In Vitro Effect of Octyl – Methoxycinnamate (OMC) on the Release of Gn-RH and Amino Acid Neurotransmitters by Hypothalamus of Adult Rats

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Key words
- UV filters
- hypothalamic Gn-RH
- amino acid neurotransmitters
- OMC

Abstract

OMC (octyl-methoxycinnamate), an endocrine disruptor having estrogenic activity, is used in sunscreen creams as UV filter. We studied its "in vitro" effects on the hypothalamic release of Gn-RH as well as on the amino acid neurotransmitter system. OMC significantly decreased Gn-RH release in normal male and female rats as well as in castrated rats with substitutive therapy. No effects were observed in castrated rats without substitutive therapy. In males OMC increases the release of GABA, decreasing the production of glutamate (GLU) while in the female decreases the excitatory amino acid aspartate (ASP) and GLU without modifications in the hypothalamic GABA release. These results suggest that OMC acting as endocrine disruptor could alter the sex hormone-neurotransmitter-Gn-RH axis relationships in adult rats.

Introduction

In the last years numerous chemicals in the environment that are able to mimic the natural hormones, thereby disrupting endocrine functions, has been described. These chemicals are able to bind to sexual hormone receptors and interfere in the hormone signaling pathways, although several of them have structures that differ substantially from the endogenous hormones (Schreurs et al., 2005). A number of absorbers of UV light, particularly those related to cinnamate, could be endocrine disruptors (Holbech et al., 2002; Ma et al., 2003; Kordon et al., 1994; Klammer et al., 2005). Between them, sun protection products contain a variety of UV-filters, among others octyl-methoxycinnamate (OMC). This compound is approved as a cosmetic ingredient in Europe and over the counter drug in the US by FDA. The highests approved concentration in Europe for OMC is 10%. (wt/wt).

In the humans, OMC is absorbed by the skin; substantial systemic absorption and urinary excretion have been detected after whole-body topical application of 2 mg/cm^2 of sunscreen formulation of 10% (w/w) OMC (Hayden et al., 1997; Janjua et al., 2004; Janjua et al., 2008).

In vitro and in vivo studies performed in rodents have reported estrogen-like activity in OMC. Under various conditions OMC was shown to have estrogenic activity in the uterus and vagina of mice and rats (Schlumpf et al., 2001; Klammer et al., 2005). In MCF-7 breast cancer cells, OMC increased cell proliferation with a concentration range similar to values known for other xenoestrogens (Klammer et al., 2005). The estrogenic activity of OMC has been also determined in vivo using the uterotrophic assay in immature rats treated with the chemical by oral administration (Schlumpf et al., 2001; Schlumpf et al., 2004). On the other hand Seidlová-Wuttke et al., 2006 have observed a very weak estrogenic effect in the uterus and a mild stimulation on serum LH levels of ovariectomised adult female rats treated with OMC in high dose by oral administration. These authors have proposed that this effect could be related with the existence of neurotransmitter(s)-involving mechanisms exerted by OMC at hypothalamic level in adult rats.

A few studies have evaluated the estrogen-like activity of OMC in humans. Janjua et al., 2004 have reported minor but statistically significant differences in testosterone, estradiol and inhibin B levels, without changes in FSH and LH after the short-term whole-body topical aplication of sunscreen formulation containing 10% (w/w) OMC. No effect on the thyroid-stimulating hormone

received 22.10.2008
first decision 22.04.2009
accepted 12.05.2009

Bibliography
DOI http://dx.doi.org/10.1055/s-0029-1224153
Published online: March 2, 2010
Exp Clin Endocrinol Diabetes 2010; 118: 298–303

ISSN 0947-7349

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This work was supported by Grants from Agencia de Promoción Científica y Técnica BID 1728 PIC 2003 and the University of Buenos Aires.

(TSH), T4 and T3 was observed in man and women submitted to similar treatment (Janjua et al., 2007).

It is a well known fact that estrogens modify gonadotrophin secretion both in male and female rats, acting at pituitary and hypothalamic levels (Kordon et al., 1994; Moguilevsky et al., 2001). The effect of sexual hormones is related with modifications in the hypothalamic neurotransmitters that regulate hypothalamic release of Gn-RH (Moguilevsky et al., 2001; Moguilevsky et al., 1995; Brann et al., 1992; Arias et al., 1993). Therefore, the estrogen-like activity of OMC could affect the reproductive function by a direct effect on the hypothalamic pituitary gonadal axis. Recently we have demonstrated that OMC in vitro, in a low micromolecular range and similar to values for other environmental chemicals identified as xenoestrogens, was able to modify the release of Gn-RH and amino acid neurotransmitters from hypothalamus of immature male and female rats (Szwarcfarb et al., 2008).

