Georg-August-Universität Göttingen

Neuroendokrine Effekte der endokrinen Disruptoren Vinclozolin und Equol in der erwachsenen männlichen Ratte

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch–Naturwissenschaftlichen Fakultät
der Georg-August-Universität zu Göttingen

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Göttingen, 2007

Georg-August-Universität Göttingen

Neuroendocrine effects of the endocrine disruptors Vinclozolin and Equol in the adult male rat

Doctoral dissertation
in partial fulfillment of the requirements
for the doctoral degree “Ph.D.”
in the Graduate Program
Center for Systems Neuroscience
Faculty of Biology, Georg-August-Universität Göttingen

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Date of the thesis defense: 19 November 2007

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Panida Loutchanwoot Göttingen, 21.09.07

To my parents and family

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1. INTRODUCTION

1.1. Background

The endogenous gonadal steroids, estradiol (E2) and testosterone (T), play pivotal roles in the differentiation, development, and maturation of the brain and its neuroendocrine function like regulation of the hypothalamo-pituitary-gonadal (HPG) axis (Kalra and Kalra, 1983; Mooradian et al., 1987; Levine et al., 1991; McEwen and Alves, 1999). The HPG axis comprises gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus, the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) producing gonadotropes in the pituitary and, in male mammalians, the T-producing Leydig cells of the testis (Fig. 1). T can directly affect the release of GnRH or LH/FSH or indirectly of its conversion to E2 by the enzyme aromatase which is expressed in the brain and pituitary (Abdelgadir et al., 1994; Lephart, 1996; Rochira et al., 2006).

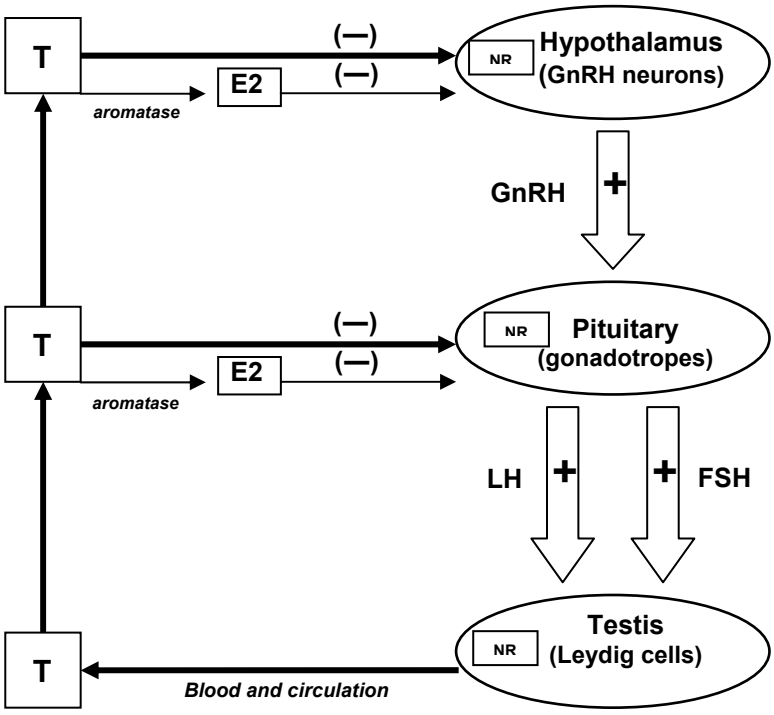


Figure 1. Schematic model of feedback control of the HPG axis by T or E2 (after local production by aromatase activity). NR = nuclear receptors. (adapted from Rochira et al., 2006).

The mechanisms of action of E2 and T are mediated through their specific nuclear receptors (NR) termed as estrogen receptors (ERs), subtypes α (ERα) and β (ERβ), and androgen receptor (AR), respectively. The two ERs are not isoforms of each other, but are the products of distinct genes located on separate chromosomes and differ in their C-terminal ligand-binding domains, suggesting that they could have different effects in some target tissues (Kuiper et al., 1996; 1997). Both ERs and AR belong to a large family of nuclear receptors

and are intracellular ligand-activated transcription factors. After binding the ligand, receptor monomers dimerize and the hormone-receptor complex is translocated into the nucleus where it binds to the specific promoter sequences designated estrogen response element (ERE) or androgen response element (ARE). Upon binding to the response elements, a number of coregulators are recruited to activate transcription of various genes relevant for the regulation of estrogenic or androgenic physiological actions (for reviews see Nilsson et al., 2001; Gelmann, 2002).

There is increasing evidence that besides the endogenous ligands E2 or T numerous anthropogenic chemicals and natural plant-derived products directly interact with ERs and/or AR. Thus, it is conceivable that any compound which is capable to bind to ERs and AR may alter the functions of those systems which are physiologically regulated by endogenous gonadal steroids like development or reproductive function. Those compounds are classified as 'Endocrine Disruptors' (EDs) (Guillette and Gunderson, 2001; Lintelmann et al., 2003; Acerini and Hughes, 2006).

EDs can be divided into three major classes: (1) antiandrogens, primarily pesticides, that are antagonists of the AR function; (2) industrial chemicals with estrogen-like structures that act as agonists or partial agonist for ERs (= xenoestrogens); and (3) plant-derived polyphenolic non-steroidal compounds with estrogen-like structure and actions (= phytoestrogens) (for review see Lintelmann et al., 2003). Several possibilities of the mechanisms of EDs action have been proposed: (a) mimicking the normal activities of endogenous hormones; (b) antagonizing the normal activities of endogenous hormones; (c) disruption of the biosynthesis and metabolism of endogenous hormones; and (d) disruption of the biosynthesis and metabolism of endogenous hormone receptors (for reviews see Sonnenschein and Soto, 1998; Lintelmann et al., 2003).

In the following, two examples of EDs with either estrogenic or antiandrogenic activity will be introduced.

1.2. Estrogenic endocrine disruption by Equol

The first described estrogenic EDs are isoflavones isolated from soy beans. These EDs were named ''Phytoestrogens''. The two major isoflavones in soy beans are genistein and daidzein. Both phytoestrogens can bind to ERα and ERβ with a slight preference to ERβ (Muthyala et al., 2004; Casanova et al., 1999). *In vivo*, isoflavones are metabolized by gut bacteria thereby being inactivated or being converted to still endocrine active or even more potent metabolites (Setchell et al., 1984; 1999; Bowey et al., 2003). For example, the soy-derived isoflavone daidzein is first transformed into dihydrodaidzein (DHD) and from there

further converted to either *O*-desmethylangolensin (*O*-DMA) (5-20%) or equol (EQ) (70%) (Chang and Nair, 1995; Bowey et al., 2003; Decroos et al., 2005) (Fig. 2). While *O*-DMA is inactive with regard to estrogenic properties, EQ is a strong estrogen which finally mediates the well known estrogenic effects of soy in mammalians (Chang et al., 1995; Schmitt et al., 2001; Setchell et al., 2002).

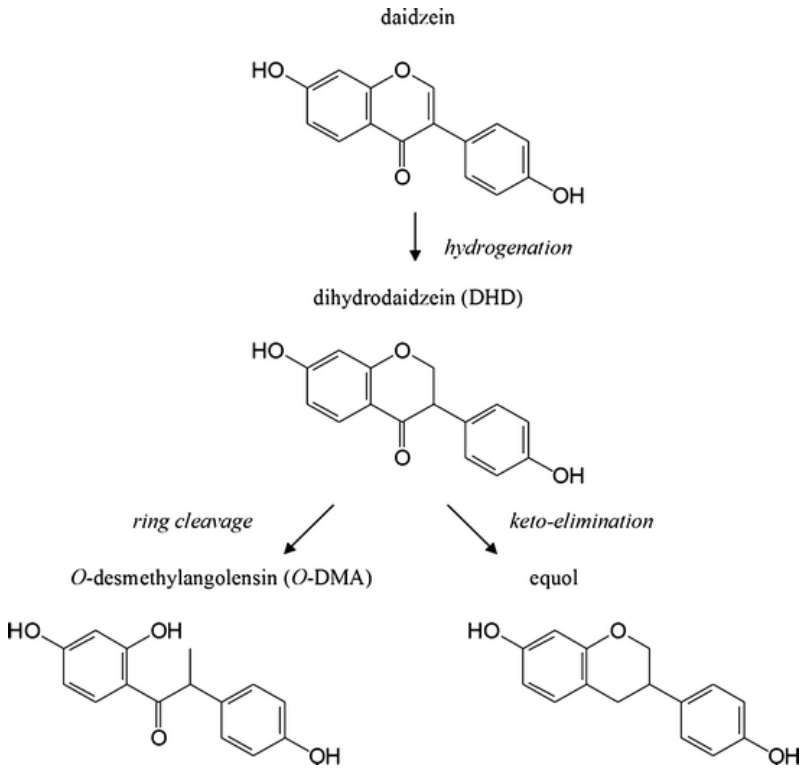


Figure 2. Biodegradation of daidzein by intestinal bacteria (modified from Decroos et al., 2005).

EQ (3, 4-dihydro-3-(4-hydroxyphenyl)-2H-1-benzopyran-7-ol; $C_{15}H_{14}O_3$) has a molecular structure similar to E2. As shown in Figure 3, the distance and orientation of the phenolic (box A) and hydroxyl (box B) groups of EQ is similar to those of the phenolic and hydroxyl moieties of E2. This is the minimum structural requirement for an estrogen-like acting molecule (Setchell et al., 1984; Jordan et al., 1984; 1985).

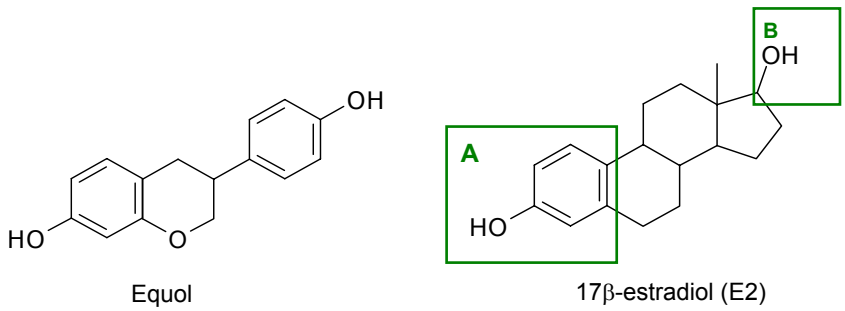


Figure 3. A comparison of the molecular structure of equol and E2 (adapted from Cos et al., 2003).

Asian diets contain high amounts of soy. It is known that in Asian countries like Japan the incidence of steroid dependent cancers is significantly lower than in Western countries (Adlercreutz et al., 1993; Magee and Rowland, 2004). High excretion of EQ in humans consuming high amounts of a soy-based diet indicated that EQ may be responsible for the beneficial role of the dietary phytoestrogens regarding the risks of breast-, uterine-, and prostate cancers in these subpopulations (Setchell et al., 1984; Adlercreutz et al., 1986a; 1986b).

Recent investigations utilizing *in vitro* assays, including competition-binding assays with both ERα and ERβ proteins and transcriptional activation in mammalian cell-based assays, have confirmed that EQ possesses estrogenic properties mediated through ERs with greater affinity to bind to ERβ than to ERα (Sathyamoorthy and Wang, 1997; Mueller et al., 2004; Muthyala et al., 2004). Furthermore, utilizing cell proliferation assays, it was demonstrated that EQ stimulated in an estrogen-like manner the growth of the ERs-positive human mammary adenocarcinoma MCF-7 cells (Sathyamoorthy and Wang, 1997; Ju et al., 2006) and of human endometrial adenocarcinoma Ishikawa cells (Lehmann et al., 2005).

A considerable number of published studies examining the *in vivo* estrogen-like effects of EQ indicated that EQ increased the uterine weight along with a dose-dependent increase of uterine epithelial proliferation and endometrial thickness in adult ovariectomized mice and rats, but with less extent than E2 (Tang and Adams, 1980; Selvaraj et al., 2004; Rachon et al., 2007c). An uterotrophic effect of EQ was also observed in the postmenopausal primate model of the ovariectomized monkey (Wood et al., 2006). Exposures of ovariectomized mice with increasing injected or dietary doses of EQ also caused dose-dependent increases in the vaginal epithelial thickness and vaginal cornification, corroborating its *in vivo* estrogenic effects (Selvaraj et al., 2004). In addition, dietary EQ displayed mild mammotrophic effects in ovariectomized monkeys (Wood et al., 2006). Chronic exposure to dietary EQ stimulated mRNA expression levels of estrogen-responsive genes in the uteri and pituitaries of adult castrated female rats (Rachon et al., 2007a; 2007c).

1.3. Antiandrogenic endocrine disruption by Vinclozolin

Vinclozolin (3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione; $C_{12}H_9Cl_2NO_3$) (VZ) is an agricultural fungicide widely used in the United States of America and the European Union for the control of fungal spore germination in grapes, strawberries, and various vegetables (Kelce et al., 1995; 1997; Gray et al., 1999). VZ is environmentally unstable because it can be further biotransformed by mammalian, plant and bacterial systems, giving rise to several metabolites. The most prominent biologically active

metabolites are 2-(3,5-dichloropheniyl)-carboxymoyl)-2-methyl-3-butenoic acid (M1) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) (Kelce et al., 1995; Sierra-Santoyo et al., 2004) (Fig. 4). VZ and its potent butenoic acid and enanilide metabolites have been found within soil, plants, wildlife, and animals exposed to VZ, and could potentially cause several adverse effects to human and animal health based on its structural similarity related to the potent and pure AR antagonistic drug flutamide (FLUT) and its hydroxylated metabolite, hydroxyflutamide (Kelce et al., 1995) (Fig. 4).

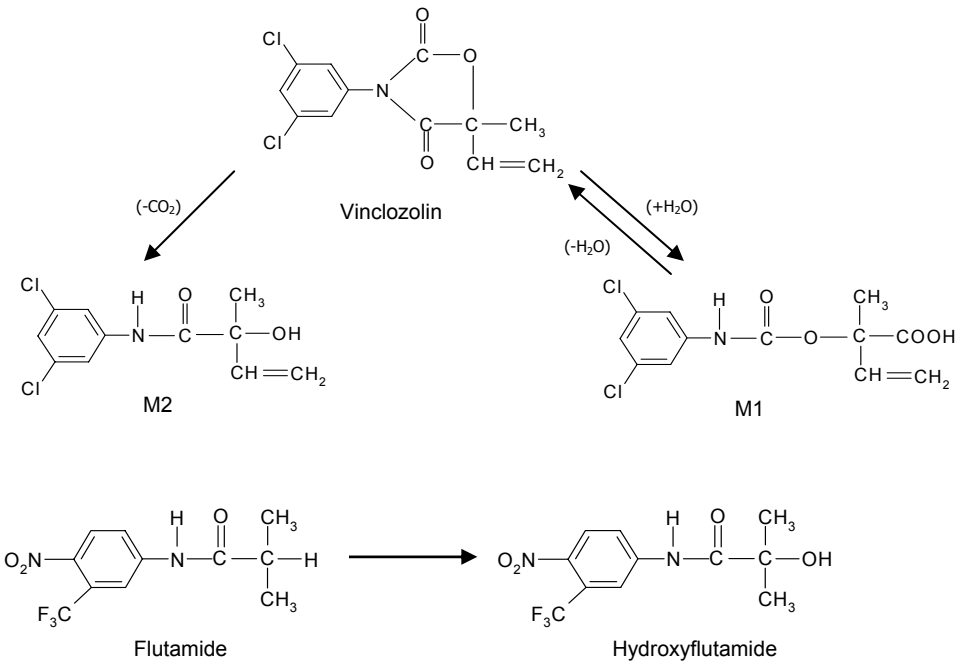


Figure 4. Molecular structures of VZ and its two primary bioactive metabolites, designated M1 and M2, which are similar to those of FLUT and its metabolite, hydroxyflutamide (modified from Kelce et al., 1994; 1995; Sierra-Santoyo et al., 2004).

There is ample evidence that VZ and its two metabolites, M1 and M2, are antiandrogenic endocrine-active substances. Using *in vitro* competition-binding assays with AR protein, or transcriptional activation in mammalian cell-based assays, it has been shown that VZ and its two hydrolysis metabolites bind to both rat and human AR and competitively inhibit endogenous androgens for AR binding, thereby inhibiting AR for binding to the ARE, and subsequently antagonize AR-induced transcriptional activation (Kelce et al., 1994; Wong et al., 1995; Nellemann et al., 2003; Molina-Molina et al., 2006). These results clearly prove that *in vitro* VZ and its two metabolites are potent AR antagonists. *In vivo* VZ exposure during pregnancy of female rats results in a pattern of urogenital malformations in the male pups (Gray et al., 1994; 1999; Matsuura et al., 2005); delays the onset of puberty, growth and development of accessory sex organs in peripubertal male rats (Gray et al., 1994; 1999; Monosson et al., 1999), ultimately resulting in reduced weights of accessory sex organs and increased levels of serum reproductive hormones (LH, FSH, T, DHT) in adult male rats

(Monosson et al., 1999; O'Connor et al., 2002; Shin et al., 2006). In adult castrated, testosterone-treated male rats, VZ causes alterations in the expression levels of AR-regulated genes in the ventral prostate (Kelce et al., 1997; Nellemann et al., 2003), as well as of androgen-dependent testicular and pituitary specific genes in adult male rats (Kubota et al., 2003). Thus, VZ and its metabolites first compete with endogenous androgens for binding to AR, resulting in inhibition of AR-binding to ARE and subsequent AR-induced transcriptional activation both *in vitro* and *in vivo*.

1.4. New endocrine features of EQ and VZ

VZ has been identified as a mixed antagonist or agonists for the AR, depending on the ligand binding affinity, concentration, and the presence of the natural androgens (Wong et al., 1995; Wilson et al., 2002; Korner et al., 2004). The overall antiandrogenic effects of VZ on the pattern of urogenital malformations, or alterations in accessory sex organ weights and serum reproductive hormone levels in adult male rats was nearly identical to that reported for the well-known antiandrogen FLUT (Gray et al., 1994; Nellemann et al., 2003; Yu et al., 2004). Recently, VZ and its two biologically active metabolites have attracted attention because *in vitro* they interact directly with both ERα and ERβ, with the greater affinity for ERα (Scippo et al., 2004; Molina-Molina et al., 2006). These data point to the possibility that the *in vitro* observed ERs agonistic action of VZ may contribute to its endocrine-disrupting properties *in vivo*. The observation of an estrogenic activity of VZ, formerly classified as a pure antiandrogen, raised the question whether EDs only interact with one type of nuclear receptors. In other words, does an estrogenic ED exert also antiandrogenic activity, i.e. is there a promiscuity regarding receptor selectivity?

Indeed, there is increasing evidence that those additional endocrine features are characteristic for EDs. It has been shown recently that EQ in addition to its estrogen-like activity has also antiandrogenic properties because *in vitro* it inhibits proliferation of human benign and malignant prostatic epithelial cells at concentrations typically found in the serum of men consuming a soy-rich diet (Hedlund et al., 2003). Lund et al. (2004) reported that the *in vitro* antiandrogenic properties of EQ are unique since EQ does not bind to the AR, but potently binds DHT (but not T), and thereby prevents DHT from binding to AR. Furthermore, subcutaneous injection of EQ (0.25 mg/kg bw/day or 0.1 mg/rat/day) to intact adult male rats fed *ad libitum* with normal rat diet for 4 days reduced weights of ventral prostate and epididymides without any alteration in testicular weight, and increased serum LH without any change in serum DHT levels (Lund et al., 2004).

1.5. Endocrine disruption and the brain

The deleterious effects of EDs on development and functions of sex steroid-regulated tissues have been investigated thoroughly in peripheral organs in particular the urogenital system in both female and male animals. However, surprisingly little efforts have been made to investigate the pharmacological actions of EDs in the central nervous system (CNS) in spite of the fact that virtually any brain area is sex steroid hormone receptive. Thus, it is clearly apparent that EDs may have the potential to alter brain function like neuroendocrine activity.

FLUT (2-Methyl-N-(4-nitro-3-(trifluoro-methyl) phenyl) propanamide; $C_{11}H_{11}F_3N_2O_3$) (Fig. 4) is well characterized as potent and pure nonsteroidal AR antagonist possessing high specificity for AR without cross-reactions with any other of sex steroid receptors (Simard et al., 1986; Laws et al., 1996; Roy et al., 2004). FLUT has been used therapeutically as an oral antiandrogenic drug primarily to treat prostate cancer or hyperplasia (Labrie, 1993; Singh et al., 2000; Gao et al., 2006), and is widely used as an positive AR antagonist in numerous recent investigations on the role of EDs in modulating androgen metabolism and function during male reproductive development (Kelce et al., 1997; Yu et al., 2004; Kang et al., 2004).

FLUT is metabolized within the gastric tract to its potent hydrolysis metabolite, hydroxyflutamide (Fig. 4) (Neri et al., 1989; Gao et al., 2006). FLUT and its metabolite compete with endogenous androgens for binding to AR, inhibit AR-ARE binding, and alter androgen-induced transcriptional activation *in vitro* (McGinnis and Mirth, 1986; Wong et al., 1995; Sohoni and Sumpter, 1998). *In vivo* perinatal exposure of pregnant rat dams with FLUT throughout gestation caused a pattern of malformations of the urogenital tract, decreased weights of ventral prostate, seminal vesicles and testes, increased serum levels of LH, FSH and T in the male rat pubs (Kassim et al., 1997; Miyata et al., 2002; 2003; Goto et al., 2004; Foster and Harris, 2005). Postnatal exposure of intact adult male rats with FLUT resulted in increased serum levels of LH, FSH, T, DHT and E2, decreased weights of ventral prostate, seminal vesicles, and epididymides (O'Connor et al., 1998a; 2002; Toyoda et al., 2000, Shin et al., 2002), and altered androgen-dependent gene expression in the testis, ventral prostate and pituitary in the adult male rat (Ohsako et al., 2003; Vinggaard et al., 2005). These data indicated that FLUT disrupts the function of the HPG axis of male rats in a typical pattern of an AR antagonist, i.e. decreases weights of androgen-dependent male accessory reproductive tissues and increases levels of serum reproductive hormones, and alters AR-regulated gene expression in the androgen-dependent organs.

Subcutaneous injection or local implants of FLUT as well as its metabolite into various brain regions including the preoptic-hypothalamus-limbic continuum of the adult castrated-T-administered male rats can adversely affect the neuroendocrine function and subsequently

effectively inhibit restoration of male sexual behavior (Sodersten et al., 1975; McGinnis and Mirth, 1986; McGinnis et al., 1996; 2002). Taken together, these data document an endocrine disrupting action of FLUT both in the CNS and its neuroendocrine activity. Surprisingly, while the neuroendocrine effects of the synthetic antiandrogenic drug FLUT are well characterized, with regard to EDs there is a clear lack of data on brain function like neuroendocrine regulation of the HPG axis function in male individuals. This prompted us to investigate whether and how VZ and EQ affect the brain and neuroendocrine function in adult male rats upon an acute treatment.

1.6. Potential action sites of VZ and EQ within the HPG axis of male rats

As described earlier, the prerequisite of the direct endocrine-disrupting action and the initial step of estrogen- and androgen-mediated gene regulation, is binding of EDs to either ERs or AR. In male rats, both ERs and AR are expressed in the urogenital tissues including the prostate, seminal vesicles, and epididymides (Pelletier, 2000; Pelletier et al., 2000; Makela et al., 2000). In addition, in areas of the brain which have been implicated either directly or indirectly in neuroendocrine regulation of the HPG axis, such as the neuroendocrine hypothalamus, particularly the medial preoptic area/anterior hypothalamus (MPOA/AH) and mediobasal hypothalamus/median eminence (MBH/ME), limbic structures (i.e., hippocampus, amygdala) and striatum (Simerly et al., 1990; Laflamme et al., 1998; Creutz and Kritzer, 2004), both ERs and AR are expressed. Also in the pituitary of male rats besides the AR, both ERs are expressed (Shughrue et al., 1998; Pelletier, 2000). Thus, it is reasonable to postulate that VZ and EQ may have potential neuroendocrine effects in these estrogen- and androgen-responsive tissues. However, in adult male rats both ERα and ERβ subtypes are differentially distributed and expressed in the CNS and urogenital system depending on the tissue types, i.e. moderate to high expression in pituitary and epididymides for ERα and hypothalamic-limbic structures, prostate, and seminal vesicles for ERβ (Kuiper et al., 1997; Makela et al., 2000; Pelletier, 2000), suggesting that they could have different and specific functions in some target tissues in response to estrogen or estrogenic compound exposure. Taken together, these data prompt us in the present study to observe both ERα and ERβ, and AR expression in the selected brain regions (MPOA/AH, MBH/ME, hippocampus, amygdala, striatum), pituitary, and male accessory sex glands (prostate, seminal vesicles, epididymides).

Regulation of the HPG axis in the rats depends upon the hypothalamic GnRH pulse generator. This neuronal network causes pulsatile release of GnRH at the ME of MBH into hypophyseal portal vessels, and subsequently regulates pulsatile pituitary gonadotropin LH release from gonadotropes, and consequently the secretion of testicular T for normal

reproductive function and development (Kalra and Kalra et al., 1983; Levine et al.,1991; Wuttke et al., 1996). It is well known that in the rat, cell bodies of GnRH neurons are located within the MPOA/AH, while their axons terminate in the MBH/ME. Since GnRH secretion cannot be measured directly, GnRH mRNA levels were determined as an indirect measure of secretion of the releasing factor (Jarry et al., 2004). GnRH binds with high affinity to the GnRH receptor (GnRHR) on the cell surface of gonadotropes, thereby activating the intracellular signal transduction pathway to affect both synthesis and pulsatile release of gonadotropins. The pituitary responsiveness to GnRH pulses correlates directly with the level of GnRHR expression (Norwitz et al., 1999). Therefore, in the present study, the MPOA/AH GnRH and the MBH/ME as well as pituitary GnRHR mRNA levels were examined.

In the pituitary, besides measurement of the ERs and AR expression, levels of the pituitary-specific, truncated isoform of rat ERα (TERP-1 and -2) mRNA expression (Friend et al., 1997; Mitchner et al., 1998; Tena-Sempere et al., 2001a) which are positively regulated by E2, but not T, were also examined in the present study. In addition, expression levels of the α- and β-subunit of LH which are known E2-regulated genes in the pituitary were also analyzed. Changes in levels of TERP-1/-2, LHβ and α-subunit mRNA expression, and serum prolactin (PRL) can be used as surrogate markers for estrogenic actions of the test compounds in the rat pituitary (Jarry et al., 2004; Klammer et al., 2005; Rachon et al., 2007a).

The classical endpoints for detection of putative antiandrogenic EDs suggested by the several international organizations like the OECD (Organization for Economic Cooperation Development) and the United States Environmental Protection Agency (U.S. EPA) task force on the EDs (EDSTAC), such as wet weights at autopsy of the androgen-dependent male accessory sex organs (prostate, seminal vesicles, and epididymides) and serum concentrations of reproductive hormones [LH, T, dihydrotestosterone (DHT)] were also included to gain a better understanding of ongoing neuroendocrine effects of VZ and EQ on the intact HPG axis in adult male rats.

An other important target of the neuroendocrine-disrupting activity of several EDs is the thyroid gland as the pituitary-thyroid axis is also regulated by E2 action. Thyroid hormones (TH), i.e. thyroxine (T_4) and triiodothyronine (T_3), are major regulators of multiple physiological processes during development, growth, differentiation, and metabolism. Alterations in TH levels affect developing organisms and can result in abnormalities in normal physiological functions of the CNS (Rivas and Naranjo, 2007; des Reis-Lunardelli et al., 2007) and urogenital system (Arambepola et al., 1998; Rao et al., 2003). It is well documented that besides effects on the reproductive neuroendocrine function, several EDs

also affect the TH system. Exposure to certain EDs, such as phytoestrogens like resveratrol and genistein (Bottner et al., 2006a), contaminants derived from the industrial products like the UV filters benzophenone 2 (BP2) (Jarry et al., 2004; Klammer et al., 2005; Schmutzler et al., 2007) and octyl-methoxycinnamate (OMC) (Klammer et al., 2007) and agricultural-derived contaminants, i.e. pesticides (Kelce and Wilson, 1997; O'Connor et al., 2002), can significantly disturb the TH- as well as steroid hormones homeostasis in rats. However, it is not known whether VZ and EQ affect the TH system. Therefore, in the present study the neuroendocrine actions of VZ and EQ were assessed by measurement of the serum levels of thyroid-stimulating hormone (TSH), thyroxine (T_4) and triiodothyronine (T_3), and of the expression of the beta subunit of TSH in the pituitary as previously described (Jarry et al., 2004; Klammer et al., 2007).

