ELSEVIER

Neurotrophic estrogens: essential profile and endpoints for drug discovery☆

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Criteria for the early recognition of selective neurotrophic action are crucial for the discovery of estrogens for supplementation therapy. The comparative characterization of 'tool' compounds in different paradigms demonstrates that estrogen-mediated CNS effects are discernible before the manifestation of changes in primary target organs. Agonist activity at, and recruitment of the coactivator SRC-1 by, the estrogen receptor α accurately reflect peripheral, but not neurotrophic, efficacy. Interaction with, and SRC-1 recruitment at, the estrogen receptor β appears to be an essential prerequisite for pronounced CNS effects. Monitoring of the hypothalamo-pituitary–adrenal axis activity and the differential organ-specific induction of estrogen-responsive proteins are helpful for early delineation of CNS efficacy. Behavioral and antioxidant efficacy are useful confirmatory readouts, with limited roles in lead selection. Finally, an algorithm for the identification of estrogens with a neurotrophic profile can be generated by assigning 'performance grades' in a multifarious test array.

Discovery of estrogens with selective neurotrophic action remains a major medical need for the management of menopausal symptoms. The continuing scientific debate elicited by the results of the Women's Health Initiative (WHI) Study [1–5] underlines the importance of the problem, and the majority of experimental [6,7] and clinical data [8] support the view that estrogens remain the remedy of choice for the treatment of neurological and mental symptoms associated with the cessation of endogenous ovarian secretions. Systemic (i.e. extracerebral) action in target organs, such as the endometrium, mammary gland, and liver are the principal issues of concern associated with the therapeutic use of estrogens. Thus, even when the therapeutic goal is restricted to alleviation of transient complaints emerging from estrogen deprivation, cautious selection of dosage and duration of estrogen administration

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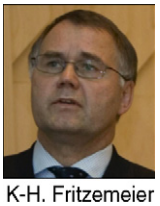

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☆ Brain-selective estrogens can be identified in the early phase of drug discovery, if looking for endpoints outside of the beaten track

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is recommended [9]. It is, however, pertinent to emphasize that the multiplicity of therapeutic goals set by current estrogen supplementation schemes (e.g. aspirations for simultaneous long-term benefits on a variety of targets, such as vasomotor regulation, vaginal lubrication, bone mineral density, mood, cognition, among others) has precluded the definition of the minimal necessary estrogen-like efficacy that satisfies the requirements of safe management of symptoms of estrogen deficiency in the central nervous system (CNS).

The issue of supplementation of estrogen deficiency and the use of estrogens in the prevention and treatment of CNS conditions in men has been sporadically addressed in the literature [10,11]. However, evidence for lower abundance of estrogen receptors in several areas of the male brain [12–14] that result from differential sex steroid exposure during ontogeny and, possibly, require higher estrogen dosage for obtaining pharmacological effects raise safety reservations (e.g. gynecomastia, gonadal suppression, prostate side effects) and difficulties as to the justification of a rationale for clinical application.

Early recognition of compounds with promising profiles in the core indication remains a crucial issue for the discovery process. Because the latter comprises a well-defined cascade of activities, the definition of reliable sorting criteria at each step will facilitate the selection of chemical entities that may ascend to the next (usually more resource-demanding) phase of examination. Currently, such 'filtering' criteria for estrogenic compounds are only vaguely defined. With dissociation between CNS-specific and systemic action being an established requirement, decisions are usually made on the basis of *in vivo* data suggestive of sufficient 'protrusion' of behavioral and/or neuroprotective effects, together with signs of attenuated efficacy in the reproductive system. Thus, the supporting or contradicting evidence is gathered in time-consuming and resource-consuming experimental designs *in vivo* that are positioned in the advanced phase of the discovery process. This statement may also apply to the circuits and mechanisms that mediate estrogen actions in the brain: their comprehensive description during the past decades has generated a host of 'all-or-none' research models, while barely addressing the issues of differential sensitivity and recognition of subtle effects. Here, we undertake an attempt to fill this gap and report on our experience with various experimental approaches for the discovery and pharmacological characterization of estrogens with dissociated efficacies (i.e. compounds that display significant neurotrophic action *in vivo* before the manifestation of typical organometric changes in primary estrogen targets). Our rationale was based on the following contemplations:

(1) Estrogen effects in the CNS represent the amalgamated outcome of actions through multiple molecular modalities (e.g. receptors with diverse structural and subcellular localization [15–17], differential signaling pathways [18] and signal amplifiers [19], interference with reactive oxygen species [7], to name just a few). Accordingly, the process of discovery and characterization should encompass experimental paradigms that scrutinize several of these aspects of estrogen action.

(2) Because neurotrophic effects of estrogens can precede the manifestation of their systemic actions *in vivo*, it is essential to select readouts of adequate sensitivity that are capable of discerning such outcomes. At best, such surrogate endpoints should be present concomitantly in the brain and peripheral estrogen target organs. In view of the intricacy and diversity of mechanisms that may account for the neurotrophic effects of estrogens, the test array should include endpoints that project onto outcomes of general importance (e.g. adaptation, resistance to adverse challenges, etc.)

(3) The reliability of endpoints that are supposed to characterize the CNS efficacy of estrogens should be verified in a comparative examination of changes induced by comparable doses of tool compounds with similar chemical structure.

We tested these assumptions using three chemically related steroids (Figure 1) as pharmacological tools, 17β-estradiol, 17α-estradiol, and 14α, 15α-methylenestra-1,3,5,(10),8-tetraen-3, 17α-diol (ZK 235244; also referred to as J 861 in earlier publications [20]), each of which has been previously reported to display significant estrogenic effects in the CNS [21–24]. The results led to the formulation of a list of criteria for discerning selective neurotrophic efficacy of estrogens, based on: (i) affinity for and agonist transcriptional activity upon the activation of individual estrogen receptor (ER) isoforms; (ii) coactivator recruitment upon ER binding; (iii) stimulation of the hypothalamo-pituitary–adrenal (HPA) axis activity in the female rat; (iv) differential induction of estrogen-regulated proteins in the brain and peripheral organs of the same individual; (v) protective efficacy against reactive oxygen species (ROS) and neurotoxins; and (vi) behavioral effects suggestive of ameliorated emotional responsiveness and learning performance. The data obtained in these studies prompted us to

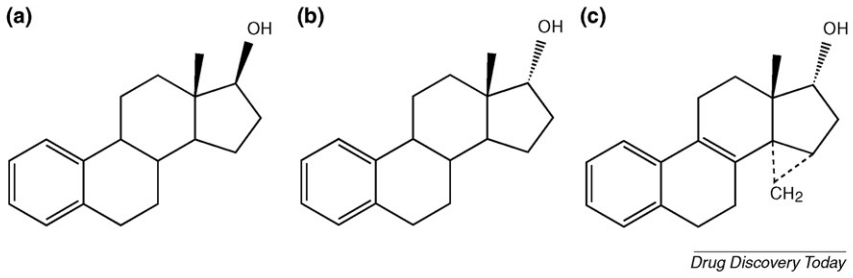

FIGURE 1

Structural formulae of the tool compounds 17β-estradiol **(a)**, 17α-estradiol **(b)**, and 14α, 15α-methylenestra-1,3,5,(10),8-tetraen-3, 17α-diol [ZK 235244 **(c)**].

TABLE 1

Relative binding affinity (RBA) for individual ER isoforms in cytosol fractions and EC_{50} of transcriptional activation of reporter constructs under the control of ERα and ERβ

Compound	ERα		ERβ	
	RBA (%)	EC_{50} [M]	RBA (%)	EC_{50} [M]
17β-Estradiol	100	1.10^{-11}	100	4.10^{-11}
17α-Estradiol	21	3.10^{-10}	23	2.10^{-8}
ZK 235244	32	2.10^{-9}	26	6.10^{-9}

Affinity for the ER was determined in competitive binding assays using [^{3}H]-labeled estradiol [61] in cytosol isolated from rabbit uterus (ERα) or rat prostate (ERβ). Transactivation assays were carried out in U2-OS cells, transiently transfected with expression vectors encoding the two isoforms of the human ER and an estrogen response element containing luciferase reporter [62].

propose an algorithm for the prediction of the neurotrophic efficacy of estrogens, while also demonstrating that the individual components of the test array differ in their capacity to discern CNS-specific actions.

Molecular interactions and their predictive power

Examination of the binding of the tool compounds to the ER isoforms α and β revealed that the affinity of 17α-Estradiol and its derivative ZK 235244 was substantially lower than that of the major natural estrogen 17β-Estradiol. Neither ZK 235244 nor its parent steroid 17α-Estradiol (17α-E_2) seemed to display binding preference for a defined ER subtype (Table 1). Specific binding to other steroid receptor classes was negligible and did not exceed 1% of those of the corresponding reference compounds (data not shown).

If predictions of the compounds' pharmacological efficacies were based on their ER-binding affinity alone, the effects of 17α-E_2 and ZK 235244 would be expected to be very similar, while significantly weaker than those of 17β-E_2. As shown below, however, this assumption appears to hold true for the systemic but not CNS effects of the test compounds.

First clues for discrimination emerge from the comparison of the transcriptional efficacy of the three steroids. All substances induced transcription of ER-driven reporter genes in a dose-dependent fashion and, in accordance with the binding affinity, the dose–response curves of ZK 235244 and 17α-E_2 indicated that the transactivating potency of these compounds was at least one order of magnitude lower when compared with 17β-E_2. It should be noted, however, that, as summarized in Table 1, 17α-E_2 displayed differential pronounced 'preference' for ERα-mediated transcriptional action.

