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Research Article

Sertraline Suppresses Testis and Adrenal Steroid Production and Steroidogenic Gene Expression While Increasing LH in Plasma of Male Rats Resulting in Compensatory Hypogonadism

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ABSTRACT

Selective serotonin reuptake inhibitors are used as first line treatment in major depressive disorder. However, selective serotonin reuptake inhibitors have also been associated with sexual disorders, abnormalities, and sexual dysfunction, although mechanisms are unclear. The aim of this project was to investigate the possible endocrine disrupting effect of sertraline (SER) on sex steroid production in male rats exposed to 3 therapeutically realistic doses of SER 1.25, 5, and 20 mg/kg/day. To achieve this, we analyzed all the major steroids in testis, adrenals, brain, and plasma using Liquid chromatography tandem mass spectrometry. Furthermore, we investigated the potential effects on gene expression on the major genes involved in testicular, adrenal and brain steroidogenesis using quantitative PCR. Moreover, plasma luteinizing hormone (LH) levels were analyzed. We observed significant reduction in steroid production, in particular on the testicular Δ-4 axis and on the adrenal CYP17-hydroxylase axis. Effects in brain and plasma were less pronounced. Testicular gene transcription was also significantly down-regulated except for the CYP19 (aromatase) gene. In contrast, no effects on the adrenal gene expression were observed, except for an up-regulation of the CYP17. Plasma LH and LH/TS were increased, in particular in the lowest exposure group, indicating some degree of compensatory hypogonadism. In conclusion, this study demonstrates extensive endocrine disruption during SER exposure in male rats, both directly on steroid production in major endocrine tissues, but also indirectly by affecting gene expression. Furthermore, increased LH levels may augment decreased sex steroid production, in particular testosterone production, inducing a state of compensatory hypogonadism.

Key words: endocrine disrupting drugs; androgens; progestagens; estrogens; corticosteroids; gonads; adrenals; plasma; luteinizing hormone.

Selective serotonin reuptake inhibitors (SSRIs) are drugs widely used against depression and other psychiatric disorders. SSRIs are the most widely prescribed drugs against major depressive disorder with much higher sales numbers than other antidepressants such as tricyclic antidepressants (TCAs) and selective noradrenalin reuptake inhibitors (Kreke and Dietrich, 2008; Nierenberg *et al.*, 2007; Werner and Covenas, 2010). Although still debated, the SSRIs are believed to exert their

therapeutic effect by blocking the serotonin reuptake transporter. This causes a decrease in the reuptake of serotonin to the presynaptic nerve thereby increasing the concentration of serotonin in the synaptic cleft. This increases the stimulation of the postsynaptic terminal, attenuating depression (Keri *et al.*, 2005; Lindqvist *et al.*, 2015). Sertraline (SER), fluoxetine and citalopram are widely used SSRIs, SER being the single most prescribed antidepressant in the United States (Grohol, 2015; IMS Institute for Healthcare Informatics, 2017).

Since the introduction of SSRIs, several side-effects have been described, some of which have been connected to the endocrine system. Sexual disorders appear to be common in SSRI users and 30%–60% of all SSRI users have been reported to suffer from some form of sexual dysfunction (Gregorian *et al.*, 2002). In adult males, the use of SSRIs has been associated with hormonal abnormalities and decreased fertility such as low levels of testosterone (TS), luteinizing hormone (LH), and follicle stimulating hormone (FSH), and elevated level of prolactin (Safarinejad, 2008).

In mammals, steroid hormones are produced in the metabolic pathway known as the steroidogenesis governing growth and reproduction. This pathway is mainly carried out in adrenals and gonads, and to a lower extent, also in the brain (Hadley and Levine, 2006; Miller and Auchus, 2011). Adrenals mainly produce progestagens and corticosteroids but low levels of androgens and estrogens may also be produced (Miller and Auchus, 2011). In contrast, the main steroids produced in the gonads are androgens (mainly TS) in testis and estrogens (mainly 17β-estradiol, βE2) in ovaries. Relatively high levels of progestagens may also be found in gonads.

The preferred pathway for the production of sex steroids is species specific. Humans, dogs (*Canis familiaris*) and pigs (*Sus scrofa*) appear to have a preference for the Δ5-pathway, meaning that pregnenolone (PREG) is converted 17-hydroxypregnenolone (OH-PREG). In other species such as rats and perhaps also polar bears (*Ursus maritimus*) and spotted hyena (*Crocuta crocuta*), steroids are mainly synthesized through the Δ4-pathway, thereby favoring the conversion of PREG to progesterone (PROG) which is then converted to sex steroids (Brock and Waterman, 1999; Conley *et al.*, 2012; Conley and Bird, 1997; Gustavson *et al.*, 2015; OECD, 2011; Sonne *et al.*, 2014; van Jaarsveld and Skinner, 1991).

The corticosteroids are synthesized mainly in the adrenals, because the Cytochrome P450 enzymes CYP21 and CYP11β1 are widely expressed in this tissue. 17α-hydroxylase/17, 20-lyase (CYP17) responsible for the production of sex steroids is expressed in both adrenals and gonads, but appears to have higher activity in gonads. The brain expresses CYP11A1, 3β-Hydroxysteroid dehydrogenase (3βHSD), CYP11β1 among others, which may form PREG, PROG, and corticosterone (COS) from cholesterol (Gazdar *et al.*, 1990; Miller and Auchus, 2011). The mammalian steroidogenesis is shown in Figure 1.

A few *in vitro* studies indicate that SSRIs have the capacity to affect steroid synthesis (Hansen *et al.*, 2017; Jacobsen *et al.*, 2015; Lupu *et al.*, 2017) although effects and mechanisms appear to be drug specific. Furthermore, SSRIs may affect the brain's regulation of steroid production. To ensure sex steroid homeostasis, the gonadal steroidogenesis is regulated by the brain via the hypothalamic-pituitary-gonadal axis (Pariante and Lightman, 2008). Gonadotropin-releasing hormone (GnRH) is released from the hypothalamus to the pituitary gland. The pituitary gland is thus activated and the 2 gonadotropins, LH, and FSH are secreted to the blood and transported to the gonads. In males, LH stimulates the testis to secrete TS (Hiller-Sturmhofel and Bartke, 1998). TS causes negative feedback on the hypothalamus, resulting in reduced release of GnRH from the hypothalamus and consequently a decrease in the release of gonadotropins from the pituitary gland, resulting in negative feedback homeostasis in sex steroid production. In males, The LH/TS ratio is a good indicator for the pituitary-testis (PT) axis activity and gonadal function.

