

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1593220> since 2018-11-06T15:29:03Z

Published version:

DOI:10.1111/andr.12222

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1
2
3 **Adult exposure to tributyltin affects hypothalamic neuropeptide Y, Y1 receptor**
4 **distribution, and circulating leptin in mice.**
5
6
7

8 **Bo E., Farinetti A., Marraudino M., Sterchele D, Eva C., Gotti S., Panzica GC.***
9

10
11
12 Dept Neuroscience "Rita Levi Montalcini", University of Torino, Italy, National Institute
13 of Neuroscience (INN, Torino), and Neuroscience Institute Cavalieri-Ottolenghi (NICO)
14
15

16
17 Running title: Effects of TBT on mice leptin-NPY system
18
19

20
21
22
23
24 Key words: TBT; NPY; food intake; arcuate nucleus; paraventricular nucleus;
25 dorsomedial nucleus; C57BL/6 mice
26
27
28
29
30
31
32

33 *Corresponding author:
34

35 Prof. GianCarlo Panzica
36

37 Laboratory of Neuroendocrinology, Neuroscience Institute Cavalieri-Ottolenghi (NICO)
38

39 Regione Gonzole, 10, 10043 – Orbassano (Torino, Italy)
40

41 Phone +39 0116706607, Fax +39 0112366607, e-mail: giancarlo.panzica@unito.it
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

ABSTRACT

Tributyltin (TBT) is a pesticide, used in antifouling paints, toxic for aquatic invertebrates. In vertebrates, TBT may act as obesogen inducing adipogenic genes transcription for adipocytes 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 NPY immunoreactivity. 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 \square 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 adipocytes 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 (Leininger, 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

1
2
3 (VMH) and DMH [for a review see (Valassi, et al., 2008)]. Acute central administration
4 of NPY induces increase of food intake (Stanley and Leibowitz, 1985), whereas chronic
5 administration produces hyperphagia, decrease of thermogenesis and obesity (Stanley, et
6 al., 1986). The action of NPY is mediated by several receptors, among them: Y1 and Y5
7 receptors are mainly related to the food control (Beck, 2006). In addition to its role to
8 regulate feeding behavior, Y1-R is involved in several NPY-induced responses, such as
9 anxiolytic effects and stress responses (Bertocchi, et al., 2011, Longo, et al., 2015),
10 ethanol drinking behavior, activation of neuroendocrine axis (for a review see Eva, et al.,
11 2006). The Y5-R subtype may have some role in the control of nesting behavior, i.e.
12 during lactation with effects on litter growth rates (Ladyman and Woodside, 2009). Thus,
13 the central nervous system plays a fundamental role in food intake regulation integrating
14 neural and endocrine factors. According to this point of view, obesity is not only
15 dependent by peripheral change of energy homeostasis, but can also be considered a
16 “neuroendocrine disease” depending on alteration of the axis leptin/hypothalamic circuits
17 controlling food intake (Austin and Marks, 2009).
18
19

20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
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 (PPAR γ) and retinoid X receptor (RXR α , RXR β , and
RXR γ), that induces *in vitro* differentiation of pre-adipocytes to adipocytes, 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

1
2
3 obesity development in vertebrates (Grun, et al., 2006) probably through its action to
4 predispose multipotent stem cells to become adipocytes (Kirchner, et al., 2010).
5 However, currently, the effects of this compound on the central nervous system have
6 received less attention (Decherf and Demeneix, 2011). *In vitro* studies demonstrated that
7 TBT may induce the expression of c-fos (Matsuoka and Igisu, 1996), apoptosis (Nakatsu,
8 et al., 2008, Nakatsu, et al., 2006, Nakatsu, et al., 2007), and inhibit dopamine
9 biosynthesis (Lee, et al., 2006) in PC12 cell cultures. In primary cultures of dissociated
10 neurons, TBT increased the Ca²⁺-activated K⁺ current induced by NMDA application
11 (Kanemoto, et al., 2002), is involved in cell death by glutamate excitotoxicity (Nakatsu,
12 et al., 2006), and it has different effects on dissociated cells from different brain regions
13 (Mitra, et al., 2014).
14
15
16
17
18
19
20
21
22

23 With respect to the *in vivo* studies, it has been demonstrated that TBT may cross the
24 placenta (Kimura, et al., 2005) and the blood-brain barrier (Hara, et al., 1994), passing
25 into the maternal milk and accumulating in pups' tissues, including the brain (Cooke, et
26 al., 2008). Therefore, the central nervous system is a potential target for its action, at least
27 for exposition during pregnancy or lactation. The exposure to TBT during the gestational
28 period induces hypothyroidism in the progeny, whereas the acute treatment of pregnant
29 females in the same period induces a dose-dependent increase of T₃-independent TRH
30 transcription levels (Decherf, et al., 2010). TBT induces oxidative damages in various
31 region of the adult rat brain, including cerebellum, hippocampus, hypothalamus, and
32 striatum (Mitra, et al., 2015). In an earlier study we demonstrated that TBT acute
33 administration induces, in short time, the expression of c-fos in ARC (Bo, et al., 2011) a
34 key hypothalamic nucleus for the control of food intake (Myers, Munzberg, Leininger
35 and Leshan, 2009). More recently, He and coworkers (He, et al., 2014) demonstrated
36 alterations in the mRNA content of hypothalamic peptides [NPY, pro-opiomelanocortin
37 (POMC)] in adult male and female rats exposed to TBT for 8 weeks. In the present study,
38 we investigated if the reported adverse effects of TBT on some physiological parameters
39 may also affect brain NPY/Y1-R circuits involved in feeding control (PVN, ARC, DMH
40 and VMH). As in previous studies in rodents (Cooke, et al., 2004, Tryphonas, et al.,
41 2004), the doses of TBT selected for this study was based on the NOEL (No Observed
42 Effect Level) dose corresponding to 0.025µg/g body weight /day (EFSA, 2004).
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 μ l 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 $22 \pm 1^\circ\text{C}$ 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

1
2
3 changes in transgene expression (Ferrara, et al., 2001, Martini, et al., 2011, Oberto, et al.,
4 2003, Oberto, et al., 2000, Oberto, et al., 2001, Oberto, et al., 1998, Zammaretti, et al.,
5 2007, Zammaretti, et al., 2001).

6 7 8 *First experiment*

9
10 A preliminary experiment was performed with 48 C57BL/6-Y1-R/LacZ male mice in
11 order to choose the correct dose. According to some previous studies made on rats
12 (Cooke, et al., 2004, Tryphonas, et al., 2004) we tried a range of 0.025, 0.25, or 2.5mg of
13 tributyltin (TBT) per Kg of body weight per day. Males were randomly divided in the
14 four experimental groups, and the experiment should originally cover 4 weeks (see below
15 for the description of the TBT administration). However, the mice of the 2.5 and 0.25
16 groups died in a few days before the end of the first week (see Table 1). The dose of
17 0.025mg/Kg of body weight (classified as NOAEL dose in the literature) was therefore
18 selected for the second experiment.
19

20 21 22 *Second experiment*

23
24 Twenty four C57BL/6-Y1-R/LacZ mice with adequate weight gain and without
25 clinical signs were divided randomly into four experimental groups and individually
26 housed: six males (M) and six females (F) were the control groups treated with vehicle
27 only (OIL), six males and six females received daily 0.025mg/kg of body weight per day
28 of TBT. The vehicle or the TBT solution was administered through a pipette with a not
29 stressful procedure (Palanza, et al., 2002). In brief, to reduce stress in animals, they were
30 manipulated for a period of ten days before starting the experiment, and then mice were
31 picked up by the skin between the shoulders and held upright. A micropipette tip was
32 placed into the mouth with the pipette tip gently touching the roof of the mouth, and the
33 oil (final volume 30 μ l) was ejected from the pipette. Mice readily consume oil. The
34 treatment was prolonged for 4 weeks.
35
36
37
38
39
40
41
42
43
44
45
46
47
48

49 50 *Feed efficiency and body weight*

51
52 Animals were fed with a standard diet 4RF21 GLP certificate (Mucedola, Italy)
53 containing 2.668 Kcal/g of metabolizable energy with 21.7 % as protein, 0.4% as fat and
54 66.5% as carbohydrate.
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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.

1
2
3 Liver, kidney and gonads were weighed and stored with the same procedure utilized
4 for the brain, for further analyses. In order to analyze the hepatic tissue integrity, livers
5 were cut with a cryostat at 10µm of thickness, collected on glass slides and then
6 processed for hematoxylin/eosin staining.
7
8
9

10 11 12 *Hormone assay*

13
14 Blood samples, collected in EDTA-treated tubes were centrifuged at 2000 rpm for 10
15 min to separate the plasma that was stored frozen at -80°C. Circulating leptin was
16 evaluated in plasma samples by using a commercial EIA kit anti-mouse leptin (SpiBio,
17 INALCO, Milano, Italy). Briefly, 100µl of diluted samples (1:2) were dispensed in
18 duplicate into wells of the microtiter plates for ELISA, and the plates were incubated for
19 1hr at room temperature, followed by washing three times with EIA-buffer. Biotin-
20 conjugated anti-leptin antibody was diluted to 1:10 in buffer containing bovine serum
21 albumin 1 mg/ml, and 100µl of the diluted antibody solution was added to each well. The
22 plates were incubated for 1hr at room temperature and washed thoroughly with EIA-
23 buffer. Streptavidin-HRP complex was added (100µl) to each well and incubated for 30
24 min at room temperature. After washing, the substrate solution (100µl) was added to each
25 well, and the plate was incubated for exactly 10 min at room temperature in the dark. To
26 stop the reaction of color development, which is dependent on the amount of leptin in
27 each well, 100µl of blocking solution were added. The intensity of color that developed
28 was measured at 490 nm with a microplate reader within 5 min from the stop of the
29 reaction. Leptin concentrations in diluted plasma samples were calculated by the standard
30 curve after subtracting the small value of nonspecific color development with a EIA
31 buffer blank.
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47

48 *Adipose tissue evaluation*

49 After brain dissection, two representative pads of White Adipose Tissue (WAT) were
50 manually dissected and weighted: adipose subcutaneous and gonadal fat pad (Cinti,
51 2011).
52
53
54
55
56
57
58
59
60

1
2
3 The fat pads were post-fixed in paraformaldehyde solution for 24 hours at 4°C,
4 cryoprotected with sucrose solution (30% in saline-phosphate buffer 0.1M), frozen in
5 liquid isopentane at -35°C, and stored at -80°C for future analysis.
6
7
8
9

10 *NPY immunohistochemistry*

11
12 Brains were cut in the coronal plane with a cryostat at 25µm of thickness, collected in
13 multiwell dishes for free-floating procedure with a cryoprotectant solution (Watson, et
14 al., 1986) and kept at -20°C. One series of sections (every fourth section, i.e. one section
15 every 100 µm) was stained for NPY immunohistochemistry. Brain sections were always
16 stained in groups containing males and females treated and non-treated, so that between
17 assays variance could not cause systematic group differences.
18
19
20
21
22

23 The procedure was previously tested and optimized in the same mouse strain (Eva, et
24 al., 2008, Oberto, et al., 2003). Briefly, after overnight washing in PBS, sections were
25 exposed to Triton X-100 (0.2% in PBS) and then treated for blocking endogenous
26 peroxidase activity [methanol/hydrogen peroxide for 20 minutes (Streefkerk, 1972)],
27 incubated with normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30
28 minutes. Then, they were incubated overnight at room temperature with a rabbit
29 polyclonal antibody against synthetic porcine NPY (a generous gift of H. Vaudry, Rouen,
30 France) diluted 1:6,000 in PBS, pH 7.3-7.4, containing 0.2% Triton X-100, followed by a
31 biotinylated anti-rat IgG (Vector, Peterborough, UK, cat. #: VC-BA-9400-MC15), diluted
32 1:250 with PBS-Triton. Sections were then processed for 1 hour in Avidin-Biotin-
33 Peroxidase Complex (Vector, Peterborough, UK) and rinsed in PBS. The peroxidase
34 activity was visualized with a solution containing 0.15 mg/ml 3,3'-diamino-benzidine
35 (DAB, Sigma-Aldrich, Europe) and 0.025% hydrogen peroxide in 0.05 M Tris-HCl
36 buffer pH 7.6. Sections were collected on chromalum-coated slides, air-dried, washed in
37 xylene, and coverslipped with Entellan (Merck, Milano, Italy).
38
39
40
41
42
43
44
45
46
47
48

49 The specificity of anti-NPY antibody has been previously reported (Pelletier, et al.,
50 1984, Pelletier, et al., 1984). This antibody was largely used for immunohistochemical
51 detection of NPY immunoreactive system in a wide range of vertebrate species, including
52 rodents and humans (Aste, et al., 1991, Danger, et al., 1991, Danger, et al., 1990,
53 Pelletier, et al., 1984, Pelletier, et al., 1984, Perroteau, et al., 1988). In addition, we have
54
55
56
57
58
59
60

1
2
3 performed the following controls in our material: a) the primary antibody was omitted or
4 replaced with an equivalent concentration of normal serum (negative controls); b) the
5 secondary antibody was omitted. In these conditions, cells and fibers were totally
6 unstained.
7
8
9

10 A second series of brain sections was Nissl-stained with toluidine blue for anatomical
11 orientation. All structures were identified using the stereotaxis atlas of the mouse brain
12 (Paxinos and Franklin, 2001).
13
14
15

16 17 *β -galactosidase histochemistry*

18
19 Y1/LacZ expression was determined by β -galactosidase staining on a third series of
20 brain coronal sections, as previously described (Oberto, et al., 2003, Oberto, et al., 1998,
21 Zammaretti, et al., 2007, Zammaretti, et al., 2001). Briefly, sections were incubated
22 overnight at 37°C in a solution containing 1mg/ml X-gal, 5mM potassium ferricyanide,
23 5mM potassium ferrocyanide, 2mM MgCl₂, 0.01% Triton-X 100 in PBS. Slices were
24 then washed in deionized water for 5min, counterstained with nuclear fast red,
25 coverslipped with Entellan (Merck, Milano, Italy) and analyzed. The transgene is
26 expressed in the neuronal cell body and is typically detected as a juxtannuclear blue dot
27 (Oberto, et. al, 1998).
28
29
30
31
32
33
34
35
36
37
38
39

40 *Quantitative analysis*

41 *1. NPY-immunoreactivity quantification*

42 For quantification we selected four nuclei involved in circuits controlling food intake
43 and showing various degrees of NPY immunoreactivity: ARC, VMH, DMH, and PVN
44 The density of NPY-immunoreactive structures was measured on three consecutive
45 sections per nucleus per animal (ARC, VMH, DMH: bregma -1.46mm, -1.58mm -
46 1.70mm; PVN: bregma -0.70mm, -0.82mm, -0.94mm, mouse brain atlas, Paxinos and
47 Franklin, 2001). All sections were observed with a Olympus BH2 microscope and
48 selected fields were then acquired by a Leica DFC320 video camera connected to a
49 Power PC G4 Macintosh. The staining density of NPY-immunoreactive structures was
50 measured in selected nuclei with Image J 1.38 software (Wayne Rasband, NIH,,
51
52
53
54
55
56
57
58
59
60

1
2
3 Bethesda, Maryland, USA) by calculating in binary transformations of the images
4 (threshold function) the fractional area (percentages of pixels) covered by
5 immunoreactive structures in predetermined fields (area of interest, AOI) as described in
6 previous studies (Oberto, et al., 2003, Pierman, et al., 2008, Plumari, et al., 2002). The
7 AOI selected for each nucleus was a box of fixed size and shape, selected to cover
8 immunoreactive material only within the boundaries of each nucleus (140,000 μm^2 for
9 VMH and DMH; 110,000 μm^2 for Arc; and 200,000 μm^2 for PVN).
10
11

12
13
14
15
16 The results for each nucleus were analyzed by two-way analysis of variance
17 (ANOVA) for repeated measures with a mixed design using the treatment and the sex as
18 independent factors and the antero-posterior neuroanatomical levels as repeated factor.
19 When preliminary analyses revealed no significant effects of anterior-posterior levels, the
20 levels were collapsed and the average number (calculated using the average values from
21 two sections) was analyzed by a two-way ANOVA (treatment and sex as independent
22 factors).
23
24
25
26
27

28 29 30 *2. β -Galactosidase histochemistry quantification*

31
32 Quantification of Y1R/LacZ transgene expression was performed by computer-
33 assisted analysis of β -galactosidase histochemical stained coronal brain sections (25 μm)
34 (Oberto, et al., 2003, Zammaretti, et al., 2007, Zammaretti, et al., 2001). Three
35 standardized sections of comparable levels of the PVN, ARC, VMH and DMH were
36 examined. Selected sections were placed on Nikon microscope and analyzed with Image
37 J software. Using a manual threshold method, dots were selected. The AOI selected for
38 each nucleus was the same box of fixed size and shape used for NPY-ir quantification.
39 For each animal and nucleus, the cumulative number of dots (corresponding to the
40 number of cell expressing the transgene) and the cumulative areas of the analyzed
41 sections were considered to obtain the density expression of the transgene expressed as
42 dots per μm^2 .
43
44
45
46
47
48
49
50
51

52 53 *Statistical analysis*

54
55 Quantitative data were examined with SPSS statistic software (SPSS inc., Chicago, USA)
56 by analysis of variance (two-way ANOVA), where sex and treatment were considered
57
58
59
60

1
2
3 independent variables. When appropriated, we performed a multivariate test (Bonferroni)
4 to compare groups. Differences between groups were considered significant for values of
5 $p \leq 0.05$
6
7
8
9

10 RESULTS

11
12
13
14 According to some previous studies on rats (Cooke, et al., 2004, Tryphonas, et al., 2004)
15 we tried a range of 0.25-2.5mg of tributyltin (TBT) per Kg of body weight, however, the
16 mice died in a few days (Table 1). The only dose compatible with long treatment was the
17 lowest one, classified as NOAEL dose in the literature (0.025mg/Kg of body weight).
18 Even in this small amount, TBT was highly aggressive by oral administration, in fact,
19 many animals developed dermatitis and hair loss.
20
21
22
23
24

25 *Body weight and Feed Efficiency (FE)*

26
27
28 No differences were observed in the percentage of body weight gain (Fig. 1A). On the
29 contrary, we observed a reduction in food consumption at the end of the treatment both in
30 TBT males and TBT females (Fig. 1B,C). The two-way ANOVA for repeated measures,
31 being sex and treatment the independent variables and weekly food consumption the
32 repeated measure, showed effects of sex ($F_{(1,1)}=20.482$ and $p \leq 0.001$) and of treatment
33 ($F_{(1,1)}=4.386$ and $p \leq 0.05$) on food consumption, but no effects of interaction between sex
34 and treatment ($F_{(1,1)}=0.136$). □ The Bonferroni's test revealed significant differences only
35 in males at the end of treatment ($p < 0.05$), whereas in females the difference was not
36 significant ($p = 0.11$).
37
38
39
40
41
42
43

44 Results for feed efficiency (Body weight/Kcal) were statistically analyzed by two-way
45 ANOVA for repeated measures (with sex and treatment as independent variables and
46 feed efficiency as repeated measure) demonstrating a global effects of sex ($F_{(1)}=7.530$
47 and $p \leq 0.05$) and treatment ($F_{(1)}=11,248$ and $p \leq 0.01$). The multiple comparisons between
48 groups displayed that, in the week before treatments no differences were observed in feed
49 efficiency between animals; while at the end of the experimental period in both TBT-
50 exposed males and females there was an increase in feed efficiency compared to controls
51 (respectively $p \leq 0.05$ for males and $p \leq 0.01$ for females) (Fig. 2A, B).
52
53
54
55
56
57
58
59
60

1
2
3 Due to the fact that, at day 0, the weight of TBT-treated females was different from that
4 of Oil-treated females, we have also analyzed the changes of the feed efficiency in
5 comparison to the values of the feed efficiency for each group at the beginning of the
6 experiment (conventionally put equal to 1). In this case, the two-way ANOVA for
7 repeated measures (with sex and treatment as independent variables and percentual
8 variations of feed efficiency as repeated measures) demonstrated effects of the
9 interactions among repeated measures and sex ($F_{(1,3)}=3.584$ and $p<0.05$) as well as among
10 repeated measures and treatment ($F_{(1,3)}=5.967$ and $p\leq 0.01$). The multiple comparisons
11 between groups displayed that no significant differences were present in the variations of
12 feed efficiency for weeks 1 to 3. At week 4 we observed an increase for both sexes, but it
13 was significant only in TBT-exposed males compared to controls ($p\leq 0.05$), whereas for
14 control females it was not significant ($p=0.1$) (Fig. 2C, D).