This trend of differential transcriptional efficacy was corroborated in the analysis of the recruitment of SRC-1, a major amplifier of nuclear receptor signaling, by the compound-activated ligand-binding domains of the two ER isoforms. The order of potency of SRC-1 recruitment following interaction with the ERα (as defined by the EC_{50}) largely reflects the relationship seen in binding affinity and transactivation: 17β-E_2 > 17α-E_2 ≈ ZK 235244, with the compounds displaying similar efficacy of SRC-1 recruitment (Figure 2a). In the paradigm using the LBD of the ERβ as 'bait', the differences in SRC-1 recruitment potency become pronounced (17β-E_2 > ZK 235244 > 17α-E_2), while the efficacy of coactivator

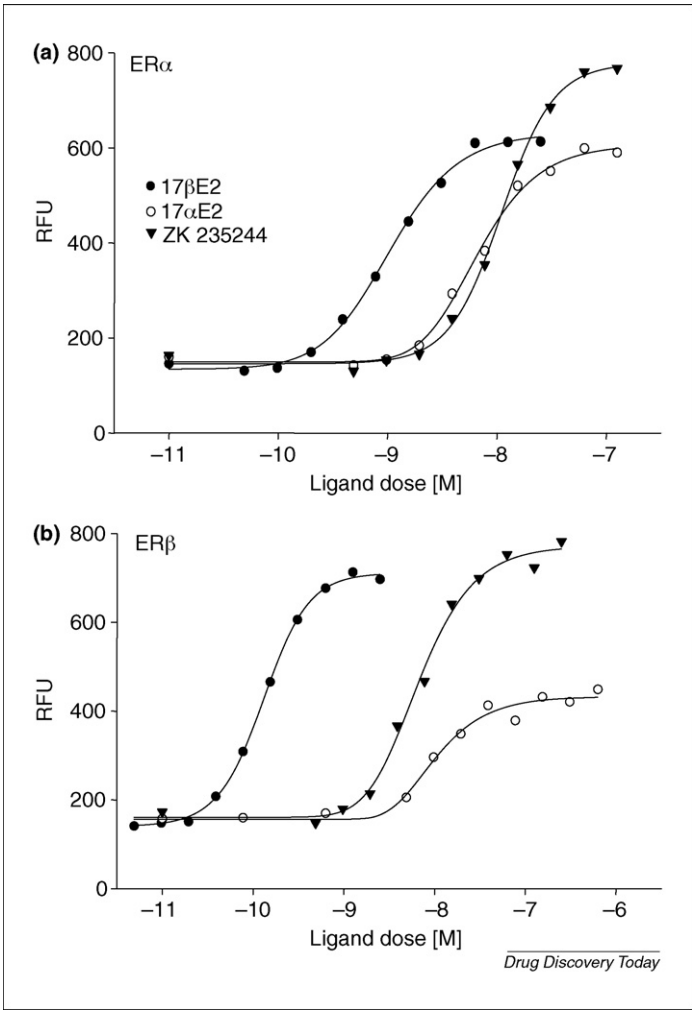

FIGURE 2

Dose–response curves of SRC-1 recruitment by the ERα (panel **a**) and ERβ (panel **b**) upon activation by the test compounds in a yeast two-hybrid assay. The ER ligand-binding domains (NP_000116, aa249-595, and NP_001428, aa211-530, respectively) and the SRC-1 fragment encoding the nuclear receptor-interacting domain (NP_003734, aa381-1441) were expressed in the haploid yeast strains AH109 and Y187. Combinations of the constructs were generated in diploid cells by mating of the respective strains. The protein interaction was quantified by fluorimetry [63]. Data depict the average values of two independent assays run in quadruplicates. The results were also confirmed in two independent experiments using full-length ER proteins (data not shown).

recruitment in the presence of 17α-E_2 barely reached 50% of that measured with 17β-E_2 and ZK 235244 (Figure 2b), In summary, the examination of compounds' interactions with the molecular target revealed that profile divergence may be based on differential transcriptional activity at individual ER isoforms and, especially, coactivator-mediated signal amplification, rather than on mere differences in the binding affinity. Although possible cell-context-dependent variability in transcriptional responses urges caution with general conclusions, the ability to mobilize sufficiently the transcriptional activity of both ER isoforms appears, as exemplified below by the compounds 17α-E_2 and ZK 235244, to be a prerequisite for the manifestation of broad-spectrum neurotrophic activity.

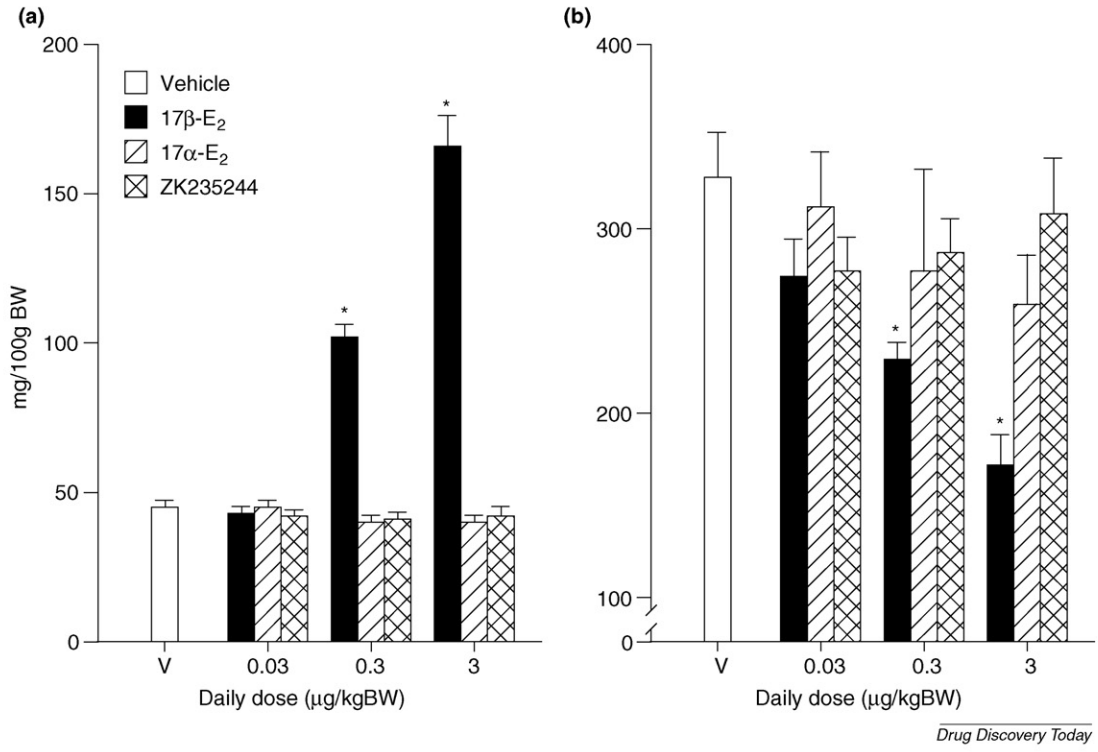

FIGURE 3

Systemic estrogen-like effects of test compounds as shown by changes in uterus (panel **a**) and thymus mass (panel **b**) in ovariectomized female rats. Continuous drug delivery over seven days was accomplished by subcutaneously implanted osmotic mini-pumps. Data are given as mean ± S.E.M.; each treatment group consists of five individuals; asterisks indicate significant differences as compared with vehicle-treated (V) animals.

The first *in vivo* application: extracting maximum information from a simple paradigm

The administration of a putative estrogen to gonadectomized female animals is a well-established paradigm for the characterization of its effects in primary target organs, such as the uterus and hypothalamo-pituitary unit; in most cases, uterine growth and suppression of (castration-induced) gonadotropin secretions fairly reflect the compound's estrogen-like potency and efficacy. Auxiliary, albeit less specific, sources of information are changes in non-reproductive organs and systems with high, but not exclusive, estrogen responsiveness (e.g. thymus and liver). Such data are usually reasonable descriptors of systemic estrogen actions, but barely usable for the characterization of CNS-specific effects.

When examined in this paradigm, the systemic estrogen effects (e.g. uterotropic and thymolytic efficacy) of the tool compounds applied at daily doses of 0.03, 0.3 and 3 μg/kg correlated with their affinity for the ER (Figure 3). Post-gonadectomy-enhanced serum LH levels were suppressed by 17β-E_2 at the highest dose tested, whereas ZK 235244 and its parent compound 17α-E_2 were ineffective on this parameter (data not shown).

If confined to these outcomes, this experiment only confirmed the expectations that the systemic estrogen-like potency of 17α-E_2 and its derivative ZK 235244 is substantially lower than that of 17β-E_2, while not revealing much about possible differences in their neurotrophic efficacy. However, such experimental settings (compound administration at different doses over several days) permit the performance of secondary tests that may well yield valuable information concerning CNS-specific activity of the compounds of interest. In rats, one suggested subarray is the examination of different aspects of the HPA axis function (e.g. basal and stress-induced secretory output, its responsiveness to exogenous glucocorticoids, and changes in the transcription of genes involved in the central regulation of the HPA axis).

Chronic administration of 17β-E_2 dose-dependently increased serum corticosterone (CORT) levels under quiescent conditions at both zenith and nadir of circadian activity. A similar trend was seen in rats receiving ZK 235244; however, only changes induced by the highest dose proved significant. By contrast, 17α-E_2 failed to influence basal CORT secretion at either time point (Figure 4a and c).

Administration of 17β-E_2 at all doses significantly augmented the amplitude of CORT increase measured 30 min after acute emotional stress. The compound ZK 235244 showed this effect only at the highest dose tested, and 17α-E_2 did not alter the response magnitude as compared with vehicle treatment (Figure 4b). Two hours after stress exposure a trend toward reinstatement of basal CORT levels was seen in all treatment groups; however, as under basal conditions, CORT concentrations remained higher in rats receiving 17β-E_2 (data not shown).

