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Effect of tributyltin on the food intake and brain neuropeptide expression in rats

Wpływ tributylocyny na przyjmowanie pokarmu i ekspresję neuropeptydów w mózgu szczurów

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Abstract

Introduction: Tributyltin (TBT) is a largely diffused environmental pollutant. Several studies have demonstrated that TBT is involved in the development of obesity. However, few studies addressing the effects of TBT on the brain neuropeptides involved in appetite and body weight homeostasis have been published.

Material and methods: Experiments were carried out on female and male Sprague-Dawley rats. Animals were exposed to TBT (0.5 μg/kg body weight) for 54 days. The hepatic triglyceride and total cholesterol were determined using commercial enzyme kits. The NPY, AgRP, POMC and CART mRNA expression in brains were quantified by real-time PCR.

Results: TBT exposure resulted in significant increases in the hepatic total cholesterol and triglyceride concentration of both male and female rats. Interestingly, increases in body weight and fat mass were only found in the TBT-treated male rats. TBT exposure also led to a significant increase in food intake by the female rats, while no change was observed in the male rats. Moreover, the neuropeptides expression was different between males and females after TBT exposure. TBT induced brain NPY expression in the female rats, and depressed brain POMC, AgRP and CART expression in the males.

Conclusions: TBT can increase food intake in female rats, which is associated with the disturbance of NPY in brains. TBT had sex-different effects on brain NPY, AgRP, POMC and CART mRNA expression, which indicates a complex neuroendocrine mechanism of TBT. **(Endokrynol Pol 2014; 65 (6): 485–490)**

Key words: *tributyltin; body weight; food intake; neuropeptides*

Streszczenie

Wstęp: Tributylocyna (TBT) jest powszechnie występującym w środowisku zanieczyszczeniem. Prowadzone dotychczas badania wykazały, że obecność TBT może mieć związek z rozwojem otyłości. Niewiele jest jednak doniesień na temat wpływu TBT na układ neuropeptydów w mózgowiu regulujących łaknienie i utrzymanie masy ciała.

Materiał i metody: Doświadczenia przeprowadzono na szczurach obu płci szczepu Sprague-Dawley. Zwierzętom podawano przez 54 dni TBT w dawce 0,5 μg/kg masy ciała. Stężenie triglicerydów i całkowite stężenie cholesterolu w wątrobie oznaczano przy użyciu komercyjnych zestawów analitycznych. Obecność mRNA NPY, AgRP, POMC i CART w mózgach szczurów oznaczano metodą PCR w czasie rzeczywistym (real time-PCR).

Wyniki: Ekspozycja na TBT powodowała istotne zwiększenie całkowitego stężenia cholesterolu i trójglicerydów w wątrobie zarówno samców, jak i samic szczura. Co ciekawe, zwiększenie masy ciała i masy tkanki tłuszczowej odnotowano jedynie u samców, którym podawano TBT. Stwierdzono także istotne zwiększenie ilości pokarmu przyjmowanego przez samice, natomiast nie obserwowano takich zmian u samców. Ponadto, odnotowano różnice w ekspresji neuropeptydów w mózgowiu samic i samców szczura, którym podawano TBT. Ekspozycja na TBT nasilała ekspresję NPY w mózgach samic, ale równocześnie zmniejszała ekspresję POMC, AgRP i CART w mózgach samców szczura.

Wnioski: Ekspozycja na TBT może zwiększać ilość pokarmu spożywanego przez samice szczura, co wiąże się z zaburzeniem układu NPY w mózgowiu. Trybutylocyna wywiera odmienny wpływ na ekspresję mRNA NPY, AgRP, POMC i CART w mózgach samców i samic szczura, co wskazuje na istnienie złożonego mechanizmu działania tej substancji na układ neuroendokrynny. **(Endokrynol Pol 2014; 65 (6): 485–490)**

Słowa kluczowe: *tributylocyna; masa ciała; przyjmowanie pokarmu; neuropeptydy*

Introduction

Organotin compounds, such as tributyltin (TBT), are used in a wide variety of consumer products, for example in agriculture and industry as biocide, heat stabiliser and chemical catalyst [1]. Although TBT has been banned from paints in the European Union since 2003 (EC Regulation 782/2003), it is still found at high levels in marine and freshwater ecosystems exceeding toxicity levels [2]. The contamination level of organotin compounds in food, particularly in fish and shellfish, remains high [3]. Recently, it was reported that TBT

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was also found in dust collected from houses [4]. High levels of butyltins were detected in human liver tissue and blood samples. For example, total butyltin concentrations in human livers collected from Poland are in the range 2.4–11 ng/g wet weight [5], and butyltin concentrations in the livers of Japanese are in the range 59-96 ng/g wet weight [6]. Kannan et al. [7] reported that TBT levels in human blood collected from the United States were up to 8.18 ng/mL.