In addition, known estrogen-regulated liver parameters, i.e. serum levels of cholesterol (CHOL), high- and low-density lipoproteins (HDL and LDL), and triglycerides (TGs) (Klammer et al., 2005; Schlecht et al., 2006; Rachon et al., 2007b) were also measured additionally to the data of liver weight which generally has to be included to assess typical toxicological effects of the test compounds.

The potential action sites and endpoint parameters selected for the present investigation of the neuroendocrine-disrupting effects of VZ and EQ on the function of the HPG axis in adult male rats are summarized in Table 1.

Table 1 Potential action sites of VZ and EQ within the HPG axis of male rats and endpoints measured in the present study

Endpoints / Organs	Organ weights	Gene expression	Serum levels
MPOA/AH		ERα; ERβ; AR; GnRH	
MBH/ME		ERα; ERβ; AR; GnRHR	
Hippocampus		ERα; ERβ; AR	
Amygdala		ERα; ERβ; AR	
Striatum		ERα; ERβ; AR	
Pituitary		ERα; ERβ; TERP-1/-2; AR; GnRHR; LHβ; α- subunit; TSHβ	LH, TSH, PRL
Thyroid			T_4 ; T_3
Testes	✓		T; DHT
Prostate	✓	ERα; ERβ; AR	
Seminal vesicles	✓	ERα; ERβ; AR	
Epididymides	✓	ERα; ERβ; AR	
Liver	✓		CHOL; TGs; HDL; LDL

1.7. Hypothesis, objectives, and experimental paradigm

As depicted in Figure 5, the hypothesis of the present study was that oral application of the endocrine active substances, VZ and EQ, may alter the brain function like neuroendocrine activity in adult male rats which is associated with altered expression of genes relevant for regulation of the HPG axis function in hypothalamic and limbic brain structures and pituitary. Therefore, the present thesis is designed to investigate *in vivo* potential neuroendocrine effects of VZ and EQ on (a) alterations of both mRNA and protein transcript levels of several genes that are involved in the regulation of androgen/estrogen-dependent non-reproductive and reproductive processes in particular areas of brain (MBH/ME, MPOA/AH, striatum, amygdala, and hippocampus) and pituitary. In addition, mRNA expression in the prostate, seminal vesicles and epididymides as the androgen-dependent reference organs was also evaluated; (b) wet weights at autopsy of ventral prostate, seminal vesicles, epididymides, and liver; (c) serum concentrations of reproductive hormones (LH, T, DHT, PRL) and thyroid parameters (TSH, T_4, T_3); (d) serum levels of cholesterol, high- and low-density lipoproteins, triglycerides, and glucose. As the reference compound, the pure antiandrogenic drug FLUT was employed.

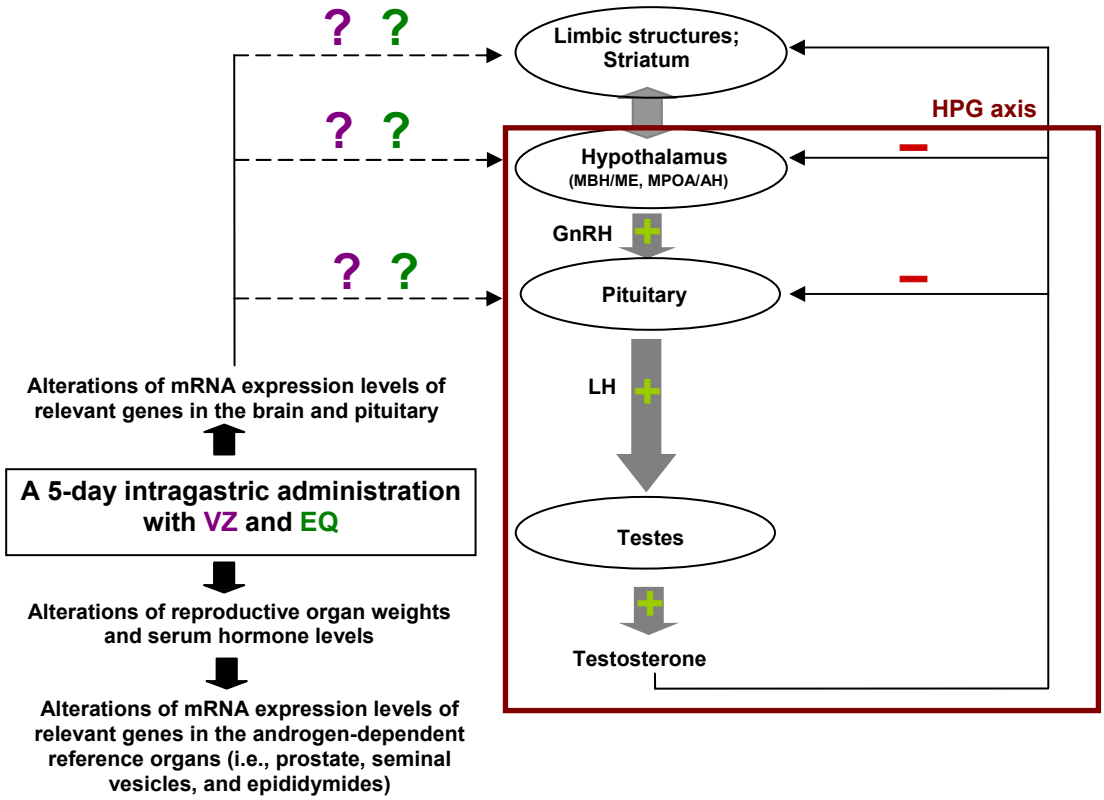


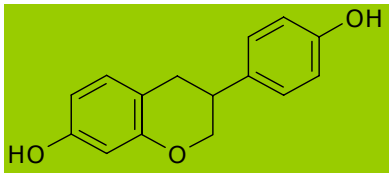
Figure 5. Schematic diagram of the *in vivo* model to investigate the possible endocrine-disrupting effects of VZ and EQ on the brain and its neuroendocrine function like regulation of the HPG axis in adult male rats.

In the present investigation adult male rat, an internationally accepted test system for detection of antiandrogenic endocrine disrupting substances with potential relevance to the human health as previously recommended by the OECD (Gray et al., 2004; O'Connor et al. 2002; Shin et al., 2006), was used. Since E2, converted from T by local aromatase, affects the HPG axis in the male rat, this animal model is also suitable to describe estrogenic effects of an ED. Male rats were treated for 5 consecutive days with the test compounds orally per gavage to imitate the major route of human and animal exposures to these EDs, and also the activity of the gut microflora and the first-pass metabolism of these compounds (Chang et al., 1995; Kelce et al., 1995; Gao et al., 2006). Thereafter animals will be sacrificed. The trunk blood was collected and brain and various peripheral steroid receptive organs were removed. Levels of mRNA and protein expression of relevant genes were determined in the brain, pituitary, and peripheral androgen-regulated organs by means of quantitative TaqMan® Real-time polymerase chain reaction (PCR) and immunocytochemistry. Serum levels of reproductive and thyroid hormones or metabolic parameters, and of the test compounds were analyzed by using rat specific radioimmunoassays (RIA) and high performance liquid chromatography (HPLC), respectively. To avoid the confounding effects of the soy-derived phytoestrogens, genistein and dadizein, which normally are contained in the regular rodent diets at concentrations that could have marked effects on the *in vivo* endpoints of endocrine action (Boettger-Tong et al., 1998; Casanova et al., 1999), we used a specialized diet without soy ingredients, such that the data in the present study result from neuroendocrine effects only of the test compounds.

2. MATERIALS AND METHODS

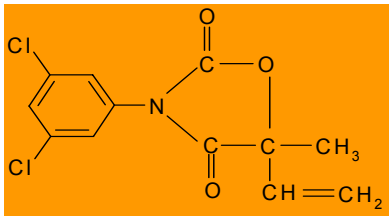
2.1. Test substances

Equol (EQ) was obtained from the Changzhou Dahua Imp. and Exp. (Group) Corp. Ltd. (Changzhou, Jiangsu, China) (Fig. 6). Vinclozolin (VZ) was purchased from Sigma-Aldrich Laborchemikalien GmbH (Taufkirchen, Germany) (Fig. 7) and flutamide (FLUT) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) (Fig. 8). The test substances were dissolved in native olive oil (Caesar&Loretz GmbH, Hilden, Germany).



Regulatory status:	Pharmaceutical agent; Natural product (metabolite)
IUPAC Name:	3,4-dihydro-3-(4-hydroxyphenyl)-2H-1-benzopyran-7-ol
Chemical Name:	(*RS*)4′,7-dihydroxyisoflavone
Chemical Class:	Flavonoid; Isoflavone
CAS Number:	531-95-3
Molecular Formula:	$C_{15}H_{14}O_3$
Pharmacological action:	Endocrine disruptor (ERs agonist)

Figure 6. The chemical structure of equol and its fact sheet.



Regulatory status:	U.S. EPA and OECD registered; General Use Pesticide (non-systemic fungicide), Toxicity Class III
IUPAC Name:	(*RS*)-3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-1,3-oxazolidine-2,4-dione
Chemical Name:	3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione
Chemical Class:	Organochlorine; Cyclic imide; Carbamate
CAS Number:	50471-44-8
Molecular Formula:	$C_{12}H_9Cl_2NO_3$
Pharmacological action:	Endocrine disruptor (AR antagonist)

Figure 7. The structural formula of vinclozolin and its descriptions.

Regulatory status:	U.S. EPA and OECD registered; Pharmaceutical agent:-non-steroidal antiandrogenic drug used for treatment of prostate cancer
IUPAC Name:	2-methyl-*N*-[4-nitro-3-(trifluoromethyl) phenyl]-propanamide
Chemical Name:	2-Methyl-N-[4-nitro-3-(trifluoro-methyl) phenyl] propanamide; α,α,α-trifluoro-2-methyl-4′-nitro-*m*-propionotoluidide; 4′-nitro-3′- trifluoromethylisobutyranilide
Chemical Class:	Amide; Anilide; Nitrobenzene
CAS Number:	13311-84-7
Molecular Formula:	$C_{11}H_{11}F_3N_2O_3$
Pharmacological action:	Endocrine disruptor (pure AR antagonist)

Figure 8. The molecular structure of flutamide and its properties.

2.2. Animals and husbandry

All animals used in this work have been treated in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123). All experiments were conducted following the GLP guidelines published by the OECD, and approved by a permit issue by the Landesamt für Verbraucherschutz, Braunschweig, Germany. Male and female Sprague-Dawley rats used in this study were either raised in the animal facility of the Universitätsmedizin Göttingen (*in vivo* experiment 1) or obtained from Winkelmann GmbH, Borchen, Germany (*in vivo* experiment 2). All animals were acclimatized to the laboratory environment for 7-14 days prior to use. Upon the day of arrival, animals were fed with soy-free chow (Ssniff GmbH, Soest, Germany) and water *ad libitum*, and housed 5-6 animals per Makrolon® cage type IV under the standardized environmental conditions (room temperature 22-24°C, relative humidity of 50-55%, illumination from 06:00 until 06:00 p.m.) throughout the study period.

2.3. Animals and treatments

2.3.1. *In vivo* experiment 1: Neuroendocrine effects of VZ in adult male rats

At the age of 4 months, male rats were weighed and divided by randomization into two treatment groups (n = 10/group), i.e. a control and a VZ group, so that there were no statistically significant differences among the group body weight means. Animals were treated orally *via* gavage once per day for 5 consecutive days with either the vehicle olive oil (1 ml/rat/day) as the negative control, or VZ (150 mg/kg bw/day in 1 ml oil). As shown in

previous *in vivo* studies, at this dose VZ alters male sexual differentiation, reproductive function and development without any adverse toxic effects or increased mortality (Gray et al., 1994; O' Connor et al., 2002). VZ suspension was prepared daily 16 h before application. The treatments were conducted at 8.00 a.m. During the 5-day treatment interval, the body weight, food intake, and clinical symptoms were recorded daily. The time line of the study design of the *in vivo* experiment 1 is shown in Fig. 9.

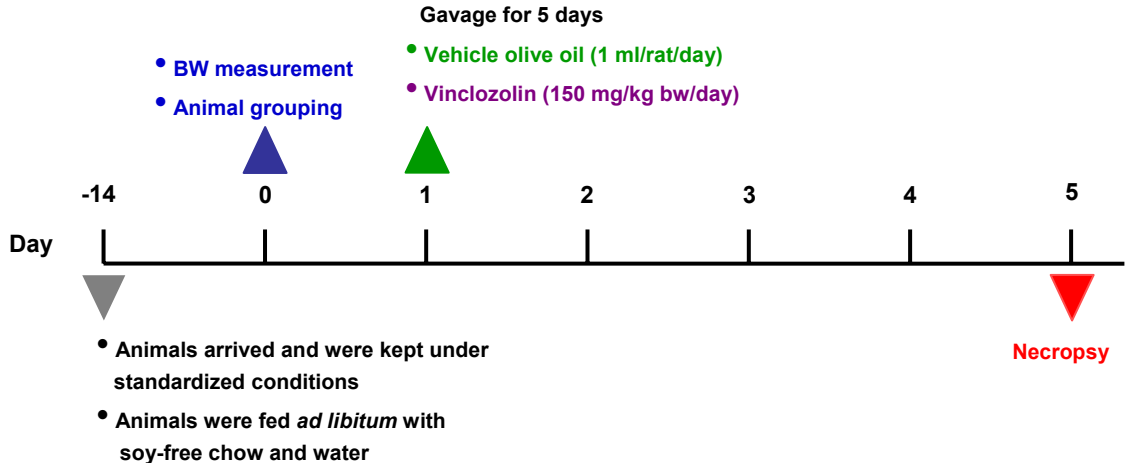


Figure 9. Time line of the study design of the *in vivo* experiment 1.

2.3.2. *In vivo* experiment 2: Neuroendocrine effects of EQ in adult male rats

At the age of 4 months, male rats were weighed and divided by randomization into four groups (n = 12/group), i.e. control-, EQ low dose-, EQ high dose-, and FLUT-group, so that there were no statistically significant differences between the group body weight means. Animals were treated orally *via* gavage once per day for 5 consecutive days with either the vehicle olive oil (1 ml/rat/day) as the negative control, EQ low dose (100 mg/kg bw/day) and high dose (250 mg/kg bw/day), or FLUT at a dose of 100 mg/kg bw/day. The low concentration of EQ was chosen to determine whether putative antiandrogenic effects of EQ could be detected at a dose which in female ovariectomized rats induced estrogen-like actions in the pituitary and uterus without adverse toxic effects or increased mortility (Rachon et al., 2007a; 2007c). The higher dose of EQ (250 mg/kg bw/day) was selected to determine potential antiandrogenic effects of EQ at a pharmacological dose. An additional group of animals was treated with FLUT at the dose of 100 mg/kg bw/day as positive antiandrogenic reference control. The dose of FLUT was selected based on previous *in vivo* studies, reporting the typical antiandrogenic effects, such as increased serum levels of reproductive hormones, and decreased the size of androgen-dependent male accessory reproductive tissues without any adverse toxic effects or increased mortality in adult male rats (O' Connor et al., 2002; Kunimatsu et al., 2004). Also, FLUT at this dose was in range that has been

previously shown to inbibit androgen-induced male sexual behavior in adult male rats (Sodersten et al., 1975; Gray, 1977; Gladue and Clemens, 1980). In the present study, all test substances were applied as a suspension in olive oil in a volume of 1 ml per animal per day. The treatments were conducted at 8.00 a.m. During a 5-day treatment period, the body weight, food intake, and clinical symptoms were recorded daily. At the end of the treatment interval, each male rat performed a mating test, thereafter the animals were sacrificed. The time line of the study design of the *in vivo* experiment 2 is shown in Fig. 10.

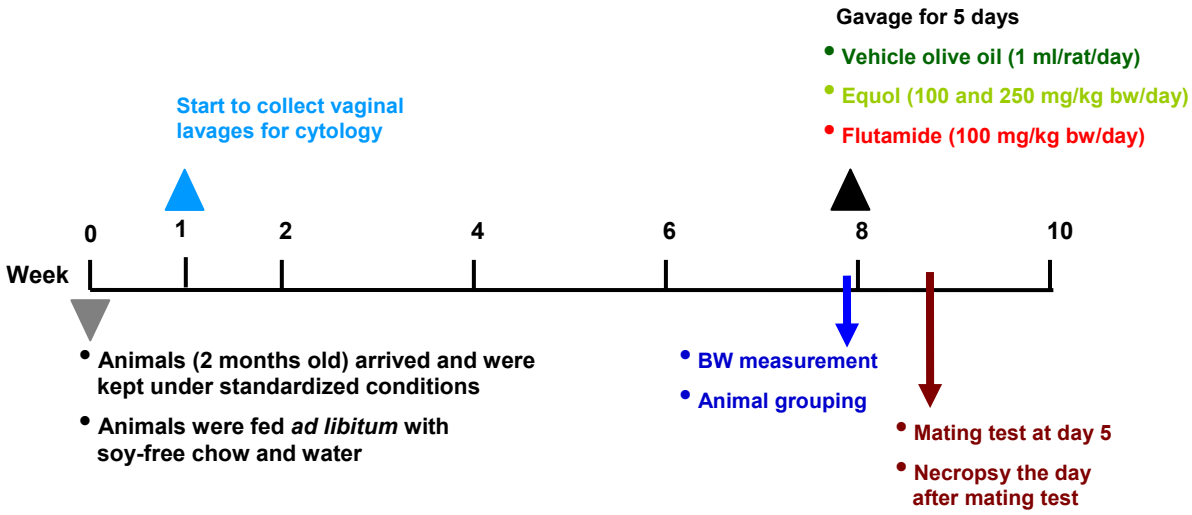


Figure 10. Time line of the experimental design of the *in vivo* experiment 2.

2.4. Vaginal cytology

Monitoring of vaginal cytology was performed with virgin female rats (2 months old) for at least 4 consecutive estrus cycles prior to the mating test. Vaginal smears were collected daily between 09:00 and 10:00 a.m. for identification of the estrus cycle phase. The mean duration of the estrus cycle of young adult female rat is 4 days, comprising the 4 phases; proestrus (P), estrus (E), diestrus 1 (D1), and diestrus 2 (D2) that are characterized *via* changes in the vaginal cytology (Fig. 11). Vaginal cells were collected with a plastic pipette tip filled with 200 µl of 0.9% NaCl. The vagina was flushed 2-3 times, or until the saline became milky. The vaginal fluid containing the suspended cells was then transferred onto a 10 well plastic plate (BIOPLATE®, Labdesign, Sweden). To determine the estrus cycle phase, the unstained native vaginal cell suspension was evaluated under a light microscope (Zeiss axioplan microscope, Zeiss, Germany) by using the 10x objective. In the vaginal samples three types of cell populations are present: -proestrus: round-shaped, nucleated, large epithelial cells (Fig. 11A); -estrus: large irregular shaped, anucleated, cornified cells (Fig. 11B); -diestrus (1 and 2): leukocytes (Fig. 11C and D).

Estradiol secretion peaks during the afternoon of proestrus, and then declines during the dark period of estrus. The LH surge occurs around 3-7 p.m. In the meantime, the sexual receptivity is increased. Therefore, in this study male rats were cohabitated with females for mating at 4 p.m. The ovulation occurs between 1-3 a.m. of estrus day (Fig. 12).

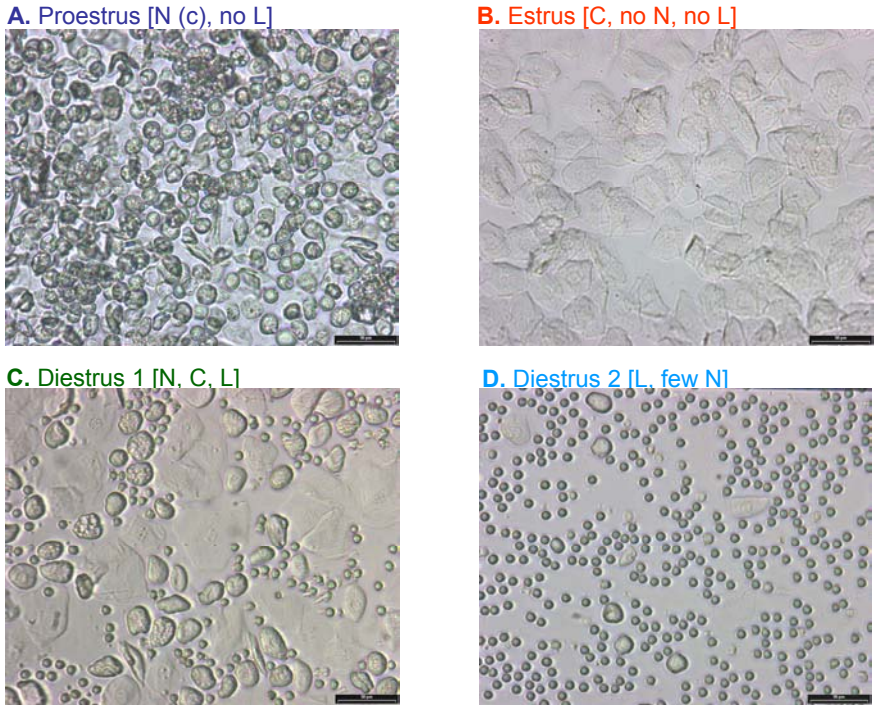


Figure 11. Photomicrographs of the native vaginal smears collected from female rats at proestrus (**A**); estrus (**B**); diestrus 1 (**C**); and diestrus 2 (**D**). All photos were taken at the same magnification (25x). 'N' = nucleated epithelial cells. 'C' = cornified epithelial cells. 'L' = leukocytes. Scale bar = 50 µm.

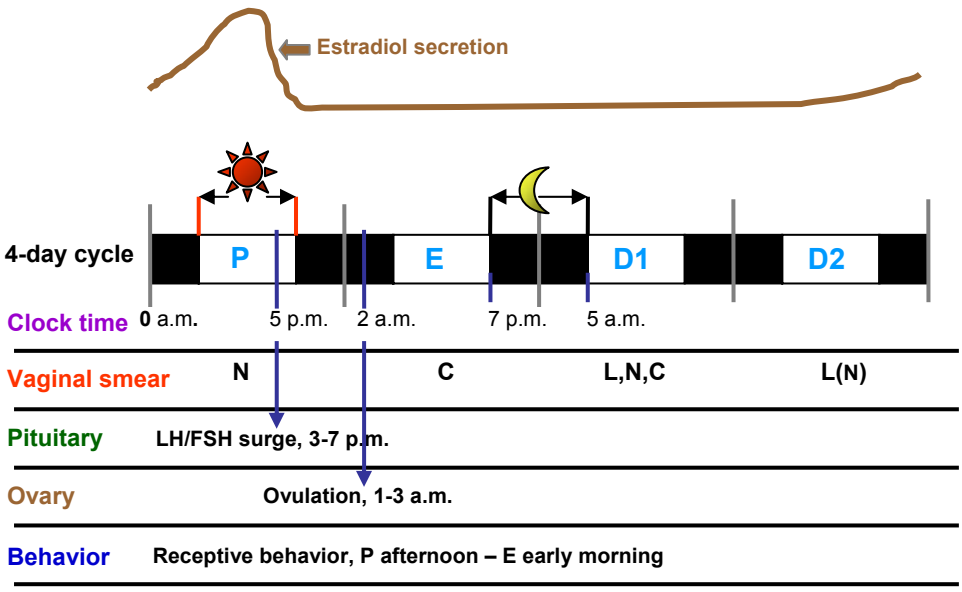


Figure 12. Behavioral rhythms of the rat's estrus cycle (modified from Maeda et al., 2000). 'N' = nucleated epithelial cells. 'C' = cornified epithelial cells. 'L' = leukocytes.

2.5. Mating behavioral testing procedure

Mating behavior was tested at the end of the 5-day treatment interval with sexually receptive females of the same age. The test was conducted at 4 p.m. on the day of proestrus. Male rats were placed separately in the females's cage, and were left overnight with the female. Next morning, females were checked for the presence of a vaginal plug, then the vaginal smear was performed to examine the presence of spermatozoa in the vaginal lavage. In this study, the presence of a vaginal plug and/or spermatozoa in the vaginal lavages was used as an indicator of positive mating behavior as previously described (Cicero et al., 2002). Following positive mating, the occurrence of female pregnancies was also recorded

2.6. Necropsy, collection of target organs, and measurement of organ weights

Two hours after the last application or after completion of the mating behavioral test, necropsy was performed. To avoid variations due to 'time-of-day' effects from each treatment group one animal was killed in consecutive order. Animals were rapidly decapitated under deep CO_2 anesthesia. Blood was collected from the trunk, stored immediately on ice and kept at 4°C for further isolation of the serum. Brains were carefully dissected and immediately frozen on dry ice, and were then kept at -70°C until RNA extraction. The pituitaries were removed from the skull, and the anterior part was immediately snapped frozen in liquid nitrogen and kept at -70°C for further RNA preparation, or immediately kept in 10% neutral buffered formalin (NBF), pH 7.0 for further histological and immunocytochemical studies. The testes, ventral prostates, seminal vesicles plus coagulating glands including luminal fluid, epididymides, and livers were dissected, carefully trimmed free of fat remnant and weighed. After weighing, all target organs were immediately snapped frozen in liquid nitrogen, and then stored at -70ºC for RNA extraction.

2.7. Serum hormone analysis

The blood samples were spun down by centrifugation at 3,000 rpm for 20 min at room temperature with an Eppendorf bench top centrifuge 5413 (Eppendorf, Hamburg, Germany) to isolate the serum. The serum samples were stored at -20°C until analysis of the concentrations of hormones, metabolic parameters, and test substances. Levels of LH, TSH, and PRL were measured with rat specific radioimmunoassays (RIA) supplied by the National Hormone and Pituitary Program of the NIH as previously described (Roth et al., 2001b). Serum concentrations of T, DHT, total T_4 and T_3 were determined with commercially available RIA kits (Diagnostic Systems Laboratories (DSL), Inc., Sinsheim, Germany). The kits were used according to instructions of the manufacturer.

2.8. Serum metabolic parameter analysis

The concentrations of the lipid metabolic parameters, cholesterol (CHOL), high- and low-density lipoproteins (HDL and LDL) and triglycerides (TGs), were analyzed using an automatic analyzer (Roche/Hitachi 902, Boehringer, Mannheim, Germany) and commercially available RIA kits (Roche). The kits were used according to the manufacturer's instructions.

