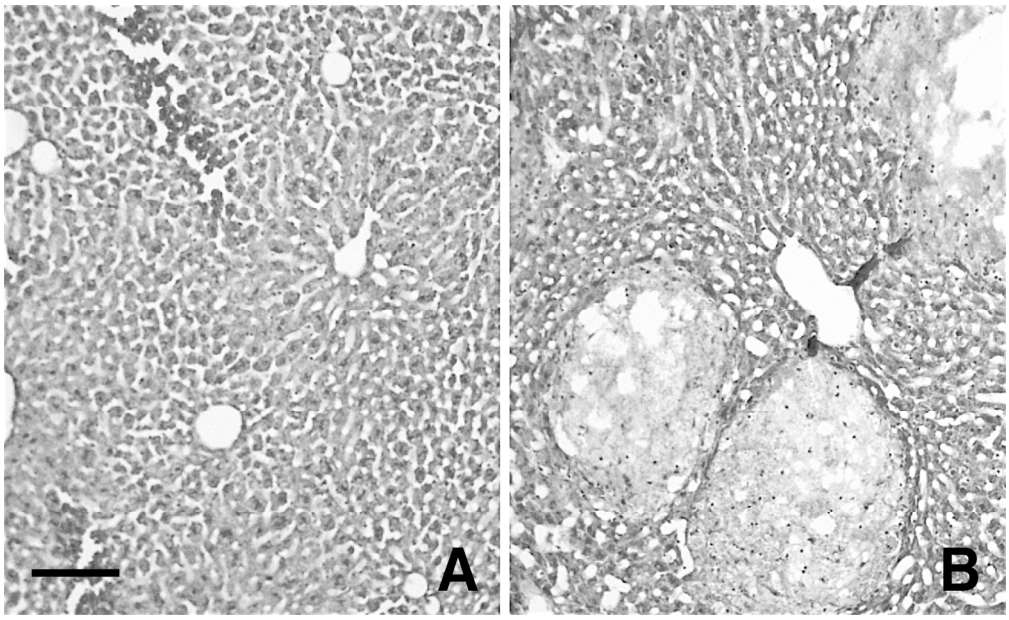

Figure 4. Microphotographs of hematoxylin-eosin stained sections of liver from control (A) and TBT-treated (B) males showing profound alterations of the parenchima. Bar represents 100 microns
180x109mm (300 x 300 DPI)