ZK 235244 and 17β-E_2 attenuated the capacity of dexamethasone to suppress the nocturnal increase in CORT secretion, whereas 17α-E_2 did not influence the responsiveness to dexamethasone at any dose tested (Figure 4d). These findings correlate with treatment-induced changes in several endpoints indicative of

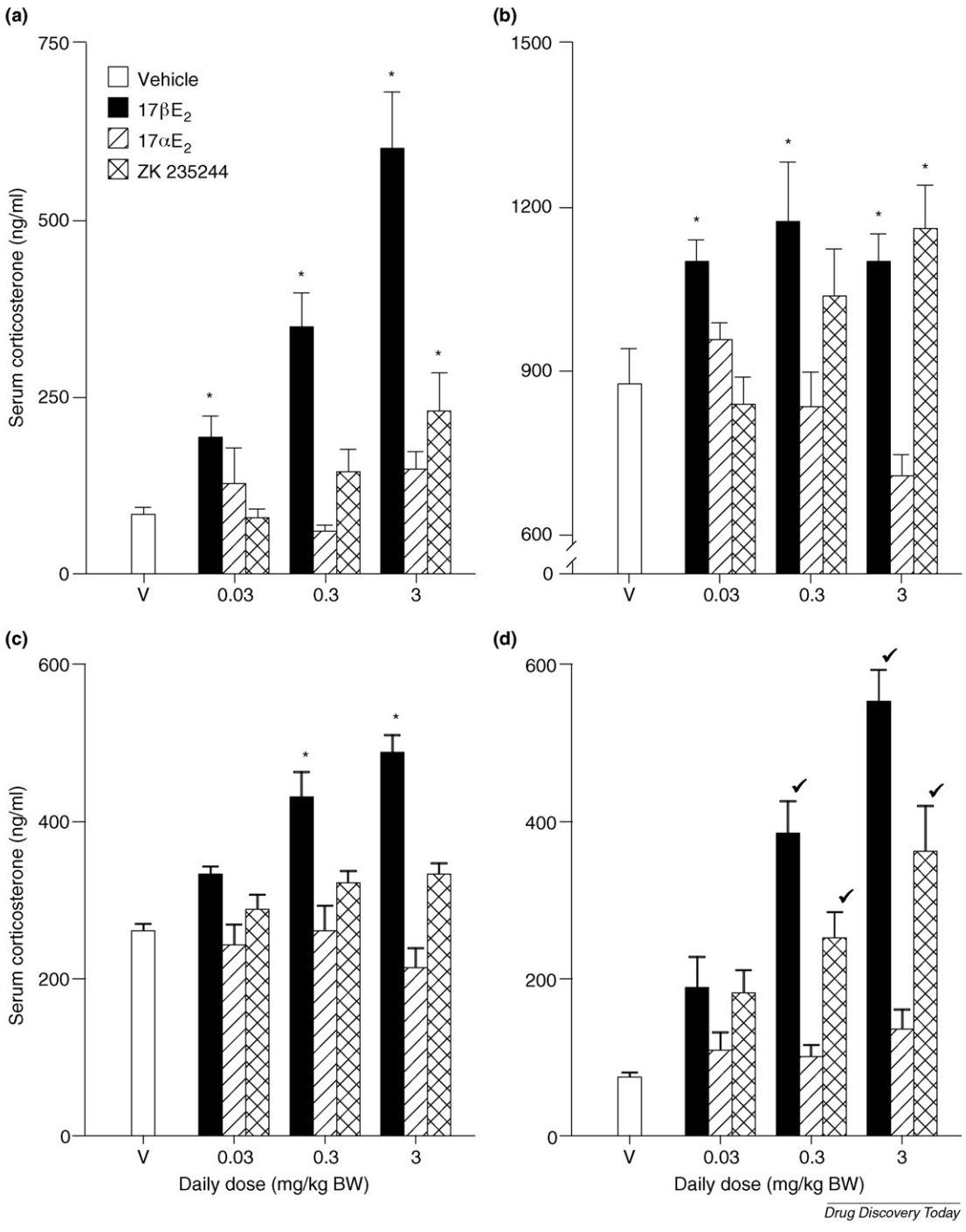

FIGURE 4

Changes in serum CORT levels in ovariectomized rats receiving continuous s.c. infusion with the test compounds at three different doses or vehicle by osmotic mini-pumps for seven consecutive days. Blood samples of 0.03 ml were collected from tail vein incisions; the entire duration of the procedure did not exceed 20 s. The endpoints of interest were examined during the last 24 h of the treatment period. Corticosterone levels under quiescent (baseline) conditions were assessed in samples collected at the time of diurnal nadir (panel **a**) and zenith (panel **c**) of pituitary–adrenal activity. Data in panel **b** (note the *y*-axis break) depict the secretory response to brief emotional stress (removal from the home cage, sampling procedure and subsequent exposure to air puffs for 1 min) measured 30 min after stress cessation; the stress-induced changes are best appreciated when compared with data in panel **a**. Asterisks in panels **a–c** denote significant differences to the matching vehicle-treated group. The effect of the test compounds on the responsiveness of the HPA axis to glucocorticoid suppression is shown on panel **d**. A single dose of dexamethasone 20 μg/kg) injected 6 h earlier is capable to restrain the nocturnal CORT peak in rats. The checkmark symbols in panel **d** denote treatment groups that failed to display a significant reduction in CORT levels shown in panel **c**. Data are presented as mean ± S.E.M.; each treatment group consists of five animals.

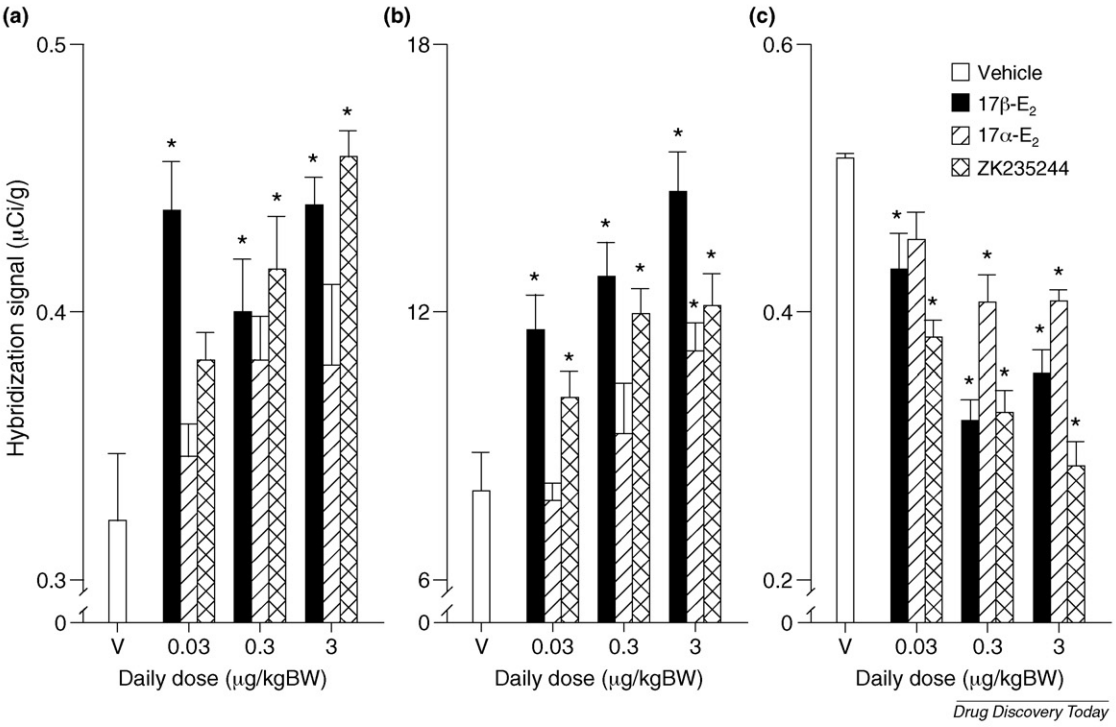

FIGURE 5

Treatment-induced changes in the density of transcripts encoding CRH (panel **a**) and oxytocin (panel **b**) in the hypothalamic PVN and GR in the hippocampal subdivision CA1 (panel **c**). The endpoints were determined by means of *in situ* hybridization histochemistry with radioactively labeled oligo-nucleotide and ribo-nucleotide probes in coronal brain sections containing the anatomic regions of interest (see [64,65] for detailed method description). The test compounds at three different doses or vehicle were applied in ovariectomized rats as continuous s.c. infusion over seven days. Data for each treatment group represent the mean ± S.E.M. of five animals; asterisks indicate significant differences to vehicle-treated rats.

altered secretory drive and sensitivity to glucocorticoid feedback in the central regulation of the HPA axis, such as increased density of CRH-coding and oxytocin-coding transcripts in the hypothalamic PVN (Figure 5a and b), and decreased GR mRNA levels in the CA1 subdivision of the hippocampus (Figure 5c). Taken together, the data gathered from this subarray indicate that two chemically closely related compounds with similar binding affinity for the ER, 17α-E_2 and ZK 235244 diverge in their capacity to influence the central mechanisms of HPA axis control in an estrogen-like fashion.

Further evidence of dichotomy between central and peripheral estrogen action was provided by the differential induction of oxytocin (OT) receptors in the brain and myometrium (Figure 6a). This parameter was examined in animals receiving the compounds at the highest dose tested (3 μg/kg), at which rats receiving 17β-E_2 displayed, in addition to uterine enlargement, clear signs of endometrial proliferation (Figure 6b). Significantly increased binding of the radiolabeled OT receptor ligand in the myometrium was documented in rats receiving 17β-E_2 and, surprisingly, 17α-E_2, but not ZK 235244 treatment. However, unlike 17β-E_2 and ZK 235244, administration of 17α-E_2 failed to influence this parameter in two brain structures, the bed nucleus of stria terminalis (BNST) and the ventromedial nucleus (VMN), which are richly endowed with estrogen-inducible OT receptors.

In summary, these data suggest that the examination of treatment-induced changes in endpoints that characterize the HPA axis function and organ-specific comparison of effects on one and the same molecular target (in this case, OT receptor induction) may provide valuable early information on dissociation between systemic and CNS effects of estrogens. Further, they underline the standpoint that structural similarity, comparable ER affinity, and transcriptional activity at the ERα, and efficacy in primary target organs are not sufficient predictors of the neurotrophic effects of estrogens.

Behavior and neuroprotection: confirmations and aberrations

Anxiolysis is an established pharmacological effect of estrogens; accordingly, the demonstration of measurable effects in anxiety paradigms belongs, also because of its relative simplicity, to the first-tier tests of neurotrophic action. When given at a dose of 3 μg/kg (but not below) all compounds produced significant anxiolytic effects in the elevated plus maze, as defined by increased number of entries (Figure 7a) and time spent in the open compartments of the device (Figure 7b). However, significant differences between individual compounds as to their efficacy on these endpoints could not be established, nor were differential treatment effects on the temporal dynamics of general motor activity manifest in the course of the experiment (Figure 7c).