2.9. HPLC analysis of serum levels of the test substances

Serum concentrations of test substances were analyzed by HPLC-UV detection after extraction and hydrolysis of the samples as previously described (Christoffel et al., 2006; Bottner et al., 2006b). In brief, 500 µl of serum from each animal was transferred into a 24 well plate, mixed with an equal volume of NH_4 acetate buffer (pH 5.0) containing 1 mg β-glucuronidase (*Helix pomatia* β-glucuronidase and sulfatase, Type H1, Sigma-Aldrich Laborchemikalien GmbH, Hannover, Germany), and incubated 12 h at 37°C to eliminate protein binding and to hydrolyze glucuronidated or sulfated metabolites of the test substances. Sample cleanup was accomplished by solid phase extraction. Columns of 60 mg bed mass/3 ml washing and elution volume (strata-X 8B-S100-UBJ, Phenomenex Ltd. Deutschland, Aschaffenburg, Germany) were used according to the manufacturer's instructions. Eluted substances were evaporated to dryness in a centrifugal concentrator (SpeedVac model SVC 200H, Savant, Alberville, USA). Samples were reconstituted with 100 µl of absolute ethanol, and filtered through a 0.45 µm PVDF membrane (Cat. No. SLHVR04NL, Millipore GmbH, Schwalbach, Germany) to remove remaining protein contaminants. 20 µl were injected into the HPLC-UV detection system (HPLC Pump K-501, Knauer, Berlin, Germany). The sample was analyzed on a NC 250 x 4.6 mm, Hypersil-ODS 5.0 µm column (Bischoff, Leonberg, Germany) protected by a precolumn 7.5 x 4.6 mm, C18 5 µm (Jasco GmbH, Gross-Umstadt, Germany). Eluent A was ddH_2O containing with 0.085% H_3PO_4, whereas eluent B was acetonitrile (100%). The flow rate was 1 ml/min with a gradient starting with *t*=0 min: A 75%, B 25%; *t*=5 min: A 75%, B 25%; *t*=10 min: A 20%, B 80%; *t*=25 min: B 100%; *t*=30 min: B 100%; *t*=32 min: A 75%, B 25%. The signal was detected with an UV/visible spectrophotometer (LC-95, Perkin-Elmer LAS (Deutschland) GmbH, Rodgau-Jügesheim, Germany) at λ 228 nm for VZ, at λ 260 nm for EQ, and at λ 286 nm for FLUT. The average retention times (min) were 15.4 (VZ), 13.2 (EQ), and 14.7 (FLUT). Serum levels of VZ, EQ, and FLUT were calculated on the basis of a standard curve utilizing serum spikes of each substance.

2.10. Microdissection of brain areas

Deeply frozen brains were mounted on a freezing microtome (model 1206, Leica Microsystems Nussloch GmbH, Wetzlar, Germany) by using Tissue-Tek® O.C.T.™ (Sakura Finetek Europe, Netherland), and warmed up to a temperature of -10°C. Then brains were sliced into serial frontal sections (600 µm thick), thaw-mounted on a conventional glass slide, immediately stored on dry ice, and then kept at -70°C until use.

Bilateral tissue micropunches of the MPOA/AH, amygdala, striatum, and hippocampus were taken from frozen coronal sections with a 1-mm-diameter stainless needle according to the method of Palkovits (1973). The stereotaxic coordinates according to the brain atlas of de Groot (Pellegrino et al., 1979) were: MPOA/AH (A 7.8, L 0.75, V -1.8); striatum (A 7.8, L 2.5, V 1.5); amygdala (A 7.8, L 4.5, V -2.0); hippocampus (A 4.2, L 2.0, V 2.3). The coordinates refer to the rostral plane of the respective section and the center of the needle (see Fig. 13 and 14). With a sharpened scalpel, the MBH/ME was dissected between optic chiasm, hypothalamic grooves and mammillary bodies with a cut depth of 2 mm as described previously (Arias et al., 1993) (Fig. 15).

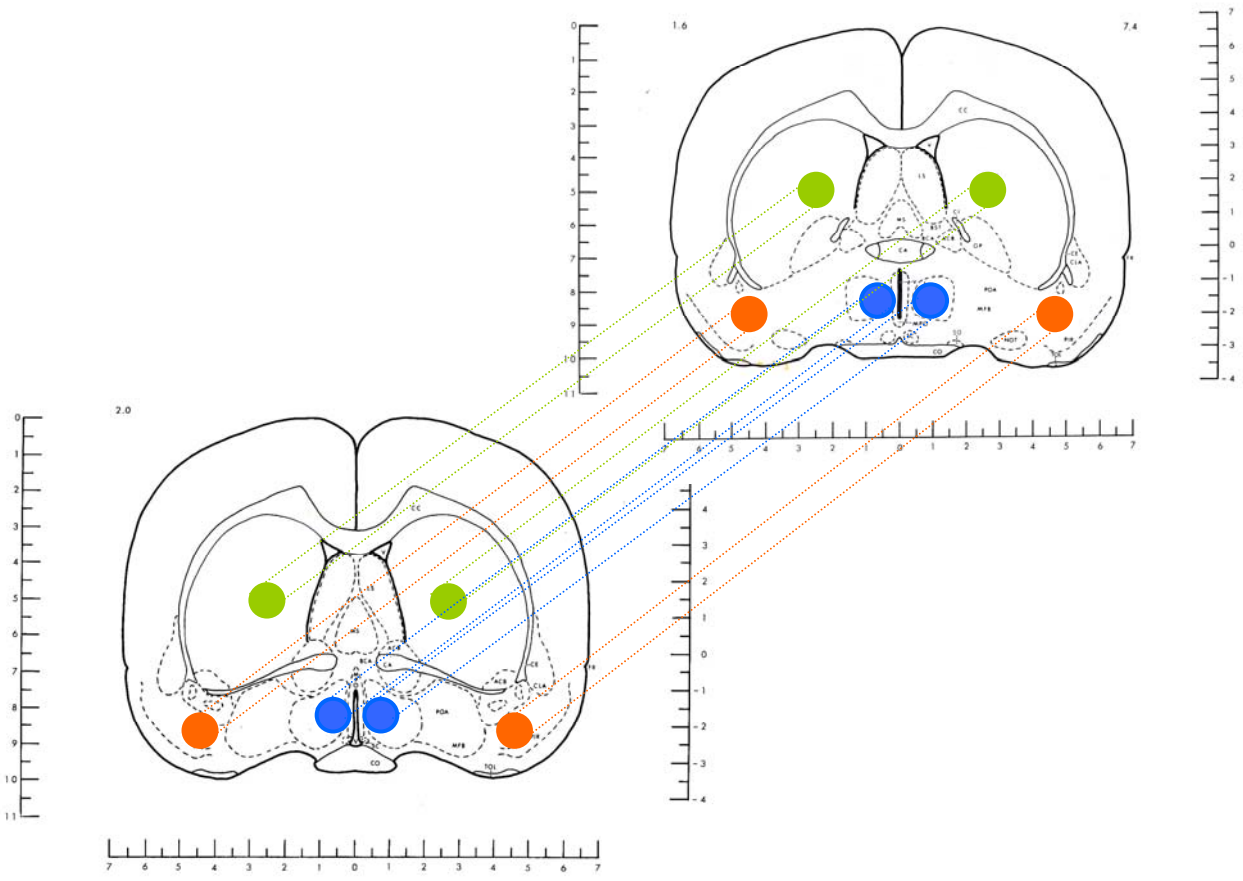


Figure 13. Microdissection of the MPOA/AH, striatum, and amygdala.

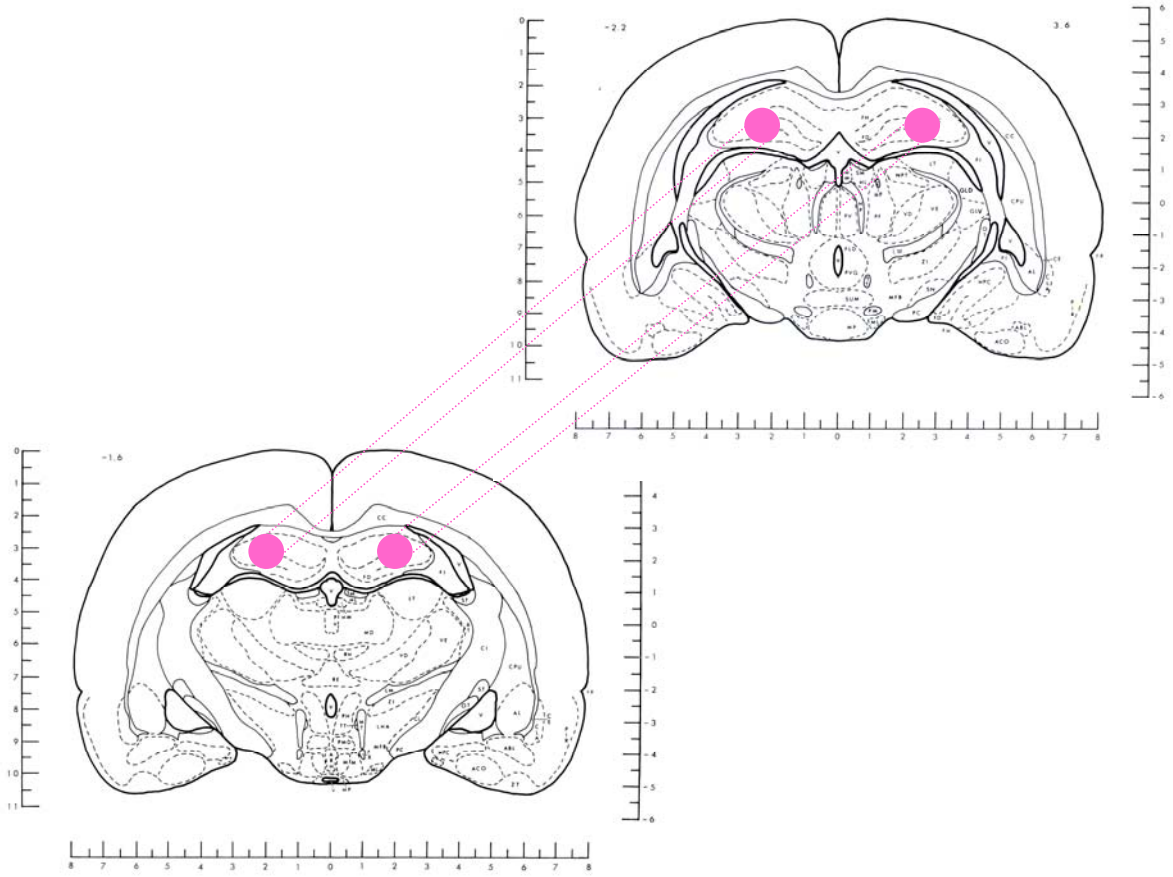


Figure 14. Microdissection of the hippocampus.

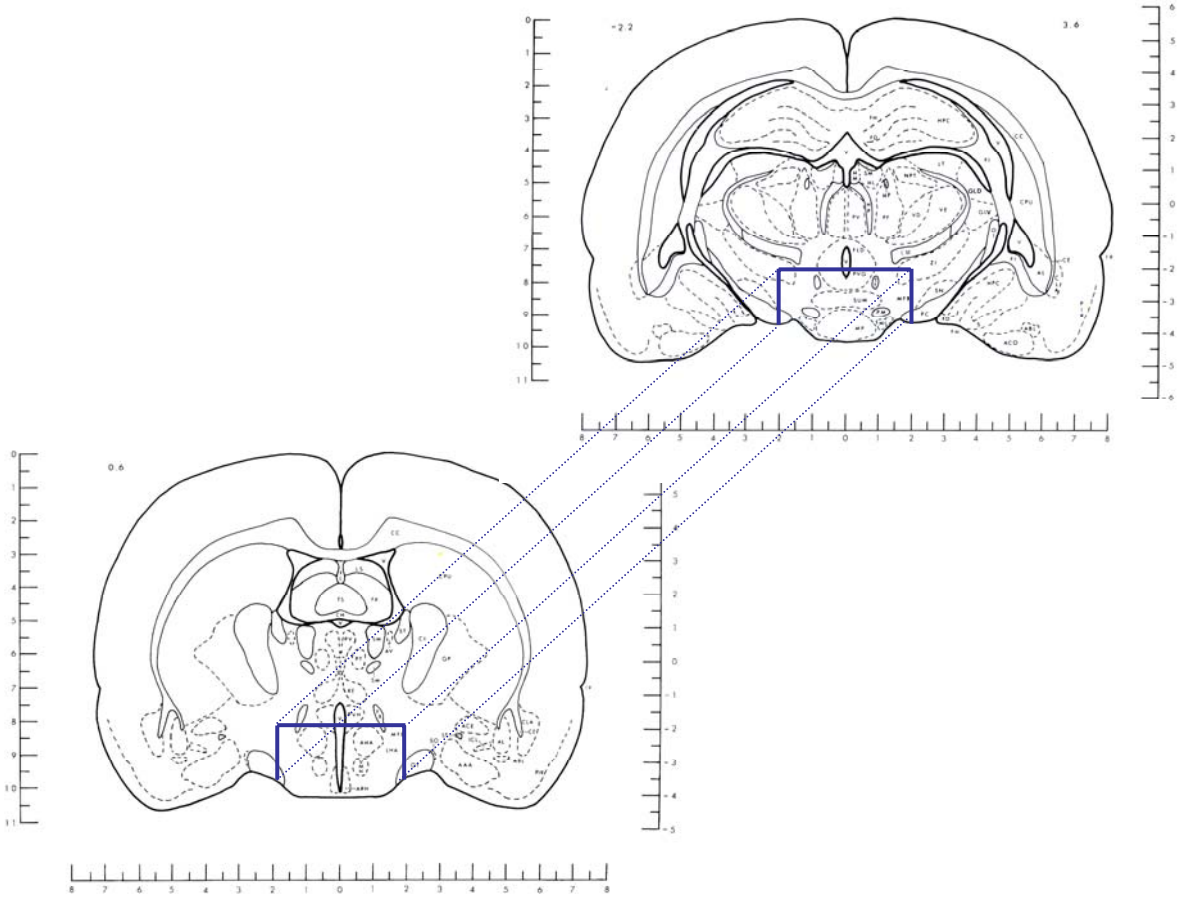


Figure 15. Micodissection of the MBH/ME.

2.11. Tissue homogenization and RNA extraction

The microdissected MBH/ME and pituitary tissues were minced with sharpened hypodermic needle in 500 µl of lysis buffer provided with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Samples were further homogenized by ultrasonication for 10 sec on ice (Sonifier® Cell disruptor model B-12, Branson Sonic Power Company, Danburg, CT, USA). Tissue micropunches of MPOA/AH, striatum, amygdala and hippocampus were directly disrupted and homogenized by ultrasonication for 10 sec on ice with an ultrasonic-cell disruptor in 250 µl of lysis buffer as previously described (Roth et al., 2001b). Prostate, seminal vesicles, and epididymis tissue samples were chilled in liquid nitrogen and pulverized with a Micro-Dismembrator (B. Braun Biotech International GmbH, Melsungen, Germany) by rapid agitation at 2,500 rpm for 20 sec. Approximately 30 mg fine powder of the prostate, seminal vesicles, and epididymis tissues were sonicated for 10 sec in 500 µl of lysis buffer.

Total RNA extraction was carried out with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. To avoid the presence of genomic DNA all samples were treated with DNase using the RNase-Free DNase I Set (Qiagen) after RNA extraction. Total RNA concentrations of the MBH/ME, pituitary, prostate, seminal vesicles, and epididymis tissue samples were determined by absorption measurement at 260 nm and 280 nm utilizing an UV spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany), then adjusted to a final concentration of 20 ng/µl with the RNase-free water, and immediately kept at -70°C for further gene expression analysis. Because of the low amount of RNA contained in the tissue micropunches of MPOA/AH, amygdala, striatum and hippocampus, the measurement of total RNA concentrations from these microdissected brain areas was performed with the VersaFluor™ fluorometer (Bio-Rad, USA) and the RiboGreen® RNA Quantitation kit (Molecular Probes Eugene, Oregon, USA) according to the manufacturer's instructions. The RNA obtained from these brain area samples was adjusted to a final concentration of 5 ng/µl with RNase-free water, and stored at -70°C until use for gene expression analysis.

2.12. Gene expression analysis

Relative changes of gene expression were determined by the *two-step* quantitative real-time reverse transcription polymerase chain reaction assay. In the first step, mRNA from the tissue samples was reversely transcribed to a complementary DNA (cDNA) by the Molony Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) enzyme, using random oligonucleotides as primers. In the second step, cDNA is amplified with the TaqMan® Real-time PCR assay which allows for the direct quantification of the PCR products in each PCR cycle.

2.12.1. Reverse transcription

Reverse transcription was performed in a thermal cycler (*T3* Thermocycler, Biometra GmbH, Göttingen). Each reaction was carried out in a total volume of 20 µl containing 1x M-MLV RT reaction buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, 50 mM dithiothreitol) (Promega, Madison WI, USA), 100 ng random primer hexamers (Invitrogen™, Karlsruhe, Germany), 0.5 mM dNTP mix (Invitrogen™), 200 U M-MLV RT, RNase H Minus, Point Mutant (Promega), 4 U Recombinant RNasin® Ribonuclease Inhibitor (Promega), and 50 ng total RNA of MPOA/AH, striatum, amygdala and hippocampus, or 200 ng total RNA of MBH/ME, pituitary, prostate, seminal vesicles, and epididymis tissues. Samples were incubated for 10 min at 22°C to allow primer annealing, reverse transcription was conducted at 42°C for 50 min, and finally RNA–cDNA hybrids and enzyme were denatured for 10 min at 95°C. Table 2 summarizes the reverse transcription components and conditions.

Table 2 Reverse transcription components and conditions per one reaction mix for cDNA synthesis

Component	Stock concentration	Volume (µl)
RNA template in a sterile RNase-free water	5 ng/µl (brain tissue) or 20 ng/µl (pituitary and reproductive tissues)	10
Random primer hexamers in 3 mM Tris-HCl (pH 7.0), 0.2 mM EDTA	0.1 µg/µl	1
RNase-free water	-	3
Total volume		14
Mix gently. Incubate the mixture at 70°C for 10 min to disrupt the secondary structures, then cool quickly on ice for 5 min. Add the following components as below.		
M-MLV Reverse Transcriptase Reaction Buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, 50 mM dithiothreitol)	5X	4
dNTP mix (PCR grade)	10 mM	1
M-MLV RT, RNase H Minus, Point Mutant	200 U/µl	1
Recombinant RNasin®, RNase Inhibitor	40 U/µl	0.1
Total volume		20
Mix gently. Incubate the mixture at 22°C for the initial 10 min, then 42°C for the final 50 min. Inactivate the reaction by heating for 10 min at 95°C. The cDNA can now be used as a template for the quantitative TaqMan® Real-time PCR Assay.		

2.12.2. Quantitative TaqMan® Real-time PCR assay

The real-time quantitative TaqMan® PCR assay was performed as described previously (Roth et al., 2001a) by using the ABI Prism®7700 Sequence Detection System (SDS) (PE Applied Biosystems, Foster City, CA, USA) and the qPCR core reagent kit (Eurogentec, Seraing, Belgium). The gene-specific TaqMan primer pairs and probes were designed with the Primer Express software (PE Applied Biosystems, Weiterstadt, Germany) and purchased from Eurogentec (Seraing, Belgium). The oligonucleotide sequences and accession numbers of each analyzed gene, including of the size of PCR products are summarized in Table 3.

Table 3 Sequences of the gene-specific TaqMan primers and probes used in the qRT-PCR assay (FAM: 6-carboxy-fluorescein; TAMRA: 6-carboxyl-tetramethyl-rhodamine)

Gene (Accession No.)	**Probe (5´−FAM−3´TAMRA)**	**Forward primer** **Reverse primer**
AR (M23264)	5'-CGCTTCTACCAGCTCACCAAGCTCCT-3'	5'-AGGAACTTGATCGCATCATTGC-3' 5'-CTGCCATCATTTCAGGAA-3'
ERα (X61098)	5'-CGTCTGGCCCAGCTCCTCCTCATC-3'	5'-AAGCTGGCCTGACTCTGCAG-3' 5'-GCAGGTCATAGAGAGGCACGA-3'
ERβ (U57439)	5'-ACGCGGTGACAGATGCCCTG-3'	5'-CTCTGTGTGAAGGCCATGAT-3' 5'-GGAGATACCACTCTTCGCAATC-3'
GnRH (NM012767)	5'-TCTGCGAGGAGCTCTGGAACGTCTG-3'	5'-GCAGAACCCCAGAACTTCGA-3' 5'-TGCCCAGCTTCCTCTTCAAT-3'
GnRHR (L07646)	5'-TTCATGCCACCATTGCGGAAAGCTG-3'	5'-AGGGATGATGAACAGGCAGC-3' 5'-TCTCGCAATGTGTGACCCAC-3'
LHβ (NM012858)	5'-CTGCCTTGCCTCCCGTGCCTCA-3'	5'-ACCTTCACCACCAGCATCTGT-3' 5'-AGCTCACGGTAGGTGCACACT-3'
α-subunit (V01252)	5'-TGCCCTGGAGAAGCAACAGCCCAT-3'	5'-TCTTGGACCTTGCGGGAGT-3' 5'-GGTGCCCCCATCTATCAGTG-3'
TERP-1 (L38931)	5'-TTGAACAGCGACCAGGCTTT-3'	5'-TTGAACAGCGACCAGGCTTT -3' 5'-AGTTAGGAGCAAACAGGAGCTTCC-3'
TERP-2 (L38931)	5'-AGATCCCAAGAGTAAGCAGAGCAGCGAGC-3'	5'-CCATTTCTTGAGCTTTGTTGAACAG-3' 5'-CCCAAAGCCGAAAAGTCAAGT-3'
TSHβ (BC058488)	5'-TCAACACACCATCTGCGCTGGG-3'	5'-GATGTACGTGGACAGGAGAGAGTGT-3' 5'-GACATCCTGAGAGAGTGCGTACTTG-3'

2.12.2.1. Set up conditions for the quantitative TaqMan® Real-time PCR assay

The preparation of the 2x ABI TaqMan™ Universal PCR Master Mix from the qPCR Core kit (Eurogentec) is summarized in Table 4. Add all components, and mix thoroughly by inversion and spin down prior to use. The PCR Master Mix can be stored at 4°C for up to 14 days.

Table 4 Components of 2x ABI TaqMan™ Universal PCR Master Mix

Component	**For 1 reaction (µl)**	**For 1000 reaction (µl)**
10x Reaction buffer	2.5	2500
50 mM $MgCl_2$	1.75	1750
5 mM dNTP mix	1	1000
HotGoldStar *Taq* polymerase	0.125	125
Uracil-N-glycocylase (UNG; 1 U/µl)	0.25	250
Ampuwa	6.875	6875
Total volume	12.5	12500

Amplification reactions were carried out in a 25 µl volume containing 1x Master Mix, 50–900 nM each of gene-specific primers, 200-225 nM gene-specific probe, and 2-5 µl cDNA samples. The preparation of a 25 µl reaction mix is shown in Table 5. All reagents were added together (except for the template) into the reaction vial, mixed thoroughly using a vortex, and then spun down. 20 (21 or 23) µl of reaction mix was pipetted into a MicroAmp Optical 96-well reaction plate (Ref/No. 72.985, SARSTEDT, Nümbrecht, Germany). 5 (4 or 2) µl of the cDNA template, 2 µl of the cDNA standard (positive control), and ampuwa (negative control) with an equal volume of the cDNA template were pipetted into each corresponding well of a reaction plate. The plate was covered with the Optical Caps (Ref/No. 65.986, SARSTEDT), and spun down for 5 min at 3,000 rpm by using the mid bench centrifuge (Labofuge 400, Heraeus Instruments, Osterrode, Germany), and then placed into the sample block of the ABI Prism®7700 Sequence Detector.

Table 5 Preparation of a 25 µl PCR Reaction Mix

Reagent	Volume (µl)	Final concentration
2x ABI TaqMan™ Universal PCR Master Mix	12.5	1X
Forward primer (10 µM)	0.125; 0.75; 2.25	50; 300; 900 nM
Reverse primer (10 µM)	0.125; 0.75; 2.25	50; 300; 900 nM
Probe (10 µM)	0.5; 0.5625	200; 225 nM
cDNA template	2; 4; 5	-
Ampuwa	8.4375; 6.4375; 5.5	-
Total volume	25	-

In the initial step, the samples were incubated for 2 min at 50°C for elimination of carryover PCR products by uracil DNA glycosylase treatment, and then for 10 min at 95°C for activation of the HotGoldStar PCR enzyme. Subsequently, samples were amplified for 40 cycles. Each cycle consists of a denaturation step of 15 sec at 95°C and a combined annealing/extension (release of fluorescent reporter) step of 1 min at 60°C. When the PCR run was completed, the output data were analyzed using the SDS software and exported into a Microsoft Excel file for analysis.

The optimized concentrations of the gene-specific TaqMan primer pairs and probes are summarized in Table 6.

Table 6 Optimized concentrations of the corresponding TaqMan primer pairs and probe of each gene investigated in the present study

Gene	Concentration (nM)		
	Forward primer	Reverse primer	Probe
AR	300	300	200
ERα	50	900	225
ERβ	300	300	225
GnRH	900	900	225
GnRHR	300	300	225
LH-β	300	300	200
α-subunit	300	300	225
TERP-1	300	300	225
TERP-2	300	300	225
TSHβ	300	300	225

2.12.2.2. Standard curves

Each PCR cycle run on an ABI Prism®7700 SDS included: (a) serial dilutions of eight duplicate cDNA samples of known DNA concentrations from which a standard curve was derived, designated as 'STND'; (b) single run of each cDNA sample from the target tissues labeled as unknown (UNKN); and (c) eight duplicate samples of every reagent required for the TaqMan PCR reaction except the target template, designated as 'no template controls' (NTC) to detect any contamination in the primer/probe mix or formation of primer dimers.

The relative quantification analysis for measurement of alterations in steady-state-levels of mRNA expression of a particular gene in the target tissue samples was achieved by using the standard curve method (Larionov et al., 2005; Holzapfel and Wickert, 2007). To generate cRNA, a cDNA was prepared from the total RNA of a tissue that expresses the gene of interest. The forward primer was extended by an upstream sequence of T7, such that the PCR product comprises the sequence of the target gene along with the upstream T7-sequence serving as the starter of the T7-RNA polymerase in the *in vitro* transcription. RNA was synthesized by *in vitro* transcription using the T7 RNA polymerase and the T7-MEGAshortscript™ high yield transcription kit (Ambion, Applera Deutschland GmbH, Darmstadt, Germany) according to the instructions of the manufacturer. Upon the completion of RNA transcription reaction, the template DNA was removed by incubation with RNase-free-DNase I (Qiagen) at 37°C for 15 min. *In vitro*-transcribed RNA transcripts were subsequently recovered and purified by phenol-chloroform extraction according to the instruction manual (Ambion). The quality of RNA was evaluated by using the 260/280 nm absorbance ratio and the size and concentration of the RNA transcripts were determined by automated capillary electrophoresis with an Agilent 2100 Electrophoresis Bioanalyzer (Agilent Technologies Sales & Service GmbH & Co. KG, Walbronn, Germany). The stock RNA solution was serially diluted 1:10 eight times with DNase-RNase-free water to obtain a

concentration range of 8 orders of magnitude. These RNA standards were then reverse transcribed using the M-MLV RT and random primer hexamers and amplified by TaqMan® PCR as already described earlier (see sections 2.12.1. and 2.12.2.). The standard curve was generated by plotting the logarithms of concentrations of the input cDNA against the corresponding cycle threshold for target amplification, i.e. the C_t-value which represents the PCR cycle at which the first increase in reporter fluorescence above the baseline signal can be detected. To determine the relative gene expression levels in a target tissue sample, the respective sample C_t-value was plotted on the standard curve and the resulting DNA amounts were calculated using the built-in ABI Prism®7700 SDS software.

2.12.3. Statistical analysis

For calculation relative gene expression values, the mean transcript count of the control group was set as 100% and the data from all treatment groups were set in correlation to the control values. Data were presented as means ± standard error of mean (SEM). Outliers of the data were excluded using the Grubbs's test as previously described (Burke, 2001). The differences between mean values of the control- and all treatment-groups were analyzed by one-way ANOVA followed with the Dunnett's multiple comparison post-hoc test or by the Student's *t*-test when only two groups were compared (GraphPad Prism 4.0, GraphPad Software, Inc., San Diego, CA, USA). *P* values <0.05 were considered statistically significant.