In this study, we investigated the endocrine disrupting effects of SER, on steroidogenesis *in vivo* using male rats. We analyzed all the major steroids in plasma, testis, adrenals and brain during exposure to realistic SER dosage regiments. Furthermore, we conducted quantitative PCR in testis, adrenals and brain to investigate the effects of SER exposure on the most important enzymes in the steroidogenesis. Finally we analyzed plasma LH levels to get an indication of the effects that SER may exert on the negative feedback system.

EXPERIMENTAL

Chemicals

Sertraline "HEXAL" 100 mg tablets (batch no. EP8713), which are commercially available on prescription were investigated in this study. Unikalk basic 400 mg tablets (batch no. 430489) is available without prescription. Heparin sodium was obtained from Applichem (Panreac, Darmstadt, CAS no. 9041-08-1). n-heptane, acetone, and methanol (MeOH) were of HPLC grade and obtained from Lab-Scan, Gliwice, Poland. The standard consisted of Androstenedione (AN), PREG, PROG, dehydroepiandrosterone (DHEA), TS, dihydrotestosterone (DHT), estrone (E1), 17β-estradiol (βE2), aldosterone (ALDO), cortisol (COR), COS, 17-hydroxyprogesterone (OH-PROG), OH-PREG, 11-deoxycorticosterone (11-deoxyCOS), 11-deoxycortisol (11-deoxyCOR) and cortisone (CORNE) and were all purchased from Sigma-Aldrich, Glostrup, Denmark. Androstenediol (ADIOL) was purchased from Toronto Research Chemicals, North York, Ontario, Canada. The internal standards (IS) d7-androstenedione (AN-d7), d4-estrone (E1-d4), d5-17β-estradiol (βE2-d5), d8-COS (COS-d8) and d8-11-deoxyCOS (11-deoxyCOS-d8) were purchased from CDN isotopes, Pointe-Claire, Qubec, Canada, while d9-progesterone (PROG-d9), TS-d3, d3-dihydrotestosterone (DHT-d3), d7-aldosterone (ALDO-d7) and d4-cortisol (COR-d4) were purchased from Toronto Research Chemicals. The following steroids were purchased from Sigma-Aldrich: d4-pregnenolone (PREG-d4), d5-11-deoxyCOR (11-deoxyCOR-d5) and DHEA-d6.

Animal Study

The experiment was approved by the animal experimentation inspectorate (protocol no. 2014−15—0201 −00031 section C). 29 Male Sprague Dawley (SPRD) rats (Harlan Laboratories B.V., Venray, The Netherlands) were purchased at 7 weeks of age with a weight of 200–250 g. They were initially housed in groups of 5–6 in type IV cages containing ordinary bedding (080114E, TAPVEI, Estonia), ASTP wooden bricks and a shelter, with food and water ad lib. Rats were housed under controlled conditions of 22°C ± 2°C and a constant 12 h/12 h light/dark cycle. The rats received chocolate spread on gnawing sticks during the first 3 days of housing to secure familiarity to taste and smell. The following 14 days, the rats were single housed when presented to a chocolate spread mixed with calcium and biscuit.

At 9 weeks of age and a mean weight of approximately 270 g, the rats were exposed to SER for a 14-day period. The rats were randomized into 5 groups each containing 6 rats except the weight control (WC) group, which contained 5 individuals.