Taking this into account, the aim of the present work was to determine the in vitro effect of OMC on the Gn-RH axis in adult rats. For this purpose we measure the in vitro hypothalamic release of Gn-RH and the amino acid neurotransmitters such us γ amino butyric acid (GABA), glutamate (GLU) and aspartate (ASP) involved in its hypothalamic regulation. These studies were performed in control, castrated and castrated plus substitutive therapy male and female rats.

Material and Methods

Animals

Adult male and female Wistar rats (weighing 260±30g and 210±20g, respectively) from the Departmento de Fisiología, Facultad de Medicina, Universidad de Buenos Aires, were used. They were kept in a light and temperature (T) controlled environment (lights on from 07.00 to 19.00h, T: 22°C. The animals had free access to a pellet diet and tap water. Adequate measures were taken to minimize pain or discomfort, in accordance with the principles and procedures outlined in the European Communities Council Directives (86/609/EEC) and FRAME Guidelines (*FRAME Reduction Committee November, 1999*). The following groups (10 rats each) were studied: 1. Normal males and diestrous females: injected with the vehicle (N); 2. Castrated males and females: castration was performed 30 days before sacrifice. Castrated males and females were injected with the vehicle 48 and 72h before this sacrificie, respectivelly. (C); 3. Castrated male rats injected with testosterone propionate 48h before killing (C+To); 4. Castrated female rats injected with estradiol benzoate 72h before sacrificie (C+E2).

Drugs

Octyl-methoxycinnamate (OMC, Eusolex 2393) was obtained from Merck (Darmstadt, Germany). Stock solution of the compound was prepared in absolute ethanol at the concentration of 10^{-2}M, stored at −20° and diluted to desired concentration in Earle's medium. OMC was added to the medium at final concentration of 2.63×10^{-7}M. This dose was chosen on the basis of those used by Schlumpf et al. (2001) in the in vitro assay to determine estrogenic activity. In previous experiments (Szwarcfarb et al., 2008) we corroborated that this is the minimal effective dose of OMC that modified Gn-RH release from rat hypothalamus. The final ethanol concentration in the medium did not exceed 0.001% (v/v). No difference in Gn-RH release was observed in control experiments with chemical-free medium or medium with 0.001% (v/v) ethanol. Therefore, we used chemical-free medium as a control.

Testosterone propionate and estradiol benzoate (Sigma Chemical Co., Saint Louis Mo.) were dissolved in sesame oil. Castrated male rats were injected subcutaneously with testosterone propionate in a single injection at a dose of 1mg/kg, 48h before sacrifice. This dose was chosen on the basis of previous experiments, in which we demonstrated that is the minimal dose which returns gonadotropin levels to normal in castrated male rats (Justo et al., 1989). Estradiol benzoate was administered to ovariectomized female animals in a single injection at a dose of 20μg/kg 72h before killing (Ponzo et al., 1999).

Tissue processing and incubation of hypothalamic fragments

Animals were sacrificed by decapitation between 16.00–17.00h. After that the brains were rapidly removed and the hypothalamic fragments dissected out with a single razor blade and weighed. Hypothalamic samples containing the anterior preoptic and medial basal areas (APOA-MBH) were dissected with the help of a stereomicroscope. The hypothalamic samples obtained were bordered laterally by the hypothalamic sulci; rostrally, 3mm anterior to the optic chiasma and caudally, by the mammillary bodies. The thickness of each sample was less than 2mm. No significant differences were found between the weights. After dissection, similar quantities of the APOA-MBH samples were put into plastic chambers containing 500μl of Earle's medium with glucose (1mg/ml) and bacitracin (20mM). The pH was adjusted to 7.4. The chambers were incubated in a Dubnoff shaker at 37°C under constant shaking (60 cyclesmin) in an atmosphere of 95% O_2 and 5% CO_2.