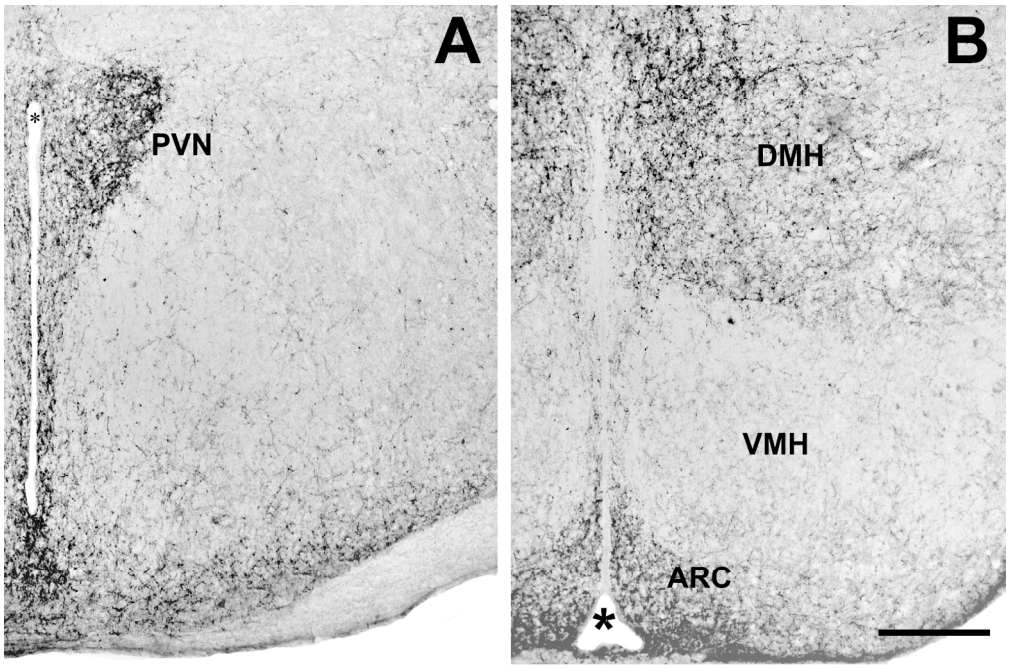

Figure 5. Microphotographs illustrating the immunohistochemical staining for NPY in the nuclei that have been considered in the present study. Microphotograps are taken at the same enlargement. Bar represents 500 microns. A- rostral section containing the paraventricular nucleus (PVN). B- More caudal section containing the dorsomedial (DMH), ventromedial (VMH) and arcuate (ARC) nuclei. * Third ventricle.
164x108mm (300 x 300 DPI)

