The effects of xenobiotics on steroidogenesis in human: *in vitro* and *in vivo* investigations

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1. Summary

Steroid hormones have a pivotal role in many physiological processes. For example, the glucocorticoids are crucial in the regulation and maintenance of sugar balance, immunity, stress response, and mood, whereas the mineralocorticoids are involved in electrolyte- and water balance thus regulating blood pressure. Androgens are crucial for muscle function, cardiovascular system and the development and maintenance of male characteristics. Therefore, the disruption of the steroidogenesis is associated with severe diseases such as cancer, metabolic syndrome, cardiovascular diseases, immune disorders, impaired brain function, and developmental dysfunctions.

In the first part of this thesis, we were interested in the *in vitro* investigation of xenobiotics affecting the human steroidogenesis. We focused on the adrenal steroidogenesis, which is rather neglected by many regulatory agencies, despite its pivotal role in humans. We provided a critical overview of the current available cell lines used to screen for potential endocrine disruptors and to study their effects on adrenal steroidogenesis. Moreover, we discussed their advantages/disadvantages, and the need for improvements of the well-established human carcinoma cell line H295R and the associated validated OECD test guideline 456, namely the "H295R steroidogenesis assay". This resulted in a refined version of the H295R steroidogenesis assay, which is distinguished from the currently used OECD protocols by analyzing multiple adrenal steroids simultaneously with exclusive separation techniques combined with mass spectrometry, as well as including additional controls, such as medium composition at the starting time and reference compounds with known mechanism. The obtained results of the steroid changes can then be further combined with the observed effects on gene expression, providing first mechanistic hints on steroidogenesis disruption. By using the newly established refined version of the H295R steroidogenesis assay, we demonstrated that exposure of H295R cells to the UV-filter octyl methoxycinnamate and the plasticizer acetyl tributylcitrate resulted in increased corticosteroid levels, as well as enhanced CYP11B2 expression, similar to the corticosteroid inducer torcetrapib (positive control). To summarize, the refined H295R steroidogenesis assay is a valuable *in vitro* tool to screen and study chemicals potentially disrupting the production of adrenal steroids and provides initial mechanistic evidence in combination with gene expression data.

Many psychoactive drugs can lead to immense increases in cortisol by stimulating the hypothalamic-pituitary-adrenal (HPA) axis. However, a comprehensive analysis of drug induced changes of several steroids, such as glucocorticoids, mineralocorticoids and adrenal androgens along with their full time courses is missing. In the second part of this thesis, we studied the effects of lysergic acid diethylamide (LSD), which has sparked a renewed interest in psychiatric research, lisdexamfetamine, a new drug for the treatment of attention deficit hyperactivity disorder (ADHD), and D-amphetamine on the circulating steroids *in vivo*. Plasma samples were obtained from two individual clinical trials, where healthy volunteers were administered a single dose of either LSD (200 µg), lisdexamfetamine dimesylate (100 mg) or immediate-release D-amphetamine sulfate (40.3 mg) at equimolar doses. Both studies were conducted using a randomized, double-blind, placebo-controlled, cross-over design and plasma steroids for the concentration–time profiles were quantified by ultra-high pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). We could demonstrate, that LSD produces significant acute

effects on circulating steroids compared to placebo in 16 healthy volunteers. The glucocorticoids cortisol, cortisone, corticosterone and 11-dehydrocorticosterone were significantly increased following LSD administration, indicating HPA axis stimulation. Cortisol and corticosterone reached the maximum concentration (c_{max}) after 2.5 h and 1.9 h of LSD administration, respectively. Evaluation of the relationship between the LSD concentration in plasma and the glucocorticoid response to LSD indicated no acute pharmacological tolerance. Furthermore, the androgens dehydroepiandrosterone (c_{max} and the area under the concentration-time curve from time 0 to 10 h (AUC_{10})) and androstenedione (AUC_{10}) were significantly increased by LSD, but not the other androgens, mineralocorticoids or progestogens compared to placebo.

We showed, that the administration of equivalent doses of lisdexamfetamine and D-amphetamine exhibit an identical pharmacokinetic profile for plasma D-amphetamine. However, lisdexamfetamine administration showed a significantly longer onset time (1.4 vs. 0.8 h) and t_{max} (4.4 vs. 3.2 h) for plasma D-amphetamine compared to D-amphetamine administration, due to the rate-limiting hydrolysis of lisdexamfetamine. Furthermore, lisdexamfetamine and D-amphetamine showed a similar enhancement of glucocorticoid production (cortisol, cortisone, corticosterone, 11-dehydrocorticosterone, and 11-deoxycortisol), increases in androgen precursors (dehydroepiandrosterone, its sulphated metabolite, and androstenedione) and adrenocorticotropic hormone (ACTH) in plasma in 24 healthy volunteers. This suggests a HPA axis stimulation. Moreover, an acute pharmacological tolerance of the drug-induced change in active glucocorticoids was demonstrated. The other circulating steroids, such as the mineralocorticoids (aldosterone and 11-deoxycorticosteone), androgens (testosterone and androsterone) and progestins (17α-hydroxyprogesterone and progesterone (but not the male progesterone levels)), were not affected by lisdexamfetamine or D-amphetamine.

In conclusion, LSD, lisdexamfetamine and D-amphetamine had an acute and profound effect on the circulating steroids, especially on the glucocorticoids, suggesting HPA stimulation. This emphasizes the need for further research to understand drug induced changes in steroid homeostasis during chronic administration of amphetamine based ADHD treatments, notably in the pediatric population. Obtained results, should then support an appropriate benefit-risk assessment of these drugs.

2. Preface

This thesis describes "*in vitro*" and "*in vivo*" investigations I undertook to address toxicological effects of xenobiotics in human steroidogenesis. The main body of the text consists of published research, a review article and work in preparation for publication. Detailed within the manuscripts is a precise description of the current understanding of the human steroidogenesis, an outline of the current needs of the research field and based on my experimental observations, future perspectives.

3. Steroidogenesis

In humans, the adrenal cortex, the gonads (testes and ovaries), and the placenta synthesize steroid hormones [1]. These are all derived from cholesterol and have a pivotal role in controlling a wide variety of physiological functions. Alterations in steroidogenesis are associated with cancer, metabolic syndrome, cardiovascular diseases, immune disorders, neurobehavioral and learning dysfunctions, and disorders of sexual differentiation, reproduction, and fertility [2-4]. Steroids are synthesized *de novo* from cholesterol and further metabolized by the cytochrome P450 (CYP) enzymes and hydroxysteroid dehydrogenases [3] in the mitochondria and smooth endoplasmic reticulum [2]. Furthermore, the produced steroids are secreted into blood circulation, where they are in an equilibrium of protein-bound and unbound steroids. To ensure an ubiquitous distribution and increased half-life, steroids are mainly bound to corticosteroid-binding globulin (CBG), sex hormone-binding globulin (SHBG), or albumin [1]. Only low concentrations of the steroids are unbound and therefore available to exert effects on their target organs. Alternatively, unbound steroids can be further metabolized in the peripheral tissues [5, 6]. Steroids are mainly excreted in the urine (75%) or feces following bile degradation (25%) [7, 8]. According to their physiological behavior [9] and the nuclear receptor to which steroids bind [2, 10], they can be classified as glucocorticoids, mineralocorticoids, androgens, estrogens and progestogens. Steroids upregulate target genes by first binding to their corresponding nuclear receptor (glucocorticoid-, mineralocorticoid-, progesterone-, androgen-, or estrogen receptor). The steroid-receptor complex then translocates into the nucleus and binds to specific response elements on the promotor of their target genes [11-13].

The hypothalamus has a pivotal role in the coordination of the endocrine system [14]. Two distinct areas of the hypothalamus, the suprachiasmatic nucleus (SCN) and the paraventricular nucleus (PVN) regulate the “biological clock” and hypothalamic-pituitary-adrenal (HPA) axis, respectively. The light-activated CLOCK system, causes the circadian release of glucocorticoids, reaching their highest concentration in the morning and the lowest in the evening [15-17]. Furthermore, the CLOCK system influences the activity of the HPA axis. This neuroendocrine axis, consisting of the PVN, the pituitary and the adrenal gland, mediates the adaptive response to stressors, besides keeping circadian activity [15]. The PVN releases corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), which induce the secretion of adrenocorticotropic hormone (ACTH) from the pituitary. Subsequently, the ACTH then stimulates adrenal synthesis of glucocorticoids in the adrenal gland. Additionally, there is a feedback loop from the circulating glucocorticoids resulting in inhibited secretion of CRH and ACTH [14, 18]. A disrupted circadian rhythm of glucocorticoids or chronic activation of the HPA axis may result in an impaired immune system, obesity/dyslipidemia, insulin resistance, alternation in mood and cognition, and cardiovascular diseases [15, 19].

The mineralocorticoid aldosterone is regulated by the renin-angiotensin-aldosterone system (RAAS), as well as serum potassium and sodium concentrations [20, 21].

The human adrenal glands, which are located above the kidney, are composed of the medulla (producing catecholamines) and the cortex (Fig. 1). The cortex can be further divided into three morphologically and biochemically distinct zones (Fig. 1) from the outermost *zona glomerulosa*, to the *zona fasciculata*, to the innermost *zona reticularis* [18, 22].

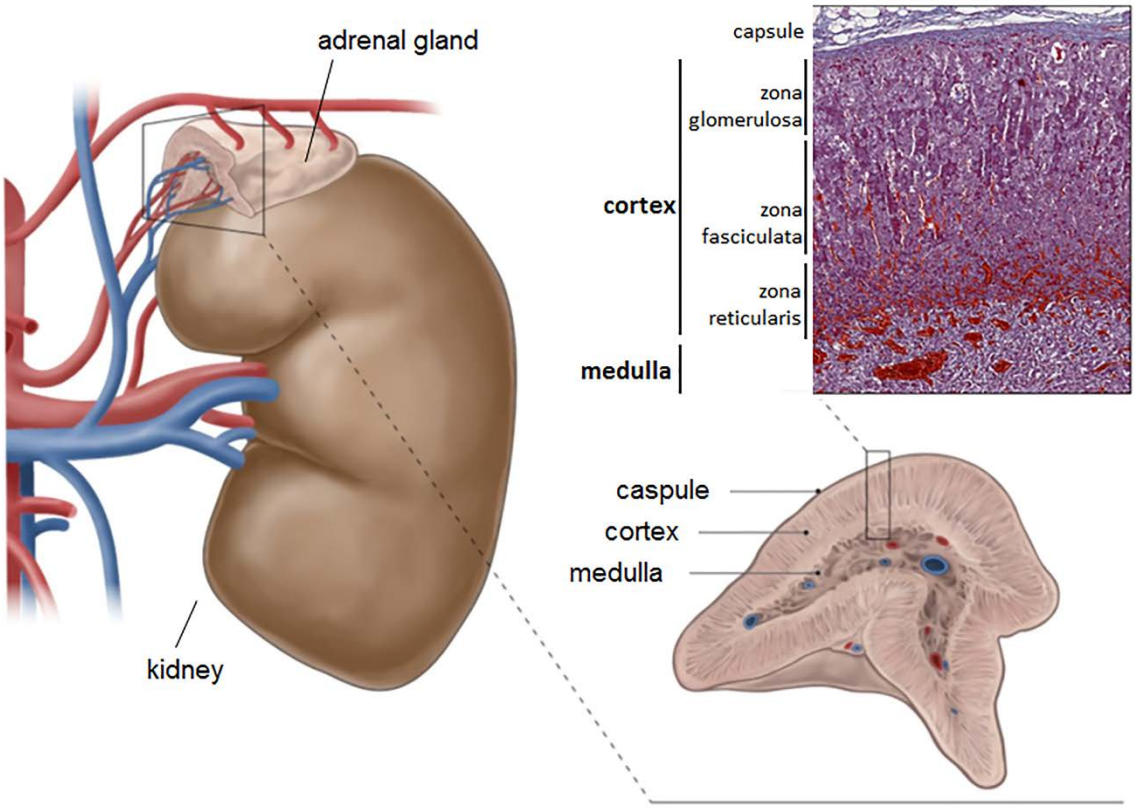

Fig. 1. Schematic overview of the adrenal gland and the different layers. Adapted from [23].

The *zona glomerulosa* synthesizes the mineralocorticoids. They are involved in electrolyte- and water balance in the kidney (reabsorption of sodium and secretion of potassium and hydrogen ions), thus regulating blood pressure [18, 24, 25]. Aldosterone is the most potent mineralocorticoid receptor activator, followed by 11-deoxycorticosterone, corticosterone, and cortisol [26]. Excessive production of aldosterone by the adrenal gland can lead to primary hyperaldosteronism [27].

Glucocorticoids produced in the *zona fasciculata* play a crucial role in regulating cellular metabolism (since they can stimulate gluconeogenesis) [1], immune system [13], modulation of the central nervous system [3, 28], cardiovascular system [18] and the stress response [3]. The main human glucocorticoid is cortisol [29], followed by corticosterone [1]. The interconversion of the active glucocorticoids, cortisol and corticosterone, to their inactive metabolites, cortisone and 11-dehydrocorticosterone, is catalyzed by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2). The reverse reaction is catalyzed by 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) [13]. An inappropriate excess of cortisol results in Cushing's syndrome, whereas Addison's disease is characterized by an insufficient production of cortisol [30].

Androgens are crucial for the development and maintenance of male characteristics [31]. The weak androgens androstenedione, and dehydroepiandrosterone (DHEA), as well as its sulphate conjugate (DHEAS), which are the most abundant steroids in the circulation, are synthesized in the *zona reticularis* [18, 32]. In the periphery, they can be further metabolized to the main androgen testosterone [18]. Moreover, the *zona reticularis* is able to produce minor amounts of testosterone [33]. Nevertheless, testosterone is mainly produced in the testis by the Leydig cells [31]. Testosterone is further converted

to the more potent androgen 5α-dihydrotestosterone in the target tissues such as prostate, skin, and hair follicles [1, 34]. Low concentrations of estrogens and progestins are produced in the adrenal cortex [35]. Estrogens, specifically estradiol and the less potent estrone, are crucial for the development and maintenance of female sexual characteristics. They are produced in the ovaries or by enzymatic conversion of androgens in the peripheral tissues [8, 36]. Progestins have a pivotal role during the menstrual cycle and pregnancy, where progesterone is the main progestin [37].

A schematic overview of the steroidogenesis (major produced steroids and involved enzymes) is depicted in Fig. 2. The steroid biosynthesis is described in detail in the published review paper "Disruption of steroidogenesis: Cell models for mechanistic investigations and as screening tools".

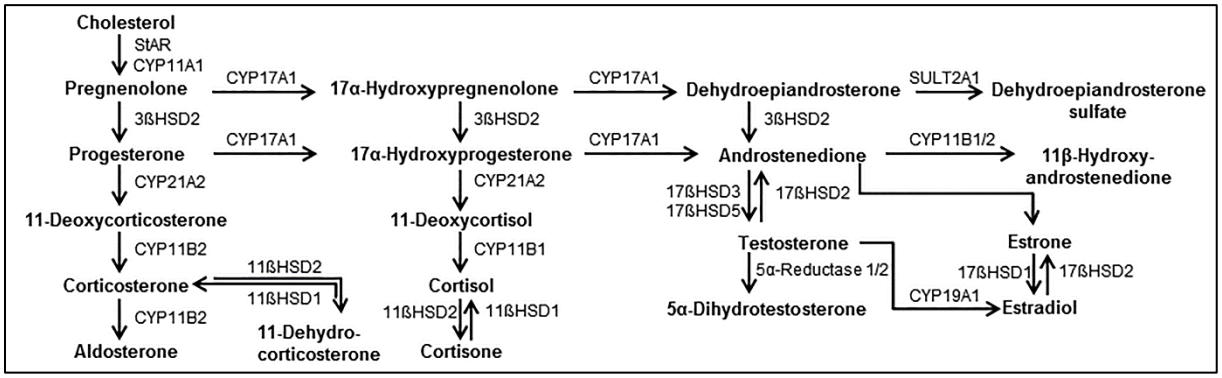

Fig. 2. Overview of steroidogenesis. Steroids are depicted in bold, the enzymes in regular and the corresponding catalyzing reactions by arrows. CYP = cytochrome P450; HSD = hydroxysteroid dehydrogenase; SULT = sulfotransferase; StAR = steroidogenic acute regulatory protein.

4. *In vitro* investigations of xenobiotics affecting human steroidogenesis

4.1 Adrenocortical endocrine disruption

There is a raised global concern in the identification, as well as hazard- and risk assessment of endocrine disruptors [20, 38, 39]. The WHO defines an endocrine disruptor as an “exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects” [20]. Endocrine disruptors can affect the endocrine system, through multiple mechanisms, such as: hormone mimicking, hormone receptor blocking, or interference with the synthesis, transport, metabolism, or excretion of an endogenous hormone [40]. Potential endocrine disruptors can be found in personal care products, cosmetics, pharmaceuticals, agricultural and industrial chemicals, additives or contaminants in food [20, 41]. They can cause adverse effects in humans and in wildlife [42].

The Organisation for Economic Co-operation and Development (OECD) has set out specific framework for testing and assessing endocrine disrupters. Importantly, this framework is only intended to be a guide and is not to be a testing strategy. It consist of levels of increasing complexity, starting at level 1 which recommends using existing data *e.g. in silico* observations and progresses to *in vitro* and *in vivo* assays in the higher levels [43]. In order to practically implement the “Three Rs” - Replacement, Reduction and Refinement in animal testing [44], a part of the strategy has be a drive to develop more predictable *in vitro* models, which are cheaper, less laborious and have a greater throughput rate compared to the *in vivo* studies. The currently available cell lines used for studying the effects of chemicals on adrenal and gonadal steroidogenesis are presented in the following section within the published review paper “Disruption of steroidogenesis: Cell models for mechanistic investigations and as screening tools“. Additionally, the limitations and required improvements of the cell systems and protocols are discussed.

Many regulatory agencies focus their endocrine disruption testing strategies towards developmental and reproductive toxicity, whereas adrenocortical function in adults is rather neglected [38, 45]. This is inconsistent with the pivotal role of the adrenal gland in the endocrine system, where the adrenal cortex synthesizes more than 30 different steroids [1] and is the exclusive endocrine organ in the production of glucocorticoids and mineralocorticoids [35]. The adrenal cortex is reported to be the most common toxicological target in the endocrine system [38, 46]. In the past it was shown, that compounds affecting the adrenal gland function, lead to therapy and drug development failures. For example, etomidate, an anesthetic induction agent used in the clinics, can induce fatal adrenocortical insufficiency [47] by CYP11B1 inhibition [48]. Moreover, torcetrapib, developed as a lipid reducer by a pharmaceutical company, increased aldosterone plasma levels, as well as it induced the expression of the enzymes CYP11B2 and CYP11B1. This resulted in an increased morbidity and mortality in torcetrapib-treated patients and therefore

termination of the clinical drug development trial [49, 50]. Altogether, this emphasizes once more the need to identify adrenocortical endocrine disruptors.

Currently, there are only limited adrenocortical cell lines available [51, 52], with the human carcinoma cell line H295R being the most well established. Additional adrenocortical cell systems (human derived cell lines: NCI-H295, H295A, the H295R clones H295R-S1, H295R-S2 and H295R-S3, as well as HAC13, HAC15 and HAC50; mouse derived cell lines: Y-1, ATC1 and ATC7-L) for testing adrenal toxicity are presented in detail in the published review paper "Disruption of steroidogenesis: Cell models for mechanistic investigations and as screening tools" together with their specific benefits and disadvantages. Due to its ability to express the majority of enzymes involved in human steroidogenesis (in contrast to the human situation, where there is a tissue and developmental stage specificity), as well as to secrete adrenal steroids, the H295R cell line provides an excellent cell model to study adrenocortical endocrine disruptors [53-55]. At present, both the OECD and the Environmental Protection Agency have issued guidelines for the H295R steroidogenesis assay to screen for chemicals affecting the steroid production. In this assay, the H295R cells are incubated with the chemical of interest for 48 h in 2.5% Nu-serum supplemented medium [56, 57]. However, these protocols are only validated for testosterone and estradiol, which are only minor products of the adrenals, and not for the glucocorticoids, mineralocorticoids and adrenal androgens. Additionally, the assay does not aim to provide mechanistic data on the mode of action.

Further limitations of the H295R cell system (*e.g.* insensitivity towards ACTH), as well as the protocol recommended by the OECD guideline, are listed in the published review paper "Disruption of steroidogenesis: Cell models for mechanistic investigations and as screening tools". Moreover, we identified specific unanswered experimental gaps in the current H295R cell system and its corresponding recommended protocol, which needed to be addressed. This included,1) usage of a stimulated cell system for studying inhibitors, 2) extended steroid profiling for a comprehensive steroid disturbance understanding, 3) inclusion of eligible controls (reference compounds, as well as medium control at the starting time of an experiment) for an enhanced data interpretation, 4) the requirement of gas- or liquid chromatography combined with mass spectrometry based detection methods instead of antibody-based approaches in the hormone pattern analysis, is addressed.

In the follow up manuscript, "Steroid profiling in H295R cells to identify chemicals potentially disrupting the production of adrenal steroids", we sequentially addressed all the points mentioned above in order to establish a refined H295R steroidogenesis assay. Moreover, the time-dependent steroid synthesis in H295R cells was studied. Our improved protocol was then used to test 31 compounds (reference and test compounds) on their ability to affect the adrenal steroidogenesis, followed by concentration-dependent experiments and steroidogenic gene expression investigations for a subset of compounds of interest.

4.2 Published review paper: Disruption of steroidogenesis: Cell models for mechanistic investigations and as screening tools

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Review

Disruption of steroidogenesis: Cell models for mechanistic investigations and as screening tools

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ABSTRACT

In the modern world, humans are exposed during their whole life to a large number of synthetic chemicals. Some of these chemicals have the potential to disrupt endocrine functions and contribute to the development and/or progression of major diseases. Every year approximately 1000 novel chemicals, used in industrial production, agriculture, consumer products or as pharmaceuticals, are reaching the market, often with limited safety assessment regarding potential endocrine activities. Steroids are essential endocrine hormones, and the importance of the steroidogenesis pathway as a target for endocrine disrupting chemicals (EDCs) has been recognized by leading scientists and authorities. Cell lines have a prominent role in the initial stages of toxicity assessment, *i.e.* for mechanistic investigations and for the medium to high throughput analysis of chemicals for potential steroidogenesis disrupting activities. Nevertheless, the users have to be aware of the limitations of the existing cell models in order to apply them properly, and there is a great demand for improved cell-based testing systems and protocols. This review intends to provide an overview of the available cell lines for studying effects of chemicals on gonadal and adrenal steroidogenesis, their use and limitations, as well as the need for future improvements of cell-based testing systems and protocols.

Contents

1. Introduction

There is an increasing interest in the identification of chemicals that interfere with the endocrine system. The Endocrine Society defines an endocrine disrupting chemical (EDC) as an "exogenous chemical or mixture of chemicals that can interfere with any aspect of hormone action" [1]. It is important, in our opinion, to distinguish between transient influences followed by adaptation and disruption of endocrine functions leading to adverse health effects. This is considered by the European Union (EU) that defines an EDC as an "exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function" [2,3]. The protection of human health and the environment is of high priority for major organizations and regulatory authorities. Regarding the large number of chemicals that need to be tested for potential endocrine disrupting effects, in programs such as REACH (Registration, Evaluation, Authorization and Restriction of Chemicals, http://ec.europa.eu/growth/sectors/chemicals/reach/index_en.htm), the EPA's EDSP (Environmental

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Protection Agency's Endocrine Disruptor Screening Program, http://www.epa.gov/endo/) or the FDA (U.S. Food and Drug Administration) guidelines for drug development (http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/), it is important to first evaluate the most relevant chemicals, *i.e.* chemicals with evidence of causing adverse effects and for which relevant exposure is known or can be expected. Besides chemicals used in industrial production, agriculture, electronics, and consumer products, the safety of pharmaceuticals and food constituents need to be assessed. Thus, a huge number of chemicals need to be tested for a wide range of possible adverse effects, including such caused by a disruption of steroid hormone action.

Amongst other endocrine hormones, steroids play crucial roles in the regulation of nearly all physiological processes. Several reports provided evidence for an association of disturbances of steroid hormone action caused by exogenous chemicals with developmental defects [4], infertility and reproductive dysfunctions [5,6], testicular, prostate and breast cancer [7–9], obesity and diabetes [10–12], immune disorders and neurobehavioral and learning dysfunctions [13,14]. Further research is needed to identify other chemicals disrupting steroid hormone action, to evaluate the mechanisms by which such chemicals disrupt steroid hormone action, and to assess the critical exposure windows and concentrations that are relevant regarding development and progression of diseases.

For the initial endocrine safety testing of a large number of chemicals, improved *in silico* and *in vitro* assays are needed to facilitate the prioritization of chemicals for further toxicological investigations. Cell-based steroidogenesis assays represent a suitable starting point to assess disturbances of steroid biosynthesis, induced by direct inhibition of steroidogenic enzymes or by affecting their expression. The advantage of the cell-based models is that several enzymes and receptors required for the synthesis of steroids, as well as the signaling pathways regulating their activities, may be covered in a single assay. In addition to the identification of potentially hazardous chemicals, the cell-based steroidogenesis assays allow first mechanistic insights into the mode-of-action of EDCs; however, the users need to be aware of the limitations of the system applied in order to avoid drawing inappropriate conclusions and over-interpretation of results. This review focuses on the cell lines that are available to study steroidogenesis, their advantages and limitations, and the existing gaps for early safety testing of chemicals disrupting steroid homeostasis.

2. Steroidogenesis

Primary organs that are producing steroids from their precursor cholesterol include the adrenal glands and the gonads, with testes in males and ovaries in females. Additionally, in females the placenta produces high amounts of progesterone during pregnancy [15]. Other organs expressing steroidogenic enzymes include the brain [16,17], the intestinal tract [18] and the skin [19]. However, the steroids produced in these tissues seem to be restricted to affect local rather than systemic levels, and the relevance of steroidogenesis in these tissues will not be discussed.

The major steroidogenic organs synthesize steroids *de novo* from cholesterol that is either produced directly by the cell from acetyl-CoA or taken up from dietary cholesterol bound to low-density lipoproteins (LDL) in the circulation (for a comprehensive review see [20]). Cholesterol can be esterified, stored in lipid droplets and be released by the activity of hormone-sensitive lipase. The rate-limiting step in adrenal and gonadal steroidogenesis is the uptake of cholesterol into the mitochondria. The steroidogenic acute regulatory protein (StAR) facilitates the transfer of cholesterol from the outer to the inner mitochondrial membrane, and its conversion to pregnenolone by the cytochrome P450 side chain cleavage enzyme (P450scc, CYP11A1) in cooperation with adrenodoxin reductase that functions as an electron transfer protein of CYP11A1 [20]. Dependent on the organ, pregnenolone is then further converted by tissue- and cell type-specific enzymes into androgens, estrogens, glucocorticoids or mineralocorticoids.

The cortex of the adult human adrenals is responsible for the production of mineralocorticoids in the *zona glomerulosa*, glucocorticoids in the *zona fasciculata* and precursors of active androgens in the *zona reticularis* (Fig. 1). The *zona reticularis* expresses high levels of CYP17A1 [21], which possesses 17α-hydroxylase activity for the formation of 17α-hydroxypregnenolone and 17,20-lyase activity for the subsequent formation of dehydroepiandrosterone (DHEA). The high expression of cytochrome b5, in the presence of cytochrome P450 reductase, allows efficient 17,20-lyase activity that is needed for the production of DHEA [20,22]. Additionally, the *zona reticularis* expresses high levels of the steroid sulfotransferase SULT2A1 that is responsible

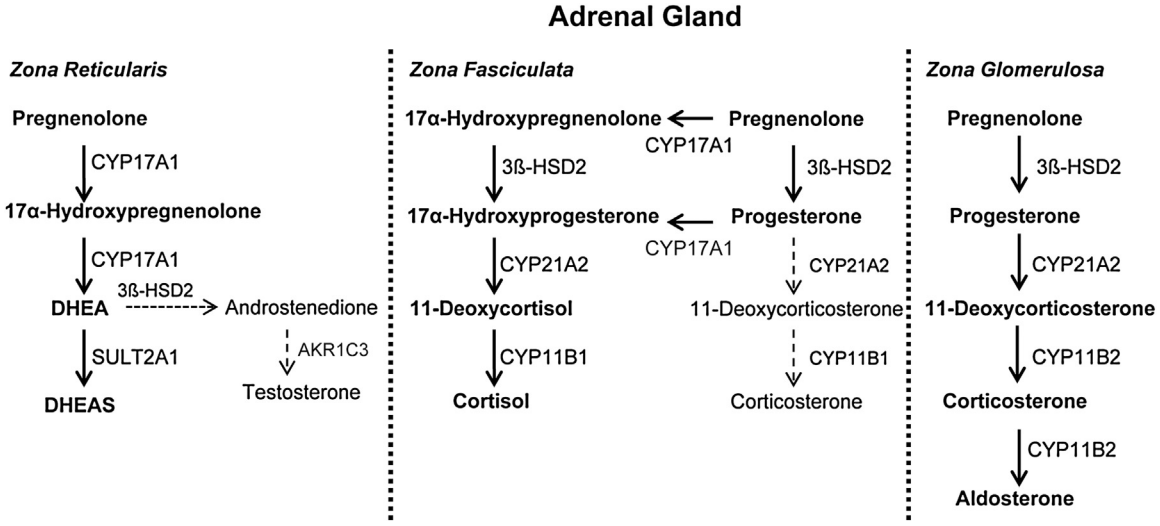

Fig. 1. Schematic overview of adrenal steroidogenesis. Major steroids produced are indicated in bold and by solid lines, minor metabolites are indicated by dashed lines.

for the formation of sulfated DHEA (DHEAS) [23], the most abundant steroid in human blood [24]. Importantly, 3β-hydroxysteroid dehydrogenase 2 (3β-HSD2) is expressed in the *zona reticularis* at very low levels, thus leading to only low amounts of Δ4-androstene-3,17-dione (androstenedione) production [20]. Since 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) is absent and 17β-HSD5 (AKR1C3) expressed at very low levels in the *zona reticularis* [21,25], only very low levels of testosterone are produced by the adrenals [26,27]. CYP21A2 is absent in the *zona reticularis*, thus no mineralocorticoids and glucocorticoids are formed in this layer [20].

In the *zona fasciculata* pregnenolone is converted to 17α-hydroxypregnenolone by CYP17A1, and pregnenolone and 17α-hydroxypregnenolone are converted to progesterone and 17α-hydroxyprogesterone, respectively, by 3β-HSD2. Most of the progesterone formed is also 17α-hydroxylated. Further metabolism by CYP21A2 leads to 11-deoxycortisol and lower amounts of 11-deoxycorticosterone that are further converted by CYP11B1, which is specifically expressed in this zone, into cortisol and corticosterone, respectively [20,28]. Cytochrome b5 is expressed at background levels in the *zona fasciculata* [21], resulting in very low CYP17A1 17,20-lyase activity and thus low amounts of DHEA formation [20]. The *zona fasciculata* expresses the melanocortin-2-receptor and is therefore responsive to adrenocorticotrophic hormone (ACTH) [20,28].

