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Tibolone Rapidly Attenuates the GABA_B Response in Hypothalamic Neurones

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Abstract

Tibolone is primarily used for the treatment of climacteric symptoms. Tibolone is rapidly converted into three major metabolites: 3 α - and 3 β -hydroxy-tibolone (3 α - and 3 β OH-tibolone), which have oestrogenic effects, and the Δ 4-isomer (Δ 4-tibolone), which has progestogenic and androgenic effects. Since tibolone is effective in treating climacteric symptoms, the effects on the brain may be explained by the oestrogenic activity of tibolone. Previously using whole-cell patch clamp recording, we found that 17 β -oestradiol (E₂) rapidly altered GABA neurotransmission in hypothalamic neurones through a membrane oestrogen receptor (mER). E₂ reduced the potency of the GABA_B receptor agonist baclofen to activate G-protein-coupled, inwardly rectifying K⁺ channels in hypothalamic neurones. Therefore, we hypothesized that tibolone may have some rapid effects through the mER and sought to elucidate the signalling pathway of tibolone's action using selective inhibitors and whole cell recording in ovariectomized female guinea pigs and mice. A sub-population of neurones was identified *post hoc* as proopiomelanocortin (POMC) neurones by immunocytochemical staining. Similar to E₂, we have found that tibolone and its active metabolite 3 β OH-tibolone rapidly reduced the potency of the GABA_B receptor agonist baclofen to activate GIRK channels in POMC neurones. The effects were blocked by the ER antagonist ICI 182,780. Other metabolites of tibolone (3 α OH-tibolone and Δ 4-tibolone) had no effect. Furthermore, tibolone (and 3 β OH-tibolone) was fully efficacious in ER α KO and ER β KO mice to attenuate GABA_B responses. The effects of tibolone were blocked by phospholipase C inhibitor U73122. However, in contrast to E₂, the effects of tibolone were not blocked by protein kinase C inhibitors or protein kinase A inhibitors. It appears that tibolone (and 3 β OH-tibolone) activates phospholipase C leading to PIP₂ metabolism and direct alteration of GIRK channel function. Therefore, tibolone may enhance synaptic efficacy through the G_q signalling pathways of mER in brain circuits that are critical for maintaining homeostatic functions.

Keywords

Tibolone; GIRK channel; GABA_B receptor; oestrogen; potassium channel

Introduction

The hypothalamus controls multiple homeostatic functions that change dramatically during the ovulatory cycle and especially during pregnancy and menopause. Fluctuations in circulating oestrogens are thought to be responsible for these changes (1–4). The drug tibolone (Livial, Organon, Roseland, NJ and Oss, The Netherlands) is used for the management of postmenopausal symptoms: it relieves hot flushes and vaginal dryness, improves mood and libido and prevents osteoporosis (5–8) (Fig. 1). In postmenopausal women, 3 α OH-tibolone is the predominant nonsulfated, free metabolite in blood, followed by 3 β OH-tibolone. Both 3-hydroxymetabolites bind to oestrogen receptors (5,9) and are responsible for the oestrogenic activity. Serum levels of tibolone and Δ 4-tibolone, which bind to progesterone and androgen receptors (9), are low and become undetectable after 4 to 6 h (10). Furthermore, it is reported that oestrogenic 3 α OH-tibolone and 3 β OH-tibolone are predominant metabolites of tibolone in brain, including the hypothalamus (11). This oestrogenic metabolite pattern is in line with the observed oestrogenic effects on the brain, i.e., a reduction in hot flushes as shown in ovariectomized monkeys (12) and postmenopausal women (7).

β -Endorphin (β -end) is the most important biologically active endogenous opioid peptide. Tibolone, like oestrogens (13), is able to increase central and peripheral levels of β -endorphin (14). In addition, circulating levels of β -endorphin are reduced in postmenopausal women (6). In the hypothalamus, POMC neurones through direct synaptic contacts modulate the excitability of hypothalamic neurones that control appetite, fluid balance, temperature, stress and reproduction (15). Also, POMC neurones project to other brain areas (e.g., midbrain) to control motivated behaviors such as sex behavior and food consumption (16).