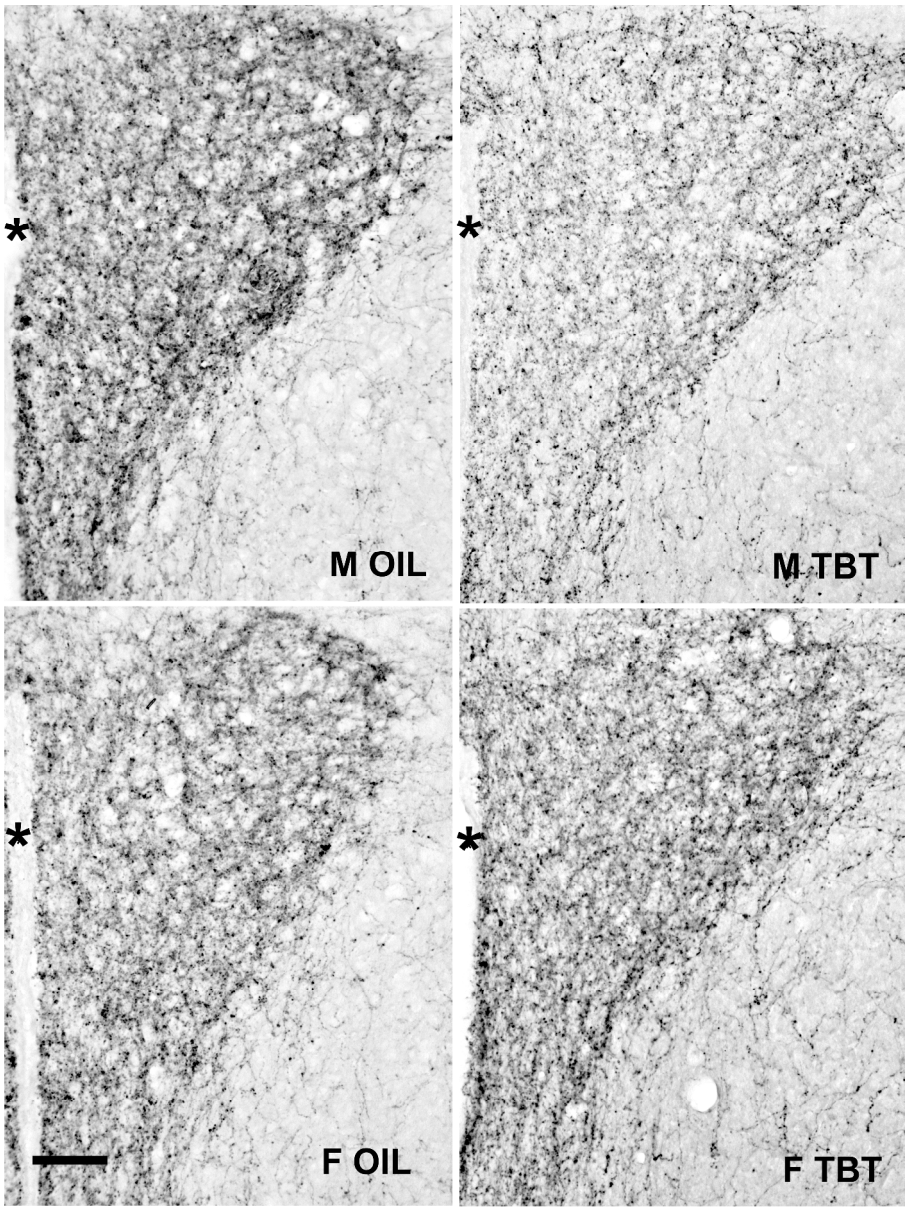

Figure 6. Sections of the paraventricular nucleus from different experimental groups immunostained for NPY. Microphotograps are taken at the same enlargement. Bar represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated male; F OIL - oil-treated female; F TBT - TBT-treated female. * Third ventricle.

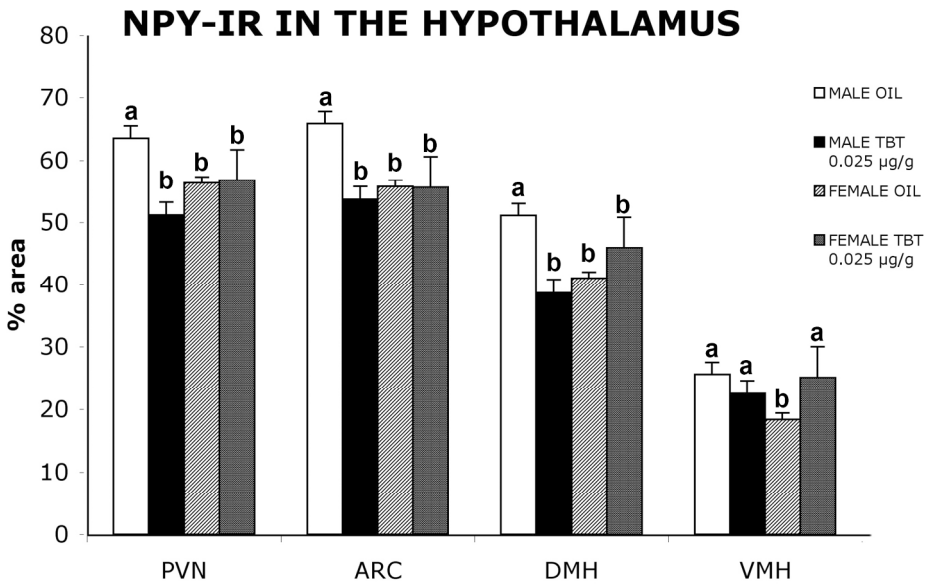

Figure 7. Histograms representing NPY-ir in hypothalamus (expressed as percentage of area covered by stained elements). The multiple comparison analysis between groups for all nuclei demonstrated: in PVN, ARC, and DMH, a significant reduction of NPY-ir in treated males compared to controls), while no significant effects were detected for females. The significant differences (ANOVA followed by the Bonferroni test at a level of $P<0.05$) are denoted by a or b.
193x120mm (300 x 300 DPI)