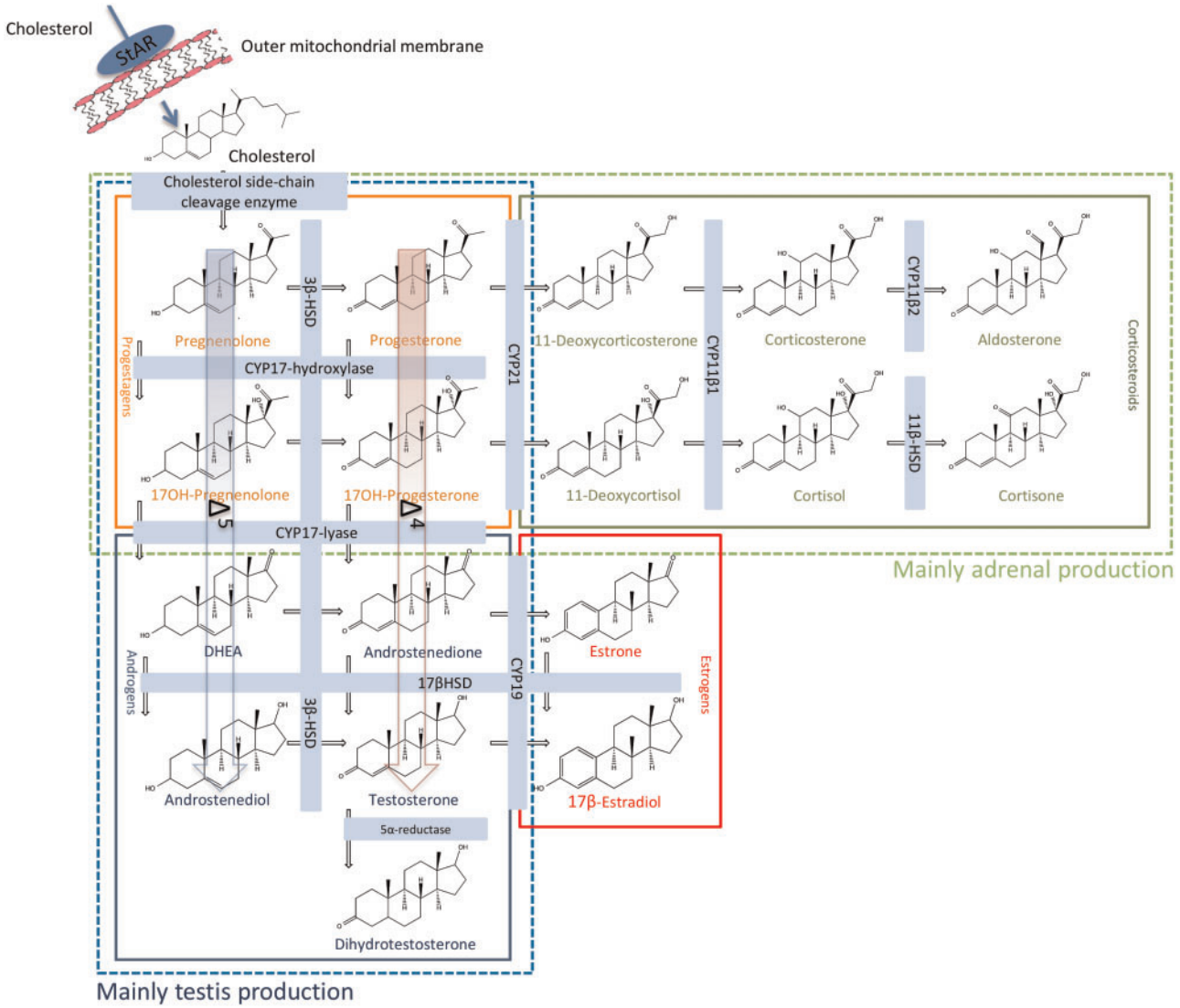


Figure 1. Schematic drawing of the steroidogenesis, with all the key enzymes (blue bars) involved in the production of the steroid hormones (chemical structure). The StAR protein mediates the transport of cholesterol from the outer mitochondrial membrane to the inner membrane. The cholesterol side-chain cleavage enzyme (CYP11A1) is the rate limiting step. The blue (left) and pink (right) arrows across some of the steroids indicate the Δ^5 and Δ^4 pathways, respectively. Steroids mainly produced in the adrenals and testis, respectively, are also indicated. PREG, pregnenolone; OH-PREG, 17-hydroxy-pregnenolone; PROG, progesterone; OH-PROG, 17-hydroxy-progesterone; DHEA, dehydroepiandrosterone; ADIOL, androstenediol; AN, androstenedione; TS, testosterone; DHT, dehydrotestosterone; deoxyCOS, 11-deoxy corticosterone; COS, corticosterone; ALDO, aldosterone; deoxyCOR, 11-deoxy cortisol; COR, cortisol; CORNE, Cortisone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The WC group only received regular food and water *ad libitum*. The control group (C) received vehicle with calcium (chocolate spread, batch no. 144485), the low dose group (L) received 1.25 mg SER/kg bw/day, the middle dose group (M) received 5.0 mg/kg bw/day and the high dose group (H) received 20.0 mg/kg bw/day. The doses used were calculated from human equivalent dose (HED) (CDER, 2005), to match a daily human dose of 50 mg/day (Danish Health Authority, 2014). The rats were dosed by mixing SER with chocolate spread on a cracker once daily. The bodyweight was controlled continuously and no deviations between the groups receiving chocolate spread on biscuits and WC were observed. The growth of the rats further followed the growth curve from vendor.

On day 15 (24 h after last dose) the rats were weighed and afterwards euthanized by a blow to the head, to ensure unconsciousness before cutting the neck. Blood was immediately collected in heparin-coated Sarstedt tubes. Brain, testis and adrenal glands were sampled and immediately frozen in liquid nitrogen. Blood samples were kept cold and centrifuged (9 min, 4°C, 5000 rpm) to separate plasma from red blood cells. All samples were stored at −80°C until further steroid and gene analysis.

Steroid Extraction and Purification

Steroid extraction and purification from testis, adrenal glands and brain was performed according to Weisser *et al.* (2016). In brief the tissue were chopped into a homogenic mass from which samples were collected in triplicates (100 mg testis, 200 mg brain), except the adrenal glands, where a single whole gland was used for each analysis. Thereafter, 75% MeOH and 50 µl (0.1 ng/µl) IS solution was added to each sample prior to homogenization with a tissue tearror. The homogenization was performed 3 times to ensure that all steroids were extracted from the tissue. The samples were centrifuged at 12 000 rpm for

6 min. The collected supernatants were transferred to C18 columns preconditioned with 3 ml *n*-heptane, 3 ml acetone, 3 ml MeOH, and 5 ml water. The loaded columns were turbo-washed with water followed by 25% MeOH. The steroids were eluted with 80% MeOH, evaporated to dryness under nitrogen and redissolved in 200 µl 20% MeOH.

Plasma samples (200 µl) were mixed with 50 µl, 0.1 µg/ml IS and diluted with 4 ml water. The diluted samples were transferred to C18 columns, preconditioned as described earlier, eluted with 5 ml 80% MeOH, evaporated to dryness and redissolved in 200 µl 20% MeOH.

Online Clean-Up and Quantification by LC-MS/MS

Further clean-up, separation, and quantification was performed according to Weisser *et al.* (2016), using a binary 1290 aglient infinity series system combined with a binary 1100 aglient HPLC series pump. The system included an autosampler set at 8°C, an in-line filter (1290 infinity in-line filter, Agilent), a TTC switching valve with 6 ports in 2 positions (left and right), and 2 columns; C18 enrichment column (µbondapak C18, 3.9× 20 mm, 10 µm, Waters) and C18 analytical column (Kinetex, 2.6 µm C_{18} 100 A, 75 × 2.1 mm, Phenomenex, USA). A constant flow of 1 ml/min 20% MeOH was generated by the 1100 pump. Two mobile phases were used, mobile phase A was composed of water with 0.1% formic acid and mobile phase B contained pure MeOH. To separate the steroids completely an elution gradient was performed. A mass spectrometer (AB SCIEX 4500 QTRAP) was used for detection, provided with an atmospheric pressure chemical ionization Turbo V source. This allowed separation of all steroids. The method was running for a total of 16 min. Further details including quality criteria and method validation is found in Weisser *et al.* (2016).

Enzyme-Linked Immunosorbent Assay

The concentration of plasma LH was determined by competitive enzyme-linked immunosorbent assay kit (Cloud-Clone Corp., Product no. CEA441Ra). The provided standard instruction manuals were followed. The plasma samples were diluted 1: 10 with phosphate buffered saline prior to detection.

Reverse Transcription Quantitative PCR

Prior to reverse transcription quantitative PCR (RT-qPCR), RNA from testis, brains and adrenals was isolated. First the tissue (testis and brain) were homogenized by grinding in liquid nitrogen to obtain homogene samples. An appropriate amount of tissue (50–100 mg for brain and testis and the whole adrenal gland) was further homogenized in 1 ml Trizol Reagent (Invitrogen) using Precellys 24 bead beater. RNA was isolated using Trizol Reagent protocol from the vendor. RNA was quantified on a nano-drop spectrophotometer and 1 µg was used for cDNA synthesis using SensiFAST cDNA synthesis Kit (Bioline) following manufactures instructions. RNA quality was not tested for individual samples, which is a limitation of the study. cDNA samples were diluted 1:2 with DEPC-treated water and stored at −80°C. RT-qPCR reactions included 2 µl cDNA, 150 nM forward and reverse primers, and SensiFast SYBR lo-ROX Q-PCR mastermix (Bioline). Transcript levels were analyzed on a Stratagene Mx3005P Q-PCR system (Agilent Technologies) with an initial 2-min step at 95°C followed by 40 cycles of 95°C for 5 s, 60°C for 20 s and 72°C for 15 s. Absence of genomic DNA was verified by –RT reactions for all RNA pools. Dissociation curves were made for each experiment to verify that only single products were amplified. Three different housekeeping genes (β-actin, hprt and glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) were tested to find the most stable reference gene for each of the 3 tissues. Hprt and β-actin showed same stability in all tissues. Consequently, it was decided to use β-actin as reference gene.