Until recently, the actions of oestrogen in the hypothalamus and other brain areas have been attributed to the activation of oestrogen receptor α (ER α) or ER β (17–20). However, we have identified a putative membrane-associated oestrogen receptor (mER) that is G_q-coupled to a phospholipase C–protein kinase C–protein kinase A (PLC–PKC–PKA) pathway (21,22). The activation of this pathway by E₂ can rapidly dis-inhibit female POMC neurones through desensitizing μ -opioid and GABA_B receptors. Since tibolone is effective in treating climacteric (female menopause) symptoms, the effects in the brain may be explained by the oestrogenic activity of tibolone. Therefore, we hypothesized that tibolone may have some rapid effects through a mER and sought to elucidate the signalling pathway of tibolone's action using selective inhibitors and whole cell recording in ovariectomized female guinea pigs and oestrogen receptor knock-out (ER α KO and ER β KO) mice.

Materials and methods

Animals and treatments

All animal procedures described in this study are in accordance with institutional guidelines based on National Institutes of Health standards. Male and female Topeka guinea pigs (400–600 gm), bred in our institutional breeding facility, and female multicolour guinea pigs (400–500 gm; Elm Hill Breeding Labs, Chelmsford, MA) were used in these experiments. The guinea pigs were maintained under constant temperature (26 C) and light (on between 6:30 A.M. and 8:30 P.M.). Animals were housed individually, with food and water provided *ad libitum*. They were ovariectomized under ketamine-xylazine anesthesia (33 and 6 mg/kg, respectively, s.c.) 5–7 d before experimentation, and they were given sesame oil vehicle (0.1 ml, s.c.) 24 hr before experimentation. Serum oestrogen concentrations were measured in the ovariectomized females by radioimmunoassay (Oregon National Primate Research Centre Radioimmunoassay Core) from trunk blood collected on the day of experimentation and were <10 pg/ml.

Wild-type C57BL/6 mice in these studies were obtained from The Jackson Laboratory (Bar Harbor, ME). ER α KO mice were generated from breeding pairs heterozygous for the disrupted ER α gene, and offspring were screened by PCR amplification of tail DNA using primers as previously described (23). ER β KO mice were generated from breeding pairs heterozygous for the disrupted ER β gene as previously described (24). Offspring were screened with two sets of primers: The first primer pair (5'ACATCCATACACCCCACTCAACC 3' and 5'AAAGAAACATGTCCTGGCAAATCA 3') produced a 600 bp product for the WT allele and no product for the KO allele. The second primer pair (5'TTCTGAGGGATCCGCTGTAAG 3' and 5'AGGCTGCTGATCTCGTTCT 3') produced a 450 bp product for the KO allele and no product for the WT allele. In addition, hypothalamic slices from WT and KO mice were tested for immunoreactive ER α using the C1355 antibody kindly provided by Dr. Margaret Shupnik. With this antibody we found high concentration of ER α immunoreactive neurones in the hypothalamus from WT, but not in the ER α KO hypothalamus. ER β expression was tested using RT-PCR of hypothalamic RNA with primer pairs designed to flank the neomycin-resistance gene insert in exon 3 of the ER β KO mice (24) (forward primer 769bp-789bp and reverse primer 947bp-968bp; GenBank accession number NM_207707). These primers produced a PCR product in WT, heterozygous and ER α KO mice, but not in ER β KO mice. All animals were maintained under controlled temperature (25 C) and photoperiod conditions (12/12 hr light/dark cycle; lights on between 6:00 A.M. and 6:00 P.M.) with food and water *ad libitum*. Adult mice were ovariectomized under ketamine-xylazine anesthesia (10 mg/kg, each, i.p.) and allowed to recover for 1 week.