25 26 27 *Blood leptin concentration*

28 Exposure to TBT induced a reduction of blood circulating leptin measured with the EIA
29 technique in both sexes. Two-ways ANOVA (sex and treatment as independent variables)
30 showed a statistically significant effect of treatment ($F_{(1)}=39.346$ and $p\leq 0.001$) on blood
31 leptin levels, but no effects of sex ($F_{(1)}=0.388$) or of the interaction between sex and
32 treatment ($F_{(1,1)}=0.654$). Multiple comparisons analysis confirmed the significant
33 reduction of blood leptin levels in treated males ($p\leq 0.001$) and females ($p\leq 0.01$)
34 compared to controls (Fig. 3B).

41 42 43 *Liver*

44 The volume of the liver was not altered in TBT-treated mice. This was confirmed by two-
45 way ANOVA (sex and treatment as independent variables, and liver weight as dependent
46 variable) showing a statistically significant effect of sex on liver weight ($F_{(1,1)}=13.821$
47 and $p\leq 0.01$), but no effects of treatment ($F_{(1,1)}=0.018$). Multiple comparison analysis
48 between groups confirmed the gender differences in control ($p\leq 0.05$) animals, whereas
49 the large variations in treated females prevented to have a significant difference with
50 treated males (Fig. 3A).

51 On the contrary, histological inspection of hematoxylin-eosin stained sections of liver
52
53
54
55
56
57
58
59
60

1
2
3 showed impairment in tissues of treated animals. In fact, in sections from TBT-treated
4 animals we observed severe congestion of the tissue and prominent cytoplasmic
5 hepatocyte degeneration while the cytoplasm seems to be empty (Fig.4).
6
7
8

9 10 *Adipose tissue*

11
12 A first qualitative analysis displayed no relevant differences of considered fat pads,
13 except for treated females that showed a reduction of the perigonadic fat deposition. This
14 was confirmed by statistical analysis. In fact, the two-way ANOVA (sex and treatment as
15 independent variables and fat weight (g) as dependent variable), showed no effects of sex
16 ($F_{(1)}=3.862$) and of treatment ($F_{(1)}=0.282$) on subcutaneous white fat. In a similar way, we
17 observed no effects of sex ($F_{(1)}=1.219$) and of treatment ($F_{(1,1)}=2.813$) for the perigonadic
18 fat, but the interaction between sex and treatment had some effects ($F_{(1,1)}=8.034$ and
19 $p\leq 0.05$). In fact, multiple comparison analysis showed statistically significant reduction
20 of perigonadic fat in TBT-exposed females ($p\leq 0.01$).
21
22
23
24
25
26
27
28
29

30 *NPY immunoreactivity*

31
32 Immunostaining for NPY in control males reported a hypothalamic distribution of
33 positive elements similar to those already reported in several previous contributions. In
34 particular, due to the lack of pretreatment with colchicine, we have only rarely observed
35 positive cell bodies, whereas a large amount of positive fibers was observed along the
36 entire hypothalamus (Fig. 5). These fibers were particularly dense within the PVN and
37 the ARC nuclei, but they were also abundant within the suprachiasmatic, supraoptic and
38 DMH nuclei. Other regions have less dense innervations, as for example the VMH.
39 Qualitative inspection of the stained sections revealed obvious differences among treated
40 and control male mice. We have therefore quantitatively analyzed NPY-immunoreactivity
41 (NPY-ir) within those nuclei that are involved in the food intake controlling circuit: PVN
42 (Fig. 6), DMH, ARC and VMH.
43
44
45
46
47
48
49
50

51
52 For all nuclei, the preliminary two-way ANOVA for repeated measures has not
53 evidenced a statistically significant effect for the anterior-posterior levels; we have
54 therefore repeated the two-way ANOVA (treatment and sex were the independent
55 factors) considering the average value of fractional area covered by immunoreactive
56
57
58
59
60

1
2
3 structures.

4
5 The quantitative analysis of stained sections indicated a decrease of NPY-ir in all the
6 considered nuclei of TBT-treated males (Fig.7). The two-way ANOVA reported
7 significant effects of the interaction between sex and treatment in DMH ($F_{(1,1)}=8.661$ and
8 $p\leq 0.05$) and VMH ($F_{(1,1)}=9.602$ and $p\leq 0.01$). In PVN and ARC the p-values for the
9 interaction were very close to significance ($p<0.07$), therefore we performed a multiple
10 comparison analysis between groups for all nuclei. These tests demonstrated a significant
11 sex differences (i.e. males showing a higher NPY-immunoreactivity than females) in OIL
12 groups (PVN, ARC and DMH, $p<0.05$, VMH $p<0.01$), this difference is abolished by the
13 treatment for all the nuclei. In particular, in PVN, ARC, and DMH we observed a
14 significant reduction of NPY-ir in treated males compared to controls ($p\leq 0.05$), while no
15 significant effects were detected for females. On the contrary, in the VMH TBT-treated
16 females have a significant increase of NPY-ir compared to control females ($p\leq 0.05$),
17 while no significant differences were found for males.
18
19
20
21
22
23
24
25
26
27
28
29

30 *β -galactosidase histochemistry*

31
32 In our specimens, the distribution of β -galactosidase activity (reflecting the activation
33 of Y1-R promoter) in the hypothalamus was comparable to earlier description (Oberto, et
34 al., 1998, Zammaretti, Panzica and Eva, 2007, Zammaretti, et al., 2001), in particular, we
35 observed a high density of positive elements in the PVN, VMH and medial preoptic area
36 (MPOA). Other regions as DMH or ARC showed a less intense positivity. Overall, the
37 amount of positive elements and their size was smaller if compared to previous works in
38 which we have not perfused the animals (Oberto, et al., 2003), but was very similar to our
39 study performed in perfused animals (Martini, et al., 2011). In part of these nuclei we
40 observed an obvious decrease of the histochemical staining in TBT-treated animals (Fig.
41 8). The subsequent quantification partly confirmed the visual inspection (Fig. 9).
42
43
44
45
46
47
48
49

50 In PVN and VMH the two-way analysis of variance showed significant effects of sex
51 (respectively $F_{(1)}=14.548$ and $p\leq 0.01$; $F_{(1)}=6.672$ and $p\leq 0.05$) and treatment (respectively
52 $F_{(1)}=17.933$ and $p\leq 0.001$; $F_{(1)}=17.768$ and $p\leq 0.001$). In VMH we observed a significant
53 effect of the interaction between the two variables ($F_{(1,1)}=5.414$ and $p\leq 0.05$). In both PVN
54 and VMH of control group, multiple comparisons showed differences in Y1R-transgene
55
56
57
58
59
60

1
2
3 expression between males and females, having females a higher number of positive
4 elements ($p \leq 0.01$). The TBT treatment induced a significant decrease in both nuclei of
5 females ($p \leq 0.01$, and $p < 0.001$ respectively). Also in male PVN and VMH nuclei we
6 observed a reduction of Y1R-transgene expression, but the p-value indicated a tendency
7 to significance ($p = 0.06$). In ARC the ANOVA showed the effect of sex ($F_{(1)} = 20.388$ and
8 $p \leq 0.001$) and treatment ($F_{(1)} = 6.876$ and $p \leq 0.05$), but no effect was found for the
9 interaction between the two variables ($F_{(1,1)} = 0.129$). In DMH there was only a significant
10 effect of treatment ($F_{(1)} = 9.207$ and $p \leq 0.05$), while nor sex ($F_{(1)} = 0.020$) neither the
11 interaction sex-treatment ($F_{(1,1)} = 0.065$) were significant. Subsequent multiple comparison
12 analysis, showed a significant dimorphism (where females have a higher expression of
13 Y1R-transgene in comparison to males) in ARC ($p \leq 0.01$), but not in DMH. Moreover
14 TBT-treated female showed a reduction in Y1R transgene expression in both nuclei; in
15 ARC was close to significant ($p = 0.07$), while in DMH this reduction was statistically
16 significant ($p \leq 0.05$).
17
18
19
20
21
22
23
24
25
26
27
28
29
30

31 DISCUSSION

32 Present data suggest that, in adult C57BL/6 mice, the oral administration of
33 $0.025 \mu\text{g/g/day}$ of TBT for a period of four weeks is able to induce profound alterations of
34 the leptin-NPY-Y1 receptor axis, even if the peripheral obesogenic effect is not strongly
35 evident. In this work, we used the NOAEL dose, instead of higher doses that were
36 applied to rats in other laboratories (Cooke, et al., 2008, Cooke, et al., 2004, et al., 2004),
37 because, in our hands, these doses ($2.5 \mu\text{g/g/day}$ or $0.25 \mu\text{g/g/day}$) were highly toxic for
38 our mice provoking animals' death in a short time (see Table 1).
39
40
41
42
43
44

45 In the present experiment, 4-weeks of TBT treatment in adult mice did not increase the
46 body weight (Fig. 1A). However, both males and females showed a decrease of food
47 intake at the end of treatment (significant only in males, see Fig. 1B.C). We have
48 therefore calculated the feed efficiency, as well as its relative changes during the
49 treatment (Fig. 2). In both cases, at the end of the treatment we observed an increase of
50 feed efficiency and of its relative changes in both TBT-treated male and female mice.
51 This increase is always significant in males, whereas the relative changes were not
52 significant in females, probably due to the differences in body weight and food
53
54
55
56
57
58
59
60

1
2
3 consumption for the two female groups. These results mean that, at the end of the
4 experiment the TBT-treated male mice had the same weight while consuming less food,
5 with a similar tendency also for females.
6
7

8
9 Previous studies demonstrated that alterations in food intake and feed efficiency can
10 lead to differences in body composition, providing a partial explanation for the obesity
11 observed in some mouse model (Djazayery, et al., 1979). Although the susceptibility of
12 the FVB mouse strain to obesity is not well studied, it was previously reported that FVB
13 mice fed with a high fat diet become obese and show an increase in circulating leptin
14 correlated with body weight (Frederich, et al., 1995, Martin, et al., 2006). In addition, 3
15 week-old males, of our transgenic strain (*Y1R/LacZ*), exposed for 8 weeks to a moderate-
16 high fat diet showed a significant increase of body weight associated with a decrease of
17 Y1R gene expression in the DMH and VMH in (Zammaretti, et al., 2007). However, in
18 the present experiment, performed in older animals (3 month-old), we have not observed
19 any significant increase of fat mass in TBT-exposed mice. This seems in contrast to
20 studies demonstrating that TBT stimulates adipocytes differentiation *in vitro* (Kanayama,
21 et al., 2005) and increases adipose mass *in vivo* (Grun, et al., 2006). In particular, *in utero*
22 exposure to TBT (with i.p. doses 2 or 20 times higher than the one we used) may increase
23 body fat mass (and not body weight), especially the fat located around the male gonadal
24 ducts (Grun, et al., 2006). It is possible that the low dosis of TBT orally administered to
25 adult mice in our experiment was not sufficient, or the period was too short to induce fat
26 deposition, even if at the end of the treatment our TBT-treated animals showed a higher
27 feed efficiency.
28
29

30
31 In contrast with the lack of effect on the weight of fat tissue we detected significant
32 effects on circulating levels of leptin (Fig. 3B). In fact, according to our results on fat
33 distribution, no significant variations of the leptin levels should occur, whereas, we
34 observed a significant decrease of the hormone. The leptin fall in TBT-treated adult mice
35 could be related to the putative xenoandrogenic action of TBT (Grote, et al., 2004), in
36 fact, estrogens increase leptin production, while androgens act in the opposite way
37 decreasing leptin levels (for a review see Mayes and Watson, 2004). The reduction of
38 leptin synthesis could be also mediated by TBT-dependent activation of PPAR γ , a
39 nuclear receptors that play important roles in lipid homeostasis and adipogenesis. In fact,
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 PPAR γ activation is known to be responsible of inhibition of genes that induce lipolysis
4 in adipocytes of white adipose tissue (Evans, et al., 2004, Ferré, 2004) and TBT was
5 shown to disrupt normal development and homeostatic controls over adipogenesis and
6 energy balance, resulting obesity through its action on PPAR γ and RXR (Grun, et al.,
7 2006).
8
9

10
11
12 The liver, which is one of the targets of the toxicant action of TBT, even if not
13 macroscopically altered (the volume was not significantly different in control or TBT-
14 treated mice) shows histological signals of hepatocytic degeneration, it seems therefore
15 that the supposed no effect dose has already a deleterious effect on this important organ
16 (Figs 3A-4).
17
18

19
20
21 As reported in the introduction, the food intake is regulated by cerebral
22 neuroendocrine circuits, mainly located in the hypothalamus, and leptin communicates
23 the levels of energy storage, inducing the state of satiety while suppressing food intake by
24 its action on these neuroendocrine circuits (Ahima, et al., 2009). However, reports on the
25 action of TBT on hypothalamic circuits are very rare and limited to studies on
26 neurotransmitters' expression and metabolisms in homogenates of discrete regions
27 (Konno, et al., 2001, Tsunoda, et al., 2004), or in cell cultures (He, et al., 2014,
28 Mizuhashi, et al., 2000, Nakatsu, et al., 2010, Nakatsu, et al., 2009). Only recently, we
29 demonstrated a short-term action of TBT in vivo, by inducing a significant increase of c-
30 fos expression in ARC elements after acute exposure to TBT (Bo, et al., 2011).
31
32
33

34
35
36 In the present experiment we studied changes induced by TBT in the expression of a
37 neuropeptide strictly related to the control of food intake, the NPY. In adult TBT-treated
38 males we observed a significant reduction of NPY expression in some of the investigated
39 hypothalamic nuclei (Figs 6-7). On the basis of the levels of circulating leptin in TBT-
40 treated animals and of the well known relationships among leptin and NPY (Myers, et al.,
41 2009, Robertson, et al., 2008), the leptin decrease should induce a parallel increase of
42 NPY-ir in ARC and, consequently, in its main targets: PVN, DMH, and VMH. On the
43 contrary, we observed, a significant reduction of NPY-immunoreactivity in male ARC,
44 PVN, and DMH, but not in females. In the VMH, a nucleus implicated both in the control
45 of energy metabolism and in the control of lordosis behavior (for reviews see Asarian and
46 Geary, 2013, Flanagan-Cato, 2011), we observed a reverse effect: no decrease in males
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 and a significant increase in TBT-treated females. Thus, there is a sex dimorphism in the
4 sensitivity of NPY-based neural circuits controlling food intake to TBT. This is probably
5 in agreement with other studies that indicate sex differences in body fat distribution as the
6 result of differences in sex hormones between males and females (for reviews see
7 Lovejoy and Sainsbury, 2009, Shi and Clegg, 2009).
8
9

10
11
12 The analysis of Y1-R transgene expression (Figs 8-9) revealed the presence of a sex
13 dimorphism in PVN, VMH and ARC. In general, TBT-treated females show a significant
14 decrease of the transgene expression (only a tendency for ARC) thus determining the
15 disappearance of the dimorphism when compared to control males. In all these nuclei the
16 exposure to TBT induced a significant decrease of Y1-R transgene expression in females,
17 whereas in males we observed a strong reduction, but this has not reached statistical
18 significance (p values 0.06-0.07) and it is probably due to the large variations in the
19 transgene expression in males. As previously demonstrated in the same transgenic strain
20 (Oberto, et al., 2003), lower levels of NPY-ir should induce an increase in Y1-R
21 transgene expression. On the contrary, in the present experiment we observed a decrease
22 of both NPY-ir and Y1-R transgene expression. Therefore, we have here a sex-oriented
23 defect in the fine-tuning among up- and down-regulation of NPY and its main receptor.
24 Previous studies have suggested that gonadal hormones may play a role in the expression
25 of Y1-R during the estrous cycle (Martini, et al., 2011) or in its regulation after exposure
26 to different diets (Zammaretti, et al., 2007). In addition, in this study we observed a sex
27 dimorphism in Y1-R expression, having females more β -galactosidase positivity than
28 males, thus the TBT is mainly acting masculinizing the Y1-R expression. It is therefore
29 possible that the decrease of Y1R expression in TBT-treated animals may depend on one
30 hand by a putative xenoandrogenic effect of TBT or, on the other hand, by the inhibition
31 of aromatase activity mediated by TBT (Cooke, 2002, Heidrich, et al., 2001, Li, et al.,
32 2015).
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

50
51 In conclusion, on the basis of the present findings, we can assume that TBT, in
52 addition to its effects on peripheral fat tissue, has some effects on neural circuits,
53 particularly directed to alter the neuroendocrine relationships among circulating leptin
54 levels and the hypothalamic circuits controlling food intake, and these effects seems to be
55
56
57
58
59
60

1
2
3 sexually differentiated. Further studies should investigate if other parts of the food intake
4 controlling system, as for example the α -MSH or the orexin circuits, may be affected by
5 TBT exposure.
6
7
8
9

10 11 12 **Acknowledgement**

13
14 This work was supported by Region Piemonte, Fondazione Cavalieri-Ottolenghi and
15 University of Torino.
16

17 18 **Disclosures**

19 The authors declare no conflict of interest
20

21 22 **Authors' contribution**

23 E.B., C.E., S.G. and G.C.P. designed and supervised the experiments; E.B., A.F., M.M.
24 and D.S. performed the experiments; E.B., A.F., S.G. and G.C.P. performed the statistical
25 analysis; E.B., S.G. and G.C.P. prepared the figures for publication; E.B., C.E., S.G. and
26 G.C.P. wrote the first draft of the manuscript; all authors read and approved the
27 manuscript.
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3
4 **Figure 5.** Microphotographs illustrating the immunohistochemical staining for NPY in
5 the nuclei that have been considered in the present study. Microphotographs are taken
6 at the same enlargement. Bar represents 500 microns. **A-** rostral section containing
7 the paraventricular nucleus (PVN). **B-** More caudal section containing the
8 dorsomedial (DMH), ventromedial (VMH) and arcuate (ARC) nuclei. * Third
9 ventricle.