After 30min of preincubation, the medium was removed by aspiration and discarded. Fresh chemical free medium was added to the control samples and fresh medium with OMC at final concentration of 2.63×10^{-7}M was incorporated to the hypothalamic fragments to test the drug. All the samples were incubated for 60min and at the end of this period the medium was collected and immediately frozen at −70°C for Gn-RH and amino acids determinations. This period of incubation was chosen on the basis of previous studies (Karanth et al., 2004; Szwarcfarb et al., 2008) in which 17 ß-estradiol and OMC at the lowest effective dose in the range of 10^{-7}M were capable to modify Gn-RH release from rats hypothalamus after 1h of incubation.

Gn-RH determination

Gn-RH was measured in the medium, in duplicate by RIA using a highly specific antibody provided by V. D. Ramirez (Department of Physiology and Biophysics, University of Illinois, Urbana, IL). [^{125}]Gn-RH was purchased from New England Nuclear (Boston, MA) and synthetic Gn-RH (Península Laboratories, Belmont, CA) served as reference. The sensitivity of the assay was 0.2pg/100μl; intra-and inter-assay coefficients of variation were 7% and 14%, respectively. The results are expressed as pgr/ml of medium.

Amino acids determination

The concentrations of GLU, ASP and GABA in the medium were determined by HPLC after derivatization with phenylisothiocyanate and UV detection at 254nm, as previously described (Jarry et al., 1992). The drugs used did not interfere in the derivatization process. Mean inter and intra-assay coefficients of variation

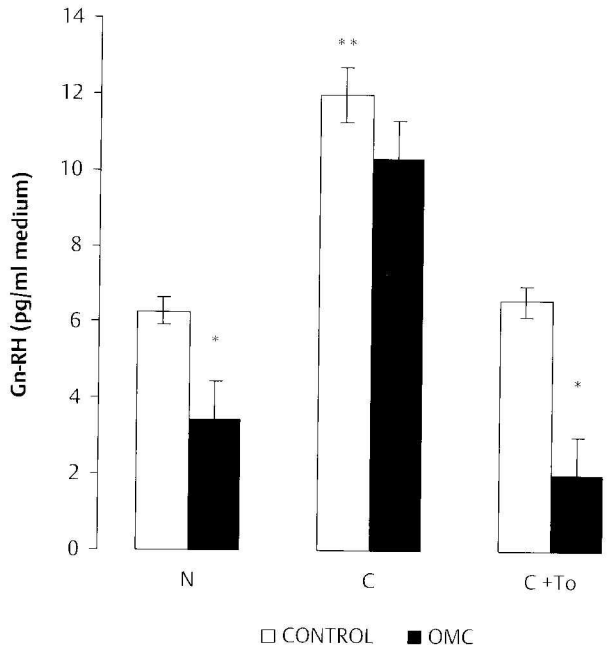

Fig. 1 Effect of OMC on the Gn-RH release by hypothalamic areas in normal (N), castrated (C) and castrated plus testosterone (C + To) male rats. Each column represents the means SEM of 10 individual determinations. OMC was added at 10–7 M to the incubation media. *p< 0.001 vs. control; **p<0.001 normal control vs. castrated control.

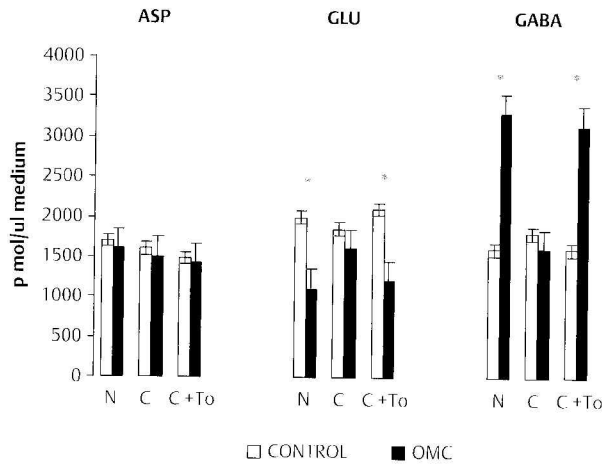

Fig. 2 Effect of OMC on the hypothalamic amino acid neurotransmitters in normal (N), castrated (C) and castrated plus testosterone (C + To) male rats. Each column represents the means SEM of 10 individual determinations. OMC was added at 10–7 M to the incubation media. *p< 0.001 vs. control.

were 4.0, 5.6 and 8.0%, respectively. The detection limit was 10 pM for GLU, 20 pM for ASP and 5 pM for GABA. The mobile phase was 0.57 M sodium acetate buffer (pH 6.5) containing 10% acetonitrile (Sintorgan, Buenos Aires, Argentina). Amino acid standards were: ASP, GLU and GABA (Sigma Chemical Co St Louis, Mo, USA.). The results are expressed as pmol/100 µl of medium.