In mammals, TBT is toxic to a number of organs [8-10]. Recently, TBT was shown to affect adipogenic differentiation by activating peroxisome proliferator-activated receptor (PPAR) γ and retinoid X receptor (RXR) *in vitro* [11]. Prenatal exposure to environmental TBT predisposes multipotent stem cells to become adipocytes in mice [12]. When pregnant mice are injected daily from gestational days 12–18 with TBT (0.05 or 0.5 mg/kg body weight intraperitoneally), an increase in lipid accumulation or an increase in mature adipocytes is observed in the pups [13]. Recent reports indicate that TBT increases body weight in male mice [14]. In our previous study, we have found that chronic and repeated exposure to low doses of TBT disturbs levels of key hormones linked to energy homeostasis [1]. These findings suggest a peripheral role of TBT on obesity. However, few studies on the effects of TBT on the central nervous system have been reported. Hypothalamus acts as the control centre for hunger and satiety. Part of the hypothalamus, the arcuate nucleus, includes neurons that coexpress peptides that stimulate food intake and weight gain, especially neuropeptide Y (NPY) and agouti related peptide (AgRP), as well as those expressing proopiomelanocortin (POMC) and cocaine- and amphetamine regulated transcript (CART) which inhibit feeding and promote weight loss. Together, these neurons and peptides control the sensations of hunger and satiety and thereby regulate appetite and energy balance [15].

Therefore, this study was designed to investigate the effects of TBT on body weight gain and determine alterations of hypothalamic expression of the neuropeptides involved in food intake regulation in rats.

Material and methods

Chemicals

TBT chloride was obtained from Fluka AG, Switzerland, with a purity of greater than 97%. All other chemicals were of analytical grade and were obtained from commercial sources. The TBT was dissolved in 100% ethanol and diluted with 0.85% (g/g) sodium chloride. The final TBT concentration was 0.1 μg/mL, and the final ethanol concentration was 0.1 ml/mL volume.

Experimental species and treatment

All animal experiments were conducted according to the research protocols approved by the Institutional Animal Care and Use Committee, Henan University of Science and Technology. Sprague-Dawley rats were purchased from Zhengzhou University (China), housed in individual °wire-mesh cages, in the same room at 24 ± 1°C under a 12-h light-dark cycle. Before the treatment, the average body weight of the males and females was 218.58 ± 18.49 and 187.85 ± 8.56 g respectively. After a quarantine period, 16 male or female rats with adequate weight gain and without clinical signs were divided randomly into two experimental groups. One group was orally administered by gavage once every three days with 0.5 μg/kg TBT, and the other group received an equal volume of vehicle (5 mL/kg). Body weight was recorded on the day of oral administration of TBT or vehicle, and actual dosing volumes were adjusted according to the body weights recorded. Food intake was determined every two days throughout the duration of the experiment. The amount of food remaining at the end of the two day period was individually weighed and subtracted from the original quantity provided. Bedding was searched thoroughly to ensure complete removal of all remaining food. All rats had ad libitum access to water.

Tissue sampling

The rats were sacrificed 54 days after the exposure began and fasted for 12 h before necropsy. The fat mass (the epididymal and retroperitoneal fat deposits) was weighed. The adiposity index was calculated as the quotient of the fat mass (g) and the final body weight of the animal (g). The isolated brains were flash frozen in liquid nitrogen for analysis of NPY, AgRP, POMC and CART mRNA expression. The isolated livers were stored at −80 °C for biochemical analysis.

Hepatic lipid

Extraction of total lipids from the livers was performed as described by Folch et al. [16] in the presence of butylated hydroxytoluene as an antioxidant. Triglyceride and total cholesterol were determined using commercial enzyme kits purchased from Nanjing Jiancheng Bioengineering Institute (China) according to the manufacturer's instructions.