2.13. Histological and immunocytochemical analyses

2.13.1. Histological procedure

The pituitary glands from 6 rats of each group were excised and fixed in 10% NBF for 24 h at room temperature. The post-fixed pituitary glands were washed with tap water for 1 h, and then processed with the automated, semi-enclosed tissue processor (Leica model TP1020, Leica Microsyteme Vertrieb GmbH, Bensheim, Germany) in a series of increasing alcohol concentrations (50% to 75% to 96% to 100%) to dehydrate the tissue, and the xylene for clearing the dehydrant from the tissue. The operating conditions for processing the tissue by the automatic tissue processor were summarized in Table 7. Thereafter, the tissue was embedded in the paraffin by using a dispenser with integrated hot plate and separated cool plate (Leica model EG1140C). 3-µm-thick serial transverse sections were cut with a rotary microtome (Leica model RM2135). The tissue sections were thaw-mounted on SuperFrost®Plus microscope slides (MENZEL-GLÄSER, Germany) and dried for 24 h at 37°C. The paraffin-embedded tissue sections were stained with hematoxylin/eosin according to standard procedures to investigate the microscopic anatomy, or immunostained with the

specific antibody by using the immunocytochemical technique to examine protein expression of the relevant genes.

Table 7 Operating conditions for processing the post-fixed tissue with the automatic tissue processor

Step	Solutions and Reagents	Incubation time (min)
1	50% EtOH	60
2	75% EtOH	60
3	75% EtOH	90
4	96% EtOH	60
5	96% EtOH	90
6	100% EtOH	60
7	100% EtOH	60
8	100% EtOH	90
9	Xylene	60
10	Xylene	60
11	Hot Paraffin (70°C)	45
12	Hot Paraffin (70°C)	45

2.13.2. Hematoxylin and Eosin (H&E) staining technique

Hematoxylin is a basic dye with an affinity for the nucleic acids of the cell nucleus, whereas eosin is an acidic dye with an affinity for cytoplasmic components of the cell. Utilizing H&E staining, in the first step the nuclei were stained blue or dark blue-violet to black by hematoxylin (Mayer's hematoxylin solution, Merck KGaA, Darmstadt, Germany). In the second step of counterstaining with eosin, the cytoplasmic components were stained pink to red. Preparation of the eosin Y stock and working solutions used in the H&E staining is shown below. Step-by-step procedure of the standard H&E staining technique used in the present study is summarized in Table 8. After completion with this multistep procedure, the tissue slide was mounted with a xylene based mounting medium (DePex mounting medium, Dibutylphthalate, Merck, Darmstadt, Germany).

Eosin Y Stock Solution:

Eosin Y 1 g
ddH_2O 20 ml
96% EtOH 80 ml
Mix to dissolve
Store at room temperature

Eosin Y Working Solution:

Stock solution 45 ml
80% EtOH 135 ml
Add 5 drops of glacial acetic acid
Mix thoroughly

Table 8 Step-by-step procedure of the H&E staining method

Step	Solutions and Reagents	Incubation time (min)
1	Xylene	5
2	Xylene	5
3	Xylene	5
4	100% EtOH	2
5	100% EtOH	2
6	100% EtOH	2
7	96% EtOH	2
8	96% EtOH	2
9	75% EtOH	2
10	Double Distilled water	3
11	Mayer's Hematoxylin Solution	5
12	Tap Water	5
13	Double Distilled water	3
14	Eosin Y Solution	1
15	Double Distilled water	1
16	75% EtOH	2
17	96% EtOH	2
18	96% EtOH	2
19	100% EtOH	3
20	100% EtOH	3
21	Xylene	5
22	Xylene	5
23	Xylene	5

2.13.3. Immunolocalization of ERα in the pituitary

The C-terminal C1355 specific rabbit polyclonal antibody to the ERα was purchased from Upstate/Chemicon Cell Signalling Solutions (Germany). This antibody is generated against the synthetic peptide corresponding to the last 15 C-terminal amino acids (586-600) of rat ERα (TYYIPPEAEGFPNTI), and does not cross-react with ERβ.

To localize ERα, 3-μm-thick serial sections of pituitaries from 6 rats from each study group were immunostained with the peroxidase–anti-peroxidase (PAP) staining technique by means of a Dako EnVision System as described previously (Shi et al., 1997; Rimoldi et al., 2006). In brief, sections were dewaxed, rehydrated, and a microwave pretreatment for antigen retrieval was performed by boiling the tissue slides twice for 5 min each in 0.01 M Citric acid buffer, pH 6.0 setting the power at 750 watts. After cooling and washing in double-distilled water and 1x PBS, endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 30 min at room temperature (RT). Sections were rehydrated in a series of decreasing alcohols (100% to 96% to 75%), washed in 1x PBS, and then preincubated with 10 μl/ml normal goat serum (Dako Denmark A/S, Denmark), and 3% BSA (Gibco, Invitrogen) in 1x PBS for 30 min at RT to reduce non-specific binding of secondary antibody. After washing in

1x PBS, sections were incubated for 30 min at RT with the primary antibody (anti-ERα C1355) diluted 1:800 (v/v) in the antibody diluent (DakoCytomation, Dako Deutschland GmbH, Germany). The negative controls were incubated with antibody diluent only without the specific primary antibody. After washing in 1x PBS, sections were incubated with goat antirabbit and antimouse IgG- horseradish peroxidase (HRP)-conjugated secondary antibody (Envision™/HRP, DakoCytomation) for 30 min at RT. After washing in 1x PBS, the sites of HRP were visualized with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate (DakoCytomation) and the Mayer's modified hematoxylin solution (Merck KGaA) as a nuclear counterstaining. Samples were then dehydrated following the step 16 to 23 (Table 8) through an ethanol series and xylene, and mounted with DePex permanent mounting medium.

Following solutions, buffers, and reagents were used for immunohistochemistry:-

- **10 mM Citrate buffer, pH 6.0**

  $C_6H_5Na_3O_7$. $2H_2O$ 2.971 g
  ddH_2O up to1 liter
  Adjust the pH to 6.0 with 1 N HCl
  Adjust the volume of the solution to 1 liter
  Store at 4°C

- **10 X PBS Buffer (stock solution)**

  NaCl 80 g
  KCl 2 g
  Na_2HPO_4 11.5 g
  KH_2PO_4 2 g
  ddH_2O up to 1 liter
  Adjust pH to pH 6.8 with 1N HCl
  Adjust the volume of the solution to 1 liter
  Store at room temperature

- **Blocking endogenous peroxidase solution**

  CH_3OH 90 ml
  H_2O_2 (30%, v/v) 10 ml
  Mix well
  Store at 4°C

- **Blocking non-specific binding of immunoglobulins solution**

  Bovine albumin (7.5%, v/v) 400 µl
  Goat normal serum 30 µl
  1x PBS 2570 µl
  Make up immediately prior to use

- **Detection reagent (DAB staining Kit)**

  DAB+ chromogen solution 20 µl
  DAB Substrate buffer 1 ml
  Make up immediately prior to use

2.13.4. Quantitative analysis of the positive immunostained cells and photography

Sections were examined with a standard light microscope (Zeiss), and micrographs of immunostained areas around sinusoid vessels in the anterior lobes of the pituitary were taken with a Zeiss CCD digital camera connected to a computer running a program of Soft Imaging System analySIS® software (Soft Imaging System GmbH, Münster, Germany).

Examination of positive immunostained cells was done by blinded preparation. An ocular micrometer with 100 squares in 0.25 × 0.25 mm was used for cell counting. Cells were considered positive if only the nuclear staining (brown color) was present. The number of positive immunostained cells regardless of intensity in five randomly selected areas of each tissue section from 6 rats per study group was counted separately at the magnification of 400x, and determined as the relative percentage to the total cell numbers as described previously (Rimoldi et al., 2006). Significant differences between the control- and all treatment-groups were analyzed as already described in section 2.12.3.

3. Results

3.1. *In vivo* Experiment 1: Neuroendocrine effects of VZ in adult male rats

3.1.1. Clinical signs, body weight gain, and food consumption

Throughout the 5-day treatment interval, each animal was examined daily for clinical symptoms of toxicity related to VZ application. No such changes were observed. As shown in Fig. 16, neither mean final body weight nor average daily food intake of the VZ-treated animals were significantly affected compared to the vehicle treated-controls. No mortality was observed in both groups and there was no evidence of abnormal behavior.

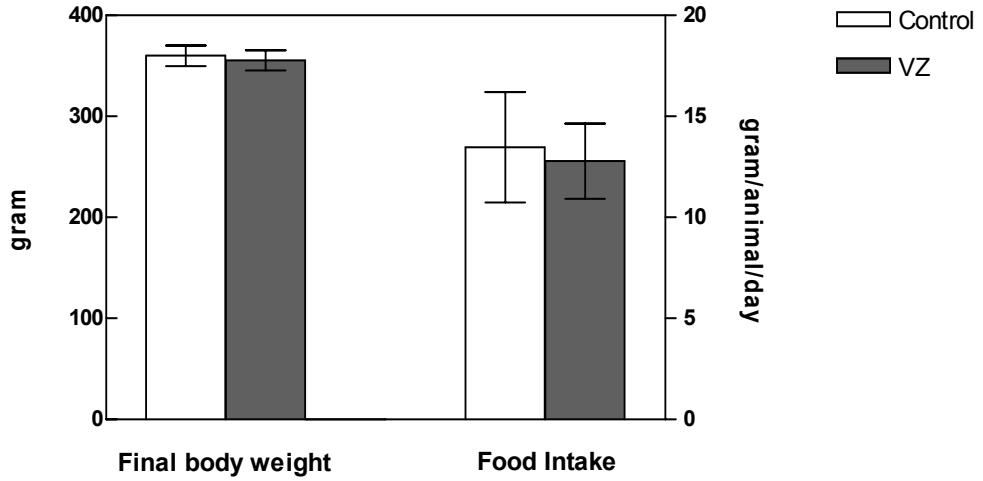


Figure 16. Mean final body weights and average daily food intake of adult (4 months old) male rats treated with VZ for 5 days. Data represent means ± SEM (*n* = 10/group).

3.1.2. Daily intake and serum concentration of VZ

The intended dose of VZ was 150 mg/kg bw/day (or 57 mg/rat/day). The achieved dose of VZ based on the measured final body weight was 148.25 mg/kg bw/day (or 55.89 mg/rat/day). The results of the HPLC analysis of the serum samples is shown in Fig. 17. The mean concentration of total VZ after extraction and hydrolysis was 15.64 ± 0.99 µg/ml or 54.65 ± 3.46 µmol/l. There was no corresponding signal detectable in the chromatograms of serum samples of the vehicle-treated controls.

3.1.3. Relative weights of reproductive and accessory sex organs

Relative organ weights were calculated by dividing the final absolute organ weight by the final body weight of each individual animal to avoid covaration between the body and organ weights at autopsy. The relative weights of testes, ventral prostate, and seminal vesicles did

not change upon VZ treatment. However, in the VZ-treated rats the relative epididymides weight was significantly decreased, while the relative liver weight was increased siginificantly (Fig. 18).

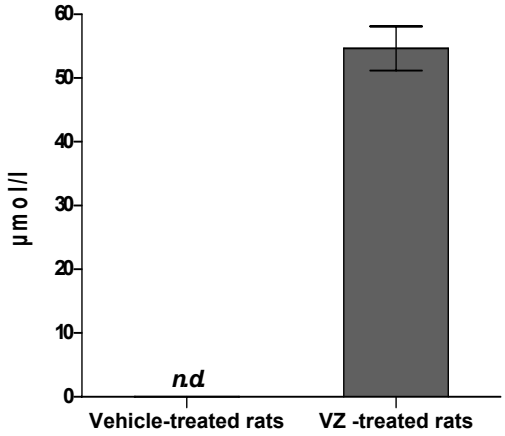


Figure 17. Mean serum levels of total VZ after extraction and hydrolysis in rats after a 5-day oral administration. Data represent means ± SEM (*n* = 10/group; *n.d.* = not detectable).

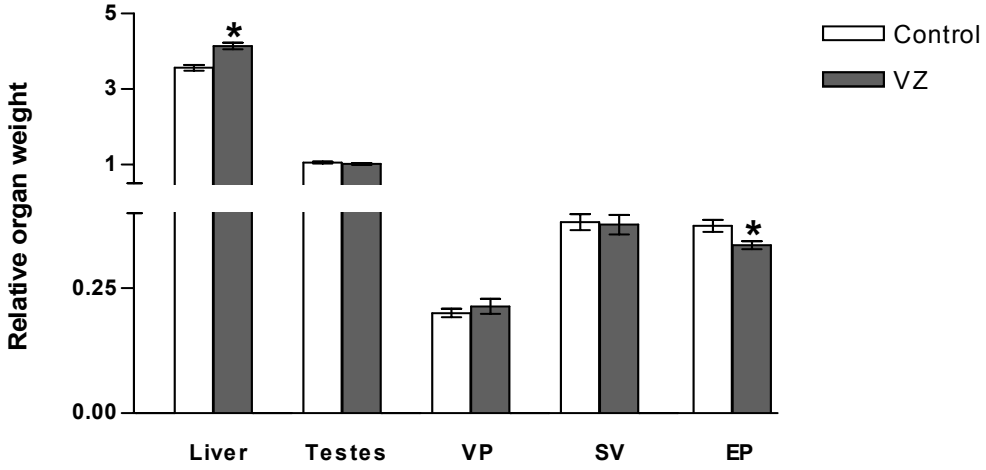


Figure 18. Relative weights of liver, testes, ventral prostate (VP), seminal vesicles (SV), and epididymides (EP) (means ± SEM, *n* = 10/group). **P* < 0.05 versus control.

3.1.4. Serum levels of reproductive and thyroid hormones

Treatment with VZ led to a significant elevation of serum levels of LH and T. Mean serum TSH and total T_4 concentrations were significantly decreased by VZ treatment, whereas total T_3 levels were not significantly altered (Fig. 19).

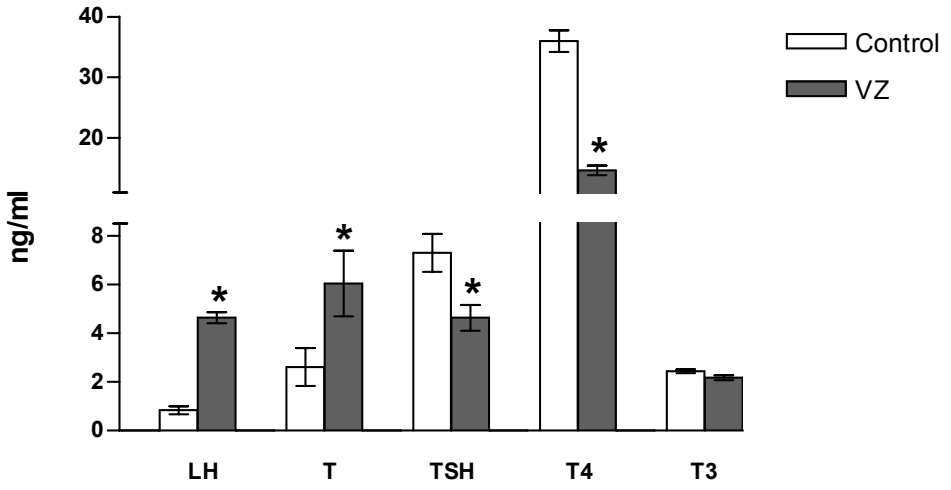


Figure 19. Mean serum levels of LH, T, TSH, total T_4 and T_3. Data represent means ± SEM, *n* = 10/group. **P* < 0.05 versus control.

3.1.5. Serum levels of metabolic parameters

Serum levels of the lipid parameters cholesterol, triglycerides, high- and low-density lipoproteins, and serum glucose levels of VZ-treated rats did not differ from the vehicle-treated controls (Fig. 20).

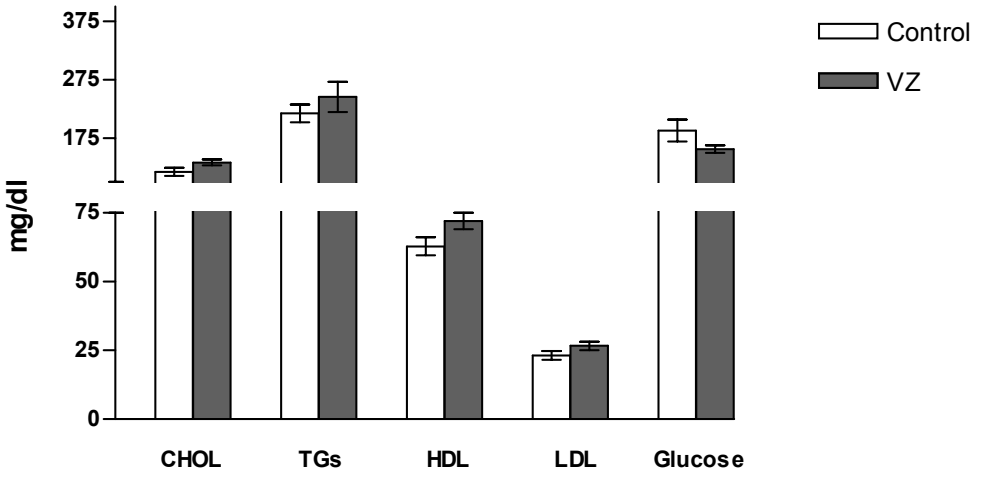


Figure 20. Mean serum levels of cholesterol (CHOL), triglycerides (TGs), high- and low-density lipoproteins (HDL and LDL), and glucose (mean ± SEM, *n* = 10/group). **P* < 0.05 versus control.

3.1.6. mRNA expression analysis in the pituitary

Relative mRNA expression levels of ERα, ERβ, and AR in the pituitary were not affected by VZ treatment. In contrast, relative levels of TERP-1 and -2 mRNA expression were significantly elevated upon VZ treatment (Fig. 21). Likewise, VZ induced significant increases

of mRNA expression levels of the GnRH receptor (GnRHR), LHβ and α-subunit, while TSHβ mRNA expression level remained unchanged (Fig. 22).

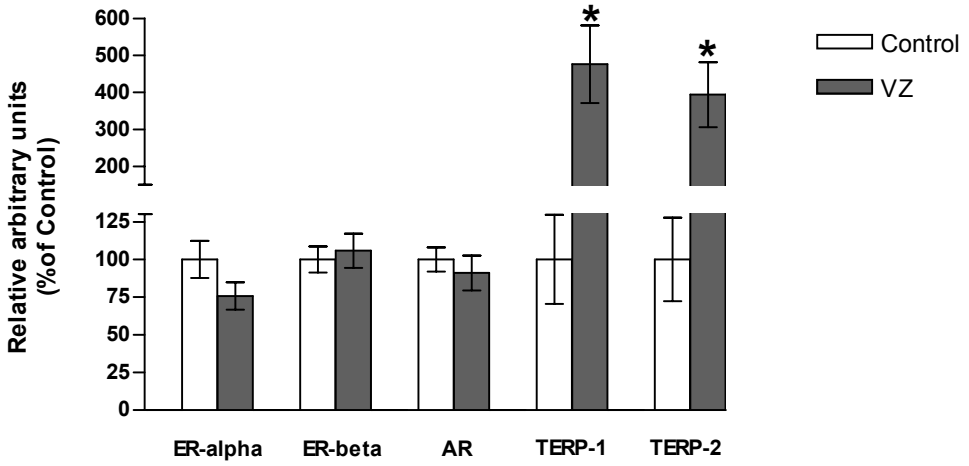


Figure 21. Relative mRNA expression levels of ERα, ERβ, AR, TERP-1 and -2 in the pituitary (means ± SEM, *n* = 10/group). **P* < 0.05 versus control.

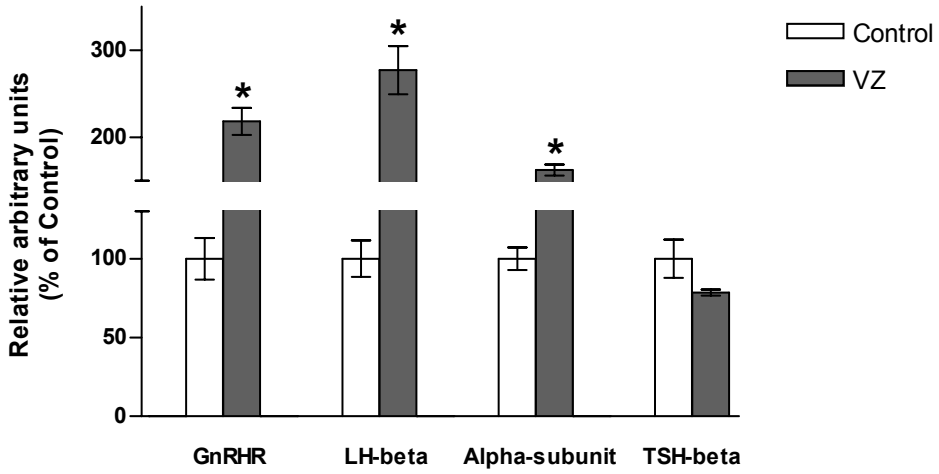


Figure 22. Relative mRNA expression levels of GnRHR, LHβ, α-subunit, and TSHβ in the pituitary. Values represent means ± SEM (*n* = 10/group). **P* < 0.05 versus control.

3.1.7. mRNA expression analysis in the MBH/ME

In the MBH/ME, relative mRNA expression of ERα, AR, and GnRH receptor were not affected by VZ treatment, while ERβ gene expression was significantly down-regulated (Fig. 23).

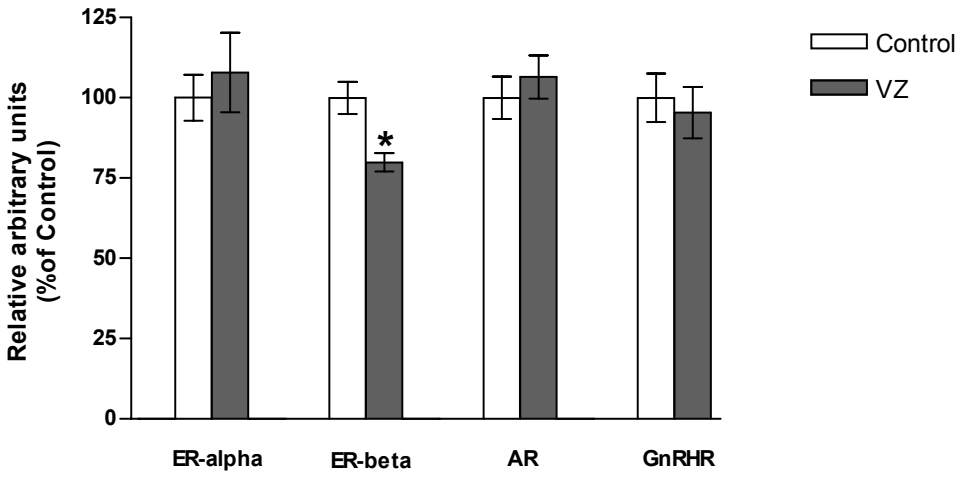


Figure 23. Relative mRNA expression levels of ERα, ERβ, AR, and GnRHR in the MBH/ME (means ± SEM, *n* = 10/group). **P* < 0.05 versus control.

3.1.8. mRNA expression analysis in the MPOA/AH

Expression analysis of ERα, ERβ, AR, and GnRH in the MPOA/AH revealed no significant effects of VZ treatment. However, tendencies towards decreased ERα and GnRH, and increased ERβ mRNA expression levels were observed (Fig. 24).

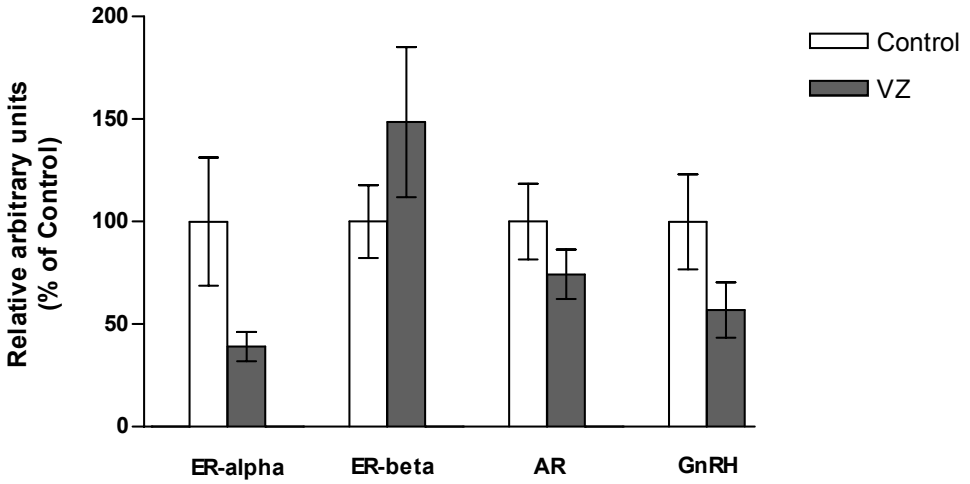


Figure 24. Relative mRNA expression levels of ERα, ERβ, AR, and GnRH in the MPOA/AH (means ± SEM, *n* = 10/group).

3.1.9. mRNA expression analysis in the striatum

In the striatum, expression of both ERα and ERβ remained unchanged, while a significant reduction of AR mRNA levels were observed. A tendency for decreased levels of ERβ mRNA was also seen (Fig. 25).

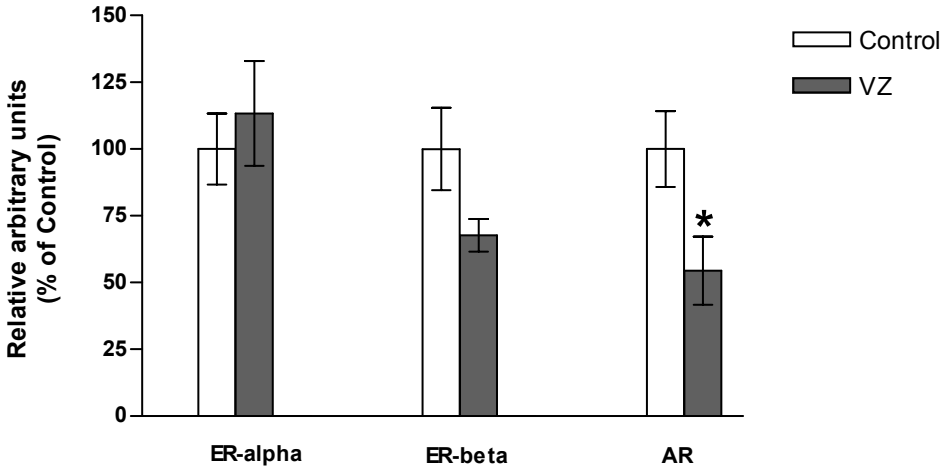


Figure 25. Relative mRNA expression levels of ERα, ERβ, and AR in the striatum (means ± SEM, *n* = 10/group). **P* < 0.05 versus control.

3.1.10. mRNA expression analysis in the hippocampus

Relative mRNA expresssion levels of ERα, ERβ, and AR in the hippocampus were not significantly affected by VZ treatment. There were tendencies for decreased ERβ mRNA expression levels in the VZ-treated rats (Fig. 26).

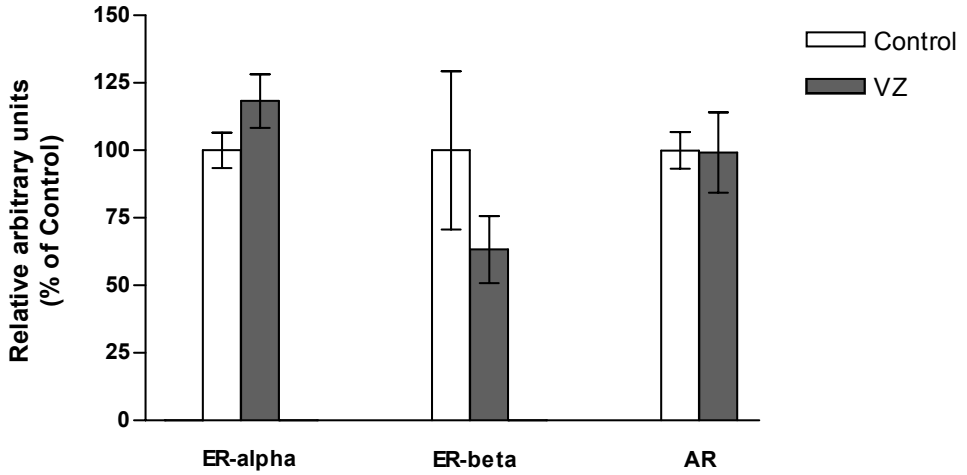


Figure 26. Relative mRNA expression levels of ERα, ERβ, and AR in the hippocampus (means ± SEM, *n* = 10/group).