The *zona glomerulosa* does not express CYP17A1, and pregnenolone is converted to progesterone by 3β-HSD2 and further to 11-deoxycorticosterone by CYP21A2, and to corticosterone and aldosterone by CYP11B2. In the adrenals, CYP11B2 expression is restricted to the *zona glomerulosa* and the production of aldosterone is regulated by angiotensin II receptors [20].

The human fetal adrenals produce high amounts of DHEAS, which is abolished soon after birth where the adrenals mainly consist of a *zona glomerulosa* and a *zona fasciculata* and thus produce mineralocorticoids and glucocorticoids [29]. The *zona reticularis* actively starts producing adrenal androgens at adrenarche at around 6–8 years of age and reaching peak levels in the third decade of life, before declining gradually [30,31].

In the testis, steroidogenesis is restricted to the Leydig cells. They convert pregnenolone by CYP17A1 into 17α-hydroxypregenenolone and further to DHEA (Fig. 2). Because of the high expression of 3β-HSD2 and 17β-HSD3 but the absence of SULT2A1, DHEA is not sulfated and therefore further converted to androstenediol, or by a lower extent to androstenedione, and subsequently to testosterone in Leydig cells [20,32]. Furthermore, CYP21A2, CYP11B1 and CYP11B2 are absent, thus no gluco- and mineralocorticoids are produced. Testicular steroidogenesis is under the control of human chorionic gonadotropin (hCG) and luteinizing hormone (LH).

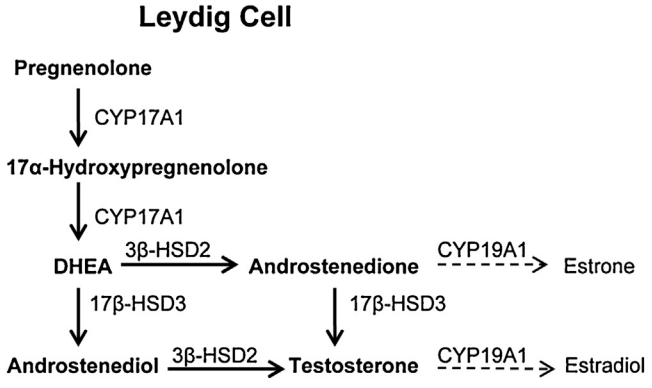

Fig. 2. Schematic overview of steroidogenesis in Leydig cells.

In the ovaries, steroidogenesis is mediated by theca and granulosa cells. The granulosa cells are located in the avascular cellular compartment surrounding the oocyte, and the theca cells reside in the ovarian stroma; these cellular compartments are separated by the basal membrane. The theca and granulosa cells both express StAR and CYP11A1 [33]. Because granulosa cells do not express CYP17A1 [34], they can synthesize pregnenolone from cholesterol and they convert it further to progesterone in the *corpus luteum* (Fig. 3) [20]. However, for the production of estrogens, pregnenolone needs to be secreted from the granulosa cells and taken up by the theca cells, or it is produced directly by the theca cells, to form DHEA. The theca cells express 3β-HSD2 and convert DHEA into androstenedione [35]. Androstenedione is then delivered back to the granulosa cells for the aromatase-dependent production of estrogens [34]. Granulosa cells also express 17β-HSD1, which is needed for the conversion of estrone into estradiol. There are cycle-dependent changes in ovarian steroidogenesis: in the luteal phase the luteinized granulosa cells are supplied with sufficient cholesterol, due to enhanced vascularization of the previously avascular compartment, and elevated LH levels enhance the expression of CYP11A1 and 3β-HSD2, resulting in the synthesis of high amounts of progesterone [33]. In the follicular phase, follicle stimulating hormone (FSH) enhances the expression of aromatase and 17β-HSD1 for the production of increased amounts of estradiol from theca cell-derived androstenedione. LH also activates LH receptors on theca cells to induce CYP17A1 expression, thereby enhancing androgen precursors for estrogen production by granulosa cells. Thus, a tight control of the cooperation of granulosa and theca cell function is essential for the appropriate regulation of estradiol synthesis.

3. Leydig cell models to investigate steroidogenesis

Three independent large epidemiological studies revealed a decline in male serum testosterone levels in the general population [36–38]. Obesity was identified as a contributing factor for some but not all observations [39]. Increasing evidence suggests that exposures to EDCs contribute to male reproductive diseases and that prevention of EDC exposures may reduce the burden of male reproductive health problems [40]. As an example, cryptorchidism is a typical impairment following exposure to antiandrogenic chemicals during male sexual development [41]. Evidence was provided that levels of polybrominated diphenyl ethers (PBDEs) in

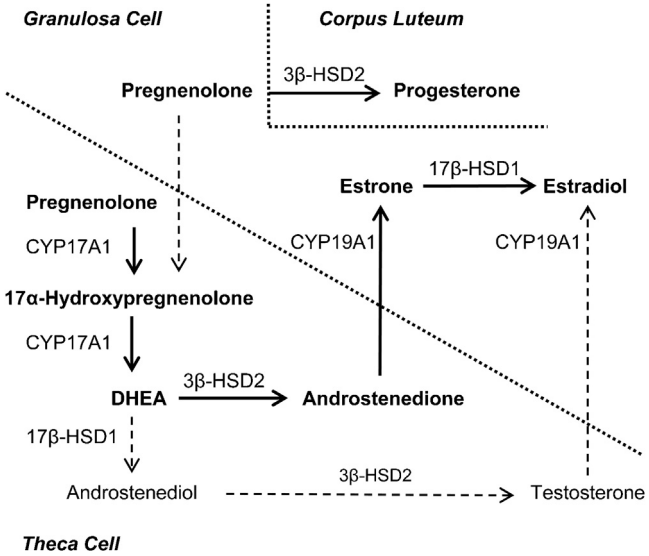

Fig. 3. Schematic overview of ovarian steroidogenesis.

human breast milk are associated with congenital cryptorchidism, although a contribution of other environmental factors cannot be excluded [42]. PDBEs have been shown in *in vitro* studies to directly antagonize AR activity (IC_{50} of approximately 5 μM for the mixture DE-71 in an MDA-kb2 cell model expressing an AR-dependent luciferase reporter), and PDBEs additively and/or synergistically acted with other AR antagonistic compounds [43]. In *in vivo* studies PDBEs were shown to cause diminished growth of androgen-dependent tissues and a delay in puberty in the male rat following a pubertal exposure to 60 and 120 mg/kg/day of the DE-71 mixture [44]. Although such high exposure levels are unlikely to be reached in humans, the fact that humans are exposed to a multitude of compounds that may exert additive or synergistic effects emphasizes the need for the screening of chemicals for potential antiandrogenic effects. Because of the high public demand to reduce animal testing [45], improved cell-based assays are needed that allow the identification of chemicals disrupting the biosynthesis of steroids and the gaining of insights into the mode-of-action of such chemicals.

There are several immortalized rodent Leydig cell lines available for studying the regulation of steroidogenesis and to assess the impact of substances on steroid hormone production. However, to our knowledge, no human Leydig cell model is currently available that can be used for screening purposes and for toxicological studies. The available rodent Leydig cell lines have been derived from spontaneous tumors, upon experimental induction, or by *in vitro* immortalization. All of these cell systems have their limitations, as some of the steroidogenic enzymes and regulatory pathways are expressed at very low levels, if at all, likely as a result of the selection of cell clones that rapidly proliferate and because of dedifferentiation and loss of initial phenotype during prolonged cultivation.

Probably the most widely used immortalized Leydig cell line is MA-10 [46]. MA-10, the related MA-12, and the frequently used mLTC-1 are all derived from a C57Bl/6 Leydig cell tumor (designated M5480) [47]. These cell lines express LH receptors, and incubation with LH/hCG induces cAMP-dependent steroidogenesis. MA-10 cells also express mouse epidermal growth factor receptor (EGFR), which suppresses the hCG-induced steroidogenesis [48]. In both, MA-10 and mLTC-1, progesterone was the main steroid being produced, in line with the observation that the original tumor M5480 secreted progesterone but only very low amounts of testosterone, and the two cell lines displayed similar functional characteristics [47]. These observations suggest that 3β-hsd1 activity is dominant over Cyp17a1; therefore, pregnenolone is mainly converted into progesterone, with only minor amounts being further converted into androstenedione and testosterone. For these reasons, we propose that, using progesterone as a read-out, MA-10 and mLTC-1 cells can serve as suitable models to detect chemicals that affect the induction of steroidogenesis, the cAMP- and PKA-dependent signaling, or that directly inhibit the activities of StAR, Cyp11a1 or 3β-hsd1. Due to the generation of only low amounts of testosterone by these cell lines, it is difficult to quantitatively assess the effect of chemicals that disrupt Cyp17a1 or 17β-hsd3 activities. Nevertheless, the mRNA expression of key steroidogenic enzymes, including StAR, Cyp11a1 and 3β-hsd1, and to a lesser extent that of Cyp17a1 and 17β-hsd3, has been detected in MA-10 and mLTC-1 cells, and has been found to be affected upon exposure to chemical modulators [49–51].

MA-10 cells are applied by many investigators to study the impact of EDCs on the regulation of steroidogenesis; only a few will be mentioned in this review as examples. Recent studies on effects of bisphenol A (BPA) and its analogs on steroidogenesis in MA-10 cells suggested that tetrabromobisphenol A (TBBPA) concentration-dependently increased testosterone production at concentrations of 3 μM and higher, while bisphenol S (BPS) had no effect and BPA and bisphenol F (BPF) induced testosterone secretion only at very high concentrations (30 and 100 μM, respectively) [49,50]. Following incubation of the cells for 48 h in the presence of 10 μM of TBBPA, BPF or BPS an increased production of progesterone, and in the case of TBBPA of 17α-hydroxyprogesterone and androstenedione, was measured. Furthermore, incubation of cells with 10 μM of BPF, BPS or TBBPA led to an elevated expression of 5α-reductase 1, indicating an increased production of 5α-androstanedione and dihydrotestosterone. Importantly, the authors provided evidence that the TBBPA-mediated increase in testosterone production may be due to an inhibition of the efflux of androgen precursors required for testosterone synthesis by the multidrug resistance proteins MRP1 and MRP4 [50]. These observations emphasize the need to include steroid transporters in the assessment of EDCs and provide a further explanation for the low amount of testosterone produced by MA-10 cells under basal conditions.

MA-10 cells were also used to study direct effects of monophthalates on testicular steroidogenesis [52]. The LH-induced production of cAMP and progesterone was significantly inhibited in MA-10 cells treated with 30 μM of mono(2-ethylhexyl) phthalate (MEHP), whereas testosterone production was significantly lowered upon incubation of the cells with 1 μM MEHP, 3 μM monobutylphthalate (MBP), 10 μM mono-*n*-oxtylphthalate (MnOP) or 3 μM monebenzylphthalate (MBeP) but not in the presence of monoethylphthalate (MEP) or monomethylphthalate (MMP) [52]. At the high concentration of 100 μM MEHP the mRNA expression levels of StAR, Cyp11A1 and Cyp17A1 were down regulated. Interestingly, in mLTC-1 cells (not induced by LH) the phthalates di-*n*-butyl phthalate (DBP), MBP, di(2-ethylhexyl) phthalate (DEHP) and MEHP seemed to increase testosterone production at low concentrations of 0.001 to 0.1 μM but inhibited at high concentrations of 100 μM. Interestingly, the mRNA expression levels of Cyp11A1, Cyp17 and 3β-HSD1 were decreased even at concentrations as low as 0.1 μM [53,54]. Also, the impact of the major metabolites of MEHP and DEHP on the expression of steroidogenic genes has been analyzed, suggesting that the metabolite 2-ethylhexanal might inhibit Leydig cell testosterone formation, although this effect was only observed at high concentrations of 100 μM [55]. The human relevance of such high concentrations are questionable and further research using lower concentrations is needed. Also, it should be noted that progesterone and testosterone were measured by ELISA in this study. Furthermore, a possible effect of phthalates on the efflux of androgen precursors or on cholesterol flux in MA-10 or in mLTC-1 cells has not been investigated.

Other studies focused on initial steps of steroidogenesis. Incubation of MA-10 and mLTC-1 cells with an organochlorine compound mixture resulted in a decreased expression of StAR, CYP11A1 and the adrenodoxin reductase, enzymes crucial for the production of pregnenolone from cholesterol [56]. The cAMP- and hCG-induced production of progesterone tended to be lower at 1 μg/ml and was significantly lower at 10 μg/ml of organochlorine mixture. Also, the UV-filter chemical 2,2′,4,4′-tetrahydroxybenzophenone (BP2), applied at 30 μM, was found to alter the expression of StAR, 3β-hsd and Cyp17a1 and had opposite effects on Leydig cell steroidogenesis than thyroid hormone signaling [57]. Moreover, MA-10 cells were employed to test pesticide formulations that are widely used in agriculture. The pesticide mixture Roundup, a broad-spectrum systemic herbicide containing glyphosate (N-phosphonomethyl-glycine), inhibited the cAMP analog-induced progesterone production at subcytotoxic concentrations of 25 μg/ml by decreasing the expression of the StAR protein [58]. Furthermore, the benzodiazepine midazolam was found to stimulate progesterone and testosterone production, measured

by radio-immunoassay, at the subcytotoxic concentrations of 30 and 150 μM in primary mouse Leydig cells and in MA-10 cells by an induction of the expression of the peripheral-type benzodiazepine receptor and StAR, probably via a pathway involving protein kinase A (PKA) and protein kinase C (PKC) [59]. Murine mLTC-1 Leydig cells were used to further investigate the reproductive toxicity of perfluorooctanic acid (PFOA) that was observed in mice treated by gavage [60]. Exposure of mLTC-1 cells with 100 μM PFOA decreased Cyp11a1 mRNA and protein expression and at 300 μM PFOA progesterone production was significantly decreased. Also, StAR protein seemed to be decreased, likely as a result of oxidative stress caused by PFOA exposure [60,61]. The mycotoxin zearalenone at concentrations of 5 μM was suggested to affect steroidogenesis in mLTC-1 cells by disrupting lipid metabolism and inducing endoplasmic reticulum stress-mediated apoptosis [62,63]. Furthermore, the polybrominated diphenyl ether BDE-47 at a concentration of 1 μM was found to decrease progesterone production via cAMP-PKA-dependent downregulation of CYP11a1 [64]. Thus, a multitude of chemicals were shown to affect inial steps of steroidogenesis by different mechanisms. It should be noted that in order to judge on the human relevance of the findings described above further investigations are required, as in most if these *in vitro* studies the concentrations applied were either much higher than concentrations measured in humans or data on such concentrations are not yet available.

Another mouse Leydig cell line, designated TTE1, was derived from transgenic mice, upon immortalization using a temperature-sensitive simian virus 40 (SV40) large T-antigen [65]. These cells can be grown at 33 °C and differentiated at a non-permissive temperature of 39 °C. The cell model was used to study genes involved in Leydig cell differentiation characteristics, and the expression of the terminal enzyme of testosterone synthesis, 17β-hsd3, was confirmed at least on the mRNA level [66]. TTE1 cells were only used in very few studies, so for example to investigate the impact of diethylstilbestrol on the expression of steroidogenic genes [67,68]. Diethylstilbestrol at concentrations of 50 nM or higher decreased Cyp11a1 expression and, furthermore, diminished apoptotic cell death pathways and DNA repair capability, suggesting an increased carcinogenic potential of the exposed cells.

Mice transgenic for the SV40 T-antigen under the control of the inhibin-α promoter were used to establish the steroidogenic Leydig cell line BLT-1 [69]. BLT-1 cells responded well to LH and hCG by increased cAMP levels and enhanced production of progesterone. As observed for MA-10 and mLTC-1, BLT-1 cells are only producing very low amounts of testosterone, as measured by enzyme immunoassay. Regarding investigations into EDCs, the BLT-1 derived cell clone BLTK1 was used to study several environmental toxicants [70]. BLTK1 cells seem to express all key steroidogenic proteins such as StAR, Cyp11a1, Cyp17a1, 3β-hsd1, 17β-hsd3 and 5α-reductase 1. These cells were shown to respond to hCG and forskolin, which resulted in enhanced cAMP production and expression of steroidogenic genes. An elevated production of progesterone and testosterone was indicated by enzyme immuno assays measurements. The antifungals prochloraz (30 μM) and triclosan (30 μM) seemed to decrease the hCG-induced testosterone production, whereas MEHP (300 μM) and atrazine (at concentrations of 30 μM or higher) promoted basal testosterone formation but inhibited the hCG-dependent testosterone synthesis. Furthermore, the triazine herbicides atrazine, simazine, propazine and terbuthylazine were reported to enhance progesterone and testosterone production in BLTK1 cells at high concentrations (with significant effects observed at 100 μM or higher), effects explained by the altered expression of steroidogenic genes [71]. However, in these studies very high concentrations of questionable human relevance were used.

The non-tumor mouse epithelial Leydig cell line TM3 was originally derived from the testis of an immature Balb/c mouse [72]. TM3 cells respond to LH, but not FSH, with an increased cAMP production [73]. Evidence was provided that LH and EGF are involved in the regulation of cyclin-dependent kinase 5 (Cdk5) expression and activity, and that this signaling pathway modulates hormonally stimulated testicular steroidogenesis [74]. Furthermore, LRH-1 was found to regulate Cyp19a1 expression via promoter II in multiple testis cell types [75]. Additionally, a role for hypoxia-inducible factor-1α by mediating hypoxia-dependent changes on steroidogenesis by regulating the transcriptional expression of 3β-hsd1 was reported [76]. The C1q and tumor necrosis factor-related protein (CTRP3) was found to induce testosterone production by increasing cAMP and phosphorylation of cAMP response element-binding protein (CREB) by PKA and subsequently enhancing the expression of StAR and Cyp11a1 [77]. TM3 cells express V1 type arginine vasopressin receptors that seem to act independent of the adenylate cyclase system [78] and calcitonin receptors, which mediate calcium influx and stimulate cAMP formation and testosterone secretion [79]. They also express inhibin/activin β-A subunits and activin receptors II and IIB [80]. A role for the Src tyrosine kinase in the regulation of phosphodiesterase PDE4 activity and the production of cAMP was reported [81]. TM3 cells mainly produce progesterone, and only minor amounts of testosterone (own observations), suggesting that they can serve as a model to study early steps of the regulation of steroidogenesis and direct inhibition of the activities of StAR, Cyp11a1 and 3β-hsd1.

The TM3 mouse Leydig cell line is frequently used to study the impact of environmental pollutants on testicular toxicity and on alterations in steroidogenesis. A study on gap junctional intercellular communication in TM3 cells showed inhibitory effects by estradiol and diethylstilbestrol via an estrogen receptor (ER)-dependent mechanism [82]. Interestingly, similar effects were observed at 10 pM and 10 μM concentrations for both diethylstilbestrol and estradiol, and these effects were fully reversed in the presence of an ER antagonist. Incubation of TM3 cells with diesel exhaust particles led to a reduced expression of ERα (at 0.1 μg/ml particle concentration) and an induction of Cyp1a1 (at 1 μg/ml) [83]. A transcriptomics analysis was performed on the impact of 1 and 5 mM methoxyacetic acid, the active metabolite of the industrial chemical ethylene glycol monomethyl ether, on TM3 cells revealing alterations in steroidogenesis, inflammation reactions and metabolic functions [84]. It needs to be noted that these concentrations are very high, and thus the human relevance is questionable. Two recent studies provided evidence for a protective role of the activation of the transcription factor Nrf2 toward the toxicity caused by the phthalate DBP, indicating the importance of the antioxidant defence system to protect Leydig cells from toxic chemicals [85,86]. Studies on chemicals affecting testosterone production in TM3 cells are rather uncertain, since these cells produce very low amounts. Furthermore, results on changes in testosterone production obtained using ELISA kits should be confirmed using quantification by GC–MS or LC–MS.

I-10 clonal Leydig cells were originally obtained from a spontaneous mouse testicular tumor [87]. Like other mouse Leydig cell lines described above, I-10 were reported to mainly produce progesterone, which was stimulated by cAMP [88], although not as efficient as in MA-10 and mLTC-1 cells. I-10 Leydig cancer cells were scarcely used for the assessment of EDCs. A study on PCBs showed enhanced CYP19a1 expression in mouse I-10 Leydig and human H295R adrenal cells following incubation for 24 h with the high concentration of 10 μM PCB126 [89]. Interestingly, this effect was blunted in hCG and cAMP analog-treated cells, and the authors proposed a role for AhR in these effects. Similarly, the mouse Leydig tumor cell line K28 was applied

only in a few studies, including the investigation of the time-dependent induction of StAR mRNA expression and progesterone production by 9-cis and all-trans of retinoic acid (increases at concentrations greater than 10 nM) [90], the impact of LH on the expression of Nur77 (NR4A1) [91], the effect of 1 μM BPA under serum-free conditions for 24 h on the induction of Nur77 expression and the production of progesterone [92], as well as the stimulating effects of 30 μM cadmium chloride on CREB protein phosphorylation and StAR expression [93].

The rat Leydig tumor interstitial cell line R2C displays high StAR expression and produces high amounts of progesterone [94–96]. The high expression of StAR, Cyp11a1 and 3β-hsd1 was confirmed by RT-PCR and Western blot [97] and the production of progesterone was detected by ELISA and RIA measurements [98,99]. The expression of Cyp17a1 and the production of testosterone have been reported [97–99]; however, a general problem with antibody-based quantification of proteins and steroids remains the often limited specificity of the antibodies used [100]. Thus, testosterone production by R2C cells should be confirmed using quantification by GC–MS or LC–MS. An interesting property of R2C cells is that they are insensitive to cAMP regulation and do not require trophic stimulation to produce progesterone, which might be explained by a constitutively activated downstream signaling pathway [95,101]. Because of the constitutive production of progesterone, these cells are suitable to test chemicals that directly inhibit the activity of StAR, Cyp11a1 or 3β-hsd1. On the other side, this cell line is not suitable to study chemicals affecting the induction of steroidogenesis due to the lack of sensitivity of the involved signaling pathways.

R2C cells were used in a comparative study with MA-10 cells to assess effects of various phthalates on testosterone production measured by ELISA [98]. The phthalates MBP and MEHP significantly inhibited testosterone synthesis at concentrations of 1 and 3 μM, with IC_{50} values of 3 and 6 μM respectively. Phthalates with shorter alkyl side chains were found to be less active or inactive. Interestingly, R2C cells express substantial levels of Cyp19a1, and this cell line has been applied to characterize aromatase inhibitors [102,103]. A study on effects of BPA (concentrations of 0.1–10 nM) on steroidogenesis suggested an up regulation of Cyp19a1 protein expression and activity, whereas testosterone synthesis was decreased [104]. Testosterone was measured by ELISA. Using R2C cells the anabolic androgenic steroids nandrolone and stanozolol (at 1 μM concentration) were shown to increase Cyp19a1 expression as well as estradiol production [105]. Further, these authors provided evidence for an additive effect of androgens and IGF-1 on R2C cell proliferation and aromatase expression. In contradiction, a recent study showed that treatment of R2C cells with the androgen mibolerone up regulated the transcription factor DAX-1 and inhibited the expression and activity of Cyp19a1, in line with observations in old Fischer rats with spontaneous Leydig cell tumors where AR and DAX-1 were down regulated and Cyp19a1 was up regulated [106]. The reason for the discrepances of the above studies remains unclear and requires further research.

A major limitation for mechanistic investigations into the regulation of steroidogenesis in Leydig cells and the assessment of the impact of potential EDCs is the fact that currently no human Leydig cell model is available. There are considerable species differences in the functions of Leydig cells. For example, it has been shown that the expression level of LH receptors is an order of magnitude higher in rat compared with human Leydig cells, and that rat Leydig cells respond with hyperplasia to hCG, whereas human Leydig cells become hypertrophic [107–110]. Furthermore, rat Leydig cells express gonadotropin-releasing hormone, whereas mouse and human Leydig cells do not [111,112].

Additionally, several studies demonstrated species-specific inhibition of testicular steroidogenesis by EDCs. Using organotypic primary culture systems, the phthalate MEHP at a concentration of 10 μM was shown to decrease testosterone production in rat but not in human fetal testis explants [113,114]. Further support for species-specific effects of phthalates was provided by studies where rat and human fetal testes were xenografted into a host mouse or rat [115,116]. Treatment with di-*n*-butylphthalate (500 mg/kg per day for four days) inhibited steroidogenesis in animals with rat but not human xenografts. Also, diethylstilbestrol did not affect human fetal testicular steroidogenesis in the xenograft model [117] and in human fetal testis explants, in contrast to rat and mouse testis cultures [118,119], a difference explained by the fact that ERα is expressed in rat and mouse but not in human fetal Leydig cells [117]. Moreover, the anti-diabetic drug metformin inhibited testosterone production at an order of magnitude higher concentrations in human compared with mouse testis explants [120]. In contrast, it was shown that BPA inhibited testosterone synthesis at 100 times lower concentrations in human compared with rat and mouse fetal testis explants [118]. These studies demonstrate important species-specific differences in the susceptibility of human, rat and mouse testes to xenobiotics and further emphasize the need to establish a human Leydig cell model for the investigations into the molecular mechanisms of steroidogenesis disruption.

4. Cell-based systems to study effects of EDCs on ovarian steroidogenesis

In the industrialized countries, there is an increasing incidence of reproductive disorders such as polycystic ovary syndrome (PCOS) [121], which is characterized by chronic anovulation and hyperandrogenism and results in hirsutism, infertility and menstrual disturbances. As with male infertility, there is evidence for the contribution of EDCs from consumer products or environmental pollutants to the increasing incidence of female reproductive disorders (for a recent comprehensive review see [122]). Several EDCs and potential EDCs have been detected in human samples, including follicular fluid, from the general population [123–127]. Exposure to EDCs likely contributes to sub-fecundity, ovarian failure and infertility, and affects reproductive behavior. Exposure to EDCs may contribute to ovulatory dysfunction by decreasing estradiol biosynthesis in granulosa cells or as abortifacients by disrupting progesterone production in luteal cells [128].

In vivo testing of EDCs for reproductive toxicity is mostly conducted in rodents, with fertility as a primary endpoint [129]. Alteration in serum steroid levels may indicate an adverse effect but it may also represent an adaptive response, thus often not providing sufficient information on the toxicity of a given chemical. Also, changes in circulating steroid levels may be due to a direct effect on steroidogenesis or an altered feedback regulatory system. *Ex vivo* tissue samples, *e.g.* whole ovaries or isolated individual follicles, can be used to study follicular development, ovulation and steroidogenesis, and assays using such samples can provide results on multiple fertility-related endpoints [130]. In order to allow high throughput analyses and to gain mechanistic insight into the action of EDCs, cultured cells are advantageous. Isolated primary theca and granulosa cells can be applied for functional studies, and they retain the normal responses and steroidogenic pathways [131]. Porcine and bovine primary cells can be isolated from ovaries obtained from the slaughterhouse or from ovaries of rodent animal models; however, there are significant species-specific differences in the steroidogenic pathways, which need to be taken into account when trying to extrapolate results to the human system. Human granulosa cells

are mostly obtained from women undergoing *in vitro* fertilization; however, these cells are usually subjected to supraphysiological concentrations of hCG and FSH, and these cells can only be cultivated for a relatively short time [132]. For these reasons, there is a great demand for suitable human theca and granulosa cell lines to investigate a large number of individual EDCs at various concentrations and incubation time as well as mixtures of EDCs. The establishment of a theca cell line was not successful so far; in contrast, several granulosa cell lines are available for investigating effects of chemicals on steroidogenesis.

Granulosa cell lines are useful to study the impact of potential EDCs on progesterone synthesis as well as on the aromatase- and 17β-HSD1-dependent production of estradiol upon incubation of these cells with androstenedione. There is a large number of human ovarian cancer cell lines available (for a recent review see [133]). Most of them express CYP19A1 and 17β-HSD1 and their proliferation is stimulated by estrogens. Additionally, immortalized granulosa cell lines from various animal species and of human origin have been described [134].

Among the rodent granulosa cell lines, KK-1, GRM01 and GRM02 were found to produce progesterone, and retain responsiveness to cAMP, FSH and LH/hCG [134]. KK-1 cells were derived from mice bearing an SV40 T-antigen driven by the inhibin-α promoter, and treatment of these cells with hCG, forskolin and FSH increased cAMP 10-fold, 40-fold and 2.6-fold, respectively, indicating enhanced steroidogenesis [135]. KK-1 cells were shown to express Cyp19a1 and 17β-hsd1 and convert androstenedione to estradiol. The KK-1 cell line was used, for example, to study effects of phthalates on the stimulation of steroidogenesis [136]. The phthalate MEHP at high concentrations of 20–100 μM stimulated basal steroid production in KK-1 granulosa cells, a finding confirmed in mLTC-1 Leydig cells. The expression of StAR and cAMP-mediated signaling did not seem to be affected, and the authors suggested that MEHP may stimulate steroidogenesis by enhanced cholesterol supply. Thus, KK-1 represents a mouse cell system to study granulosa steroidogenesis. GRM01 and GRM02 granulosa cell lines were established by transfection of murine granulosa cells with v-myc [137]. Both cell lines retained steroidogenic activity and were shown to express 3β-hsd2 and 17β-hsd1 [138]. GRM01 was able to produce both progesterone and estradiol *de novo*, whereas GRM02 produced progesterone but not estradiol [139]. However, aromatase activity was also demonstrated in GRM02 upon the addition of androstenedione or testosterone to the culture medium. Steroid production was induced in both GRM01 and GRM02 by LH/hCG, FSH, forskolin and cAMP analogs. Both cell lines also express inhibin-α, which has a role in feedback regulation by inhibiting pituitary FSH secretion. They represent alternative murine cell models to study the impact of potential EDCs that act as direct inhibitors of enzymes involved in progesterone or estradiol production or of the signaling pathways involved in steroidogenesis in granulosa cells.

There are several human granulosa cell lines that are useful for the investigation of endogenous regulators of steroidogenesis as well as pathways involved in metabolic regulation, the regulation of cell proliferation and apoptotic pathways. The cell lines HGP53, HO23, HGL5, HTOG and SVOG were primarily used to study signaling pathways, which are involved in the regulation of steroidogenesis and effects on apoptosis as well as cell proliferation [140–145]. The immortalized human granulosa cell line COV434, initially isolated from a primary granulosa cell tumor, was shown to express FSH receptor and CYP19A1. In FSH supplemented medium COV434 was able to produce estradiol from androstenedione [146]. FSH and forskolin both stimulated steroidogenesis by induction of cAMP. Pharmacological inhibition of the FSH receptor was found to inhibit COV434 cell proliferation [147]. Furthermore, incubation of COV434 cells with soy isoflavones, considered to act as phytoestrogens, promoted cell proliferation [148]. Incubation with 5–50 μM genistein led to increased expression of ERα and enhanced cell proliferation by repressing proapoptotic genes. The human relevance of these observations remain uncertain because of the low bioavailablity of oral intake of isoflavones.