Statistical Analysis

The results given in the text were expressed as the mean ± S. E. M. Significance was assessed by analysis of variance (ANOVA) and Tukey's multiple range test (Tukey, 1994). When appropriate, Student's t test was used for the comparison of two treatments; $p < 0.05$ was considered significant.

Results

Male rats

Fig. 1, shows the effect of OMC on Gn-RH release by the hypothalamic area in male rats. OMC 10^{-7} M significantly ($p < 0.001$) decreased the hypothalamic release of Gn-RH in normal rats and this effect was not observed in the castrated ones. On the other hand substitutive therapy in castrated male rats not only decreased the Gn-RH liberation at similar values to normal ones but restored the inhibitory effect of OMC on Gn-RH synthesis as well.

Fig. 2 shows the effect of OMC on hypothalamic amino acids release in normal, castrated and castrated male rats pretreated with substitutive therapy. While in normal animals OMC significantly decreased hypothalamic release of GLU increasing GABA liberation ($p < 0.001$) no modifications in the hypothalamic release of these amino acids were observed in castrated male rats injected with the vehicle. However the pretreatment with testosterone was capable to restore the modificationes produced by OMC in normal rats, i.e. decreases GLU and increases GABA hypothalamic release.

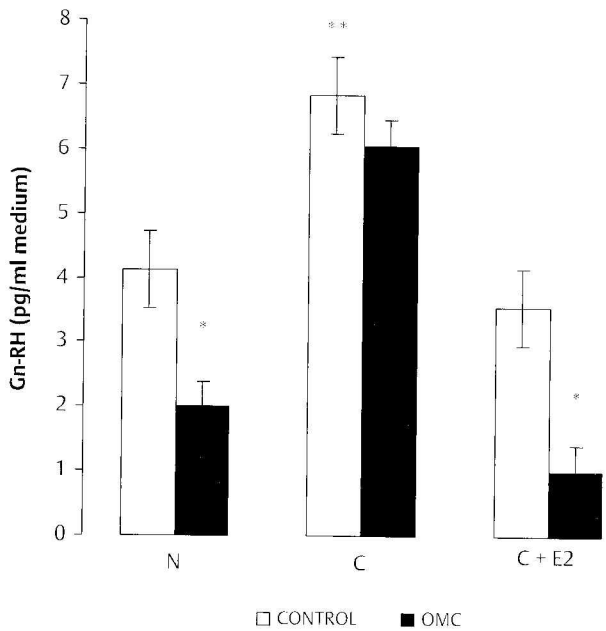

Fig. 3 Effect of OMC on the Gn-RH release by hypothalamic areas in normal (N), castrated (C) and castrated plus estradiol (C + E2) female rats. Each column represents the means SEM of 10 individual determinations. OMC was added at 10–7 M to the incubation media. *p<0.001 vs. control; **p<0.001 castrated control vs. normal control.

Female rats

Fig. 3 shows the effect of OMC on Gn-RH release by the hypothalamic areas in female rats. OMC significantly ($p < 0.001$) decreased the hypothalamic release of Gn-RH in normal diestrous female rats and in ovariectomized rats injected with

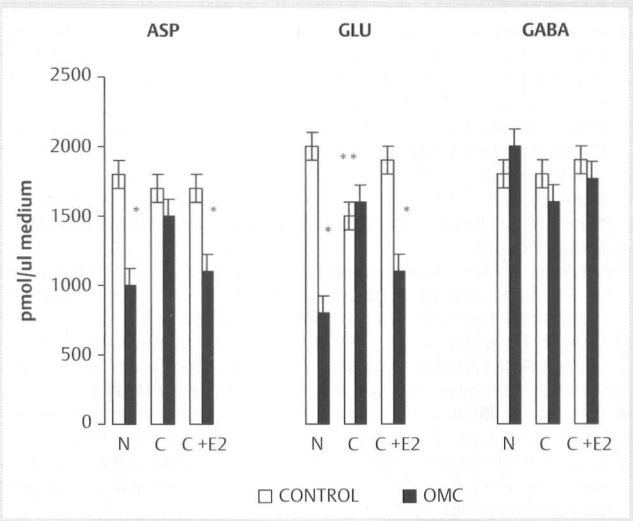

Fig. 4 Effect of OMC on the hypothalamic amino acid neurotransmitters in normal (N), castrated (C) and castrated plus estradiol (C+E2) female rats. Each column represents the means SEM of 10 individual determinations. OMC was added at 10–7 M to the incubation media. *p<0.001 vs. control; **p<0.01 castrated control vs. normal control.