Reverse-transcriptional real-time PCR (RT-PCR)

Levels of NPY, AgRP, POMC and CART mRNA were determined by quantitative real-time PCR using SYBR Green chemistry on a Rotor-Gene 3000 (Applied Biosystems, USA) using the housekeeping gene β-actin as internal control according to the methods of our laboratory [17]. The Relative Expression Software Tool

Table I. *Sequences of forward and reverse primers used for real-time RT-PCR*
Tabela I. *Sekwencje starterów (lewy starter i prawy starter) stosowanych do analizy PCR w czasie rzeczywistym (RT-PCR)*

Target	Sequence	Accession number	PCR efficiency (%)
NPY	(F)5′ CGCTCTATCCCTGCTCGTGT 3′	NM_012614	91.7
	(R)5′ GGTCTTCAAGCCTTGTTCTGG 3′		
AGRP	(F)5′ AAGAAGAACCGGAACAAATGC 3′	NM_033650	93.1
	(R)5′ GCAGGACTCGTGCAGCCTTA 3′		
POMC	(F)5′ AACGGAGATGAACAGCCCTTGAC 3′	NM_139326	90.0
	(R)5′ CGACTCGTTCTCGGCGACATT 3′		
CART	(F)5′ GCCAAGTCCCCATGTGTGAC 3′	NM_017110	94.1
	(R)5′ CACCCCTTCACAAGCACTTCA 3′		
β-actin	(F)5′ CCGTAAAGACCTCTATGCCAACA 3′	NM_031144	98.0
	(R)5′ CGGACTCATCGTACTCCTGCT 3′		

(F) — forward primer; (R) — reverse primer

(REST 2008©-version 2) was used to calculate the relative expression. The real time quantitative PCR primers and the PCR efficiencies are shown in Table I.

Data processing

Results were reported as means ± standard error of measurement (S.E.M.). Significant differences between means were analysed with one-way analysis of variance (ANOVA) followed by Dunnett's post hoc using statistical software, SPSS Version 11.0 (SPSS Inc., Chicago, IL, USA) for Windows, except mRNA expression, which was performed using the Pair Wise Fixed Reallocation Randomization Test© [18]. In all cases, a value of $p < 0.05$ was used to indicate significant differences.

Results

Body weight

The average body weights of rats after TBT exposure for 54 days are shown in Figure 1. Significant increases of body weights at days 6, 12, 18, 24 and 30 were found in the male rats exposed to TBT compared to the control (Fig. 1A). However, in the female rats, no significant change of body weight was observed in the TBT exposure group compared to the control (Fig. 1B).

Fat mass

In the male rats, TBT exposure resulted in a significant increase (by 1.56-fold) in fat mass compared to the control (Table II). However, in the female rats, there was no significant difference of fat mass between the exposure group and the control (Table II). Although increases of adiposity index were found in both male and female rats after TBT exposure, no significant changes were found (Table II).

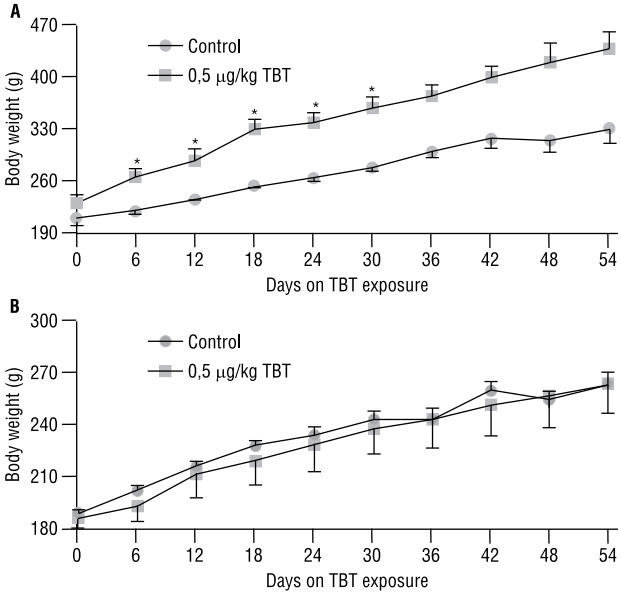

Figure 1. *The body weight gain of control and TBT-treated male* **(A)** *and female* **(B)** *rats. Data is presented as mean ± S.E.M. (n = 8). Asterisks (*) indicate that the values are significantly different from those of the control group at $p < 0.05$*