The most widely used human ovarian granulosa-like tumor cell line is KGN. Progesterone production as well as CYP19A1- and 17β-HSD1-dependent estradiol formation from androstenedione supplemented culture medium was found to be induced by FSH *via* induction of IGF-1 in KGN granulosa cells [149]. Additionally, several endogenous regulators, such as steroidogenic factor-1 (SF-1) [150], liver receptor homolog-1 (LRH-1) [151], AMP-kinase (AMPK)/sirtuin-1 (SIRT1) [152], oocyte-derived growth differentiation factor and bone morphogenic protein 15 [153], the Notch signaling patway [154] and the Hippo pathway [155], were shown to affect progesterone production and CYP19A1- and 17β-HSD1-dependent estradiol synthesis.

Several investigators used the KGN granulosa cell line to address the impact of xenobiotics on steroid synthesis. Bisphenol-A (BPA) was found to activate peroxisome proliferator-activated receptor (PPAR)γ and inhibit the FSH-stimulated insulin-like growth factor-1 (IGF1)-dependent induction of CYP19A1 expression and estradiol synthesis in KGN cells and in primary granulosa cells [156]. A significant blunting of the FSH-induced CYP19A1 expression was seen at 40 μM whereas estradiol production was reduced after treatment with 80 μM of BPA. The BPA concentrations applied are very high and human relevance of this findings remains uncertain. Another study found that BPA concentration-dependently down regulated CYP19A1 expression in KGN cells as well as in human fetal osteoblastic cells, with significant effects seen at 5 μM [157]. Additionally, DEHP (5 μM) and TCDD (10 nM) were found to inhibit the FSH-induced estradiol synthesis and to enhance the AhR expression in a PPAR-dependent manner [158]. Another study found that atrazine and simazine at 10 μM enhanced the stimulatory effect of transfected SF-1 on aromatase mRNA expression and activity in KGN cells [159]. Recently, the pesticide simazine was found to shorten anogenital distance and to decrease whole body, ovarian and uterine weights in offspring of pregnant mice treated with 5–500 μg/kg of this pesticide [160]. Simazine at a concentration of 1 nM diminished the viability and proliferation of KGN granulosa cells. Interestingly, a U-shaped curve was observed, whereby concentrations of 100–1000 nM no longer inhibited cell viability and proliferation.

Currently, most studies on EDCs affecting ovarian steroidogenesis are conducted using tumor cell lines of granulosa origin, where several pathways may be altered compared with normal granulosa cells. This limitation needs to be considered in the interpretation of results. Also, most cell lines are cultivated in medium containing high glucose concentrations and fetal bovine serum as well as under hyperoxia, a situation clearly distinct from that of the physiological context and likely to affet metabolic pathways and steroid production. Another limitation is that currently no suitable human theca cell line is available. Since the production of steroids by the ovaries requires a tight cooperation of granulosa and theca cells, ideally a co-culture system of granulosa and theca cells should be applied for the investigation of ovarian steroidogenesis.

5. Adrenal cell models to investigate disruption of steroidogenesis

The adrenal glands play an essential role in the regulation of electrolyte and energy homeostasis [161]. An over production of glucocorticoids by the adrenal glands ultimately causes Cushing's syndrome, which is characterized by increased visceral adipose tissue, insulin resistance, skin and skeletal muscle atrophy, and impaired wound healing. In contrast, insufficient glucocorticoid

production causes Addison's disease, characterized by hypotension, fatigue, muscle weakness, loss of body weight and depression. The clinical observations emphasize the importance of including the assessment of chemicals applied to humans (drugs, chemicals in food and personal care products) or released at high amounts into the environment for potential adrenal toxicity. In this respect, fatal adrenal isufficiency, due to unexpected severe adverse drug effects [162–165], is a known clinical problem that has been recognized by the FDA [166]. In contrast to the investigations of the safety of chemicals regarding reproductive and developmental endpoints, with a major focus on the disruption of sex steroid hormone action, the adrenal gland has been neglected in EDCs regulatory testing strategy, as recently discussed by Harvey [167]. However, there are several chemicals, *e.g.* drugs, chemicals contained in consumer products and environmental pollutants, that were shown to cause adrenal toxicity (for reviews see [163,167,168]), further emphasizing the necessity of testing chemicals for potential adrenal toxicity.

Nevertheless, regarding the use of cell-based testing systems, there is a widely used human adrenal cell line, *i.e.* H295R. The OECD (Organization for Economic Cooperation and Development) published a guideline for the testing of chemicals using this cell line [169]. The H295R cell line was derived from the NCI-H295 cell line that was established from an adrenocortical carcinoma of a female patient [170]. The use of NCI-H295 cells was limited by the slow proliferation and the fact that they formed cell clusters in culture. Using GC-MS analysis and radio-immuno assays (RIA) NCI-H295 cells were shown to produce about 30 different steroids [170–174]. Importantly, this cell line was shown to express most of the major steroidogenic genes; it also expresses CYP11B2 and has the ability to produce aldosterone, mainly upon stimulation with angiotensin II or potassium. The parental NCI-H295 cells were used to derive the H295A cells [175] as well as the H295R cells [173]. H295R cells can further be distinguished as H295R-S1, H295R-S2 and H295R-S3 clones, depending on the cultivation conditions. H295R-S1 are cultivated in a medium containing Nu-serum, H295R-S2 in a medium with the serum substitute Ultroser-G and H295R-S3 in a medium containing Cosmic calf serum [176]. Furthermore, three additional clones were derived from NCI-H295, namely HAC13, HAC15 and HAC50 [176–178]. The NCI-H295 derived clonal cell lines all grow as adherent monolayers but show significant differences in the expression of steroidogenic enzymes, the response to endogenous regulators and the amounts of steroids synthesized, emphasizing the importance of the culture medium composition. Nevertheless, the NCI-H295 cell lines respond to angiotensin II and potassium by increased aldosterone production; however, their response to ACTH is either absent or very weak [179]. Besides the NCI-H295 clonal cell lines, no other human adrenal cell line with substantial steroidogenic properties has been reported to date.

Based on the secreted steroids and mRNA analyses the NCI-H295 clonal cell lines appeared to express all of the adrenocortical enzymes that were present in the original tumor including StAR, 3β-HSD2, CYP11A1, CYP17A1, CYP21A1, CYP11B1, CYP11B2, 3β-hydroxysteroid sulfotransferase and low levels of CYP19A1 [173,178]. The expression pattern and steroids produced indicates that these cells represent characteristics of the different adrenal zones. It needs to be noted that the basal production of cortisol and aldosterone in H295R cells is low, indicating a low expression of CYP11B1 and CYP11B2 in the absence of inducers. However, treatment with endogenous regulators can enhance some zone-specific effects. Forskolin and cAMP analogs enhance the production of adrenal androgens (DHEA, DHEAS, androstenedione) and glucocorticoids (cortisol, 11-deoxycortisol, corticosterone), whereas angiotensin II, the primary regulator of the renin-angiotensin-aldosterone system, and potassium induce the production of aldosterone in H295R cells [171,172,180,181]. It was shown that H295R cells mediate angiotensin II effects through angiotensin receptor 1 (AT1) [172,179,182–184]. In contrast, H295A do not express substantial levels of AT1 and lack sensitivity to angiotensin II [185]. NCI-H295 clonal cell lines show weak or absent response to ACTH due to the very low expression of melanocortin 2 receptor (MC2R) [177]. Interestingly, in H295R cells ACTH induced a transient increase in aldosterone but not in cortisol production. Thus, depending on whether the cells are used in the basal state or upon stimulation with various effective agonists, the adrenal cell lines may be used to study the effect of EDCs on the functions of the different adrenocortical zones.

Besides the human NCI-H295 clonal cell lines, mouse adrenal cell lines have been used in several studies on adrenal steroidogenesis. The mouse Y-1 cells were reported to exhibit characteristics of both *zona fasciculata* and *zona glomerulosa*, and they are able to produce corticosterone and aldosterone [173,186–188]. Y-1 cells were shown to respond to ACTH with increased expression of steroidogenic genes and enhanced corticosterone production; however, the stimulatory effect was rather modest compared with that of isolated primary mouse adrenal cells [179,189]. Later, two other cell lines, designated ATC1 and ATC7-L, established from adrenal tumors of two transgenic mice expressing the SV40 large T-antigen under the control of the akr1b7 promoter, have been described [190]. Both cell lines exhibited a typical phenotype of the *zona fasciculata*. They produced high amounts of corticosterone and retained responsiveness to ACTH. Incubation of these cells with ACTH increased SF-1 and decreased DAX-1 expression, providing an explanation for the observed stimulation of corticosterone production. Thus, ATC1 and ATC7-L represent useful cell models to study *zona fasciculata* specific function.

In contrast to the testicular and ovarian cell models, there is a human adrenal cell model (H295R) that has been recognized by the regulators for toxicity screening and resulted in an OECD test guideline for the evaluation of EDCs [169]. Therefore, a large number of studies applied the H295R cell model for the assessment of chemicals that cause disturbances of steroidogenesis, including pharmaceuticals, consumer products, food constituents and environmental pollutants [191–198], and it is out of the scope of this review to cover the findings of these studies. Currently, the OECD guideline only focuses on the use of H295R cells in their basal state and on the production of estradiol and testosterone as endpoints [199], two hormones not typically produced by the adrenals. Thus, there are limitations of the current protocol as well as in the use of the H295R cells and the exploitation of this cell model could be significantly extended. Interestingly, the measurement of the main adrenal steroids, *i.e.* adrenal androgens, glucocorticoids and mineralocorticoids, is currently not covered by the OECD guideline and an extended protocol to include the quantification of DHEA, cortisol and aldosterone needs to be validated [200,201]. Other important steroids such as progesterone, 17α-hydroxyprogesterone, 11-deoxycorticosterone and 11-deoxycortisol should also be determined simultaneously with the major adrenal steroids in order to obtain a broader picture of disturbances caused by a given chemical.

Since in their basal state H295R cells produce only low amounts of cortisol and aldosterone, the cells should be used in the basal state to detect chemicals that induce steroidogenesis and upon treatment with specific agonists such as ACTH, angiotensin II and potassium [202] in order to detect chemicals that inhibit steroidogenesis. For the latter, the time point of adding a chemical is important. The pre-incubation or simultaneous addition of a chemical with an inducer may allow to identify chemicals that disrupt regulatory pathways of steroidogenesis. Incubation of a chemical following stimulation of the cells will allow to identify

compounds that directly inhibit steroidogenic enzymes. Thus, different protocols need to be applied depending on the mode-of-action of a given compound. Also, inclusion of appropriate reference compounds (positive and negative controls) and time course analysis of the steroid production would aid the interpretation of the data. Another important issue is the inclusion of measurements of the steroid concentrations in the complete medium at the time of the start of the experiment, as the amounts of these steroids are influenced by the composition of the serum used. Furthermore, the availability of LC–MS based methods allows to simultaneously quantify several steroid hormones and specific steroid pattern analysis can be performed for reference compounds and individual EDCs [201,203–205]. In many recent studies antibody-based detection methods have still been used for quantification of steroids. These methods often are lacking specificity due to cross-reactivity of the antibodies. Thus, GC–MS and LC–MS methods should not only allow more accurate quantification but allow the simultaneous assessment of multiple steroids.

A major limitation of the H295R cell system is the insensitivity toward ACTH. Thus, the establishment of an additional human adrenal cell line is required. Regarding ACTH response, murine ATC1 and ATC7-L cells may represent useful alternatives for testing until a suitable human cell system is available; however, species-specific differences in signaling pathways need to be taken into account.

6. Conclusions and outlook

Cell-based steroidogenesis models are highly valuable for mechanistic studies of chemicals disrupting steroidogenesis and allow an initial medium to high throughput assessment of the potential endocrine toxicity of chemicals. In contrast, to adrenal steroidogenesis, there is no commonly used cell line or standardized procedure to assess effects of chemicals on steroidogenesis in Leydig cells and ovarian cells. Future efforts should therefore aim at establishing a human Leydig cell line with the capability to respond to LH and produce testosterone. To investigate ovarian steroidogenesis, a human theca cell model is needed and, ideally, a theca granulosa co-culture cell system responding to FSH should ideally be established, with the capability for *de novo* steroid synthesis up to the final step of estradiol production. Moreover, there is a need for an ACTH-sensitive human adrenal cell line.

In order to extend and improve the current cell-based testing protocols for studying chemicals that disrupt steroidogenesis and to facilitate the comparison of results from different laboratories, several general issues should be considered: (1) the description of experiments using steroidogenic cell lines should include passage number, cell density, incubation time and the composition of the complete medium used, including glucose concentration, possible use of antibiotics, amount of serum as well as the amount of steroids contained in the complete medium. Cells should only be used within certain passage numbers to guarantee comparable steroidogenic activity and responsiveness of the involved signaling pathways; (2) Ideally, the same positive and negative controls should be included in every experiment to verify the responsiveness of the cell batch used; (3) The cells should be used in the basal state as well as upon stimulation with specific inducers. Ideally, the same inducers, concentrations and conditions should be applied in different laboratories and experiments to allow a direct comparison of the results. The chemicals to be tested should be added prior to stimulation or simultaneously with the inducer in order to investigate whether the response to an inducer is blunted or potentiated, as well as following stimulation in order to detect direct effects on steroidogenic enzymes; (4) The quantification of steroid metabolites should be performed by GC–MS or LC-MS to assure specificity of the results. The major steroids should be quantified rather than a single steroid; and (5) another key issue remains the experimental concentration of a given chemical to be tested. A drawback of cell-based studies is the short duration of the incubation compared with humans who might be exposed for a long period of time. Also, often human exposure data is not available and concentrations of a given compound can vary significantly from its tissue concentration. Usually concentrations chosen for *in vitro* experiments are higher than those observed in humans. Nevertheless, it has to be distinguished between studies aiming at providing mechanistic information and studies for risk assessment. For the latter, it is crucial to choose concentrations that realistically can be reached after occupational exposure or in case of environmental toxicants after exposure in the general population. As suggested by Teeguarden and Hanson-Drury toxicity study exposures should be directly compared to human exposure if such data are available and qualification of a study as “low dose” in the absence of reliable human exposure data should be avoided [206].

Thus, there is still considerable room for improvement of the currently available cellular testing systems and the protocols for measurements of chemical-induced disturbances of steroidogenesis.

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4.3 Published paper: Steroid profiling in H295R cells to identify chemicals potentially disrupting the production of adrenal steroids

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Steroid profiling in H295R cells to identify chemicals potentially disrupting the production of adrenal steroids

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ABSTRACT

The validated OECD test guideline 456 based on human adrenal H295R cells promotes measurement of testosterone and estradiol production as read-out to identify potential endocrine disrupting chemicals. This study aimed to establish optimal conditions for using H295R cells to detect chemicals interfering with the production of key adrenal steroids. H295R cells' supernatants were characterized by liquid chromatography–mass spectrometry (LC–MS)-based steroid profiling, and the influence of experimental conditions including time and serum content was assessed. Steroid profiles were determined before and after incubation with reference compounds and chemicals to be tested for potential disruption of adrenal steroidogenesis. The H295R cells cultivated according to the OECD test guideline produced progestins, glucocorticoids, mineralocorticoids and adrenal androgens but only very low amounts of testosterone. However, testosterone contained in Nu-serum was metabolized during the 48 h incubation. Thus, inclusion of positive and negative controls and a steroid profile of the complete medium prior to the experiment ($t = 0$ h) was necessary to characterize H295R cells' steroid production and indicate alterations caused by exposure to chemicals. Among the tested chemicals, octyl methoxycinnamate and acetyl tributylcitrate resembled the corticosteroid induction pattern of the positive control torcetrapib. Gene expression analysis revealed that octyl methoxycinnamate and acetyl tributylcitrate enhanced CYP11B2 expression, although less pronounced than torcetrapib. Further experiments need to assess the toxicological relevance of octyl methoxycinnamate- and acetyl tributylcitrate-induced corticosteroid production. In conclusion, the extended profiling and appropriate controls allow detecting chemicals that act on steroidogenesis and provide initial mechanistic evidence for prioritizing chemicals for further investigations.

1. Introduction

The adrenals produce active glucocorticoids (*i.e.* cortisol, corticosterone) and mineralocorticoids (*i.e.* aldosterone, 11-deoxycorticosterone), which are essential for the regulation of electrolyte balance, blood pressure, immune system and energy homeostasis. They also produce progestins (*i.e.* progesterone, 17α-hydroxyprogesterone) and adrenal androgens (*i.e.* Δ4-androstene-3,17-dione(androstenedione), dehydroepiandrosterone) that serve as precursors for peripheral formation of active sex steroids. Impaired adrenal steroidogenesis has been associated with cardiometabolic, immune and psychiatric diseases (Gallo-Payet and Battista, 2014; Miller and Auchus, 2011). In Addison's disease, insufficient glucocorticoid synthesis leads to hypotension, fatigue, muscle weakness, weight loss and depression. In Cushing's disease, an over production of glucocorticoids results in visceral obesity, insulin resistance, skin and skeletal muscle atrophy and impaired wound healing. An over function of the adrenal cortex can also

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDCs, endocrine disrupting chemicals; LC–MS, liquid chromatography–mass spectrometry; LOD, limit of detection; LLOQ, lower limit of quantification; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OECD, Organization for Economic Co-operation and Development; REACH, Registration, Evaluation, Authorization and Restriction of Chemicals.

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cause hyperaldosteronism, associated with cardiovascular disease, and hyperandrogenism, associated with hirsutism. This emphasizes the importance to evaluate or test for adrenal toxicity chemicals that are contained in pharmaceuticals, food and consumer products or are released into the environment.

Drug-induced adrenal toxicity is a well-recognized clinical problem; however, in contrast to the investigations into endocrine disruption affecting sex steroid action, disruption of adrenal steroidogenesis has been neglected in the endocrine disrupting chemicals (EDCs) regulatory testing strategy despite compelling evidence for the existence of chemicals contained in consumer products and environmental pollutants that can cause adrenal toxicity (FDA, 2013; Harvey, 2016; Harvey and Everett, 2003; Harvey et al., 2007; Harvey and Sutcliffe, 2010; Hinson and Raven, 2006; Martinez-Arguelles and Papadopoulos, 2015). Also, the interference of industrial chemicals besides PCBs (Johansson et al., 1998, 2005), arsenic (Gosse et al., 2014; Kaltreider et al., 2001) and the organotin dibutyltin (Gumy et al., 2008) with glucocorticoid and mineralocorticoid hormone action requires further research (Macikova et al., 2014; Neel et al., 2013; Odermatt and Gumy, 2008; Stavreva et al., 2012).

The human adrenal cortical cell line H295R exhibits the main steroidogenic properties (Gazdar et al., 1990; Gracia et al., 2006) and has been validated to assess chemical effects on testosterone and estradiol production (Hecker et al., 2011; OECD, 2011). For the identification of EDCs, this protocol can be significantly extended by including progestins, adrenal androgens, glucocorticoids and mineralocorticoids (for a general overview of steroid synthesis see Fig. 1) (Odermatt et al., 2016). In fact, several studies addressed this issue and included several steroid metabolites in their analytical method (Feng et al., 2016; Karmaus et al., 2016; Mangelis et al., 2016; Nakano et al., 2016; Rijk et al., 2012; Saito et al., 2016; Tonoli et al., 2015; van den Dungen et al., 2015; Wang et al., 2015). Besides, the OECD testing guideline does not explicit the analytical method to be used as well as determined the read-out after a 48 h incubation period without inclusion of a control at the start of the experiment. Because improved testing protocols are especially important for situations where no clinical studies are performed, *e.g.* chemicals contained in cosmetics, UV-filters, food additives, drugs of abuse and designer steroids, the aim of the present study was to establish conditions for the LC–MS-based detection of changes of a series of progestins, adrenal androgens, glucocorticoids and mineralocorticoids upon exposure to reference chemicals and to chemicals that based on evidence from the literature might exert endocrine disrupting effects by disturbance of steroid hormone action.

2. Materials and methods

2.1. Chemicals and reagents

Test and reference compounds (angiotensin II (CAS Nr. 4474-91-3), forskolin (CAS Nr. 66575-29-9), prochloraz (CAS Nr. 67747-09-5), etomidate (CAS Nr. 33125-97-2), abiraterone acetate (CAS Nr. 154229-18-2), formestane (CAS Nr. 566-48-3), trilostan (CAS Nr. 13647-35-3), torcetrapib (CAS Nr. 262352-17-0), abietic acid (CAS Nr. 514-10-3), benzophenone-1 (CAS Nr. 131-56-6), chlorophene (CAS Nr. 120-32-1), enoxolone (CAS Nr. 471-53-4), escitalopram oxalate (CAS Nr. 219861-08-2), genistein (CAS Nr. 446-72-0), mitotane (CAS Nr. 53-19-0), rofecoxib (CAS Nr. 162011-90-7), sotalol (CAS Nr. 959-24-0), triclocarban (CAS Nr. 101-20-2), valproic acid sodium salt (CAS Nr. 1069-66-5), yohimbine hydrochloride (CAS Nr. 65-19-0), zidovudine (CAS Nr. 30516-87-1), octocrylene (CAS Nr. 6197-30-4), octyl methoxycinnamate (CAS Nr. 5466-77-3), acetyl tributylcitrate (CAS Nr. 77-90-7), linuron (CAS Nr. 330-55), digoxin (CAS Nr. 20830-75-5), digitoxin (CAS Nr. 71-63-6), amiodarone hydrochloride (CAS Nr. 19774-82-4), clofazimine (CAS Nr. 2030-63-9), sulforaphane (CAS Nr. 142825-10-3), and CDDO methyl ester (CAS Nr. 218600-53-4)) of the highest grade available were obtained from Sigma–Aldrich (Buchs, Switzerland). Stock solutions (10 mM) were prepared in dimethyl sulfoxide (DMSO). UHPLC-grade purity methanol, acetonitrile and formic acid were obtained from Biosolve (Dieuze, France). Aldosterone, 11-deoxycorticosterone, corticosterone, dehydroepiandrosterone-3-sulfate, androstenedione, testosterone, pregnenolone and [2,2,4,6,6,21,21-$^{2}H_{7}$]-aldosterone (98% isotopic purity) were purchased from Sigma-Aldrich. 11-Dehydrocorticosterone, dehydroepiandrosterone, progesterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone, 11-deoxycortisol, cortisol and cortisone were purchased from Steraloids (Newport, RI). [1,2-$^{2}H_{2}$]-Testosterone (98% isotopic purity), [2,2,4,6,6,16,16-$^{2}H_{7}$]-4-androstene-3,17-dione (98% isotopic purity) and [2,2,4,6,6,17α,21,21-$^{2}H_{8}$]-corticosterone (98% isotopic purity) were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Canada). Deuterated analogues [2,2,4,6,6,21,21,21-$^{2}H_{8}$]-17α-hydroxyprogesterone and [9,11,12,12-$^{2}H_{4}$]-cortisol were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Stock solutions (10 mM and/or 1 mM) of above mentioned steroids were prepared in ethanol or methanol.

2.2. Cell culture and H295R steroidogenesis assay

The human adrenocortical carcinoma cell line H295R was obtained from American Type Culture Collection (ATCC, Manassas, USA) and grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (1:1, v/v) (Life Technologies, Zug, Switzerland), supplemented with 1% (v/v) IST + Premix (BD Bioscience, Bedford, MA, USA), 2.5% (v/v) Nu-serum (Lot: 2342913, BD Bioscience, Bedford, MA, USA), 15 mM HEPES buffer and 1% (v/v) penicillin–streptomycin (Sigma–Aldrich) at 37 °C with a humidified 5% CO_2 atmosphere. The Nu-serum consists of 25% newborn calf serum and 75% of a proprietary formulation containing epidermal growth factor, endothelial cell growth supplement, insulin, transferrin, triiodothyronine, progesterone, estradiol, testosterone, cortisol, selenous acid, o-phosphorylethanolamine, glucose, amino acids, vitamins and other trace elements and nutrients in its Ham's F12 medium base. The concentrations of the supplements are not declared by the supplier.

The H295R steroidogenesis assay was performed according to the OECD test guideline (OECD, 2011). Briefly, cells at passages between 5 and 10 were seeded in 24-well plates at a density of 200,000 cells/ml in complete medium. The medium was replaced 24 h later with fresh medium containing test and reference compounds where indicated. DMSO (0.1% (v/v)) served as vehicle control. For studying time-dependent steroid production, cells were incubated for either 4, 8, 24 or 48 h in separate wells, and culture supernatants were collected and frozen at −20 °C until further analysis. For the other experiments, cells were incubated for

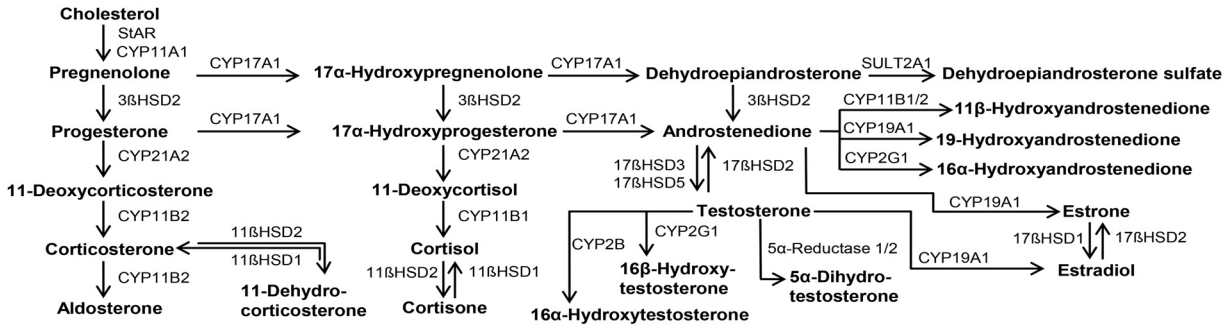


Fig. 1. Overview of steroidogenesis. Steroids are indicated in *bold*, the key enzymes in *regular* and the corresponding catalyzing reactions by *arrows*. CYP = cytochrome P450; HSD = hydroxysteroid dehydrogenase; SULT = sulfotransferase; StAR = steroidogenic acute regulatory protein.

48 h. Complete medium prior to adding to the cells ($t = 0$ h) served as control. To study the impact of serum, the complete medium was replaced 24 h after seeding by Nu-serum-free medium, followed by incubation for 48 h and collection of culture supernatants. All experiments were performed three times independently and in triplicates, with the exception of the untargeted analysis of steroid changes upon treatment with test and reference compounds; this experiment was performed two times independently, each in triplicates.

2.3. Assessment of cytotoxicity

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate possible effects of the test compounds on cellular metabolic activity. Briefly, H295R cells were seeded in 96-well plates (30,000 cells/100 μl complete medium). The medium was replaced after 24 h and cells were incubated with fresh medium containing the compounds of interest in a range of 0.04–50 μM, as indicated in the figures and tables. After 48 h, cells were inspected under the microscope. None of the treatments shown in this study, for both H295R cells kept in complete medium and cells kept in Nu serum-free medium, resulted in morphological changes. Then, 20 μl of MTT (5 mg/ml) was added to the medium, followed by incubation for another 3 h. For dissolving of the formed formazan crystals, the medium was aspirated and 100 μl of Sorenson's glycine buffer was added to each well. The plates were analyzed after 5 min at 565 and 650 nm (reference wavelength). The MTT assay was performed three times independently with technical triplicates. The conditions and concentrations used for the experiments presented in this study did not result in reduced cellular metabolic activity (values lower than 80% compared to vehicle control). Signs of cytotoxicity were observed, however, when incubating cells in the absence of Nu-serum with 10 μM forskolin. For this reason, the lower forskolin concentration of 5 μM was used for experiments with Nu-serum free medium.

2.4. Targeted steroid quantification

Targeted analysis of steroid hormone levels in H295R culture supernatants was performed as previously described with minor adaptations (Strajhar et al., 2016). Briefly, for solid-phase extraction, 1 ml of each H295R cell supernatant was mixed with 0.1 ml of protein precipitation solution (0.8 M zinc sulfate in water/methanol 50/50, v/v) that contained deuterium-labeled aldosterone, corticosterone, androstenedione and testosterone as internal standards. After incubating the samples in a shaker for 10 min at 4 °C with thorough shaking (1300 rotations/min), they were centrifuged for 10 min at 16,000 × g at 4 °C. The supernatants (950 μl) were transferred to Oasis HLB SPE cartridges, preconditioned with methanol and water. Steroids were eluted with 1 ml of methanol after washing once with 1 ml of water and twice with 1 ml of methanol/water (10/90, v/v). The samples were evaporated to dryness and then reconstituted in 25 μl of methanol. The separation and quantification of the steroids was performed by ultra-high pressure LC–MS/MS (UHPLC–MS/MS) using an Agilent 1290 UPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer equipped with a jet-stream electrospray ionization interface. The steroids were separated using a reverse-phase column (Waters Acquity UPLC BEH C18, 1.7 μm, 2.1 mm × 150 mm) and a mobile phase A and B, consisting of water–acetonitrile–formic acid (95/5/0.1; v/v/v) and (5/95/0.1; v/v/v), respectively. For data acquisition and analysis, Mass Hunter software (Agilent Technologies) was used. Lower limits of quantification (LLOQ) are shown in Table 1. The steroid levels measured in all supernatants of incubated H295R cells were above the LLOQ. Recoveries for all steroids analyzed were between 80% and 120%.