The inference that could be drawn from these data was that, as exemplified by 17α-E_2 and ZK 235244, the anxiolytic effects of estrogens may become manifest at doses that do not affect the reproductive organs. Apparently, once a certain threshold dose is exceeded, estrogen-induced anxiolysis becomes manifest regard-

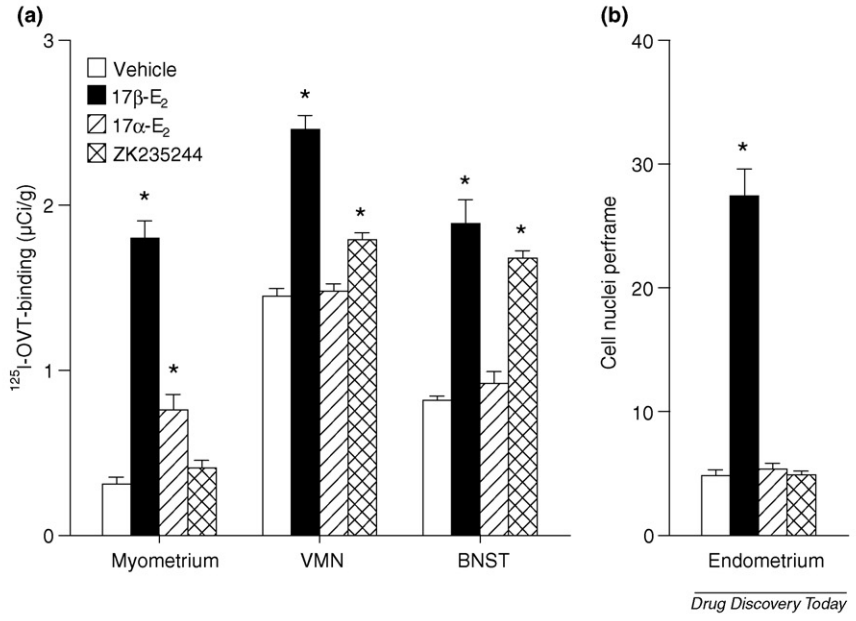

FIGURE 6

Oxytocin receptor densities (panel **a**) in the myometrium and two brain regions, the ventromedial nucleus (VMN) and the bed nucleus of stria terminals (BNST), and morphometric assessment of endometrial proliferation (panel **b**) following s.c. test compound administration at a dose of 3 μg/kg for seven days. Oxytocin receptors were visualized autoradiographically with the [^{125}I]-labeled OT receptor antagonist d(CH$_2$)$_5$[Tyr(Me)^2, Thr^4, Tyr-NH$_2$^{9}] ornithine vasotocin, and the signal intensity was quantified by densitometric image analysis of the anatomic regions of interest within rectangular frames of pre-set size (see [66] for method details). Signs of endometrial proliferation were assessed in hematoxylin–eosin-stained transverse sections at the level of the uterine horn bifurcation. For each animal, endometrial cell nuclei were counted in two frames positioned at diametrically opposed sites of the microscopic image. Asterisks indicate significant differences as compared with vehicle-treated rats; each treatment group consists of four to five animals; data are displayed as mean ± S.E.M.

less of the specific drug profile; also it seems that the readouts of this routinely used paradigm may not provide the discrimination power necessary for comparisons of compound potency.

The ability of estrogens to rescue nerve cells from the deleterious impact of various endogenous products that are implicated in the pathogenesis of neurodegenerative diseases [e.g. reactive oxygen species (ROS) or beta-amyloid (Aβ) fragments] has coined the term 'neuroprotection', which is still widely used as an argument in favor of estrogen supplementation in menopause and aging. Although it has proved difficult to demonstrate such effects in clinical settings, especially in conditions associated with irreversible loss of neural substrate, demonstration of neuroprotective effects still belongs to the standard arsenal used to characterize the CNS action of estrogens. Here it is certainly worthy to mention that most experimental paradigms of inflicting neurotoxic injury, and especially the surrogate models *in vitro*, employ drastic challenges that do not emulate the pathological process in humans but rather rapidly produce readouts of irreversible damage. Accordingly, the doses of toxic agents and protective compounds used in such settings with a time-lapse course are, by far, higher than those applicable to the real condition, and the results often document all-or-nothing responses.

In our *in vitro* studies, exposure of cells to Aβ(1-42) resulted in a dramatic increase in ROS production and disturbed mitochondrial integrity. Pre-treatment with the compounds of interest dose-dependently attenuated ROS production, with ZK 235244 displaying a significantly stronger effect at the highest dose tested (Figure 8a), and being the only one that significantly counteracted signs of mitochondrial damage (Figure 8b).

Two further indicators of detrimental cellular consequences of ROS-induced lipid peroxidation, hydroxynonenal (HNE)-modified mitochondrial proteins [25] with molecular mass of 70 and 52 kDa were isolated from cells exposed to Aβ(1-42). Pre-treatment with the compounds of interest differentially decreased the abundance of these proteins, with 17β-E$_2$ influencing only the 70 kDa protein, and 17α-E$_2$ and ZK 235244 preventing the appearance of both protein fractions (Figure 9).

These findings corroborate numerous previous observations that estrogens not only attenuate ROS production and oxidative damage of nerve cells, but also demonstrate that individual compounds may differ with regard to the magnitude, as well as the quality of the protective effect. As already mentioned, the data also show that the estrogen doses required for the manifestation of robust effects exceed those applicable *in vivo*.

The capacity of estrogens to exert neuroprotective effects *in vivo* was also examined in a paradigm, in which selective damage of cholinergic neuronal populations by the neurotoxic agent (AF64A) through a variety of mechanisms, also involving ROS formation [26], is associated with the impairment of acquisition and retention of a new behavioral repertoire and spatial navigation. However, the results showed that, in addition to differential potency, individual compounds may affect different behavioral aspects. Thus, apart from significantly improving the task acquisition at a dose of 40 μg/kg, the less potent estrogens 17α-E$_2$ and ZK 235244 displayed,

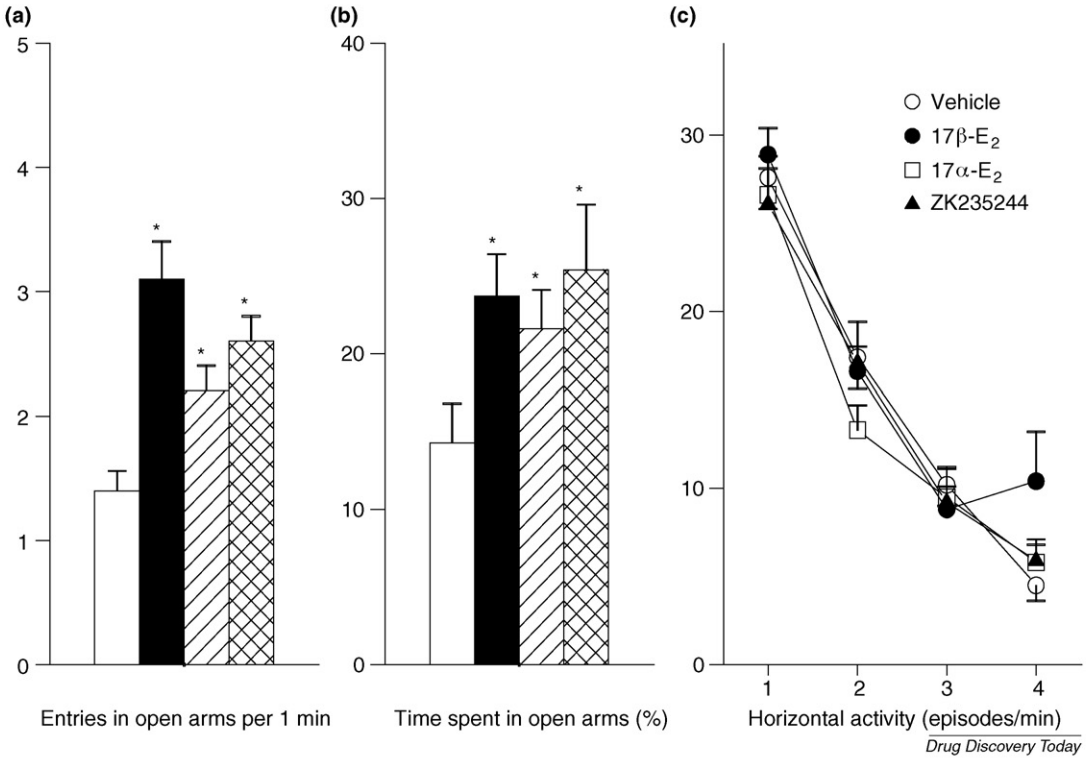

FIGURE 7

Effects of daily s.c. injection of the test compounds at a dose of 3 μg/kg on behavioral measures of anxiety (panels **a** and **b**) and locomotor activity (panel **c**) in the elevated plus-maze (see ref. [67] for details). Increased exploration of the open compartments of the device is indicative of anxiolysis. For each rat video records of 5 min duration were generated and the behavior displayed during the first 4 min was assessed by an unbiased investigator. Data in panel **a** show the averaged individual entry scores per minute; panel **b** displays the fraction of time spent during the entire observation period. Asterisks in panels **a** and **b** denote significant differences to vehicle treatment (open bars); solid bars, 17β-E_2; hatched bars, 17α-E_2; cross-hatched bars, ZK 235244. The locomotor episodes (panel **c**) were scored for each minute of the test; the overlapping slopes of activity decrease suggest that habituation to the novel environment is not significantly influenced by the drugs used. Data are presented as mean ± S.E.M.; each treatment group consists of seven rats.

unlike 17β-E_2, a visibly clear trend of performance amelioration also at the lower dose tested (Figure 10a). The compound 17α-E_2 was the only one that positively influenced the retention of the behavioral response at the lower, but unable to maintain this effect at the higher dose tested, at which the protective action of 17β-E_2 and ZK 235244 became manifest (Figure 10b). The trend of 'weaker' estrogens producing more impressive behavioral effects was evident also in the examination of spatial navigation in the water maze: significant improvement of AF64A-impaired performance was recorded in rats receiving 17α-E_2 and ZK 235244 at a dose of 40 μg/kg, whereas, surprisingly, treatment with 17β-E_2 did not result in major changes in this parameter (Figure 10c).