Drugs

All drugs were purchased from Calbiochem (La Jolla, CA) unless otherwise specified. Tetrodotoxin (TTX) (Alomone Labs, Jerusalem, Israel) was dissolved in Milli-Q H₂O and further diluted with 0.1% acetic acid (final concentration, 1 mM), pH 4–5. The anti-oestrogen ICI 182,780 (10 mM; Tocris Cookson, Ballwin, MO), was dissolved in 100% ethanol. The phospholipase C inhibitor U73122 (20 mM) and the less active analog U73343 (20 mM) were dissolved in DMSO. The protein kinase C inhibitor bisindolylmaleimide I hydrochloride (BIS) (100 μ M) and the protein kinase A inhibitor Rp-cAMPS (50 mM), were dissolved in H₂O. The following compounds were supplied by NV Organon, Oss, The Netherlands: tibolone (Org OD 14) [(7 α ,17 α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one]; Δ 4-tibolone [(7 α ,17 α)-17-hydroxy-7-methyl-19-norpregn-4-en-20-yn-3-one]; 3 α -hydroxytibolone [(3 α ,7 α ,17 α)-7-methyl-19-norpregn-5(10)-en-20-yne-3,17-diol]; 3 β -hydroxytibolone [(3 β ,7 α ,17 α)-7-methyl-19-norpregn-5(10)-en-20-yne-3,17-diol]. These compounds were dissolved in 100% ethanol. Aliquots of the stock solutions were stored at –20 C until needed.

Electrophysiology

Adult guinea pigs or mice were ovariectomized 6–10 days before each experiment. Each animal was quickly killed by decapitation, the brain rapidly removed from the skull and a block containing the hypothalamus immediately dissected. The hypothalamic block was submerged in cold (4 C) oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (aCSF) containing the following constituents, in mM: NaCl, 124; KCl, 5; NaHCO₃, 26; NaH₂PO₄, 2.6; dextrose, 10; HEPES, 10; MgSO₄, 2; CaCl₂, 2 at 4 °C. Coronal slices (300–350 μ m) through the caudal-rostral extent of the arcuate nucleus were cut with the aid of a vibratome. The slices were transferred to a multiwell auxiliary chamber containing oxygenated aCSF and kept there until electrophysiological recording after ~2 hr. During recording, slices were maintained in a chamber perfused via a peristaltic pump with warmed (35 C) oxygenated aCSF at a rate of 1.5 ml/min. Microelectrodes (resistances 3–6 MO) were fabricated from borosilicate glass pipettes (1.5 mm O.D) and filled with an internal solution (pH 7.30) containing the following constituents, in mM: K-Gluconate, 128; NaCl, 10; MgCl₂, 2; EGTA, 11; HEPES, 10; ATP, 1; GTP, 0.25, and 0.25% biocytin. Standard whole-cell voltage clamp procedures were followed

using an Axopatch 1D amplifier (2 kHz lowpass filter; Molecular Devices, Foster City, CA). Signals were digitized with a Digidata 1322A, and analyzed using pClamp software (version 9.2; Molecular Devices). The liquid junctional potential of -10 mV was corrected in the data analysis. Current and voltage traces were also recorded on an analog chart recorder (Gould Instruments). Following the formation of a >1 G Ω seal, intracellular access was achieved by suction, and only those cells that showed less than 10% change in access resistance throughout the recording were included in this study. All the responses to baclofen were measured in voltage clamp as outward currents ($V_{\text{hold}} = -60$ mV). For the electrophysiology analysis, only cells with gigaohm or better seals were included in this study.

The protocol for drug administration in the whole-cell patch voltage-clamp experiments (V_{hold} , -60 mV) was as described in a previous publication (22). After seals were formed and the whole-cell configuration was obtained, slices were perfused with TTX (1 μ M) for 5 min. The first GABA_B receptor-mediated response was generated by perfusing baclofen (at EC₅₀ concentration of 5 μ M) until a steady-state outward current was obtained (R_1). After drug washout, the current returned to its predrug resting level. The cells were then treated with tibolone or its metabolites and/or other drugs for 15 min, baclofen (5 μ M) was perfused again, and R_2 was measured. The effects of tibolone or other drugs on the baclofen response are expressed as a percentage of R_2 over R_1 .