2.5. Steroid quantification using an untargeted acquisition mode

Protein precipitation was performed adding 0.5 volume of protein precipitation solution in cell culture supernatant (1 ml). Precipitation solution was prepared adding 0.4 ml of 17α-hydroxyprogesterone-d8, cortisol-d4 at 1 μg/ml in methanol, and 0.2 ml of testosterone-d3 at 1 μg/ml in methanol (final concentrations in the cellular medium equal to 4, 4 and 2 ng/ml, respectively) into 49 ml of 6% perchloric acid solution in water. Samples were mixed for 10 min at 1400 rotations/min and 15 °C with a Thermomixer (Vaudaux-Eppendorf, Buchs, Switzerland). Samples underwent then centrifugation for 10 min at 4 °C and 12,000 × g. Supernatant was then loaded onto Oasis HLB cartridges (Waters, Milford, MA, USA) in 96-well plate format (30 mg, 30 μm particle size). Prior to supernatant deposition, cartridges were conditioned with 1 ml of methanol, dried for 10 min at 10 in Hg, and equilibrated with two times 1 ml of water. Cartridges were then washed three times successively with 1 ml of water/methanol (90:10, v/v) and dried for 1 min at 10 in Hg before elution with 1 ml of methanol. Fractions were then transferred into 1.5 ml polypropylene tubes and evaporated to dryness under vacuum with a centrifugal evaporator (RC1022, Jouan, Instrumenten Gesellschaft AG, Zürich, Switzerland). Samples were then reconstituted in 50 μl of water + 0.1% formic acid/acetonitrile + 0.1% formic acid (90:10, v/v) and homogenized in a Thermomixer for 10 min at 20 °C and 1400 rotations/min (Vaudaux-Eppendorf, Buchs, Switzerland) before 10 μl injection into the LC–MS system. Separation was performed with an UHPLC Acquity H-Class (Waters) including a quaternary solvent manager (QSM), a sample manager (SM-FTN) and a column manager (CM-A). The separation was performed on a Kinetex C18 column (2.1 mm × 150 mm, 1.7 μm) (Phenomenex, Torrance, CA) with a SecurityGuard ULTRA C18 (2.1 mm × 2 mm) (Phenomenex). Mobile phase A was water + 0.1% formic acid, and mobile phase B was acetonitrile + 0.1% formic acid. The flowrate was set at 300 μl/min. The composition in mobile phase B was increased linearly from 5% up to 80% in 14 min, then up to 90% in 0.5 min (hold for 1.5 min) and equilibrated back to original mobile phase conditions in 0.1 min for 6 min. Total analysis time was of 22.1 min per sample. Column was kept at 30 °C during the analysis while samples were kept at 8 °C in the autosampler. The automatic calibration procedure was performed as described by Tonoli et al. (2015). MaXis 3G QTOFMS (Bruker, Bremen, Germany) was equipped with an electrospray ionization source operated in positive mode. Source parameters were as follows: end plate offset was set at −500 V, nebulizer pressure at 1.8 bar, dry gas flowrate at 5.5 l/min, and temperature at 225 °C. Capillary voltage was set at −4.7 kV. Accumulation time was set at 1 s and mass range monitored was from m/z 50 to m/z 1000. Acquisition was performed in profile mode. Data were acquired using Compass v1.5 SR3 software suite from Bruker and HyStar v 3.2 SR2. UHPLC was controlled using plug-in for Waters Acquity UPLC v.1.5.

Table 1
Targeted quantification of steroids by LC–MS. Subset of 11 steroids used for quantification with their respective lower limits of quantification (LLOQ) values in nM.

Analyte	LLOQ (nM)
Progesterone	0.05
17α-Hydroxyprogesterone	0.78
11-Deoxycorticosterone	0.78
Aldosterone	0.20
Corticosterone	0.98
11-Deoxycortisol	0.78
Cortisol	1.95
Dehydroepiandrosterone	3.91
Dehydroepiandrosterone 3-sulfate	19.5
Androstenedione	0.78
Testosterone	0.39

2.6. Analysis of mRNA expression

Following a medium change and 48 h of incubation of H295R cells with the respective compounds, RNA was extracted using Tri-reagent (Sigma–Aldrich) and purified with the Direct-Zol RNA Mini Prep kit (Zymo research, Irvine, CA, USA) according to the manufacturer's instructions. RNA quality and yield was assessed using a Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies). Complementary DNA (cDNA) was synthesized from RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) as previously described (Chantong et al., 2014). RT-qPCR was performed using KAPA SYBR FAST qPCR kit (Kapasystems, Boston, MA, USA) with the primers for the genes *CYP11B2*, *CYP17A1*, *CYP21A2*, *HSD3B2*, *CYP11A1*, *StAR*, and *CYP19A1* (Hilscherova et al., 2004), *CYP11B1* (Xu et al., 2006), *GAPDH* (Tanaka et al., 2008), and *HSD17B1*, *HSD17B2*, *HSD17B3*, *AKR1C3* (sequences of oligonucleotide primers are listed in Table S1 in the Supporting Information) and using the rotor-gene 6000 (Corbett Research, Sydney, Australia). Relative gene expression compared with the internal control GAPDH was determined using the 2-(ΔCt sample-ΔCt control) method. GAPDH was chosen as reference gene because its expression did not change between the various experimental conditions and time points applied in the present study. S18 RNA and PPIA were also analyzed but did not fulfill the quality criteria to be used as reference in these experiments (Taylor et al., 2010). Each sample was analyzed in triplicate.

2.7. Statistics

Computational analysis was performed in MATLAB® 8 environment (The MathWorks, Natick, USA). For analysis of data obtained from three independent steroid profiling experiments each performed in triplicate ($n = 9$), Shapiro–Wilk test was used to verify the normality of data. One-way analysis of variance (ANOVA) and Dunnett's multiple-comparison test were performed to evaluate differences between chemical treatments compared to the solvent control. Differences in gene expression were evaluated using a one-sample t-test of the fold changes after log2 transformation. Differences with $p < 0.05$ were considered to be significant. For analysis of data obtained from a representative experiment performed in triplicate ($n = 3$), Kruskal–Wallis test followed by Dunn's test was used.

3. Results

According to the OECD test guideline 456, H295R cells are incubated in medium containing 2.5% Nu-serum with the chemical of interest for 48 h, followed by determination of testosterone and estradiol as read-out and expression of the results as relative changes in hormone production compared with the solvent

controls (Hecker et al., 2011; OECD, 2011). In the present study, an untargeted LC–MS-based acquisition mode was chosen for the initial characterization of the H295R cell model and for a qualitative assessment of changes in the profile of 14 steroids upon exposure to various reference and test chemicals. In a second step, a targeted LC–MS-based method was used for quantification of steroid profiles upon exposure of cells to selected chemicals. This methods covered 11 adrenal steroids, including progestins, adrenal androgens, glucocorticoids and mineralocorticoids; however, not estrogens as they did not properly ionize under the conditions applied.

3.1. Time-dependent production of steroids in H295R cells

As a first analytical step, untargeted signal acquisition led to the detection of approximately 130 steroid-like metabolites annotated automatically based on exact mass. Starting from this panel of candidate compounds, 14 main steroids were unambiguously

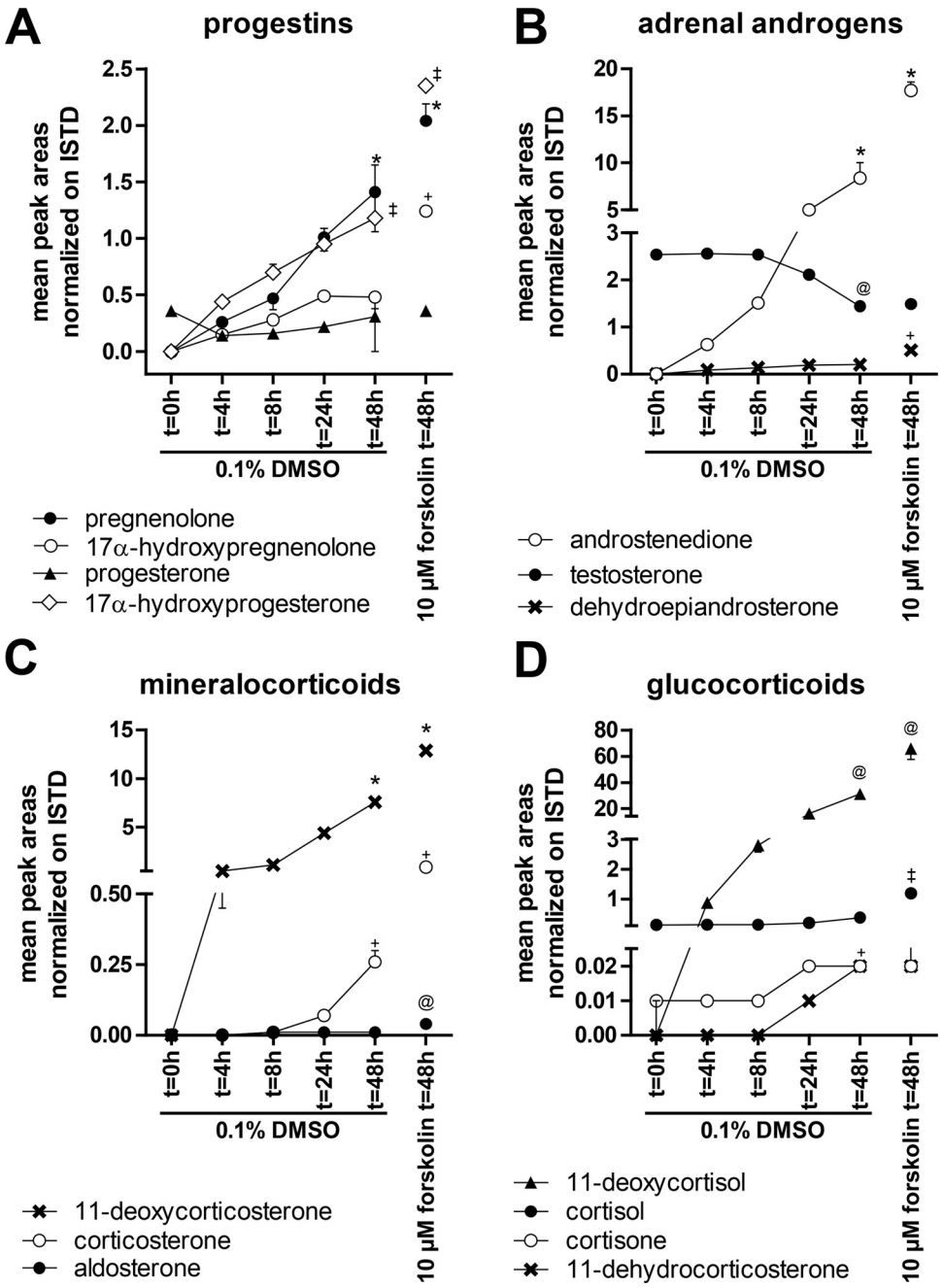


Fig. 2. Time-dependent steroid synthesis in H295R cells. H295R cells were cultivated following a medium change for 48 h in complete medium in the presence of vehicle (0.1% DMSO) or 10 μM forskolin as positive control to stimulate steroidogenesis. The secretion of (A) progestins, (B) adrenal androgens, (C) mineralocorticoids and (D) glucocorticoids into the medium was measured in culture supernatants by LC–MS. Kruskal–Wallis test followed by Dunn's test was used for statistical analysis. Data represent peak areas normalized to internal standard (ISTD), median with range, from one (out of three) representative experiment, performed in triplicate ($n = 3$). Significant differences ($p < 0.05$) for hormones (pregnenolone (*), 17α-hydroxypregnenolone (+), 17α-hydroxyprogesterone (‡), 11-deoxycorticosterone (*), corticosterone (+), aldosterone (@), 11-deoxycortisol (@), cortisol (‡), cortisone (+), androstenedione (*), testosterone (@), and dehydroepiandrosterone (+)) compared to vehicle (0.1% DMSO) at starting time point ($t = 0$ h) are indicated by the respective symbols.

identified by comparison with reference standards. Based on mean peak areas, a relative abundance comparison was conducted at 0, 4, 8, 24 and 48 h of incubation of the cells in complete medium containing 2.5% Nu-serum (Fig. 2). Additionally, steroidogenesis was stimulated in H295R cells by adding 10 μM forskolin and incubating the cells for 48 h.

Inclusion of steroid measurements of the complete medium prior to adding it to the cells at the start of the experiment ($t = 0$ h) revealed the presence of steroids that are added by the producer of Nu-serum (BD Bioscience), *i.e.* progesterone, testosterone and cortisol (estradiol is also added but was not measured by the method applied in this study) as well as cortisone in the complete medium (Fig. 2). While progesterone levels were not significantly altered upon incubation for up to 48 h, even in the presence of forskolin, the progestins pregnenolone, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone showed time-dependent increases and were further enhanced upon treatment for 48 h with forskolin (Fig. 2A). A similar time-dependent increase was observed for the androgens androstenedione and, much less pronounced, for dehydroepiandrosterone (Fig. 2B), as well as for the corticosteroids 11-deoxycorticosterone, corticosterone, 11-deoxycortisol, and, less pronounced, for aldosterone, cortisone and cortisol (Fig. 2C and D). An exception was testosterone that was lower after 48 h of incubation (Fig. 2B). Also, the presence of forskolin did not stimulate the production of testosterone, and, although not reaching significance, a trend decrease was observed. These results show that H295R cells, in line with adrenal steroidogenesis, produce progestins, adrenal androgens, mineralocorticoids and glucocorticoids, reaching significant levels under the conditions applied after 48 h of incubation. In contrast, the testosterone contributed by addition of the Nu-serum seemed to be metabolized by the H295R cells.

3.2. De novo *synthesis of androstenedione but very low amounts of testosterone*

The production of androgens was further studied in H295R cells incubated for 48 h in the presence or absence of Nu-serum. This confirmed the presence of testosterone in the Nu-serum (compare DMSO control at $t = 0$ h in Fig. 3A and B) and that only very low amounts were produced *de novo* by the H295R cells (Fig. 3B). After 48 h of incubation in the absence of Nu-serum the amount of

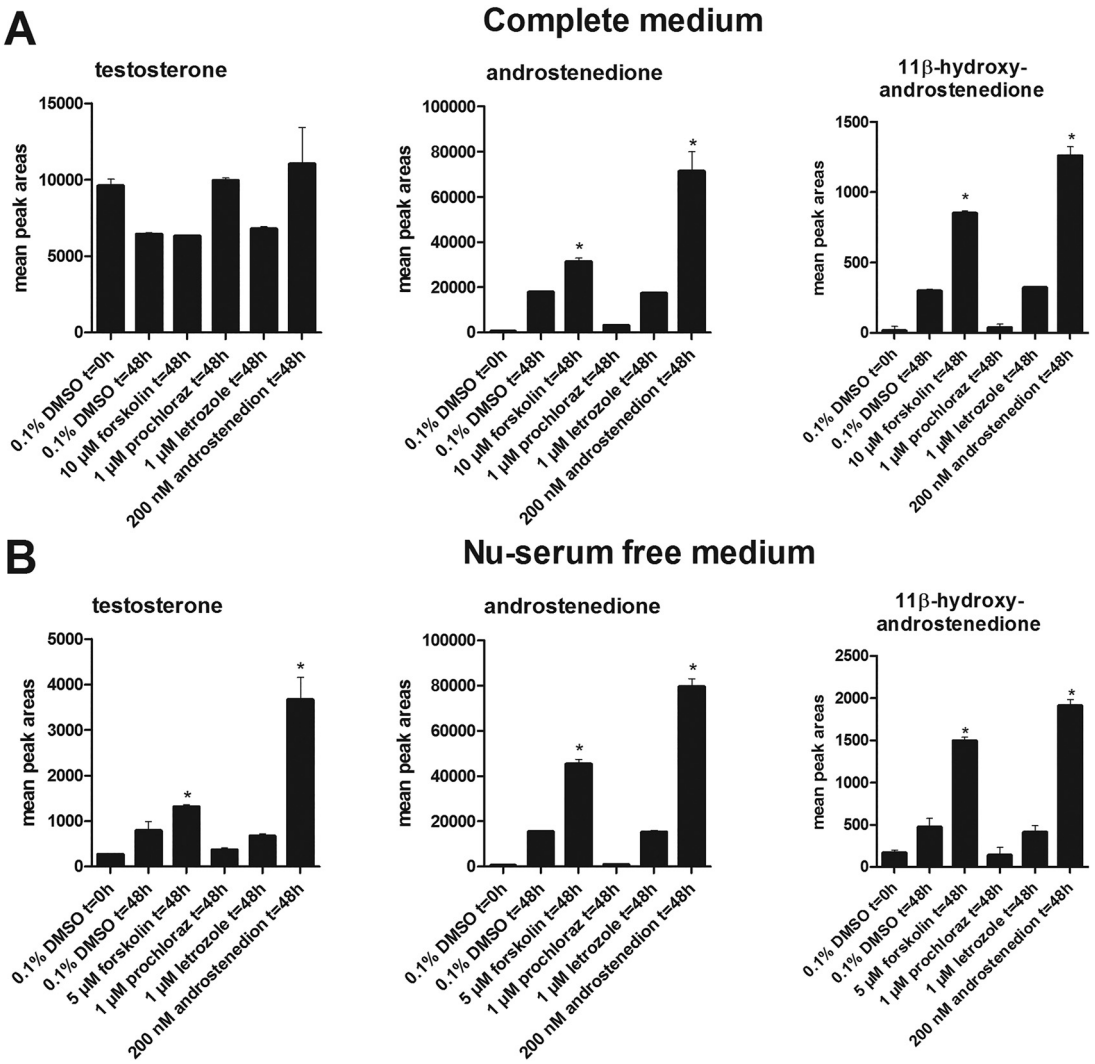


Fig. 3. *De novo* synthesis of testosterone, androstenedione and 11β-hydroxyandrostenedione by H295R cells. Steroids were quantitated in culture supernatants of H295R cells incubated either in complete medium (A) or in Nu-serum-free medium (B) for 48 h with vehicle (0.1% DMSO solvent control), 10 μM forskolin, 1 μM prochloraz, 1 μM letrozole or 200 nM androstenedione. Controls for complete medium and Nu-serum-free medium ($t = 0$ h) were included for comparison. Steroids (mean peak areas) were measured by LC–MS and represent median with range from one (out of three) representative experiment, performed in triplicate ($n = 3$). Kruskal–Wallis test followed by Dunn's test was used to analyze significant difference ($p < 0.05$) of solvent control at $t = 0$ to chemical treatment at $t = 48$ h (*).

testosterone produced was approximately 10-fold lower than the level present in the complete medium containing Nu-serum, thus masking *de novo* testosterone synthesis in cells kept in complete medium. Interestingly, forskolin did not affect the amount of testosterone in cells kept in the complete medium compared to vehicle control (Fig. 3A, DMSO control at $t = 48$ h), despite of an activation of testosterone formation observed in serum-free medium (Fig. 3B). The metabolism of testosterone from Nu-serum in cells kept in complete medium (Fig. 3A, also seen in Fig. 2B) could be prevented by the CYP17A1/CYP21A2 inhibitor prochloraz but not by the CYP19A1 inhibitor letrozole, suggesting that metabolism to estradiol had at best a minor contribution to the observed decrease of testosterone from the complete medium. However, formation of estrone and estradiol was not analyzed in this study because the LC–MS method applied was not designed to quantify these estrogens. Addition of androstenedione led to a trend increase (1.7-fold) in testosterone amounts in cells kept in complete medium, but a more pronounced increase (4.5-fold) that was further enhanced upon forskolin treatment (7.5-fold) in cells in the absence of serum, demonstrating the capability of H295R cells to produce testosterone (Fig. 3B). A comparison of H295R cells cultivated in complete medium with cells kept in Nu-serum-free medium revealed that androstenedione was synthesized *de novo* and that its production was further enhanced by forskolin (Fig. 3A and B). Also, 11β-hydroxyandrostenedione, a steroid produced by the human adrenals and found at higher concentration than androstenedione in adrenal vein sampling (Rege et al., 2013; Swart et al., 2013), was produced *de novo* by the H295R cells and was further enhanced upon forskolin treatment. While prochloraz abolished the formation of androstenedione and 11β-hydroxyandrostenedione, letrozole had no effect on the amounts of these steroids, suggesting that CYP19A1 plays a minor role under these conditions and that these androgens were not converted in substantial amounts to estrogens.

The adrenals can also form other hydroxylated metabolites of androstenedione and testosterone (Ford et al., 1975; Wang et al., 2010). For example, as shown in Fig. S1 (see Supplementary information), 16α- and 19α-hydroxyandrostenedione were found to be produced by the H295R cells and further increased by forskolin treatment, both in the presence and absence of Nu-serum. Also, 16α- and 16β-hydroxytestosterone were produced by the H295R cells and further enhanced by forskolin, and these metabolites were not present at substantial amounts in the complete medium. Thus, hydroxylation of testosterone by cytochrome P450 enzymes provides a possible explanation for the observed decrease of testosterone added by the complete medium upon incubation of H295R cells.

3.3. Impact of Nu-serum and forskolin on mRNA expression of steroidogenic genes

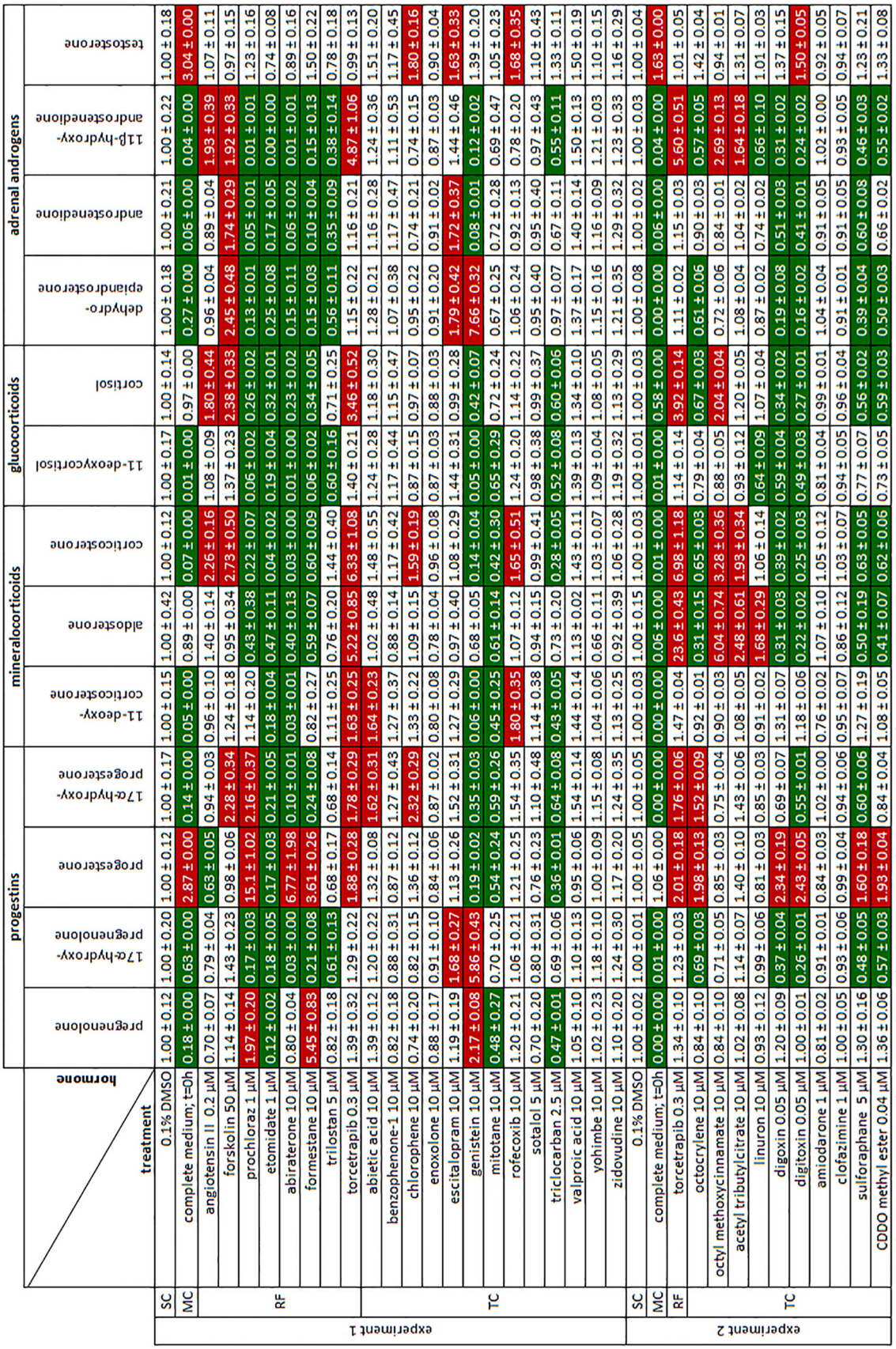
In order to assess the impact of Nu-serum removal on steroidogenic gene expression, H295R cells were subjected to a medium change and incubated in Nu-serum-free medium for 48 h in the presence of 0.1% DMSO. The comparison of mRNA expression levels with cells kept in complete medium revealed only minor differences in basal gene expression with CYP11B2 and StAR being expressed at about 2-fold lower levels (Table 2). Next, the stimulation of steroidogenesis by forskolin was analyzed. The most pronounced increase in mRNA expression was observed for 3β-HSD2 followed by CYP21A2, CYP11B2 and CYP19A1. All genes involved in steroidogenesis were induced at least 2-fold upon treatment with forskolin. Additionally, the expression of genes involved in the final steps of testosterone and estradiol synthesis were measured. 17β-HSD3 (converting androstenedione to testosterone), 17β-HSD1 (converting estrone to estradiol) and 17β-HSD2 (converting estradiol to estrone, testosterone to androstenedione) were not expressed at substantial levels. AKR1C3 mRNA was well expressed; thus, the enzyme responsible for the last step of *de novo* testosterone formation in H295R cells seems to be AKR1C3 and not 17β-HSD3, the key enzyme in Leydig cell testosterone formation (Miller and Auchus, 2011). AKR1C3 decreased 2-fold upon forskolin treatment in both cells kept in complete medium and in cells incubated in Nu-serum-free medium. Forskolin stimulation of steroidogenesis was in general more pronounced in Nu-serum-free medium, which may be explained by higher free concentrations of the compound in the absence of binding to serum proteins. At forskolin concentrations of 10 μM and higher, morphological changes indicating cytotoxicity were observed in cells incubated in Nu-serum-free medium (not shown); therefore a forskolin concentration of 5 μM, which did not lead to morphological changes, changes in the MTT assay, or changes in the expression levels of the GAPDH control, was chosen for these experiments.

3.4. Qualitative effects of reference and test compounds on the steroid profile in H295R cells

In the present study, H295R cells were incubated in medium containing 2.5% Nu-serum (complete medium) with the compounds of interest for 48 h, followed by collection of culture supernatants, according to the OECD test guideline. Relative amounts of the identified steroids, including the main adrenal steroids plus testosterone, were compared to those of cells incubated with vehicle (0.1% DMSO) (Fig. 4). The steroid profile of the complete medium at the start of the experiment ($t = 0$ h) was

Table 2
Impact of serum and forskolin on mRNA expression of steroidogenic genes. H295R cells were cultivated for 48 h in complete medium or in Nu-serum free medium in the presence or absence of 10 μM or 5 μM of forskolin, respectively, followed by quantification of mRNA expression by qPCR. Data represent mean ± SD from three independent experiments ($n = 9$), each performed in triplicates. GOIs = genes of interest; GAPDH = glycerinaldehyde-3-phosphatedehydrogenase; Ct = cycle threshold. Differences in gene expression were evaluated using a one-sample *t*-test of the fold changes after log2 transformation. Differences with $p < 0.05$ were considered to be significant.

	Complete medium		Nu-serum-free medium	
	Ct value DMSO 0.1%	Fold change GOI/GAPDH Forskolin 10 μM	Ct value DMSO 0.1%	Fold change GOI/GAPDH Forskolin 5 μM
GAPDH	13.7 ± 0.5	1.0 ± 0.4	13.6 ± 0.7	1.0 ± 0.1
StAR	15.9 ± 0.5	2.2 ± 0.4*	17.0 ± 0.4	5.6 ± 1.1*
CYP11A1	17.5 ± 0.5	2.4 ± 0.8*	17.9 ± 0.6	3.1 ± 0.3*
3β-HSD2	24.6 ± 0.6	27.9 ± 5.2*	25.0 ± 0.2	25.8 ± 10*
CYP17A1	19.5 ± 0.4	3.4 ± 0.6*	20.0 ± 0.5	7.3 ± 2.3*
CYP21A2	20.0 ± 0.7	8.0 ± 1.9*	20.8 ± 0.8	19 ± 5.9*
CYP11B1	29.1 ± 0.7	2.9 ± 1.0*	29.1 ± 0.9	5.3 ± 3.2*
CYP11B2	25.9 ± 0.6	9.1 ± 2.1*	26.9 ± 0.5	14.3 ± 5.7*
CYP19A1	23.4 ± 0.5	7.7 ± 1.6*	23.0 ± 0.3	10.5 ± 1.1*
AKR1C3	21.6 ± 0.6	0.5 ± 0.1*	22.0 ± 0.5	0.5 ± 0.0*