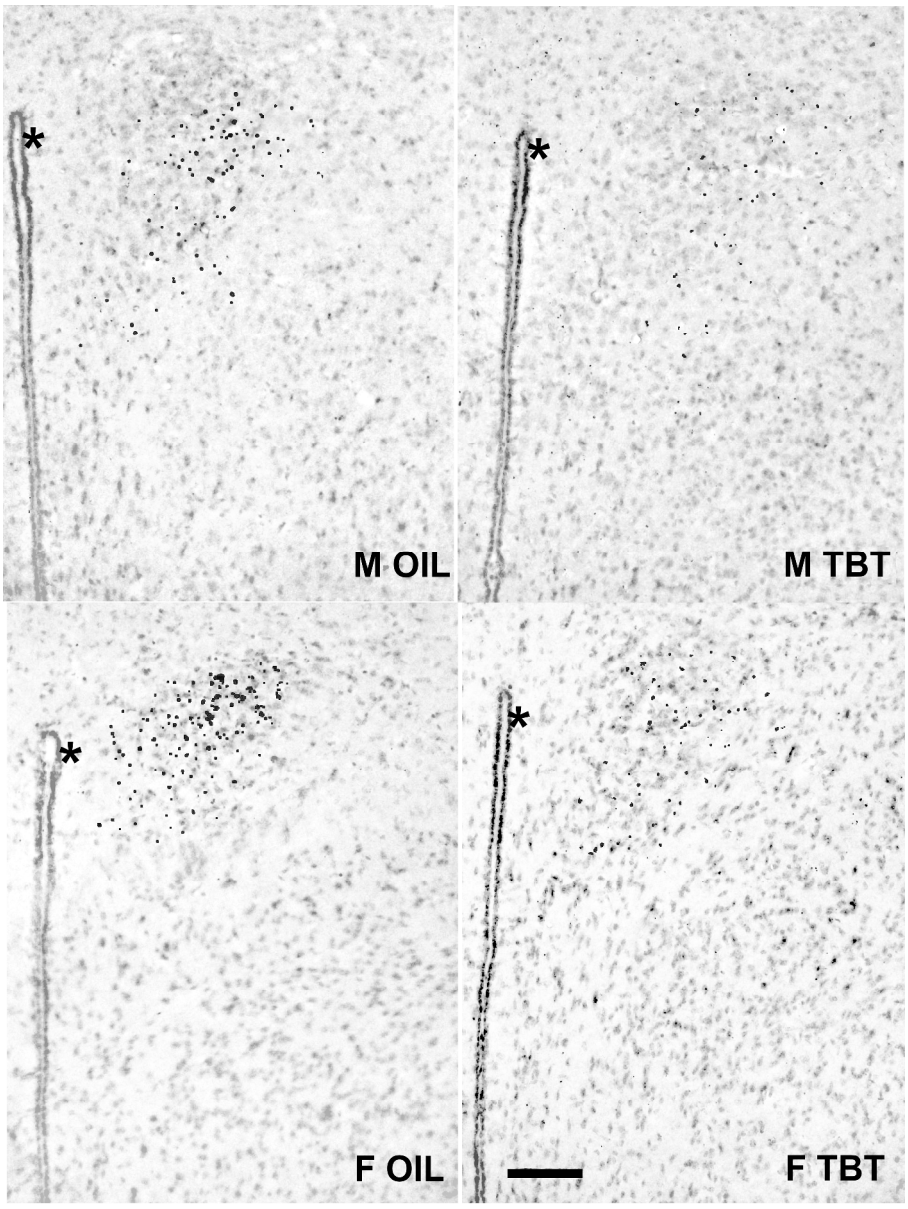

Figure 8. Histochemical detection of beta-galactosidase activity (expression of Y1R-LacZ transgene) in sections of the paraventricular nucleus from different experimental groups. Microphotograps are taken at the same enlargement. Bar represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated male; F OIL - oil-treated female; F TBT - TBT-treated female. * Third ventricle.