Primer sequences were: β-actin, (F: 5'—ACT ATC GGC AAT GAG CGG TTC C and R: 5'—CTG TGT TGG CAT AGA GGT CTT TAC G); steroidogenic acute regulatory protein (StAR), (F: 5'—TTC TCA ACT GGA AGC AAC ACT CTA C and R 5'—ACC TGG CAC CAC CTT ACT TAG C); Cytochrome P450 17A1 (CYP17A1), (F: 5'—CTA TCC GAG AAG TGC TGC GTA TC and R: 5'—CTG CGT GGG TGT AAT GAG ATG G); CYP11A1, (F: 5'—GGG CTT TGG CTG GGG TGT TC and R: 5'—AGG CTG GAA GTT GAA GAA GAT AGG C) (Zhang *et al.*, 2013); GAPDH, (F: 5'—GGT GAT GCT GGT GCT GAG TA and R: 5'—GGA TGC AGG GAT GAT GTT CT) (Shi *et al.*, 2013); Cytochrome P450 19 (CYP19), (F: 5' TGC ACA GGC TCG AGT ATT TCC and R: 5'—ATT TCC ACA ATG GGG CTG TCC) (Hapon *et al.*, 2010); Cytochrome P450 21A1 (CYP21A1), (F: 5' CAA GAA ACT CTC TCG CTC AGC CCT and R: 5' CAG CAA AGT GCT GTC CTG CTT GT) (Hu *et al.*, 2007); 3βHSD, (F: 5' GTT CTA CTA CAT CTC AGA TGA CAC C and R: 5' TAT AGT TGT AAA ATG GAC GCA GC) (Khodadadi *et al.*, 2012).

Data Processing and Statistical Analysis

Data obtained from LC-MS/MS were examined using the MultiQuant 3.0 Software. Chromatograms from all samples were inspected and peaks were manually integrated if necessary. Afterwards, the raw data were transferred and processed in Microsoft Excel. All data were analyzed using GraphPad Prism vers. 7. All datasets of each steroid hormone were gathered and a Grubb's test for outliers was performed. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was performed to test if responses from test concentrations were significantly different from control samples. Differences were considered significant at $p \leq .05$. RT-qPCR data were analyzed by the comparative Ct method, described by Schmittgen and Livak (2008). All data were analyzed using GraphPad Prism vers. 7. Data were reported as fold change relative to control samples. Statistically analyses of differences in gene expression were assessed by ANOVA followed by Dunnett's multiple comparison test. A $p \leq .05$ was considered as significant.

RESULTS

Effect of Sertaline on Steroid Production

The effects of SER on the production of steroid hormones were examined in testis, adrenals, plasma, and brain.

Testis. In total 12 steroids were quantified in testis: 4 progestagens, 3 corticosteroid, and 5 androgens (Figure 2). An overall decrease of steroid production was observed in the testis after exposure to SER. Most pronounced was the decrease in the steroid production on the Δ-4 axis, in particular in group L and M. The steroids (PROG, OH-PROG, AN, TS, DHT) decreased significant in the range of 28%–71% ($p < .05$–.0001). The effect on the production of steroids on the CYP17-hydroxylase axis (PREG, PROG, 11deoxyCOS, and COS) was similar and a significant decrease of 36%–71% ($p < .01$–.0001) was observed particular in the L and M group. A decrease was also observed for the androgen ADIOL in all test groups ($p < .001$– .0001). No changes were observed for DHEA.

Adrenals. Changes in steroid production in the adrenals can be categorized into 2 groups, the decrease on the hydroxylase axis and the increase on Δ-4 axis. Most pronounced is the decrease