3.1.11. mRNA expression analysis in the ventral prostate

In the ventral prostate, VZ significantly increased and decreased levels of AR and ERβ mRNA expression, respectively (Fig. 27).

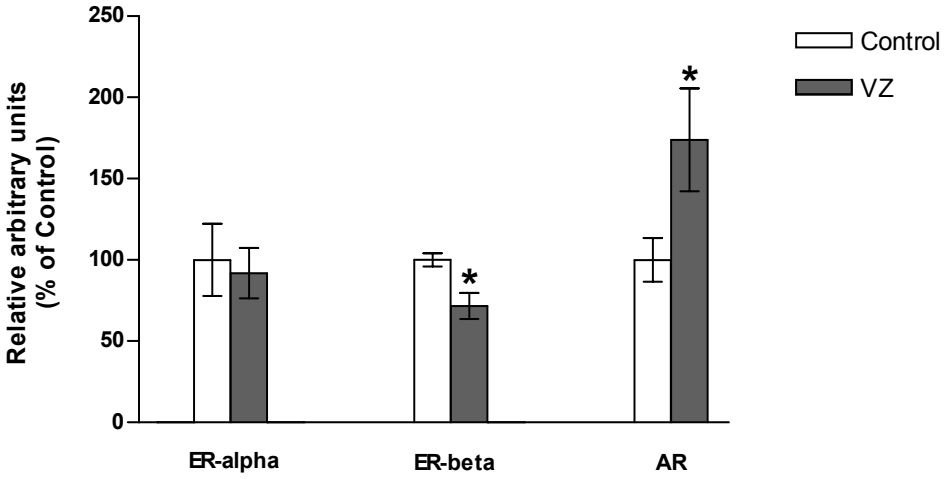


Figure 27. Relative mRNA expression levels of ERα, ERβ, and AR in the ventral prostate (means ± SEM, *n* = 10/group). **P* < 0.05 versus control.

3.1.12. mRNA expression analysis in the seminal vesicles

In the seminal vesicles, levels of ERα and AR mRNA expression were significantly up-regulated, while ERβ mRNA levels tended to be elevated upon VZ treatment (Fig. 28).

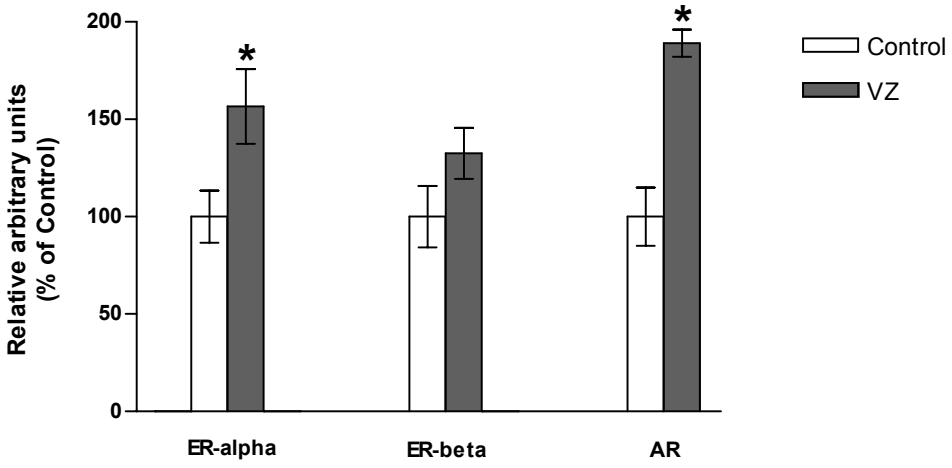


Figure 28. Relative mRNA expression levels of ERα, ERβ, and AR in the seminal vesicles (means ± SEM, *n* = 10/group). **P* < 0.05 versus control.

3.1.13. mRNA expression analysis in the epididymis

In the epididymis, the levels of relative ERβ and AR mRNA expression were up-regulated, while the relative ERα gene expression level was not affected by VZ treatment (Fig. 29).

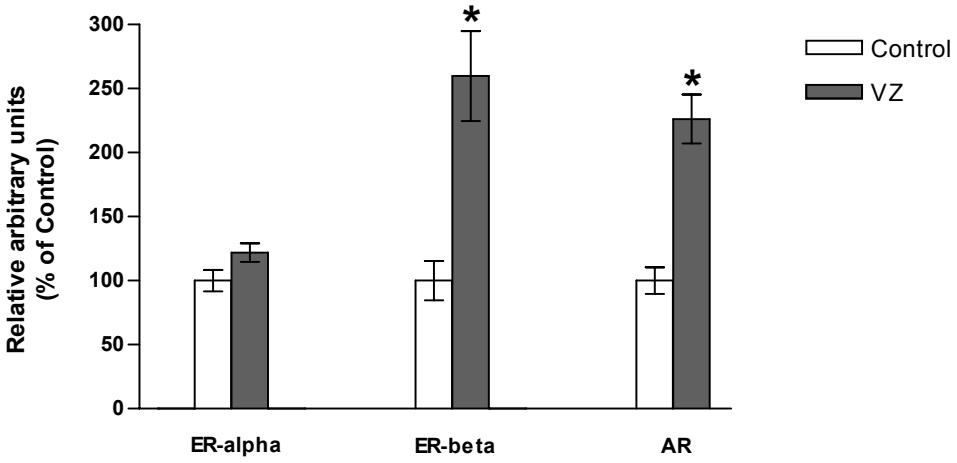


Figure 29. Relative mRNA expression levels of ERα, ERβ, and AR in the epididymis (means ± SEM, *n* = 10/group). **P* < 0.05 versus control.

A compilation of effects of a 5-day oral treatment of adult (4 months old) male rats with VZ in comparison with vehicle control is summarized in Table 9.

Table 9 A compilation of effects of VZ on the brain and the intact HPG axis after a 5-day oral treatment in adult male rats

Endpoints	VZ-treated males
Body weight, food consumption	Terminal body weights➡; Daily food intake➡
Organ weights	Testes➡; Epididymides⬇; Prostate➡; Seminal vesicles➡; Liver⬆
Serum hormone levels	LH⬆; T⬆; TSH⬇;T_4⬇; T_3➡
Serum metabolic parameter levels	CHOL➡; TGs➡; HDL➡; LDL➡, Glucose➡
Pituitary, gene expression	ERα➡; ERβ➡; AR➡; TERP-1⬆; TERP-2⬆; GnRHR⬆; LHβ⬆; α-subunit⬆; TSHβ➡
MBH/ME, gene expression	ERα➡; ERβ⬇; AR➡; GnRHR➡
MPOA/AH, gene expression	ERα➡; ERβ➡; AR➡; GnRH➡
Striatum, gene expression	ERα➡; ERβ➡; AR⬇
Hippocampus, gene expression	ERα➡; ERβ➡; AR➡
Prostate, gene expression	ERα➡; ERβ⬇; AR⬆
Seminal vesicles, gene expression	ERα⬆; ERβ➡; AR⬆
Epididymis, gene expression	ERα➡; ERβ⬆; AR⬆

Note: ⬆/⬇= significant increase/decrease compared with the controls.
➡ = no significant change compared with the controls.

3.2. *In vivo* experiment 2: Neuroendocrine effects of EQ in adult male rats

3.2.1. Clinical signs, body weight gain, and food consumption

Throughout the 5-day treatment interval, each individual animal was examined daily for the presence of any clinical signs of toxicity related to the EQ and FLUT treatments. Final mean body weights of animals in the EQ low and high dose and FLUT treatment group were slightly but significantly decreased, achieving the mean values of 96%, 95% and 97% of the vehicle-treated controls, respectively (Fig. 30A). All treatments reduced the daily food intake (Fig. 30B). No mortality was observed in all treatment- and control-groups and there was no evidence for abnormal behavior.

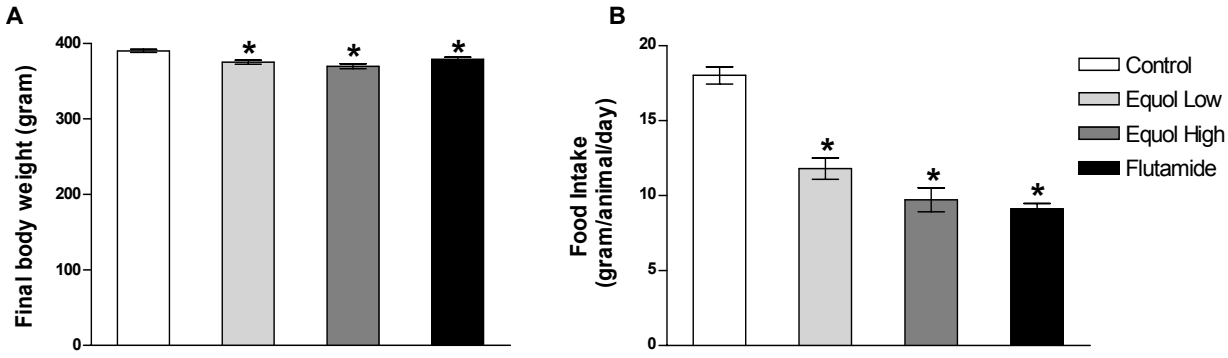


Figure 30. Effects of the 5-day oral administration with either EQ at low and high doses, or FLUT on (**A**) mean final body weights, and (**B**) average daily food intakes of adult (4 months old) male rats. Each bar represents the mean ± SEM (*n* = 12/group). **P* < 0.05 versus control.

3.2.2. Daily intake and serum concentrations of the test substances

The intended dosages of EQ were 100 and 250 mg/kg bw/day. The intended dose of FLUT was 100 mg/kg bw/day. Based on the measured final body weights, EQ low dose-treated rats received an average dose of 99.98 mg/kg bw/day (or 37.99 mg/rat/day), while EQ high dose-treated rats received 250.49 mg/kg bw/day (or 95.19 mg/rat/day). The achieved dose of FLUT was 98.96 mg/kg bw/day (or 37.60 mg/rat/day).

Utilizing HPLC analysis after extraction and hydrolysis of serum samples, the determined mean concentrations of EQ in rats treated at low and high doses were 2.72 ± 0.44 µg/ml (11.24 ± 1.81 µmol/l) and 6.55 ± 0.74 µg/ml (27.02 ± 3.06 µmol/l), respectively. In rats treated with FLUT the serum levels were 41.00 ± 2.31 µg/ml (148.4 ± 8.36 µmol/l) (Fig. 31). No corresponding signals of EQ and FLUT were detectable in the chromatograms of serum samples of the vehicle-treated controls.

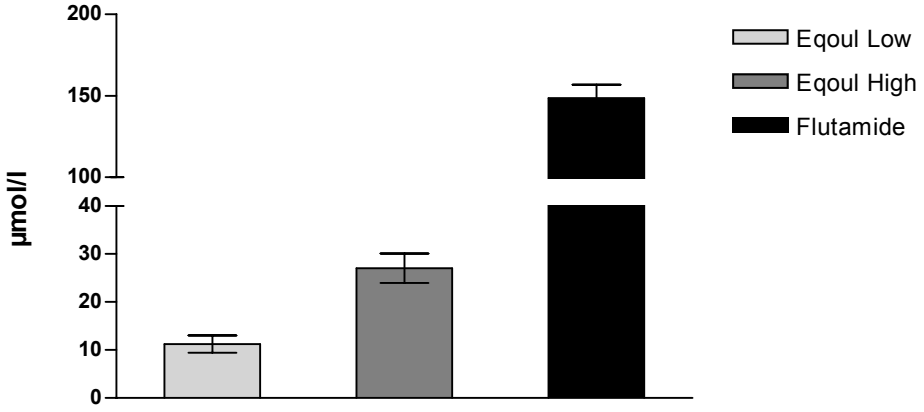


Figure 31. Mean serum levels of EQ and FLUT. Each bar represents the mean ± SEM (*n* = 12/group).

3.2.3. Effects of the test substances on expression of male mating behavior

In the present study, male sexual behavior was determined by successful mating with sexually receptive females as indicated by the presence of a vaginal plug, or spermatozoa in the vaginal lavages after overnight mating.The effects of the 5-day oral administration of adult male rats with either EQ or FLUT on mating behavior are summarized in Table 10. All of the vehicle-treated controls showed successful mating behavior (100%) (Fig. 32), whereas all of the FLUT-exposed rats failed to mate with sexually receptive females (0%) (Fig. 33). Comparable to the results of the vehicle-treated controls, all male rats treated with EQ either at low- or high-dose showed successful mating behavior (100%) (Fig. 34 and 35). Sub-acute treatment of male rats with EQ both at low and high doses did not affect the pregnancy outcome of their respective female partners. None of the female rats mated with the FLUT-treated males became pregnant (Table 10).

Table 10 Effects of a 5-day oral administration of adult (4 months old) male rats with either EQ low and high doses, or FLUT on male mating behavior and female pregnancy outcome

Parameters	Vehicle-treated males	EQ Low-treated males	EQ High-treated males	FLUT-treated males
Cohabitations	10	10	11	10
Positive vaginal lavages	10	10	11	0
Successful mating (%)	100	100	100	0
Pregnancies with positive matings	8	8	9	0

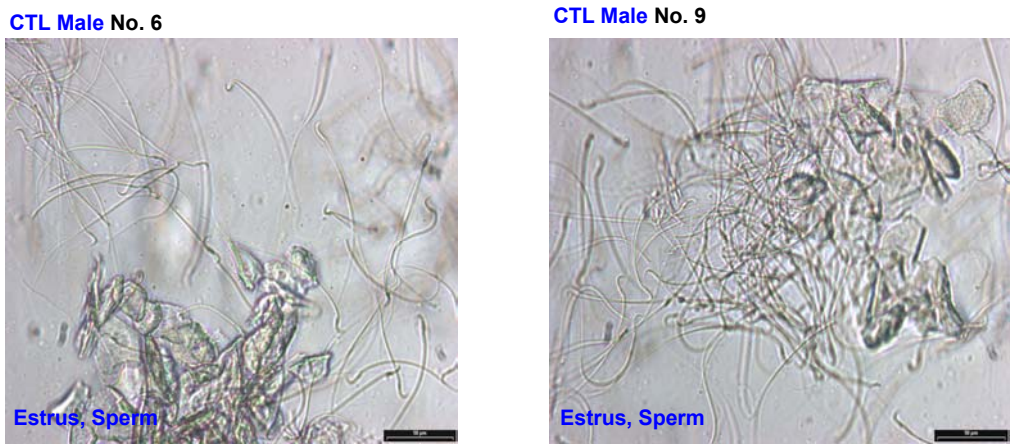


Figure 32. Two representative photomicrographs (25x) showing the presence of spermatozoa in vaginal lavages of sexually receptive female rats after mating with the vehicle-treated males (*n* = 10) at day 5 of treatment. Scale bar = 50 µm.

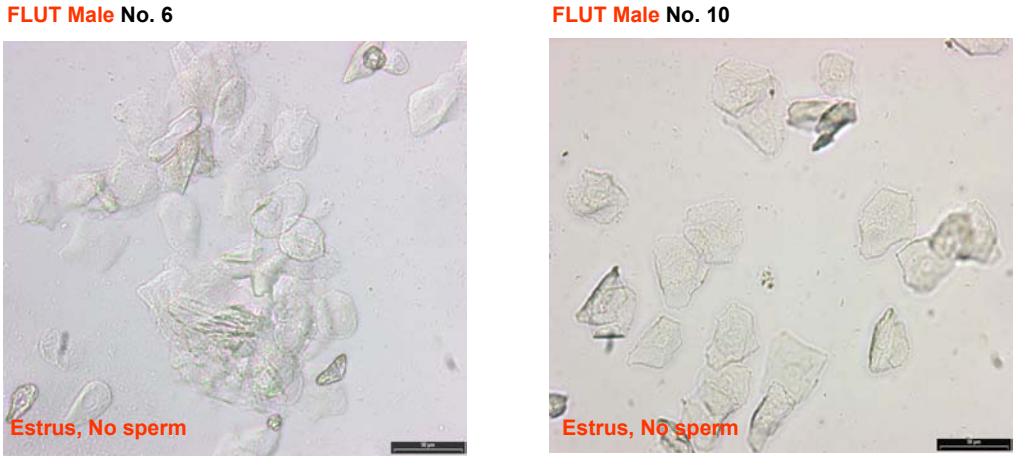


Figure 33. Two representative photomicrographs (25x) showing the absence of spermatozoa in vaginal lavages of sexually receptive females after mating with the FLUT-exposed male rats (*n* = 10) at day 5 of treatment. Scale bar = 50 µm.

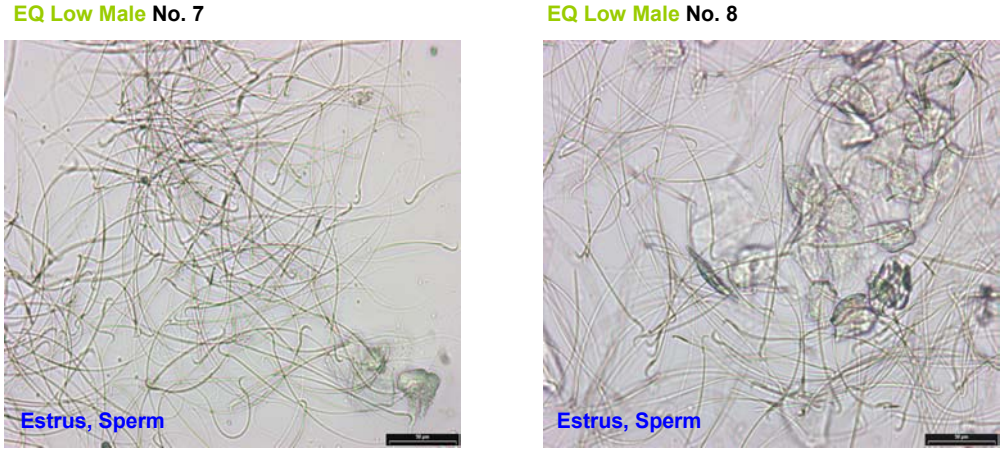


Figure 34. Two representative photomicrographs (25x) of positive vaginal lavages of sexually receptive female rats after mating with the EQ low dose-treated males (*n* = 10) at day 5 of treatment. Scale bar = 50 µm.

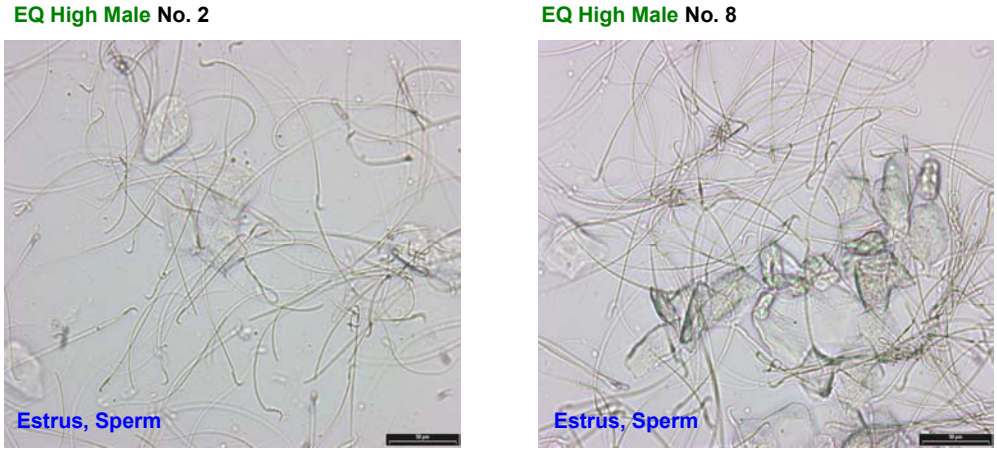


Figure 35. Two representaive photomicrographs (25x) of positive vaginal lavages of sexually receptive females after mating with the EQ high dose-treated male rats (*n* = 11) at day 5 of treatment. Scale bar = 50 µm.

It has been reported that EQ at a dose of 0.25 mg/kg bw/day reduced prostate size in adult male rats after a 4-day treatment (Lund et al., 2004). This prompted us to examine whether EQ at this extremely low dose affects male sexual behavior. According to the protocol of Lund et al. (2004), adult (4 months old) male rats were *s.c.* injected for 4 days with either EQ (0.25 mg/kg bw/day), or DMSO (0.5 ml/rat/day, vehicle control). At day 4 of treatment, all rats performed the mating test with sexually receptive female rats of the same age. 100% of male rats treated with EQ at this dose showed successful mating behavior (Fig. 36). Similarly, daily vehicle injections had no adverse effect on male mating behavior (data not shown).

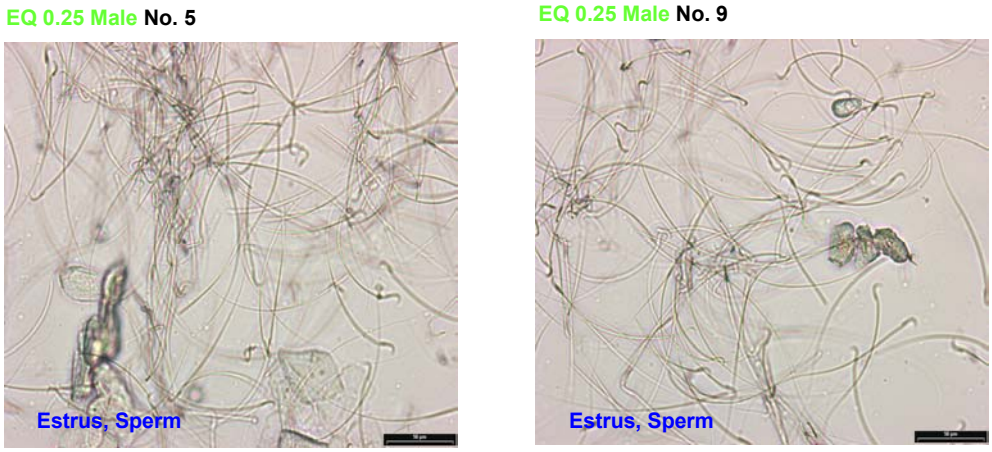


Figure 36. Two representative photomicrographs (25x) showing the presence of spermatozoa in vaginal lavages of sexually receptive female rats after mating with the males *s.c.* injected with EQ at a dose of 0.25 mg/kg bw/day (*n* = 10) at day 4 of treatment. Scale bar = 50 µm.

An additionally mating test was conducted to examine the reversibility of the inhibitory effects of FLUT on male sexual behavior. After 5 days of cessation with FLUT treatment, all rats were mated with sexually receptive females of the same age. 60% of FLUT-treated rats showed successful mating behavior (Table 11).

Table 11 Mating behavior of adult male rats in response to 5 days after cessation with FLUT treatment and pregnancy rate of the mated females

Parameters	FLUT-treated males
Cohabitations	10
Positive vaginal lavages	6
Successful mating (%)	60
Pregnancies with positive matings	3

3.2.4. Relative weights of reproductive and accessory sex organs

Since the mean final body weights were different between the animal groups, relative organ weights are reported. Figure 37 shows the relative weights of testes, epididymides, ventral prostate, seminal vesicles, and liver of adult male rats after oral application of either EQ at low and high doses, or FLUT for 5 consecutive days. EQ both at low and high doses, and FLUT significantly increased the relative liver weight. EQ at high dose as well as FLUT markedly decreased relative weight of seminal vesicles. EQ did not exert any effects on the relative weights of ventral prostate and epididymides, while FLUT significantly decreased the relative weights of these organs. Both the EQ and FLUT treatments did not significantly affect the relative testicular weight.

3.2.5. Serum levels of reproductive and thyroid hormones

EQ at two dose applied reduced serum concentrations of LH, T and DHT, and increased serum PRL levels in a dose-dependent manner. In contrast, FLUT profoundly increased serum LH, T, and DHT levels without altering PRL concentration (Fig. 38).

Mean serum TSH concentrations remained unaffected in all treatment groups. However, T_4 and T_3 levels were significantly reduced by EQ at both doses and FLUT (Fig. 39).

3.2.6. Serum concentrations of metabolic parameters

EQ caused dose-dependent reductions in mean concentrations of serum cholesterol, high- and low-density lipoproteins. In addition, EQ at high dose slightly but significantly reduced serum triglycerides levels. In contrast, FLUT caused statistically significant increases in mean serum concentrations of cholesterol, high- and low-density lipoproteins, without causing any significant alteration in serum triglycerides levels (Fig. 40).

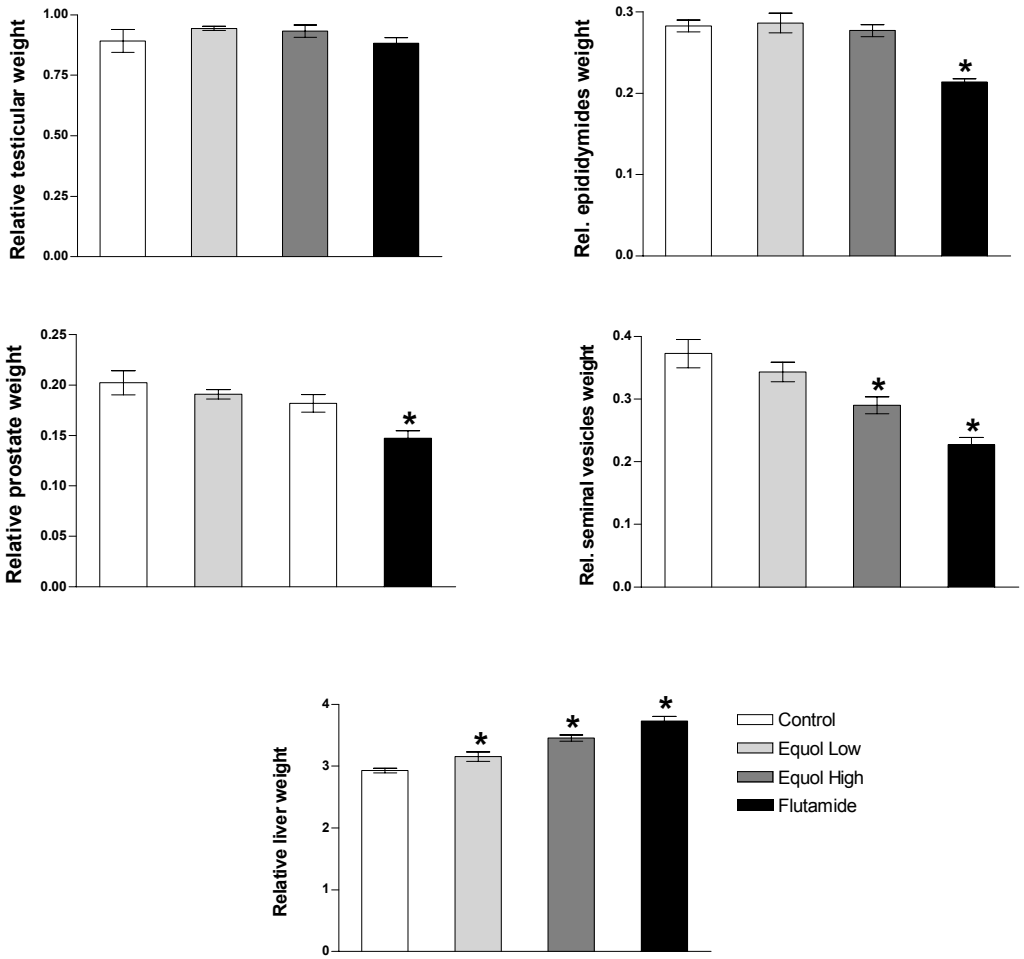


Figure 37. Relative weights of testes, epididymides, ventral prostate, seminal vesicles, and liver (means ± SEM, *n* = 12/group). **P* < 0.05 versus control.