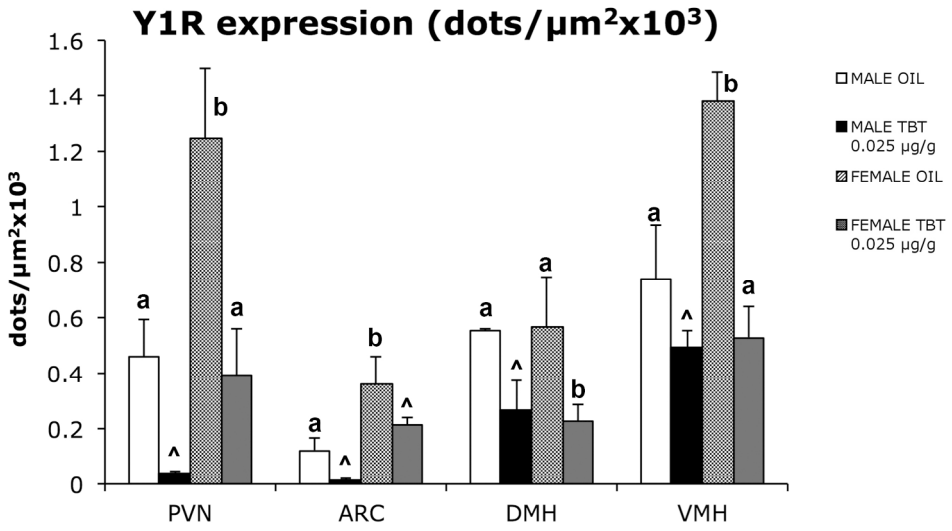

Figure 9. Histograms representing Y1R-transgene expression in hypothalamus (expressed as dots/µm2). In females the treatment significantly reduced Y1R expression in PVN and VMH. Also in males, TBT reduced Y1R-transgene expression in both nuclei but the statistical p-value is only close to significance. The significant differences (ANOVA followed by the Bonferroni test at a level of P<0.05) are denoted by a or b, ^ indicates a close to significant difference (P<0.07).
193x113mm (300 x 300 DPI)

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Torino, March 29, 2016

Dear Dr. Rajpert-De Meyts
,

I have pleasure in submitting to your journal ***Andrology*** the revision of our manuscript entitled: *Adult exposure to tributyltin affects hypothalamic neuropeptide Y, Y_1 receptor distribution, and circulating leptin in mice*.

This original work has been presented as one of the invited lectures at the COW 2015 meeting in Copenhagen.

We have carefully followed the suggestions of the two reviewers (see annexed Answers to reviewers) and we believe that the final version of the manuscript is now greatly improved

I hope that the manuscript will be favourably considered for the publication in this special issue.

Best regards

Prof. GianCarlo Panzica

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ANSWERS TO REVIEWERS

Reviewer: 1

1. Comments to the Author: The article Adult exposure to tributyltin affects hypothalamic neuropeptide Y, Y1 receptor distribution, and circulating leptin in mice is an interesting and original study that shows the effects of tributyltin on hypothalamic nuclei involved in feeding regulation. Results show how a NOAEL dose of this compound produces alterations in feed efficiency, liver histology, blood leptin concentration, and in NPY and Y1 receptor expression in several hypothalamic structures.

2. General comment: This kind of studies are very useful at present because their results stress the importance of scientific research about the alterations that endocrine disrupting chemicals produce on the nervous system, even when other significant damages are no detected at low doses.

Reply to 1 and 2. We thank the reviewer for his/her appreciation of our paper

Minor comments: (corrections according to reviewer 1 are in yellow in the text)

3. - Page 10 and 11: β-galactosidase histochemistry and β-galactosidase histochemistry quantification. Although the method is described, as author indicates, in a previous report, it would be recommended that in Materials and Methods section the staining of β-galactosidase histochemistry would be described briefly to explain what the staining dots represent. -
Reply: on page 11 we have added a short description of the staining, on page 12 we have explained that the number of dots correspond to the number of cells expressing the transgene.

4. Page 14: Adipose tissue section. In the first three lines is indicated a significant reduction of perigonadic fat deposition and that this is confirmed by statistical analysis. However in the following lines is written that no effects were observed either of sex or treatment in this parameter.
Reply: we added, at the end of the section (page 15) the results of the statistical analysis for the perigonadal fat.

5.- Page 15, line 27: In which direction are showed the sex differences in OIL

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groups? It can be seen in the graph but it should be clear also in the text.
Reply: we have specified that males show higher immunoreactivity than females.