Immunocytochemistry

Following electrical recording, the slices were prepared for fluorescence immunocytochemistry as described previously (22). Briefly, the slices were fixed with 4% paraformaldehyde in Sorensen's phosphate buffer, pH 7.4, for 120 min, immersed overnight in 20% sucrose dissolved in Sorensen's buffer, and then frozen in OCT embedding medium and prepared for immunocytochemistry as described previously (25). Coronal sections (20 μ m) were cut on a cryostat (model 1720 Digital Cryostat; Leitz, Wetzlar, Germany) and mounted on Fisher SuperFrost Plus slides. Sections were washed for 5 min with 0.1 M sodium phosphate buffer, pH 7.4, and then streptavidin-Cy₂ ($1:7,500$ – $1:10,000$; Jackson ImmunoResearch, West Grove, PA) was applied for 2 hr. The reaction was terminated by washing with buffer. The slices were scanned for the injected neurone with a Nikon (Melville, NY) Eclipse 800 fluorescence microscope. After localization of the biocytin-filled neurones, the slides containing the appropriate sections were processed for the presence of β -endorphin using fluorescence immunohistochemistry as described previously (26). The sections with the biocytin-identified neurones were first incubated overnight with a polyclonal β -endorphin antibody (kindly provided by Dr. Robert Eskay, NIH) at $1:5000$ and washed in 0.1 M phosphate buffer, followed by incubation with a donkey anti-rabbit IgG-Cy₃ at $1:500$ (Jackson ImmunoResearch). Then, the sections were washed with sodium phosphate buffer and coverslips were applied using a glycerolglycine buffer ($2:1$; pH 8.6) containing 5% N-propylgallate (Sigma-Aldrich) to reduce photobleaching. Immunostained cells were analyzed and photographed using a Nikon E800 microscope equipped with a digital Nikon DS camera.

Statistical analyses

Comparisons between groups were performed using a one-way ANOVA with *post hoc* Newman-Keuls paired analysis. Differences were considered statistically significant if the probability of error was $<5\%$.

Results

Whole-cell recordings were made in arcuate neurones ($n = 109$) from OVXed female guinea pigs. A subgroup of these neurones ($n = 28$) was identified using dual-labeling immunocytochemistry (Fig. 2), and 40% of the neurones were β -endorphin-positive (i.e.,

POMC neurones). For the electrophysiology analysis, only cells with gigaohm or better seals were included in this study. The mean resting membrane potential (RMP) for female guinea pig neurones was -55.0 ± 0.8 mV at a 0 pA holding current and the mean input resistance was 1.1 ± 0.15 G Ω ($n = 79$). For the C57BL/6 background mice, the RMP was -54.3 ± 1.4 mV ($n = 12$) for wild-type; -54.4 ± 2.2 mV ($n = 16$) for ER α KO; -54.5 ± 2.3 mV ($n = 13$) for ER β KO mice. The mean input resistance for the mouse arcuate neurones was 0.93 ± 0.11 G Ω ($n = 38$). Only cells that gave a reversible baclofen response of at least 10 pA were included in the study.

Tibolone and 3 β OH-tibolone, but not 3 α OH-tibolone and Δ 4-tibolone, rapidly attenuated the baclofen response

We used the whole-cell recording method to measure the effects of tibolone and its metabolites on the activation of the GIRK conductance by the GABA $_B$ receptor agonist baclofen. For assessing the modulation of the GABA $_B$ response by tibolone and its metabolites, we used an EC $_{50}$ concentration (5 μ M) of baclofen and the protocol described previously (22). A robust outward current was measured in response to baclofen that subsided after washout of the GABA $_B$ agonist (Fig. 2a). The application of baclofen 20 min later elicited the same robust response, suggesting that desensitization and rundown did not occur in response to successive applications of 5 μ M baclofen. However, if tibolone (1 μ M) was applied during the interim period (i.e., after the washout of the first application of baclofen), there was a significant ($p < 0.01$) decrease of 30% in the response to a second application of baclofen (Fig. 2a and 2b). Tibolone and its metabolites alone had no effect on the holding potential. Current-voltage relationships generated before and during the application of tibolone and its metabolites showed that they did not change the reversal potential for the baclofen-mediated response: control E_{baclofen} , -91.0 ± 3.0 mV ($n = 7$) versus after tibolone and its metabolites, E_{baclofen} , -90.0 ± 3.8 mV ($n = 7$). In addition, 3 β OH-tibolone (1 μ M) also significantly inhibited the baclofen response. However, 3 α OH-tibolone (1 μ M) and Δ 4-tibolone (1 μ M) were inactive ($p > 0.05$ vs control). Furthermore, the inhibitory effects of tibolone and 3 β OH-tibolone on the baclofen response were blocked by ICI 182,780 (10 μ M) (Fig. 2a and 2b), indicating these drug actions were oestrogenic. Treatment with ICI 182,780 alone had no effect on the baclofen response ($93.3 \pm 5.3\%$, $n = 5$).