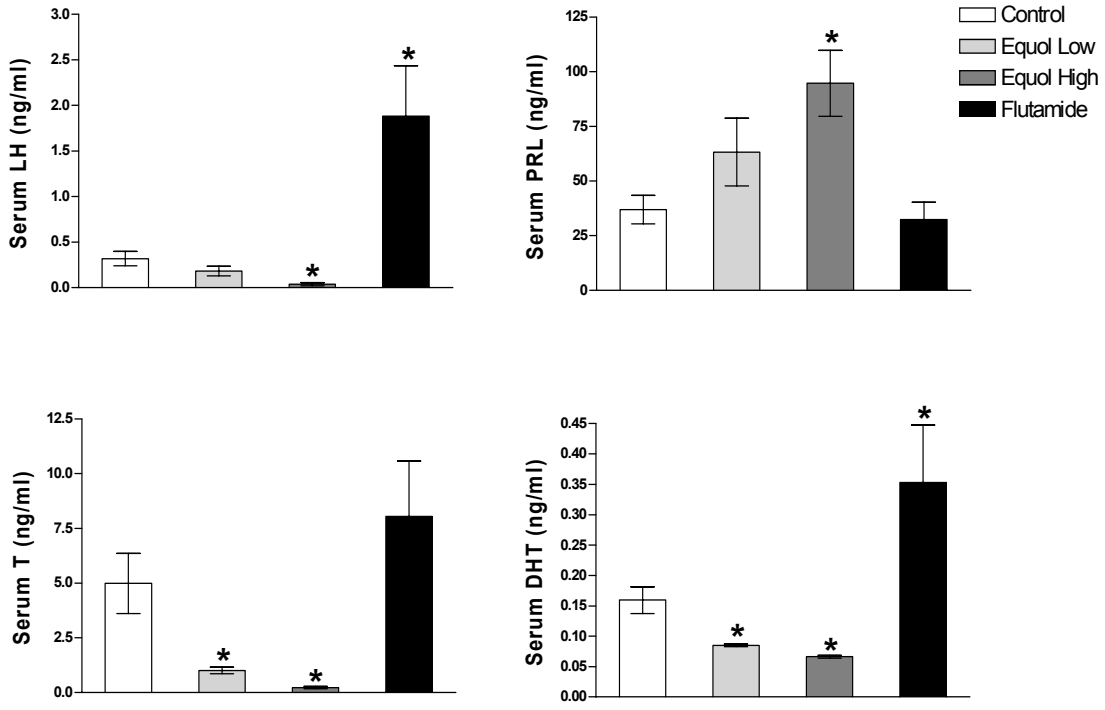


Figure 38. Mean serum concentrations of LH, PRL, T, and DHT. Data represent means ± SEM (*n* = 12/group). **P* < 0.05 versus control.

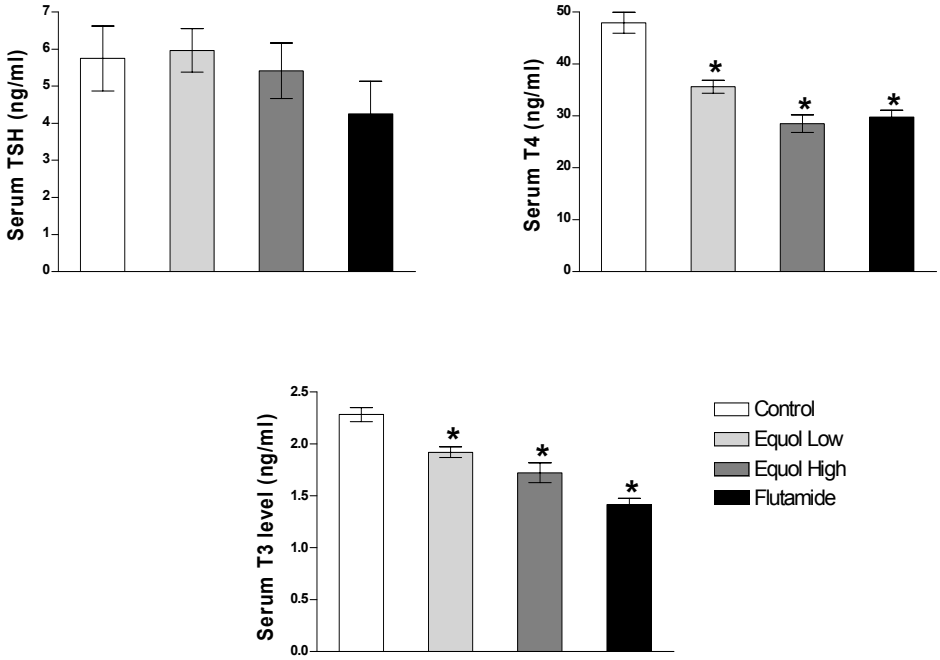


Figure 39. Mean serum levels of TSH, total T_4 and T_3. Each bar represents the mean ± SEM (*n* = 12/group). **P* < 0.05 versus control.

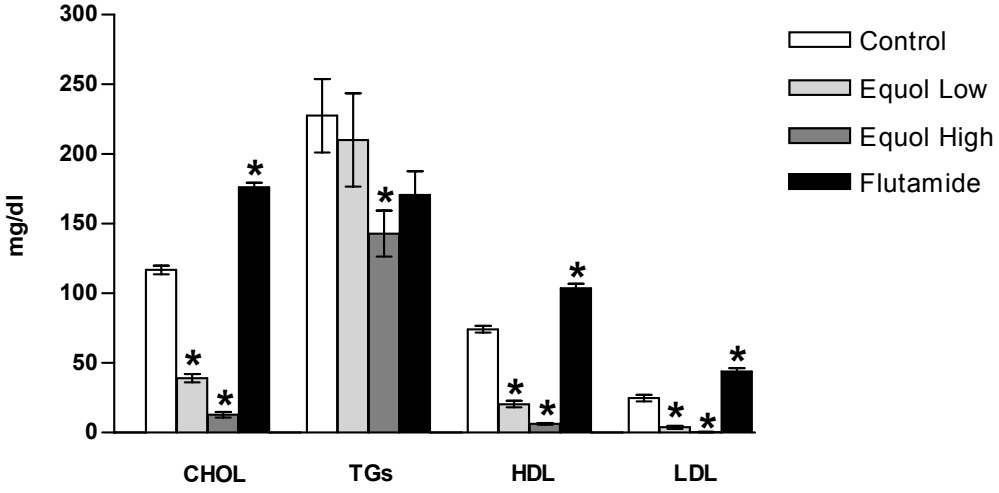


Figure 40. Mean serum levels of cholesterol (CHOL), triglycerides (TGs), high- and low-density lipoproteins (HDL and LDL) (means ± SEM, *n* = 12/group). **P* < 0.05 versus control.

3.2.7. mRNA expression analysis in the pituitary

Expression of the ERα-splice variants, TERP-1 and -2, in the pituitary was profoundly up-regulated by EQ in a dose-dependent manner, whereas FLUT did not affect the levels of mRNA transcripts of those genes (Fig. 41A). At high dose EQ caused a significant up-regulation of ERα mRNA expression, while FLUT did not change the relative ERα mRNA levels. Neither ERβ nor AR mRNA expression levels were significantly altered in any

treatment group (Fig. 41A). EQ at high dose significantly down-regulated, whereas FLUT dramatically up-regulated the levels of GnRH receptor mRNA transcripts (Fig. 41B). EQ both at low and high doses did not significantly alter LHβ mRNA expression levels, whereas FLUT doubled the increased levels of LHβ mRNA (Fig. 41B). EQ at low dose significantly down-regulated gonadotropin α-subunit mRNA expression, while EQ at high dose caused a tendency towards decreased the relative α-subunit mRNA levels. In contrast, FLUT increased the levels of α-subunit mRNA transcripts significantly (Fig. 41B). EQ as well as FLUT down-regulated TSHβ mRNA expression in a similar fashion (Fig. 41B).

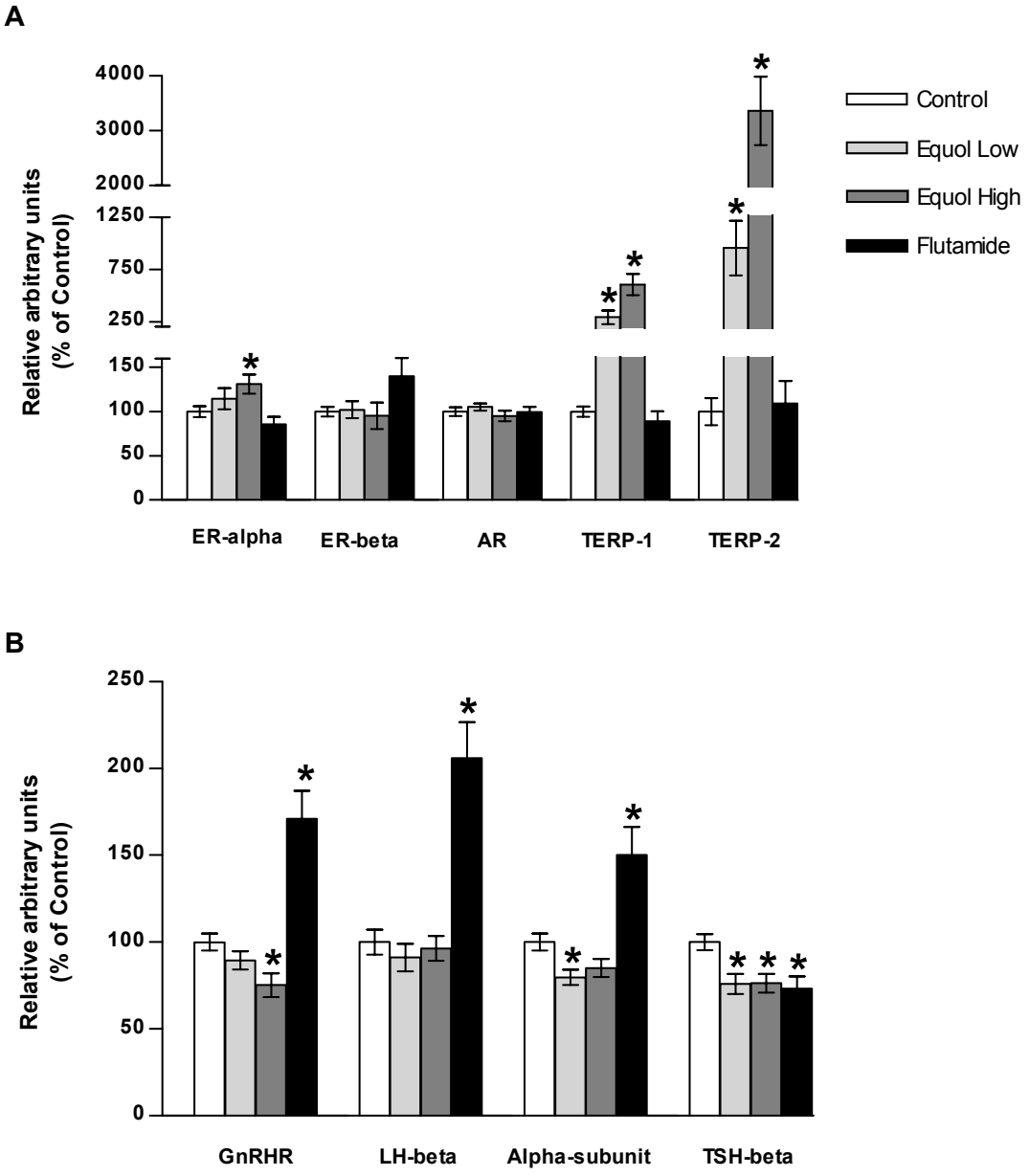


Figure 41. Relative mRNA expression levels of ERα, ERβ, AR, TERP-1 and -2 (**A**) and GnRHR, LHβ, α-subunit, and TSHβ (**B**) in the pituitary. Data represent means ± SEM. (*n* = 12/group). **P* < 0.05 versus control.

3.2.8. mRNA expression analysis in the MBH/ME

In the MBH/ME, neither EQ nor FLUT caused any statistically significant alterations in the relative ERα and AR mRNA expression levels. Only EQ at high dose decreased significantly the levels of ERβ mRNA transcripts. EQ low dose as well as FLUT up-regulated the levels of GnRH receptor mRNA expression (Fig. 42).

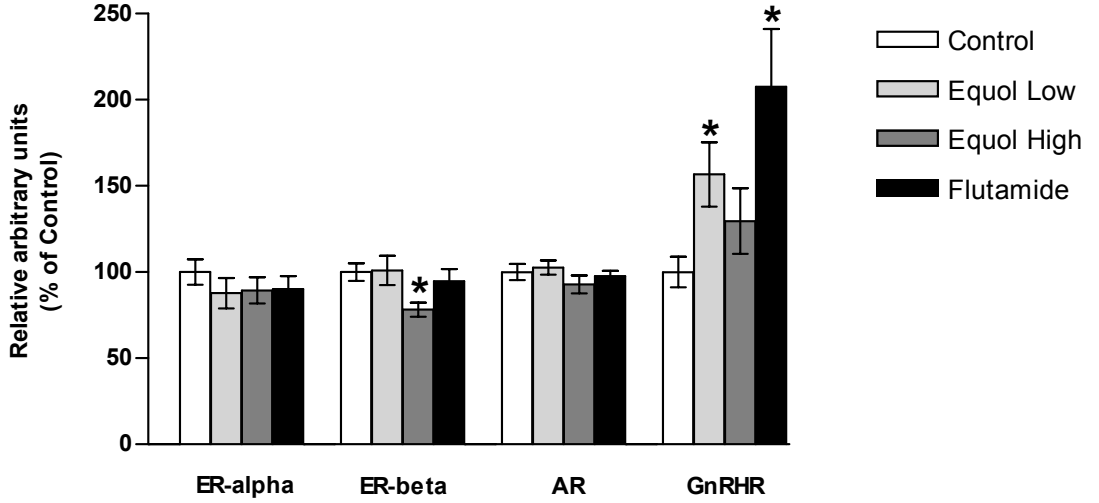


Figure 42. Relative mRNA expression levels of ERα, ERβ, AR, and GnRHR in the MBH/ME (means ± SEM, *n* = 12/group). **P* < 0.05 versus control.

3.2.9. mRNA expression analysis in the MPOA/AH

In the MPOA/AH, mRNA expression levels of ERα, ERβ, and AR remained unaffected by either EQ or FLUT treatment. Only FLUT caused a significant down-regulation of GnRH mRNA levels (Fig. 43).

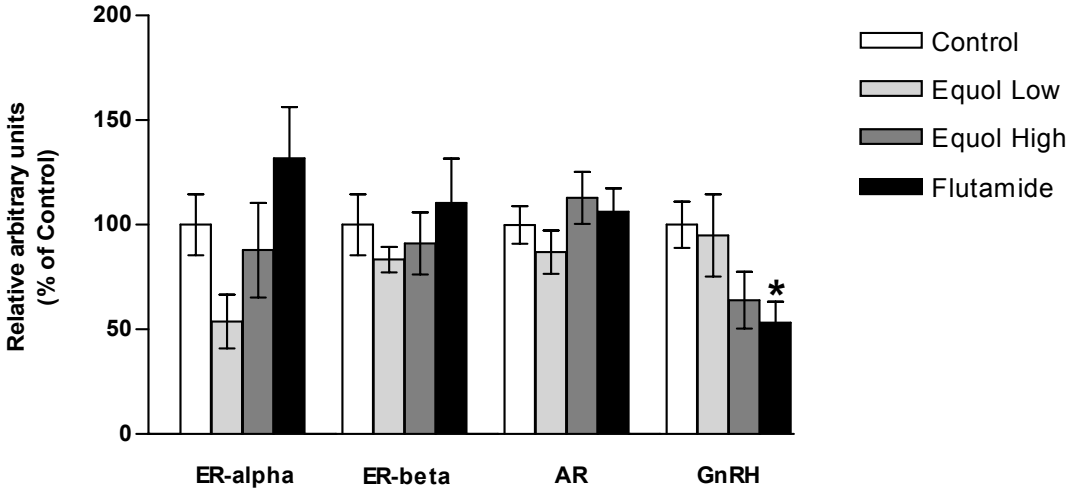


Figure 43. Relative mRNA expression levels of ERα, ERβ, AR, and GnRH in the MPOA/AH (means ± SEM, *n* = 12/group). **P* < 0.05 versus control.

3.2.10. mRNA expression analysis in the amygdala and hippocampus

In the amygdala and hippocampus the mRNA expression of ERα and AR were measured. Neither treatment induced changes of expression of both nuclear receptors in these brain areas (Fig. 44).

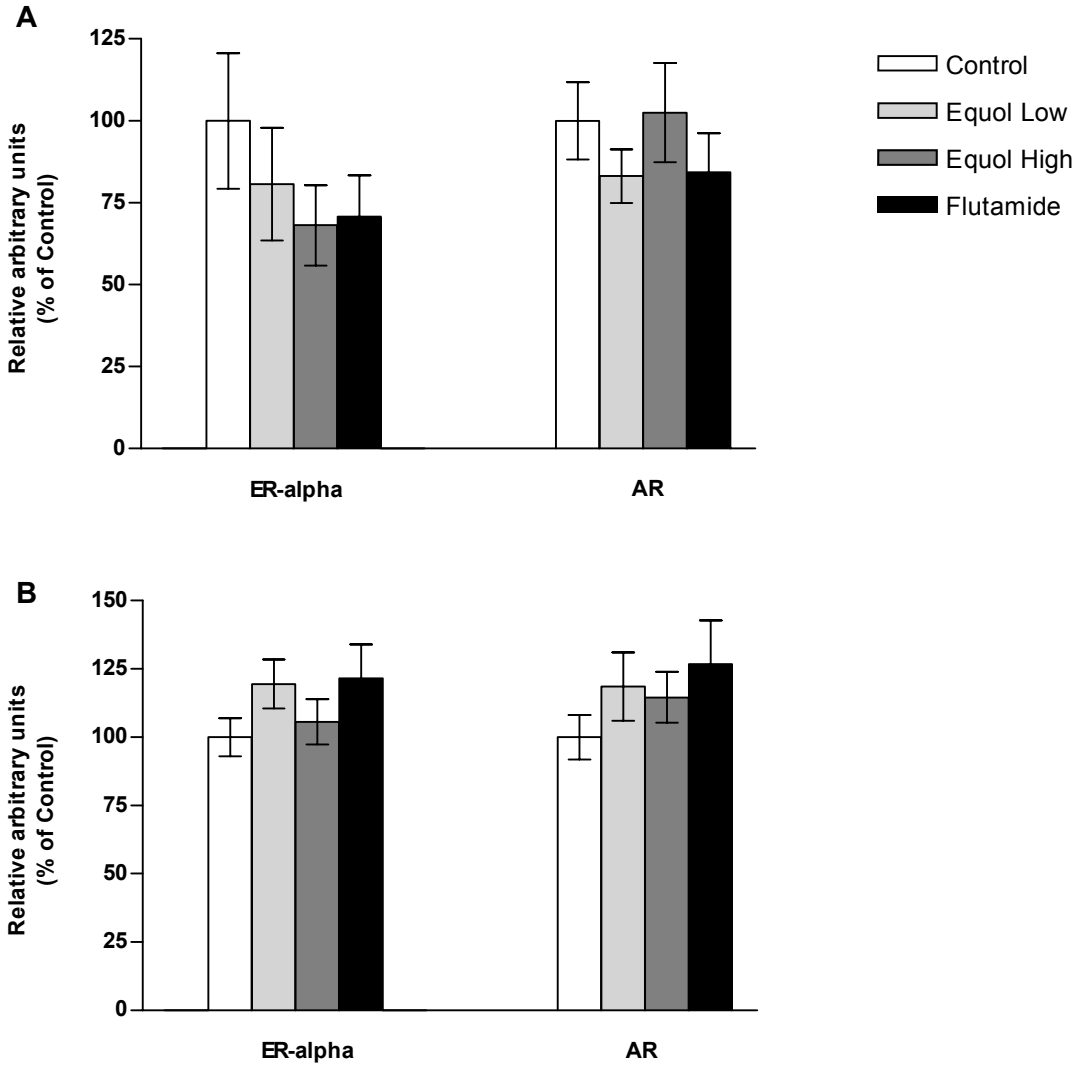


Figure 44. Relative mRNA expression levels of ERα and AR in the amygdala (**A**) and hippocampus (**B**) (means ± SEM, *n* = 12/group).

3.2.11. mRNA expression analysis in the ventral prostate

Figure 45 summarizes the effects of EQ and FLUT in the reference androgen-dependent organ, ventral prostate. Levels of AR mRNA were significantly up-regulated by both the EQ high dose and FLUT treatments. Likewise, expression levels of ERα mRNA were also elevated by both the EQ high dose and FLUT treatments, but the significant increase was only observed with the FLUT treatment. ERβ mRNA expression was not affected by either compound.

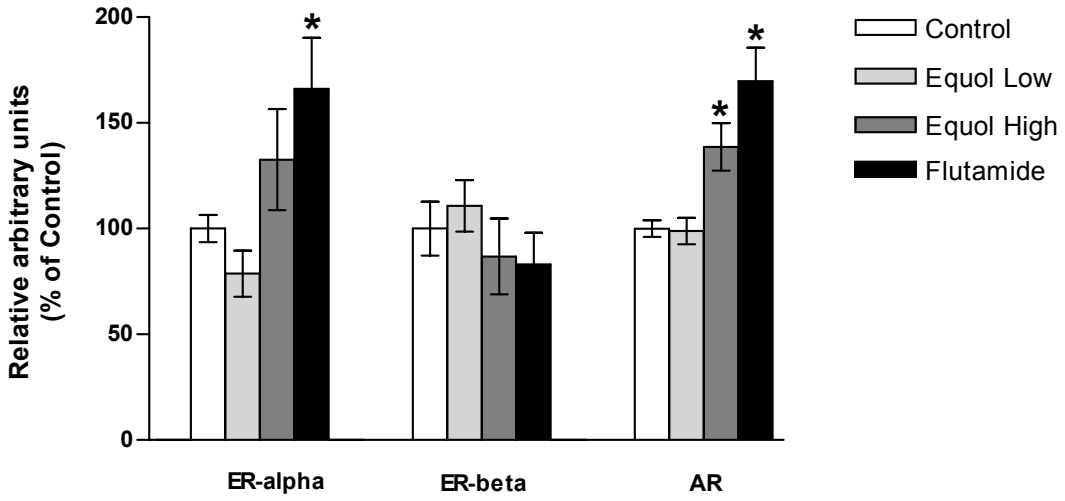


Figure 45. Relative mRNA expression levels of ERα, ERβ, and AR in the ventral prostate (means ± SEM, *n* = 12/group).

3.2.12. ERα immunolocalization in the pituitary

EQ significantly upregulated the mRNA levels of ERα and the ERα splice variant TERP in the pituitary. To investigate whether this elevated mRNA expression is detectable at protein level, an immunohistochemical analysis of the anterior pituitary was performed. Utilizing an ERα antibody which also recognizes TERP-1 and -2, the number of ''ERα''-positive cells was determined. Nuclear staining for ERα was detected in a large population of anterior lobe cells, but not in the intermediate or posterior lobe (Fig. 46).

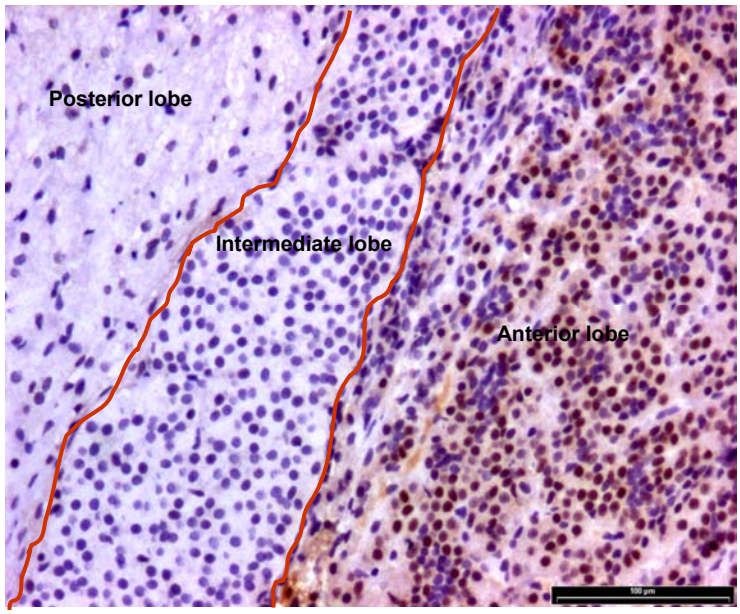


Figure 46. Representative section showing the immunolocalization of ERα in the pituitary of adult male rat. Nuclear staining for ERα was present in a large population of anterior lobe cells, but not in the intermediate or posterior lobe. x 5. Scale bar = 100 µm.

The ERα immunoreactive cells were counted in 5 fields in each section from 6 rats per group, and then determined as the relative percentage of total cells. Representative micrographs of stainings from each group are shown in Fig. 47. The number of immunoreactive ERα cells in the anterior pituitary of male rats treated with EQ at high dose was significantly increased compared to the controls, whereas no significant changes were observed from those rats treated with EQ at low dose and FLUT (Fig. 48).

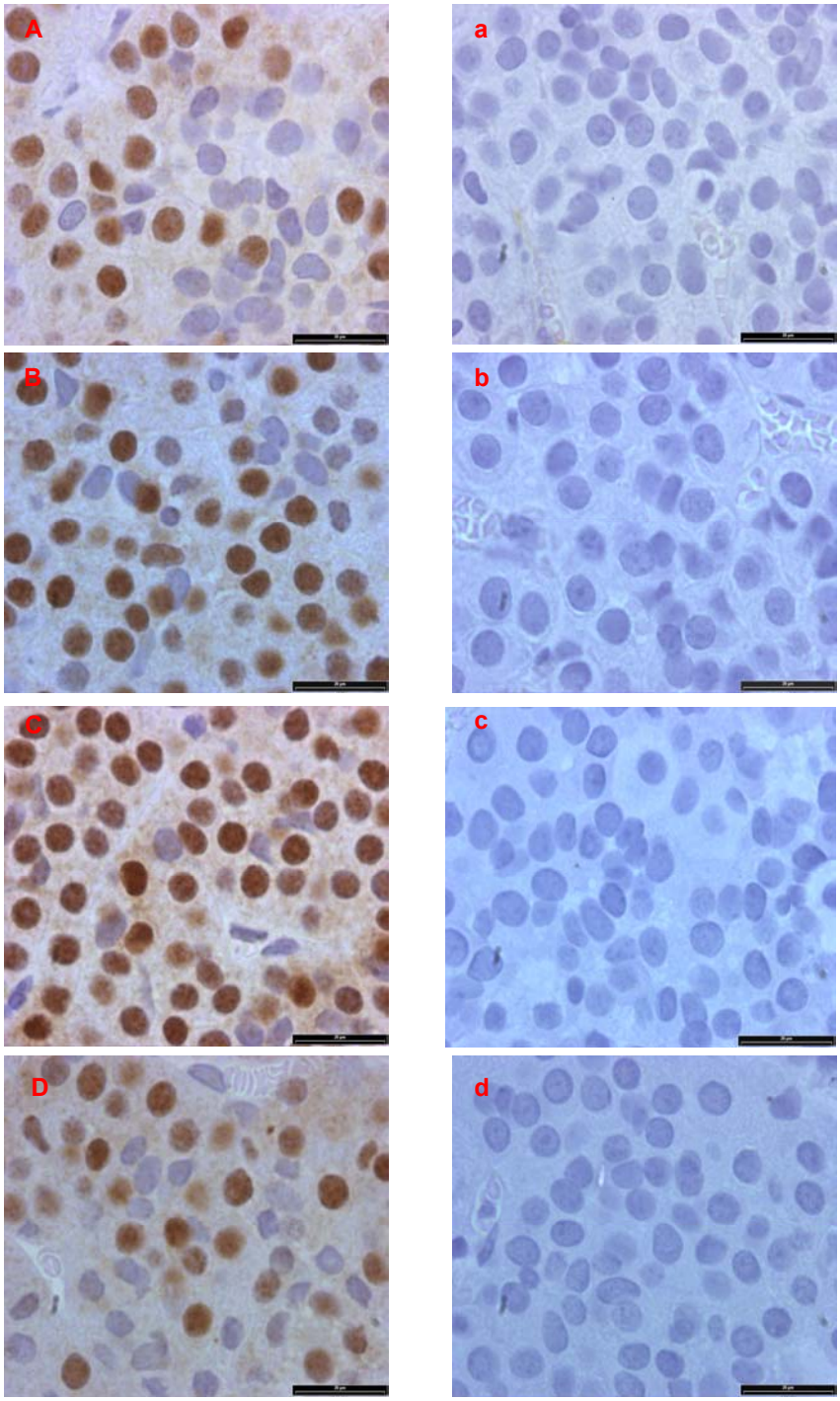


Figure 47. Representative sections demonstrating the immunolocalization of ERα in the pituitary of adult male rats treated by gavage for 5 days with either vehicle olive oil (A;a), EQ at low (B;a) and high doses (C;c), or FLUT (D;d). All micrographs (100x) at the right panel were taken from the negative control sections, whereas those at the left panel were taken from the ERα-immunostained sections from the same rat in each treatment. Scale bar = 20 μm.