10
11
12
13
14
15
16 **Figure 6.** Sections of the paraventricular nucleus from different experimental groups
17 immunostained for NPY. Microphotographs are taken at the same enlargement. Bar
18 represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated male; F
19 OIL - oil-treated female; F TBT - TBT-treated female. * Third ventricle.

20
21
22
23
24
25 **Figure 7.** Histograms representing NPY-ir in hypothalamus (expressed as percentage of
26 area covered by stained elements). The multiple comparison analysis between groups
27 for all nuclei demonstrated: in PVN, ARC, and DMH, a significant reduction of
28 NPY-ir in treated males compared to controls), while no significant effects were
29 detected for females. The significant differences (ANOVA followed by the
30 Bonferroni test at a level of $P < 0.05$) are denoted by **a** or **b**.

31
32
33
34
35
36
37 **Figure 8.** Histochemical detection of beta-galactosidase activity (expression of Y1R-
38 LacZ transgene) in sections of the paraventricular nucleus from different
39 experimental groups. Microphotographs are taken at the same enlargement. Bar
40 represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated male; F
41 OIL - oil-treated female; F TBT - TBT-treated female. * Third ventricle.

42
43
44
45
46
47
48 **Figure 9.** Histograms representing Y1R-transgene expression in hypothalamus
49 (expressed as dots/ μm^2). In females the treatment significantly reduced Y1R
50 expression in PVN and VMH. Also in males, TBT reduced Y1R-transgene
51 expression in both nuclei but the statistical p-value is only close to significance. The
52 significant differences (ANOVA followed by the Bonferroni test at a level of
53 $P < 0.05$) are denoted by **a** or **b**, ^ indicates a close to significant difference ($P < 0.07$).

REFERENCES

- Ahima RS, Saper CB, Flier JS, Elmquist JK. Leptin regulation of neuroendocrine systems. *Frontiers in Neuroendocrinology*. 2000;21:263-307.
- Asarian L, Geary N. Sex differences in the physiology of eating. *Am J Physiol Regul Integr Comp Physiol*. 2013;305:R1215-67.
- Aste N, Viglietti-Panzica C, Fasolo A, Andreone C, Vaudry H, Pelletier G, Panzica GC. Localization of neuropeptide Y (NPY) immunoreactive cells and fibres in the brain of the Japanese quail. *Cell and Tissue Research*. 1991;265:219-230.
- Austin J, Marks D. Hormonal regulators of appetite. *International journal of pediatric endocrinology*. 2009;2009:141753.
- Banks WA, Kastin AJ. Passage of peptides across the blood-brain barrier: Pathophysiological perspectives. *Life Sci*. 1996;59:1923-1943.
- Bates SH, Stearns WH, Dundon TA, Schubert M, Tso AW, Wang Y, Banks AS, Lavery HJ, Haq AK, Maratos-Flier E, Neel BG, Schwartz MW, Myers MG, Jr. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. *Nature*. 2003;421:856-9.
- Beck B. Neuropeptide Y in normal eating and in genetic and dietary-induced obesity. *Philos Trans R Soc Lond B Biol Sci*. 2006;361:1159-85.
- Bertocchi I, Oberto A, Longo A, Mele P, Sabetta M, Bartolomucci A, Palanza P, Sprengel R, Eva C. Regulatory functions of limbic Y1 receptors in body weight and anxiety uncovered by conditional knockout and maternal care. *Proc Natl Acad Sci U S A*. 2011;108:19395-400.
- Bo E, Viglietti-Panzica C, Panzica GC. Acute exposure to tributyltin induces c-fos activation in the hypothalamic arcuate nucleus of adult male mice. *Neurotoxicology*. 2011;32:277-80.
- Cinti S. Between brown and white: novel aspects of adipocyte differentiation. *Ann Med*. 2011;43:104-15.
- Cooke GM. Effect of organotins on human aromatase activity in vitro. *Toxicol Lett*. 2002;126:121-30.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- Cooke GM, Forsyth DS, Bondy GS, Tachon R, Tague B, Coady L. Organotin speciation and tissue distribution in rat dams, fetuses, and neonates following oral administration of tributyltin chloride. *J Toxicol Environ Health A*. 2008;71:384-95.
- Cooke GM, Tryphonas H, Pulido O, Caldwell D, Bondy GS, Forsyth D. Oral (gavage), in utero and postnatal exposure of Sprague-Dawley rats to low doses of tributyltin chloride. Part 1: Toxicology, histopathology and clinical chemistry. *Food Chem Toxicol*. 2004;42:211-20.
- Danger JM, Breton B, Vallarino M, Fournier A, Pelletier G, Vaudry H. Neuropeptide Y in the trout brain and pituitary: localization, characterization, and action on gonadotropin release. *Endocrinol*. 1991;128:2360-2368.
- Danger JM, Tonon MC, Basille C, Jenks BG, Saint Pierre S, Martel JC, Fasolo A, Quirion R, Pelletier G, Vaudry H. Neuropeptide Y: localization in the central nervous system and neuroendocrine functions. *Fundamental and Clinical Pharmacology*. 1990;4:307-340.
- Decherf S, Demeneix BA. The obesogen hypothesis: a shift of focus from the periphery to the hypothalamus. *J Toxicol Environ Health B Crit Rev*. 2011;14:423-48.
- Decherf S, Seugnet I, Fini JB, Clerget-Froidevaux MS, Demeneix BA. Disruption of thyroid hormone-dependent hypothalamic set-points by environmental contaminants. *Mol Cell Endocrinol*. 2010;323:172-82.
- Djazayery A, Miller DS, Stock MJ. Energy balances in obese mice. *Nutr Metab*. 1979;23:357-67.
- EFSA. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission to assess the health risks to consumers associated with exposure to organotins in foodstuffs. *EFSA Journal*. 2004;102:1-119.
- Eva C, Mele P, Collura D, Nai A, Pisu MG, Serra M, Biggio G. Modulation of neuropeptide Y and Y1 receptor expression in the amygdala by fluctuations in the brain content of neuroactive steroids during ethanol drinking discontinuation in Y1R/LacZ transgenic mice. *J Neurochem*. 2008;104:1043-54.
- Eva C, Serra M, Mele P, Panzica GC, Oberto A. Physiology and gene regulation of the brain NPY Y1 receptor. *Frontiers in Neuroendocrinology*. 2006;27:308-339.

- 1
2
3 Evans RM, Barish GD, Wang YX. PPARs and the complex journey to obesity. *Nat Med.*
4 2004;10:355-61.
5
6
7 Ferrara G, Serra G, Zammaretti F, Pisu MG, Panzica GC, Biggio G, Eva C. Increased
8 expression of the neuropeptide Y receptor Y1 gene in the medial amygdala of
9 transgenic mice induced by long-term treatment with progesterone or
10 allopregnanolone. *Journal of Neurochemistry.* 2001;79.
11
12
13 Ferré P. The biology of peroxisome proliferator-activated receptors: relationship with
14 lipid metabolism and insulin sensitivity. *Diabetes.* 2004;53 Suppl 1:S43-50.
15
16
17 Flanagan-Cato LM. Sex differences in the neural circuit that mediates female sexual
18 receptivity. *Front Neuroendocrinol.* 2011;32:124-36.
19
20
21 Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS. Leptin levels
22 reflect body lipid content in mice: evidence for diet-induced resistance to leptin
23 action. *Nat Med.* 1995;1:1311-4.
24
25
26 Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature.*
27 1998;395:763-770.
28
29
30 Frye C, Bo E, Calamandrei G, Calza L, Dessi-Fulgheri F, Fernandez M, Fusani L, Kah O,
31 Kajta M, Le Page Y, Patisaul HB, Venerosi A, Wojtowicz AK, Panzica GC.
32 Endocrine Disrupters: A Review of Some Sources, Effects, and Mechanisms of
33 Actions on Behaviour and Neuroendocrine Systems. *J Neuroendocrinol.*
34 2012;24:144-159.
35
36
37 Grote K, Stahlschmidt B, Talsness CE, Gericke C, Appel KE, Chahoud I. Effects of
38 organotin compounds on pubertal male rats. *Toxicology.* 2004;202:145-58.
39
40
41 Grun F. The obesogen tributyltin. *Vitam Horm.* 2014;94:277-325.
42
43
44 Grun F, Blumberg B. Environmental obesogens: organotins and endocrine disruption via
45 nuclear receptor signaling. *Endocrinology.* 2006;147:S50-55.
46
47
48 Grun F, Watanabe H, Zamanian Z, Maeda L, Arima K, Cubacha R, Gardiner DM, Kanno
49 J, Iguchi T, Blumberg B. Endocrine-disrupting organotin compounds are potent
50 inducers of adipogenesis in vertebrates. *Mol Endocrinol.* 2006;20:2141-55.
51
52
53 Hara K, Yoshizuka M, Doi Y, Fujimoto S. Effect of bis (tributyltin) oxide on
54 permeability of the blood-brain barrier: a transient increase. *Occup Environ Med.*
55 1994;51:735-8.
56
57
58
59
60

- 1
2
3 He K, Zhang J, Chen Z. Effect of tributyltin on the food intake and brain neuropeptide
4 expression in rats. *Endokrynol Pol.* 2014;65:485-90.
5
6
7 Heidrich DD, Steckelbroeck S, Klingmuller D. Inhibition of human cytochrome P450
8 aromatase activity by butyltins. *Steroids.* 2001;66:763-9.
9
10 Heindel JJ, Vom Saal FS, Blumberg B, Bovolin P, Calamandrei G, Ceresini G, Cohn BA,
11 Fabbri E, Gioiosa L, Kassotis C, Legler J, La Merrill M, Rizzir L, Machtinger R,
12 Mantovani A, Mendez MA, Montanini L, Molteni L, Nagel SC, Parmigiani S,
13 Panzica G, Paterlini S, Pomatto V, Ruzzin J, Sartor G, Schug TT, Street ME, Suvorov
14 A, Volpi R, Zoeller RT, Palanza P. Parma consensus statement on metabolic
15 disruptors. *Environ Health.* 2015;14:54.
16
17
18 Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS. Increased adipose tissue in
19 male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci U S A.*
20 2000;97:12729-34.
21
22
23 Kanayama T, Kobayashi N, Mamiya S, Nakanishi T, Nishikawa J. Organotin compounds
24 promote adipocyte differentiation as agonists of the peroxisome proliferator-activated
25 receptor gamma/retinoid X receptor pathway. *Mol Pharmacol.* 2005;67:766-74.
26
27
28 Kanemoto Y, Ishibashi H, Matsuo S, Oyama Y, Akaike N. Modification of NMDA
29 responses by tri-n-butyltin in rat brain neurons. *Br J Pharmacol.* 2002;136:201-6.
30
31
32 Kimura K, Kobayashi K, Naito H, Suzuki Y, Sugita-Konishi Y. Effect of lactational
33 exposure to tributyltin chloride on innate immunodefenses in the F1 generation in
34 mice. *Biosci Biotechnol Biochem.* 2005;69:1104-10.
35
36
37 Kirchner S, Kieu T, Chow C, Casey S, Blumberg B. Prenatal exposure to the
38 environmental obesogen tributyltin predisposes multipotent stem cells to become
39 adipocytes. *Molecular endocrinology (Baltimore, Md).* 2010;24:526-539.
40
41
42 Konno N, Tsunoda M, Nakano K, Liu Y. Effect of tributyltin on the N-methyl-D-
43 aspartate (NMDA) receptors in the mouse brain. *Arch Toxicol.* 2001;75:549-54.
44
45
46 Ladyman SR, Woodside B. Regulation of maternal food intake and mother-pup
47 interactions by the Y5 receptor. *Physiol Behav.* 2009;97:91-7.
48
49
50 Lee JJ, Kim YM, Park SK, Lee MK. Effects of tributyltin chloride on L-DOPA-induced
51 cytotoxicity in PC12 cells. *Arch Pharm Res.* 2006;29:645-50.
52
53
54
55
56
57
58
59
60

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- Leinninger GM, Jo YH, Leshan RL, Louis GW, Yang H, Barrera JG, Wilson H, Opland DM, Faouzi MA, Gong Y, Jones JC, Rhodes CJ, Chua S, Jr., Diano S, Horvath TL, Seeley RJ, Becker JB, Munzberg H, Myers MG, Jr. Leptin acts via leptin receptor-expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine system and suppress feeding. *Cell Metab.* 2009;10:89-98.
- Leung KM, Kwong RP, Ng WC, Horiguchi T, Qiu JW, Yang R, Song M, Jiang G, Zheng GJ, Lam PK. Ecological risk assessments of endocrine disrupting organotin compounds using marine neogastropods in Hong Kong. *Chemosphere.* 2006;65:922-38.
- Li ZH, Zhong LQ, Wu YH, Mu WN. Alteration of cytochrome P450 1 regulation and HSP 70 level in brain of juvenile common carp (*Cyprinus carpio*) after chronic exposure to tributyltin. *Fish Physiol Biochem.* 2015.
- Lima D, Castro LFC, Coelho I, Lacerda R, Gesto M, Soares J, André A, Capela R, Torres T, Carvalho AP, Santos MM. Effects of Tributyltin and Other Retinoid Receptor Agonists in Reproductive-Related Endpoints in the Zebrafish (*Danio rerio*). *Journal of toxicology and environmental health Part A.* 2015;78:747-60.
- Longo A, Oberto A, Mele P, Mattiello L, Pisu MG, Palanza P, Serra M, Eva C. NPY-Y1 coexpressed with NPY-Y5 receptors modulate anxiety but not mild social stress response in mice. *Genes Brain Behav.* 2015;14:534-42.
- Lovejoy JC, Sainsbury A. Sex differences in obesity and the regulation of energy homeostasis. *Obes Rev.* 2009;10:154-67.
- Martin TL, Alquier T, Asakura K, Furukawa N, Preitner F, Kahn BB. Diet-induced obesity alters AMP kinase activity in hypothalamus and skeletal muscle. *J Biol Chem.* 2006;281:18933-41.
- Martini M, Sica M, Gotti S, Eva C, Panzica GC. Effects of estrous cycle and sex on the expression of neuropeptide Y Y1 receptor in discrete hypothalamic and limbic nuclei of transgenic mice. *Peptides.* 2011;32:1330-4.
- Matsuoka M, Igisu H. Induction of c-fos expression by tributyltin in PC12cells: involvement of intracellular Ca²⁺. *Environmental Toxicology and Pharmacology.* 1996;2:373-380.

- 1
2
3 Mayes JS, Watson GH. Direct effects of sex steroid hormones on adipose tissues and
4 obesity. *Obes Rev.* 2004;5:197-216.
5
6
7 Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Trayhurn P.
8 Localization (Ob-Rb) of leptin receptor mRNA and the long form splice variant in
9 mouse hypothalamus and adjacent brain regions by in situ hybridization. *FEBS Lett.*
10 387, 113-116. *FEBS Letters.* 1996;387:113-116.
11
12
13
14 Michel C, Levin BE, Dunn-Meynell AA. Stress facilitates body weight gain in
15 genetically predisposed rats on medium-fat diet. *Am J Physiol Regul Integr Comp*
16 *Physiol.* 2003;285:R791-9.
17
18
19
20 Mitra S, Siddiqui WA, Khandelwal S. Early cellular responses against tributyltin chloride
21 exposure in primary cultures derived from various brain regions. *Environ Toxicol*
22 *Pharmacol.* 2014;37:1048-59.
23
24
25
26 Mitra S, Siddiqui WA, Khandelwal S. Differential susceptibility of brain regions to
27 tributyltin chloride toxicity. *Environ Toxicol.* 2015;30:1393-405.
28
29
30 Mizuhashi S, Ikegaya Y, Nishiyama N, Matsuki N. Cortical astrocytes exposed to
31 tributyltin undergo morphological changes in vitro. *Jpn J Pharmacol.* 2000;84:339-46.
32
33
34
35 Montague CT, Prins JB, Sanders L, Zhang J, Sewter CP, Digby J, Byrne CD, O'Rahilly
36 S. Depot-related gene expression in human subcutaneous and omental adipocytes.
37 *Diabetes.* 1998;47:1384-91.
38
39
40
41 Myers MG, Jr., Munzberg H, Leininger GM, Leshan RL. The geometry of leptin action
42 in the brain: more complicated than a simple ARC. *Cell Metab.* 2009;9:117-23.
43
44
45
46 Nakatsu Y, Kotake Y, Hino A, Ohta S. Activation of AMP-activated protein kinase by
47 tributyltin induces neuronal cell death. *Toxicol Appl Pharmacol.* 2008;230:358-63.
48
49
50
51 Nakatsu Y, Kotake Y, Komasa K, Hakozaiki H, Taguchi R, Kume T, Akaike A, Ohta S.
52 Glutamate excitotoxicity is involved in cell death caused by tributyltin in cultured rat
53 cortical neurons. *Toxicol Sci.* 2006;89:235-42.
54
55
56
57 Nakatsu Y, Kotake Y, Ohta S. Tributyltin-induced cell death is mediated by calpain in
58 PC12 cells. *Neurotoxicology.* 2006;27:587-93.
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

- 1
2
3 Nakatsu Y, Kotake Y, Takai N, Ohta S. Involvement of autophagy via mammalian target
4 of rapamycin (mTOR) inhibition in tributyltin-induced neuronal cell death. *J Toxicol*
5 *Sci.* 2010;35:245-51.
6
7
8
9 Nakatsu Y, Kotake Y, Takishita T, Ohta S. Long-term exposure to endogenous levels of
10 tributyltin decreases GluR2 expression and increases neuronal vulnerability to
11 glutamate. *Toxicol Appl Pharmacol.* 2009;240:292-8.
12
13
14 Newbold RR, Padilla-Banks E, Snyder RJ, Phillips TM, Jefferson WN. Developmental
15 exposure to endocrine disruptors and the obesity epidemic. *Reprod Toxicol.*
16 2007;23:290-6.
17
18
19 Nguyen AD, Herzog H, Sainsbury A. Neuropeptide Y and peptide YY: important
20 regulators of energy metabolism. *Curr Opin Endocrinol Diabetes Obes.* 2011;18:56-
21 60.
22
23
24 Oberto A, Mele P, Zammaretti F, Panzica GC, Eva C. Evidence of Altered Neuropeptide
25 Y Content and Neuropeptide Y1 Receptor Gene Expression in the Hypothalamus of
26 Pregnant Transgenic Mice. *Endocrinology.* 2003;144:4826-4830.
27
28
29 Oberto A, Panzica G, Altruda F, Eva C. Chronic modulation of the GABA_(A) receptor
30 complex regulates Y₁ receptor gene expression in the medial amygdala of transgenic
31 mice. *Neuropharmacology.* 2000;39:227-34.
32
33
34 Oberto A, Panzica GC, Altruda F, Eva C. GABAergic and NPY-Y1 network in the
35 medial amygdala: a neuroanatomical basis for their functional interaction.
36 *Neuropharmacology.* 2001;41:639-642.
37
38
39 Oberto A, Tolosano E, Brusa R, Altruda F, Panzica G, Eva C. The murine Y1 receptor 5'
40 upstream sequence directs cell-specific and developmentally regulated LacZ
41 expression in transgenic mice CNS. *European Journal of Neuroscience.*
42 1998;10:3257-68.
43
44
45 Palanza P, Morellini F, Parmigiani S, vom Saal FS. Ethological methods to study the
46 effects of maternal exposure to estrogenic endocrine disruptors: a study with
47 methoxychlor. *Neurotoxicol Teratol.* 2002;24:55-69.
48
49
50 Panzica GC, Bo E, Martini MA, Miceli D, Mura E, Viglietti-Panzica C, Gotti S.
51 Neuropeptides and Enzymes are Targets for the Action of Endocrine Disrupting
52
53
54
55
56
57
58
59
60