		treatment \ hormone	pregnenolone	17α-hydroxy-pregnenolone	progesterone	17α-hydroxy-progesterone	11-deoxy-corticosterone	aldosterone	corticosterone	11-deoxycortisol	cortisol	dehydro-epiandrosterone	androstenedione	11β-hydroxy-androstenedione	testosterone
experiment 1	SC	0.1% DMSO	1.00 ± 0.12	1.00 ± 0.20	1.00 ± 0.12	1.00 ± 0.17	1.00 ± 0.15	1.00 ± 0.42	1.00 ± 0.12	1.00 ± 0.17	1.00 ± 0.14	1.00 ± 0.18	1.00 ± 0.21	1.00 ± 0.22	1.00 ± 0.18
	MC	complete medium; t=0h	0.18 ± 0.00	0.63 ± 0.00	2.87 ± 0.00	0.14 ± 0.00	0.05 ± 0.00	0.89 ± 0.00	0.07 ± 0.00	0.01 ± 0.00	0.97 ± 0.00	0.27 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	3.04 ± 0.00
	RF	angiotensin II 0.2 µM	0.70 ± 0.07	0.79 ± 0.04	0.63 ± 0.05	0.94 ± 0.03	0.96 ± 0.10	1.40 ± 0.14	2.26 ± 0.16	1.08 ± 0.09	1.80 ± 0.44	0.96 ± 0.04	0.89 ± 0.04	1.93 ± 0.39	1.07 ± 0.11
		forskolin 50 µM	1.14 ± 0.14	1.43 ± 0.23	0.98 ± 0.06	2.28 ± 0.34	1.24 ± 0.18	0.95 ± 0.34	2.73 ± 0.50	1.37 ± 0.23	2.38 ± 0.33	2.45 ± 0.48	1.74 ± 0.29	1.92 ± 0.33	0.97 ± 0.15
		prochloraz 1 µM	1.97 ± 0.20	0.17 ± 0.03	15.1 ± 1.02	2.16 ± 0.37	1.14 ± 0.20	0.43 ± 0.38	0.22 ± 0.07	0.06 ± 0.02	0.26 ± 0.02	0.13 ± 0.01	0.05 ± 0.01	0.01 ± 0.01	1.23 ± 0.16
		etomidate 1 µM	0.12 ± 0.02	0.18 ± 0.05	0.17 ± 0.03	0.21 ± 0.05	0.18 ± 0.04	0.47 ± 0.11	0.04 ± 0.02	0.19 ± 0.04	0.32 ± 0.01	0.25 ± 0.08	0.17 ± 0.05	0.00 ± 0.00	0.74 ± 0.08
		abiraterone 10 µM	0.80 ± 0.04	0.03 ± 0.00	6.77 ± 1.98	0.10 ± 0.01	0.03 ± 0.01	0.40 ± 0.13	0.03 ± 0.00	0.01 ± 0.00	0.23 ± 0.02	0.15 ± 0.11	0.06 ± 0.02	0.01 ± 0.01	0.89 ± 0.16
		formestane 10 µM	5.45 ± 0.83	0.21 ± 0.08	3.61 ± 0.26	0.24 ± 0.08	0.82 ± 0.27	0.59 ± 0.07	0.60 ± 0.09	0.06 ± 0.02	0.34 ± 0.05	0.15 ± 0.03	0.10 ± 0.04	0.15 ± 0.13	1.50 ± 0.22
		trilostan 5 µM	0.82 ± 0.18	0.61 ± 0.13	0.68 ± 0.17	0.68 ± 0.14	1.11 ± 0.25	0.76 ± 0.20	1.44 ± 0.40	0.60 ± 0.16	0.71 ± 0.25	0.56 ± 0.11	0.35 ± 0.09	0.38 ± 0.14	0.78 ± 0.18
	TC	torcetrapib 0.3 µM	1.39 ± 0.32	1.29 ± 0.22	1.88 ± 0.28	1.78 ± 0.29	1.63 ± 0.25	5.22 ± 0.85	6.33 ± 1.08	1.40 ± 0.21	3.46 ± 0.52	1.15 ± 0.22	1.16 ± 0.21	4.87 ± 1.06	0.99 ± 0.13
		abietic acid 10 µM	1.39 ± 0.12	1.20 ± 0.22	1.32 ± 0.08	1.62 ± 0.31	1.64 ± 0.23	1.02 ± 0.48	1.48 ± 0.55	1.24 ± 0.28	1.18 ± 0.30	1.28 ± 0.21	1.16 ± 0.28	1.24 ± 0.36	1.51 ± 0.20
		benzophenone-1 10 µM	0.82 ± 0.18	0.88 ± 0.31	0.87 ± 0.12	1.27 ± 0.43	1.27 ± 0.37	0.88 ± 0.14	1.17 ± 0.42	1.17 ± 0.44	1.15 ± 0.47	1.07 ± 0.38	1.17 ± 0.47	1.11 ± 0.53	1.17 ± 0.45
		chlorophene 10 µM	0.74 ± 0.20	0.82 ± 0.15	1.36 ± 0.12	2.32 ± 0.29	1.33 ± 0.22	1.09 ± 0.15	1.59 ± 0.19	0.87 ± 0.15	0.97 ± 0.07	0.95 ± 0.22	0.74 ± 0.21	0.74 ± 0.15	1.80 ± 0.16
		enoxolone 10 µM	0.88 ± 0.17	0.91 ± 0.10	0.84 ± 0.06	0.87 ± 0.02	0.80 ± 0.08	0.78 ± 0.04	0.96 ± 0.08	0.87 ± 0.03	0.88 ± 0.03	0.91 ± 0.20	0.91 ± 0.02	0.87 ± 0.03	0.90 ± 0.04
		escitalopram 10 µM	1.19 ± 0.19	1.68 ± 0.27	1.13 ± 0.26	1.52 ± 0.31	1.27 ± 0.29	0.97 ± 0.40	1.08 ± 0.29	1.44 ± 0.31	0.99 ± 0.28	1.79 ± 0.42	1.72 ± 0.37	1.44 ± 0.46	1.63 ± 0.33
		genistein 10 µM	2.17 ± 0.08	5.86 ± 0.43	0.19 ± 0.02	0.35 ± 0.03	0.06 ± 0.00	0.68 ± 0.05	0.14 ± 0.04	0.05 ± 0.00	0.42 ± 0.07	7.66 ± 0.32	0.08 ± 0.01	0.12 ± 0.02	1.39 ± 0.20
		mitotane 10 µM	0.48 ± 0.27	0.70 ± 0.25	0.54 ± 0.24	0.59 ± 0.26	0.45 ± 0.25	0.61 ± 0.14	0.42 ± 0.30	0.65 ± 0.29	0.72 ± 0.24	0.67 ± 0.25	0.72 ± 0.28	0.69 ± 0.47	1.05 ± 0.23
		rofecoxib 10 µM	1.20 ± 0.21	1.06 ± 0.21	1.21 ± 0.25	1.54 ± 0.35	1.80 ± 0.35	1.07 ± 0.12	1.65 ± 0.51	1.24 ± 0.20	1.14 ± 0.22	1.06 ± 0.24	0.92 ± 0.13	0.78 ± 0.20	1.68 ± 0.35
		sotalol 5 µM	0.70 ± 0.20	0.80 ± 0.31	0.76 ± 0.23	1.10 ± 0.48	1.14 ± 0.38	0.94 ± 0.15	0.99 ± 0.41	0.98 ± 0.38	0.99 ± 0.37	0.95 ± 0.40	0.95 ± 0.40	0.97 ± 0.43	1.10 ± 0.43
		triclocarban 2.5 µM	0.47 ± 0.01	0.69 ± 0.06	0.36 ± 0.01	0.64 ± 0.08	0.43 ± 0.05	0.73 ± 0.20	0.28 ± 0.05	0.52 ± 0.08	0.60 ± 0.06	0.97 ± 0.07	0.67 ± 0.11	0.55 ± 0.11	1.33 ± 0.11
		valproic acid 10 µM	1.05 ± 0.10	1.10 ± 0.13	0.95 ± 0.06	1.54 ± 0.14	1.44 ± 0.14	1.13 ± 0.02	1.43 ± 0.11	1.39 ± 0.13	1.34 ± 0.10	1.37 ± 0.17	1.40 ± 0.14	1.50 ± 0.13	1.50 ± 0.19
		yohimbe 10 µM	1.02 ± 0.23	1.18 ± 0.10	1.00 ± 0.09	1.15 ± 0.08	1.04 ± 0.06	0.66 ± 0.11	1.03 ± 0.07	1.09 ± 0.04	1.08 ± 0.05	1.15 ± 0.16	1.16 ± 0.09	1.21 ± 0.03	1.10 ± 0.15
		zidovudine 10 µM	1.10 ± 0.20	1.24 ± 0.30	1.17 ± 0.20	1.24 ± 0.35	1.13 ± 0.25	0.92 ± 0.39	1.06 ± 0.28	1.19 ± 0.32	1.13 ± 0.29	1.21 ± 0.35	1.29 ± 0.32	1.23 ± 0.33	1.16 ± 0.29
experiment 2	SC	0.1% DMSO	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.15	1.00 ± 0.03	1.00 ± 0.01	1.00 ± 0.03	1.00 ± 0.08	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.04
	MC	complete medium; t=0h	0.00 ± 0.00	0.01 ± 0.00	0.06 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.58 ± 0.00	0.00 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	1.63 ± 0.00
	RF	torcetrapib 0.3 µM	1.34 ± 0.10	1.23 ± 0.03	2.01 ± 0.18	1.76 ± 0.06	1.47 ± 0.04	23.6 ± 0.43	6.98 ± 1.18	1.14 ± 0.14	3.92 ± 0.14	1.11 ± 0.02	1.15 ± 0.03	5.60 ± 0.51	1.01 ± 0.05
	TC	octocrylene 10 µM	0.84 ± 0.10	0.69 ± 0.03	1.98 ± 0.13	1.52 ± 0.09	0.92 ± 0.01	0.31 ± 0.15	0.65 ± 0.03	0.79 ± 0.04	0.67 ± 0.03	0.61 ± 0.06	0.90 ± 0.03	0.57 ± 0.05	1.42 ± 0.04
		octyl methoxycinnamate 10 µM	0.84 ± 0.10	0.71 ± 0.05	0.85 ± 0.03	0.75 ± 0.04	0.90 ± 0.03	6.04 ± 0.74	3.28 ± 0.36	0.88 ± 0.05	2.04 ± 0.04	0.72 ± 0.06	0.84 ± 0.01	2.69 ± 0.13	0.94 ± 0.01
		acetyl tributylcitrate 10 µM	1.02 ± 0.08	1.14 ± 0.07	1.40 ± 0.10	1.43 ± 0.06	1.08 ± 0.05	2.48 ± 0.61	1.93 ± 0.34	0.93 ± 0.12	1.20 ± 0.05	1.08 ± 0.04	1.04 ± 0.02	1.64 ± 0.18	1.31 ± 0.07
		linuron 10 µM	0.93 ± 0.12	0.99 ± 0.06	0.81 ± 0.03	0.85 ± 0.03	0.91 ± 0.02	1.68 ± 0.29	1.06 ± 0.14	0.64 ± 0.09	1.07 ± 0.04	0.87 ± 0.02	0.74 ± 0.02	0.66 ± 0.10	1.01 ± 0.03
		digoxin 0.05 µM	1.20 ± 0.09	0.37 ± 0.04	2.34 ± 0.19	0.69 ± 0.07	1.31 ± 0.07	0.31 ± 0.03	0.39 ± 0.02	0.59 ± 0.04	0.34 ± 0.02	0.19 ± 0.08	0.51 ± 0.03	0.31 ± 0.02	1.37 ± 0.15
		digitoxin 0.05 µM	1.00 ± 0.01	0.26 ± 0.01	2.43 ± 0.05	0.55 ± 0.01	1.18 ± 0.06	0.22 ± 0.02	0.25 ± 0.03	0.49 ± 0.03	0.27 ± 0.01	0.16 ± 0.02	0.41 ± 0.01	0.24 ± 0.02	1.50 ± 0.05
		amiodarone 1 µM	0.81 ± 0.02	0.91 ± 0.01	0.84 ± 0.03	1.02 ± 0.00	0.76 ± 0.02	1.07 ± 0.10	1.05 ± 0.12	0.81 ± 0.04	0.99 ± 0.01	1.04 ± 0.04	0.91 ± 0.05	1.02 ± 0.00	0.92 ± 0.05
		clofazimine 1 µM	1.00 ± 0.05	0.93 ± 0.06	0.99 ± 0.04	0.94 ± 0.06	0.95 ± 0.07	0.86 ± 0.12	1.03 ± 0.07	0.94 ± 0.05	0.96 ± 0.04	0.91 ± 0.01	0.91 ± 0.05	0.93 ± 0.05	0.94 ± 0.07
		sulforaphane 5 µM	1.30 ± 0.16	0.48 ± 0.05	1.60 ± 0.18	0.60 ± 0.06	1.27 ± 0.19	0.50 ± 0.19	0.63 ± 0.05	0.77 ± 0.07	0.56 ± 0.02	0.39 ± 0.04	0.60 ± 0.08	0.46 ± 0.03	1.23 ± 0.21
		CDDO methyl ester 0.04 µM	1.36 ± 0.06	0.57 ± 0.03	1.93 ± 0.04	0.84 ± 0.04	1.08 ± 0.05	0.41 ± 0.07	0.62 ± 0.06	0.73 ± 0.05	0.59 ± 0.03	0.50 ± 0.03	0.66 ± 0.02	0.55 ± 0.02	1.33 ± 0.08

SC: solvent control; MC: medium control; RF: reference compound; TC: test compound

Fig. 4. Qualitative analysis of effects of reference and test chemicals on the H295R steroid profile. H295R cells were incubated following a medium change in complete medium for 48 h with vehicle (0.1% DMSO) or the respective reference or test compound at the indicated concentration. Changes in steroid levels were measured by LC–MS. Data are expressed as a fold change relative to the solvent control and represent mean ± SD from one (out of two) representative experiment, performed in triplicate ($n = 3$). Steroid metabolites down regulated by 1.5-fold or more are indicated in green and steroids up regulated 1.5-fold or higher are depicted in red in order to indicate trend changes. The complete medium control was taken at the start of the experiment ($t = 0$ h). RF: reference compound; TC: test compound; SC: solvent control; MC: medium control.

included to distinguish steroids produced by the cells from steroids contributed by the Nu-serum. Progesterone, testosterone and cortisol were mainly contributed by the Nu-serum. Aldosterone was present at very low levels in unstimulated cells and varied in the medium control of the two experiments, thereby affecting the fold increase upon stimulation.

Next, in a qualitative experiment, the effects of various reference compounds were analyzed. As expected, angiotensin II and forskolin both enhanced the production of corticosterone, cortisol as well as the CYP11B product 11β-hydroxyandrostenedione (Fig. 4). Forskolin additionally enhanced the levels of 17α-hydroxyprogesterone, dehydroepiandrosterone and androstenedione, in line with an overall stimulation of the steroidogenesis. Prochloraz, which besides CYP17A1 also inhibits CYP21A2 (Ohlsson et al., 2009), prevented the further metabolism of progesterone, resulting in its accumulation, and led to reduced levels of corticosteroids and adrenal androgens. As mentioned above, the metabolism of testosterone from the Nu-serum was not affected by prochloraz treatment. Etomidate, at the concentration used in the present study, was found to inhibit CYP11B1, CYP11B2 and CYP11A1 (Hahner et al., 2010), explaining the almost complete block of steroidogenesis observed. Abiraterone, a known inhibitor of CYP17A1 and 3β-HSD2 (Jarman et al., 1998; Li et al., 2012), resulted in the simultaneous increase in progesterone and decrease in adrenal androgens and corticosteroids. Formestane at 10 μM exhibited a similar inhibition pattern with the exception of enhanced pregnenolone levels. The 3β-HSD inhibitor trilostane (Cooke, 1996) led to reduced adrenal androgen production. Since one of the aims of the present study was to establish conditions to identify chemicals disrupting corticosteroid synthesis, torcetrapib was used as a positive control. Torcetrapib was initially developed as a lipid lowering drug for treatment of cardiovascular disease but failed in phase III clinical trials due to excessive production of corticosteroids (Clerc et al., 2010; Hu et al., 2009). As expected, incubation of H295R cells with torcetrapib enhanced the CYP11B1/CYP11B2 products aldosterone, corticosterone, cortisol and 11β-hydroxyandrostenedione. The steroid profiles obtained for these reference compounds should facilitate classifying effects of test chemicals that yield a similar pattern, providing initial mechanistic insight and helping to prioritize further investigations.

Numerous test compounds were then analyzed for potential disruption of adrenal steroidogenesis. Several of them did not affect the steroid profile and/or presented very moderate effects. A general reduction of steroid production was observed for triclocarban, as reported earlier (Tonoli et al., 2015), and for mitotane, although the effects were moderate. Genistein led to enhanced pregnenolone, 17α-hydroxypregnenolone and dehydroepiandrosterone, but reduced progesterone, 17α-hydroxyprogesterone, corticosteroids and 3β-HSD-dependent androgens, in line with earlier findings of an inhibition of 3β-HSD activity (Sirianni et al., 2001). Five compounds, the UV-filter octocrylene, the cardiac glycosides digoxin and digitoxin, and the Nrf2 activators sulforaphane and CDDO methyl ester, resembled the steroid profile of abiraterone, suggesting inhibition and/or down regulation of CYP17A1 as an underlying mechanism. Two compounds, the UV-filter oxtyl methoxycinnamate and the phthalate replacement compound acetyl tributylcitrate, resembled the steroid profile of torcetrapib, suggesting an induction of CYP11B1 and CYP11B2 expression and activity.

3.5. Concentration-response study of selected chemicals on steroid production and impact on gene expression

To confirm the impact on the steroidogenesis of various concentrations of three important compounds, namely octocrylene, acetyl tributylcitrate and octyl methoxycinnamate, a supplementary set of experiments was conducted. For this purpose, targeted analyses on a QqQ system were then carried out to complement the first screening and determination of the extended steroid profiles with quantitative data. The use of targeted MS analysis of 11 reference steroid compounds allowed to unambiguously identify the concentration-dependent effects of the selected compounds on the alteration of steroid production. Incubation of H295R cells in complete medium containing 10 μM of octocrylene confirmed the increased progesterone and 17α-hydroxyprogesterone levels but suggested only a weak trend decrease for the corticosteroids 11-deoxycortisol, cortisol and corticosterone (Fig. 5). Exposure of H295R cells to acetyl tributylcitrate tended to increase the synthesis of the corticosteroids corticosterone and aldosterone and increased 11-deoxycorticosterone production. As seen with torcetrapib, acetyl tributylcitrate enhanced progesterone and 17α-hydroxyprogesterone levels. Octyl methoxycinnamate enhanced corticosterone and aldosterone and tended to increase cortisol levels. Hence, these results suggest that acetyl tributylcitrate and octyl methoxycinnamate increase mineralocorticoid production at concentrations of 10 μM, while none of these steroid metabolites were altered at lower concentrations.

As the initial goal of this study was to search for chemicals enhancing corticosteroid production and thereby contributing to hyperaldosteronism and hypercortisolism, the effect of acetyl tributylcitrate and octyl methoxycinnamate on the expression of key steroidogenic genes was determined (Fig. 6). As positive control, torcetrapib led to a profound up regulation of the expression of CYP11B2 and 3β-HSD2 and a more moderate induction of CYP11B1, CYP21A2, StAR, CYP11A1 and CYP17A1. The elevated expression of 3β-HSD2 explains the increased production of progesterone and 17α-hydroxyprogesterone, whereas the enhanced CYP11B1, CYP11B2 and CYP21A2 are responsible for the observed increase in aldosterone, cortisol, corticosterone and 11β-hydroxyandrostenedione. At concentrations of 10 μM, octyl methoxycinnamate and acetyl tributylcitrate both increased CYP11B2, 3β-HSD2 and CYP21A2 expression. They also increased or tended to increase CYP11B1 expression, thus explaining the torcetrapib-like effects previously observed in the qualitative assessment on the extended steroid profile.

4. Discussion

The OECD test guideline 456 describes a steroidogenesis assay validated for using the H295R cell line in its basal state to detect chemicals affecting the levels of testosterone and estradiol as endpoints (Hecker et al., 2006; OECD, 2011). According to the test guideline, the cells are incubated with the chemicals for 48 h. Testosterone and estradiol levels are being compared between cells exposed to vehicle and cells exposed to the test chemical (relative concentration determination). The steroidogenesis inhibitor prochloraz (1 μM) and the inducer forskolin (10 μM) are suggested as relevant controls. The present study proposes several suggestions for improvement of using H295R cells to detect chemicals interfering with adrenal steroid production. Using MS-based methods an extended panel of adrenal steroids can be quantified in culture supernatants of H295R cells at the start of the experiment ($t = 0$ h) and upon incubation ($t = 48$ h), allowing distinguishing between steroids produced by the cells and steroids contributed by the serum. Additionally, comparison of the observed changes caused by a given test chemical in the steroid profile with that of reference compounds acting on specific steroidogenic enzymes can provide initial mechanistic information.

First, because H295R cells and the complete medium with 2.5% Nu-serum represent a complex biological matrix already

treatment \ hormone	progestins		mineralocorticoids			glucocorticoids		adrenal androgens			
	progesterone	17α-hydroxyp-rogesterone	11-deoxy-corticosterone	aldosterone	corticosterone	11-deoxycortisol	cortisol	dehydro-epiandrosterone	dehydro-epiandrosterone sulfate	androstenedione	testosterone
0.1% DMSO	1.41 ± 0.75	7.93 ± 0.51	14.8 ± 5.97	0.29 ± 0.14	3.61 ± 1.47	226.6 ± 71.5	22.8 ± 1.40	5.57 ± 0.78	53.2 ± 13.3	43.4 ± 7.59	3.77 ± 0.55
complete medium; t=0	1.39 ± 0.75	1.37 ± 0.85*	1.10 ± 0.72*	0.25 ± 0.17	<LLOQ	<LLOQ	14.3 ± 6.12	<LLOQ	<LLOQ	<LLOQ	6.44 ± 2.24*
torcetrapib 0.3 µM	4.00 ± 0.76*	14.4 ± 3.70*	33.4 ± 7.12*	2.45 ± 0.92*	37.0 ± 9.24*	376.2 ± 87.2*	64.0 ± 23.04*	7.17 ± 3.13	48.6 ± 26.6	54.5 ± 9.84*	4.12 ± 0.86
octocrylene 10 µM	3.20 ± 0.82*	12.2 ± 2.92*	18.0 ± 3.79	0.25 ± 0.18	2.70 ± 0.64	201.0 ± 25.5	17.5 ± 4.83	6.21 ± 3.10	50.0 ± 36.1	38.5 ± 3.54	7.01 ± 1.11*
octocrylene 5 µM	2.70 ± 0.46*	9.90 ± 0.81	19.5 ± 3.26	0.26 ± 0.22	5.12 ± 2.56	234.9 ± 22.1	20.4 ± 2.47	5.91 ± 0.46	37.4 ± 16.1	42.6 ± 4.87	5.16 ± 0.54*
octocrylene 1 µM	1.86 ± 0.51	9.77 ± 0.82	19.9 ± 2.32	0.27 ± 0.21	4.53 ± 0.17	267.8 ± 10.4	20.6 ± 4.5	6.04 ± 0.69	41.5 ± 16.8	43.6 ± 4.80	4.46 ± 0.32
octocrylene 0.5 µM	2.01 ± 0.62	10.4 ± 2.04	18.4 ± 1.76	0.27 ± 0.17	3.73 ± 0.99	277.9 ± 43.2	19.4 ± 4.3	5.86 ± 0.38	41.9 ± 16.0	47.2 ± 11.4	4.35 ± 0.30
octocrylene 0.1 µM	2.18 ± 0.77	9.90 ± 0.80	19.2 ± 3.57	0.25 ± 0.19	3.25 ± 0.76	263.7 ± 31.9	19.7 ± 3.36	5.64 ± 1.20	39.7 ± 14.4	42.4 ± 5.17	4.62 ± 0.59
octyl methoxycinnamate 10 µM	1.37 ± 0.43	7.32 ± 2.85	17.5 ± 3.98	0.87 ± 0.27*	13.5 ± 2.96*	242.6 ± 62.6	34.2 ± 5.72	6.81 ± 4.06	34.1 ± 25.6	37.4 ± 8.53	4.04 ± 0.78
octyl methoxycinnamate 5 µM	1.65 ± 0.75	8.37 ± 1.14	16.0 ± 4.41	0.30 ± 0.17	3.48 ± 0.59	249.5 ± 24.0	19.8 ± 5.48	5.63 ± 0.45	41.3 ± 18.6	40.9 ± 5.51	4.10 ± 0.81
octyl methoxycinnamate 1 µM	1.97 ± 0.75	9.27 ± 1.13	18.1 ± 4.35	0.31 ± 0.16	3.43 ± 1.14	254.3 ± 18.1	19.6 ± 3.9	5.58 ± 0.57	40.5 ± 17.4	42.1 ± 5.70	4.34 ± 0.46
octyl methoxycinnamate 0.5 µM	2.07 ± 0.74	9.88 ± 0.81	19.1 ± 4.09	0.30 ± 0.16	3.32 ± 1.04	261.2 ± 7.38	19.4 ± 4.03	5.79 ± 0.91	41.3 ± 20.1	42.6 ± 4.78	4.41 ± 0.48
octyl methoxycinnamate 0.1 µM	1.71 ± 0.46	10.0 ± 0.48	19.6 ± 4.80	0.29 ± 0.16	3.12 ± 0.80	263.2 ± 4.36	18.3 ± 6.36	5.80 ± 1.26	42.1 ± 20.6	42.1 ± 2.95	4.58 ± 0.78
acetyl tributylcitrate 10 µM	2.34 ± 1.12*	12.1 ± 3.17*	20.6 ± 4.67*	0.56 ± 0.46	6.93 ± 2.96	273.2 ± 89.3	21.8 ± 5.83	6.66 ± 0.51	37.4 ± 26.3	49.9 ± 11.8	5.82 ± 0.75*
acetyl tributylcitrate 5 µM	1.98 ± 0.89	10.9 ± 2.23*	17.9 ± 3.81	0.36 ± 0.25	3.91 ± 1.36	272.9 ± 36.4	21.9 ± 2.45	6.66 ± 0.67	42.8 ± 18.4	45.0 ± 4.46	4.47 ± 0.49
acetyl tributylcitrate 1 µM	2.03 ± 0.81	10.1 ± 0.92	18.9 ± 4.14	0.31 ± 0.17	3.63 ± 0.71	268.7 ± 26.4	19.9 ± 3.85	5.93 ± 0.82	39.7 ± 16.7	44.2 ± 3.39	4.40 ± 0.43
acetyl tributylcitrate 0.5 µM	2.08 ± 0.83	10.3 ± 0.98	19.2 ± 4.32	0.30 ± 0.16	3.60 ± 0.95	273.6 ± 32.0	19.7 ± 3.76	5.86 ± 0.73	40.7 ± 15.3	43.7 ± 3.89	4.23 ± 0.59
acetyl tributylcitrate 0.1 µM	2.25 ± 0.75	10.3 ± 0.38	19.7 ± 3.44	0.30 ± 0.16	3.45 ± 0.75	276.3 ± 18.2	20.3 ± 4.15	5.71 ± 0.83	42.9 ± 18.6	43.4 ± 6.34	4.43 ± 0.76

SC: solvent control; MC: medium control; RF: reference compound; TC: test compound

Fig. 5. Concentration-dependent effects of octocrylene, octyl methoxycinnamate and acetyl tributylcitrate on the H295R steroid profile. H295R cells were incubated following a medium change for 48 h with 0.1% DMSO (solvent control), 0.3 µM torcetrapib (reference compound) or the test compounds at the concentrations indicated. A complete medium control ($t = 0$ h) was included for comparison. Targeted quantification of steroids was performed by LC–MS. Data depicted in nM (mean ± SD; $n = 9$) were obtained from three independent experiments, each performed in triplicates. Steroid levels are shown as absolute values in nM. Values are depicted in a color code; where down regulation (>1.5-fold) is indicated in green and up regulation (>1.5-fold) in red compared to vehicle control. Shapiro–Wilk test was used to verify the normality of data.

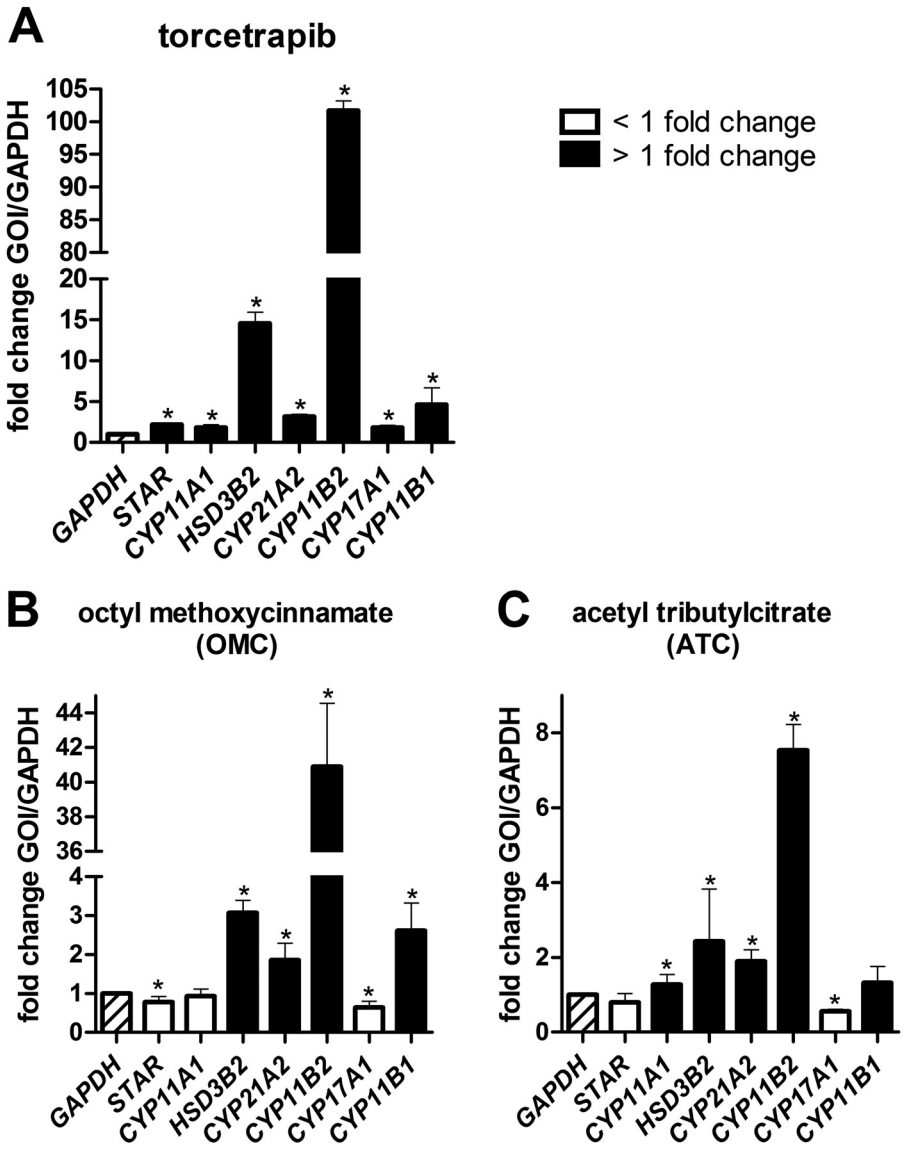


Fig. 6. Impact of torcetrapib, octyl methoxycinnamate and acetyl tributyl citrate on steroidogenic gene expression. H295R cells were incubated for 48 h with 0.3 μM torcetrapib, 10 μM octyl methoxycinnamate (OMC) or 10 μM acetyl tributyl citrate (ATC), followed by determination of the mRNA expression of steroidogenic genes of interest (GOIs). Values were normalized to glycerinaldehyde-3-phosphatedehydrogenase (GAPDH) and represent fold change over DMSO control (solid bars: up regulation; open bars: down regulation). Data, obtained from three independent experiments each performed in triplicates, are expressed as mean ± SD; $n = 9$. Differences in gene expression were evaluated using a one-sample *t*-test of the fold changes after log2 transformation. Differences with $p < 0.05$ were considered to be significant. $^{*}p < 0.05$ compared to control.

containing about 30 different steroids (Gazdar et al., 1990; Rainey et al., 1993, 1994), appropriate analytical methods, *i.e.* LC–MS and GC–MS, should be applied to obtained a sufficient analytical selectivity and the specific quantification of individual steroids. Antibody-based methods often fail to discriminate between similar steroid metabolites, resulting in an over estimation of the concentration of an individual steroid metabolite. For example, antibodies recognizing testosterone might also bind 6-, 11-, 16- and 19-hydroxylated or 5α-reduced metabolites, thus explaining the higher values obtained compared to MS-based quantification methods (Handelsman et al., 2015). The specificity analysis of commercially available antibody-based steroid quantification kits usually includes only a few steroid metabolites, thus the application of such kits should be restricted to well-defined samples. Also, a comparison of antibody-based kits revealed heterogeneity regarding recovery and linearity of steroid quantification (Buttler et al., 2013; Haisenleder et al., 2011; Handelsman and Wartofsky, 2013; Rosner et al., 2007). These issues emphasize the use of hyphenated approaches such as LC–MS and GC–MS methods for quantification of steroids, already considered as routine use determination in other important scientific fields such as doping analysis (Badoud et al., 2011). Moreover, untargeted LC–MS acquisition allows the retrospective analysis of the data without the need to reprocess the samples (Boccard et al., 2011). Such an approach constitutes therefore an appealing alternative for future developments of an extended profiling of the molecular

One-way analysis of variance (ANOVA) and Dunnett's multiple-comparison test were performed to evaluate differences between chemical treatments compared to the solvent control. Differences with $p < 0.05$ were considered to be significant. $^{*}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

actors involved in steroidogenesis, to complement the OECD reference guideline.