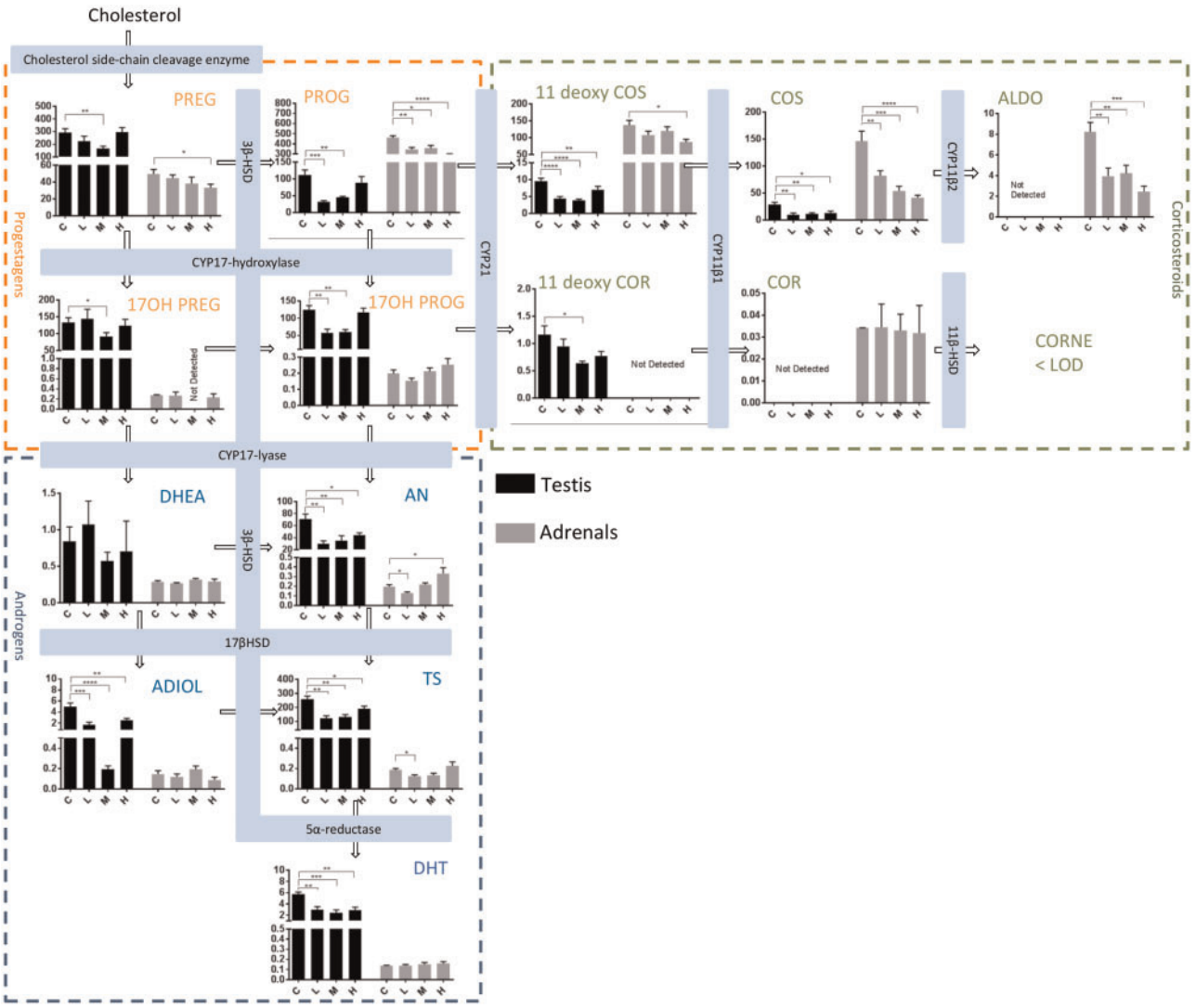


Figure 2. Steroidogenic overview of the effects of SER on steroid production in the testis (ng/g tissue) and adrenal glands (ng/gland). Black bars, testis; Gray bars, adrenal gland. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$. The control (C) group received vehicle only, low (L) dose group received 1.5 mg SER/kg bw/day, middle (M) dose group received 5.0 mg SER/kg bw/day and high (H) dose group received 20.0 mg SER/kg bw/day. Error bars are SEM ($n = 6$ in L, M, and H group, $n = 12$ in C group. Symbols and legends otherwise as in Figure 1).

on the hydroxylase axis (Figure 2). For PROG, COS and ALDO a significant decrease was observed in all test groups by 22%–72% ($p < .05$–$.0001$) compared with the control. For PREG and 11deoxyCOR only the H group ($p < .05$) was significantly decreased although both steroids showed decreasing tendencies in the remaining groups. A tendency to increase in the range 17%–69% in H group was observed for the androgens on the Δ-4 axis (AN, TS, and DHT), this increase was significant for AN ($p < .05$). No changes in steroid production were observed for the steroids on the Δ-5 axis (17OH-PREG, DHEA, and ADIOL) or the detected steroids on the CYP17-lyase axis (17OH-PROG and COR).

Plasma. Changes in steroid levels from plasma samples collected at day 15 are presented in Figure 3A, and in general steroid hormone levels were low except for COS. The following 8 steroids were detected: PREG, PROG, OH-PROG, 11-deoxyCOS, COS, AN, TS, and DHT. The 11deoxyCOS and COS levels decreased significantly for all groups when compared with control. The observed decrease was in the range from 64% to 81% ($p < .05$–$.001$). The 3 detected androgens all decreased after exposure to SER except in group H, where no effect was observed. The decrease was around 50% for AN and DHT in group L and M ($p < .01$–$.001$). No pronounced effects were observed for the progestagens.

Brain. Nine steroids were quantified in the brain after exposure to SER, and data are presented in Figure 3B. In general, all steroids decreased, except the androgens on the Δ^4-axis (AN, TS, and DHT) in group H which had a tendency to slightly increase. Further AN showed a decrease by 84% ($p < .05$) in group M. All determined steroids on the CYP17-hydroxylase axis decreased. PREG decreased by 64% ($p < 0.01$) in group L, PROG by 69% ($p < .05$) in group H, 11deoxyCOS and COS by 62%–80% ($p < .05$–$.01$) in all groups. ADIOL by 31% ($p < .05$) in group L and 91% ($p < .0001$) in group H. No significant changes were observed for DHEA.

An overview of the effects of SER on steroid production in the different tissue and compartments can be seen in Table 2. In summary, SER exerts a general inhibitory effect on steroidogenesis for the organs and concentration range tested, the

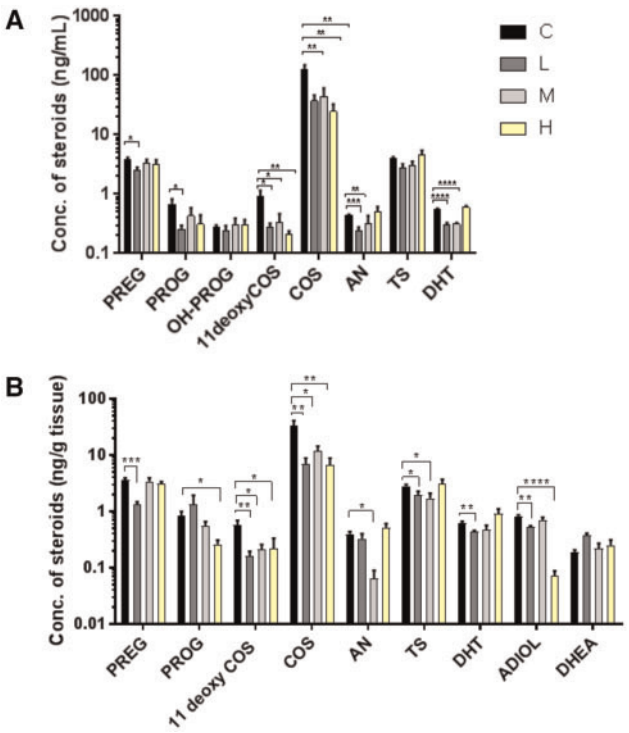
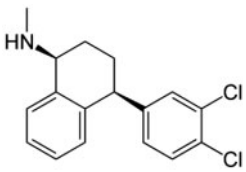


Figure 3. A, Steroid levels (ng/ml) in rat plasma from day 15. B, Steroid levels (ng/g tissue) in brain. Black, control; dark gray, low dose group; light gray, middle dose group; and yellow, high dose group. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$. Error bars are SEM ($n = 6$ in L, M, and H group, n=12 in c group. Symbols and legends otherwise as in Figure 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1. Overview of the Chemical Structure, Selected Physiochemical, and Pharmacokinetic Properties for SER.

	SER
Chemical structure	
Molecular mass, g/mol	306.229
Log(P)	5.06
Therapeutic dose (mg/day)	50–200[a]
Cmax, ng/ml (μM)	55–250[b] (0.16–0.73)
Solubility in DMSO, mg/ml	>25[c]

[a]Marken and Munro (2000).
[b]Winek *et al.* (2001).
[c]SCBT (2014).

decrease in testicular androgens being most pronounced. The adrenal increases in Δ-4 steroids in the highest dose could indicate a compensatory effect due to decreased sex steroid production in the testis.

Effect of Sertaline on Gene Expression

The effects of SER on gene expression of 1 important transporter (StAR) and 5 key steroidogenic enzymes (CYP11A1, CYP17, CYP21, CYP19, and 3β-HSD), were examined in testis, adrenals, and brain. No significant changes in gene expression were observed in the brain (data not shown).