- 1
2
3 Chemicals in the Vertebrate Brain. *J Toxicol Environ Health B Crit Rev*.
4 2011;14:449-72.
5
6
7 Panzica GC, Viglietti-Panzica C, Mura E, Quinn Jr MJ, Palanza P, Ottinger MA. Effects
8 of xenoestrogens on the differentiation of behaviorally relevant neural circuits.
9 *Frontiers in Neuroendocrinology*. 2007;28:179-200.
10
11
12 Paxinos G, Franklin KBJ. *The Mouse Brain in Stereotaxic Coordinates*. Second Edition
13 ed. San Diego: Academic Press; 2001.
14
15
16 Pelletier G, Desy L, Kerkerian L, Cote J. Immunocytochemical localization of
17 neuropeptide Y (NPY) in the human hypothalamus. *Cell Tissue Res*. 1984;238:203-5.
18
19 Pelletier G, Guy J, Allen YS, Polak JM. Electron microscopic immunocytochemical
20 localization of neuropeptide Y (NPY) in the rat brain. *Neuropeptides*. 1984;4:319-
21 324.
22
23
24 Perroteau I, Danger JM, Biffo S, Pelletier G, Vaudry H, Fasolo A. Distribution and
25 characterization of neuropeptide Y immunoreactivity in the brain of the crested newt.
26 *Journal of Comparative Neurology*. 1988;275:309-325.
27
28
29 Pierman S, Sica M, Allieri F, Viglietti-Panzica C, Panzica GC, Bakker J. Activational
30 effects of estradiol and dihydrotestosterone on social recognition and the arginine-
31 vasopressin immunoreactive system in male mice lacking a functional aromatase
32 gene. *Horm Behav*. 2008;54:98-106.
33
34
35 Plumari L, Viglietti Panzica C, Allieri F, Honda S, Harada N, Absil P, Balthazar J,
36 Panzica GC. Changes in the Arginine-Vasopressin Immunoreactive Systems in Male
37 Mice Lacking a Functional Aromatase Gene. *Journal of Neuroendocrinology*.
38 2002;14:971-978.
39
40
41 Rantakokko P, Main KM, Wohlfart-Veje C, Kiviranta H, Airaksinen R, Vartiainen T,
42 Skakkebaek NE, Toppari J, Virtanen HE. Association of placenta organotin
43 concentrations with growth and ponderal index in 110 newborn boys from Finland
44 during the first 18 months of life: a cohort study. *Environ Health*. 2014;13:45.
45
46
47 Rantakokko P, Main KM, Wohlfart-Veje C, Kiviranta H, Airaksinen R, Vartiainen T,
48 Skakkebaek NE, Toppari J, Virtanen HE. Association of placenta organotin
49 concentrations with congenital cryptorchidism and reproductive hormone levels in
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 280 newborn boys from Denmark and Finland. *Human reproduction* (Oxford,
4 England). 2013;28:1647-60.
5
6
7 Robertson SA, Leininger GM, Myers MG, Jr. Molecular and neural mediators of leptin
8 action. *Physiol. Behav.* 94, 637–642. *Physiology and Behavior.* 2008;94:637-642.
9
10 Shi H, Clegg DJ. Sex differences in the regulation of body weight. *Physiol Behav.*
11 2009;97:199-204.
12
13 Stanley BG, Kyrkpouli SE, Lampert S, Leibowitz SF. Neuropeptide Y injected into the
14 hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. *Peptides.*
15 1986;7:1189-1192.
16
17 Stanley BG, Leibowitz SF. Neuropeptide Y injected in the paraventricular hypothalamus:
18 a powerful stimulant of feeding behavior. *Proceedings of the National Academy of*
19 *Sciences of the United States of America.* 1985;82:3940-3943.
20
21 Streefkerk JG. Inhibition of erythrocyte peroxidase activity by treatment with hydrogen
22 peroxide following methanol. *Journal of Histochemistry and Cytochemistry.*
23 1972;20:829-831.
24
25 Tryphonas H, Cooke G, Caldwell D, Bondy G, Parenteau M, Hayward S, Pulido O. Oral
26 (gavage), in utero and post-natal exposure of Sprague-Dawley rats to low doses of
27 tributyltin chloride. Part II: effects on the immune system. *Food Chem Toxicol.*
28 2004;42:221-35.
29
30 Tsunoda M, Konno N, Nakano K, Liu Y. Altered metabolism of dopamine in the
31 midbrain of mice treated with tributyltin chloride via subacute oral exposure. *Environ*
32 *Sci.* 2004;11:209-19.
33
34 Valassi E, Scacchi M, Cavagnini F. Neuroendocrine control of food intake. *Nutrition,*
35 *Metabolism & Cardiovascular Diseases.* 2008;18:158-168.
36
37 Watson RE, Wiegand SJ, Clough RW, Hoffman GE. Use of cryoprotectant to maintain
38 long-term peptide immunoreactivity and tissue morphology. *Peptides.* 1986;7:155-
39 159.
40
41 Zammaretti F, Panzica G, Eva C. Sex-dependent regulation of hypothalamic
42 neuropeptide Y-Y1 receptor gene expression in moderate/high fat, high-energy diet-
43 fed mice. *J Physiol.* 2007;583:445-54.
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Zammaretti F, Panzica GC, Eva C. Fasting, leptin treatment and glucose administration differentially regulate Y1 receptor gene expression in the hypothalamus of transgenic mice. *Endocrinology*. 2001;142:3774-3782.

For Peer Review

1
2
3 **Adult exposure to tributyltin affects hypothalamic neuropeptide Y, Y1 receptor**
4 **distribution, and circulating leptin in mice.**
5
6
7

8 **Bo E., Farinetti A., Marraudino M., Sterchele D, Eva C., Gotti S., Panzica GC.***
9

10
11
12 Dept Neuroscience "Rita Levi Montalcini", University of Torino, Italy, National Institute
13 of Neuroscience (INN, Torino), and Neuroscience Institute Cavalieri-Ottolenghi (NICO)
14
15

16
17 Running title: Effects of TBT on mice leptin-NPY system
18
19

20
21
22
23
24 Key words: TBT; NPY; food intake; arcuate nucleus; paraventricular nucleus;
25 dorsomedial nucleus; C57BL/6 mice
26
27
28
29
30
31
32

33 *Corresponding author:
34

35 Prof. GianCarlo Panzica
36

37 Laboratory of Neuroendocrinology, Neuroscience Institute Cavalieri-Ottolenghi (NICO)
38

39 Regione Gonzole, 10, 10043 – Orbassano (Torino, Italy)
40

41 Phone +39 0116706607, Fax +39 0112366607, e-mail: giancarlo.panzica@unito.it
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

ABSTRACT

Tributyltin (TBT) is a pesticide, used in antifouling paints, toxic for aquatic invertebrates. In vertebrates, TBT may act as obesogen inducing adipogenic genes transcription for adipocytes 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 NPY immunoreactivity. 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 adipocytes 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 (Leininger, 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

1
2
3 (VMH) and DMH [for a review see (Valassi, et al., 2008)]. Acute central administration
4 of NPY induces increase of food intake (Stanley and Leibowitz, 1985), whereas chronic
5 administration produces hyperphagia, decrease of thermogenesis and obesity (Stanley, et
6 al., 1986). The action of NPY is mediated by several receptors, among them: Y1 and Y5
7 receptors are mainly related to the food control (Beck, 2006). In addition to its role to
8 regulate feeding behavior, Y1-R is involved in several NPY-induced responses, such as
9 anxiolytic effects and stress responses (Bertocchi, et al., 2011, Longo, et al., 2015),
10 ethanol drinking behavior, activation of neuroendocrine axis (for a review see Eva, et al.,
11 2006). The Y5-R subtype may have some role in the control of nesting behavior, i.e.
12 during lactation with effects on litter growth rates (Ladyman and Woodside, 2009). Thus,
13 the central nervous system plays a fundamental role in food intake regulation integrating
14 neural and endocrine factors. According to this point of view, obesity is not only
15 dependent by peripheral change of energy homeostasis, but can also be considered a
16 “neuroendocrine disease” depending on alteration of the axis leptin/hypothalamic circuits
17 controlling food intake (Austin and Marks, 2009).
18
19

20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 (PPAR γ) and retinoid X receptor (RXR α , RXR β , and RXR γ), that induces *in vitro* differentiation of pre-adipocytes to adipocytes, 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

1
2
3 obesity development in vertebrates (Grun, et al., 2006) probably through its action to
4 predispose multipotent stem cells to become adipocytes (Kirchner, et al., 2010).
5 However, currently, the effects of this compound on the central nervous system have
6 received less attention (Decherf and Demeneix, 2011). *In vitro* studies demonstrated that
7 TBT may induce the expression of c-fos (Matsuoka and Igisu, 1996), apoptosis (Nakatsu,
8 et al., 2008, Nakatsu, et al., 2006, Nakatsu, et al., 2007), and inhibit dopamine
9 biosynthesis (Lee, et al., 2006) in PC12 cell cultures. In primary cultures of dissociated
10 neurons, TBT increased the Ca²⁺-activated K⁺ current induced by NMDA application
11 (Kanemoto, et al., 2002), is involved in cell death by glutamate excitotoxicity (Nakatsu,
12 et al., 2006), and it has different effects on dissociated cells from different brain regions
13 (Mitra, et al., 2014).

14
15
16
17
18
19
20
21
22
23 With respect to the *in vivo* studies, it has been demonstrated that TBT may cross the
24 placenta (Kimura, et al., 2005) and the blood-brain barrier (Hara, et al., 1994), passing
25 into the maternal milk and accumulating in pups' tissues, including the brain (Cooke, et
26 al., 2008). Therefore, the central nervous system is a potential target for its action, at least
27 for exposition during pregnancy or lactation. The exposure to TBT during the gestational
28 period induces hypothyroidism in the progeny, whereas the acute treatment of pregnant
29 females in the same period induces a dose-dependent increase of T₃-independent TRH
30 transcription levels (Decherf, et al., 2010). TBT induces oxidative damages in various
31 region of the adult rat brain, including cerebellum, hippocampus, hypothalamus, and
32 striatum (Mitra, et al., 2015). In an earlier study we demonstrated that TBT acute
33 administration induces, in short time, the expression of c-fos in ARC (Bo, et al., 2011) a
34 key hypothalamic nucleus for the control of food intake (Myers, Munzberg, Leininger
35 and Leshan, 2009). More recently, He and coworkers (He, et al., 2014) demonstrated
36 alterations in the mRNA content of hypothalamic peptides [NPY, pro-opiomelanocortin
37 (POMC)] in adult male and female rats exposed to TBT for 8 weeks. In the present study,
38 we investigated if the reported adverse effects of TBT on some physiological parameters
39 may also affect brain NPY/Y1-R circuits involved in feeding control (PVN, ARC, DMH
40 and VMH). As in previous studies in rodents (Cooke, et al., 2004, Tryphonas, et al.,
41 2004), the doses of TBT selected for this study was based on the NOEL (No Observed
42 Effect Level) dose corresponding to 0.025µg/g body weight /day (EFSA, 2004).
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 μ l 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 $22 \pm 1^\circ\text{C}$ 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

1
2
3 changes in transgene expression (Ferrara, et al., 2001, Martini, et al., 2011, Oberto, et al.,
4 2003, Oberto, et al., 2000, Oberto, et al., 2001, Oberto, et al., 1998, Zammaretti, et al.,
5 2007, Zammaretti, et al., 2001).

6 7 8 *First experiment*

9
10 A preliminary experiment was performed with 48 C57BL/6-Y1-R/LacZ male mice in
11 order to choose the correct dose. According to some previous studies made on rats
12 (Cooke, et al., 2004, Tryphonas, et al., 2004) we tried a range of 0.025, 0.25, or 2.5mg of
13 tributyltin (TBT) per Kg of body weight per day. Males were randomly divided in the
14 four experimental groups, and the experiment should originally cover 4 weeks (see below
15 for the description of the TBT administration). However, the mice of the 2.5 and 0.25
16 groups died in a few days before the end of the first week (see Table 1). The dose of
17 0.025mg/Kg of body weight (classified as NOAEL dose in the literature) was therefore
18 selected for the second experiment.

19 20 21 *Second experiment*

22
23 Twenty four C57BL/6-Y1-R/LacZ mice with adequate weight gain and without
24 clinical signs were divided randomly into four experimental groups and individually
25 housed: six males (M) and six females (F) were the control groups treated with vehicle
26 only (OIL), six males and six females received daily 0.025mg/kg of body weight per day
27 of TBT. The vehicle or the TBT solution was administered through a pipette with a not
28 stressful procedure (Palanza, et al., 2002). In brief, to reduce stress in animals, they were
29 manipulated for a period of ten days before starting the experiment, and then mice were
30 picked up by the skin between the shoulders and held upright. A micropipette tip was
31 placed into the mouth with the pipette tip gently touching the roof of the mouth, and the
32 oil (final volume 30µl) was ejected from the pipette. Mice readily consume oil. The
33 treatment was prolonged for 4 weeks.

34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 *Feed efficiency and body weight*

50
51 Animals were fed with a standard diet 4RF21 GLP certificate (Mucedola, Italy)
52 containing 2.668 Kcal/g of metabolizable energy with 21.7 % as protein, 0.4% as fat and
53 66.5% as carbohydrate.
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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.

1
2
3 Liver, kidney and gonads were weighed and stored with the same procedure utilized
4 for the brain, for further analyses. In order to analyze the hepatic tissue integrity, livers
5 were cut with a cryostat at 10 μ m of thickness, collected on glass slides and then
6 processed for hematoxylin/eosin staining.
7
8
9

10 11 12 *Hormone assay*

13
14 Blood samples, collected in EDTA-treated tubes were centrifuged at 2000 rpm for 10
15 min to separate the plasma that was stored frozen at -80°C. Circulating leptin was
16 evaluated in plasma samples by using a commercial EIA kit anti-mouse leptin (SpiBio,
17 INALCO, Milano, Italy). Briefly, 100 μ l of diluted samples (1:2) were dispensed in
18 duplicate into wells of the microtiter plates for ELISA, and the plates were incubated for
19 1hr at room temperature, followed by washing three times with EIA-buffer. Biotin-
20 conjugated anti-leptin antibody was diluted to 1:10 in buffer containing bovine serum
21 albumin 1 mg/ml, and 100 μ l of the diluted antibody solution was added to each well. The
22 plates were incubated for 1hr at room temperature and washed thoroughly with EIA-
23 buffer. Streptavidin-HRP complex was added (100 μ l) to each well and incubated for 30
24 min at room temperature. After washing, the substrate solution (100 μ l) was added to each
25 well, and the plate was incubated for exactly 10 min at room temperature in the dark. To
26 stop the reaction of color development, which is dependent on the amount of leptin in
27 each well, 100 μ l of blocking solution were added. The intensity of color that developed
28 was measured at 490 nm with a microplate reader within 5 min from the stop of the
29 reaction. Leptin concentrations in diluted plasma samples were calculated by the standard
30 curve after subtracting the small value of nonspecific color development with a EIA
31 buffer blank.
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47

48 *Adipose tissue evaluation*

49 After brain dissection, two representative pads of White Adipose Tissue (WAT) were
50 manually dissected and weighted: adipose subcutaneous and gonadal fat pad (Cinti,
51 2011).
52
53
54
55
56
57
58
59
60

1
2
3 The fat pads were post-fixed in **paraformaldehyde** solution for 24 hours at 4°C,
4 cryoprotected with sucrose solution (30% in saline-phosphate buffer 0.1M), frozen in
5 liquid isopentane at -35°C, and stored at -80°C for future analysis.
6
7
8
9

10 *NPY immunohistochemistry*

11
12 Brains were cut in the coronal plane with a cryostat at 25µm of thickness, collected in
13 multiwell dishes for free-floating procedure with a cryoprotectant solution (Watson, et
14 al., 1986) and kept at -20°C. One series of sections (every fourth section, i.e. one section
15 every 100 µm) was stained for NPY immunohistochemistry. Brain sections were always
16 stained in groups containing males and females treated and non-treated, so that between
17 assays variance could not cause systematic group differences.
18
19
20
21
22

23 The procedure was previously tested and optimized in the same mouse strain (Eva, et
24 al., 2008, Oberto, et al., 2003). Briefly, after overnight washing in PBS, sections were
25 exposed to Triton X-100 (0.2% in PBS) and then treated for blocking endogenous
26 peroxidase activity [methanol/hydrogen peroxide for 20 minutes (Streefkerk, 1972)],
27 incubated with normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30
28 minutes. Then, they were incubated overnight at room temperature with a rabbit
29 polyclonal antibody against synthetic porcine NPY (a generous gift of H. Vaudry, Rouen,
30 France) diluted 1:6,000 in PBS, pH 7.3-7.4, containing 0.2% Triton X-100, followed by a
31 biotinylated anti-rat IgG (Vector, Peterborough, UK, cat. #: VC-BA-9400-MC15), diluted
32 1:250 with PBS-Triton. Sections were then processed for 1 hour in Avidin-Biotin-
33 Peroxidase Complex (Vector, Peterborough, UK) and rinsed in PBS. The peroxidase
34 activity was visualized with a solution containing 0.15 mg/ml 3,3'-diamino-benzidine
35 (DAB, Sigma-Aldrich, Europe) and 0.025% hydrogen peroxide in 0.05 M Tris-HCl
36 buffer pH 7.6. Sections were collected on chromalum-coated slides, air-dried, washed in
37 xylene, and coverslipped with Entellan (Merck, Milano, Italy).
38
39
40
41
42
43
44
45
46
47
48

49 The specificity of anti-NPY antibody has been previously reported (Pelletier, et al.,
50 1984, Pelletier, et al., 1984). This antibody was largely used for immunohistochemical
51 detection of NPY immunoreactive system in a wide range of vertebrate species, including
52 rodents and humans (Aste, et al., 1991, Danger, et al., 1991, Danger, et al., 1990,
53 Pelletier, et al., 1984, Pelletier, et al., 1984, Perroteau, et al., 1988). In addition, we have
54
55
56
57
58
59
60

1
2
3 performed the following controls in our material: a) the primary antibody was omitted or
4 replaced with an equivalent concentration of normal serum (negative controls); b) the
5 secondary antibody was omitted. In these conditions, cells and fibers were totally
6 unstained.
7
8
9