Second, to distinguish between changes in levels of steroids produced by the H295R cells from changes of steroids contributed by the Nu-serum, a control sample of the complete medium at the start of the experiment ($t = 0$ h) should be included. The composition of Nu-serum shows batch-dependent variations that are not defined by the vendor; besides varying concentrations of testosterone and other steroids (own observations), other components such as growth factors may show Nu-serum batch-dependent differences, which may explain some of the inter-laboratory differences of steroid values and responses reported for this cell line (LeBaron et al., 2014). The Nu-serum used in the present study contained a relatively high concentration of testosterone (6.4 nM in complete medium containing 2.5% Nu-serum), and upon incubation with the cells part of this exogenously added testosterone was metabolized by enzyme(s) that could be inhibited by prochloraz (Fig. 3A and Fig. S1), likely involving CYP enzymes. In an earlier report, Zhang et al. reported the presence of testosterone in the culture medium (1260 pg/ml, corresponding to 4.4 nM) (Zhang et al., 2011); however, in their study they observed testosterone formation in complete medium (470 pg/ml, 1.6 nM) and forskolin led to a 2.9-fold increase in testosterone production. In the present study, the H295R cells in the absence of Nu-serum produced 0.55 nM testosterone, which was increased to 0.87 nM (158%) upon forskolin treatment, in line with the OECD guideline standard for induction/inhibition of testosterone synthesis (OECD, 2011). However, the H295R cells used in this study seemed to be less responsive to forskolin and they seemed to have a higher capacity to metabolize testosterone than the cells used by Zhang et al. (2011).

A limitation of the present study includes that estrone and estradiol, generated from androstenedione and testosterone by CYP19A1, were not quantified. Nevertheless, an induction of CYP19A1 by forskolin was observed, as reported in other studies focusing on CYP19A1 expression and activity (Caron-Beaudoin et al., 2016; Sanderson et al., 2000, 2002; Zhang et al., 2011). Compared with androstenedione synthesis, the capacity of H295R cells to produce estrogens seems to be rather low. For example Zhang et al. found approximately 50% lower amounts of estradiol produced compared to testosterone (Zhang et al., 2011). The present study showed that androstenedione was synthesized by the H295R cells, reaching an estimated concentration of 39 nM after incubation for 48 h in Nu-serum free medium (Fig. 3B). This compares with the much lower levels of testosterone generated under Nu-serum free conditions (0.55 nM), indicating inefficient 17-oxoreduction, and, as indicated by the study of Zhang et al. (2011) of estradiol, thus providing an explanation why incubation with the potent CYP19A1 inhibitors letrozole and formestane did not result in an accumulation of androstenedione and testosterone upon blocking the conversion to the corresponding estrogens.

Whilst comparison of treatment with vehicle *versus* chemical allows detecting compounds that cause changes in steroid levels, the inclusion of a complete medium control ($t = 0$ h) within any experimental design is mandatory to distinguish between steroids produced by the cells and steroids from the Nu-serum that then might be metabolized by the cells, thereby providing mechanistic information. For example, in the present study in the presence of Nu-serum, treatment of cells with prochloraz resulted in higher testosterone levels (Fig. 3A), which could be misinterpreted as a testosterone inducing chemical effect if no $t = 0$ h control were included and if no comparison with cells incubated in Nu-serum-free medium would have been analyzed. Thus, the use of H295R cells in the absence of serum represents a useful alternative for testing of chemicals interfering with steroidogenesis. Nevertheless, due to the higher unbound fraction of chemicals in the absence of serum, cytotoxicity of test compounds might be higher and should be excluded under these conditions.

Third, the simultaneous quantification of a panel of progestins, adrenal androgens, glucocorticoids and mineralocorticoids can provide initial mechanistic insight into the effects of a new test chemical. Measuring a group of important steroids provides more reliable information than determination of a single steroid. For example, ratios between selected compounds could be used to improve analytical reproducibility. Upon stimulation of CYP11B1 expression, the corticosteroids corticosterone, cortisol and 11β-hydroxyandrostenedione are expected to increase, whereas the progestins progesterone, 17α-hydroxyprogesterone, pregnenolone and 17α-hydroxypregnenolone remain unchanged or tend to decrease. A modulation of adrenal androgens is indicated if dehydroepiandrosterone, its sulfated form, androstenedione and its 11β-hydroxylated form are altered. Testosterone appears not to be a reliable marker, since it is present at a substantial level in Nu-serum (Zhang et al., 2011) and can be metabolized, thereby masking the production by the H295R cells. Furthermore, inhibition of the initial steps of steroidogenesis, *i.e.* StAR or CYP11A1, is indicated by a pattern resembling that of the complete medium at the start of the experiment, thus further emphasizing the inclusion of this important control.

Fourth, the use of several reference compounds with known mechanisms allows classifying effects of new test chemicals. As shown in this study, for the UV-filter chemical octocrylene a steroid profile similar to that of abiraterone (Mangelis et al., 2016; Rijk et al., 2012), with enhanced progesterone but slightly decreased corticosteroids and adrenal androgens was observed (Fig. 4), suggesting further mechanistic studies on whether octocrylene might inhibit and/or down regulate the expression of CYP17A1 and 3β-HSD2. The toxicological relevance of the observed effects of octocrylene needs to be investigated in a follow-on study. The observed inhibitory concentration was high and reliable concentrations in exposed individuals need to be established; however, mixtures of UV-filter chemicals need also to be considered in such follow-on studies. Similarly, future experiments should investigate whether the cardiac glycosides digoxin and digitoxin and the Nrf2 activators sulforaphane and CDDO methyl ester indeed act on CYP17A1. For this purpose, the use of forskolin stimulated cells should be considered because H295R cells produce rather moderate levels of adrenal androgens and corticosteroids in their basal state. The use of stimulated cells will facilitate the identification of chemicals inhibiting different steps of steroidogenesis. Also, the use of reference inhibitor compounds such as etomidate (Hahner et al., 2010; Rijk et al., 2012; Ulleras et al., 2008) and abiraterone (Mangelis et al., 2016; Rijk et al., 2012) can be optimized to use concentrations where a more selective inhibition of CYP11B1/CYP11B2 and CYP17A1, respectively, is achieved.

Using a corticosteroid inducer such as torcetrapib as a reference compound (Clerc et al., 2010; Hu et al., 2009), the two chemicals octyl methoxycinnamate and acetyl tributylcitrate were found to have a similar pattern. A supplementary concentration-dependence experiment revealed that only the highest concentration of 10 μM showed corticosteroid inducing effects (Fig. 5). Although it seems unlikely that such high concentrations are reached *in vivo* in the adrenals, a significant contribution of this compound when present in mixtures cannot be excluded and further studies should address this important issue of potential synergistic effect between EDCs. Furthermore, torcetrapib may serve as a useful reference compound to induce corticosteroid production and search for chemicals that are associated with hypocortisolism and hypoaldosteronism by inhibiting corticosteroid production. Determination of incubation time and type of inducer is important for such studies. In a recent study, Karmaus et al. used H295R cells that

were stimulated for 48 h with forskolin prior to incubation with the test chemicals for profiling a large number of chemical effects on steroidogenesis, aiming at the categorization of action (Karmaus et al., 2016).

Finally, as shown in the present study, the steroid profile changes induced by a given chemical should ideally be confirmed or at least combined with gene expression analysis. As demonstrated for octyl methoxycinnamate and acetyl tributylcitrate, the torcetrapib-like steroid pattern with increased corticosteroids could be explained by elevated expression of CYP11B2 and 3β-HSD2 mRNA levels (Fig. 6). Thus, these compounds do not directly modulate the activity of these enzymes but rather alter their expression levels. Follow-on investigations need to show whether L-type calcium channels might be involved in the mode of action and whether the increased CYP11B2 expression is a result of enhanced activation of the nuclear receptor NR4A2 as reported for torcetrapib expressed H295R cells (Clerc et al., 2010). Often, mRNA expression does not translate into protein expression; thus, determination of protein expression and/or enzyme activity measurements can complement mRNA expression analysis.

5. Conclusion

H295R cells represent an invaluable tool for the detection of hazardous chemicals interfering with steroidogenesis. The simultaneous measurements of a panel of progestins, adrenal androgens, glucocorticoids and mineralocorticoids by separation techniques hyphenated to MS such as LC–MS or GC–MS, as well as inclusion of a complete medium control at the start of the experiment allow identifying chemicals altering adrenal steroid production and provide initial mechanistic insight into the effects of such chemicals. Comparison with the steroid profiles of suitable reference compounds further allows classifying new test chemicals, thereby facilitating the prioritization of follow-on *in vitro* and *in vivo* experiments.

The results of the test chemicals suggest that the UV-filter octocrylene, the cardiac glycosides digoxin and digitoxin and the Nrf2 activators sulforaphane and CDDO methyl ester affect steroidogenesis by inhibiting or down regulating CYP17A1. Further, octyl methoxycinnamate and acetyl tributylcitrate increase corticosteroid production *via* induction of CYP11B2 and 3β-HSD2 expression. Further studies need to address the toxicological relevance of these observations. Finally, additional investigations of the untargeted steroid profiles will be carried out to extend the number of potential biomarkers and offer a more complete picture of the biochemical events resulting from H295R exposure to possible EDCs.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

The http://dx.doi.org/10.1016/j.tox.2017.02.010 associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2017.02.010.

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4.4 Achieved knowledge and future perspectives

The first goal of this thesis was to provide a critical overview of the current cell lines available to test for potential endocrine disruptors (published review paper “Disruption of steroidogenesis: Cell models for mechanistic investigations and as screening tools”), as well as addressing their advantages/disadvantages. We then outlined recommendations for improvements in the H295R steroidogenesis assay. My work then specifically focused on experimentally addressing the key issues raised in the review with respect to the OECD/EPA steroidogenesis assay. This resulted in the establishment of the refined version of the H295R steroidogenesis assay compared to the currently used protocols published by the OECD and the EPA.

Briefly, the five major improvements in the usage of the H295R cell system and the current guidelines, as detailed in the published manuscript “Steroid profiling in H295R cells to identify chemicals potentially disrupting the production of adrenal steroids”, include: 1) monitoring of an enlarged adrenal steroid profile, 2) exclusive hormone analysis by separation techniques combined with MS, 3) inclusion of the medium composition at the starting time of an experiment leading to an improved data interpretation as well as 4) simultaneous testing of reference compound with a known mode of action to further classify the effect of the test compounds, and 5) performance of gene expression investigations indicating first mechanistic hints of changes in steroid profiles.

As previously mentioned, humans may be exposed to endocrine disruptors from numerous sources such as consumer products (cosmetics and UV-filters used in sun creams), agricultural and industrial chemicals, plant constituents, food additives, synthetic hormones and designer drugs [20]. For chemicals where no clinical trial or *in vivo* studies [58] can be performed, such as for cosmetics, the *in vitro* cell systems are the only biological tool available for investigative screening. Based on evidence from the literature, I selected 31 reference and test compounds based on their endocrine disrupting properties and tested for their potential to disturb the key adrenal steroids in the enhanced H295R steroidogenesis assay.

In addition, concentration-dependent experiments with the UV-filters octocrylene (OC), octyl methoxycinnamate (OMC) and the plasticizer acetyl tributylcitrate (ATC) were conducted, revealing significant changes in hormone production only at the highest test concentration (10 µM). These findings and its toxicological relevance need to be further assessed. However, it remains unclear at which concentration a chemical should be tested. Certainly, the research question mainly influences the chosen test concentration. For screening approaches, high concentrations (without cytotoxic effect) are frequently used to identify hits [59-61]. Subsequently, these chemicals can then be selected for concentration-dependent experiments to help identify the concentrations by which they can affect enzymes involved in steroidogenesis. This is complex, since a chemicals can selectively affect its target enzyme at low concentrations, whereas at high concentrations can affect multiple enzymes in steroidogenesis [59]. For example, etomidate inhibits CYP11B1 at low concentrations (IC_{50} 15 nM), whilst higher concentrations (IC_{50} 400 nM) also blocked CYP11A1 [62]. Likewise, the enzymes CYP17A1 (EC_{50} values in the range 0.26–0.82 µM) and CYP11A1 (EC_{50} value of 1.60 µM) exert a concentration dependent sensitivity towards

ketoconazole treatment [63]. It is essential to consider, that mixtures of low potent endocrine disruptors can also lead to considerable changes in steroid profiles. Importantly, in the risk assessment, concentrations close to the predicted human exposure should be evaluated. However, predicting exposure in humans is complex and influenced by numerous factors such as different concentration in biofluids, tissue distribution, accumulation, population heterogeneity (age, gender, state of health, nutrition, and previous exposures), route of chemical exposure (oral, dermal, inhalation), compound metabolism, external measurements (levels in water, food), and collecting/analysis design [20].

Studies often combine steroid hormone measurements with gene expression of selected steroidogenic enzymes [22, 63-66] providing initial mechanistic hints of how the chemicals can affect steroidogenesis. The enzymes involved in steroidogenesis are considered as essential targets for adrenocortical endocrine disruptors [59]. We could show that treatment with ATC (10 µM) and OMC (10 µM), similar to torcetrapib (0.3 µM), upregulated the expression of CYP11B2, CYP11B1 and CYP21A2, consistent with the increase of corticosterone and aldosterone. Nevertheless, it was reported that there is not always a direct relationship between changes in steroid production and gene expression [66]. The mRNA levels determined by RT-qPCR offer a robust method to investigate gene expression [67], however when possible, should be supplemented with the protein expression and enzyme activity analysis. In our screening approach using the H295R cells, treatment with cardiac glycosides (digoxin, digitoxin), UV-filter (octocrylene) and Nrf2 activators (sulforaphane and CDDO methyl ester) results in similar steroid profile changes as seen with the CYP17A1 inhibitor abiraterone. Therefore, it would be interesting to investigate the effect of these chemicals on the mRNA, protein and activity levels.

The use of stimulated cell systems would favor the identification of adrenocortical endocrine disruption [59], which may be missed in the basal state, where the H295R produces only low levels of corticosteroids and adrenal androgens. For cell system stimulation, we suggest using torcetrapib or forskolin. Torcetrapib shows a profound stimulation of corticosteroids and forskolin additionally stimulates adrenal androgens. Furthermore, it would be helpful to test potential adrenocortical endocrine disruptors using an *in silico* approach, thus increasing the positive hit rate and potentially reducing cell based testing.

Our refined H295R steroidogenesis assay needs further analytical improvements by including estradiol, estrone, and the 11-hydroxylated adrenal androgen metabolites 11β-hydroxytestosterone and 11β-hydroxyandrostenedione in the hormone analysis. The strategy of increasing the numbers of steroidogenic analytes in this assay will help to delineate the effects of chemicals on adrenal steroidogenesis.

In our studies, we observed a batch-to-batch variability in steroid concentrations derived from the Nu-serum, which is a medium additive used in the H295R steroidogenesis assay. To avoid misinterpretation in hormone changes, the medium composition (with or without Nu-serum, as well as its exact steroid content) has to be strictly defined prior experimental usage.

It would also be interesting to investigate chemically induced time dependent disruption of steroidogenesis, as shown by others [68, 69]. This may lead to the identification of time-dependent sensitive steroids, which

may be used as markers to delineate different steroidogenic pathways, similar to the approach described by Tonoli et al. on concentration dependent sensitivity [41]. The H295R cell line could also be used to investigate receptor-mediated effects. For example, a study in the H295R cells showed the glucocorticoid receptor, regulated steroid production by an autocrine positive feedback loop [70].

In the past, a major limitation of the H295R cell line was its poor response to the most potent physiological modulator of steroidogenesis ACTH [70, 71]. This was overcome following treatment of forskolin or cAMP analogues [72] or by using H295RA, an ACTH-responsive human adrenocortical cell line, recently developed by genetic manipulation of the H295R cell line. ACTH stimulation of H295RA cells results in an increase of the major adrenal steroids [73]. However, the H295R steroidogenesis assay is not a useful tool to comprehensively study chemicals affecting the HPA. Currently, assays to assess the disruption of HPA by endocrine disruptors are, due to its high complexity, regulation, and numerous potential targets, poorly developed and no approved regulatory protocols are available [74]. Therefore, *in vivo* studies are unavoidable. In rodent studies, HPA disturbance can be identified by corticosterone and ACTH measurements [74]. Moreover, an increased ACTH stimulation of the adrenal gland can be observed by adrenocortical hypertrophy. Here, it is important to distinguish between stress related changes from direct adrenal toxicity (adrenocortical steroidogenesis inhibition) [45].

The H295R steroidogenesis assay is not an appropriate assay to study the effect of chemicals on the circadian rhythm of adrenal glucocorticoids, as it lacks a HPA regulation and feedback mechanism. Nevertheless, a peripheral clock system in H295R cells was demonstrated, where the glucocorticoids affect the periodic oscillations of clock genes [75]. This is consistent with other reports, where an adrenal intrinsic mechanism is suggested in the local adrenal clockwork [17]. However, the H295R cell line is not suitable to study circadian rhythm on the steroid levels, based on our time-dependent steroid synthesis experiments in H295R cells, where no oscillations in steroid levels were observed.

Furthermore, the H295R can be used to study signaling mechanisms involved in steroidogenesis pathways. For example, Krug et al. addressed the association between body weight and inadequately increased aldosterone levels. By using the H295R cells they showed that adipokines can directly stimulate aldosterone secretion, mediated via ERK1/2-dependent upregulation of StAR [69]. In addition, complex steroidogenic interactions can be studied by using a co-culture model. For instance, H295R co-cultured with an estrogen receptor-positive breast cancer cell line, such as MCF7 [76], can be used to investigate aromatase inhibitors on hormone dependent breast cancer cell proliferation [77]. Moreover, co-cultures of H295R cells and the human choriocarcinoma BeWo cells provide an *in vitro* model to investigate the steroidogenic interactions between placenta and fetus, thereby enabling to screen for potential endocrine disrupting chemicals during pregnancy [78]. In our studies, the H295R cells express the enzyme AKR1C3 but not 17β-HSD3 (both enzymes catalyze the conversion from androstenedione to testosterone). However, we demonstrated that H295R cells only have minor testosterone production capacity. This could be overcome with stable transfection of human 17β-HSD3 in H295R cells.

Currently, no human adrenocortical zone specific cell lines are available. Due to the low prevalence of adrenocortical carcinoma, which can affect every single zone in the adrenal cortex in humans [79], isolating primary cells from zone specific adrenocortical carcinoma is challenging. Nevertheless, combinations of steroidogenic inducers or inhibitors in the H295R cell line could be tested to mimic zone specific steroid expression patterns. For instance, exposure of H295R to the 3β-HSD inhibitor trilostane [80] could block forskolin induced glucocorticoid elevation, favoring the production of adrenal androgens, as seen in the *zona reticularis*. The steroid pattern of the *zona fasciculata* could be replicated in H295R cells with the combined treatment of the aldosterone inducing angiotensin II [81] and CYP17 inhibitor abiraterone [80] preventing the production of glucocorticoids and adrenal androgens. In addition to their utility in studies of adrenal steroidogenesis, the H295R cell line can be a useful tumor model for studying adrenocortical carcinoma and for *in vitro* screening of chemotherapeutic agents [51, 82]. Due to experimental limitations, such as short life span and lack of proliferation, possible contamination with non-steroidogenic cells, or donor dependent variability, primary cultures from adrenal cortex [83] are not the preferred *in vitro* model to screen for chemicals potentially disrupting the production of adrenal steroids [84].

In general, it remains challenging to translate the information obtain from the H295R assay to the *in vivo* situation. The H295R assay, due to its limited incubation time with a chemical, cannot mimic the repeated acute or chronic exposure as seen in humans. Moreover, it is also complex, to incorporate data obtained from the H295R steroidogenesis assay into animal studies, due to important species differences. For example, the main glucocorticoid in rodents (due to lack in CYP17) is corticosterone whereas in humans it is cortisol [45]. Furthermore, the human adrenals produce high amounts of adrenal androgens, whereas mouse and rat adrenals produce very low amounts of androgens [85].

In contrast, as shown previously in the case of “torcetrapib” [49, 50], the H295R cell line can be an excellent tool to study the underlying mechanisms of action of clinically manifested endocrine disrupting effects.

In conclusion, the improved H295R steroidogenesis assay described in this thesis, is an important *in vitro* tool to investigate adrenocortical disruptors. First mechanistic hints of chemically disrupted steroidogenesis can be observed by linking the changes in steroid hormone levels to gene expression of the steroidogenic enzymes.

5. *In vivo* investigations of xenobiotics affecting human steroid homeostasis

5.1 Acute effects of psychoactive drugs on steroids in healthy volunteers

Human steroidogenesis can be affected by many drugs. As a consequence, the disruption of steroid homeostasis can either be clinically intended, as seen with the CYP19A1 inhibitor formestane in the treatment of breast cancer [86] or it can be a result of an off-target effect, for example in case of the anesthetic agent etomidate that induced fatal adrenocortical insufficiency due to CYP11B1 inhibition [47]. Previously, it has been reported that many psychoactive substances, such as 3,4-methylenedioxymethamphetamine (MDMA) and cocaine, increase the level of glucocorticoids by activating the hypothalamic-pituitary-adrenal (HPA) axis [4, 87-89]. A perturbation of the glucocorticoid circadian rhythm is associated with learning, memory and behavioral deficits, mood disorders, impaired immune system, and development of metabolic syndrome [17, 19, 20, 90, 91]. Since there was limited data in the literature monitoring the effects of psychotropic drugs on circulating steroids, we sought to close this knowledge gap with respect to the psychoactive drugs lysergic acid diethylamide (LSD), lisdexamfetamine, and D-amphetamine.

LSD is a direct serotonin agonist [92, 93], which is used recreationally or in psychiatric research [94, 95]. Animal studies showed that LSD affects steroid homeostasis. Upon LSD administration, the 17-hydroxy-corticosteroide and 17-ketosteroid urine levels in rats were increased, and in zebrafish the levels of cortisol were augmented [96, 97]. In humans, LSD led to increases in 17-ketosteroid levels in urine [98, 99]. In a more recent study [94], which was a double-blind, placebo-controlled, cross-over clinical trial in 16 healthy volunteers, it was shown, that LSD significantly increases plasma cortisol levels, indicating HPA axis activation. In the same study, cortisol levels were measured up to 3 h after LSD administration [94] in order to study the stress response. In our study, we aimed to further characterize the full steroid profile of the same volunteers following LSD exposure over a 24 hour time course. Our new analysis included glucocorticoids, mineralocorticoids, progestins, and androgens. These results were published in the paper “Acute Effects of Lysergic Acid Diethylamide on Circulating Steroid Levels in Healthy Subjects”.

D-amphetamine, is a psychostimulant [100, 101] used in the clinic to treat attention deficit hyperactivity disorder (ADHD) or is a substance of recreational abuse [102]. D-amphetamine increases the levels of norepinephrine (NE) and dopamine (DA) in the brain, by releasing NE and DA in the synaptic gap, and by inhibiting their corresponding transporters NET and DAT [100, 103]. Lisdexamfetamine is D-amphetamine covalently bound to the amino acid L-lysine [104-106]. In the blood circulation, lisdexamfetamine has first to be hydrolyzed in the erythrocytes to become the active D-amphetamine (for the rest of the text, I will refer to the D-amphetamine measured in the plasma after lisdexamfetamine or D-amphetamine administration as ‘amphetamine’) [107, 108]. Lisdexamfetamine has been marketed in the USA (Vyvanse®) and

Switzerland (Elvanse®) since 2007 and 2014, respectively. In the USA it has been clinically used to treat ADHD and binge-eating disorder [109], and in Switzerland for the treatment of ADHD in patients not responding to methylphenidate [110]. The activation step for lisdexamfetamine, results in altered pharmacokinetics of amphetamine compared to the administration of immediate-release D-amphetamine. For example, amphetamine has a lower maximum concentration (C_{max}) and a prolonged t_{max} after lisdexamfetamine compared to immediate-release D-amphetamine administration. However, the total plasma exposure (AUC) of amphetamine after lisdexamfetamine is similar to D-amphetamine at equimolar doses [105, 111-113]. Importantly, the appearance of amphetamine in the brain is delayed after lisdexamfetamine [105], which is critical for its market ability, since it reduces abuse potential and extends the length of therapeutic effect compared to D-amphetamine [104, 105, 111]. Furthermore, it is well documented that D-amphetamine administration increases cortisol concentrations [101, 114-119], but the effect on other steroids remains unclear. We first, compared the effects of lisdexamfetamine and immediate-release D-amphetamine at equimolar doses on multiple steroids, such as corticosteroids, progestins and androgens. Second, we tested whether lisdexamfetamine, due to its altered pharmacokinetic profile, shows an attenuated endocrine response compared to D-amphetamine administration. Additionally, we monitored the subjective effects and vital signs after lisdexamfetamine and D-amphetamine administration. Therefore, we set up a randomized, double-blind, placebo-controlled, cross-over study, where 24 healthy volunteers (12 women, 12 men) were orally administered with single oral doses of either lisdexamfetamine dimesylate (100 mg), D-amphetamine sulfate (40.3 mg), and placebo in three experimental sessions. The results of this study are described in detail in the paper draft “Acute effects of D-amphetamine and lisdexamfetamine on plasma steroid concentrations in healthy subjects” and the submitted manuscript “Pharmacokinetics and pharmacodynamics of lisdexamfetamine compared with D-amphetamine in healthy subjects”.

5.2 Published paper: Acute effects of lysergic acid diethylamide on circulating steroid levels in healthy subjects

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ORIGINAL ARTICLE

Acute Effects of Lysergic Acid Diethylamide on Circulating Steroid Levels in Healthy Subjects

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Lysergic acid diethylamide (LSD) is a serotonin 5-hydroxytryptamine-2A (5-HT_{2A}) receptor agonist that is used recreationally worldwide. Interest in LSD research in humans waned after the 1970s, although the use of LSD in psychiatric research and practice has recently gained increasing attention. LSD produces pronounced acute psychedelic effects, although its influence on plasma steroid levels over time has not yet been characterised in humans. The effects of LSD (200 μg) or placebo on plasma steroid levels were investigated in 16 healthy subjects using a randomised, double-blind, placebo-controlled, cross-over study design. Plasma concentration–time profiles were determined for 15 steroids using liquid-chromatography tandem mass-spectrometry. LSD increased plasma concentrations of the glucocorticoids cortisol, cortisone, corticosterone and 11-dehydrocorticosterone compared to placebo. The mean maximum concentration of LSD was reached at 1.7 h. Mean peak psychedelic effects were reached at 2.4 h, with significant alterations in mental state from 0.5 h to > 10 h. Mean maximal concentrations of cortisol and corticosterone were reached at 2.5 h and 1.9 h, and significant elevations were observed 1.5–6 h and 1–3 h after drug administration, respectively. LSD also significantly increased plasma concentrations of the androgen dehydroepiandrosterone but not other androgens, progestogens or mineralocorticoids compared to placebo. A close relationship was found between plasma LSD concentrations and changes in plasma cortisol and corticosterone and the psychotropic response to LSD, and no clockwise hysteresis was observed. In conclusion, LSD produces significant acute effects on circulating steroids, especially glucocorticoids. LSD-induced changes in circulating glucocorticoids were associated with plasma LSD concentrations over time and showed no acute pharmacological tolerance.

Key words: lysergic acid diethylamide, serotonin, steroid, glucocorticoid

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Lysergic acid diethylamide (LSD) was discovered in 1943 and is the prototypic serotonergic hallucinogen (1,2). LSD was used in psychiatric research in the 1950s to 1970s to study psychotic-like states (i.e. model psychosis) and as an adjunct to psychotherapy (1) before its widespread recreational use. Today, LSD is still frequently used for personal and spiritual purposes. Additionally, renewed interest has been seen in the use of LSD in psychiatric research (3) and practice (4). Pharmacologically, LSD mainly acts as an agonist at serotonin 5-hydroxytryptamine-1 (5-HT_1) and 5-HT_2 receptors, although it also interacts with dopamine D_1, D_2 and D_3 receptors and adrenergic α_1 receptors (2). By contrast to stimulants such as amphetamines or cocaine, LSD does not interact with monoamine transporters (2). In humans, LSD induces alterations in perception, methylenedioxymethamphetamine (MDMA)-like empathogenic mood effects and moderate sympathomimetic stimulation (3).

Many psychoactive substances activate the hypothalamic-pituitary-adrenal (HPA) axis, leading to the release of adrenocorticotrophic hormone (ACTH) and glucocorticoids (5,6). However, limited data have been reported on the effects of LSD on the HPA axis. In rats, LSD increased 17-hydroxy-ketosteroid and 17-ketosteroid levels in urine, which is consistent with HPA axis activation (7), although effects on circulating corticosterone could not be shown

(8). LSD was reported to increase cortisol levels in zebrafish (9). Early studies in humans found that LSD increased 17-ketosteroid excretion in urine (10,11), although effects on circulating steroid levels were not investigated. LSD also blunted the normal increase in 17-ketosteroid after ACTH administration (10). We recently found that LSD significantly increased plasma cortisol 180 min after LSD administration in humans (3), which is also consistent with HPA axis activation. However, we previously determined the concentrations of cortisol only (and not of other steroids) and only up to 180 min (3) despite the much longer effects of LSD. A more comprehensive analysis of the effects of LSD on circulating levels of different steroids and including full time courses is still missing.

Corticosteroids, androgens and progestogens may all contribute to or modulate psychotropic drug actions (12,13). For example, amphetamine or MDMA-induced increases in plasma cortisol levels were associated with subjective drug effects (14,15) and stress-induced increases in plasma cortisol levels correlated with euphoric responses to amphetamine (16). Testosterone plays a role in social behaviour (17) that is enhanced by MDMA (18). Testosterone and progesterone both reduced cocaine self-administration in female rhesus monkeys (19) and progesterone is known to be associated with reductions of subjective responses to and the use of psychostimulants in women (13,20,21). Plasma dehydroepiandrosterone (DHEA) levels correlated with the subjective response to MDMA (14). Increases in DHEA and progesterone were also observed after γ-hydroxybutyrate administration (22).

Glucocorticoids are involved in the stress response and the modulation of behaviour. In humans, inactive cortisone and active cortisol are the main glucocorticoids (23). Inactive 11-dehydrocorticostrone and active corticosterone (i.e. the major glucocorticoids in rodents) are present at lower concentrations than cortisone and cortisol in human plasma. However, corticosterone, which also has additional mineralocorticoid properties, presents a higher brain/plasma concentration ratio than cortisol (24). Mineralocorticoids are involved in the regulation of sodium absorption and blood pressure (25) and they also play a role in modulating the immune response (26). 11-Deoxycortisol is a precursor of cortisol (27). Aldosterone is the most important mineralocorticoid. 11-Deoxycorticosterone is a precursor of corticosterone and aldosterone and has mineralocorticoid activity (28).

To characterise the influence of LSD on plasma steroid levels, we newly evaluated the acute effects of LSD on the plasma concentrations of a series of steroids over 24 h in healthy subjects. We also explored the effects of LSD on a wide range of other steroids not previously measured. The plasma LSD concentration–steroid effect response curves over time were also plotted and compared with the LSD exposure-psychotropic effect relationship. The psychotropic effects of LSD were reported previously (3) and selected effects were included here to determine associations with steroid levels.