substitutive therapy. An increase in the hypothalamic release of Gn-RH was observed in castrated animals but no changes in the levels of the releasing hormone were produced by OMC.

Fig. 4 shows the in vitro effect of OMC 10^{-7} M on the hypothalamic amino acids neurotransmitters. In normal diestrus female rats, OMC significantly ($p<0.001$) decreased the hypothalamic release of ASP and GLU (the excitatory amino acids involved in NMDA neurotransmission) without changes in GABA levels. No modifications were produced by OMC in castrated female rats injected with the vehicle. On the other hand substitutive therapy administered to ovariectomized animals restored the changes induced by OMC in normal rats, i.e. decrease in the hypothalamic release of ASP and GLU.

Discussion

Certain UV filters used in cosmetics are absorbed by the skin and can be detected in blood and urine a few minutes after its application (Hyden et al., 1997; Janjua et al., 2004; Janjua et al., 2008). They have estrogenic activity and could affect the normal functioning of the gonadal axis (Janjua et al., 2004; Ma et al., 2003; Schlumpf et al., 2002; Klammer et al., 2005). On these bases we explored the possibility that such endocrine disruptors could also affect the hypothalamic reproductive function by a direct action on the hypothalamic synthesis of Gn-RH, (the primary hormone of the hypothalamic-pituitary gonadal axis), as well as on the amino acid neurotransmitters involved in its regulation (Kordon et al., 1994; Moguilevsky et al., 2001; Brann et al., 1992).

It is important to note that little is known about the absorption of the sunscreems through the rat skin. In the abscense of toxicokinetic in vivo data, it is not possible to exactly determine the dose of OMC will pass the blood barrier to reach finally at which concentration hypothalamic neurons. For this reason we use in vitro experiments to study the direct effect of OMC on Gn-RH and neurotransmitters release from hypothalamus of rats.

The results of the present work showed that the addition of OMC 10^{-7} M to the incubation medium of hypothalamic tissue significantly decreased the Gn-RH release in both male and female normal adult rats. These findings are in accordance with the in vitro inhibitory effects of OMC on Gn-RH release from hypothalamus of immature male and female rats, previouslly reported by us (Szwarcfarb et al., 2008). On the other hand, it is a well known that the castration increases Gn-RH release by inhibition of the negative feed-back mechanism. In our experiments the suppressing effects of OMC on Gn-RH release were not observed in cultures of hypothalmic slices derived from castrated control rats. However, the administration of testosterone to male and estradiol to female castrated rats, in addition to decreasing Gn-RH release as compared with castrated control rats, restored the inhibitory effect of OMC on the hypothalamic Gn-RH release. It could be indicated that an adequate endogenous hormonal environment is a relevant point for the inhibitory effect of OMC on the Gn-RH release. The estrogen-like activity of OMC in adittion to the effect of the endogenous or exogenous sexual homones could exert a negative feed-back mechanism on Gn-RH release in normal or castrated rats, respectivelly. On the other hand sexual steroids modulate the mechanism by which the amino acid neurotransmitters regulate their own release and the relaese of Gn-RH at hypothalamic level. (Carbone et al., 1992, Moguilevsky et al., 1995). On this basis OMC could also modulate the effects of sexual hormones on the release of neurotransmitters and modying Gn-RH release from hypothalamus of normal and castrated rats treated with substitutive therapy.

It is a well known fact that the hypothalamic amino acid neurotransmitter system plays a very important role in the regulation of Gn-RH neurons activity and consequently on Gn-RH release from the hypothalamus. (Moguilevsky et al., 2001; Brann et al., 1992; Arias et al., 1993; Moguilevsky et al., 1995). The excitatory amino acids ASP and GLU stimulate the Gn-RH release while GABA has an inhibitory effect in normal male rats (Brann et al., 1992; Moguilevsky et al., 1995; Brann et al., 1992).