10 A second series of brain sections was Nissl-stained with toluidine blue for anatomical
11 orientation. All structures were identified using the stereotaxis atlas of the mouse brain
12 (Paxinos and Franklin, 2001).
13
14
15

16 *β -galactosidase histochemistry*

17
18 Y1/LacZ expression was determined by β -galactosidase staining on a third series of
19 brain coronal sections, as previously described (Oberto, et al., 2003, Oberto, et al., 1998,
20 Zammaretti, et al., 2007, Zammaretti, et al., 2001). Briefly, sections were incubated
21 overnight at 37°C in a solution containing 1mg/ml X-gal, 5mM potassium ferricyanide,
22 5mM potassium ferrocyanide, 2mM MgCl₂, 0.01% Triton-X 100 in PBS. Slices were
23 then washed in deionized water for 5min, counterstained with nuclear fast red,
24 coverslipped with Entellan (Merck, Milano, Italy) and analyzed. The transgene is
25 expressed in the neuronal cell body and is typically detected as a juxtannuclear blue dot
26 (Oberto, et. al, 1998).
27
28
29
30
31
32
33
34
35
36
37
38
39

40 *Quantitative analysis*

41 *1. NPY-immunoreactivity quantification*

42 For quantification we selected four nuclei involved in circuits controlling food intake
43 and showing various degrees of NPY immunoreactivity: ARC, VMH, DMH, and PVN
44 The density of NPY-immunoreactive structures was measured on three consecutive
45 sections per nucleus per animal (ARC, VMH, DMH: bregma -1.46mm, -1.58mm -
46 1.70mm; PVN: bregma -0.70mm, -0.82mm, -0.94mm, mouse brain atlas, Paxinos and
47 Franklin, 2001). All sections were observed with a Olympus BH2 microscope and
48 selected fields were then acquired by a Leica DFC320 video camera connected to a
49 Power PC G4 Macintosh. The staining density of NPY-immunoreactive structures was
50 measured in selected nuclei with Image J 1.38 software (Wayne Rasband, NIH,,
51
52
53
54
55
56
57
58
59
60

1
2
3 Bethesda, Maryland, USA) by calculating in binary transformations of the images
4 (threshold function) the fractional area (percentages of pixels) covered by
5 immunoreactive structures in predetermined fields (area of interest, AOI) as described in
6 previous studies (Oberto, et al., 2003, Pierman, et al., 2008, Plumari, et al., 2002). The
7 AOI selected for each nucleus was a box of fixed size and shape, selected to cover
8 immunoreactive material only within the boundaries of each nucleus (140,000 μm^2 for
9 VMH and DMH; 110,000 μm^2 for Arc; and 200,000 μm^2 for PVN).
10
11

12
13
14
15
16 The results for each nucleus were analyzed by two-way analysis of variance
17 (ANOVA) for repeated measures with a mixed design using the treatment and the sex as
18 independent factors and the antero-posterior neuroanatomical levels as repeated factor.
19 When preliminary analyses revealed no significant effects of anterior-posterior levels, the
20 levels were collapsed and the average number (calculated using the average values from
21 two sections) was analyzed by a two-way ANOVA (treatment and sex as independent
22 factors).
23
24
25
26
27
28
29

30 2. *β -Galactosidase histochemistry quantification*

31
32 Quantification of Y1R/LacZ transgene expression was performed by computer-
33 assisted analysis of β -galactosidase histochemical stained coronal brain sections (25 μm)
34 (Oberto, et al., 2003, Zammaretti, et al., 2007, Zammaretti, et al., 2001). Three
35 standardized sections of comparable levels of the PVN, ARC, VMH and DMH were
36 examined. Selected sections were placed on Nikon microscope and analyzed with Image
37 J software. Using a manual threshold method, dots were selected. The AOI selected for
38 each nucleus was the same box of fixed size and shape used for NPY-ir quantification.
39 For each animal and nucleus, the cumulative number of dots (corresponding to the
40 number of cell expressing the transgene) and the cumulative areas of the analyzed
41 sections were considered to obtain the density expression of the transgene expressed as
42 dots per μm^2 .
43
44
45
46
47
48
49
50
51

52 *Statistical analysis*

53
54 Quantitative data were examined with SPSS statistic software (SPSS inc., Chicago, USA)
55 by analysis of variance (two-way ANOVA), where sex and treatment were considered
56
57
58
59
60

1
2
3 independent variables. When appropriated, we performed a multivariate test (Bonferroni)
4 to compare groups. Differences between groups were considered significant for values of
5 $p \leq 0.05$
6
7
8
9

10 RESULTS

11
12
13 According to some previous studies on rats (Cooke, et al., 2004, Tryphonas, et al., 2004)
14 we tried a range of 0.25-2.5mg of tributyltin (TBT) per Kg of body weight, however, the
15 mice died in a few days (Table 1). The only dose compatible with long treatment was the
16 lowest one, classified as NOAEL dose in the literature (0.025mg/Kg of body weight).
17 Even in this small amount, TBT was highly aggressive by oral administration, in fact,
18 many animals developed dermatitis and hair loss.
19
20
21
22
23
24
25

26 *Body weight and Feed Efficiency (FE)*

27
28 No differences were observed in the percentage of body weight gain (Fig. 1A). On the
29 contrary, we observed a reduction in food consumption at the end of the treatment both in
30 TBT males and TBT females (Fig. 1B,C). The two-way ANOVA for repeated measures,
31 being sex and treatment the independent variables and weekly food consumption the
32 repeated measure, showed effects of sex ($F_{(1,1)}=20.482$ and $p \leq 0.001$) and of treatment
33 ($F_{(1,1)}=4.386$ and $p \leq 0.05$) on food consumption, but no effects of interaction between sex
34 and treatment ($F_{(1,1)}=0.136$). □ The Bonferroni's test revealed significant differences only
35 in males at the end of treatment ($p < 0.05$), whereas in females the difference was not
36 significant ($p = 0.11$).
37
38
39
40
41
42
43

44 Results for feed efficiency (Body weight/Kcal) were statistically analyzed by two-way
45 ANOVA for repeated measures (with sex and treatment as independent variables and
46 feed efficiency as repeated measure) demonstrating a global effects of sex ($F_{(1)}=7.530$
47 and $p \leq 0.05$) and treatment ($F_{(1)}=11,248$ and $p \leq 0.01$). The multiple comparisons between
48 groups displayed that, in the week before treatments no differences were observed in feed
49 efficiency between animals; while at the end of the experimental period in both TBT-
50 exposed males and females there was an increase in feed efficiency compared to controls
51 (respectively $p \leq 0.05$ for males and $p \leq 0.01$ for females) (Fig. 2A, B).
52
53
54
55
56
57
58
59
60

1
2
3 Due to the fact that, at day 0, the weight of TBT-treated females was different from that
4 of Oil-treated females, we have also analyzed the changes of the feed efficiency in
5 comparison to the values of the feed efficiency for each group at the beginning of the
6 experiment (conventionally put equal to 1). In this case, the two-way ANOVA for
7 repeated measures (with sex and treatment as independent variables and percentual
8 variations of feed efficiency as repeated measures) demonstrated effects of the
9 interactions among repeated measures and sex ($F_{(1,3)}=3.584$ and $p<0.05$) as well as among
10 repeated measures and treatment ($F_{(1,3)}=5.967$ and $p\leq 0.01$). The multiple comparisons
11 between groups displayed that no significant differences were present in the variations of
12 feed efficiency for weeks 1 to 3. At week 4 we observed an increase for both sexes, but it
13 was significant only in TBT-exposed males compared to controls ($p\leq 0.05$), whereas for
14 control females it was not significant ($p=0.1$) (Fig. 2C, D).

25 26 *Blood leptin concentration*

27 Exposure to TBT induced a reduction of blood circulating leptin measured with the EIA
28 technique in both sexes. Two-ways ANOVA (sex and treatment as independent variables)
29 showed a statistically significant effect of treatment ($F_{(1)}=39.346$ and $p\leq 0.001$) on blood
30 leptin levels, but no effects of sex ($F_{(1)}=0.388$) or of the interaction between sex and
31 treatment ($F_{(1,1)}=0.654$). Multiple comparisons analysis confirmed the significant
32 reduction of blood leptin levels in treated males ($p\leq 0.001$) and females ($p\leq 0.01$)
33 compared to controls (Fig. 3B).

34 35 *Liver*

36 The volume of the liver was not altered in TBT-treated mice. This was confirmed by two-
37 way ANOVA (sex and treatment as independent variables, and liver weight as dependent
38 variable) showing a statistically significant effect of sex on liver weight ($F_{(1,1)}=13.821$
39 and $p\leq 0.01$), but no effects of treatment ($F_{(1,1)}=0.018$). Multiple comparison analysis
40 between groups confirmed the gender differences in control ($p\leq 0.05$) animals, whereas
41 the large variations in treated females prevented to have a significant difference with
42 treated males (Fig. 3A).

43 On the contrary, histological inspection of hematoxylin-eosin stained sections of liver
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 showed impairment in tissues of treated animals. In fact, in sections from TBT-treated
4 animals we observed severe congestion of the tissue and prominent cytoplasmic
5 hepatocyte degeneration while the cytoplasm seems to be empty (Fig.4).
6
7
8

9 10 *Adipose tissue*

11
12 A first qualitative analysis displayed no relevant differences of considered fat pads,
13 except for treated females that showed a reduction of the perigonadic fat deposition. This
14 was confirmed by statistical analysis. In fact, the two-way ANOVA (sex and treatment as
15 independent variables and fat weight (g) as dependent variable), showed no effects of sex
16 ($F_{(1)}=3.862$) and of treatment ($F_{(1)}=0.282$) on subcutaneous white fat. In a similar way, we
17 observed no effects of sex ($F_{(1)}=1.219$) and of treatment ($F_{(1,1)}=2.813$) for the perigonadic
18 fat, but the interaction between sex and treatment had some effects ($F_{(1,1)}=8.034$ and
19 $p\leq 0.05$). In fact, multiple comparison analysis showed statistically significant reduction
20 of perigonadic fat in TBT-exposed females ($p\leq 0.01$).
21
22
23
24
25
26
27
28
29

30 *NPY immunoreactivity*

31
32 Immunostaining for NPY in control males reported a hypothalamic distribution of
33 positive elements similar to those already reported in several previous contributions. In
34 particular, due to the lack of pretreatment with colchicine, we have only rarely observed
35 positive cell bodies, whereas a large amount of positive fibers was observed along the
36 entire hypothalamus (Fig. 5). These fibers were particularly dense within the PVN and
37 the ARC nuclei, but they were also abundant within the suprachiasmatic, supraoptic and
38 DMH nuclei. Other regions have less dense innervations, as for example the VMH.
39 Qualitative inspection of the stained sections revealed obvious differences among treated
40 and control male mice. We have therefore quantitatively analyzed NPY-immunoreactivity
41 (NPY-ir) within those nuclei that are involved in the food intake controlling circuit: PVN
42 (Fig. 6), DMH, ARC and VMH.
43
44
45
46
47
48
49
50

51
52 For all nuclei, the preliminary two-way ANOVA for repeated measures has not
53 evidenced a statistically significant effect for the anterior-posterior levels; we have
54 therefore repeated the two-way ANOVA (treatment and sex were the independent
55 factors) considering the average value of fractional area covered by immunoreactive
56
57
58
59
60

1
2
3 structures.

4
5 The quantitative analysis of stained sections indicated a decrease of NPY-ir in all the
6 considered nuclei of TBT-treated males (Fig.7). The two-way ANOVA reported
7 significant effects of the interaction between sex and treatment in DMH ($F_{(1,1)}=8.661$ and
8 $p\leq 0.05$) and VMH ($F_{(1,1)}=9.602$ and $p\leq 0.01$). In PVN and ARC the p-values for the
9 interaction were very close to significance ($p<0.07$), therefore we performed a multiple
10 comparison analysis between groups for all nuclei. These tests demonstrated a significant
11 sex differences (i.e. males showing a higher NPY-immunoreactivity than females) in OIL
12 groups (PVN, ARC and DMH, $p<0.05$, VMH $p<0.01$), this difference is abolished by the
13 treatment for all the nuclei. In particular, in PVN, ARC, and DMH we observed a
14 significant reduction of NPY-ir in treated males compared to controls ($p\leq 0.05$), while no
15 significant effects were detected for females. On the contrary, in the VMH TBT-treated
16 females have a significant increase of NPY-ir compared to control females ($p\leq 0.05$),
17 while no significant differences were found for males.
18
19
20
21
22
23
24
25
26
27
28
29

30 *β -galactosidase histochemistry*

31
32 In our specimens, the distribution of β -galactosidase activity (reflecting the activation
33 of Y1-R promoter) in the hypothalamus was comparable to earlier description (Oberto, et
34 al., 1998, Zammaretti, Panzica and Eva, 2007, Zammaretti, et al., 2001), in particular, we
35 observed a high density of positive elements in the PVN, VMH and medial preoptic area
36 (MPOA). Other regions as DMH or ARC showed a less intense positivity. Overall, the
37 amount of positive elements and their size was smaller if compared to previous works in
38 which we have not perfused the animals (Oberto, et al., 2003), but was very similar to our
39 study performed in perfused animals (Martini, et al., 2011). In part of these nuclei we
40 observed an obvious decrease of the histochemical staining in TBT-treated animals (Fig.
41 8). The subsequent quantification partly confirmed the visual inspection (Fig. 9).
42
43
44
45
46
47
48
49

50 In PVN and VMH the two-way analysis of variance showed significant effects of sex
51 (respectively $F_{(1)}=14.548$ and $p\leq 0.01$; $F_{(1)}=6.672$ and $p\leq 0.05$) and treatment (respectively
52 $F_{(1)}=17.933$ and $p\leq 0.001$; $F_{(1)}=17.768$ and $p\leq 0.001$). In VMH we observed a significant
53 effect of the interaction between the two variables ($F_{(1,1)}=5.414$ and $p\leq 0.05$). In both PVN
54 and VMH of control group, multiple comparisons showed differences in Y1R-transgene
55
56
57
58
59
60

1
2
3 expression between males and females, having females a higher number of positive
4 elements ($p \leq 0.01$). The TBT treatment induced a significant decrease in both nuclei of
5 females ($p \leq 0.01$, and $p < 0.001$ respectively). Also in male PVN and VMH nuclei we
6 observed a reduction of Y1R-transgene expression, but the p-value indicated a tendency
7 to significance ($p = 0.06$). In ARC the ANOVA showed the effect of sex ($F_{(1)} = 20.388$ and
8 $p \leq 0.001$) and treatment ($F_{(1)} = 6.876$ and $p \leq 0.05$), but no effect was found for the
9 interaction between the two variables ($F_{(1,1)} = 0.129$). In DMH there was only a significant
10 effect of treatment ($F_{(1)} = 9.207$ and $p \leq 0.05$), while nor sex ($F_{(1)} = 0.020$) neither the
11 interaction sex-treatment ($F_{(1,1)} = 0.065$) were significant. Subsequent multiple comparison
12 analysis, showed a significant dimorphism (where females have a higher expression of
13 Y1R-transgene in comparison to males) in ARC ($p \leq 0.01$), but not in DMH. Moreover
14 TBT-treated female showed a reduction in Y1R transgene expression in both nuclei; in
15 ARC was close to significant ($p = 0.07$), while in DMH this reduction was statistically
16 significant ($p \leq 0.05$).
17
18
19
20
21
22
23
24
25
26
27
28
29
30

31 DISCUSSION

32 Present data suggest that, in adult C57BL/6 mice, the oral administration of
33 $0.025 \mu\text{g/g/day}$ of TBT for a period of four weeks is able to induce profound alterations of
34 the leptin-NPY-Y1 receptor axis, even if the peripheral obesogenic effect is not strongly
35 evident. In this work, we used the NOAEL dose, instead of higher doses that were
36 applied to rats in other laboratories (Cooke, et al., 2008, Cooke, et al., 2004, et al., 2004),
37 because, in our hands, these doses ($2.5 \mu\text{g/g/day}$ or $0.25 \mu\text{g/g/day}$) were highly toxic for
38 our mice provoking animals' death in a short time (see Table 1).
39
40
41
42
43
44

45 In the present experiment, 4-weeks of TBT treatment in adult mice did not increase the
46 body weight (Fig. 1A). However, both males and females showed a decrease of food
47 intake at the end of treatment (significant only in males, see Fig. 1B.C). We have
48 therefore calculated the feed efficiency, as well as its relative changes during the
49 treatment (Fig. 2). In both cases, at the end of the treatment we observed an increase of
50 feed efficiency and of its relative changes in both TBT-treated male and female mice.
51 This increase is always significant in males, whereas the relative changes were not
52 significant in females, probably due to the differences in body weight and food
53
54
55
56
57
58
59
60

1
2
3 consumption for the two female groups. These results mean that, at the end of the
4 experiment the TBT-treated male mice had the same weight while consuming less food,
5 with a similar tendency also for females.
6
7

8
9 Previous studies demonstrated that alterations in food intake and feed efficiency can
10 lead to differences in body composition, providing a partial explanation for the obesity
11 observed in some mouse model (Djazayery, et al., 1979). Although the susceptibility of
12 the FVB mouse strain to obesity is not well studied, it was previously reported that FVB
13 mice fed with a high fat diet become obese and show an increase in circulating leptin
14 correlated with body weight (Frederich, et al., 1995, Martin, et al., 2006). In addition, 3
15 week-old males, of our transgenic strain (*Y1R/LacZ*), exposed for 8 weeks to a moderate-
16 high fat diet showed a significant increase of body weight associated with a decrease of
17 *Y1R* gene expression in the DMH and VMH in (Zammaretti, et al., 2007). However, in
18 the present experiment, performed in older animals (3 month-old), we have not observed
19 any significant increase of fat mass in TBT-exposed mice. This seems in contrast to
20 studies demonstrating that TBT stimulates adipocytes differentiation *in vitro* (Kanayama,
21 et al., 2005) and increases adipose mass *in vivo* (Grun, et al., 2006). In particular, *in utero*
22 exposure to TBT (with i.p. doses 2 or 20 times higher than the one we used) may increase
23 body fat mass (and not body weight), especially the fat located around the male gonadal
24 ducts (Grun, et al., 2006). It is possible that the low dosis of TBT orally administered to
25 adult mice in our experiment was not sufficient, or the period was too short to induce fat
26 deposition, even if at the end of the treatment our TBT-treated animals showed a higher
27 feed efficiency.
28
29

30
31 In contrast with the lack of effect on the weight of fat tissue we detected significant
32 effects on circulating levels of leptin (Fig. 3B). In fact, according to our results on fat
33 distribution, no significant variations of the leptin levels should occur, whereas, we
34 observed a significant decrease of the hormone. The leptin fall in TBT-treated adult mice
35 could be related to the putative xenoandrogenic action of TBT (Grote, et al., 2004), in
36 fact, estrogens increase leptin production, while androgens act in the opposite way
37 decreasing leptin levels (for a review see Mayes and Watson, 2004). The reduction of
38 leptin synthesis could be also mediated by TBT-dependent activation of PPAR γ , a
39 nuclear receptors that play important roles in lipid homeostasis and adipogenesis. In fact,
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 PPAR γ activation is known to be responsible of inhibition of genes that induce lipolysis
4 in adipocytes of white adipose tissue (Evans, et al., 2004, Ferré, 2004) and TBT was
5 shown to disrupt normal development and homeostatic controls over adipogenesis and
6 energy balance, resulting obesity through its action on PPAR γ and RXR (Grun, et al.,
7 2006).
8