The comparison of estrogen effects in peripheral target organs at the end of the treatment period revealed that the potency relationships seen in female rats also persist in males: dose-dependent enlargement of the prostate and seminal vesicles and thymus involution were documented in both treatment groups receiving 17β-E_2, whereas ZK 235244 and 17α-E_2 were virtually ineffective at the above endpoints (data not shown).

Explaining the rationale and its limitations

The comprehensive comparison of several features of three 'tool' estrogens with different pharmacological potency, 17β-E_2, 17α-E_2 and its derivative ZK 235244, can be summarized in the following key messages: (i) several aspects of CNS-specific efficacy of estrogens do not necessarily correlate with, and are not directly deducible from, their interaction with the cognate molecular target (e.g. ER) and potency in models based on the reproductive system; (ii) balanced agonist activity at both major ER isoforms and efficient recruitment of molecular 'amplifiers' of ER signaling (e.g. SRC-1) expand the neurotrophic profile of estrogens; (iii) the crucial objective of dissociation between neurotrophic ad systemic actions of estrogens appears achievable, as CNS effects are discernible at doses considerably below those that affect targets in the reproductive system; (iv) the exceptional estrogen sensitivity of several compartments of the female rat HPA axis suggests its suitability as a model for revealing CNS-selective estrogen effects; (v) monitoring of anxiolysis can serve as a simple and productive method to validate the CNS efficacy of estrogens with subdued systemic action profile; (vi) estrogen-mediated neuroprotection is a multifarious outcome that may provide confirmatory evidence for the neurotrophic efficacy, but lacks the sensitivity needed for the early discrimination of CNS-selective estrogens. Ample experimental evidence justifies the selection of these parameters for the discovery of CNS-selective estrogens; however, as shown here, their testifying power seems unequal.

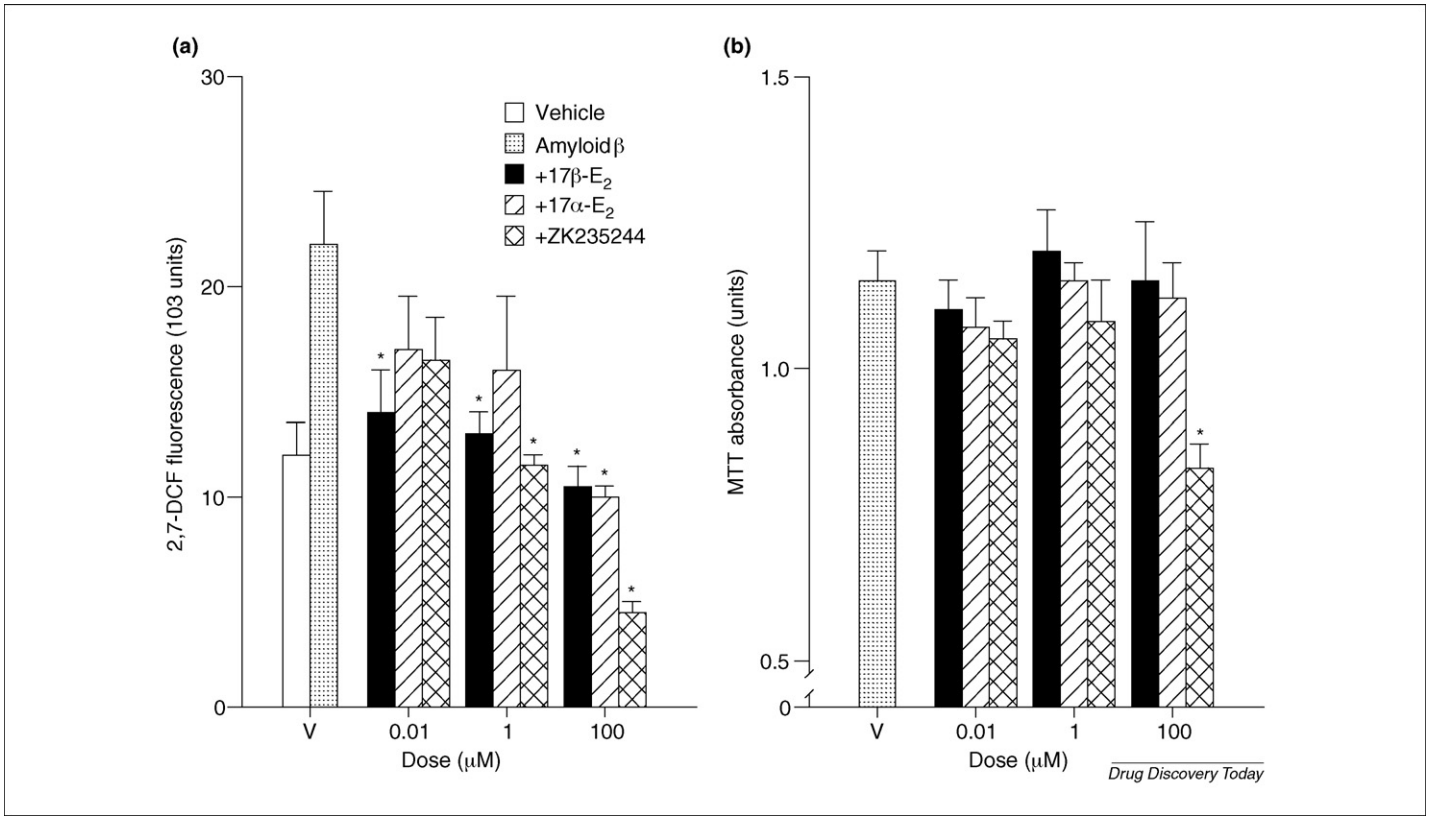

FIGURE 8

Effects of pre-treatment with the test compounds on amyloid-β induced production of reactive oxygen species (panel **a**) and mitochondrial deterioration (panel **b**) in PC12 cells, as disclosed by DCF reaction (see [68]) and MTT reduction (see [69]), respectively. The cells were pre-treated for 48 h with different concentrations of the test drugs, before exposure to 50 μM of amyloid-β_{1-42} protein (Aβ) for 6 h. Data represent mean ± S.D. values generated from three independent experiments; asterisks denote significant differences versus treatment with Aβ alone.

The discrete regional distribution of ER isoforms in the CNS is, meanwhile, well established [16,17], and research on their distinct role in the regulation of neurophysiological process other than reproductive control is gaining momentum. Phenotype scrutiny in animals with targeted disruption of ER isoforms [27], and pharmacological studies using ER isoform-selective ligands [28,29], point to the significant participation of ERβ in behavioral repertoire and neuroendocrine responses of adaptive significance. Thus, ERβ-mediated signaling appears to be an important determinant of the spectrum of CNS actions of estrogens. As exemplified by the chemically related steroids 17α-E_2 and ZK 235244, differential potency in ERβ-mediated transactivation is reflected by disparate efficacy on endpoints of CNS action. This assumption is further supported by the comparison of the capacity to attract SRC-1 upon ER binding. The role of SRC-1 as an amplifier of ER receptor signaling has been demonstrated for both isoforms [30,31]; its abundance in estrogen-sensitive brain regions [32,33], responsiveness to changing estrogen levels [33], and involvement in behavioral and neuroendocrine effects of estrogens underlines its importance as a useful screening criterion for the discovery of CNS-selective estrogens. In view of the remarkably diminished efficacy of 17α-E_2 in recruiting SRC-1 upon binding to the ERβ, we speculate that, *quod erat demonstrandum* by several *in vivo* data, the inferior capacity of 17α-E_2 to influence ERβ-signaling might curb the spectrum of its neurotrophic effects. It should be noted, however, that physiological readouts indicative of ERβ-specific signaling in the CNS remain to be established. On the basis of the descriptions of ERβ distribution in the rat brain [16,17] and behavioral observations [27–29], we suppose that changes in neuropeptide gene expression in the hypothalamic PVN and anxiety may reflect estrogen effects that are predominantly mediated through ERβ activation. However, some discrepant effects of 17α-E_2, a compound with subdued ERβ activity, on transcriptional and behavioral endpoints indicate that, owing to the complexity of mechanisms involved in estrogen signaling in the CNS, caution applies to the canonic interpretation of data obtained in complex (i.e. behavioral) paradigms.

Virtually all results obtained with the natural estrogen 17β-E_2 in this study reproduce previous observations on its effects in peripheral estrogen-sensitive tissues and the CNS. Stimulation of uterine growth, suppression of post-castration-elevated gonadotropins, and thymolysis [34] are standard endpoints for the evaluation of systemic estrogen action. Stimulation of the gene expression of hypothalamic ACTH secretagogues [35] and decreased abundance of hippocampal corticosteroid receptors [36] following estrogen treatment precipitate in increased basal and stress-induced adrenocortical output and diminished sensitivity of glucocorticoid-mediated negative feedback on HPA activity in the female rat. Implementation of the latter concept proved helpful in the process of evaluation of the neurotrophic properties

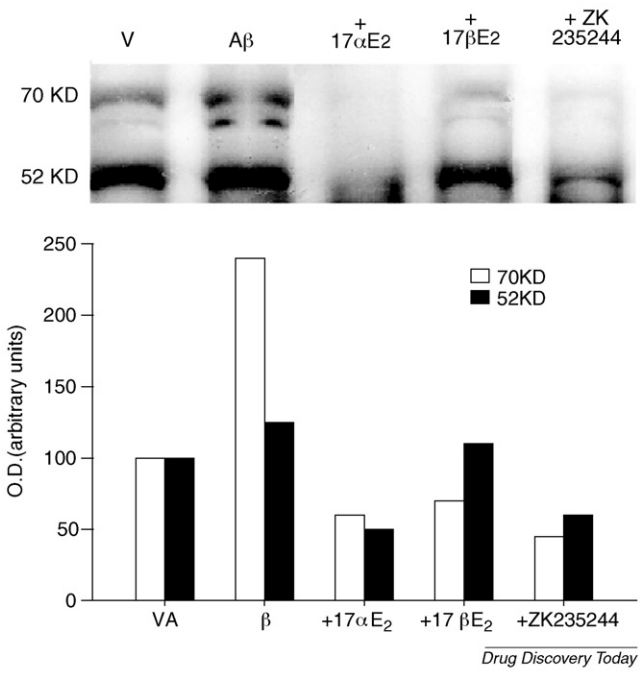

FIGURE 9

Representative western blot and densitometric quantification of HNE-modified mitochondrial proteins in PC12 cells following 6 h of exposure to Aβ fragment$_{1-42}$ alone or after pre-treatment with the test compounds at a concentration of 100 μM for 48 h. Upon isolation, solubilization and electrophoretic separation (see [70,71] for method description) of mitochondrial proteins, HNE-modified proteins were detected with a specific anti-HNE antibody (see [72] for specifications) and visualized with a peroxidase-coupled secondary antiserum. The bar charts depict average optical density values from two independent experiments.