In general, significant decreasing effects were observed for the testicular gene expression as shown in Figure 4A. StAR,

Table 2. Overview of Effects of SER on Steroid Production in Testis, Adrenals, Brain, and Plasma.

Steroid Group	Steroid	Testis	Adrenals	Brain	Plasma
Progestagens	PREG	→	→	↓	↓
	OH-PREG	→	→	–	–
	PROG	↓	↓	↓	↓
	OH-PROG	↓	↑	—	→
Androgens	DHEA	→	→	→	—
	ADIOL	↓	→	↓	—
	AN	→	↑	↓	↓
	TS	↓	↑	↓	→
	DHT	↓	→	↓	↓
Corticoids	deoxyCOS	↓	↓	↓	↓
	COS	↓	↓	↓	↓
	ALDO	—	↓	—	—
	deoxyCOR	→	—	—	—
	COR	—	→	—	—
	CORNE	—	—	—	—

↑, increase in steroid production; ↓, decrease in steroid production; →, no significant change in steroid production and — not detected. PREG, pregnenolone; OH-PREG, 17-hydroxy-pregnenolone; PROG, progesterone; OH-PROG, 17-hydroxy-progesterone; DHEA, dehydroepiandrosterone, ADIOL, androstenediol; AN, androstenedione, TS, testosterone, DHT, dehydrotestosterone; deoxyCOS, 11-deoxy corticosterone; COS, corticosterone; ALDO, aldosterone; deoxyCOR, 11-deoxy cortisol; COR, cortisol; CORNE, Cortisone.

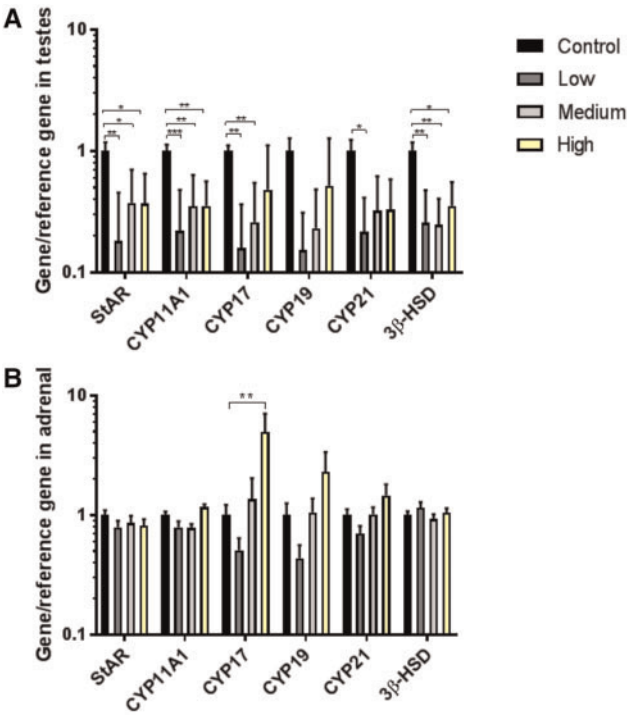


Figure 4. The effect of SER on gene expression of selected key enzymes, expressed as fold changes compared to control (A) in testis and (B) in adrenals. Black, control; dark gray, low dose group; light gray, middle dose group; yellow, high dose group. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. Error bars are SEM ($n = 6$ in low, middle, and high groups, $n = 12$ in control group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

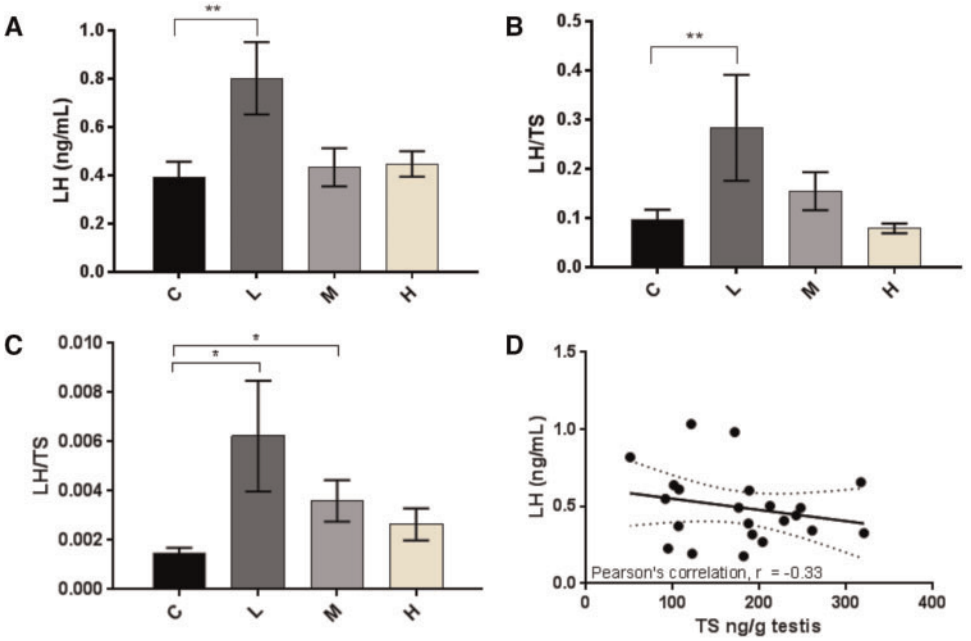


Figure 5. A, LH levels in plasma (ng/ml) at day 15. B, LH/TS in plasma ratio as a function of dose group (C) LH/TS in testis ratio as a function of dose group (D) LH level as a function of TS concentration in the testis (ng/g tissue). C, control group; L, low dose group; M, middle dose group; H, high dose group, error bars are represented by SEM, $n = 6$ in each group. $^{*}p < .05$, $^{**}p < .01$.

CYP11A1 and 3β-HSD were significant decreased at all test doses ($p < .05$–$.001$). The expression of CYP17 was significant decreased in the L and M group ($p < .01$), whereas the decrease in CYP21 expression was only significant in group L ($p < .05$). As expected, no significant effects were observed on the expression of CYP19. In the adrenals, only a significant ($p < .01$) up-regulation of CYP17 in the H group was observed (Figure 4B). This corresponds well with the observed increase in adrenal Δ-4 steroids and could indicate a compensatory effect of the adrenals due to the decreased production of Δ-4 steroids in the testis.