9
10
11
12 The liver, which is one of the targets of the toxicant action of TBT, even if not
13 macroscopically altered (the volume was not significantly different in control or TBT-
14 treated mice) shows histological signals of hepatocytic degeneration, it seems therefore
15 that the supposed no effect dose has already a deleterious effect on this important organ
16 (Figs 3A-4).
17

18
19
20
21 As reported in the introduction, the food intake is regulated by cerebral
22 neuroendocrine circuits, mainly located in the hypothalamus, and leptin communicates
23 the levels of energy storage, inducing the state of satiety while suppressing food intake by
24 its action on these neuroendocrine circuits (Ahima, et al., 2009). However, reports on the
25 action of TBT on hypothalamic circuits are very rare and limited to studies on
26 neurotransmitters' expression and metabolisms in homogenates of discrete regions
27 (Konno, et al., 2001, Tsunoda, et al., 2004), or in cell cultures (He, et al., 2014,
28 Mizuhashi, et al., 2000, Nakatsu, et al., 2010, Nakatsu, et al., 2009). Only recently, we
29 demonstrated a short-term action of TBT in vivo, by inducing a significant increase of c-
30 fos expression in ARC elements after acute exposure to TBT (Bo, et al., 2011).
31
32
33
34
35
36
37
38

39 In the present experiment we studied changes induced by TBT in the expression of a
40 neuropeptide strictly related to the control of food intake, the NPY. In adult TBT-treated
41 males we observed a significant reduction of NPY expression in some of the investigated
42 hypothalamic nuclei (Figs 6-7). On the basis of the levels of circulating leptin in TBT-
43 treated animals and of the well known relationships among leptin and NPY (Myers, et al.,
44 2009, Robertson, et al., 2008), the leptin decrease should induce a parallel increase of
45 NPY-ir in ARC and, consequently, in its main targets: PVN, DMH, and VMH. On the
46 contrary, we observed, a significant reduction of NPY-immunoreactivity in male ARC,
47 PVN, and DMH, but not in females. In the VMH, a nucleus implicated both in the control
48 of energy metabolism and in the control of lordosis behavior (for reviews see Asarian and
49 Geary, 2013, Flanagan-Cato, 2011), we observed a reverse effect: no decrease in males
50
51
52
53
54
55
56
57
58
59
60

1
2
3 and a significant increase in TBT-treated females. Thus, there is a sex dimorphism in the
4 sensitivity of NPY-based neural circuits controlling food intake to TBT. This is probably
5 in agreement with other studies that indicate sex differences in body fat distribution as the
6 result of differences in sex hormones between males and females (for reviews see
7 Lovejoy and Sainsbury, 2009, Shi and Clegg, 2009).

8
9
10
11
12 The analysis of Y1-R transgene expression (Figs 8-9) revealed the presence of a sex
13 dimorphism in PVN, VMH and ARC. In general, TBT-treated females show a significant
14 decrease of the transgene expression (only a tendency for ARC) thus determining the
15 disappearance of the dimorphism when compared to control males. In all these nuclei the
16 exposure to TBT induced a significant decrease of Y1-R transgene expression in females,
17 whereas in males we observed a strong reduction, but this has not reached statistical
18 significance (p values 0.06-0.07) and it is probably due to the large variations in the
19 transgene expression in males. As previously demonstrated in the same transgenic strain
20 (Oberto, et al., 2003), lower levels of NPY-ir should induce an increase in Y1-R
21 transgene expression. On the contrary, in the present experiment we observed a decrease
22 of both NPY-ir and Y1-R transgene expression. Therefore, we have here a sex-oriented
23 defect in the fine-tuning among up- and down-regulation of NPY and its main receptor.
24 Previous studies have suggested that gonadal hormones may play a role in the expression
25 of Y1-R during the estrous cycle (Martini, et al., 2011) or in its regulation after exposure
26 to different diets (Zammaretti, et al., 2007). In addition, in this study we observed a sex
27 dimorphism in Y1-R expression, having females more β -galactosidase positivity than
28 males, thus the TBT is mainly acting masculinizing the Y1-R expression. It is therefore
29 possible that the decrease of Y1R expression in TBT-treated animals may depend on one
30 hand by a putative xenoandrogenic effect of TBT or, on the other hand, by the inhibition
31 of aromatase activity mediated by TBT (Cooke, 2002, Heidrich, et al., 2001, Li, et al.,
32 2015).

33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52 In conclusion, on the basis of the present findings, we can assume that TBT, in
53 addition to its effects on peripheral fat tissue, has some effects on neural circuits,
54 particularly directed to alter the neuroendocrine relationships among circulating leptin
55 levels and the hypothalamic circuits controlling food intake, and these effects seems to be
56
57
58
59
60

1
2
3 sexually differentiated. Further studies should investigate if other parts of the food intake
4 controlling system, as for example the α -MSH or the orexin circuits, may be affected by
5 TBT exposure.
6
7
8
9

10 11 12 **Acknowledgement**

13
14 This work was supported by Region Piemonte, Fondazione Cavalieri-Ottolenghi and
15 University of Torino.
16

17 18 **Disclosures**

19 The authors declare no conflict of interest
20

21 22 **Authors' contribution**

23 E.B., C.E., S.G. and G.C.P. designed and supervised the experiments; E.B., A.F., M.M.
24 and D.S. performed the experiments; E.B., A.F., S.G. and G.C.P. performed the statistical
25 analysis; E.B., S.G. and G.C.P. prepared the figures for publication; E.B., C.E., S.G. and
26 G.C.P. wrote the first draft of the manuscript; all authors read and approved the
27 manuscript.
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3
4 **Figure 5.** Microphotographs illustrating the immunohistochemical staining for NPY in
5 the nuclei that have been considered in the present study. Microphotographs are taken
6 at the same enlargement. Bar represents 500 microns. **A-** rostral section containing
7 the paraventricular nucleus (PVN). **B-** More caudal section containing the
8 dorsomedial (DMH), ventromedial (VMH) and arcuate (ARC) nuclei. * Third
9 ventricle.

10
11
12
13
14
15
16 **Figure 6.** Sections of the paraventricular nucleus from different experimental groups
17 immunostained for NPY. Microphotographs are taken at the same enlargement. Bar
18 represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated male; F
19 OIL - oil-treated female; F TBT - TBT-treated female. * Third ventricle.

20
21
22
23
24
25 **Figure 7.** Histograms representing NPY-ir in hypothalamus (expressed as percentage of
26 area covered by stained elements). The multiple comparison analysis between groups
27 for all nuclei demonstrated: in PVN, ARC, and DMH, a significant reduction of
28 NPY-ir in treated males compared to controls), while no significant effects were
29 detected for females. The significant differences (ANOVA followed by the
30 Bonferroni test at a level of $P < 0.05$) are denoted by **a** or **b**.

31
32
33
34
35
36
37 **Figure 8.** Histochemical detection of beta-galactosidase activity (expression of Y1R-
38 LacZ transgene) in sections of the paraventricular nucleus from different
39 experimental groups. Microphotographs are taken at the same enlargement. Bar
40 represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated male; F
41 OIL - oil-treated female; F TBT - TBT-treated female. * Third ventricle.

42
43
44
45
46
47
48 **Figure 9.** Histograms representing Y1R-transgene expression in hypothalamus
49 (expressed as dots/ μm^2). In females the treatment significantly reduced Y1R
50 expression in PVN and VMH. Also in males, TBT reduced Y1R-transgene
51 expression in both nuclei but the statistical p-value is only close to significance. The
52 significant differences (ANOVA followed by the Bonferroni test at a level of
53 $P < 0.05$) are denoted by **a** or **b**, ^ indicates a close to significant difference ($P < 0.07$).

REFERENCES

- Ahima RS, Saper CB, Flier JS, Elmquist JK. Leptin regulation of neuroendocrine systems. *Frontiers in Neuroendocrinology*. 2000;21:263-307.
- Asarian L, Geary N. Sex differences in the physiology of eating. *Am J Physiol Regul Integr Comp Physiol*. 2013;305:R1215-67.
- Aste N, Viglietti-Panzica C, Fasolo A, Andreone C, Vaudry H, Pelletier G, Panzica GC. Localization of neuropeptide Y (NPY) immunoreactive cells and fibres in the brain of the Japanese quail. *Cell and Tissue Research*. 1991;265:219-230.
- Austin J, Marks D. Hormonal regulators of appetite. *International journal of pediatric endocrinology*. 2009;2009:141753.
- Banks WA, Kastin AJ. Passage of peptides across the blood-brain barrier: Pathophysiological perspectives. *Life Sci*. 1996;59:1923-1943.
- Bates SH, Stearns WH, Dundon TA, Schubert M, Tso AW, Wang Y, Banks AS, Lavery HJ, Haq AK, Maratos-Flier E, Neel BG, Schwartz MW, Myers MG, Jr. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. *Nature*. 2003;421:856-9.
- Beck B. Neuropeptide Y in normal eating and in genetic and dietary-induced obesity. *Philos Trans R Soc Lond B Biol Sci*. 2006;361:1159-85.
- Bertocchi I, Oberto A, Longo A, Mele P, Sabetta M, Bartolomucci A, Palanza P, Sprengel R, Eva C. Regulatory functions of limbic Y1 receptors in body weight and anxiety uncovered by conditional knockout and maternal care. *Proc Natl Acad Sci U S A*. 2011;108:19395-400.
- Bo E, Viglietti-Panzica C, Panzica GC. Acute exposure to tributyltin induces c-fos activation in the hypothalamic arcuate nucleus of adult male mice. *Neurotoxicology*. 2011;32:277-80.
- Cinti S. Between brown and white: novel aspects of adipocyte differentiation. *Ann Med*. 2011;43:104-15.
- Cooke GM. Effect of organotins on human aromatase activity in vitro. *Toxicol Lett*. 2002;126:121-30.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- Cooke GM, Forsyth DS, Bondy GS, Tachon R, Tague B, Coady L. Organotin speciation and tissue distribution in rat dams, fetuses, and neonates following oral administration of tributyltin chloride. *J Toxicol Environ Health A*. 2008;71:384-95.
- Cooke GM, Tryphonas H, Pulido O, Caldwell D, Bondy GS, Forsyth D. Oral (gavage), in utero and postnatal exposure of Sprague-Dawley rats to low doses of tributyltin chloride. Part 1: Toxicology, histopathology and clinical chemistry. *Food Chem Toxicol*. 2004;42:211-20.
- Danger JM, Breton B, Vallarino M, Fournier A, Pelletier G, Vaudry H. Neuropeptide Y in the trout brain and pituitary: localization, characterization, and action on gonadotropin release. *Endocrinol*. 1991;128:2360-2368.
- Danger JM, Tonon MC, Basille C, Jenks BG, Saint Pierre S, Martel JC, Fasolo A, Quirion R, Pelletier G, Vaudry H. Neuropeptide Y: localization in the central nervous system and neuroendocrine functions. *Fundamental and Clinical Pharmacology*. 1990;4:307-340.
- Decherf S, Demeneix BA. The obesogen hypothesis: a shift of focus from the periphery to the hypothalamus. *J Toxicol Environ Health B Crit Rev*. 2011;14:423-48.
- Decherf S, Seugnet I, Fini JB, Clerget-Froidevaux MS, Demeneix BA. Disruption of thyroid hormone-dependent hypothalamic set-points by environmental contaminants. *Mol Cell Endocrinol*. 2010;323:172-82.
- Djazayery A, Miller DS, Stock MJ. Energy balances in obese mice. *Nutr Metab*. 1979;23:357-67.
- EFSA. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission to assess the health risks to consumers associated with exposure to organotins in foodstuffs. *EFSA Journal*. 2004;102:1-119.
- Eva C, Mele P, Collura D, Nai A, Pisu MG, Serra M, Biggio G. Modulation of neuropeptide Y and Y1 receptor expression in the amygdala by fluctuations in the brain content of neuroactive steroids during ethanol drinking discontinuation in Y1R/LacZ transgenic mice. *J Neurochem*. 2008;104:1043-54.
- Eva C, Serra M, Mele P, Panzica GC, Oberto A. Physiology and gene regulation of the brain NPY Y1 receptor. *Frontiers in Neuroendocrinology*. 2006;27:308-339.

- 1
2
3 Evans RM, Barish GD, Wang YX. PPARs and the complex journey to obesity. *Nat Med.*
4 2004;10:355-61.
5
6
7 Ferrara G, Serra G, Zammaretti F, Pisu MG, Panzica GC, Biggio G, Eva C. Increased
8 expression of the neuropeptide Y receptor Y1 gene in the medial amygdala of
9 transgenic mice induced by long-term treatment with progesterone or
10 allopregnanolone. *Journal of Neurochemistry.* 2001;79.
11
12
13
14 Ferré P. The biology of peroxisome proliferator-activated receptors: relationship with
15 lipid metabolism and insulin sensitivity. *Diabetes.* 2004;53 Suppl 1:S43-50.
16
17
18 Flanagan-Cato LM. Sex differences in the neural circuit that mediates female sexual
19 receptivity. *Front Neuroendocrinol.* 2011;32:124-36.
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
- Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nat Med.* 1995;1:1311-4.
- Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature.* 1998;395:763-770.
- Frye C, Bo E, Calamandrei G, Calza L, Dessi-Fulgheri F, Fernandez M, Fusani L, Kah O, Kajta M, Le Page Y, Patisaul HB, Venerosi A, Wojtowicz AK, Panzica GC. Endocrine Disrupters: A Review of Some Sources, Effects, and Mechanisms of Actions on Behaviour and Neuroendocrine Systems. *J Neuroendocrinol.* 2012;24:144-159.
- Grote K, Stahlschmidt B, Talsness CE, Gericke C, Appel KE, Chahoud I. Effects of organotin compounds on pubertal male rats. *Toxicology.* 2004;202:145-58.
- Grun F. The obesogen tributyltin. *Vitam Horm.* 2014;94:277-325.
- Grun F, Blumberg B. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology.* 2006;147:S50-55.
- Grun F, Watanabe H, Zamanian Z, Maeda L, Arima K, Cubacha R, Gardiner DM, Kanno J, Iguchi T, Blumberg B. Endocrine-disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. *Mol Endocrinol.* 2006;20:2141-55.
- Hara K, Yoshizuka M, Doi Y, Fujimoto S. Effect of bis (tributyltin) oxide on permeability of the blood-brain barrier: a transient increase. *Occup Environ Med.* 1994;51:735-8.

- 1
2
3 He K, Zhang J, Chen Z. Effect of tributyltin on the food intake and brain neuropeptide
4 expression in rats. *Endokrynol Pol.* 2014;65:485-90.
5
6
7 Heidrich DD, Steckelbroeck S, Klingmuller D. Inhibition of human cytochrome P450
8 aromatase activity by butyltins. *Steroids.* 2001;66:763-9.
9
10 Heindel JJ, Vom Saal FS, Blumberg B, Bovolin P, Calamandrei G, Ceresini G, Cohn BA,
11 Fabbri E, Gioiosa L, Kassotis C, Legler J, La Merrill M, Rizzir L, Machtinger R,
12 Mantovani A, Mendez MA, Montanini L, Molteni L, Nagel SC, Parmigiani S,
13 Panzica G, Paterlini S, Pomatto V, Ruzzin J, Sartor G, Schug TT, Street ME, Suvorov
14 A, Volpi R, Zoeller RT, Palanza P. Parma consensus statement on metabolic
15 disruptors. *Environ Health.* 2015;14:54.
16
17
18 Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS. Increased adipose tissue in
19 male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci U S A.*
20 2000;97:12729-34.
21
22
23 Kanayama T, Kobayashi N, Mamiya S, Nakanishi T, Nishikawa J. Organotin compounds
24 promote adipocyte differentiation as agonists of the peroxisome proliferator-activated
25 receptor gamma/retinoid X receptor pathway. *Mol Pharmacol.* 2005;67:766-74.
26
27
28 Kanemoto Y, Ishibashi H, Matsuo S, Oyama Y, Akaike N. Modification of NMDA
29 responses by tri-n-butyltin in rat brain neurons. *Br J Pharmacol.* 2002;136:201-6.
30
31
32 Kimura K, Kobayashi K, Naito H, Suzuki Y, Sugita-Konishi Y. Effect of lactational
33 exposure to tributyltin chloride on innate immunodefenses in the F1 generation in
34 mice. *Biosci Biotechnol Biochem.* 2005;69:1104-10.
35
36
37 Kirchner S, Kieu T, Chow C, Casey S, Blumberg B. Prenatal exposure to the
38 environmental obesogen tributyltin predisposes multipotent stem cells to become
39 adipocytes. *Molecular endocrinology (Baltimore, Md).* 2010;24:526-539.
40
41
42 Konno N, Tsunoda M, Nakano K, Liu Y. Effect of tributyltin on the N-methyl-D-
43 aspartate (NMDA) receptors in the mouse brain. *Arch Toxicol.* 2001;75:549-54.
44
45
46 Ladyman SR, Woodside B. Regulation of maternal food intake and mother-pup
47 interactions by the Y5 receptor. *Physiol Behav.* 2009;97:91-7.
48
49
50 Lee JJ, Kim YM, Park SK, Lee MK. Effects of tributyltin chloride on L-DOPA-induced
51 cytotoxicity in PC12 cells. *Arch Pharm Res.* 2006;29:645-50.
52
53
54
55
56
57
58
59
60