Tibolone attenuation of the GABA $_B$ response depends on activation of PLC, but not on activation of PKC and PKA

Recent studies have shown that the mER in arcuate neurones is coupled to a G α_q protein and activates PLC (22,27). Therefore, we next examined the involvement of specific signalling proteins in the tibolone-mediated modulation. Because mER receptors in arcuate neurones are G α_q protein-coupled and PLC is in the downstream pathway, we first examined whether activation of PKC is critical for tibolone modulation of the GABA $_B$ response using a PKC inhibitor. If activation of the PKC pathway is involved, then the effect of tibolone on GABA $_B$ responses should be blocked by inhibiting PKC. We have established that the PKC inhibitor BIS (100 nM) and the PKA inhibitor Rp-cAMPS block the inhibition of baclofen responses by E_2 (22). To test this, we applied BIS, which is a selective inhibitor of PKC that does not distinguish between the conventional, novel, and atypical isoforms of PKC. But as shown in Fig. 3, after 15 min of dialysis with BIS (100 nM) and Rp-cAMPS, the tibolone-induced reduction of the GABA $_B$ response was not attenuated.

In light of the above results for a primary role for G α_q in tibolone-mediated inhibition, we tested whether the activation of PLC might also play a role. Because the specific PKC inhibitor BIS did not block the effects of tibolone, this indicated that PLC, which is downstream from the activation of G α_q protein and a well known G α_q effector, may be responsible for the tibolone effect. Therefore, we focused on pathways downstream of PLC to further elucidate the

tibolone-mediated signalling pathway. To determine whether the activation of PLC is required for the tibolone-induced inhibition of the GABA_B response, neurones were treated with the broad-spectrum PLC inhibitor U73122 (10 μ M), which was perfused in the extracellular bathing media. Under these conditions, there was no difference between the first baclofen response and second response in the presence of U73122 (U73122-treated versus control group, 95.6 ± 2.4 %, $n = 3$ and 96.5 ± 5.9 %, $n = 5$), but the tibolone-mediated reduction of the GABA_B response was blocked (Fig. 3b), whereas the less active PLC inhibitor, U73343, at the same concentration had no effect (data not shown). Therefore, it appears that tibolone (and 3 β OH-tibolone) activate PLC leading to PIP₂ metabolism and direct alteration of GIRK channel function. Furthermore, these results indicate that the suppression of the GABA_B response by tibolone requires PLC activation but not the activation of PKC and PKA. Although the effects of tibolone were blocked by the PLC inhibitor U73122, the effects of tibolone, in contrast to E₂, were not blocked by inhibitors of PKC and PKA.

Tibolone and 3 β OH-tibolone rapidly attenuated the baclofen response in wild type and ER α and ER β knock-out mice

As described previously (28), ER α is abundantly expressed in guinea pig arcuate neurones. Also, ER β mRNA and protein are expressed in the rodent arcuate nucleus but to a much lesser extent than ER α (29–31). Therefore, to verify that neither ER α nor ER β are involved in the rapid desensitization of the GABA_B response by tibolone, we compared the effects of tibolone on the GABA_B response in arcuate neurones including POMC neurones from ER α knock-out, ER β knock-out, and wild-type (C57BL/6) mice (Fig. 4). As observed in ovariectomized female guinea pigs, we found that tibolone significantly attenuated the GABA_B response in arcuate neurones from ovariectomized ER α KO and ER β KO female mice. Moreover, the efficacy of the response was equivalent in wild-type (C57BL/6) and KO mice, indicating that the effects of tibolone are mediated by a membrane ER (mER) distinct from ER α or ER β (Fig. 4).