6.- Page 19, second paragraph: The NPY-ir decrease that males showed in all hypothalamic nuclei studied is discussed but not the increase of the NPY-ir in the VMH of the females. A mention and a possible explanation about this different pattern of the VMH should be included.
Reply: on page 19-20 we have now mentioned the reverse effect on the VMH. At the moment we have no explanation for this effect.

7.- Some orthographic mistakes: Page 5, line 14: "coltures" Page 5, line 23: "may to cross" Page 5 line 37: "Cerebellum, Hyppocampus..." no capital letters are needed Page 5, line 39: "In a earlier study"
Page 6, line 47: "Mice belongs" Page 7, line 48: "weight,." Page 9, line 14: "parafomaldeyde"
Reply: we have corrected all the orthographic mistakes.

Reviewer: 2

Comments to the Author: The authors previously showed that acute TBT exposure in adults led to c-fos expression in the arcuate nucleus and set out there to test the hypothesis that adult TBT exposure may alter the function of neuroendocrine circuits involved in the regulation of appetite and satiety. They found that a 4 week administration of TBT at the established NOAEL did not produce changes in body weight or fat deposition, but did lead to increased feeding efficiency and decreased circulating leptin levels. Concurrently they observed a significant decrease in the expression of trangenes reporting NPY and Y1 activity and concluded that TBT does indeed interfere with neural circuits involved in the stimulation of food intake.

The manuscript presents data that are a valuable contribution to our understanding of how TBT, and other obesogens with similar modes of action could alter feeding behavior leading to weight gain, although these authors did not observe weight gain with the experimental design chosen.
Reply: we thank the reviewer for his/her comments on our paper

Major revisions: (corrections according to reviewer 2 are in green in the text)

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1. In the materials and methods the authors state that the "energy intake was obtained by multiplying daily food intake by caloric value of the chow". And in the discussion, the authors state that after the exposure to TBT, animals "had the same weight while consuming less food". However, there is no description about how food intake was measured or graph showing the results of these measurements. This information is critical considering the hypothesis and discussion of the manuscript.
Reply: thanks to the reviewer for pointing this problem. In fact this was a mistake!
We have now added a paragraph in the methods (page 8) describing the way in which we have measured food consumption (see also our study Zammaretti et al., 2007). In addition we have reported the data and the statistical analysis in the results section (page 13) and we added also two graphs for male and female food consumption in the new figure 1.

2. In both males and females, the feed efficiency on week 0 is not statistically significant among groups. However, in females both groups do not start at the same basal level, which makes the comparison inappropriate. The authors should discuss why females from control and treated groups show this difference if they have been randomly assigned to each group. In order to make a proper comparison between groups, the authors should represent the feed efficiency as the percentage of the increase between week 0 and week 4.
Reply: We thank again the reviewer for this comment. We have no explanation for the difference among control and treated females at the week 0. They were randomly assigned to the groups and we have not noticed that some of the females were smaller than others and by chance they were grouped in the same group- However, we agree with the comment and we added a second measure by calculating the changes of the feed efficiency from the beginning of the experiment (conventionally put equal to 1). This was added in the methods (page 8), in the results (page 14) and in two drawings in the new Figure 2. We have taken in the text both the results for the classical feed efficiency and the new data regarding the proportional changes. In males we conserved the significant difference for the week 4, but in the females this disappeared (only a tendency with $p<0.1$). We have discussed the new data on food consumption as well those relative to feed efficiency on pages 17-18-

3. The authors state in the discussion that "TBT exposure induced a significant decrease in Y1-R transgene expression". As indicated in the results, most of these comparisons are not significant ($p > 0.05$). Therefore, these results should be interpreted and discussed with an appropriate amount of caution.