- 1
2
3 Leininger GM, Jo YH, Leshan RL, Louis GW, Yang H, Barrera JG, Wilson H, Opland
4 DM, Faouzi MA, Gong Y, Jones JC, Rhodes CJ, Chua S, Jr., Diano S, Horvath TL,
5 Seeley RJ, Becker JB, Munzberg H, Myers MG, Jr. Leptin acts via leptin receptor-
6 expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine
7 system and suppress feeding. *Cell Metab.* 2009;10:89-98.
8
9
10
11 Leung KM, Kwong RP, Ng WC, Horiguchi T, Qiu JW, Yang R, Song M, Jiang G, Zheng
12 GJ, Lam PK. Ecological risk assessments of endocrine disrupting organotin
13 compounds using marine neogastropods in Hong Kong. *Chemosphere.* 2006;65:922-
14 38.
15
16
17
18
19 Li ZH, Zhong LQ, Wu YH, Mu WN. Alteration of cytochrome P450 1 regulation and
20 HSP 70 level in brain of juvenile common carp (*Cyprinus carpio*) after chronic
21 exposure to tributyltin. *Fish Physiol Biochem.* 2015.
22
23
24
25 Lima D, Castro LFC, Coelho I, Lacerda R, Gesto M, Soares J, André A, Capela R, Torres
26 T, Carvalho AP, Santos MM. Effects of Tributyltin and Other Retinoid Receptor
27 Agonists in Reproductive-Related Endpoints in the Zebrafish (*Danio rerio*). *Journal*
28 *of toxicology and environmental health Part A.* 2015;78:747-60.
29
30
31
32 Longo A, Oberto A, Mele P, Mattiello L, Pisu MG, Palanza P, Serra M, Eva C. NPY-Y1
33 coexpressed with NPY-Y5 receptors modulate anxiety but not mild social stress
34 response in mice. *Genes Brain Behav.* 2015;14:534-42.
35
36
37
38 Lovejoy JC, Sainsbury A. Sex differences in obesity and the regulation of energy
39 homeostasis. *Obes Rev.* 2009;10:154-67.
40
41
42
43 Martin TL, Alquier T, Asakura K, Furukawa N, Preitner F, Kahn BB. Diet-induced
44 obesity alters AMP kinase activity in hypothalamus and skeletal muscle. *J Biol Chem.*
45 2006;281:18933-41.
46
47
48
49 Martini M, Sica M, Gotti S, Eva C, Panzica GC. Effects of estrous cycle and sex on the
50 expression of neuropeptide Y Y1 receptor in discrete hypothalamic and limbic nuclei
51 of transgenic mice. *Peptides.* 2011;32:1330-4.
52
53
54
55 Matsuoka M, Igisu H. Induction of c-fos expression by tributyltin in PC12cells:
56 involvement of intracellular Ca²⁺. *Environmental Toxicology and Pharmacology.*
57 1996;2:373-380.
58
59
60

- 1
2
3 Mayes JS, Watson GH. Direct effects of sex steroid hormones on adipose tissues and
4 obesity. *Obes Rev.* 2004;5:197-216.
5
6
7 Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Trayhurn P.
8 Localization (Ob-Rb) of leptin receptor mRNA and the long form splice variant in
9 mouse hypothalamus and adjacent brain regions by in situ hybridization. *FEBS Lett.*
10 387, 113-116. *FEBS Letters.* 1996;387:113-116.
11
12
13
14 Michel C, Levin BE, Dunn-Meynell AA. Stress facilitates body weight gain in
15 genetically predisposed rats on medium-fat diet. *Am J Physiol Regul Integr Comp*
16 *Physiol.* 2003;285:R791-9.
17
18
19
20 Mitra S, Siddiqui WA, Khandelwal S. Early cellular responses against tributyltin chloride
21 exposure in primary cultures derived from various brain regions. *Environ Toxicol*
22 *Pharmacol.* 2014;37:1048-59.
23
24
25
26 Mitra S, Siddiqui WA, Khandelwal S. Differential susceptibility of brain regions to
27 tributyltin chloride toxicity. *Environ Toxicol.* 2015;30:1393-405.
28
29
30 Mizuhashi S, Ikegaya Y, Nishiyama N, Matsuki N. Cortical astrocytes exposed to
31 tributyltin undergo morphological changes in vitro. *Jpn J Pharmacol.* 2000;84:339-46.
32
33
34
35 Montague CT, Prins JB, Sanders L, Zhang J, Sewter CP, Digby J, Byrne CD, O'Rahilly
36 S. Depot-related gene expression in human subcutaneous and omental adipocytes.
37 *Diabetes.* 1998;47:1384-91.
38
39
40
41 Myers MG, Jr., Munzberg H, Leininger GM, Leshan RL. The geometry of leptin action
42 in the brain: more complicated than a simple ARC. *Cell Metab.* 2009;9:117-23.
43
44
45
46 Nakatsu Y, Kotake Y, Hino A, Ohta S. Activation of AMP-activated protein kinase by
47 tributyltin induces neuronal cell death. *Toxicol Appl Pharmacol.* 2008;230:358-63.
48
49
50
51 Nakatsu Y, Kotake Y, Komasa K, Hakozaiki H, Taguchi R, Kume T, Akaike A, Ohta S.
52 Glutamate excitotoxicity is involved in cell death caused by tributyltin in cultured rat
53 cortical neurons. *Toxicol Sci.* 2006;89:235-42.
54
55
56
57 Nakatsu Y, Kotake Y, Ohta S. Tributyltin-induced cell death is mediated by calpain in
58 PC12 cells. *Neurotoxicology.* 2006;27:587-93.
59
60
61
62 Nakatsu Y, Kotake Y, Ohta S. Concentration dependence of the mechanisms of
63 tributyltin-induced apoptosis. *Toxicol Sci.* 2007;97:438-47.

- 1
2
3 Nakatsu Y, Kotake Y, Takai N, Ohta S. Involvement of autophagy via mammalian target
4 of rapamycin (mTOR) inhibition in tributyltin-induced neuronal cell death. *J Toxicol*
5 *Sci.* 2010;35:245-51.
6
7
8
9 Nakatsu Y, Kotake Y, Takishita T, Ohta S. Long-term exposure to endogenous levels of
10 tributyltin decreases GluR2 expression and increases neuronal vulnerability to
11 glutamate. *Toxicol Appl Pharmacol.* 2009;240:292-8.
12
13
14 Newbold RR, Padilla-Banks E, Snyder RJ, Phillips TM, Jefferson WN. Developmental
15 exposure to endocrine disruptors and the obesity epidemic. *Reprod Toxicol.*
16 *2007;23:290-6.*
17
18
19 Nguyen AD, Herzog H, Sainsbury A. Neuropeptide Y and peptide YY: important
20 regulators of energy metabolism. *Curr Opin Endocrinol Diabetes Obes.* 2011;18:56-
21 60.
22
23
24 Oberto A, Mele P, Zammaretti F, Panzica GC, Eva C. Evidence of Altered Neuropeptide
25 Y Content and Neuropeptide Y1 Receptor Gene Expression in the Hypothalamus of
26 Pregnant Transgenic Mice. *Endocrinology.* 2003;144:4826-4830.
27
28
29 Oberto A, Panzica G, Altruda F, Eva C. Chronic modulation of the GABA_(A) receptor
30 complex regulates Y₁ receptor gene expression in the medial amygdala of transgenic
31 mice. *Neuropharmacology.* 2000;39:227-34.
32
33
34 Oberto A, Panzica GC, Altruda F, Eva C. GABAergic and NPY-Y1 network in the
35 medial amygdala: a neuroanatomical basis for their functional interaction.
36 *Neuropharmacology.* 2001;41:639-642.
37
38
39 Oberto A, Tolosano E, Brusa R, Altruda F, Panzica G, Eva C. The murine Y1 receptor 5'
40 upstream sequence directs cell-specific and developmentally regulated LacZ
41 expression in transgenic mice CNS. *European Journal of Neuroscience.*
42 *1998;10:3257-68.*
43
44
45 Palanza P, Morellini F, Parmigiani S, vom Saal FS. Ethological methods to study the
46 effects of maternal exposure to estrogenic endocrine disruptors: a study with
47 methoxychlor. *Neurotoxicol Teratol.* 2002;24:55-69.
48
49
50 Panzica GC, Bo E, Martini MA, Miceli D, Mura E, Viglietti-Panzica C, Gotti S.
51 Neuropeptides and Enzymes are Targets for the Action of Endocrine Disrupting
52
53
54
55
56
57
58
59
60

- 1
2
3 Chemicals in the Vertebrate Brain. *J Toxicol Environ Health B Crit Rev*.
4 2011;14:449-72.
5
6
7 Panzica GC, Viglietti-Panzica C, Mura E, Quinn Jr MJ, Palanza P, Ottinger MA. Effects
8 of xenoestrogens on the differentiation of behaviorally relevant neural circuits.
9 *Frontiers in Neuroendocrinology*. 2007;28:179-200.
10
11 Paxinos G, Franklin KBJ. *The Mouse Brain in Stereotaxic Coordinates*. Second Edition
12 ed. San Diego: Academic Press; 2001.
13
14 Pelletier G, Desy L, Kerkerian L, Cote J. Immunocytochemical localization of
15 neuropeptide Y (NPY) in the human hypothalamus. *Cell Tissue Res*. 1984;238:203-5.
16
17 Pelletier G, Guy J, Allen YS, Polak JM. Electron microscopic immunocytochemical
18 localization of neuropeptide Y (NPY) in the rat brain. *Neuropeptides*. 1984;4:319-
19 324.
20
21 Perroteau I, Danger JM, Biffo S, Pelletier G, Vaudry H, Fasolo A. Distribution and
22 characterization of neuropeptide Y immunoreactivity in the brain of the crested newt.
23 *Journal of Comparative Neurology*. 1988;275:309-325.
24
25 Pierman S, Sica M, Allieri F, Viglietti-Panzica C, Panzica GC, Bakker J. Activational
26 effects of estradiol and dihydrotestosterone on social recognition and the arginine-
27 vasopressin immunoreactive system in male mice lacking a functional aromatase
28 gene. *Horm Behav*. 2008;54:98-106.
29
30 Plumari L, Viglietti Panzica C, Allieri F, Honda S, Harada N, Absil P, Balthazar J,
31 Panzica GC. Changes in the Arginine-Vasopressin Immunoreactive Systems in Male
32 Mice Lacking a Functional Aromatase Gene. *Journal of Neuroendocrinology*.
33 2002;14:971-978.
34
35 Rantakokko P, Main KM, Wohlfart-Veje C, Kiviranta H, Airaksinen R, Vartiainen T,
36 Skakkebaek NE, Toppari J, Virtanen HE. Association of placenta organotin
37 concentrations with growth and ponderal index in 110 newborn boys from Finland
38 during the first 18 months of life: a cohort study. *Environ Health*. 2014;13:45.
39
40 Rantakokko P, Main KM, Wohlfart-Veje C, Kiviranta H, Airaksinen R, Vartiainen T,
41 Skakkebaek NE, Toppari J, Virtanen HE. Association of placenta organotin
42 concentrations with congenital cryptorchidism and reproductive hormone levels in
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 280 newborn boys from Denmark and Finland. *Human reproduction* (Oxford,
4 England). 2013;28:1647-60.
5
6
7 Robertson SA, Leininger GM, Myers MG, Jr. Molecular and neural mediators of leptin
8 action. *Physiol. Behav.* 94, 637–642. *Physiology and Behavior.* 2008;94:637-642.
9
10 Shi H, Clegg DJ. Sex differences in the regulation of body weight. *Physiol Behav.*
11 2009;97:199-204.
12
13 Stanley BG, Kyrkpouli SE, Lampert S, Leibowitz SF. Neuropeptide Y injected into the
14 hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. *Peptides.*
15 1986;7:1189-1192.
16
17 Stanley BG, Leibowitz SF. Neuropeptide Y injected in the paraventricular hypothalamus:
18 a powerful stimulant of feeding behavior. *Proceedings of the National Academy of*
19 *Sciences of the United States of America.* 1985;82:3940-3943.
20
21 Streefkerk JG. Inhibition of erythrocyte peroxidase activity by treatment with hydrogen
22 peroxide following methanol. *Journal of Histochemistry and Cytochemistry.*
23 1972;20:829-831.
24
25 Tryphonas H, Cooke G, Caldwell D, Bondy G, Parenteau M, Hayward S, Pulido O. Oral
26 (gavage), in utero and post-natal exposure of Sprague-Dawley rats to low doses of
27 tributyltin chloride. Part II: effects on the immune system. *Food Chem Toxicol.*
28 2004;42:221-35.
29
30 Tsunoda M, Konno N, Nakano K, Liu Y. Altered metabolism of dopamine in the
31 midbrain of mice treated with tributyltin chloride via subacute oral exposure. *Environ*
32 *Sci.* 2004;11:209-19.
33
34 Valassi E, Scacchi M, Cavagnini F. Neuroendocrine control of food intake. *Nutrition,*
35 *Metabolism & Cardiovascular Diseases.* 2008;18:158-168.
36
37 Watson RE, Wiegand SJ, Clough RW, Hoffman GE. Use of cryoprotectant to maintain
38 long-term peptide immunoreactivity and tissue morphology. *Peptides.* 1986;7:155-
39 159.
40
41 Zammaretti F, Panzica G, Eva C. Sex-dependent regulation of hypothalamic
42 neuropeptide Y-Y1 receptor gene expression in moderate/high fat, high-energy diet-
43 fed mice. *J Physiol.* 2007;583:445-54.
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Zammaretti F, Panzica GC, Eva C. Fasting, leptin treatment and glucose administration
4 differentially regulate Y1 receptor gene expression in the hypothalamus of transgenic
5 mice. *Endocrinology*. 2001;142:3774-3782.
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

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.

For Peer Review

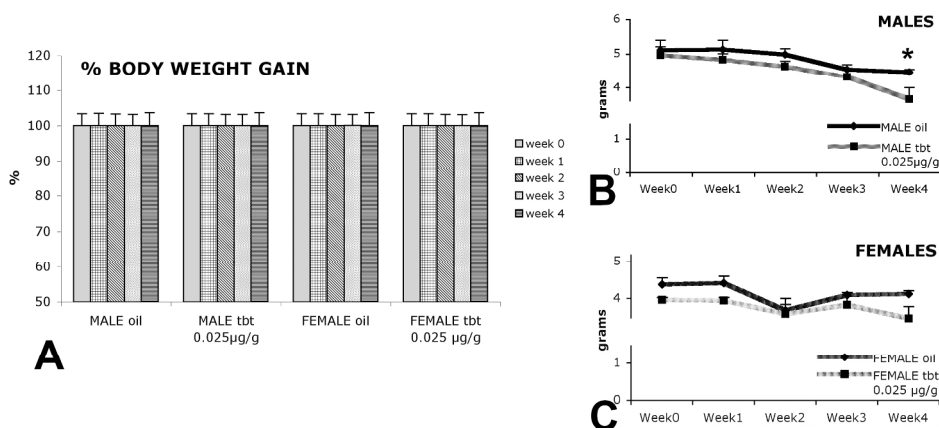


Figure 1. A- Histograms represent body weight gain (expressed as percentage) during the treatment. No statistically significant differences were found between groups. B-C - The lines represents variations of the amount (in grams) of food intake during the treatment in control and treated males (B) and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

Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

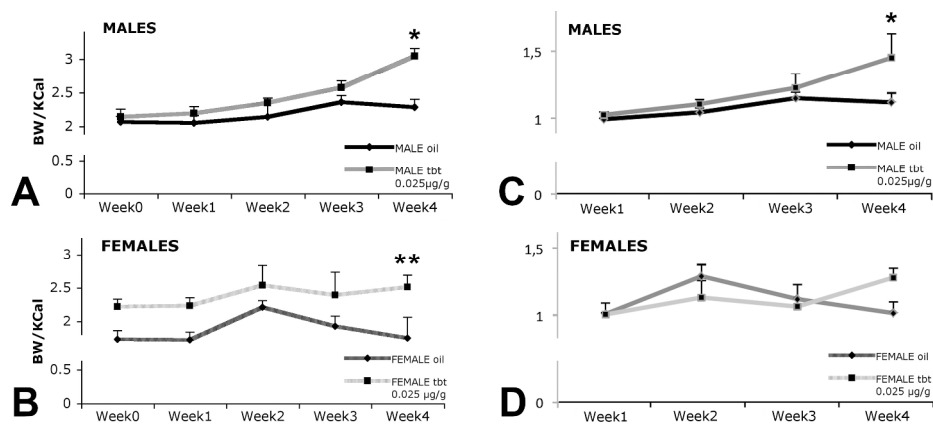


Figure 2. - Changes of feed efficiency (expressed as index of body weight/Kcal introduced) calculated during the period of treatment in control and TBT-treated males (A) and females (B). Feed efficiency is significantly increased at the end of treatment in TBT mice: * $p < 0.05$, ** $p < 0.01$ (Bonferroni's 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)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

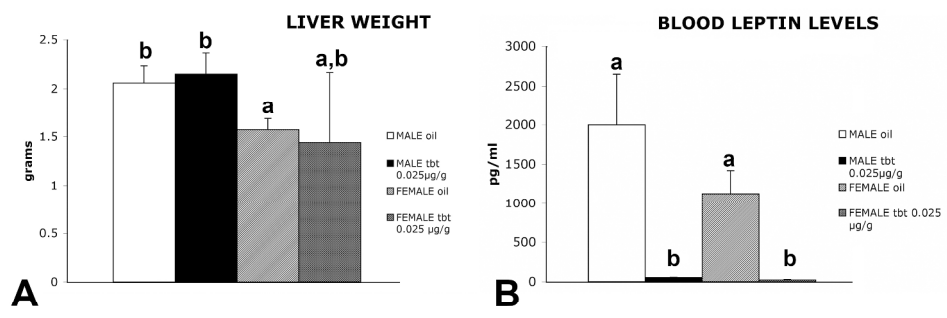


Figure 3- A - Histograms represent the liver's weight at the day of sacrifice. No statistically significant differences were found between treated and control groups, a sex difference is observed among control groups. The significant differences (ANOVA followed by the Bonferroni's test at a level of $P < 0.05$) 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)

Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

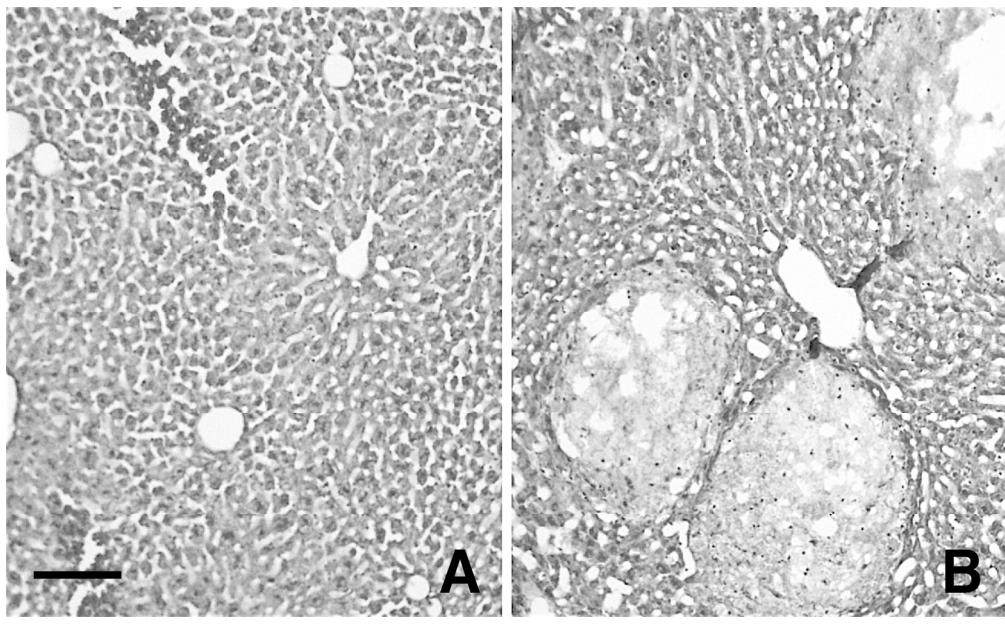


Figure 4. Microphotographs of hematoxylin-eosin stained sections of liver from control (A) and TBT-treated (B) males showing profound alterations of the parenchyma. Bar represents 100 microns 180x109mm (300 x 300 DPI)