of 17α-E_2 and its synthetic derivative ZK 235244. The latter, otherwise weak, estrogen displayed clear dose-dependent estrogen-like effects in the female rat HPA axis at most readouts. However, the differential efficacy of 17α-E_2 and ZK 235244 does not correlate with the endpoints describing the interaction of the compounds with the ERα. Also, examination of the biotransformation and pharmacokinetics *in vivo* (Schering AG, unpublished data) is not supportive of the assumption that these differential CNS effects may be ascribed to the generation of metabolites with differential estrogen-like activity. Thus, the pronounced CNS efficacy of ZK 235244 apparently reflects consequences of the structural modification of the parent compound 17α-E_2, most probably resulting in a coactivator-friendly agonist conformation of the ERβ ligand-binding domain. This conclusion is also underpinned by the comparison of organ-selective induction of oxytocin receptors in the same individual; the natural estrogen 17α-E_2, the capacity of which to activate ERα-mediated transcription is superior to that of ZK 235244, increased OT receptor densities in the ERα-rich myometrium, while being virtually ineffective on this parameter in brain regions with mixed ER isoform populations.

As these observations corroborate previous and recent evidence [37–39] that several aspects of the HPA axis function in the gonadectomized female rat show distinct dose-dependent responses to systemic subthreshold estrogen amounts, it is pertinent to mention that (i) this effect profile may substantially differ following supraphysiological estrogen doses [40,41] and (ii) the inconsistent responsiveness of the human HPA axis to estrogens [42,43] advocates caution as to the value of these endpoints in clinical settings.

Diminution of behavioral symptoms of anxiety is a well-established, albeit not undisputed, consequence of estrogen treatment in the female rat [29,44]. In this study, all compounds displayed anxiolytic activity at the highest dose tested and, unlike at other endpoints, the efficacy of 17α-E_2 was commensurable with that of the remaining test compounds. In view of reports suggesting that neurotrophic effects of 17α-E_2 may employ pathways other than ER-mediated transcription [15,18,21], this observation is certainly intriguing. However, as the specific contribution of these non-conventional pathways of estrogen signaling to the control of anxiety is far from being elucidated, speculations are hardly justified. The interpretation of estrogen effects on behavioral acquisition and retention also urges circumspection: the view that estrogens unequivocally improve the cognitive performance may not apply to fear-based experimental paradigms [45]. Thus, the meaning of the present data should be restricted to a situation in which the behavioral performance is primarily determined by the toxic damage of relevant neuronal populations.

The capacity of steroidal estrogens to eliminate ROS by virtue of their phenolic A ring is a well-documented phenomenon [7,46] and has shaped (for a long time) the opinion on potential benefits of estrogen therapy, especially with regard to prevention or attenuation of neurodegeneration. The efficacy of the compound ZK 235244 in paradigms based on ROS-inflicted damage in nerve cells was predictable, as previous studies have documented its capacity to eliminate free radicals [47], protect nerve cells from oxidative stress with a superior efficacy than 17β-E_2 and 17α-E_2 [24] and induce the expression of anti-apoptotic genes in the hippocampus (V. Patchev, unpublished data). While the results of the present *in vitro* experiments underline previous evidence for the efficacy of estrogens in attenuating the detrimental consequences of cell exposure to ROS and Aβ [7,46], the effects on cognitive performance suggest that ZK 235244 and its natural precursor 17α-E_2 are superior to 17β-E_2 in counteracting neurotoxin-induced damage in cholinergic neurons. This observation is, to a certain extent, unexpected, as earlier reports have underscored the efficacy of 17β-E_2 in similar settings [48,49]. One possible explanation of this discrepancy is the sex of the subjects in our study. These experiments were deliberately carried out in males, aiming to explore the protective effects of estrogens in a setting that is not entirely dominated by the transcriptional consequences of their interaction with the ER. Sex-hormone-dependent brain differentiation during early development accounts for the establishment of differential estrogen sensitivity, as exemplified by sex dichotomy in the control of reproduction and adaptive responses [50]. It can be assumed that, owing to the autologous downregulation of ER in the male brain during early development [51,52], mechanisms other than ER-mediated signaling (e.g. ROS elimination) may primarily account for estrogen-based neuroprotection in the male brain. Thus, the use of male subjects appears to be a useful approach to reveal neuroprotective effects of estrogens that are based on mechanisms other than interactions with the cognate molecular target—the ER. As the neurotoxic damage inflicted by

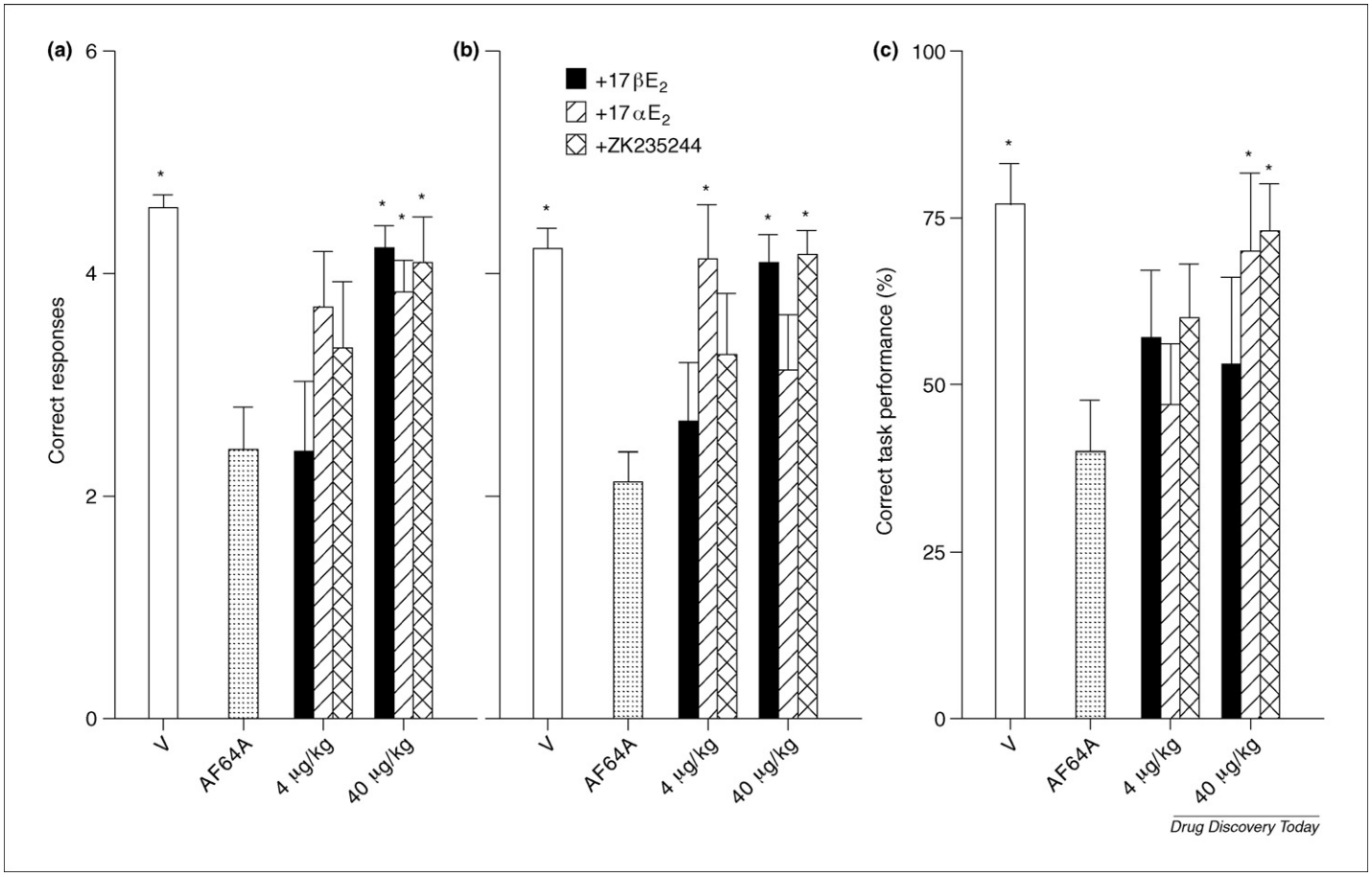

FIGURE 10

Effects of treatment of castrated male rats with the test compounds on the acquisition (panel **a**) and retention (panel **b**) of conditioned avoidance behavior, and spatial navigation in the water maze (panel **c**). Subcutaneous injections at daily doses of 4 and 40 μg/kg commenced immediately after orchidectomy. One week later, selective damage of the basal forebrain cholinergic neurons was inflicted by stereotaxic infusion of the cholinergic neurotoxin ethylcholine aziridinium (AF64A) into the lateral cerebral ventricles. Control animals received i.c.v. infusion with artificial cerebrospinal fluid. Acquisition and retention of conditioned active avoidance behavior were tested in the shuttle-box paradigm (see [73] for details) in two consecutive sessions one week after the neurotoxic damage and 16–17 days of test compound treatment. Rats were subjected to two sessions of 35 learning/retention trials each. Correct responses were defined as escape within 5 s after the presentation of the conditioned stimulus and scored during the last 15 trials of the acquisition and first 15 trials of the retention session. The same animals were subsequently used for examination of the spatial navigation ability in the Morris water maze (see [74] for method details). One training and three retrieval sessions (eight trials each) were conducted on days 10–14 after AF64A application and 18–21 of continuous test compound treatment. Correct task performance was defined as goal detection latency below 120 s and expressed as percentage from all trials in the retrieval sessions. The treatment groups consisted of 10 (test compounds) or 20 animals (vehicle, AF64A alone). Data represent mean ± S.E.M.; asterisks indicate significant differences to rats receiving the neurotoxin AF64A alone.