Discussion

In the present study, we have characterised the signalling pathway of tibolone in hypothalamic (POMC) arcuate neurones. It appears that tibolone (and 3 β OH-tibolone) activates PLC but not PKA or PKC, which leads to PIP₂ metabolism and direct alteration of GIRK channel function, thereby desensitizing the GABA_B receptor mediated response.

The membrane oestrogen receptor mediates the rapid effects of tibolone

Tibolone has been used in postmenopausal women as an alternative to oestrogen-based hormone therapy (HT). Tibolone is a synthetic steroid with oestrogenic, androgenic, and progestagenic properties. In humans, tibolone is quickly metabolized after administration into 3 α OH-tibolone and 3 β OH-tibolone, which solely bind the oestrogen receptor (ER), and into a Δ -4-isomer with affinity for the progesterone receptor and androgen receptor but not for ER. The two hydroxytibolones are the main metabolites in human circulation (32) and clear oestrogenic-like effects are seen on the brain, vagina and bone, but no stimulation of the breast and the endometrium is seen (33). In the brain the predominant metabolites of tibolone are the 3-hydroxytibolones and these are thought to be responsible for the observed reduction of hot flushes and sweats (8,11,12,34–38). Indeed, we found that tibolone and 3 β OH-tibolone were effective to attenuate the GABA_B response. Therefore, we hypothesize that like testosterone which is aromatized to oestrogen in the slice (39), tibolone is converted to its active metabolite 3 β OH-tibolone within minutes in the slice, but this needs to be addressed in the future experiments.

For the first time we have identified that tibolone targets a mER, which activates a membrane-delimited pathway in arcuate (POMC) neurones, although we found that other arcuate neurones

also express this mER signalling pathway (22). The mER pathway is involved in heterologous desensitization of the μ -opioid (autoreceptor) and GABA_B receptors in arcuate (POMC) neurones and therefore would play a critical role in dictating the level of excitability of these neurones. Although we have focused on the postsynaptic actions of tibolone and its metabolites, the excitability of POMC neurons can be augmented by an increase in EPSCs or a decrease in IPSCs. Indeed, recently it has been reported that E₂ can increase the mEPSC frequency in POMC neurons (40,41), and it would be interesting to investigate the presynaptic effects of tibolone (and metabolites) in future experiments.

Similar to E₂, we have found that tibolone and its active metabolite 3 β OH-tibolone rapidly reduced the potency of the GABA_B receptor agonist baclofen to activate GIRK channels in POMC neurones. The effects were blocked by the ER antagonist ICI 182,780 indicating an "oestrogenic" action of tibolone and 3 β OH-tibolone in POMC neurones. Other metabolites of tibolone (3 α OH-tibolone and Δ 4-tibolone) had no effect. Interestingly, the 3 β OH-metabolite is active but the 3 α OH-tibolone is not, indicating that 3 β OH-tibolone has some specificity and the mER is distinct from intracellular oestrogen receptor. Moreover, tibolone is fully efficacious in ER α KO mice and ER β KO mice to attenuate GABA_B responses. The fact that tibolone is fully efficacious in activating this signalling pathway in oestrogen receptor knock-out mice is further evidence for the existence of a novel mER that is involved in critical physiological processes. Therefore, it appears that tibolone can alter the excitability of hypothalamic neurones that are involved in regulating numerous homeostatic functions including reproduction, stress responses, feeding and motivated behaviors.

G α_q mediates the inhibition of the GABA_B response by tibolone through PLC

Our data show that the G α_q /PLC-coupled pathway of mER by tibolone causes an inhibition of the baclofen response, and we provide evidence that depletion of PIP₂, rather than the phosphorylation of the channel, is a key step in this pathway based on the following: First, PKC inhibitors did not block the inhibition of the baclofen response by tibolone. Second, the tibolone-mediated reduction of the GABA_B response was significantly attenuated by the PLC inhibitor U73122 compared to cells perfused with the less active inhibitor U73343. Thus, we conclude that the tibolone-mediated inhibition of GIRK channels involves PLC activation by G α subunits of the G α_q family and that the tibolone-mediated hydrolysis of plasma membrane PIP₂ is the critical mediator (Fig. 5).