Rycina 1. *Przyrost masy ciała w grupie kontrolnej oraz u samców* **(A)** *i samic* **(B)** *po ekspozycji na TBT. Przedstawiono wartości średnie ± S.E.M. (błąd standardowy średniej) (n = 8). Gwiazdką (*) oznaczono istotne różnice w porównaniu z grupą kontrolną, dla $p < 0,05$*

Hepatic lipid

In both male and female rats, TBT exposure resulted in significant increases (by 1.44- and 1.31-fold, respectively) of the hepatic total cholesterol (Table III). The hepatic triglyceride also increased significantly (by 1.86- and 1.24-fold, respectively) after exposure to TBT in both male and female rats (Table III).

Table II. *Effects of TBT on the fat mass and adiposity index in male and female rats*

Tabela II. *Wpływ TBT na masę tkanki tłuszczowej i wskaźnik otłuszczenia u samców i samic*

	Fat mass (g)		Adiposity index	
	Male	**Female**	**Male**	**Female**
Control	7.98 ± 0.96	9.09 ± 1.47	2.06 ± 0.51	3.09 ± 0.40
TBT	12.3 ± 1.94*	7.69 ± 1.08	3.56 ± 0.72	3.58 ± 0.34

Data is presented as mean ± S.E.M. (n = 8). Asterisks (*) indicate that the values are significantly different from those of the control group at $p < 0.05$

Table III. *Effects of TBT on the hepatic total cholesterol and triglyceride in male and female rats*

Tabela III. *Wpływ TBT na całkowite stężenie cholesterolu i triglicerydów u samców i samic*

	Total cholesterol [mmol/L]		Triglyceride [mmol/L]	
	Male	**Female**	**Male**	**Female**
Control	0.84 ± 0.24	1.18 ± 0.15	0.23 ± 0.11	0.17 ± 0.11
TBT	1.21 ± 0.13*	1.55 ± 0.32*	0.43 ± 0.08*	0.21 ± 0.08*

Note: Data is presented as mean ± S.E.M. (n = 8). Asterisks (*) indicate that the values are significantly different from those of the control group at $p < 0.05$

Table IV. *Effects of TBT on the food intake (g/2 day/rat) in male and female rats*

Tabela IV. *Wpływ TBT na ilość przyjmowanego pokarmu (g/2 × dziennie/zwierzę) u samców i samic*

	Food intake/body weight		Food intake (g)	
	Male	**Female**	**Male**	**Female**
Control	0.094 ± 0.002	0.094 ± 0.001	29.15 ± 1.68	20.17 ± 0.30
TBT	0.096 ± 0.003	0.104 ± 0.004*	28.25 ± 0.43	22.52 ± 0.87*

Note: Data is presented as mean ± S.E.M. (n = 8). Asterisks (*) indicate that the values are significantly different from those of the control group at $p < 0.05$

Food intake

In the male rats, no significant changes of food intake and the ratio of food intake and body weight were found between the exposure group and the control (Table IV). However, in the female rats, TBT exposure resulted in a significant increase (by 1.50- and 1.12-fold) in food intake and the ratio of food intake and body weight compared to the control (Table IV).

Neuropeptides expression

No significant change in the brain expression of NPY was observed in the male rats (Fig. 2A). However, in fe-