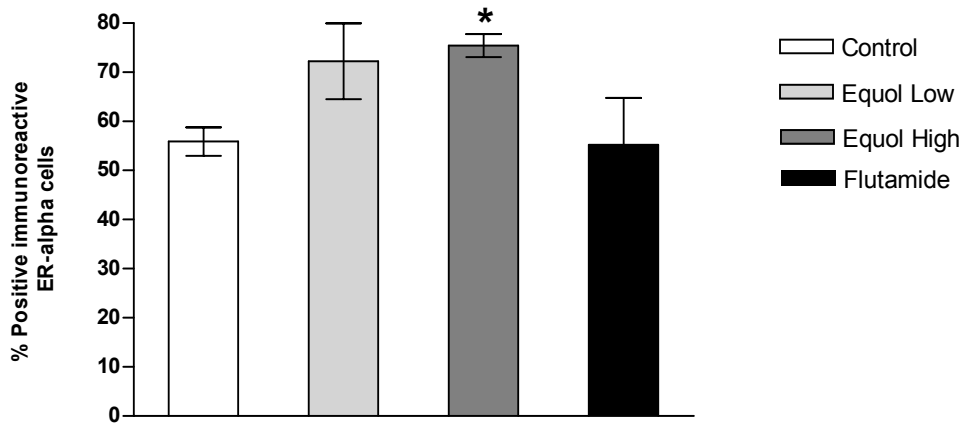


Figure 48. Relative percentages of the positive ERα immunoreactive cells in the anterior pituitary. **P*<0.05 versus control.

An overview of effects of the 5-day oral treatment of adult (4 months old) male rats with EQ in comparison with FLUT is summarized in Table 12.

Table 12 An overview of effects of EQ compared to that of FLUT on the brain and its neuroendocrine regulation of the HPG axis and control of sexual behavior in adult male rats.

Parameters	Equol Low-treated males	Equol High-treated males	Flutamide-treated males
Body weight gain and Food consumption			
Final body weight	↓	↓	↓
Daily food intake	↓	↓	↓
Relative organ weights			
Testes	→	→	→
Epididymides	→	→	↓
Seminal vesicles	→	↓	↓
Prostate	→	→	↓
Liver	↑	↑	↑
Serum hormone levels			
LH	→	↓	↑
T	↓	↓	→
DHT	↓	↓	↑
PRL	→	↑	→
TSH	→	→	→
T_4	↓	↓	↓
T_3	↓	↓	↓
Serum metabolic parameter levels			
Cholesterol	↓	↓	↑
HDL	↓	↓	↑
LDL	↓	↓	↑
Triglycerides	→	↓	→

Table 12 An overview of effects of EQ compared to that of FLUT on the brain and its neuroendocrine regulation of the HPG axis and control of sexual behavior in adult male rats (continued).

Parameters	Equol Low-treated males	Equol High-treated males	Flutamide-treated males
Pituitary, gene expression			
ERα	➡	⬆	➡
ERβ	➡	➡	➡
AR	➡	➡	➡
TERP-1	⬆	⬆	➡
TERP-2	⬆	⬆	➡
GnRHR	➡	⬇	⬆
LHβ	➡	➡	⬆
α-subunit	⬇	➡	⬆
TSHβ	⬇	⬇	⬇
MBH/ME, gene expression			
ERα	➡	➡	➡
ERβ	➡	⬇	➡
AR	➡	➡	➡
GnRHR	⬆	➡	⬆
MPOA/AH, gene expression			
ERα	➡	➡	➡
ERβ	➡	➡	➡
AR	➡	➡	➡
GnRH	➡	➡	⬇
Amygdala, gene expression			
ERα	➡	➡	➡
AR	➡	➡	➡
Hippocampus, gene expression			
ERα	➡	➡	➡
AR	➡	➡	➡
Prostate, gene expression			
ERα	➡	➡	⬆
ERβ	➡	➡	➡
AR	➡	⬆	⬆
Behavioral response and female parameter			
Male mating behavior	Positive	Positive	Failed
Female pregnancy outcome	Normal	Normal	Failed

Note: ⬆/⬇= significant increase/decrease compared with the controls.
➡ = no significant change compared with the controls.

4. DISCUSSION

4.1. *In vivo* Experiment 1: Neuroendocrine effects of VZ in adult male rats

This is the first study to investigate possible impacts of VZ on the brain and neuroendocrine activity of the HPG axis in adult male rats. As endpoint parameters mRNA expression levels of genes relevant for the activity of the HPG axis, i.e. sex steroid receptors and sex steroid-regulated genes, were determined in selected areas of the brain and pituitary. To evaluate antiandrogenic effects of VZ, gene expression in accessory sex organs was also evaluated.

4.1.1. Achieved dosage of VZ after 5 days oral application

The intended dose of VZ was 150 mg/kg bw/day. Due to the slightly reduced final body weight, the achieved dose of VZ was 148.25 mg/kg bw/day. The HPLC analysis of serum samples collected about 2 h after the last application revealed a mean concentration of total (i.e. after extraction and hydrolysis) VZ of 15.64 ± 0.99 µg/ml or 54.65 ± 3.46 µmol/l. Recently, Sierra-Santoyo et al. (2004) reported serum concentrations of VZ 4 h after an oral application of 100 mg/kg bw to adult male rats. With the method reported in this recent study, the authors achieved a mean VZ concentration of 20 µM. We applied 150 mg VZ/kg bw and determined mean serum concentration of about 50 µM. Thus, our extraction method and the HPLC analysis ensure a high recovery of VZ and a reliable measurement of VZ in serum samples.

4.1.2. Effects of VZ on the reproductive organ weights and hormone levels

The wet weights of accessory reproductive organs (prostate, seminal vesicles, epididymides) and serum levels of reproductive hormones (LH, T) are proposed by the OECD as reliable measures to detect antiandrogenic actions of endocrine-disrupting chemicals (O'Connor et al., 2002; Shin et al., 2006). The present results show that sub-acute administration of VZ resulted in the significant decrease in relative epididymides weight, and elevation of serum LH and T levels. These results confirm and extend those recently reported by O'Connor et al. (2002), who demonstrated decreased epididymides weight and elevated serum LH and T in response to oral VZ treatment of intact adult male Sprague-Dawley rats for 15 days at the same dose as applied in the present study. However, our short-term 5 days *in vivo* oral treatment regimen is already sufficient to alter the weight of an androgen-dependent organ and serum reproductive hormone levels. Therefore, a 5-day treatment should be sufficient to unravel an antiandrogenic property of a test compound. It should be emphasized that we purposely used a short application period because we knew from our own behavioral studies with male rats that a 5-day oral application of the antiandrogen FLUT completely suppresses sexual behavior, i.e. already after 5 days brain function is clearly affected. Thus, the results

revealed that VZ with regard to reproductive organ weights and hormone levels acts as a typical AR antagonist.

4.1.3. Effects of VZ on the hypothalamic GnRH pulse generator

VZ increased LH levels after a 5-day oral administration which is either due to a direct stimulatory effect on pituitary gonadotropes or to enhanced activity of hypothalamic GnRH neurons. A direct effect on gonadotropes is unlikely since experiments with rat pituitary cells *in vitro* revealed no effect of VZ on LH release under both basal and GnRH stimulated conditions (Konstantin Svechnikov, personal communication). Thus, increased LH release upon VZ treatment should result from increased activity of GnRH neurons. Surprisingly, elevated LH release was associated with decreased rather than enhanced GnRH mRNA expression in the MPOA/AH, where the GnRH perikaya are predominantly located. In the MBH/ME where the majority of GnRH axon terminals are located, expression of the GnRH receptor remains unaffected. However, in the pituitary the GnRH receptor mRNA levels were dramatically up-regulated by VZ treatment, which may be caused by enhanced GnRH release into the pituitary portal vessels. Hence, it is very likely that GnRH mRNA levels in the MPOA/AH do not reflect the processing and secretion of the neuropeptide. It has been shown previously that depending upon the physiological conditions, in the rat the regulation of hypothalamic GnRH release may be controlled at the levels of GnRH perikaya and/or their axon terminals, and does not reflect always in change of the preoptic GnRH mRNA levels (Leonhardt et al., 1999; 2000; Kang et al., 2000).

It is well known that expression of the GnRH receptor in the pituitary is controlled by the pulsatile exposure to GnRH. Depending on GnRH pulse frequency and amplitude, the GnRH receptor expression levels are increased or decreased (Kaiser et al., 1997). Expression of the GnRH receptor in the pituitary is up-regulated. Since LH release is not affected by VZ directly at the gonadotropes, it is suggested that VZ at the dose applied in the present study apparently increased the activity of GnRH neurons which results in up-regulation of pituitary GnRH receptor expression. In contrast, the GnRH receptor expression in the MBH/ME remains unchanged which is indicative that these receptors regulate other functions in the CNS than GnRH release. This hypothesis is supported by a previous study in which application of a GnRH agonist affected expression of the GnRH receptor in the pituitary, MBH/ME, and MPOA/AH in a different manner (Roth et al., 2001a). No significant changes of levels of AR and ERα mRNA expression were observed in the hypothalamic structures, while ERβ expression in the MBH/ME was reduced. Thus, the hypothalamic mechanisms involved in the possible effects of VZ may be due to changes in the GnRH release and ERβ sensitivity.

4.1.4. Effects of VZ on the extrahypothalamic regions

The striatum and hippocampus are the extrahypothalamic sites that mediate the sex steroidal effects on brain function like regulation of the HPG axis (Kalra and Kalra, 1983; Levine et al., 1991; Sagrillo et al., 1996) due to the presence of receptors (Kerr et al., 1995; Kuppers and Beyer, 1999; Creutz and Kritzer, 2004). Therefore, they may be potential action sites of VZ either directly *via* binding to ERs or AR, or indirectly *via* altering sex steroid levels. Considering that within the rat brain the AR is autologously regulated, i.e. when the circulating androgens levels are high, the AR expression will be down-regulated (Burgess and Handa, 1993; Handa et al., 1996), we indeed observed a significant down-regulation of the AR in the striatum of VZ-treated rats, while in the hippocampus AR expression remained unaffected. Whether the reduction of striatal AR levels is due to a direct interaction of VZ or its metabolites with the receptor or whether it is caused indirectly *via* increased serum T levels remains yet unknown. It is interesting to note that no significant changes of levels of ERα and ERβ mRNA expression were observed in the extrahypothalamic brain areas, while ERβ mRNA levels in the MBH/ME was significantly down-regulated. Thus, these data are the first evidence that VZ may affect expression of nuclear receptors in a brain area-specific manner.

4.1.5. Effects of VZ on the pituitary function

Consistent with previous reports, we demonstrate an interference of VZ with the negative feedback mechanism of T on pituitary LH secretion in adult male rats as indicated by increased serum LH and T levels which is due to its known antiandrogenic properties (O'Connor et al., 2002; Kubota et al., 2003). In line with the elevated LH secretion is the up-regulation of LHβ mRNA expression and the significant increases in pituitary GnRH receptor and α-subunit mRNA expression following VZ treatment, which were not examined in previous studies (O'Connor et al., 2002; Kubota et al., 2003). Apparently, VZ enhances pituitary responsiveness to hypothalamic GnRH pulses, and therefore, the biosynthesis of GnRH receptor and LHβ- and α-subunits are increased which, in turn, induces activation of testicular steroidogenesis and release.

While VZ did not alter the levels of ERα, ERβ, and AR mRNA levels in the pituitary, mRNA expression of TERP-1 and -2, which are the pituitary-specific, estrogen-induced, truncated ERα isoforms, were significantly elevated. Although the physiological roles of TERP-1/-2 are still largely unknown, it has been demonstrated that TERP-1 suppresses estrogen-induced transactivation by dimerization with both ERα and ERβ (Schreihofer et al., 1999; Resnick et al., 2000). In turn, in both female and male rats, TERP expression is profoundly up-regulated

by *in vivo* estradiol treatment (Friend et al., 1997; Mitchner et al., 1998) which may represent an additional tuning mechanism for estrogen actions in the rat pituitary (Tena-Sempere et al., 2001a). Hence, the stimulatory effect of VZ or its metabolites, M1 and M2, on TERP expression may be attributed to an estrogenic action in the pituitary. Such an estrogen-like action of VZ has been shown only *in vitro* previously (Scippo et al., 2004; Molina-Molina et al., 2006). Thus, this is the first evidence for a putative estrogenic action of VZ or its metabolites *in vivo*, and therefore at least in the pituitary of adult male rat, VZ has to be considered as a mixed AR antagonist/ERs agonist, not as a 'pure' anti-androgenic as has been concluded from previous *in vivo* investigations.

4.1.6. Effects of VZ on the thyroid hormone homeostasis

According to the classical negative feedback concept, low serum T_4 levels should lead to higher TSH secretion from the pituitary. In the present study, however, despite lower circulating thyroid hormone concentrations, the levels of serum TSH and in tendency also TSHβ expression were reduced by VZ. This hormonal constellation is reminiscent of changes observed in central tertiary hypothyroidism or thyroid hormone metabolism known as non-thyroidal illness (Fliers et al. 2006). Since our animals did not show any signs of illness or abnormalities of behavior, the simultaneously reduced serum TSH and T_4 levels may result from a direct action of VZ on the thyrotropes, resulting in reduced thyrotropin release. Notably, our findings of decreased serum T_4 levels along with increased relative liver weights of VZ-treated rats are in line with a previous study demonstrating that the lowered serum T_4 levels in response to oral VZ treatment of intact adult male Sprague-Dawley rats for 15 days at the same dose as applied in the present study may be secondary due to the increased hepatic clearance of thyroid hormones following liver enzyme induction, and this was nearly identical to that reported for the well-known antiandrogen FLUT (O'Connor et al., 2002).

4.1.7. Effects of VZ on the reproductive and accessory sex organs

The prostate, seminal vesicles and epididymides are known androgen-dependent organs. In the present study, they were selected as ''reference'' organs for an anti-androgenic action of VZ. Expression of AR in the prostate, seminal vesicles, and epididymis was up-regulated by VZ, i.e. a 5-day treatment is sufficient to unravel an antiandrogenic property of a test compound. Furthermore, expression levels of ERβ in the ventral prostate were down- and in the epididymis up-regulated, whereas ERα gene expression was not affected by VZ treatment in the prostate and epididymis while in the seminal vesicles it was increased. Since VZ has been demonstrated to directly bind to both ERα and ERβ (Scippo et al., 2004; Molina-Molina et al., 2006), and the alteration of ERs expression is a surrogate marker for an estrogen-modulated response (Shupnik et al., 1989; Mitchner et al., 1998; Tena-Sempere et

al., 2000; Prange-Kiel et al., 2003), it is suggested that VZ might exert estrogen-like effects in these organs. To the best of our knowledge, these results are the first data demonstrating that VZ acts as the mixed AR antagonist/ERs agonist in the prostate, seminal vesicles, and epididymis of adult male rats rather than as a 'pure' antiandrogen as concluded from previous *in vivo* studies. The results obtained so far clearly prove that data of gene expression analysis are reliable endpoints to further evaluate mechanisms of VZ action that could contribute to its *in vivo* deleterious effects in development and reproductive function.

4.2. *In vivo* Experiment 2: Neuroendocrine effects of EQ in adult male rats

Based on the results of the VZ-study, which revealed yet unknown estrogenic effects of the formerly classified antiandrogen VZ, it was investigated whether EQ, a known estrogenic compound, may exert antiandrogenic actions in the HPG axis in the male rat. The measures employed were alterations of mRNA expression of selected androgen- and estrogen-responsive genes in particular areas of brain, pituitary, and the resulting impact on androgen-induced male mating behavior. In addition, mRNA expression in the prostate as an androgen-dependent reference organ was also evaluated. The known antiandrogen FLUT was employed as the reference compound.

4.2.1. Effects of EQ on mating behavior in the male rat

Sub-acute oral administration of EQ both at low and high doses to adult male rats did not affect mating behavior. In a pilot study, we also examined the effects of EQ at the dose of 0.25 mg/kg bw/day on mating behavior to confirm its antiandrogenic effects reported by Lund et al. (2004) who observed the reduced prostate size in intact adult male rats treated for 4 days with EQ at this dose. This experiment was conducted with a subset of adult male rats *s.c.* injected for 4 days with either EQ or vehicle DMSO as previously described (Lund et al., 2004). After 4 days treatment, all of these male rats performed the mating test with the sexually receptive females. All of them showed successful mating behavior. Thus, the previously reported antiandrogenic effects of EQ at a putative low dose of 0.25 mg/kg bw/day do not reflect in mating behavior. We therefore decide to repeat the experiment with two higher doses of EQ. However, even at dose of 100 or 250 mg/kg bw/day, EQ did not affect mating behavior. In contrast, sub-acute administration of FLUT at the dose used in this present study resulted in complete impairment of mating behavior in adult male rats which is in line with previous reports (Sodersten et al., 1975; Gray, 1977; Gladue and Clemens, 1980; McGinnis and Mirth, 1986). To the best of our knowledge, there are no current data available regarding to the effects of EQ on male mating behavior. Thus, the present data demonstrate that EQ did not exert antiandrogenic effects on mating behavior in adult male rats. In addition, the results of FLUT treatment demonstrate that a 5-day *in vivo* oral administration

utilizing intact adult male rats should be a powerful tool to characterize putative antiandrogenic compounds regarding male sexual behavioral effects.

It may be argued that the applied dose of EQ was too low to affect mating behavior. However, serum concentrations of EQ measured in rats treated with 100 mg EQ/kg bw/day were in the range found in Asian men consuming a traditional soy-rich diet (Morton et al., 2002; Hedlund et al., 2003). Serum concentrations of rats treated with 250 mg EQ /kg bw/day were at a pharmacological level ($\approx 3 \times 10^{-5}$ M). At this concentration EQ profoundly inhibits proliferation of steroid receptive breast and prostate cancer cells (unpublished own data). Therefore, the EQ concentration achieved in the serum is clearly sufficient to induce biological effects. Thus, it may be possible that even at this high level EQ does not cross the blood brain barrier (BBB). This interpretation is substantiated by the finding that we observed only in the MBH/ME an effect of EQ on gene expression. The collected tissue sample contains the eminentia mediana which is outside the BBB which is accessible for EQ while those brain areas which control behavior, however, are not. Thus, it is unlikely that in human males EQ at concentrations measured in serum of men consuming soy affect brain function directly.

4.2.2. Effects of EQ on the reproductive organ weights and hormone levels

Of the OECD classical endpoints examined in the present study, FLUT revealed the anticipated typical pattern of antagonistic effects both at the peripheral and central AR functions, i.e. decreased weights of ventral prostate, seminal vesicles, and epididymides, and increased levels of serum LH, T, and DHT without any alteration in serum PRL levels as previously described in the intact adult male rat assay (O'Connor et al., 1998a; 2002; Andrews et al., 2001; Kunitmatsu et al., 2004). The effects of EQ on alterations in serum concentrations of reproductive hormones were completely opposite to those exerted by FLUT. EQ at high dose partially induced an antiandrogenic effect by reducing the relative weight of seminal vesicles with no changes of relative weights of ventral prostate and epididymides. Thus, on a molar base EQ is a weak antiandrogen compared to FLUT which becomes apparent only in the very androgen-sensitive seminal vesicles. It is therefore concluded that EQ and FLUT interfere with the activity of HPG axis by different mechanisms of action, and EQ did not completely exert antiandrogenic effects on the reproductive organ weights and serum reproductive hormone levels. As demonstrated for FLUT, our results, in addition, indicated that the 5-day intact adult male rat assay should be a powerful tool to characterize putative antiandrogenic EDs regarding neuroendocrine regulation of the HPG axis function.

4.2.3. Effects of EQ on the hypothalamic GnRH pulse generator

In the MPOA/AH where most of the GnRH perikaya are located, EQ at high dose decreased levels of GnRH mRNA transcripts, however, this effect did not reach significance. Since we suggest that EQ does not cross the BBB, the effect of EQ on GnRH expression must be indirect, possibly *via* the significantly reduced T-levels. In the MBH/ME where most of the GnRH axons are terminated, EQ low dose significantly increased levels of GnRH receptor mRNA expression, while in the pituitary EQ reduced GnRH receptor mRNA levels. It is well known that in the rat the regulation of the GnRH receptor depends on pulsatile exposure to GnRH (Norwitz et al., 1999; Roth et al., 2001a). EQ reduced LH secretion which is either due to a direct effect at the gonadotropes or indirect *via* reduced activity of GnRH neurons. The latter explanation would be in line with the reduced expression of the GnRH receptor in the pituitary. Thus, it is very likely that GnRH mRNA levels in the MPOA/AH do not always reflect the processing and secretion of the neuropeptide as reported previously (Leonhardt et al., 1999; Kang et al., 2000). In contrast, FLUT treatment resulted in decreased levels of GnRH mRNA expression in the MPOA/AH, and dramatically up-regulated GnRH receptor mRNA levels in the MBH/ME and pituitary. These results suggested that in adult male rats FLUT increases hypothalamic GnRH- and pituitary LH-release due to the relief of the AR-mediated negative feedback effects of endogenous androgens on the hypothalamus and pituitary. However, it was curious that FLUT did not alter the levels of AR mRNA expression in the MPOA/AH and MBH/ME. Our findings were nevertheless similar to those of McAbee and Doncarlos (1999), who found that treatment of intact male rats with FLUT at doses that significantly decreased accessory sex organ weights did not alter AR mRNA expression in either the MPOA or ventromedial hypothalamus, and suggested that AR mRNA expression in these brain regions at adulthood are not affected by FLUT. Taken together, our results revealed that the hypothalamic effects of EQ and FLUT comprised changes of GnRH and GnRH receptor expression.

4.2.4. Effects of EQ on the extrahypothalamic structures

In the extrahypothalamic brain regions (i.e., amygdala, hippocampus) that mediate the androgen and estrogen effects on the regulation of the HPG axis and male sexual behavior (Kostarczyk, 1986; Burgess and Handa, 1993; Kerr et al., 1995; McGinnis et al., 1996), both EQ and FLUT did not significantly alter mRNA expression levels of AR and ERα. However, the present finding that the hippocampal AR mRNA levels did not significantly increase by FLUT treatment is surprising. Since previous studies have demonstrated that the expression of AR mRNA in hippocampus of adult male rats was generally up-regulated following short-term castration, and this effect was reversed by treatment with androgens (Quarmby et al., 1990; Burgess and Handa, 1993; Handa et al., 1996). A possible explanation may be that the

decrease in the androgenic stimulus following FLUT treatment was not sufficient and would be more evident in the castrated rat model (Turner et al., 2001). Our finding of unchanged AR mRNA expression levels in the amygdala upon both treatments is consistent to those previously reported by Burgess and Handa (1993) in which in the amygdala of adult male rats the AR mRNA levels were not influenced by either castration, DHT, or estradiol administration. Taken together, our results revealed that the extrahypothalamic mechanisms of EQ and FLUT actions share changes of ERα and AR expression. In addition, these results demonstrated that the regulation of ERα and AR in the hippocampus and amygdala upon sub-acute EQ or FLUT exposure are not related to male mating behavior, since similar pattern of changes of those gene expression were found in the EQ- and FLUT-treated rats, while only FLUT suppressed mating behavior.

4.2.5. Effects of EQ on the pituitary function

EQ had notable effects on LH and PRL secretion. It is well known that estradiol is a stimulator of PRL secretion both *in vitro* and *in vivo* (Lieberman et al., 1981; Jordan et al., 1984; Jarry et al., 1986), whereas androgens exert opposite effects (Giguere et al., 1982; Tong et al., 1989). In support of the estrogenic effects of EQ on pituitary LH and PRL secretion, there are several reports describing a decrease in serum LH and increase in serum PRL levels in response to estradiol exposure of adult male rats (O'Connor et al., 1998b; Tena-Sempere et al., 2001a; 2001b). To our knowledge, direct effects of EQ on male rat pituitary cells have not been investigated yet. The present data provides therefore a first indication for direct estrogenic effects of EQ on both gonadotropes and lactotropes in adult male rats.

The EQ-induced changes on LH and PRL release may be mediated *via* ERα and TERP because EQ increased levels of ERα and TERP mRNA transcripts in a dose-dependent manner. To support this hypothesis, the present results revealed that ERα is localized to most anterior pituitary cells as previously described (Mitchner et al., 1998; Shughrue et al., 1998; Schlecht et al., 2004) and the EQ treatment significantly increased the nuclear ERα mRNA/protein transcripts as have been previously demonstrated with the estradiol treatment (Shughrue et al., 1998; Rachon et al., 2007a). Also, ERα has been previously reported to mediate the direct effects of estrogen in pituitary cells, since ERα knockout mice displayed markedly reduction in PRL mRNA levels and elevated gonadotropin mRNA levels (Scully et al., 1997). Furthermore, it has been demonstrated that in female and male rats the expression levels of the pituitary-specific, truncated isoform of rat ERα, TERP-1, is profoundly elevated by estradiol treatment (Friend et al., 1997; Mitchner et al., 1998; Tena-Sempere et al., 2001a), suggesting that the TERP-1 is an important mediator of the effects of

estrogen in the pituitary (Friend et al., 1995). In agreement with our results, acute treatment with estradiol (Mitchner et al., 1998), and chronic EQ exposure (Rachon et al., 2007a) did not affect pituitary ERβ expression in adult ovariectomized rats, but TERP-1 mRNA expression levels were dramatically increased. In contrast, FLUT tended to up-regulate ERβ mRNA expression in the pituitary, whereas it did not affect the expression levels of ERα and TERP. To our surprise, pituitary AR mRNA expression was not changed by FLUT treatment, but nevertheless, this result is in agreement with previous data reported by Ohsako et al. (2003).