Review

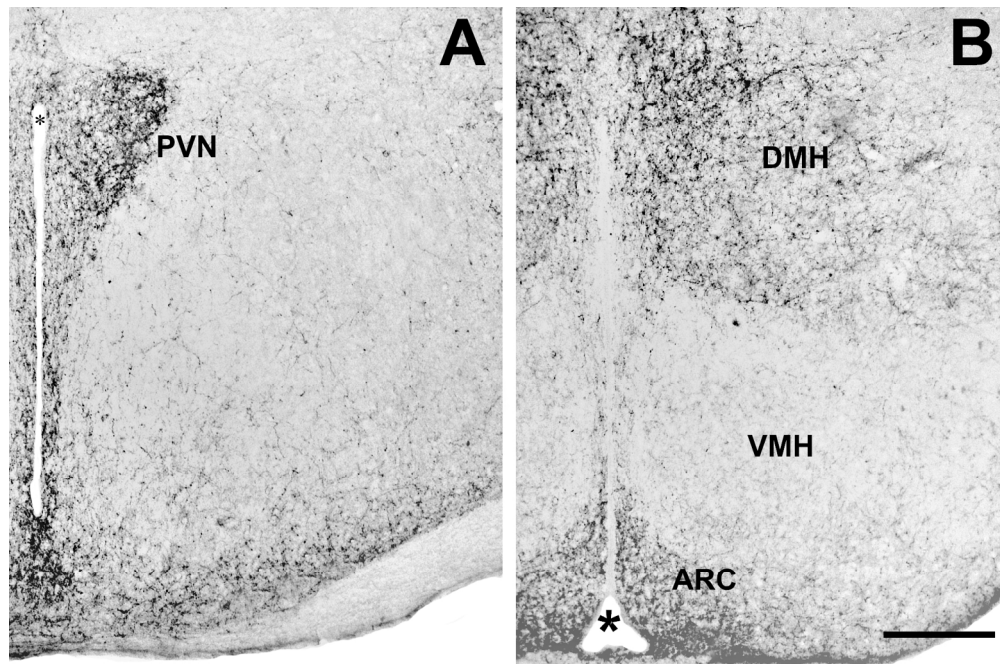


Figure 5. Microphotographs illustrating the immunohistochemical staining for NPY in the nuclei that have been considered in the present study. Microphotographs are taken at the same enlargement. Bar represents 500 microns. A- rostral section containing the paraventricular nucleus (PVN). B- More caudal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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

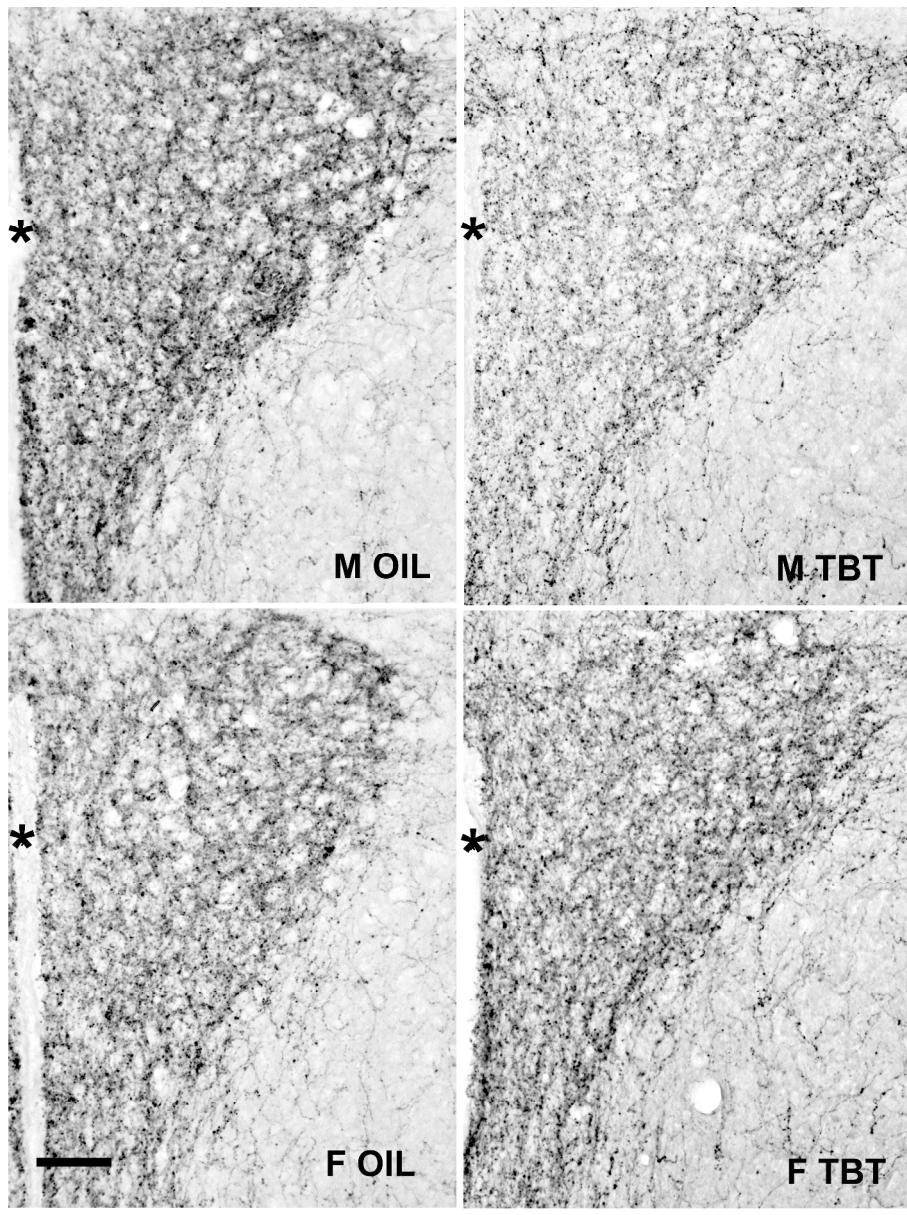


Figure 6. Sections of the paraventricular nucleus from different experimental groups immunostained for NPY. Microphotographs are taken at the same enlargement. Bar represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated male; F OIL - oil-treated female; F TBT - TBT-treated female. * Third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

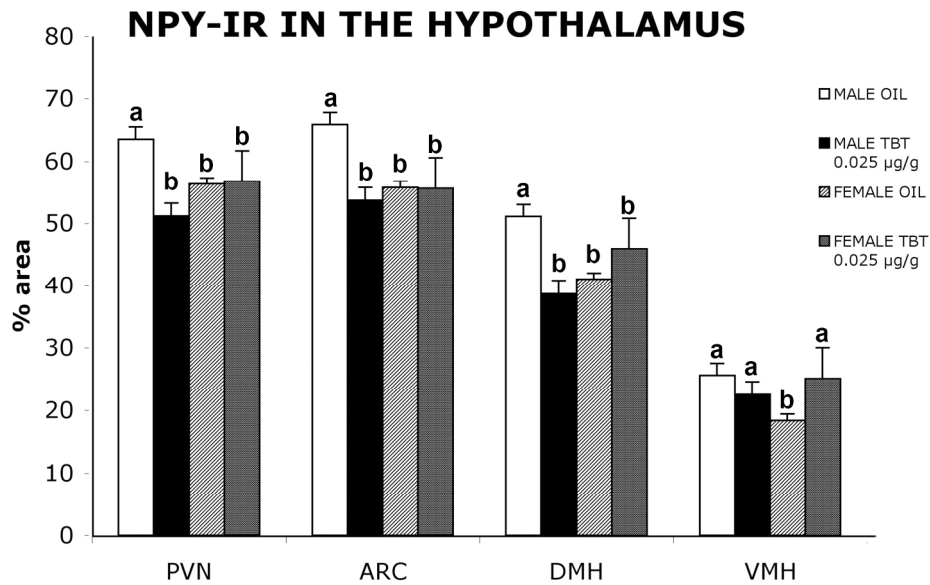


Figure 7. Histograms representing NPY-ir in hypothalamus (expressed as percentage of area covered by stained elements). The multiple comparison analysis between groups for all nuclei demonstrated: in PVN, ARC, and DMH, a significant reduction of NPY-ir in treated males compared to controls), while no 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)

Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

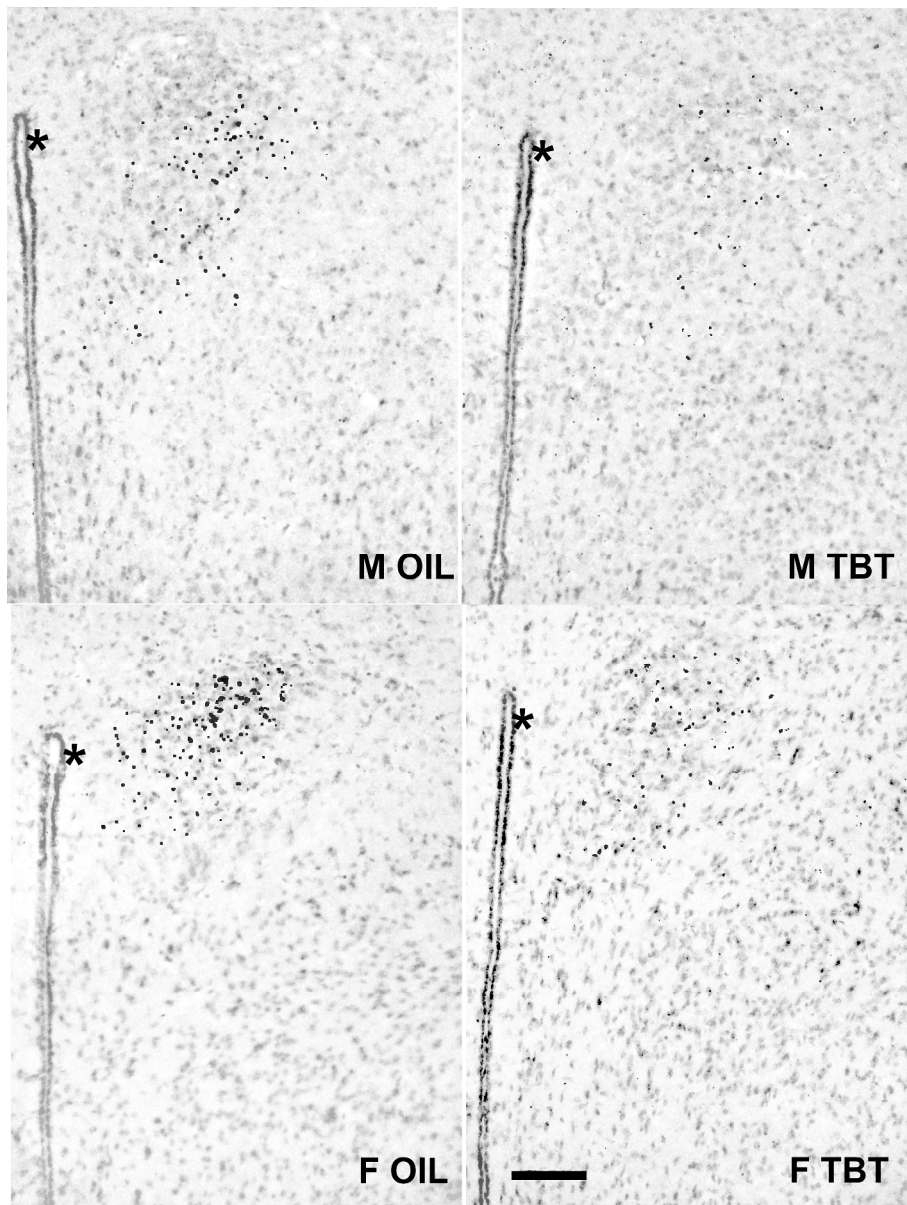
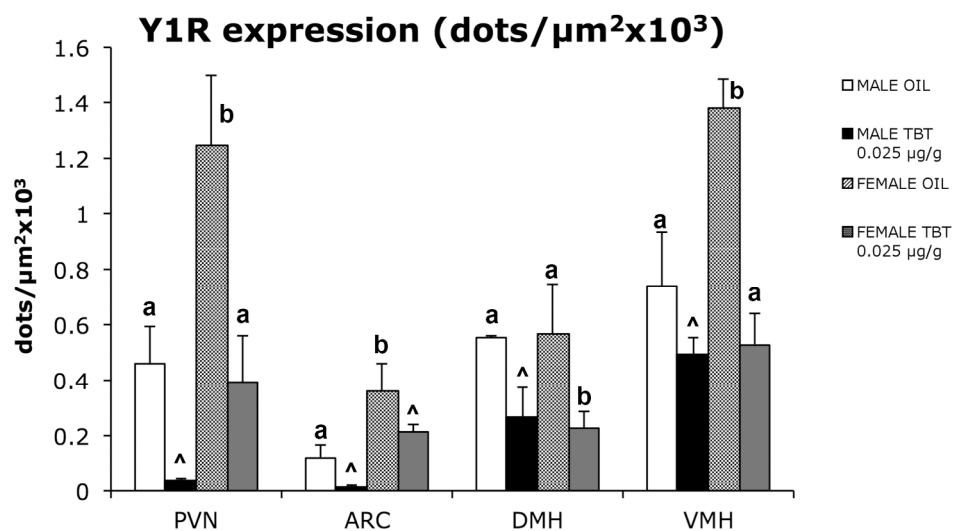


Figure 8. Histochemical detection of beta-galactosidase activity (expression of Y1R-LacZ transgene) in sections of the paraventricular nucleus from different experimental groups. Microphotographs are taken at the same enlargement. Bar represents 100 microns. M OIL - oil-treated male; M TBT - TBT-treated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



27 Figure 9. Histograms representing Y1R-transgene expression in hypothalamus (expressed as dots/ μm^2). In
 28 females the treatment significantly reduced Y1R expression in PVN and VMH. Also in males, TBT reduced
 29 Y1R-transgene expression in both nuclei but the statistical p-value is only close to significance. The
 30 significant differences (ANOVA followed by the Bonferroni test at a level of $P < 0.05$) are denoted by a or b, ^
 31 indicates a close to significant difference ($P < 0.07$).
 32 193x113mm (300 x 300 DPI)

33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Review



UNIVERSITA' DEGLI STUDI DI TORINO

DIPARTIMENTO DI NEUROSCIENZE

"Rita Levi Montalcini"

Segreteria Amministrativa

Via Cherasco 15 – 10126 Torino Tel. 011- 6636327 Fax 011 -6963487

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, Y₁ 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

A handwritten signature in black ink, appearing to read 'GianCarlo Panzica', written over a horizontal line.

Prof. GianCarlo Panzica, Direttore

Dipartimento di Neuroscienze, Polo Anatomico, corso M. D'Azeglio 52, 10126 Torino e
Neuroscience Institute Cavalieri-Ottolenghi (NICO), Regione Gonzole 10, Orbassano (Torino)
Tel. +39 011 6706607 Fax +39 011 2366607 e-mail giancarlo.panzica@unito.it



UNIVERSITA' DEGLI STUDI DI TORINO

DIPARTIMENTO DI NEUROSCIENZE

"Rita Levi Montalcini"

Segreteria Amministrativa

Via Cherasco 15 – 10126 Torino Tel. 011- 6636327 Fax 011 -6963487

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 not 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

Prof. GianCarlo Panzica, Direttore

Dipartimento di Neuroscienze, Polo Anatomico, corso M. D'Azeglio 52, 10126 Torino e

Neuroscience Institute Cavalieri-Ottolenghi (NICO), Regione Gonzole 10, Orbassano (Torino)

Tel. +39 011 6706607 Fax +39 011 2366607 e-mail giancarlo.panzica@unito.it



UNIVERSITA' DEGLI STUDI DI TORINO

DIPARTIMENTO DI NEUROSCIENZE

"Rita Levi Montalcini"

Segreteria Amministrativa

Via Cherasco 15 – 10126 Torino Tel. 011- 6636327 Fax 011 -6963487

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 transgenes 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)

Prof. GianCarlo Panzica, Direttore

Dipartimento di Neuroscienze, Polo Anatomico, corso M. D'Azeglio 52, 10126 Torino e

Neuroscience Institute Cavalieri-Ottolenghi (NICO), Regione Gonzole 10, Orbassano (Torino)

Tel. +39 011 6706607 Fax +39 011 2366607 e-mail giancarlo.panzica@unito.it



UNIVERSITA' DEGLI STUDI DI TORINO

DIPARTIMENTO DI NEUROSCIENZE

"Rita Levi Montalcini"

Segreteria Amministrativa

Via Cherasco 15 – 10126 Torino Tel. 011- 6636327 Fax 011 -6963487

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57

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.

Prof. GianCarlo Panzica, Direttore

Dipartimento di Neuroscienze, Polo Anatomico, corso M. D’Azeglio 52, 10126 Torino e
Neuroscience Institute Cavalieri-Ottolenghi (NICO), Regione Gonzole 10, Orbassano (Torino)
Tel. +39 011 6706607 Fax +39 011 2366607 e-mail giancarlo.panzica@unito.it



UNIVERSITA' DEGLI STUDI DI TORINO

DIPARTIMENTO DI NEUROSCIENZE

"Rita Levi Montalcini"

Segreteria Amministrativa

Via Cherasco 15 – 10126 Torino Tel. 011- 6636327 Fax 011 -6963487

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 reorganized in 3 figures (1-2-3)

Prof. GianCarlo Panzica, Direttore

Dipartimento di Neuroscienze, Polo Anatomico, corso M. D'Azeglio 52, 10126 Torino e

Neuroscience Institute Cavalieri-Ottolenghi (NICO), Regione Gonzole 10, Orbassano (Torino)

Tel. +39 011 6706607 Fax +39 011 2366607 e-mail giancarlo.panzica@unito.it



UNIVERSITA' DEGLI STUDI DI TORINO

DIPARTIMENTO DI NEUROSCIENZE

"Rita Levi Montalcini"

Segreteria Amministrativa

Via Cherasco 15 – 10126 Torino Tel. 011- 6636327 Fax 011 -6963487

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 acronyms 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 acronyms 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

Prof. GianCarlo Panzica, Direttore

Dipartimento di Neuroscienze, Polo Anatomico, corso M. D'Azeglio 52, 10126 Torino e
Neuroscience Institute Cavalieri-Ottolenghi (NICO), Regione Gonzole 10, Orbassano (Torino)
Tel. +39 011 6706607 Fax +39 011 2366607 e-mail giancarlo.panzica@unito.it



UNIVERSITA' DEGLI STUDI DI TORINO

DIPARTIMENTO DI NEUROSCIENZE

"Rita Levi Montalcini"

Segreteria Amministrativa

Via Cherasco 15 – 10126 Torino Tel. 011- 6636327 Fax 011 -6963487

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.

Prof. GianCarlo Panzica, Direttore

Dipartimento di Neuroscienze, Polo Anatomico, corso M. D'Azeglio 52, 10126 Torino e
Neuroscience Institute Cavalieri-Ottolenghi (NICO), Regione Gonzole 10, Orbassano (Torino)
Tel. +39 011 6706607 Fax +39 011 2366607 e-mail giancarlo.panzica@unito.it