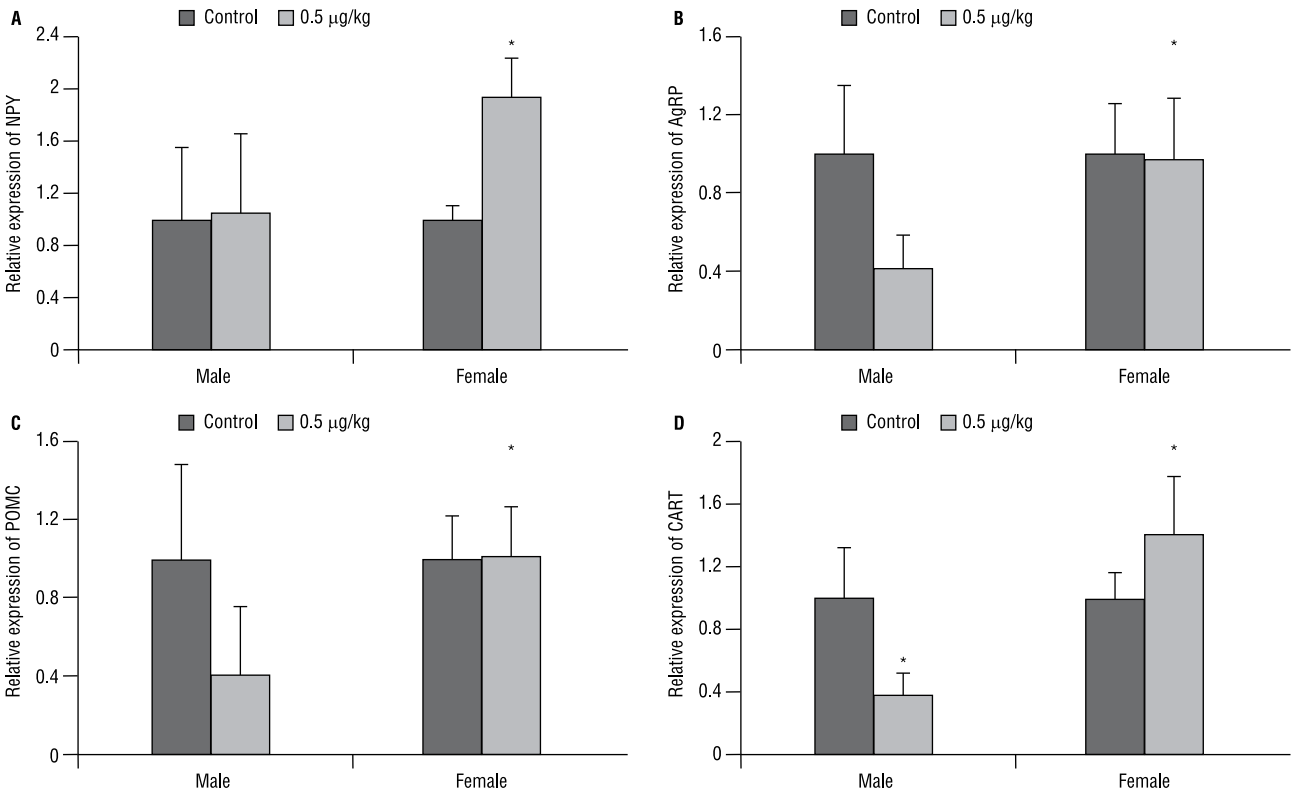

Figure 2. *The brain mRNA expression of NPY* **(A)**, *AgRP* **(B)**, *POMC* **(C)**, *and CART* **(D)** *of control and TBT-treated rats. Data is presented as mean ± S.E.M. (n = 8). Asterisks (*) indicate that the values are significantly different from those of the control group at $p < 0.05$*

Rycina 2. *Ekspresja mRNA NPY* **(A)**, *AgRP* **(B)**, *POMC* **(C)** *i CART* **(D)** *w mózgowiu szczurów w grupie kontrolnej i u zwierząt po ekspozycji na TBT. Przedstawiono wartości średnie ± S.E.M.(błąd standardowy średniej) (n = 8). Gwiazdką (*) oznaczono istotne różnice w porównaniu z grupą kontrolną dla $p < 0,05$*

male rats, the brain expression of NPY was significantly increased (by 1.93-fold) in the TBT exposure group compared to the control (Fig. 2A).

TBT exposure significantly decreased (by 0.42-fold) the brain expression of AgRP in male rats compared to the control (Fig. 2B). No significant change of AgRP expression in the brain was observed in the female rats (Fig. 2B).

Although a decrease of brain POMC expression was found in the male rats after TBT exposure, no significant changes were found (Fig. 2C). No significant change of brain POMC expression was also observed in the female rats (Fig. 2C).

TBT exposure significantly decreased (by 0.38-fold) the brain expression of CART in male rats compared to the control (Fig. 2D). No significant change of CART expression in the brain was observed in the female rats (Fig. 2D).