Materials and methods

Study design

The present study used a double-blind, placebo-controlled, cross-over design with two experimental test sessions in balanced order. The washout periods between sessions were at least 7 days. The study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Guidelines in Good Clinical Practice and was approved by the Ethics Committee of the Canton of Basel, Switzerland, and the Swiss Agency for Therapeutic Products (Swissmedic). The administration of LSD to healthy subjects was authorised by the Swiss Federal Office for Public Health, Bern, Switzerland. The study was registered at ClinicalTrials.gov (NCT01878942). All of the subjects provided their written informed consent after being given written and oral descriptions of the study, the procedures involved, and the effects and possible risks of LSD administration.

Participants

Sixteen healthy subjects (eight men and eight women; mean ± SD age : 28.6 ± 6.2 years; range 25–51 years) were included. The exclusion criteria were pregnancy, personal or family (first-degree relative) history of psychotic or major affective disorder, regular use of medications, chronic or acute physical illness, lifetime prevalence of illicit drug use > 10 times (except for tetrahydrocannabinol), illicit drug use within the last 2 months and illicit drug use during the study as reported in detail elsewhere (3). The subjects were asked to abstain from excessive alcohol consumption between test sessions and particularly to limit their use to one drink on the day before the test sessions. Additionally, the participants were not allowed to drink caffeine-containing liquids after midnight before the study day. Three subjects were light smokers (< 10 cigarettes/day) and were told to maintain their usual smoking habits but not to smoke during the sessions. We performed urine drug tests at screening and before each test session using TRIAGE 8 (Biosite, San Diego, CA, USA). Safety recommendations for high-dose hallucinogen research were followed (29).

Study procedures

The test sessions began at 08.00 h. A urine sample was taken to confirm abstinence from drugs of abuse, and a pregnancy test was performed in women. An indwelling intravenous catheter was placed in an antecubital vein for blood sampling and the subjects completed baseline measurements. A single dose of LSD (200 μg) or placebo was administered orally at 09.00 h. A standardised lunch and dinner were served at 13.30 h and 17.30 h, respectively. The subjects were sent home the next day at 09.30 h after the 24 h blood sample collection. The sessions were conducted in a calm laboratory environment. The subjects did not engage in any physical activity and were resting in hospital beds during the test session. Blood samples for the analysis of plasma steroid hormone levels were collected in lithium heparin tubes 1 h before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 24 h after LSD or placebo administration. Blood samples were immediately centrifuged, and plasma was rapidly stored at −20 °C. Plasma LSD concentrations were determined using liquid-chromatography tandem mass-spectrometry (LC-MS/MS) (30). The pharmacokinetics of LSD have been reported previously (31) and LSD concentrations are included here to describe the LSD exposure–steroid response effect relationship. The subjective and autonomic effects of LSD were also recorded in the present study and have been reported previously (3). The subjective effects of LSD over time were repeatedly recorded at the times of blood sampling using visual analogue scales (VAS) as reported previously (3,31). VAS items included 'any subjective drug effects', (reflecting the overall subjective response in a single scale), 'good drug effects' 'bad drug effects', 'fear' and 'stimulation'. VAS items were presented as 100-mm horizontal lines (0–100%) marked 'not at all' on the left and 'extremely' on the right (3,32).

Steroid quantification

Plasma steroid hormone levels [cortisol, cortisone, corticosterone, 11-dehydrocorticosterone, 11-deoxycorticosterone, aldosterone, DHEA, DHEA sul-

phate (DHEAS), Δ4-androstene-3,17-dione (androstenedione), testosterone, 11-deoxycortisol, progesterone, 5α-dihydrotestosterone, androsterone and 17α-hydroxyprogesterone] were determined as described previously with minor adaptations (6). A detailed description of the materials, procedure, and method validation is included in the Supporting information supplemental methods and Tables S1–S5.

Briefly, for solid-phase extraction, 700 μl of each plasma sample was mixed with 100 μl of protein precipitation solution (0.8 M zinc sulphate in water/methanol; 50/50, v/v) that contained deuterium-labeled aldosterone, corticosterone, androstenedione, androsterone, 5α-dihydrotestosterone and testosterone as internal standards, and diluted to a final volume of 1 ml with water. The samples were incubated in a thermoshaker for 10 min at 4 °C with thorough shaking (1300 rotations/min). The samples were then centrifuged for 10 min at 16 000 *g* at 4 °C, and 700 μl of the supernatants was transferred to Oasis HBL SPE cartridges (Waters, Milford, MA, USA), preconditioned with methanol and water. After washing once with 1 ml of water and twice with 1 ml of methanol/water (10/90, v/v), the steroids were eluted with 1 ml of methanol and evaporated to dryness. The samples were then reconstituted in 25 μl of methanol. The steroids were separated and quantified by ultra-pressure LC-MS/MS (UPLC-MS/MS) using an Agilent 1290 UPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer equipped with a jet-stream electrospray ionisation interface (Agilent Technologies, Santa Clara, CA, USA). Analyte separation was achieved using a reverse-phase column (1.7 μm, 2.1 × 150 mm; Acquity UPLC BEH C18; Waters). MASSHUNTER software (Agilent Technologies) was used for data acquisition and analysis. As described in detail in the Supporting information (Table S5), the variation coefficient was < 15% and accuracy was between 85% and 115% tested at three concentrations for all analytes. The recovery of control samples was in the range 80–120%.

Drugs

Gelatin capsules that contained 100 μg of LSD (D-lysergic acid diethylamide hydrate; Lipomed AG, Arlesheim, Switzerland) and corresponding placebo capsules were prepared with authorisation from the Swiss Federal Office for Public Health. LSD was administered in a single absolute dose of 200 μg, corresponding to a dose of 2.8 ± 0.1 μg/kg body weight (mean ± SEM). The same dose was previously used in LSD-assisted psychotherapy in a clinical study (4). The dose was in the upper range of doses that are taken for recreational purposes and is expected to induce robust effects in humans (1).

Statistical analysis

To determine differences between LSD and placebo, maximum concentration (C_{max}) values and areas under the concentration–time curve (AUCs) were compared for each steroid using repeated-measures ANOVA, with drug (LSD versus placebo) as the within-subject factor. Sex differences were determined by including sex (male versus female) as a between-subject factor in the ANOVA. To test how long the subjective and endocrine responses last over time, data were also analysed using two-way ANOVAs with drug and time as factors and Tukey's test was used for post-hoc comparisons between corresponding time points. C_{max} was determined directly from the concentration–time curves. AUC values were determined from time 0.5 h to 10 h (AUC_{10}) using the trapezoidal method. The LSD exposure–steroid concentration response relationships were explored by plotting the LSD response as a function of steroid concentration after LSD administration minus the individual time-matched concentration after placebo as a function of LSD plasma concentrations at each time point (hysteresis curves). Correlations between mean LSD concentrations and mean LSD-induced subjective (five scales) or endocrine responses (cortisol and corticosterone) over time and correlations between subjective and endocrine responses over time (n = 12 time points) within the 16 subjects were then analysed using Spearman's rank correlations. $P < 0.05$ was considered statistcally significant. Seventeen correlations were tested, giving a Bonferroni-corrected statistical threshold of $P < 0.003$. The statistical analyses were performed using STATISTICA, version 12 (StatSoft, Tulsa, OK, USA).

Results

The plasma concentration–time curves of the different steroid hormones after LSD and placebo administration are shown in Figs 1 and 2. Peak steroid concentrations, total steroid exposure over time (AUC_{10} values) and statistics are presented in Table 1. LSD significantly increased the plasma concentrations of the glucocorticoids cortisol, cortisone, corticosterone and 11-dehydrocorticosterone compared to placebo (Fig. 1B–E). LSD also significantly increased the sums of cortisol + cortisone and corticosterone + 11-dehydrocorticosterone and the cortisol/cortisone and corticosterone/11-dehydrocorticosterone ratios (Table 1), indicating elevated glucocorticoid production. LSD had no effect on plasma concentrations of the cortisol precursor 11-deoxycortisol (Fig. 1A), the mineralocorticoid aldosterone (Fig. 1G) or the moderate mineralocorticoid 11-deoxycorticosteone (Fig. 1F). LSD significantly increased plasma concentrations of DHEA compared to placebo (Fig. 2A) and also increased the plasma exposure (AUC_{10} but not C_{max}) of androstenedione compared to placebo (Fig. 2C). By contrast, LSD did not alter plasma concentrations of the androgens DHEAS (Fig. 2B), testosterone (Fig. 2D,F), 5α-dihydrotestosterone or androsterone (Table 1). Similarly, LSD had no effect on plasma concentrations of the progestogens progesterone (Fig. 2G) and 17α-hydroxyprogesterone (Fig. 2E). As expected, testosterone levels were higher in men than in women, although no sex differences in testosterone levels in response to LSD were found compared to placebo. Similarly, no other drug and sex interaction effects on any of the steroid levels were observed.

LSD exposure–steroid concentration response relationships are shown in Fig. 3. Pharmacokinetic data on LSD from the present study have been reported in detail elsewhere (31). The C_{max} of LSD was reached 1.7 ± 1 h (mean ± SD) after LSD administration (Fig. 3A,B). The peak psychotropic effect was reached at 2.4 ± 0.8 h, with significant alterations in mental state from 0.5 to 10 h after LSD administration (Fig. 3A,B). Maximum concentrations of cortisol (Fig. 3C,D) and corticosterone (Fig. 3E,F) were reached at 2.5 ± 0.8 h and 1.9 ± 0.5 h (mean ± SD), and significant elevations were observed 1.5–6 h and 1–3 h after LSD administration, respectively. Thus, plasma levels of corticosterone increased more rapidly and fell more rapidly back to baseline levels compared to cortisol levels (Fig. 3E,F). Counterclockwise hysteresis was observed for subjective 'any drug effects' and cortisol, which is consistent with an initial delay between increases in plasma LSD concentration and drug effects that was attributable to drug absorption/distribution up to 2.5 h (Fig. 3B,D). After maximal drug effects were reached at 2.5 h, the psychotropic effects and changes in plasma cortisol levels decreased slowly, paralleling the steady decrease in the plasma levels of LSD (Fig. 3A,C) and presenting a close concentra-

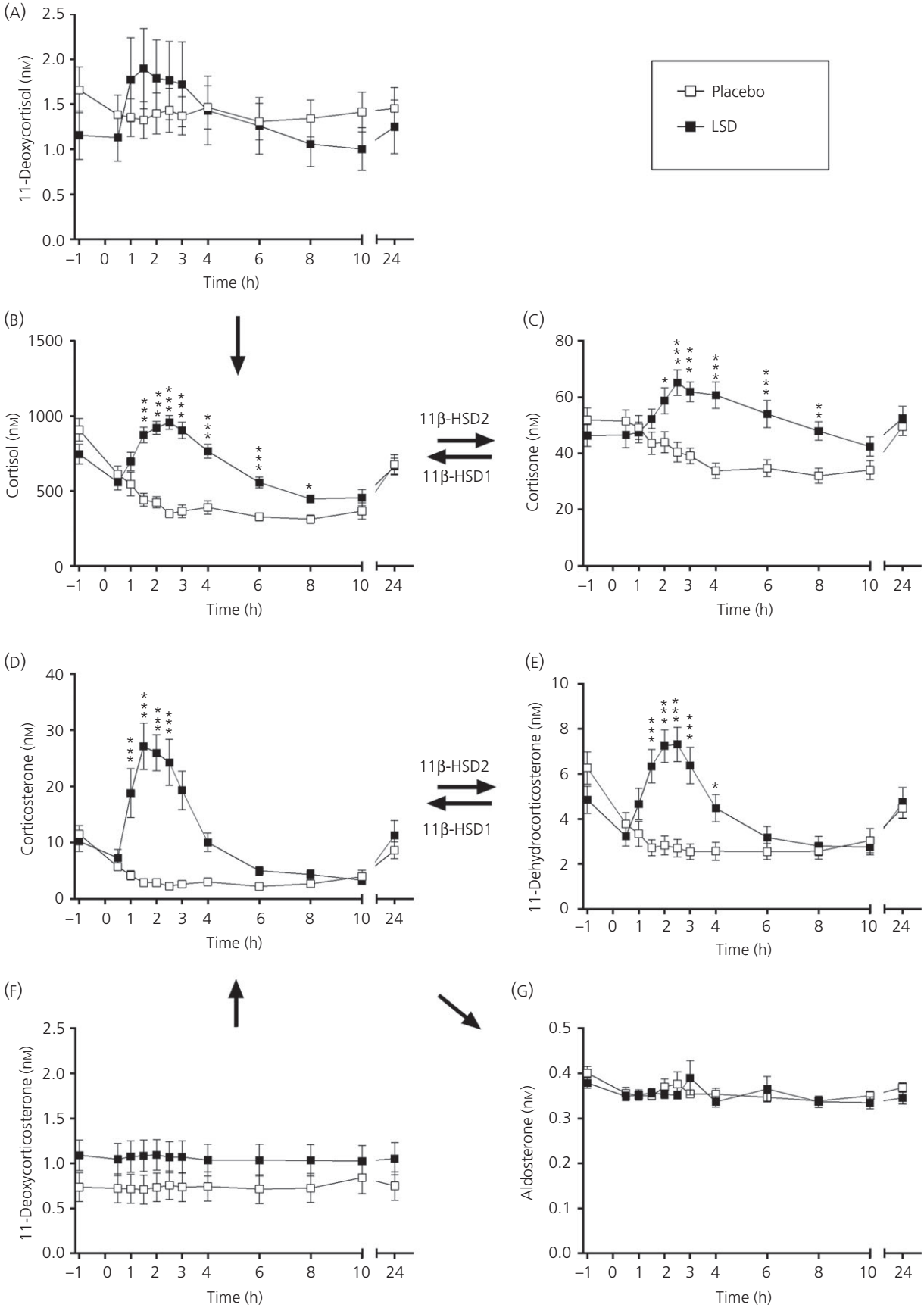

Fig. 1. Plasma concentration–time profiles of glucocorticoids and mineralocorticoids following lysergic acid diethylamide (LSD) or placebo administration. The values, obtained from 16 subjects, are expressed as the mean ± SEM. LSD or placebo was administered at t = 0 h. LSD significantly increased the plasma concentrations of the glucocorticoids cortisol (B), cortisone (C), corticosterone (D) and 11-dehydrocorticosterone (E) compared to placebo. LSD did not alter plasma concentrations of the cortisol precursor 11-deoxycortisol (A) or the mineralocorticoids 11-deoxycorticosterone (F) and aldosterone (G). 11β-HSD, 11β-hydroxysteroid dehydrogenase; CYP, cytochrome P450. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the time-matched placebo concentration (Tukey's test based on significant drug × time interactions in the two-way analysis of variance).

tion–effect relationship up to 24 h (Fig. 3B,D). The average plasma level of LSD was strongly correlated with the average subjective 'any drug effects' and the average level of cortisol over time (R_s = 0.94, P < 0.001 and R_s = 0.97, P < 0.001, respectively). The relationship between 'subjective any drug effect' and circulating glucocorticoids was explored by plotting the LSD-induced subjective 'any subjective drug effect' as a function of changes in the plasma concentrations of cortisol and corticosterone (Fig. 3G,H). After LSD administration, subjective drug effects increased together with plasma corticosterone levels but more rapidly than plasma cortisol levels. At 1 h after LSD administration, 80% of the average maximal subjective drug effect was reached, with more than 50% of the maximal corticosterone response but less than 50% of the maximal cortisol response. Thus, the psychotropic effects of LSD appeared to emerge faster than the LSD-induced changes in plasma cortisol levels. Nevertheless, the average subjective 'any drug effect' was closely related to the levels of cortisol and corticosterone (R_s = 0.97, P < 0.001 and R_s = 0.90, P < 0.001, respectively). LSD produced pronounced subjective 'good drug effects' and 'stimulation' but induced only small increases in subjective 'bad drug effects' and 'fear' compared to placebo, as reported previously (3). Average subjective 'good drug effects' and 'stimulation' were both strongly associated with the plasma levels of LSD over time (R_s = 0.88, P < 0.001 and R_s = 0.84, P < 0.001, respectively). Average 'good drug effects' and 'stimulation' were both also associated with the plasma levels of cortisol (R_s = 0.93, P < 0.001 and R_s = 0.82, P < 0.001, respectively) and corticosterone (R_s = 0.86, P < 0.001 and R_s = 0.84, P < 0.001, respectively). By contrast, LSD-induced 'bad drug effects' or 'fear' did not correlate with LSD-induced increases in cortisol or corticosterone over time. The Supporting information (Fig. S1) shows the concentration–effect curves of MDMA (125 mg) for 'any drug effects', cortisol and corticosterone based on our previous study in 16 healthy subjects (6,33) for comparison with the concentration–effect curves of LSD (Fig. 3B,D,F). The MDMA concentration–effect relationships for the psychotropic effects and glucocorticoid responses exhibited clockwise hysteresis, indicating acute pharmacological tolerance (see Supporting information, Fig. S1A–C). Consistently, the average subjective 'any drug effects' did not significantly correlate with the average plasma levels of MDMA over time. After MDMA administration, the subjective drug effects increased faster and particularly decreased faster than the plasma levels of cortisol (see Supporting information, Fig. S1D) and corticosterone (see Supporting information, Fig. S1E) (i.e. clockwise hysteresis). Thus, MDMA-induced changes in plasma glucocorticoid levels over time did not reflect the psychotropic effects of the drug very well, in contrast to LSD, for which no tolerance was observed.

Discussion

The present study provides insights into the acute effects of LSD on the plasma levels of a series of steroids in healthy humans. LSD increased circulating glucocorticoid levels, with the levels of both inactive 11-dehydrocorticosterone and cortisone and active corticosterone and cortisol being elevated compared to placebo, indicating HPA axis stimulation. The LSD-induced changes in circulating cortisol and corticosterone had, in contrast to the glucocorticoid response to MDMA, a close relationship with both the plasma concentrations of LSD and the psychotropic response to LSD. No clockwise hysteresis in the LSD concentration–effect plots was observed, thus indicating no acute tolerance to the effects of LSD on glucocorticoid concentrations or subjective drug effects, in contrast to the pronounced acute tolerance observed with MDMA. LSD also significantly increased plasma concentrations of the androgens DHEA (AUC_{10}, C_{max}) and androstenedione (AUC_{10}), although the concentration of testosterone was unaltered, and the ratio of active to inactive androgens (testosterone/androstenedione) decreased. Other androgens, as well as progestogens and mineralocorticoids, were unaffected by LSD.

The LSD-induced relative increase in corticosterone was greater than the increase in cortisol. The brain penetration of corticosterone is greater compared to cortisol because of differential transport by P-glycoprotein at the blood–brain barrier (24). Thus, the effect of LSD on brain corticosterone concentrations may be more prominent. Additionally, the LSD-induced changes in circulating corticosterone in the present study also more closely reflected psychotropic alterations over time, in which plasma cortisol levels increased later in time than the subjective effects of LSD after drug administration.

Stimulation of the HPA axis by LSD has previously been demonstrated in animals (7,9), as well as in a preliminary study in humans (10). The present study in humans provided a more comprehensive analysis of plasma concentration-over-time profiles up to 24 h after drug administration and of a series of different steroids. LSD is a prototypic serotonergic hallucinogen that mainly acts as a potent serotonin 5-HT_1 and 5-HT_2 receptor agonist. It also less potently binds to dopamine D_{1-3} and adrenergic α_1 receptors but does not inhibit monoamine transporters (2,34). In the present study, LSD also increased plasma levels of prolactin (3), which is a marker of increased serotonergic activity (35,36). Similar to LSD in the present study, the hallucinogen psilocybin increased plasma levels of cortisol in healthy humans, along with increases in prolactin and ACTH (37). Importantly, psilocybin (psilocin) activates 5-HT receptors similar to LSD but does not exhibit relevant binding to D_{1-3} and α_1 receptors, unlike LSD (34), indicating that HPA axis activation by serotonergic hallucinogens including LSD involves

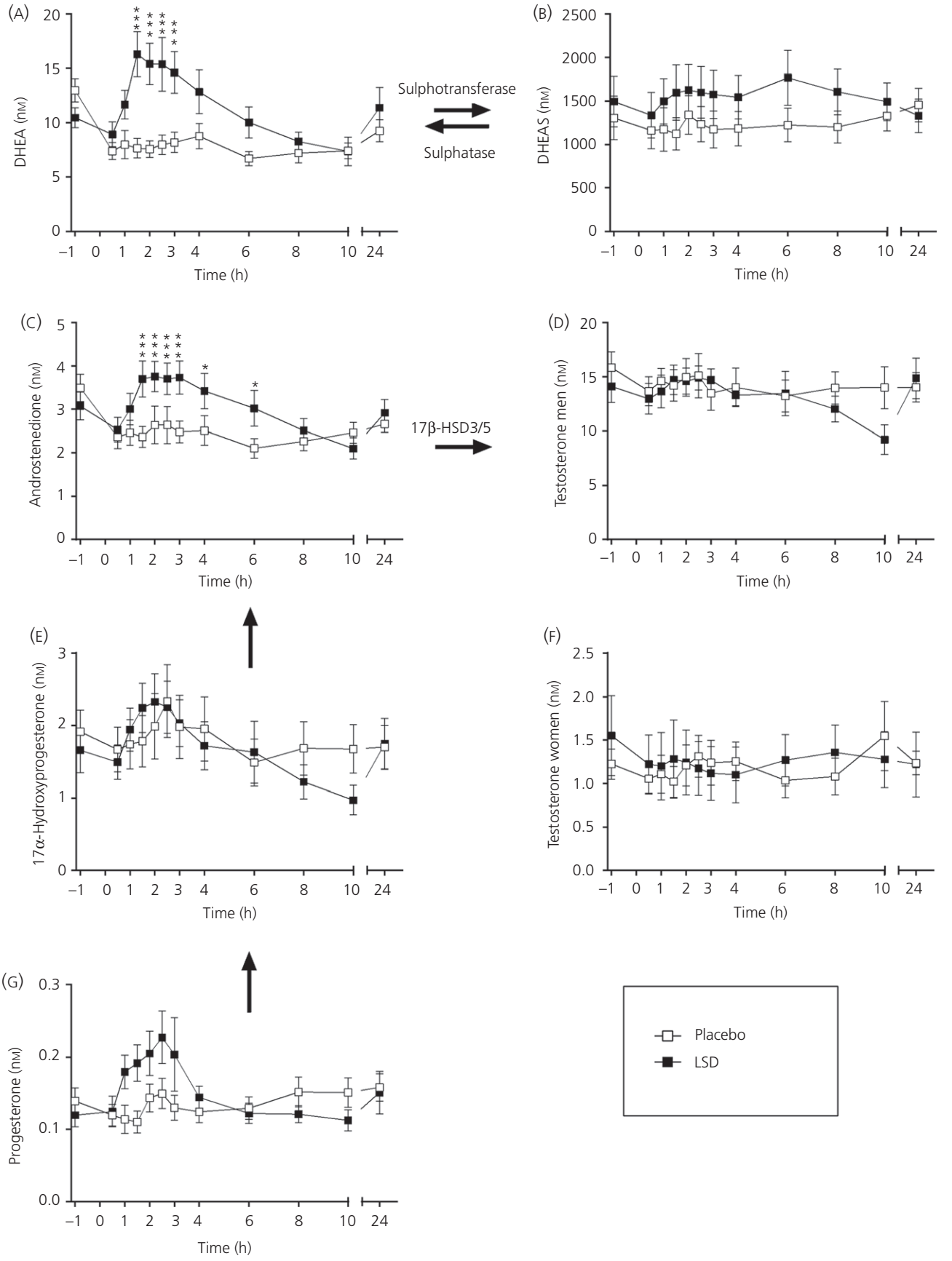
(A)
DHEA (nM)
Time (h)
Sulphotransferase
Sulphatase
(B)
DHEAS (nM)
Time (h)
(C)
Androstenedione (nM)
Time (h)
17β-HSD3/5
(D)
Testosterone men (nM)
Time (h)
(E)
17α-Hydroxyprogesterone (nM)
Time (h)
(F)
Testosterone women (nM)
Time (h)
(G)
Progesterone (nM)
Time (h)
Placebo
LSD

Fig. 2. Plasma concentration–time profiles of androgens and progestogens following lysergic acid diethylamide (LSD) or placebo administration. The values, obtained from 16 subjects (eight per sex for testosterone), are expressed as the mean ± SEM. LSD or placebo was administered at t = 0 h. LSD significantly increased plasma concentrations of dehydroepiandrosterone (DHEA) compared to placebo (A). LSD also increased the area under the concentration–time curve but not the maximal concentration of androstenedione compared to placebo (C). By contrast, LSD did not alter plasma concentrations of dehydroepiandrosterone sulphate (DHEAS) (B) or testosterone (D, F). Similarly, LSD did not change plasma levels of the progestogens progesterone (G) and 17α-hydroxyprogesterone (E). 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP, cytochrome P450. *P < 0.05 and ***P < 0.001 compared to the time-matched placebo concentration (Tukey's test based on significant drug × time interactions in the two-way analysis of variance).

Table 1. Plasma Steroid Concentrations Following Lysergic Acid Diethylamide (LSD) or Placebo Adminstration.

	C_{max}				AUC_{10}			
	Placebo	LSD	*$F_{1,15}$	P value	Placebo	LSD	*$F_{1,15}$	P value
Glucocorticoids								
Cortisol (nM)	691 ± 77	1060 ± 40	19.78	< 0.001	3545 ± 247	6160 ± 256	45.85	< 0.001
Cortisone (nM)	58.3 ± 4.2	73.1 ± 4.9	6.17	< 0.05	349 ± 23	507 ± 31	25.05	< 0.001
Corticosterone (nM)	7.43 ± 1.0	38.2 ± 3.6	57.60	< 0.001	27.9 ± 2.2	101 ± 9.8	48.66	< 0.001
11-Dehydrocorticosterone (nM)	4.43 ± 0.7	8.70 ± 0.7	13.97	< 0.01	25.7 ± 3.4	39.8 ± 4.4	4.16	NS
Cortisol + cortisone	737 ± 81	1119 ± 41	18.96	< 0.001	3886 ± 260	6666 ± 267	45.86	< 0.001
Cortisol/cortisone ratio	13.9 ± 1.1	20.1 ± 1.3	32.76	< 0.001	99.4 ± 7.4	120 ± 7.7	10.89	< 0.01
11-Deoxycortisol (precursor of cortisol) (nM)	1.67 ± 0.3	2.53 ± 0.5	2.09	NS	13.0 ± 2.0	13.0 ± 3.1	0.00	NS
Corticosterone + 11-dehydrocorticosterone	11.7 ± 1.6	46.4 ± 4.2	46.00	< 0.001	53.6 ± 5.2	141 ± 13.5	27.67	< 0.001
Corticosterone/11-dehydrocorticosterone ratio	2.26 ± 0.3	5.13 ± 0.4	34.11	< 0.001	13.4 ± 2.3	21.2 ± 1.5	7.47	< 0.05
Mineralocorticoids								
Aldosterone (nM)	0.42 ± 0.03	0.41 ± 0.04	0.08	NS	3.33 ± 0.1	3.33 ± 0.13	0.00	NS
11-Deoxycorticosterone (nM)	0.93 ± 0.16	1.16 ± 0.17	0.50	NS	7.03 ± 1.5	9.94 ± 1.7	0.99	NS
Androgens								
DHEA (nM)	11.1 ± 1.3	19.1 ± 2.3	12.12	< 0.01	71.9 ± 6.8	106 ± 12.1	10.33	< 0.01
DHEAS (nM)	1761 ± 234	2070 ± 318	1.03	NS	11551 ± 1751	15224 ± 2509	1.94	NS
Androsterone (nM)	4.03 ± 0.3	3.59 ± 0.2	1.53	NS	26.3 ± 1.1	27.0 ± 0.9	0.23	NS
Androstendione (nM)	3.68 ± 0.5	4.37 ± 0.5	2.03	NS	22.5 ± 2.2	28.8 ± 3.0	8.59	< 0.01
Testosterone (nM)	10.2 ± 2.3	9.15 ± 2.1	0.80	NS	71.8 ± 17.1	71.3 ± 15.8	0.54	NS
Testosterone in women (nM)	2.07 ± 0.4	1.58 ± 0.3	1.30	NS	11.2 ± 1.7	11.9 ± 3.4	0.10	NS
Testosterone in men (nM)	18.3 ± 1.9	16.7 ± 1.6	0.44	NS	132.4 ± 14.1	123.3 ± 9.9	0.65	NS
Testosterone/androstendione ratio	4.50 ± 1.0	3.85 ± 0.9	3.62	NS	31.7 ± 7.1	24.7 ± 5.7	9.43	< 0.01
5α-Dihydrotestosterone (nM)	1.86 ± 0.3	2.12 ± 0.5	2.40	NS	8.55 ± 1.8	8.03 ± 1.6	0.04	NS
Progestins								
Progesterone (nM)	0.22 ± 0.03	0.31 ± 0.05	2.05	NS	1.28 ± 0.1	1.40 ± 0.1	0.36	NS
17α-Hydroxyprogesterone (nM)	2.99 ± 0.5	2.96 ± 0.4	0.00	NS	16.8 ± 3.4	15.6 ± 2.6	0.17	NS

Values are the mean ± SEM in 16 subjects. DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; C_{max}, peak plasma concentration; AUC_{10}, area under the concentration–time curve up to 10 h. NS, not significant.
*$F_{1,7}$ if only men or women.

mainly 5-HT receptors. Consistently, it has been shown that 5-$HT_{2A/C}$ receptors stimulate ACTH and corticosterone release and activate corticotrophin-releasing factor-expressing cells in the hypothalamic periventricular nucleus (38,39).