EQ did not affect the levels of LHβ mRNA transcripts, but slightly reduced α-subunit expression. Dalkin et al. (1989; 2001) demonstrated that pulsatile GnRH release can selectively increase the levels of gonadotropin subunit gene expression and release in male rat pituitary, and that a lack of this stimulation decrease gonadotropin subunit mRNA levels. Hence, the decreased levels of α-subunit mRNA expression by the EQ treatment could be caused by the decreased GnRH stimulation of gonadotrope cells as mentioned already above. Consequently, the levels of serum LH were substantially decreased, which in turn reduced the testicular steroidogenesis, and thereby decreased serum levels of T and DHT. Opposite effects were observed following FLUT treatment. Consistent with previous studies (Ohsako et al., 2003; Kubota et al., 2003), FLUT stimulated levels of LHβ, α-subunit, and GnRH receptor mRNA transcripts which can be explained that FLUT could enhance GnRH pulses due to the disruption of negative feedback of endogenous androgens on the hypothalamic GnRH pulse generator. Taken together, effects of EQ and FLUT in the pituitary were different due to the opposite changes in ERα-, TERP-1/-2-, GnRH receptor-, LHβ-, and α-subunit expression.

4.2.6. Effects of EQ on thyroid hormone system

Both EQ and FLUT at the dose applied decreased serum T_4 and T_3 without any significant alteration in serum TSH levels, while TSHβ gene expression was significantly down-regulated in all treatment groups. This discrepancy cannot be explained at present, however, our findings of decreased serum T_4/T_3 levels along with increased relative liver weights of FLUT-treated rats were consistent with previous studies demonstrating that the lowered serum T_4/T_3 levels following FLUT exposure to adult male rats was due to a secondary mechanism induced by the increased hepatic clearance of thyroid hormones following liver enzyme induction (O'Connor et al., 1998a; 2002; Andrews et al., 2001). However, it is also possible that EQ inhibits the thyrotropes such that they cannot compensate low T_4 levels *via* increased TSH biosynthesis. Such an effect has been described recently for the endocrine disruptor octyl-methoxycinnamate (OMC) (Klammer et al., 2007). As demonstrated for FLUT, it is reasonable to postulate that EQ may also induce liver enzyme activities, giving rise to the

increased liver weights along with decreased serum T_4/T_3 levels in a similar manner to FLUT. To our knowledge, this is the first study demonstrating an adverse effect of EQ on thyroid hormone homeostasis in adult male rats.

4.2.7. Effects of EQ on the metabolic parameters

Recent studies from our laboratory have demonstrated that sub-acute treatment of adult ovariectomized rats with natural estrogen or estrogen-like compounds resulted in the dramatic reductions in serum concentrations of lipid parameters, i.e. cholesterol, HDL, and LDL (Klammer et al., 2005; Schlecht et al., 2006; Rachon et al., 2007b). According to these studies, EQ clearly shows estrogen-like effects in adult male rats by decreasing serum levels of lipid parameters. Moreover, EQ at high dose caused a significant decrease in serum concentration of triglycerides, and this result is in agreement with the previous study of our group demonstrating that chronic EQ treatment has profound effects on serum triglycerides levels in adult ovariectomized rats (Rachon et al., 2007b). The decreased levels of serum lipid parameters reflect the decreased lipid metabolism that could contribute to a significant reduction in the terminal body weights of EQ-treated rats as previously demonstrated (Rachon et al., 2007b). In contrast, treatment with FLUT caused significant increases in circulating cholesterol, HDL, and LDL, without changing triglycerides levels. Comparable to our study, it has been previously reported that the concentrations of total plasma cholesterol and phospholipids were highly significant increased in adult male rats treated with FLUT at the same dose as used in this study (Kunimatsu et al., 2004; Mannaa et al., 2005), and this may also contribute to the increased relative liver weight. Hence, our data demonstrated that EQ and FLUT exert their regulatory influences on lipid metabolism through the different modes of action.

4.2.8. Effects of EQ on the prostate function

Up-regulation of AR mRNA expression in intact adult rat ventral prostate is a robust marker for antiandrogenic effect of FLUT within this androgen-dependent tissue (Kumar et al., 1997; 2002). Similarly, an up-regulation of the AR was also observed in the ventral prostate of EQ high dose-treated males. However, in this study, the EQ high dose treatment did not decrease the relative ventral prostate weight. Since EQ does not bind to AR (Lund et al., 2004), our results may point to a different mechanism underlying the up-regulation of AR in the ventral prostate by EQ or FLUT. A decrease in circulating T levels causes activation of AR gene and up-regulation of AR mRNA levels (Kumar et al., 1997; 2002). Since the EQ high dose treatment resulted in very low levels of circulating T *via* reduction of LH secretion, this data suggest that the up-regulation of AR mRNA expression by EQ high dose treatment may be due to the estrogenic effect of EQ on LH release.

It is interesting to note that FLUT caused the up-regulation of ERα mRNA levels in the prostate, whereas EQ did not. This data was somehow unexpected as the observed results of FLUT exposure caused clear-cut effects on hormone responsiveness, prostate weight, and prostatic AR gene expression in a typical pattern of a potent AR antagonist. Also, FLUT has neither estrogenic nor antiestrogenic effects both *in vitro* and *in vivo* (McGinnis and Mirth, 1986; Laws et al., 1996; O'Connor et al., 1998a; Kim et al., 2002). Thus, the up-regulation of ERα mRNA levels upon FLUT treatment, may therefore be causally related to the net temporal increase in estrogenic stimulus, perhaps as a result of elevated E2 levels following the aromatic conversion of elevated T to E2, that may still exist within the prostate due to the attenuation of the androgenic stimulus upon FLUT treatment as described previously (Simard et al., 1986; O'Connor et al., 1998a; Kim et al., 2002).

4.3. Conclusion

In conclusion, the present study provided the first *in vivo* data demonstrating that:

1. VZ is not a ‘pure’ antiandrogen, since it exerts mixed AR antagonistic/ERs agonistic actions observed at the levels of mRNA expression of selected AR- and ERs-regulated genes in the brain, pituitary, and male accessory sex organs;
2. EQ displays a clear-cut endocrine activity within the HPG axis which is attributed to be estrogenic;
3. EQ does not exert any antiandrogenic effects on brain, pituitary and prostate functions, and on male mating behavior;
4. FLUT exerts its potent antiandrogenic actions in the brain and its neuroendocrine regulation of the HPG axis, and control of androgen-induced male mating behavior;
5. As demonstrated for FLUT, a short-term (5 days) *in vivo* oral administration utilizing intact adult male rats should be a powerful tool to characterize putative antiandrogenic EDs with regard to neuroendocrine and male sexual behavioral aspects.

5. SUMMARY

Numerous anthropogenic chemicals and natural plant-derived products can mimic or disrupt the normal function of endogenous sex steroid hormones by direct interaction with their specific nuclear receptors. These compounds were classified as 'Endocrine Disruptors' (EDs). However, there is increasing evidence that at least some EDs are rather promiscuous with regard to the sex steroid receptor selectivity, i.e. they bind to more than one nuclear receptor. An example of such promiscuous EDs is fungicide Vinclozolin (VZ) which can exert androgen receptor (AR) antagonistic as well as estrogen receptors (ERs) agonistic actions. Likewise, Equol (EQ), a metabolite of the isoflavonoid daidzein, interacts with ERs, however, as suggested recently, EQ may also exert antiandrogenic activity. Despite the proven adverse effects of EDs on development and reproductive function, however, little efforts have been made to investigate the potential pharmacological actions of VZ and EQ on a variety of brain functions, particularly the neuroendocrine regulation of the HPG (hypothalamo-pituitary-gonadal) axis function when male individuals are exposed to these EDs. Therefore, the present study is designed to investigate whether and how VZ and EQ affect the brain and neuroendocrine function in adult male rats upon 5-days oral treatment.

Examined parameters were potential effects of VZ and EQ on gene expression in the brain (medial preoptic area/anterior hypothalamus (MPOA/AH), mediobasal hypothalamus/median eminence (MBH/ME), striatum, hippocampus and amygdala), pituitary, prostate, seminal vesicles, and epididymis. In addition, the impact of equol on male mating behavior was examined. As reference compound, the pure antiandrogenic drug flutamide (FLUT) was employed. At the end of treatment interval or after completion with mating test, animals were sacrificed. The trunk blood was collected and brains and pituitaries were removed. Prostates, seminal vesicles, epididymides, testes, and livers were dissected and weighed. Changes in levels of serum hormones (LH, T, DHT, TSH, T_4, T_3) and mRNA expressions of relevant genes (sex steroid receptors, sex steroid-regulated genes) were measured by RIA and qRT-PCR, respectively. Serum concentrations of test substances were analyzed by HPLC-UV detection.

The present study revealed that VZ decreased weight of epididymides and increased serum levels of LH and T. In the hypothalamic brain areas, VZ affected the expression of GnRH and both ERs subtypes ERα and ERβ. In the extrahypothalamic brain areas, VZ altered expression of both AR and ERs. In the pituitary, VZ up-regulated expression of GnRH receptor, LHβ, α-subunit, and TERP-1/-2. In the prostate, VZ increased and decreased levels of AR and ERβ mRNA, respectively. In the seminal vesicles, VZ increased levels of AR and ERα mRNA expression. In the epididymis, VZ up-regulated AR and ERβ mRNA levels. Upon

the pituitary-thyroid axis, VZ exerted direct effects on the pituitary thyrotropes resulting in decreased serum TSH and T_4 levels.

While FLUT displayed the typical pattern of an AR antagonist, such as decreased weights of ventral prostate, seminal vesicles, and epididymides, and increased levels of serum LH, T and DHT without effects on serum PRL levels, EQ exerted opposite effects. The hypothalamic effects of EQ and FLUT comprised changes of GnRH and GnRH receptor expression, while in the extrahypothalamic areas both compounds altered ERα and AR expression in a similar manner. In contrast, effects of EQ and FLUT in the pituitary were different due to the opposite changes in ERα-, TERP-1/-2-, GnRH receptor-, LHβ-, and α-subunit expression. In the prostate, EQ and FLUT both affected ERα and AR expression. EQ did not modulate the expression of male mating behavior, whereas FLUT completely inhibited it. The similar reductions in serum T_4 and T_3 levels caused by EQ and FLUT treatments was primary due to the direct action on the thyrotropes, and secondary to the hepatic clearance of thyroid hormones following liver enzyme induction as assessed by the increased liver weight.

In summary, the present study provided the first *in vivo* data demonstrating that:

1. VZ is not a ‘pure’ antiandrogen, since it exerts mixed AR antagonistic/ERs agonistic actions observed at the levels of mRNA expression of selected AR- and ERs-regulated genes in the brain, pituitary, and male accessory sex organs;
2. EQ displays a clear-cut endocrine activity within the HPG axis which is attributed to be estrogenic;
3. EQ does not exert any antiandrogenic effects on brain, pituitary and prostate functions, and on male mating behavior;
4. FLUT exerts its potent antiandrogenic actions in the brain and its neuroendocrine regulation of the HPG axis, and control of androgen-induced male mating behavior;
5. As demonstrated for FLUT, a short-term (5 days) *in vivo* oral administration utilizing intact adult male rats should be a powerful tool to characterize putative antiandrogenic EDs with regard to neuroendocrine and male sexual behavioral aspects.

6. ZUSAMMENFASSUNG

Zahlreiche anthropogene Chemikalien und Pflanzeninhaltsstoffe können die Funktion der endogenen Sexualsteroide durch eine direkte Interaktion mit deren spezifischen nukleären Rezeptoren beeinflussen. Derartige Substanzen werden als „Endokrine Disruptoren (EDs)“ bezeichnet. Es mehren sich aber die Hinweise, dass zumindest einige EDs hinsichtlich der Interaktion mit Steroidrezeptoren eine geringe Selektivität aufweisen, i.e. diese promisken EDs binden an mehr als einen nukleären Rezeptor.

Ein Beispiel für einen derartigen promisken endokrinen ED ist das Fungizid Vinclozolin (VZ), welches am Androgenrezeptor (AR) antagonistische, am Östrogenrezeptor (ER) hingegen agonistische Wirkungen ausüben kann. Ein zweites Beispiel ist Equol (EQ), ein Metabolit des Isoflavonoids Daidzein. Es ist vielfach gezeigt worden, dass Equol mit dem ER interagiert, aber neueste Daten weisen auf eine mögliche Androgene Wirkung von EQ hin.

Trotz der eindeutigen negativen Wirkungen von EDs auf die Entwicklung und Funktion reproduktiver Organe gibt es keine Untersuchungen zur möglichen Wirkung von VZ und EQ auf die Funktion des ZNS, insbesondere die neuroendokrine Regulation der hypothalomo-hypophysio-gonadalen Achse (HPG-Achse). Daher ist die Zielsetzung der vorliegenden Studie die Untersuchung, ob und wie VZ und EQ die neuroendokrine Regulation der HPG-Achse in erwachsenen, männlichen Ratten nach einer fünftägigen oralen Behandlung beeinflussen.

Als Zielparameter wurde die Genexpression in der medialen, präoptischen Region des vorderen Hypothalamus (MPOA/AH), des mediobasalen Hypothalamus/Eminentia mediana (MBH/ME) sowie im Striatum, Hippocampus, Amygdala sowie der Hypophyse untersucht. Als Referenzorgane für steroidregulierte Gewebe wurden Prostata, Samenblase und Nebenhoden gewählt. Im Fall des Equols wurde zusätzlich der Effekt diese EDs auf das Sexualverhalten der männlichen Ratten untersucht. Als Goldstandard für ein reine Antiandrogen wurde Flutamid (FLUT) verwendet. Nach Beendigung der Behandlung, bzw, des Verhaltenstests, wurden die Tiere tierschutzgerecht getötet, das Blut gesammelt und die oben genannten Organe präpariert. Von der Prostata, der Samenblase, dem Nebenhoden und der Leber wurde das Frischgewicht bestimmt. In der Blutprobe wurden die Konzentrationen der Hormone LH, Testosteron (T), Dihydrotestosteron (DHT), TSH, T4, T3 sowie die Konzentrationen der Testsubstanzen selbst durch Radioimmunoassay bzw. HPLC bestimmt. Die Genexpression von Steroidrezeptoren sowie von steroidrezeptor-regulierten Genen in den oben genannten Organen wurde per Taqman-PCR gemessen.

Die VZ-Behandlung resultierte in einem signifikant geringeren Gewicht der Nebenhoden und erhöhten Serumspiegeln von LH und T. Im Hypothalamus beeinflusste VZ die Expression von GnRH und von ERα und ERß. In den extrahypothalamischen Arealen verursachte VZ eine Veränderung der Expression sowohl des ARs als auch der ERs. Die hypophysäre Expression von LH, von TERP 1/2 sowie des GnRH-Rezeptors wurde durch VZ hochreguliert. In der Prostata hingegen reduzierte VZ die Expression des ERß und erhöhte die mRNA-Spiegel des AR. In der Samenblase bewirkte die Behandlung mit VZ eine erhöhte Expression des AR und des ERα. Im Nebenhoden erhöhte sich unter VZ-Behandlung die Expression des AR und ERß. Die reduzierten Serumwerte von TSH und T4 werden wahrscheinlich durch einen direkten Effekt von VZ auf die thyreoptropen Zellen der Hypophyse hervorgerufen.

FLUT erzeugte das erwartete Muster von Effekten eines AR-Antagonisten, i.e. reduzierte Gewichte der Prostata, der Samenblase und des Nebenhodens und erhöhte Serumspiegel von LH, T und DHT ohne Effekte auf die Prolaktinspiegel. EQ hingegen induzierte gegensätzliche Effekte. EQ als auch FLUT veränderten im Hypothalamus die Expression von GnRH und des GnRH-Rezeptors während in den extrahypothlamischen Arealen beide Substanzen die Expression von ERα und AR in identischer Weise veränderten. In der Hypophyse hingegen waren unter EQ bzw. FLUT-Behandlung gegenläufige Effekte auf die Expression von ERαm TERP 1/2, des GnRH-Rezeptors, der LHα und ß-Untereinheit zu messen. Die Expression des ERα und des AR in der Prostata wurde sowohl durch EQ als auch durch FLUT beeinflusst.

Die fünftägige Behandlung der männlichen Ratten mit drei verschiedenen Dosierungen von EQ führte in keiner Behandlungsgruppe zu einer Veränderung des Sexualverhaltens. FLUT hingegen unterdrückte das Sexualverhalten in allen getesteten Tieren komplett.

Die Serum T4- und T3- Spiegel wurden durch EQ und FLUT signifikant reduziert, was auf eine direkte Wirkung der Testsubstanzen auf die thyreotrophen Zellen der Hypophyse hinweist. Möglicherweise trägt auch der erhöhte hepatische Metabolismus der Schilddrüsenhormone zu deren reduzierten Serumspiegeln bei. Diese Interpretation einer Enzyminduktion steht im Einklang mit den erhöhten Lebergewichten.

Zusammenfassend können aus der vorliegenden Studie folgende Schlussfolgerungen gezogen werden:

1. *In vivo* ist VZ kein reines Antiandrogen, sondern besitzt sowohl AR-antagonistische als ER-agonistische Eigenschaften im Gehirn, der Hypophyse und akzessorischen Geschlechtsorganen.
2. Equol hat eine ausgeprägte ED-Aktivität auf die HPG-Achse, die ausschließlich auf eine östrogene Wirkung zurückzuführen ist. Weder im Gehirn, der Hypophyse noch in peripheren Organen wie der Prostata konnten eine signifikante antiandrogene Wirkung von EQ nachgewiesen werden.
3. Die Referenzsubstanz FLUT erwies sich als ein starkes Antiandrogen hinsichtlich der neuroendokrinen Regulation der HPG-Achse und des androgeninduzierten männlichen Sexualverhaltens.

7. ACKNOWLEDGEMENTS

This study was carried out at the Department of Clinical and Experimental Endocrinology, Faculty of Medicine, Georg-August-University of Goettingen during September 2004 to November 2007. I am extremely grateful to all those who have contributed to this work and to my friends and family for making this a great period of time. Of course, this thesis would not have been completed without a nice gesture from many persons. I would especially like to take this opportunity to thank the following persons:

I would like to express my deepest sense of gratitude and sincerest appreciation to my thesis advisor and sponsor for pursuing my PhD study at the PhD program of Center for Systems Neuroscience, Professor. Dr. med Wolfgang Wuttke, the Head and Executive director of the Department of Clinical and Experimental Endocrinology, for giving me the opportunity to join in his research group, his valuable encouragement and support and providing the best research facilities to do independently the research works for my PhD study under his supervision and guidance. Moreover, he always makes very positive contributions to the success of my PhD study and also to my participations at any international conferences relevant to my research area to get much more knowledge and scientific networks.

My sincerest appreciation and gratefulness are also expressed to my thesis supervisor and committee, Professor. Dr. Hubertus Jarry, the Executive director of the Department of Clinical and Experimental Endocrinology, who not only gave me an interesting topic and encouraged and guided me throughout the work, but also offered me an opportunity to pursue my own ideas and to work independently as a young scientist under his excellent supervision. I really appreciate his wide knowledge on both theory and practice of biochemistry and neurosciences, his capacity of precise, fast and logical thinking and his enthusiasm when he becomes really interested in something and/or with some doubt. I also appreciate with his endless patience in a not-always-easy way when I was too much concerned about my plans and results. I also gratefully acknowledge him for his positive and substantial contributions to the success of my PhD project and study. Without his extreme encouragement and excellent support, my publications and thesis would never have been completed. I will remember him as an excellent thesis supervisor whom I have ever met.

Together with this, I would like to gratefully and sincerely thank Professor Dr. Stefan Treue and Professor Dr. med. Hannelore Ehrenreich, the Board members of the PhD program of Center for Systems Neuroscience, for both of their great interest in my work and endless helps for my participation in highly motivating PhD program, and also for their serving as my official thesis advisor and committee.

Also, I am most grateful to Dr. med. Dana Seidlová-Wuttke for her instruction, advice, guidance and very helpful encouragement at the beginning period of my study.

Grateful thank is conveyed to Dr. vet. med. Guillermo Rimoldi, for his important contribution to the beginning time and the first part of my work with the laboratory rats, and for guiding me into the research areas of immunocytochemistry and histopathology.

My sincere gratitude is especially expressed to Dr. med. Dominik Ráchon, who gave invaluable help and valuable comments with interpretation of the results concerning the medical and physiological aspects. His fascinating personality and enthusiasm were most inspiring me and his expertise is gratefully appreciated.

Special thanks are also due to Dr. Sari Mäkelä, Institute of Biomedicine, Department of Anatomy, University of Turku, Finland, for giving me the opportunity to visit her laboratory and guiding me into the world of immunocytochemistry and its relation to pathophysiological studies.

Likewise, sincere thanks are also expressed to all of my friends in the laboratory of the Department of Clinical and Experimental Endocrinology, Damina Balmer, Julie Christoffel, Holger Klammer, Christiane Schlecht, Tina Vortherms, and Nguyen Ba Tiep for their very friendly assistance and friendship, and for making the days in the lab enjoyable and productive as well.

I owe my warmest gratitude to the senior technicians of the Department of Clinical and Experimental Endocrinology, Maria Metten, Sabine Lüdemann, Claudia Nietzel, Christel Düls, Annette Witt, Martina Bremer, Antje Ahrbecker, Christine Klein, Horst Schikola and Heide Brüggeman-Meyer for their skillful and excellent technical supports, and helpful advice about the materials and instruments. I particularly wish to address special thanks to Maria Metten, Sabine Lüdemann, and Claudia Nietzel for always being ready to help despite their own duties, and for their cordial relationship.

My acknowledgements are also expressed to Dr. vet. med. Klaus Nebendahl, the Head of the animal experimental facilities at Faculty of Medicine, University of Goettingen, and also his colleagues, Martina Radtke, Melanie Bernhardt, Christiane Bode, Hartmut Danneberg, for their effective cooperation, invaluable help with bringing and preparing the animal models of this study.

I recognize and gratefully acknowledge the Corroborative Research Network (CRN) scholarship awarded by the Commission on Higher Education, Ministry of Education, Thai Government, Thailand, for the financial support during the full-time period of my PhD study.

I owe my deepest gratitude and loving thanks to my father Mr. Wirachai Loutchanwoot, my mother Mrs. Chittraporn Pinyarattana, my younger sisters Ms. Potchana Loutchanwoot and Mrs. Potchanee Loutchanwoot, and my younger brother Mr. Theerathep Loutchanwoot, for their warmful love, nice support and encouragement, and also for their believing in me.

Finally, my deepest love and heartful thanks belong to Dr. Prayook Srivilai for his beautiful and warmful love, care, support and understanding which never be forgotten. I am privileged to have the most wonderful boyfriend, who not only brings joy, happiness and safety into my life, but also has generously helped me in dealing with any problems.

This work was financially supported in part by the EU-funded “CASCADE” Network of Excellence.

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9. LIST OF ABBREVIATIONS

α-subunit	=	gonadotropin alpha-subunit
ANOVA	=	analysis of variances
AR	=	androgen receptor
ARE	=	androgen response element
BSA	=	bovine serum albumin
bw	=	body weight
°C	=	degree celcius
cDNA	=	DNA complementary to RNA
CHOL	=	cholesterol
CNS	=	central nervous system
Ct	=	cycle threshold
ddH_2O	=	double distilled water
DHT	=	dihydrotestosterone
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
DMSO	=	dimethylsulfoxide
dNTP	=	deoxyribonucleoside triphosphate
EDs	=	endocrine disruptors
EDSTAC	=	U.S. EPA task force on the EDs
EQ	=	equol
ERα	=	estrogen receptor subtype alpha
ERβ	=	estrogen receptor subtype beta
ERE	=	estrogen response element
ERs	=	estrogen receptors
et al.	=	et alii or ‘and others’
EtOH	=	ethyl alcohol or ethanol
E2	=	17β-estradiol
FAM	=	6-carboxy-fluorescein
FLUT	=	flutamide
FSH	=	follicle-stimulating hormone
g	=	gram
GnRH	=	gonadotropin-releasing hormone
GnRHR	=	gonadotropin-releasing hormone receptor
h	=	hour
HDL	=	high density lipoprotein
H&E	=	hematoxylin and eosin

HPG	=	hypothalamo-pituitary-gonadal axis
HPLC	=	high performance liquid chromatography
HRP	=	horseradish (*Armoracia rusticana*) peroxidase
i.e.	=	id est or ‘that is’ or ‘in other words’
kg	=	kilogram
λ	=	lambda (wavelength)
l	=	liter
LDL	=	low density lipoprotein
LH	=	luteinizing hormone
LHβ	=	beta subunit of luteinizing hormone
M	=	molar
MBH/ME	=	mediobasal hypothalamus/median eminence
μg	=	microgram
μl	=	microliter
μM	=	micromolar
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimolar
M-MLV RT	=	Moloney Murine Leukemia Virus Reverse Transcriptase
MPOA/AH	=	medial preoptic area/anterior hypothalamus
mRNA	=	messenger ribonucleic acid
NIH	=	National Institute of Health
nM	=	nanomolar
nm	=	nanometer
NTC	=	no template control
OECD	=	Organization for Economic Cooperation Development
PBS	=	phosphate-buffered saline
PCR	=	polymerase chain reaction
pH	=	potential of hydrogen
′ (prime)	=	denotes a truncated gene at the indicated side
PRL	=	prolactin
qRT-PCR	=	quantitative TaqMan®Real-time PCR
RIA	=	radioimmunoassay
RNA	=	ribonucleic acid
RNase	=	ribonuclease
RNasin	=	ribonuclease inhibitor
rpm	=	round per minute

SEM	=	standard of the mean
sec	=	second
s.c.	=	subcutaneous
STDN	=	standard
T	=	testosterone
TAMRA	=	6-carboxy-tetramethyl-rhodamine
Taq	=	*Thermus aquaticus* DNA polymerase
TERP-1	=	truncated estrogen receptor product-1
TERP-2	=	truncated estrogen receptor product-2
TGs	=	triglycerides
TSHβ	=	beta subunit of thyroid-stimulating hormone
T_3	=	triiodothyronine
T_4	=	tetraiodothyronine or thyroxine
U	=	unit
UNG	=	uracil-N-glycocylase
UNKN	=	unknown
U.S. EPA	=	United States Environmental Protection Agency
UV	=	ultraviolet
VZ	=	vinclozolin
v/v	=	volume per volume

10. CURRICULUM VITAE

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CONFERENCE CONTRIBUTIONS:

Loutchanwoot P, Hubertus J, and Wolfgang W. Neuroendocrine and sexual behavioral effects of the endocrine disruptor Equol in young-adult male rats. Poster Abstract. ‘’CASCADE’’ 3rd Annual Meeting, 17–19 April 2007, Helsinki, Finland. p. 32

Loutchanwoot P, Wolfgang W, and Hubertus J. Effects of a 5-day treatment with vinclozolin on the hypothalamo-pituitary axis in young-adult male rats. Poster Abstract. Neurizon 2007 Interdisciplinary Meeting on the Neurosciences, 31 May–2 June 2007, Göttingen, Germany. p. 53

POSTER PRESENTATIONS:

Loutchanwoot P, Wolfgang W, Hubertus J. Effects of a 5-day treatment with vinclozolin on the hypothalamo-pituitary axis in adult male rats. Poster Session No.53B. Neurizon 2007 Interdisciplinary Meeting on the Neurosciences, 31 May–2 June 2007, Göttingen, Germany

Loutchanwoot P, Hubertus J, Wolfgang W. Neuroendocrine and sexual behavioral effects of the endocrine disruptor, Equol, in adult male rats. Poster Session No.20. ‘’CASCADE’’ 3rd Annual Meeting, 17–19 April 2007, Helsinki, Finland

Loutchanwoot P, Hubertus J, Wolfgang W. Effects of equol and vinclozolin on the hypothalamo-pituitary-gonadal axis in male rats. Poster Session No.13, Bregenz Summer School on Endocrinology of the ‘’CASCADE’’ Network of Excellence, 31 July–3 August 2006, Bregenz, Austria

LIST OF PUBLICATIONS:

Loutchanwoot P, Wolfgang W, Hubertus J. Effects of a 5-day treatment with vinclozolin on the hypothalamo-pituitary-gonadal axis in adult male rats. (Accepted by “Toxicology”)

Loutchanwoot P, Wolfgang W, Hubertus J. Neuroendocrine and sexual behavioral effects of equol in adult male rats. (Submitted to “Reproductive Toxicology”)