Discussion

TBT is a largely diffused environmental pollutant, and the level in food, particularly in fish and shellfish, remains high. The average intake of TBT by humans from market-bought seafood has been estimated to vary worldwide between 0.18 and 2.6 μg per day per person [19]. It is reported that mean concentrations of TBT in fish muscle collected from Taiwanese harbours is 308.7 ng/g wet weight [20]. Using the average seafood consumption data of 0.067 kg/day provided by Lee et al. [21], the dose of TBT used in the present study is below those doses to which people may be exposed. Recently, a human epidemiological study showed that placenta TBT is associated with increased weight gain during the first three months of life [22], which indicated a role for TBT pollution in the development of obesity. In the present study, 0.5 μg/kg TBT treatment already produced a prominent obesity-related effect. However, the Tolerable Daily Intake of organotins established by the European Food Safety Authority is 0.250 μg/kg body weight [23]. Therefore, the health impact of TBT on human might be underestimated.

TBT is considered as a kind of obesogen, chiefly for its action on fat tissue inducing the differentiation of preadipocytes from adipocytes [11, 13, 24]. In our previous study, hepatic dysfunction, as well as a rise in plasmatic levels of insulin, leptin, and resistin, was observed [1]. In the present study, TBT exposure resulted in significant increases of the hepatic total cholesterol and triglyceride in both male and female rats. The liver plays a central role in coordinating whole body metabolism. Changes in hepatic lipid metabolism might contribute to the obesity effect of TBT exposure. Interestingly, the increases of fat mass and body weight were only found in the male rats. It has been reported that male mice are more susceptible to obesity than female mice, and ovarian hormones might provide protection against weight gain [25]. In addition, the ovary is one of the most dynamic endocrine organs in females. A substantial energy source is necessary for its activity in puberty. In our previous study, TBT exposure induced an increase of interstitial ectopic lipid accumulation and total lipids in the ovaries of fish [26]. Thus, no change of fat mass and body weight in females might be also associated with the high-energy demand of ovaries.

Obesity is not a purely metabolic disease and its genesis is also due to an imbalance of neuroendocrine mechanisms acting under the control of specific neural circuits located in the hypothalamus [27]. TBT can be transported to the brains of fish by axonal transport [28]. In rats, it has been reported that TBT disrupted blood-brain barrier and increased Sn accumulation in the brain regions [29]. Therefore, the brain might be a potential target organ of TBT. Neurotoxicological studies have demonstrated that the levels of brain dopamine, norephinephrine and serotonin decrease in a dose-dependent manner after ingestion of high doses of TBT in mice [30]. It is also reported that acute exposure to TBT induces c-fos activation in the hypothalamic arcuate nucleus of adult male mice [31].

It is well known that hypothalamic orexigenic (*i.e.* NPY and AgRP) and anorexigenic (*i.e.* POMC and CART) neuropeptides play an important role in the regulation of appetite and body weight homeostasis [15]. In the present study, we observed that TBT exposure leads to a significant increase of NPY expression in the female rats, which could explain the observed increase of food intake in the females. However, no change of food intake was observed in the male rats, which might be associated with the opposite effects on appetite of decrease of AgRP and CART. The increase in body weight in the male rats may be caused by complex factors, not only by food intake. In addition, except NPY, the brain expression of AgRP, POMC and CART was depressed by TBT exposure in male rats. It is reported that TBT induces oxidative damage, inflammation and apoptosis via disturbance in the blood–brain barrier and metal homeostasis in rat brains [32]. In our previous study, TBT induced brain astrocyte apoptosis in rockfish [33]. Thus, the no change or depression of neuropeptides expression in male rats would be due to the cytotoxicity of TBT. There is some evidence showing that NPY-expressing neurons in the hypothalamus concentrate 17β-oestradiol and the sex steroid oestrogen may play a role in the regulation of NPY synthesis [34,35]. Therefore, we suspect that the sex-different effects of TBT on brain neuropeptides expression might be due to the neuro-protection of 17β-oestradiol in the brain, where females have higher 17β-oestradiol levels compared to males [36].

Conclusions

TBT can increase the food intake in female rats, which is associated with the disturbance of NPY in brains. TBT had sex-different effects on brain NPY, AgRP, POMC and CART mRNA expression, which indicates a complex neuroendocrine mechanism of TBT. The obesity induced by TBT might be caused by complex factors, not only by food intake. Elucidation of this mechanism requires further study.

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