Regulation-PT Feedback

Changes in LH levels in the plasma at day 15 can be seen in Figure 5A. A significant increase in group L by 105% ($p < .01$) was observed. No significant changes were observed for groups M and H. Figures 5B and 5C shows the changes in the LH/TS ratio for plasma and testis, respectively. For plasma, increases by 66%–179% in the L and M groups were observed, though only significant in the L group ($p < .01$). For testis, an increase in the LH/TS ratio of 76%–281% was observed, being significant for groups L and M ($p < .05$). A tendency to a negative correlation between TS levels in the testis and LH levels can be seen in Figure 5C, with a Pearson coefficient of, $r = -0.33$, though not significant.

DISCUSSION

This study provides evidence that SER has the capacity to disrupt steroidogenesis by decreasing steroid production, both in testis and in adrenals (Figure 3). The study also suggests a potential mechanism by which this disruption occurs, ie, an inhibition of the transcription of the genes regulating steroidogenesis and metabolizing steroids (Figure 4). We have not been able to identify any studies investigating the effects of SER on steroidogenic gene expression, and we have not found other *in vivo* studies on SER interactions with CYP gene expression and/or CYP enzymes and further studies are needed. However, it is also clear, in particular from the effects on adrenals, that effects on transcription alone cannot explain the extensive decrease in steroid levels and direct effects on steroid metabolizing enzymes associated with steroidogenesis seem likely. This is supported by the findings of Jacobsen *et al.* (2015) who investigated the direct inhibition of 5 different SSRIs using a recombinant CYP19 assay, and found IC50 values for SER in the range 13–91 μM. Thus, SER may inhibit steroidogenesis both indirectly by inhibiting steroidogenic gene transcription and directly by inhibiting steroidogenic CYP enzymes.

Based on the considerations above, future studies may therefore include the investigation of changes in protein levels for the most rate-limiting enzymes in the steroidogenesis and also Leydig cell transcripts such as insulin-like factor 3. This study also indicates effects of SER on Leydig cells and it may be speculated that the morphology of these cells were compromised. This study, however, was not designed to study testicular histology. In order to conduct histology, samples must be preserved in PFA or even better in Bouin's solution. This is not possible once the samples are frozen in −80, which is the case here, since we wanted to secure the RNA for qPCR. Thus, future studies should also include histological and histochemical end points.

SER is known to also inhibit several non-steroidogenic CYP enzymes *in vitro*. For example, SER is a rather potent *in vitro* inhibitor of CYP2D6 (Crewe *et al.*, 1992; DeVane *et al.*, 2002) and may also moderately inhibit CYP2C11 and CYP3A4 (Masubuchi and Kawaguchi, 2013; Wójcikowski *et al.*, 2013). Other CYP enzymes such as CYP2A/B/C may also be inhibited to a lesser extent (DeVane *et al.*, 2002; Ghosh *et al.*, 2015). All in all, it seems likely that SER has some potential to directly interact with CYP enzymes, but these observations need to be confirmed *in vivo*.

Importantly, this study also indicates a state of compensatory primary hypogonadism, in which the negative feedback, communicated via the PT-axis (Hiller-Sturmhofel and Bartke 1998; Kotsuji *et al.*, 1988), is augmenting decreased TS levels by increased LH levels (Figure 5). In a recent study by Atli *et al.* (2017) male rats were exposed to 5, 10, and 20 mg/kg/day for 28 days. Thus, the exposure scenario by Atli *et al.* (2017) was similar to this study, although other end points were investigated. Similar to this study, these authors also found TS levels to be lowest in the low exposure group, and highest in the high exposure group. In fact, both TS and LH levels were significantly higher in the highest exposure group. The authors do not mention this directly, but this could indicate a state of compensatory hypogonadism following SER exposure, in which TS levels are maintained or even slightly overcompensated by increased LH levels (Atli *et al.*, 2017). This may potentially also explain why we observed a lower effect on TS levels in H group than in the L and M groups. Thus, the 2 studies combined indicate that exposure to SER in therapeutically realistic concentrations may induce compensatory hypogonadism in male rats.

The question then remains whether a state of compensatory hypogonadism could also occur in human males during exposure to SER and other drugs disrupting TS production. Kristensen *et al.* (2018) provides evidence that this may indeed be the case. Exposing young and healthy men to ibuprofen for 6 weeks, Kristensen *et al.* (2018) observed increases plasma LH levels compared with TS with increasing levels of plasma ibuprofen concentrations. The authors show that the likely reason for the LH increase is a direct effect of ibuprofen on the endocrine capabilities of the testis shown by the use of *ex vivo* testis culture. The consequences of such pharmaceutical induced increased levels of LH compared with TS is not known but compensated hypogonadism has been associated with decreased sexual desire, depression, coronary heart disease/heart attack, autoimmune diseases like arthritis, and all-cause mortality (Baillargeon *et al.*, 2016; Hintikka *et al.*, 2009; Holmboe *et al.*, 2015; Kloner *et al.*, 2016). Thus, it is concerning, that 1 of several severe side-effects of SER treatment against depression may be primary hypogonadism, speculated to cause depression. This may potentially be one of the reasons as to why the clinical effects of SER and other SSRIs appear to be rather low, although significant (Jakobsen *et al.*, 2017).

In a previous study by Hansen *et al.* (2017), 5 SSRIs including SER were investigated for their endocrine disrupting potential using the H295R steroidogenesis assay. In this study, all progestagens, corticosteroids, and androgens decreased during SER exposure, indicating that effects on the top part of the steroidogenesis may occur prior to PREG formation. This is in accordance with this study in which PREG levels were decreased in both testis and adrenals. Furthermore, the gene expression for StAR and CYP11A1 in testis was significantly down-regulated. SER may therefore not only affect the steroidogenic pathway itself, but also its regulation.

In total Eight steroids were identified in plasma samples whereas 9 steroids were detected in the brain (Figure 2). Overall, a decrease in steroids was observed, although the patterns were less clear than that observed in testis and adrenals. In general, observed levels are in accordance with previous studies. For example, the levels of PREG, TS, and COS in plasma were 3, 4, and 110 ng/ml, respectively. In comparison, the levels of PREG and COS were determined to be 3 and 117 ng/ml, respectively, in a study by Serra et al. (2001). The plasma levels of TS in this study were also in agreement with 2 other studies where levels were reported to 6 ng/ml (Hansen *et al.*, 2011) and 2.6 ng/ml (Zorrilla *et al.*, 2009). A study by Meffre *et al.* (2007) using GC/MS investigated the baseline levels of 8 steroids in the brain of male SPRD rats of which 3 were also identified in this study. COS, PREG, and TS concentrations were 50, 4.9, and 1.4 ng/g, respectively (Meffre *et al.*, 2007). This was in agreement with this study where the levels of group C were found to be 36, 3.8, and 2.7 ng/g respectively.