It is interesting that GABA_B, μ -opioid, and 5-HT_{1A} receptors are all expressed in POMC neurones (22,42,43) and present findings). All of these receptors are G $\alpha_{i/o}$ -coupled to activation of GIRK channels, which uniformly inhibit POMC neuronal activity. It has been reported that the inhibition of GIRK channels in neurons by many GPCRs is mediated by PIP₂ depletion through G α_q -PLC activation and that PIP₂-channel interactions is the final common mechanism responsible for the inhibition(44). Similar to our findings with the tibolone-mediated desensitization of the GABA_B response in POMC neurones, activation of 5-HT_{2C} receptors can desensitize GABA_B receptors and increase the excitability of POMC neurones through this mechanism (39). Therefore, the PLC-mediated PIP₂ depletion maybe a common signalling pathway for G α_q -coupled receptors in hypothalamic neurones.

It is noteworthy that the downstream signalling pathway of baclofen inhibition by tibolone in arcuate (POMC) neurones is different from oestrogen, which desensitizes the GABA_B response via a G α_q -PLC-PKC δ -PKA pathway (22). These differences may be due to the differential conformations of the receptors which dictate different ligand-selectivity and different signalling. Therefore, mER's may be preassembled into different complexes with different downstream effectors that converge on GIRK channels. It has been reported that the GnRH receptor type I, assumes different conformations and has different selectivity for GnRH analogs and intracellular signalling proteins. This ligand-induced selective-signalling recruits certain

pathways while by-passing others (45). We hypothesize that the mER can also recruit different signalling molecules depending on the ligand.

Functional significance of rapid membrane effects of tibolone in CNS neurones

Previous studies have shown that tibolone is able to reduce hot flushes in cynomolgous monkeys similarly to ethinyl oestradiol (12). Treatment of postmenopausal women with oestrogens (10,46–48) or tibolone (7,34) also reduces the number and severity of hot flushes and sweats. It appears that the oestrogenic activity of tibolone is mainly responsible for the reduction of the severity and frequency of the hot flushes and sweats. It is hypothesized that the effects of hormone therapy on vasomotor and subjective psychobehavioral symptoms may be mediated by its action on an opioidergic pathway (6). β -endorphin is the most important and biologically active endogenous opioid peptide that exerts behavioral, analgesic, thermoregulatory and neuroendocrine actions (49,50). Oestrogens are able to increase central and peripheral levels of β -endorphin in ovariectomized female rats (13) and increase the number of neurones expressing β -end in the arcuate nucleus in female guinea pigs (51). In postmenopausal women, transdermal oestradiol induces a significant increase in plasma β -endorphin levels to premenopausal values and restores the β -endorphin response to naloxone and clonidine challenges, which are impaired in postmenopausal women (47). Similarly, tibolone has been shown to increase central and peripheral levels of β -endorphin in rodents (14). Therefore, the activation of POMC neurones by tibolone is congruent with the significant role of POMC peptides in homeostatic functions.

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Figure 1.
Chemical structures of tibolone, its metabolites and 17 β -oestradiol

Figure 2. Tibolone and 3βOH-tibolone, but not 3αOH-tibolone and Δ4-tibolone, rapidly attenuated the baclofen response in hypothalamic arcuate (POMC) neurones in ovariectomized guinea pigs a. Representative traces of the baclofen response in the presence of vehicle, tibolone (1 μM), 3αOH-tibolone (1 μM), 3βOH-tibolone (1 μM), Δ4-tibolone (1 μM) and the oestrogen receptor antagonist ICI 182,780 (10 μM) in arcuate neurones are shown. b. Bar graphs summarizing the effects of tibolone, 3αOH-, 3βOH- and Δ4-tibolone on the baclofen response in the presence of ICI 182,780 (ICI) or vehicle. Tibolone (1 μM) and 3βOH-tibolone (1 μM) rapidly attenuated the outward (GIRK) current induced by the GABA_B receptor agonist baclofen by 30 %. The inhibitory effects of tibolone and 3βOH-tibolone on the baclofen response were blocked by ICI 182,780 (10 μM). Bars represent the mean ± SEM of 4–12 cells tested per group. *p < 0.05, **p < 0.01, versus vehicle control group. c. Double labeling of POMC neurones that responded to tibolone from ovariectomized guinea pigs. Arcuate neurones were filled with biocytin during the whole-cell recording. Left panel, Biocytin-streptavidin-Cy₂ labeling of a small fusiform arcuate neurone. Scale bar, 20 μm. Right panel, Immunocytochemical staining of β-endorphin in the same neurone (arrow). Eleven of the twenty-eight responsive neurones (40 %) were identified as β-endorphin.