AF64 includes a strong ROS component [26], the protective action of weak estrogens, such as ZK 235244 and 17α-E_2, could be ascribed to their superior ability to interfere with this pathogenic component. However, in view of the multiplicity of neurotrophic actions of estrogens, mechanisms involving stimulation of acetylcholine biosynthesis [53] in intact neurons or compensatory activation of non-cholinergic transmitter pathways originating from distant neuronal populations [54] should also be taken into consideration. Other mechanisms of plausible neurotrophic relevance are the interactions of estrogens with growth factor signaling on convergent intracellular pathways [55] and a broad array of mechanisms of neurotransmission [56,57]. Exploration of these intriguing aspects may elucidate the contribution of signal transducers, other than the cognate nuclear receptors, to the neuroprotective effects of estrogens. In general, and in the context of the present study, however, it is prudent to consider that the applicability of estrogens is confined to prevention, and not reversal, of organic damage, thus austerely admitting that the neuroprotective use of estrogens is limited to prophylaxis, rather than restorative therapy.

Conclusions

Evidence from numerous experimental and clinical observations in the past has underpinned the importance of estrogen signaling in several physiological aspects of CNS function. Understandably, in the aftermath of the WHI Study, a vivid discussion on the pros and cons of estrogen supplementation and the reasons for the controversial clinical outcomes continues to send out its repercussions [4,5,58,59]. The realization that, at least in a sizeable subpopulation, estrogen supplementation is the only efficient approach to the management of symptoms associated with the menopausal transition has now shifted the focus of this debate from the issue of necessity to those of therapy timing, duration, and drug selection [9,60]. Thus, the need for therapeutic principles

TABLE 2

Retrospective assignment of 'performance grades' (+++ high, ++ medium, + fair) to endpoints used for the demonstration of dissociated CNS effects of the test compounds

Endpoint	Profile relevance	Discrimination power
Binding affinity for ERα	+	
Binding affinity for ERβ	++	
Transactivation at ERα	+	
Transactivation at ERβ	+++	
Coactivator recruitment (efficacy) by ERα	+	
Coactivator recruitment (efficacy) by ERβ	+++	
Marginal or absent uterotropic effect	+++	n.a.
Antigonadotropic action	+	+
Increased amplitude of stress-induced CORT secretion	++	+
Escape from dexamethasone suppression	+	++
Induction of CRH expression in the PVN	++	++
Induction of oxytocin expression in the PVN	++	++
Dissociated induction of oxytocin receptors in the brain and myometrium	+++	+++
Anxiolysis	+++	+
Improved acquisition/retention of conditioned behavior	+++	+
Improved spatial navigation	+++	+
Counteraction of ROS-induced damage *in vitro*	++	+
Attenuation of neurotoxic damage *in vivo*	++	+

'Profile relevance' denotes the probability that the fulfillment of the criterion will be associated with a pronounced neurotrophic profile; 'discrimination power' describes the capacity of the readout to distinctly respond to subthreshold estrogen doses.

capable of selectively alleviating CNS symptoms of estrogen deprivation in an estrogen-like mode remains topical, even if evidence-based medicine urges caution concerning the applicability of such principles as first-tier medication for the treatment and prevention of neurodegenerative and mental conditions [9,60].

The results of the studies described in this Foundation review indicate that criteria for the prediction of neurotrophic effects of steroidal estrogens can be elaborated under consideration of some distinct aspects of estrogen signaling in the CNS. In the early phase of compound discovery it is prudent to consider that neurotrophic actions of estrogens largely reflect transcriptional changes occurring with the participation of both major ER isoforms. The differential abundance of ERβ in 'classic' target organs and the brain also suggests that activation of this isoform and concomitant mobilization of molecular amplifiers of transcription might be important component of estrogen action in the CNS.

Demonstration of dissociation between neurotrophic and systemic pharmacological effects of estrogens is a crucial criterion in drug discovery. Usually, evidence originates from sophisticated examination of behavioral-cognitive and histological endpoints in experiments employing broad dose ranges. Our studies show that estrogens can produce significant changes in endpoints indicative of neurotrophic activity at doses below those required for the occurrence of measurable organometric effects in peripheral targets. The present data demonstrate that changes in descriptors of basal and stress-induced HPA axis activity in the ovariectomized female rat may reveal the occurrence of dissociated CNS-specific estrogen effects in a less invasive and time-consuming fashion. As safety remains a crucial issue in estrogen therapy, it should be noted that this phenomenon of dissociated action is also achievable with compounds that are not marked by either exceptional affinity for, or powerful transcriptional activity at the ER.

Estrogens exert a wide array of behavioral effects that employ various and, to some extent, less-investigated cellular and molecular pathways in highly complex and heterogeneous neuronal populations. In view of this multiplicity, recommendations for the preferential use of defined behavioral paradigms should be left at the discretion of the investigator. Proof of efficacy in behavioral models that emulate key pathogenic components of the envisaged medical indication remains the most convincing argument. In the general framework of knowledge on the neurotrophic actions of estrogens, surrogate tests addressing the fine-tuning between neural systems involved in emotional responsiveness (e.g. anxiety), forestalling of endogenous damage (e.g. ROS elimination) and compensation for substrate loss by mobilization of alternative/redundant neurochemical mechanisms may provide reliable readouts for compound selection. However, it is pertinent to note that the ongoing scientific debate has already heralded a shift in the therapeutic priorities that merits consideration in the process of drug discovery. Thus, demonstration of a drug candidate's efficacy in models of menopausal vasomotor symptoms, together with attenuated estrogen-like efficacy in reproductive organs, would considerably boost its developmental prospects.

The leading principle in the discovery of neurotrophic estrogens remains confirmation of CNS efficacy in conjunction with evidence that their systemic effects do not exceed an arbitrarily defined acceptable degree. Regrettably, the present drug spectrum contains only a few approved (and, frequently, progestin containing) formulations. Despite previous and current debates on the

medical necessity of estrogen supplementation in the course of the menopausal transition, several discoveries in this field during the past 10 years have outlined new frameworks for the definition of estrogens with pronounced neurotrophic efficacy. In Table 2, we have summarized our experience with the profile demarcation of neuroactive estrogens and appropriateness of individual experimental approaches for the discovery of such compounds. On the basis of the present data, here we undertake an attempt to classify the importance of individual endpoints for the definition of essential features of steroidal estrogens with a pronounced neurotrophic profile and their relative power to reveal the occurrence of dissociation between CNS and systemic effects. The multiplicity of estrogen effects in the CNS and accumulating knowledge on the molecular principles of estrogen signaling and pharmacology of isoform-selective agonists and modulators of the ER warrants the continuous extension and rearrangement of the discovery criteria in this list.