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Reply: we changed the text of the discussion according to this observation (page 20)

4. In Table 1 the authors indicate the surviving animals after exposing the mice to different concentrations of TBT. The figure legend states that this information represents only information from male mice. If that is the case, the numbers do not match the information provided in the methods (6 Oil- and 6 TBT-treated males=12). If, on the contrary, the data represents both males and females, the figure legend should be modified and the information about males and females should be separated to show any potential sexual dimorphism in the detrimental effect of high doses of TBT. Additionally, the table represents three doses of TBT but in the methods the only dose included is the lower dose. Authors should indicated in the methods all doses tested, included the toxic ones.
Reply: we have now fully described our preliminary experiment. We added it as First experiment in methods at page 7. For this experiment we used 48 males exposed to different doses of TBT. We have also clarified the TABLE 1 and reported the surviving of animals exposed to different doses of TBT in the first 5 days from the beginning of the treatment.

Minor revisions:

5. Page 9 lines 9-12. Why is the differential expression of the androgen receptor in the two different fat pads important for the discussion of the manuscript? If the authors decide to maintain this statement it should be introduced in the results or the discussion rather than in the methods and the significance of this statement should be extended.
Reply; we accepted the suggestion of the reviewer and removed the paragraph

6. The order the figures are called throughout the text does not follow the order the figures are presented which makes it a bit more difficult for the reader to follow the arguments.
Reply: to follow this suggestion we have moved the section about the circulating leptin (fig. 3) in the results before (page 14) the liver section (fig.3 and 4). In addition we have split the histograms for NPY and Y1 receptor in two figures. One follows the NPY immunohistochemistry photos, and the second is now following the Y1 photos.

7. In general figures are not well described in the figure legends.
Figure 1. Figure legend does not describe panel E.
Reply: The figure 1 is now totally reaorganized in 3 figures (1-2-3)

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Figure 2 does not have scale bars but the figure legend mentions the scale bar.▪ −
Reply: this is now figure 4 and we added the scale bar

Figure 3. Are the pictures taken with the same magnification? Please clarify that point. The scale bar is only present in one of the pictures. What do the asterisks mean? Additionally, this picture should be introduced as supplemental material since it shows the justification for the choice of the regions to be analyzed but it is not adding any information to test the original hypothesis.
Reply: this is now Figure 5. We have clearly stated that the photos are at the same magnification, therefore we don't need multiple scale bars. We don't agree to put this figure as supplemental material, we think that it is important to show i) the quality of the staining and ii) the regions that we have considered.

Figure 4. What does the asterisk mean? What does M and F mean?
Reply: this is now figure 6 and we explained asterisks and acronims in the legend

Figure 6A should be part of Figure 4 since they belong to the same result.▪ −
Reply: This is now figure 7 (separate from the histograms of Y1 receptor). We prefer to have it as a separate figure, with a separate legend. the publisher will decide if putting figures 6 ad 7 together or separate.

Figure 5. What does the asterisk mean? What does M and F mean?
Reply: this is now figure 8 and we explained asterisks and acronims in the legend

Figure 6B should be part of Figure 5 since they belong to the same result.□•
Reply: This is now figure 9 (separate from the histograms of NPY immunoreactivity). We prefer to have it as a separate figure, with a separate legend. the publisher will decide if putting figures 8 ad 9 together or separate.

8.

Page 5 line 6: Please define the acronym NMDA for non-specialist readers
Page 5 line 14: coltures should be changed for cultures□•
Page 5 line 46: Please define the acronym POMC for non-specialist readers.□•
Page 9 line 7: gonadic should be replaced by gonadal□•
Page 10 line 53: Arc in low case while throughout the text is always

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indicated in capital letters•
Page 15 line 49: Please define the acronym MPOA for non-specialist readers•
C57/BL6 should be replaced for C57BL/6 throughout the text
Reply: we have performed all the requested changes.

9. Page 19 lines 25-27. This statement needs a reference.
Reply: we added two relatively recent reviews on this argument

10. In the discussion, it would be helpful to have an indication of which figure they are discussing within the text.
Reply: we agree with this observations and we added in the discussion the references to the specific figures.

Final comment to the editor, we have also added at the end of the text, after the Acknowledgement (in grey), a paragraph on the conflict of interest (no conflict) and another one with the authors' contributions.

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