Figure 3. Tibolone attenuation of the GABA_B response depends on activation of PLC, but not on activation of PKC and PKA in hypothalamic arcuate (POMC) neurones

a. Two representative traces of the baclofen response in the presence of tibolone (1 μ M) and BIS or U73122 in arcuate neurones. Cells were dialyzed for 15 min before the first baclofen application with or without Rp-cAMPS (200 μ M) or BIS (100 nM), which were included in the patch pipette solution. b. Bar graphs summarizing the effects of protein kinase A inhibitor Rp-cAMPS, the broad PKC inhibitor BIS and PLC inhibitor U73122. U73122 (10 μ M) blocked the attenuation of the baclofen response by tibolone, however, BIS and Rp-cAMPS did not block the attenuation. Bars represent the mean $\pm$ S.E.M. of 4–12 cells tested per group. * p < 0.05, ** p < 0.01 versus vehicle control.

Figure 4. Tibolone and 3βOH-tibolone rapidly attenuated the baclofen response in wild type and ERα and ERβ knock-out mice

a. Two representative traces of the baclofen response in the presence of 3βOH-tibolone (1 μM) in arcuate neurones from ERαKO (upper) and ERβKO (below) mice are shown. b. Bar graphs summarizing the effects of tibolone and 3βOH-tibolone in arcuate (POMC) neurones from ovariectomized, wild type, ERαKO and ERβKO mice. tibolone (1 μM) and 3βOH-tibolone (1 μM) rapidly attenuated the outward (GIRK) current induced by the GABA_B receptor agonist baclofen in arcuate (POMC) neurones from ovariectomized wild type (C57BL/6), ERαKO and ERβKO mice. Bars represent the mean ± S.E.M. of 3–12 cells. **p<0.01, tibolone treated wild-type group versus vehicle wild-type control; *p<0.05, **p<0.01, tibolone or 3β-tibolone treated ERαKO or ERβKO groups versus vehicle ERKO control. The data for the ERαKO and ERβKO control responses were combined in one bar graph (ERKO).

Figure 5. A cellular model of the rapid signalling of oestrogen and tibolone in hypothalamic neurones

Schematic overview showing the membrane oestrogen receptor (mER) -mediated modulation of neurotransmitter regulated, G protein-coupled receptors in arcuate (POMC) neurones. Black colours are used to summarize the results of the current experiments. The cellular cascade activated by oestrogen (see Qiu et al., 2003) is shown in grey. Oestrogen activates a membrane-associated ER that is $G_{\alpha_{q/11}}$ -coupled to activation of phospholipase C that catalyzes the hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG). Calcium is released from intracellular stores (endoplasmic reticulum) by IP_3 , and DAG activates protein kinase C δ (PKC δ). Through phosphorylation, adenylyl cyclase (ACVII) activity is upregulated by PKC δ . The generation of cAMP activates PKA, which can rapidly uncouple $GABA_B$ and μ -opioid (μ) receptors from their effector system through phosphorylation of a downstream effector molecule (e.g. the inwardly rectifying K^+ channel, or GIRK). Tibolone (or 3β OH-tibolone) binds to mER and activates a different signalling pathway that involves PLC stimulation by $G\alpha$ subunits of the $G_{\alpha_{q/11}}$ family and receptor-mediated hydrolysis of plasma membrane PIP_2 (PIP_2 depletion) that leads to inhibition of GIRK activity. Therefore, tibolone (or 3β OH-tibolone) through the mER intracellular signalling pathway dis-inhibit arcuate (POMC) neurones regulating the homeostasis.