Disclosure statement

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References

1 Shumaker, S.A. *et al.* (2004) Conjugated equine estrogens and incidence of probable dementia and mild cognitive impairment in postmenopausal women: Women's Health Initiative Memory Study. *JAMA* 291, 2947–2958
2 Espeland, M.A. *et al.* (2004) Conjugated equine estrogens and global cognitive function in postmenopausal women: Women's Health Initiative Memory Study. *JAMA* 291, 2959–2968
3 Klein, K.P. and Rapp, S.R. (2004) Women's cognitive health: postmenopausal dementia and the Women's Health Initiative Memory Study. *Womens Health Issues* 14, 71–74
4 Morrison, J.H. *et al.* (2006) Estrogen, menopause, and the aging brain: how basic neuroscience can inform hormone therapy in women. *J. Neurosci.* 26, 10332–10348
5 Toran-Allerand, C.D. (2006) Reply to 'Hormone in the hot seat'. *Nat. Med.* 12, 379–380
6 Garcia-Segura, L.M. *et al.* (2001) Neuroprotection by estradiol. *Prog. Neurobiol.* 63, 29–60
7 Behl, C. (2002) Oestrogen as a neuroprotective hormone. *Nat. Rev. Neurosci.* 3, 433–442
8 Sherwin, B.B. (2003) Estrogen and cognitive functioning in women. *Endocr. Rev.* 24, 133–151
9 (2003) North American Menopause Society. Amended report from the NAMS Advisory Panel on Postmenopausal Hormone Therapy. *Menopause* 10, 6–12
10 Gooren, L.J.G. and Toorians, A.W.F.T. (2003) Significance of oestrogens in male (patho)physiology. *Ann. Endocrinol.* 64, 126–135
11 Stein, D.G. (2001) Brain damage, sex hormones and recovery: a new role for progesterone and estrogen? *Trends Neurosci.* 24, 386–391
12 Lauber, A.H. *et al.* (1991) Estrogen receptor messenger RNA expression in rat hypothalamus as a function of genetic sex and estrogen dose. *Endocrinology* 129, 3180–3186
13 Kühnemann, S. *et al.* (1994) Sex differences in the development of estrogen receptors in the rat brain. *Horm. Behav.* 28, 483–491
14 Kruijver, F.P.M. *et al.* (2002) Estrogen receptor-α distribution in the human hypothalamus in relation to sex and endocrine status. *J. Comp. Neurol.* 454, 115–139
15 Toran-Allerand, C.D. (2004) Minireview: a plethora of estrogen receptors in the brain: where will it end? *Endocrinology* 145, 1069–1074
16 Osterlund, M.K. *et al.* (2000) Estrogen receptor β (ERβ) messenger ribonucleic acid (mRNA) expression within the human forebrain: distinct distribution pattern to ERα mRNA. *J. Clin. Endocrinol. Metab.* 85, 3840–3846
17 Shughrue, P.J. *et al.* (1997) Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *J. Comp. Neurol.* 388, 507–525
18 Rønnekleiv, O.K. *et al.* (2007) Membrane-initiated signaling of estrogen in the brain. *Semin. Reprod. Med.* 25, 165–177
19 Charlier, T.D. and Balthazart, J. (2005) Modulation of hormonal signaling in the brain by steroid receptor coactivators. *Rev. Neurosci.* 16, 339–357
20 Siemann, H.J. *et al.* (1995) A novel synthesis of 14α, 15α-methylene estradiol (J 824). *Steroids* 60, 308–315
21 Balthazart, J. and Ball, G.F. (2006) Is brain estradiol a hormone or a neurotransmitter? *Trends Neurosci.* 29, 241–249
22 Toran-Allerand, C.D. *et al.* (2005) 17α-estradiol: a brain-active estrogen? *Endocrinology* 146, 3843–3850
23 Honjo, H. *et al.* (2003) Estrogen and non-feminizing estrogen for Alzheimer's disease. *Endocr. J.* 50, 361–367
24 Blum-Degen, D. *et al.* (1998) Scavestrogens protect IMR 32 cells from oxidative stress-induced cell death. *Toxicol. Appl. Pharmacol.* 152, 49–55
25 Grune, T. and Davies, K.J.A. (2003) The proteasomal system and HNE-modified proteins. *Mol. Aspects Med.* 24, 195–204
26 Hanin, I. (1996) The AF64A model of cholinergic hypofunction: an update. *Life Sci.* 58, 1955–1964
27 Bodo, C. and Rissman, E.F. (2006) New roles for estrogen receptor β in behavior and neuroendocrinology. *Front. Neuroendocrinol.* 27, 217–232
28 Lund, T.D. *et al.* (2005) Novel actions of estrogen receptor-β on anxiety-related behaviours. *Endocrinology* 146, 797–807
29 Walf, A.A. and Frye, C.A. (2006) A review and update of mechanisms of estrogen in the hippocampus and amygdala for anxiety and depression behaviour. *Neuropsychopharmacology* 31, 1097–1111
30 Robyr, D. *et al.* (2000) Nuclear hormone receptor coregulators in action: diversity for shared tasks. *Mol. Endocrinol.* 14, 329–347
31 Hall, J.M. and McDonnell, D.P. (2005) Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. *Mol. Interv.* 5, 343–357
32 Meijer, O.C. *et al.* (2000) Different expression and regional distribution of steroid receptor coactivators SRC-1 and SRC-2 in brain and pituitary. *Endocrinology* 141, 2192–2199
33 Mitev, Y.A. *et al.* (2003) Developmental expression profiles and distinct regional estrogen responsiveness suggest a novel role for the steroid receptor coactivator SRC-1 as discriminative amplifier of estrogen signaling in the rat brain. *FASEB J.* 17, 518–519
34 Luster, M.I. *et al.* (1984) Estrogen immunosuppression is regulated through estrogenic responses in the thymus. *J. Immunol.* 133, 110–116
35 Patchev, V.K. *et al.* (1995) Implications of estrogen-dependent brain organization for gender differences in hypothalamo-pituitary–adrenal regulation. *FASEB J.* 9, 419–423
36 Patchev, V.K. and Almeida, O.F.X. (1998) Gender specificity in the neural regulation of the response to stress. *Mol. Neurobiol.* 16, 63–77
37 Burgess, L.H. and Handa, R.J. (1992) Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. *Endocrinology* 131, 1261–1269
38 Carey, M.P. *et al.* (1995) The influence of ovarian steroids on hypothalamic-pituitary–adrenal regulation in the female rat. *J. Endocrinol.* 144, 311–321
39 Figueiredo, H.F. *et al.* (2007) Estrogen potentiates adrenocortical responses to stress in female rats. *Am. J. Physiol. Endocrinol. Metab.* 292, E1173–E1182
40 Redei, E. *et al.* (1994) Fast glucocorticoid feedback inhibition of ACTH secretion in the ovariectomized rat: effect of chronic estrogen and progesterone. *Neuroendocrinology* 60, 113–123
41 Ferrini, M. *et al.* (1995) Estradiol abolishes autologous down regulation of glucocorticoid receptors in brain. *Life Sci.* 57, 2403–2412

42 Kirschbaum, C. *et al.* (1996) Short-term estradiol treatment enhances pituitary–adrenal axis and sympathetic responses to psychosocial stress in healthy young men. *J. Clin. Endocrinol. Metab.* 81, 3639–3643

43 Kudielka, B.M. and Kirschbaum, C. (2005) Sex differences in HPA axis responses to stress: a review. *Biol. Psychol.* 69, 113–132

44 Morgan, M.A. *et al.* (2004) Estrogens and non-reproductive behaviors related to activity and fear. *Neurosci. Biobehav. Rev.* 28, 55–63

45 Toufexis, D.J. *et al.* (2004) The effect of gonadal hormones and gender on anxiety and emotional learning. *Horm. Behav.* 50, 539–549

46 Singh, M. *et al.* (2006) Novel mechanisms for estrogen-induced neuroprotection. *Exp. Biol. Med. (Maywood)* 231, 514–521

47 Römer, W. *et al.* (1996) Novel 'scavestrogens' and their radical scavenging effects, iron-chelating, and total antioxidative activities: $\Delta^{8,9}$-dehydro derivatives of 17α-estradiol and 17β-estradiol. *Steroids* 62, 304–310

48 Nilsen, J. *et al.* (2006) Estrogen protects neuronal cells from amyloid beta-induced apoptosis via regulation of mitochondrial proteins and function. *BMC Neurosci.* 7, 74

49 Harms, C. *et al.* (2001) Differential mechanisms of neuroprotection by 17β-estradiol in apoptotic versus necrotic neurodegeneration. *J. Neurosci.* 21, 2600–2609

50 Simerly, R.B. (2002) Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. *Annu. Rev. Neurosci.* 25, 507–536

51 Yokosuka, M. *et al.* (1997) Postnatal development and sex difference in neurons containing estrogen receptor-α immunoreactivity in the preoptic brain, the diencephalon, and the amygdala in the rat. *J. Comp. Neurol.* 389, 81–93

52 Ikeda, Y. *et al.* (2003) Sexually dimorphic and estrogen-dependent expression of estrogen receptor β in the ventromedial hypothalamus during rat postnatal development. *Endocrinology* 144, 5098–5104

53 Gibbs, R.B. (1994) Estrogen and nerve growth factor-related systems in brain. Effects on basal forebrain cholinergic neurons and implications for learning and memory processes and aging. *Ann. N. Y. Acad. Sci.* 743, 165–196

54 Norbury, R. *et al.* (2003) The neuroprotective effects of estrogen on the aging brain. *Exp. Gerontol.* 38, 109–117

55 Garcia-Segura, L.M. *et al.* (2007) Estradiol, insulin-like griwth factor-I and brain aging. *Psychoneuroendocrinology* 32, S57–S61

56 Morissette, M. *et al.* (2008) Contribution of estrogen receptors alpha and beta to the effects of estradiol in the brain. *J. Steroid Biochem. Mol. Biol.* 108, 327–338

57 Brann, D.W. *et al.* (2007) Neurotrophic and neuroprotective actions of estrogen: basic mechanisms and clinical implications. *Steroids* 72, 381–405

58 Craig, M.C. *et al.* (2005) The Women's Health Initiative Memory Study: findings and implications for treatment. *Lancet Neurol.* 4, 190–194

59 Henderson, V.W. (2004) Hormone therapy and Alzheimer's disease: benefit or harm? *Expert Opin. Pharmacother.* 5, 389–406

60 NIH state-of-the-science conference statement on management of menopause-related symptoms. *NIH Consens. State Sci. Statements* 22, 1–28

61 Johnson, R.B. *et al.* (1975) Simplified Scatchard-plot assay for estrogen receptor in human breast tumor. *Clin. Chem.* 21, 1725–1730

62 Hillisch, A. *et al.* (2004) Dissecting physiological roles of estrogen receptor alpha and beta with potent selective ligands from structure-based design. *Mol. Endocrinol.* 18, 1599–1609

63 Albers, M. *et al.* (2006) A novel principle for partial agonism of liver X receptor ligands. Competitive recruitment of activators and repressors. *J. Biol. Chem.* 281, 4920–4930

64 Fischer, D. *et al.* (1995) Lactation as a model for naturally reversible hypercorticalism plasticity in the mechanisms governing hypothalamo-pituitary–adrenocortical activity in rats. *J. Clin. Invest.* 96, 1208–1215

65 Patchev, V.K. and Almeida, O.F.X. (1996) Gonadal steroids exert facilitating and 'buffering' effects on glucocorticoid-mediated transcriptional regulation of corticotropin-releasing hormone and corticosteroid receptor genes in rat brain. *J. Neurosci.* 16, 7077–7084

66 Patchev, V.K. *et al.* (1994) The neurosteroid tetrahydroprogesterone counteracts corticotropin-releasing hormone-induced anxiety and alters the release and gene expression of corticotropin-releasing hormone in the rat hypothalamus. *Neuroscience* 62, 265–271

67 Patchev, V.K. *et al.* (1993) Oxytocin binding sites in rat limbic and hypothalamic structures: site-specific modulation by adrenal and gonadal steroids. *Neuroscience* 57, 537–543

68 Goodman, Y. and Mattson, M.P. (1994) Secreted forms of β-amyloid precursor protein protect hippocampal neurons against amyloid β-peptide-induced oxidative injury. *Exp. Neurol.* 128, 1–12

69 Lecanu, L. *et al.* (2004) Identification of naturally occurring spirostenols preventing β-amyloid-induced neurotoxicity. *Steroids* 69, 1–16

70 Yao, Z.X. *et al.* (1999) Free radicals and lipid peroxidation do not mediate β-amyloid-induced neuronal cell death. *Brain Res.* 847, 203–210

71 Krueger, K.E. and Papadopoulos, V. (1990) Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells. *J. Biol. Chem.* 265, 15015–15022

72 Uchida, K. *et al.* (1993) Immunochemical detection of 4-hydroxynonenal protein adducts in oxidized hepatocytes. *Proc. Natl. Acad. Sci. U. S. A.* 90, 8742–8746

73 Lermontova, N. *et al.* (1998) Effects of tacrine on deficits in active avoidance performance induced by AF64A in rats. *Mol. Chem. Neuropathol.* 33, 51–61

74 Mukhina, T.V. *et al.* (2001) Versatile computerized system for tracking and analysis of water maze tests. *Behav. Res. Methods Instrum. Comput.* 33, 371–380