Steroids are able to pass the blood-brain barrier and the brain takes up steroids from circulation. However, evidence is mounting that the brain is also capable of *de novo* synthesis of a variety of steroids although there appear to be no specific site for steroidogenesis and steroid synthesis may supplement local action (Miller and Auchus, 2011). For example, the brain contains the mRNA coding for CYP11β1 enabling the synthesis of the corticosteroids (Miller and Auchus, 2011), as indicated by the presence of high levels of 11-deoxy-COS and COS in this study, but it is unclear whether the brain is actually a site of corticosteroid biosynthesis, or if these steroids are produced by the adrenals and taken up from the blood.

The role of steroids in the mammalian brain is quite complex and includes the synthesis of several steroids rarely found in other steroidogenic tissues. One major pathway is the conversion of PREG to allopregnanolone (ALLO) via PROG and dihydroprogesterone (DHP) (Miller and Auchus, 2011). It is well known that patients suffering from depression also have low levels of neuroactive steroids such as ALLO. However, SSRI including SER treatment appears to normalize ALLO levels. The mechanism is assumed to be due to a 10–30 time reduction in K_m for the conversion of DHP into ALLO by 3α-HSD, one of the key neurosteroid enzymes of the human brain (Griffin and Mellon, 1999; Pinna 2010; Schüle *et al.*, 2006). Thus, other studies indicate beneficial effects of SER on the brain steroidogenesis, whereas this study suggests decreased levels of several steroids which may have negative effects. However, it is worth mentioning that several highly important brain neurosteorids such as PREG, TS, and DHT did not decrease significantly in the brain during SER exposure, although these steroids were significantly affected in adrenals and particularly in testis. This could indicate an ability of the brain to maintain neurosteroid levels by de novo synthesis when synthesis in other organs may be compromised. This aspect should be studied further.

The pronounced decrease in testicular Δ-4 steroids confirms the notion that the rat mainly synthesizes TS and DHT via the Δ-4 axis (Conley *et al.*, 2012; Conley and Bird, 1997; OECD, 2011) and this axis also appears to be significantly affected by SER exposure. Interestingly, the CYP17 gene transcription was the only gene significantly up-regulated in the adrenals, and this corresponded with an increase in all Δ-4 steroids downstream from the CYP17-hydroxylase reaction. This is preliminary evidence that the sex steroid production of adrenals may be up-regulated if testicular sex steroid production is compromised, but this aspect of sex steroid regulation awaits further investigation.

We have not been able to identify other studies analyzing all the steroids on the testicular Δ-4 axis of male rats, but other studies report decreasing TS levels in plasma and serum following SER exposure. Erdemir *et al.* (2014) administered 10 mg SER/kg/day (corresponding to 100 mg/day in a standard 70 kilo human male) to Wistar rats for 2 months. The study showed a 61% decrease in serum TS levels compared with the control group. This is similar to the 53% decrease we observed in testis in this study in rats exposed to 5 mg/kg/day SER and a general decrease on the Δ-4 axis, and indicates the pronounced effects SER appear to have on TS production in male rats. Tanrikut *et al.* (2010)

suggest that a 28% decrease in TS levels may have negative impact on human male spermatogenesis. Using the Johnsen's Score, Atli *et al.* (2017) demonstrated decreased sperm count, abnormal sperm morphology increased DNA damage and degeneration of cellular-tubular structures in rats during SER exposure in the concentration range 5–20 mg/kg/day. Collectively, these studies suggest significant effects on steroid production and associated reproductive end points at therapeutically realistic doses.

The dosages used in this *in vivo* study were 1.25, 5, and 20 mg/kg/day for group L, M, and H, respectively. Taking the HED into account, this corresponds to human doses of 12, 50, and 200 mg/day. Thus the dosages used in group M represent the starting dose in humans, which is 50 mg SER/day (pro.medicin.dk, 2015). Significant effects were observed in this group and also in the group L, which received SER 4 times below the starting dose, thus effects must be regarded as clinically relevant. However, conclusions based on rat studies cannot be directly transferred to humans due to variations between species. For example, sex steroids are transported in the human blood by the sex hormone-binding globulin (SHBG) which is produced by the liver and essentially determine the tissue bioavailability of sex steroids. However, the rodent liver appears not to express SHBG and no SHBG is found in rodent blood. Instead SHBG is expressed in sertoli cells of rodent testis (Sáez-López *et al.*, 2015). Thus, transport and regulation of tissue bioavailability in rodents may be fundamentally different from that of humans. Nevertheless, Ye *et al.* (2011) argued that the testicular steroidogenic enzymes of the endocrine system may be affected in a somewhat comparable way in rats and humans. Regardless, a possible endocrine disrupting effect was found *in vivo* using pharmacological relevant dosages, and it shows the need for further investigation. It is especially concerning that SSRI exposure causes a drastic decrease in TS levels in rats, since TS is an important regulator of normal spermatogenesis (Walker, 2011).

Based on the studies mentioned earlier, it is possible that SSRI exposure at therapeutically relevant doses leads to decreased semen quality in rats, and likely also in human males and a 3 months clinical trial study with 60 male patients confirms these findings (Akasheh *et al.*, 2014). This study showed a significant reduction in sperm concentration, sperm morphology, and an increase in DNA fragmentation when treated with 25 mg/day SER demonstrating that SER adversely affects fertility. In another study, 27 infertile men were treated with the aromatase inhibitor letrozole. The men exhibited an increased TS/βE2 ratio, which increased the sperm motility and sperm count (Gregoriou *et al.*, 2012; Saylam *et al.*, 2011). This suggests a connection between sex steroid levels and sperm count, and that increased TS levels may improve fertility.

CONCLUSIONS

This *in vivo* study revealed significant effects on both testicular and adrenal steroidogenesis when exposing male rats to SER in therapeutically realistic doses. This was evident both when investigating steroid production but also when investigating gene expression for the major genes in the steroidogenesis. These effects were observed even at the lowest administrated dosage of 1.25 mg/kg, which corresponds to a dosage 4 times below starting dose in humans. The most significant effects were observed on testicular sex steroid production, in particular on the Δ-4 axis and TS production was significantly decreased in all 3 exposure groups. This is especially concerning as decreased TS production is associated with low sperm production, low fertility, and low reproductive potential in the human male. This study also indicates a state of compensatory hypogonadism, in particular in the lowest exposure groups. In the human male, hypogonadism is associated with depression, and have been suggested to be involved in increased incidences of cancer and over-all mortality. This study may therefore suggest that men suffering from depression and treated with SER should be monitored carefully for any side-effects on sexual dysfunction, and further investigations on this field are needed to clarify potential effects in patients.

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