Our studies about the effect of OMC on the hypothalamic release of amino acid neurotransmitters in normal male rats showed that this drug has an inhibitory effect on hypothalamic release of GLU and a stimulatory one on the release of GABA. On the other hand, in normal female rats OMC decreases the hypothalamic release of both excitatory amino acids (ASP and GLU) without modifications in the hypothalamic release of GABA. The present results are in accordance with previous one in immature rats, in which we have detected sexual differences in the effect of OMC on hypothalamic amino acids neurotransmitters release (Szwarcfarb et al., 2008). It is very probable that these dissimilar neuroendocrine mechanisms, by which OMC decreases Gn-RH in male and female rats, are connected with the sexual differences in the control of the hypothalamic hormone, previously described in normal rats (Moguilevsky et al., 1991; Moguilevsky et al., 1995).

Similar actions to those produced by OMC in both normal male and female neurotransmitters system rats were founded in castrated animals treated with sexual hormones. No changes in the release of ASP, GLU and GABA were found in castrated animals without substitutive therapy.

It is interesting to note that the decrease in Gn-RH levels produced by OMC was accompanied with changes in the release of amino acid neurotransmitters which were different in both sexes. Assuming that the inhibitory effect of OMC on Gn-RH release is directly connected with the changes observed in the

excitatory and inhibitory amino acid neurotransmitters in normal rats and castrated animals treated with sexual hormones, it is clear that the lack of the effect of OMC on Gn-RH release in castrated rats could be the consequence of the fact that this drug did not modify this neurotransmission system in the absence of an adequate hormonal environment.

Our findings could indicate that OMC is capable to exert its inhibitory effect in the hyppothalmic hormone release by disrupting the normal neuroendocrine mechanisms in a sex dependent manner. It is very probable that these dissimilar mechanisms by which OMC decreases Gn-RH in male and female rats, are connected with the sexual differences in the control of the hypothalamic hormone, previously described in normal rats (Moguilevsky et al., 1991; Moguilevsky et al., 1995). It could be related with a dissimilar sexual pattern in the maturation of the amino acid neutransmitter receptors in the hypothalamus, probably induced by a different exposure to sexual hormones during the pubertal development (Szwarcfarb et al., 1994). In addition, others studies have reported a sexually dimorphic expression of the different subtypes of GABA receptors in rat hypothalamus and a modulatory effect of the androgens in its maturation (Szwarcfarb et al., 1991; Bianchi et al., 2004).

According to the presented results, the presence of sexual hormones is a relevant point for the inhibitory effect of OMC on the hypothalamic amino acid neurotransmitter system and consequently on the Gn-RH release. The experimental evidences have demonstrated that the sexual hormones modulate the activity of amino acid neurotransmitter system (Kanamaru et al., 2001; Lariviere et al., 2005; Weilan, 1992).

On this basis it could be postulated that the lack of sexual hormones modify the sensitivity of this system to the effect of different substances acting through changes in amino acid neurotransmitter activity. It is also demonstrated that the regulatory effects of amino acids neurotransmitter system on Gn-RH neurons is conditioned by the presence of sexual hormones (Arias et al., 1993; Brann et al., 1992; Carbone et al., 1995). In summary, the results of the present paper shows that OMC in vitro induced modifications in the hypothalamic release of the Gn-RH as well as in the amino acid neurotransmitter system regulating the hypothalamic hormone. Apparently the inhibition of the hypothalamic release of Gn-RH induced by OMC in both male and female adult rats could be connected with changes related to gender differences in the hypothalamic control of this hormone by the amino acid neurotransmitter system. In adition a direct modulatory action estrogen-like of OMC on Gn-RH and aminoacidergic neurons could be involved in its effect.

Our results support the need for in vivo testing of OMC after identification of the neuroendocrine activity in vitro. They also suggest that the potential benefits of extensive UV screen use to protect the skin against the adverse effects of exposure to ultraviolet radiation as well the clinical implications of the absorption and bioacumulation of this chemical may have to be reconsidered from a medical and ecological perspective. The design of nonpenetrating sunscreens for cosmetic and pharmaceutical formulationns could be a good clinical strategy to reduce the risk of the exposure to OMC (Touitou et al., 2008).

Conflict of interest: None.

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