Many psychotropic drugs activate the HPA axis (5). Acute administration of serotonin transporter inhibitors (35,40), but not dopamine transporter inhibitors (6,41,42), increases plasma cortisol levels, indicating that serotonin rather than dopamine mediates HPA axis stimulation. Cocaine inhibits presynaptic serotonin, dopamine and norepinephrine reuptake transporters, and increases ACTH and cortisol in humans (12,43). Amphetamine activates the norepinephrine and dopamine but not serotonin systems and increases cortisol (15,42), although to a lesser extent than the serotonergic drugs LSD and MDMA. One speculation is that the stimulant-induced increase in cortisol may depend on dopamine-mediated HPA axis stimulation (15,43). However, the stimulatory effects of amphetamines on ACTH secretion are mediated by adrenergic receptors (44) and not by dopamine (45). Additionally, methylphenidate activates the dopamine system and produces effects of stimulation and euphoria that are similar to those produced by amphetamines (33,46), although methylphenidate did not increase plasma cortisol levels (6,42) or only to a small extent (46). Furthermore, the MDMA-induced increase in circulating cortisol was reduced by pharmacologically blocking the MDMA-induced release

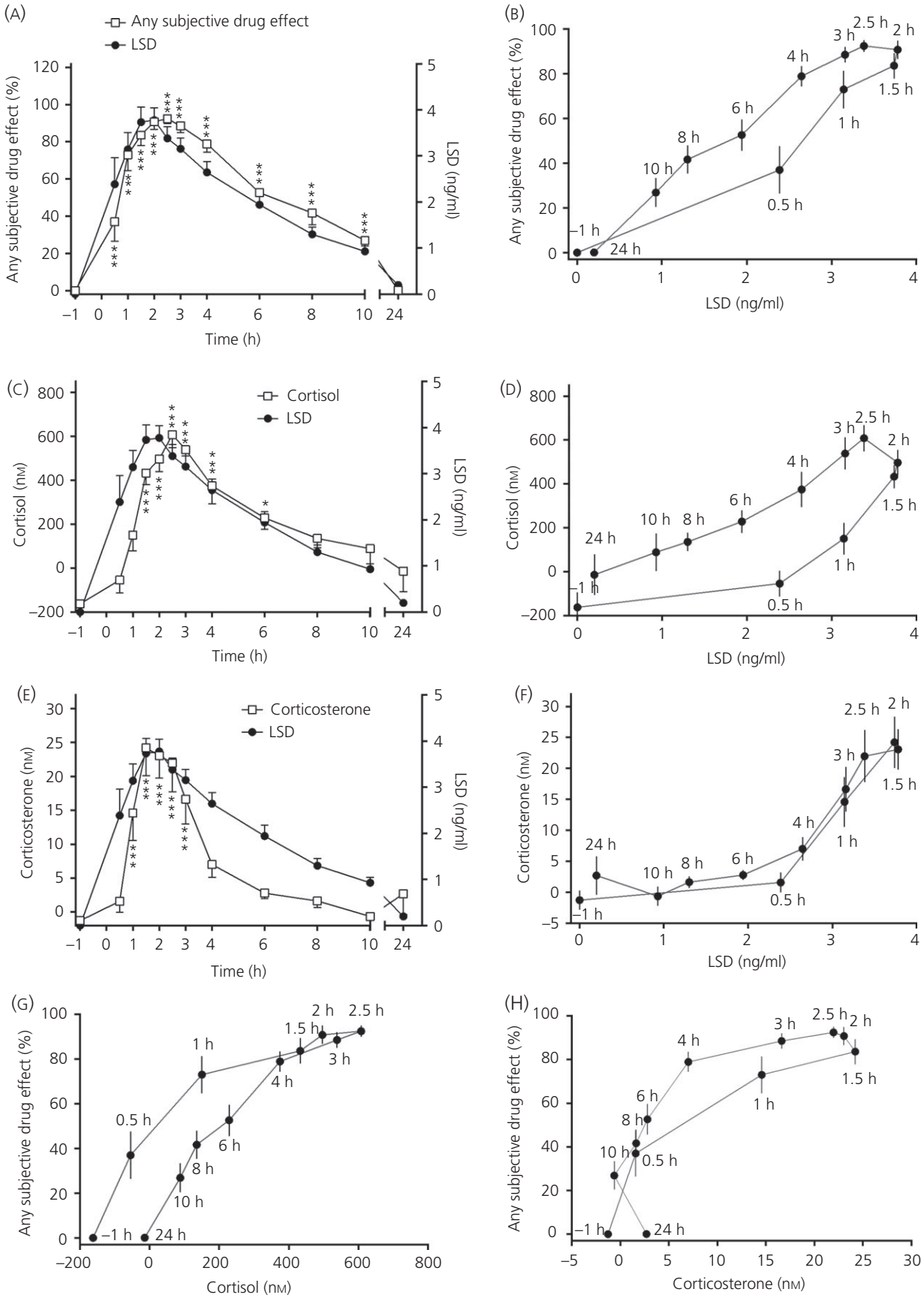

Fig. 3. Lysergic acid diethylamide (LSD) exposure–response relationships. LSD responses are shown as LSD effect (item 'any subjective drug effect' reflecting the overall subjective response to LSD, cortisol or corticosterone concentration) minus the individual time-matched effect of placebo. Any subjective responses to LSD (A) and LSD-induced changes in cortisol (C) and corticosterone (E) over time are presented with the corresponding LSD concentrations over time (mean ± SEM) in 16 subjects. LSD or placebo was administered at t = 0 h. Subjective responses to LSD (B) and LSD-induced changes in cortisol (D) and corticosterone (F) concentrations (mean ± SEM) are plotted as a function of mean LSD plasma concentrations (hysteresis curves). The time of sampling is noted next to each point (in hours after LSD administration). The maximum concentration of LSD was reached 1.7 ± 1 h after LSD administration (A, B). The peak psychotropic effect was reached at 2.4 ± 0.8 h, with significant alterations in mental state from 0.5 h to > 10 h after LSD administration (A, B) [drug × time interaction in the two-way analysis of variance (ANOVA): $F_{11,165} = 41.39$; $P < 0.001$]. Maximum concentrations of cortisol (C, D) and corticosterone (E, F) were reached at 2.5 ± 0.8 h and 1.9 ± 0.5 h (mean ± SD), with significant elevations from 1.5 to 6 h and from 1 to 3 h after LSD administration, respectively ($F_{11,165} = 17.71$; $P < 0.01$ and $F_{11,165} = 13.35$, $P < 0.001$, respectively). Counterclockwise hysteresis was observed for any drug effects (B) and cortisol (D), which is consistent with an initial delay between plasma concentration and an effect that was attributable to drug absorption. Beyond 2 h after LSD administration, the psychotropic effects (A) and changes in plasma cortisol levels (C) decreased slowly, in parallel with the plasma levels of LSD, exhibiting a close concentration–effect relationship up to 24 h (B, D). LSD significantly increased plasma levels of cortisol 1.5–6 h after LSD administration (C). By contrast, plasma levels of corticosterone increased more rapidly but fell more quickly back to baseline levels, resulting in significant differences in plasma levels 1–3 h after LSD administration and compared to placebo (E). There was no evidence of acute pharmacological tolerance (clockwise hysteresis) for any of the effects of LSD. After drug administration, subjective drug effects increased together with plasma levels of corticosterone but more rapidly than plasma levels of cortisol (G, H). *$P < 0.05$ and ***$P < 0.001$ compared to the time-matched placebo concentration (Tukey's test based on significant drug × time interactions in the two-way ANOVAs).

of serotonin and norepinephrine (47,48) but not when dopamine release was blocked (41). The greater effects of amphetamine on cortisol release compared to methylphenidate are thus likely attributable to its greater noradrenergic versus dopaminergic properties compared to methylphenidate (42,49,50). Nonetheless, the present study shows that stimulation of the serotonin system by LSD increased cortisol levels similarly to MDMA, which has more amphetamine-type properties and stimulates both the serotonin and norepinephrine systems.

Stimulation of the HPA axis involves serotonin and norepinephrine systems (45). Similar to LSD, the serotonin and norepinephrine releaser MDMA increased the plasma concentrations of the glucocorticoids cortisol, corticosterone and 11-dehydrocorticosterone (6). Unlike LSD and MDMA, methylphenidate, which activates dopamine and norepinephrine systems but not the serotonin system, did not significantly alter plasma steroid levels in humans (6,42), further supporting a role for serotonin receptors in drug-induced HPA axis stimulation. Unexpectedly, the glucocorticoid response was more pronounced after LSD administration than after MDMA administration (6). This is consistent with the greater psychotropic response to LSD compared to MDMA (33) (see Supporting information, Fig. S1A). By contrast, MDMA produced more stimulant-type effects, including greater increases in blood pressure and heart rate (33). The greater glucocorticoid response after LSD compared to MDMA indicates that the direct serotonergic stimulation of postsynaptic 5-HT_1 and 5-HT_2 receptors by LSD similarly or even more effectively stimulated the HPA axis compared to the release of both serotonin and norepinephrine by MDMA (48). The relatively similar time courses of the glucocorticoid response and the psychotropic effects of LSD, together with the greater glucocorticoid and psychotropic responses to LSD compared to MDMA, raise the issue of whether the subjective effects of LSD contribute to or further enhance HPA axis stimulation by LSD. We observed a close relationship between LSD-induced subjective drug effects and changes in plasma corticosterone levels. Associations between amphetamine-induced increases in cortisol and subjective arousal and euphoria have been reported previously (15,42). The covariance of the psychological and endocrine drug responses indicates that both are mediated by the same transmitter, likely norepinephrine in the case of amphetamine (42) and serotonin in the case of LSD. It is unlikely that glucocorticoids critically mediate the psychotropic drug response because the subjective effects of methamphetamine (51) and cocaine (52) are unaltered when the drug-induced cortisol response is pharmacologically augmented or blocked. On the other hand, the psychotropic effects of LSD might have contributed to the endocrine stress response. Indeed, the subjective effects occurred faster than the cortisol response to LSD. However, only the subjective 'good drug effects' and 'stimulation' induced by LSD and not the 'bad drug effects' or 'fear' correlated with the steroid response over time. Thus, the endocrine changes in response to LSD appear be related to the positive and stimulant subjective LSD effects but not to anxiety.

Both cortisol and prolactin levels increase when the serotonin system is pharmacologically activated (35,36,53). Interestingly, the prolactin response was greater after MDMA administration (33) than after LSD administration (3), whereas the glucocorticoid response was less, indicating differential effects of LSD and MDMA on markers of serotonergic activity, and further supporting the view that the LSD-induced increase in glucocorticoids may have been enhanced by the more pronounced subjective effects of LSD.

A striking difference was found between the plasma concentration–effect curves of LSD in the present study and the plasma concentration–effect curves of MDMA in our previous study (33). Specifically, the plasma concentration–effect curve of MDMA showed pronounced clockwise hysteresis for the psychotropic effects of MDMA (33) and also for the cortisol and corticosterone responses (see Supporting information, Fig. S1A–C), suggesting acute tolerance to the effects of MDMA. By contrast, we observed no tolerance to the effects of LSD. This means that the effects of LSD on the HPA axis are longer-lasting than those of MDMA, although MDMA has a longer plasma half-life than LSD (31,33). The finding could be explained by the pharmacological mechanisms of MDMA and LSD. MDMA releases endogenous serotonin and norepinephrine from presynaptic terminals (48), whereas LSD directly interacts with postsynaptic 5-HT receptors (2). Indeed, the MDMA-induced cortisol response was blocked after duloxetine pre-

treatment, which prevents MDMA from interacting with the serotonin and norepinephrine transporters (47). In the case of cocaine, cocaine-induced euphoria is also short-lasting and exhibits acute tolerance (12), which is similar to MDMA, whereas the cortisol concentration–time curve is concordant with the cocaine–plasma concentration time curve (12), similar to LSD.

Unlike LSD, MDMA also increased the mineralocorticoids 11-deoxycorticosterone and aldosterone. Mineralocorticoids promote sodium retention and increase extracellular fluid volume, thereby increasing blood pressure (25). The MDMA-induced increase in mineralocorticoids may thus contribute to the greater increase in blood pressure after MDMA administration (33) compared to LSD (3). The mechanisms that underlie the differential effects of MDMA and LSD on mineralocorticoid production remain unclear.

LSD increased DHEA. DHEA is a precursor of many other steroids and may itself modulate γ-aminobutyric acid-ergic and glutamatergic neurotransmission (54). DHEA has well-documented anxiolytic and antidepressant effects (54–57). An interesting line of investigation would be to evaluate further the role of DHEA in the potential anxiolytic effects of LSD that are reported in terminally ill patients (4).

The present study has limitations. First, only a single dose and single administration of LSD were used. However, a relatively high dose of LSD was administered, which produced pronounced psychotropic effects and was within the range of doses used clinically (4) and recreationally (1,2). Additionally, we present LSD exposure–effect relationships that can partially substitute for a multiple dose-level study. Second, only psychiatrically and somatically healthy subjects with limited previous experience with hallucinogenic drugs were included. LSD may differentially affect steroid profiles in chronic LSD or polydrug users. Third, we did not assess concentrations of corticotrophin-releasing factor or ACTH to describe the effects of the drug on other mediators within the HPA axis.

In conclusion, LSD induced significant effects on plasma glucocorticoids, which is consistent with HPA axis stimulation via serotonergic receptors. Plasma levels of cortisol and particularly corticosterone covaried in close relationship to the plasma levels of LSD over time. The corticosterone response was also closely related to the subjective effects of LSD. The glucocorticoid response to LSD showed no acute pharmacological tolerance, in contrast to the response to MDMA.

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Supporting Information

The following supplementary material is available:

Supplemental Methods. Quantification of steroid hormones in human plasma samples.

Fig. S1. Methylenedioxymethamphetamine (MDMA) exposure–response relationship.

Table S1. Optimised ion source conditions and analytical parameters.

Table S2. Multiple reaction monitoring (MRM) analyte transitions.

Table S3. Limit of detection (LLOD), lower limit of quantification (LLOQ), signal-to-noise ratio (S/N), retention time (RT), linearity and calibration range.

Table S4. Recovery (QC high, QC medium, QC low).

Table S5. Reproducibility: nominal and measured concentration, standard deviation (SD), imprecision (CV%) and inaccuracy (RE%) of QC high, QC medium and QC low.

5.3 Paper draft: Acute effects of D-amphetamine and lisdexamfetamine on plasma steroid concentrations in healthy subjects

This manuscript still requires major revision in order to avoid redundant information and for optimal completion of the submitted manuscript «Pharmacokinetics and pharmacodynamics of lisdexamfetamine compared with D-amphetamine in healthy subjects».

Moreover, currently some calculations are undertaken for comparison of drug induced changes in steroid homeostasis after administration of D-amphetamine with methylphenidate, 3,4-methylenedioxymethamphetamine (MDMA), and LSD.

Original Paper

Effects of lisdexamfetamine compared with D-amphetamine on plasma steroid concentrations in healthy subjects

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Running title: D-amphetamine, lisdexamfetamine, and steroids

Figures: 3, Table: 2, Supplement online: Figure S1

ABSTRACT

Introduction: Lisdexamfetamine is a novel prodrug of D-amphetamine used for the treatment of attention-deficit/hyperactivity disorder (ADHD). D-amphetamine releases dopamine and norepinephrine and stimulates the hypothalamic-pituitary-adrenal (HPA) axis, which may contribute to its reinforcing effects and risk of abuse. The goal of the present study was to assess effects of lisdexamfetamine on circulating steroids in comparison with classic D-amphetamine. **Method**: Equimolar doses of D-amphetamine (40 mg) and lisdexamfetamine (100 mg), and placebo were administered in 24 healthy subjects in a randomized, double-blind, placebo-controlled, cross-over study. Plasma concentrations of steroids and D-amphetamine were determined up to 24 h.

Results: D-amphetamine plasma concentrations increased and reached peak levels later after administration of lisdexamfetamine compared with D-amphetamine, but maximal concentrations and total exposure (AUC) were similar. Lisdexamfetamine and D-amphetamine significantly increased plasma concentrations of ACTH, the glucocorticoids cortisol, cortisone, corticosterone, 11-dehydrocorticosterone, 11-deoxycortisol, the androgens dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstendione, and of progesterone (only in men) compared with placebo. Steroid concentration-time curves were shifted to the right due to the significantly later onset after lisdexamfetamine compared with D-amphetamine, but maximal plasma concentrations and AUC value of the steroids did not differ between treatments. None of the treatments changed plasma concentrations of the mineralocorticoids aldosterone and 11-deoxycorticosterone or of testosterone. The effect of amphetamines on glucocorticoid production was similar to those previously shown for methylphenidate (60 mg) but weaker than those of the serotonin releaser 3,4-methylenedioxymethamphetamine (MDMA, 125 mg) or the serotonin agonist lysergic acid diethylamide (LSD, 0.2 mg).

Conclusion: Lisdexamfetamine produced comparable HPA axis activation and pharmacokinetics compared with D-amphetamine with the exception of a later onset time. Serotonin (MDMA, LSD) may more effectively stimulate the HPA axis than dopamine and norepinephrine (D-amphetamine).

Keywords: lisdexamfetamine, D-amphetamine, steroids, glucocorticoids, mineralocorticoids, pharmacokinetics, psychostimulants

INTRODUCTION

Lisdexamfetamine is a prodrug of D-amphetamine [1-3] and both are used for the treatment of attention-deficit/hyperactivity disorder (ADHD) similar to methylphenidate. However, amphetamines and methylphenidate are also misused recreationally to induce euphoria or to stay awake [4-8]. After oral administration, the conversion of lisdexamfetamine to D-amphetamine is thought to occur gradually in the circulation [9, 10], reportedly resulting in a prolonged pharmacokinetic profile with low peak but sustained plasma amphetamine concentrations [11]. Such a prolonged pharmacokinetic profile is considered to be associated with slower effects on dopamine (DA) release, lower euphoric effects, and a possibly lower risk of misuse [11-13]. Indeed, in rats, the peak plasma concentration (C_{max}) of amphetamine was lower after lisdexamfetamine, and it produced a gradual and sustained increase in dopamine efflux and much less locomotor activity compared with D-amphetamine [14]. In humans, 100 mg lisdexamfetamine produced lower subjective “drug liking” than an equivalent dose of 40 mg D-amphetamine [11]. Amphetamines and methylphenidate not only enhance subjective mood, concentration and wakefulness but also act as acute pharmacological stressors and stimulate the hypothalamic-pituitary-adrenal (HPA) axis to elevate concentrations of circulating stress hormones including adrenocorticotropic hormone (ACTH), cortisol, epinephrine, and norepinephrine (NE) [15-19]. The effects of lisdexamfetamine on the HPA axis are unknown. In particular, it has not been studied whether lisdexamfetamine would produce smaller HPA axis stimulation than D-amphetamine based on its reportedly prolonged kinetic characteristics [11-13]. Animal studies indicate that HPA axis stimulation may be associated with a greater risk of drug abuse. Specifically, rats that show greater HPA axis reactivity or were administered corticosterone were more likely to self-administer D-amphetamine [20, 21]. Lisdexamfetamine may have a reduced risk of oral abuse compared with D-amphetamine because of a slowed increase in plasma amphetamine but also because of a consequently reduced HPA response. Therefore, we directly compared the plasma concentrations of ACTH and steroids after administration of equivalent and relatively high doses of D-amphetamine and lisdexamfetamine. The study hypothesis was that lisdexamfetamine would produce lower C_{max} and longer time to C_{max} (T_{max}) values for plasma amphetamine and steroids compared with classic immediate release D-amphetamine. Lisdexamfetamine and D-amphetamine were expected to result in equivalent area under the plasma concentration-time curve (AUC) values for amphetamine and steroids confirming the use of equivalent doses.

The present study used relatively high doses of lisdexamfetamine and D-amphetamine. D-Amphetamine at low oral doses of 10-20 mg has repeatedly been shown to increase concentrations of plasma or saliva cortisol [19, 22-28] while no effect on plasma cortisol were also reported [29]. Few studies used higher doses of D-amphetamine that would better reflect stimulant misuse. One study showed increases in plasma cortisol compared to baseline after 34 mg D-amphetamine [30]. However, this study did not include a placebo control condition. Therefore, the present study investigated the effect of relatively high doses of D-amphetamine (40 mg) and lisdexamfetamine (100 mg) and placebo on plasma concentrations of ACTH and cortisol and other steroids not previously measured.

Both D-amphetamine and methylphenidate enhance DA and NE neurotransmission. While D-amphetamine releases DA and NE from presynaptic terminals and inhibits their reuptake [31], methylphenidate only inhibits their reuptake without inducing transporter-mediated release [32]. Although methylphenidate stimulates DA and NE systems similar to D-amphetamine, methylphenidate seems to produce only very moderate stimulating effects on the HPA axis. Single oral doses of 10-20 mg methylphenidate had no significant effect on plasma cortisol concentrations compared with placebo [26] and a single oral dose of 40 mg methylphenidate only moderately increased plasma cortisol levels [17]. A dose of 60 mg methylphenidate only non-significantly increases plasma levels of cortisol, cortisone, corticosterone, and 11-dehydrocorticosterone compared with placebo [16, 18]. Interestingly, the relatively high dose of 60 mg methylphenidate produced at lease similar subjective liking to 30 mg D-amphetamine [18, 33] indicating that methylphenidate may induce lower HPA axis stimulation than D-amphetamine at equivalently psychostimulant doses. This view is supported by one study that directly compared cortisol plasma concentrations after 10-20 mg doses of both D-amphetamine and methylphenidate [26] but higher doses of both drugs have not been compared. Therefore, the present study also indirectly compared the effects of a high dose of 40 mg D-amphetamine with those of a high dose of 60 mg methylphenidate previously tested in the same laboratory and using the same clinical and analytical methods [16]. Based on previous data [16, 18, 26] the hypothesis was that D-amphetamine would produce greater HPA axis activation than methylphenidate.

A final goal of the present study was to explore the role of the different monoamine neurotransmitters in regulating HPA activity. D-amphetamine releases DA and NE and may release

cortisol mainly via NE [34]. The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA) mainly releases 5-HT and NE [31, 35, 36]. Therefore, MDMA and D-amphetamine can be used as pharmacological tools to study the role of 5-HT versus DA release in HPA axis stimulation. Accordingly, we indirectly compared the effects of D-amphetamine on the plasma concentrations of steroids with those of 125 mg oral MDMA previously tested in the same laboratory and using the same clinical and analytical methods [16]. To further study the role of 5-HT versus DA and NE release in HPA axis stimulation by psychoactive substances in humans, we also compared the effects of D-amphetamine in the present study with similar historic data [37] on the direct 5-HT receptor agonist LSD [38]. We hypothesized that MDMA and LSD would produce greater cortisol increases in humans than D-amphetamine indicating a more prominent role of 5-HT compared to DA and NE in the stimulation of this major steroid by psychoactive substances.

MATERIALS AND METHODS

Study design

The study used a double-blind, placebo-controlled, cross-over design with three experimental test days (D-amphetamine, lisdexamfetamine, and placebo) in balanced order. The washout periods between sessions were at least 7 days. The study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization Guidelines in Good Clinical Practice and approved by the Ethics Committee northwest/central Switzerland (EKNZ) and the Swiss Agency for Therapeutic Products (Swissmedic). The study was registered at ClinicalTrials.gov (NCT02668926). All of the subjects provided written informed consent prior to participating in the study.

Participants

Twenty-four healthy subjects (twelve men and twelve women; mean age ± SD: 25.3 ± 3.0 years; range: 21-34 years) were included. The inclusion criteria were age between 18 and 45 years, body mass index between 18 and 27 kg/m^2, and birth control for women. The exclusion criteria were chronic or acute medical conditions including clinically relevant abnormality in physical exam, laboratory values, or ECG, personal or family (first-degree relative) history of psychotic or major affective disorder, lifetime prevalence of illicit drug use > 5 times (except for tetrahydrocannabinol),

illicit drug use within the last 2 months, pregnancy, regular use of medications, smoking (>10 cigarettes/day), and alcohol consumption of alcoholic drinks (>10/week). The subjects were asked to abstain from excessive alcohol consumption between test sessions and not to drink caffeine-containing liquids after midnight before the study day. A urine drug tests were performed at study inclusion and before each test session using TRIAGE 8 (Biosite, San Diego, CA, USA).

Drugs

Gelatin capsules either containing lisdexamfetamine dimesylate (100 mg salt; Opopharma, Rümlang, Switzerland) or D-amphetamine sulfate (40.3 mg salt; Hänseler, Herisau, Switzerland), both corresponding to a dose of 29.6 mg D-amphetamine, as well as the placebo capsules (mannitol) were prepared by the pharmacy of the University Hospital Basel according to Good Manufacturing Practice.

Study procedures

Before the test session, a urine sample was taken to verify abstinence from drugs of abuse, and a pregnancy test was performed in women. At 8:00 AM the test session began by placing an indwelling intravenous catheter in an antecubital vein for blood sampling. At 9:00 AM a single dose of lisdexamfetamine, D-amphetamine, or placebo was administered orally. During the test session the subject did not engage in any physical activity, were resting in hospital beds in a calm standard hospital room, and were served a standardized lunch and dinner at 11:30 AM and 6:30 PM, respectively. For the analysis of hormone and D-amphetamine concentrations in plasma, blood samples were collected in lithium heparin tubes 1 h before and 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, and 24 h after drug administration. The blood samples were immediately centrifuged and the plasma stored at -20°C. For the determination of adrenocorticotropic hormone (ACTH) concentrations, blood samples were drawn into EDTA-containing tubes 1 h before and 3.5 h after drug administration. The test session ended at 9:00 PM. Subjects returned home and returned to the following day at 9:00 AM and for drawing the final 24h blood sample. Subjective, autonomic, and adverse responses were also assessed and are published elsewhere.

Steroid quantification in plasma

The following plasma steroid hormones with the corresponding lower limit of quantification

(LLOQ) (values in brackets) were determined using a previously published UHPLC-MS/MS method [37] with minor adaptations: cortisol (1.95 nM), cortisone (1.95 nM), corticosterone (0.98 nM), 11-dehydrocorticosterone (0.98 nM), 11-deoxycorticosterone (0.78 nM), aldosterone (0.2 nM), DHEA (3.91 nM), DHEA sulfate [DHEAS] (19.53 nM), ∆4-androstene-3,17-dione [androstenedione] (0.78 nM), testosterone (0.39 nM), 11-deoxycortisol (0.78 nM), progesterone (0.05 nM), androsterone (3.91 nM), and 17α-hydroxyprogesterone (0.78 nM)) The accuracy was between 85 and 115% and the variation coefficient was <15% tested at three concentrations for all analytes. The recovery of control samples was in the range of 80-120%. The used method in detail and its validation are reported previously [37]. Briefly, after protein precipitation, the plasma samples (containing deuterium-labeled aldosterone, corticosterone, androstenedione, androsterone, and testosterone as internal standards) were solid-phase extracted. After evaporation and reconstitution in methanol, the steroids were separated and quantified by ultra-pressure LC-MS/MS (UPLC-MS/MS) using an Agilent 1290 UPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer equipped with a jet-stream electrospray ionization interface. Analyte separation was achieved using a reverse-phase column (Waters Acquity UPLC BEH C18, 1.7 µm, 2.1 x 150 mm). Mass Hunter software (Agilent Technologies) was used for data acquisition and analysis.

Quantification of adrenocorticotropic hormone (ACTH) in human plasma samples

ACTH was determined by a chemiluminescent immunometric assay (Immulite 2000 ACTH; Siemens, Erlangen, Germany).

Quantification of amphetamine (D-amphetamine) concentrations in blood plasma

Plasma concentrations of D-amphetamine were measured using an Ultra-High pressure LC-MS/MS (UHPLC-MS/MS). Materials, procedures, and method validation are described in detail in the Supplementary Material. The method had a lower limit of detection (LOD) of 0.26 ng/mL, respectively a lower limit of quantification (LLOQ) of 0.78 ng/mL for D-amphetamine and was validated over the range of 0.78 to 200 ng/mL for D-amphetamine. Plasma concentrations of D-amphetamine were primarily measured to confirm the use of bioequivalent lisdexamfetamine and D-amphetamine doses regarding total D-amphetamine exposure and to assess the D-amphetamine-steroid response relationships. The comprehensive pharmacokinetic data from this study are shown elsewhere.

Statistical analyses

Maximum concentrations (C_{max}) and time to reach C_{max} (t_{max}) values were derived directly from the observed data. Time to reach 10% of C_{max} (t_{onset}), and areas under the concentration-time curve from time 0 to 24 h (AUC_{24}) were calculated using the linear trapezoidal method in Phoenix WinNonlin (Version 6.4, Pharsight, St. Louis, MO). Statistical analyses were performed using STATISTICA 12 software (StatSoft, Tulsa, OK, USA). Kinetic parameters of the steroids were compared using repeated-measures analysis of variance (ANOVA), with drug (D-amphetamine, lisdexamfetamine, and placebo) as the within-subject factor, followed by Tukey post hoc tests. Sex differences were assessed by adding sex as between-subject factor to the analysis. Plasma amphetamine concentrations after administration of lisdexamfetamine and D-amphetamine were compared using paired t-tests The amphetamine concentration-effect relationships were studied by plotting the endocrine responses as difference from time-matched placebo against the amphetamine concentration for each time point. Selected peak endocrine effects of D-amphetamine and lisdexamfetamine calculated as differences from placebo were then compared with the effects of 60 mg methylphenidate [16], 125 mg MDMA [16] and 200 µg LSD [37] obtained in previous identical studies in healthy subjects in the same laboratory using ANOVA and drug as between-subject factor followed by Tukey post hoc tests. The use of placebo-corrected values accounted for between-subject differences in baseline steroid levels and circadian within-subject changes.

RESULTS

Blood could not be drawn from one subject under the D-amphetamine condition and therefore complete datasets were available for D-amphetamine and lisdexamfetamine for 23 and 24 subjects, respectively.

Plasma levels of amphetamine after administration of D-amphetamine and lisdexamfetamine

The amphetamine plasma concentration-time curves were identical after administration of D-amphetamine and lisdexamfetamine with the exception of significantly longer T_{onset} and T_{max} values after lisdexamfetamine compared with D-amphetamine (Figure 2). The C_{max} and AUC_{24} values were similar (Table 1).

Effects of D-amphetamine and lisdexamfetamine *on plasma steroid concentrations*

Effects of D-amphetamine, lisdexamfetamine and placebo on the plasma steroid hormone concentrations are depicted in Figure 1 and 2. Table 1 shows the corresponding T_{max}, C_{max} and AUC values and comparative statistics. Both active treatments significantly and similarly increased the plasma concentrations of the glucocorticoids cortisol, cortisone, corticosterone, 11-dehydrocorticosterone, and 11-deoxycortisol compared with placebo (Fig. 1A-E, Table 1). Elevated glucocorticoid production was evidenced by the significantly increased sums of cortisol + cortisone and corticosterone + 11-dehydrocorticosterone as well as cortisol/cortisone and corticosterone/11-dehydrocorticosterone ratios (Table 1). Only T_{onset} and T_{max} values differed between D-amphetamine and lisdexamfetamine while C_{max} and AUC_{24} values and the shape of the concentration-time curves were practically identical (Fig. 2E, Table 1). Neither the mineralocorticoids aldosterone (Fig. 1F) and 11-deoxycorticosteone, nor the progestins 17α-hydroxyprogesterone (Fig. 2C) and progesterone were changed by lisdexamfetamine or D-amphetamine compared with placebo. An exception was progesterone concentration in men, where increased C_{max} and AUC values were seen compared with placebo (Table 1). The plasma concentration of DHEA and DHEAS and androstenedione (Fig. 2A, B, D, F) were significantly increased by the two active drugs compared with placebo. Likewise, lisdexamfetamine and D-amphetamine had an effect on C_{max} and AUC values of the sum of androstenedione + testosterone in women, but not in men. In contrast, lisdexamfetamine and D-amphetamine had no effect on the concentrations of androgens testosterone and androsterone (Table 1). The plasma concentrations of 11-deoxycorticosterone were above the LOD but below the LLOQ and therefore the quantification of this steroid was not validly possibly.

Relationship between amphetamine and steroid plasma concentrations after administration of D-amphetamine and lisdexamfetamine

Selected drug exposure-steroid concentration response relationships are shown in Supplemental Figure S1. Clockwise hysteresis was observed indicating acute pharmacological tolerance. Plasma concentrations of ACTH are shown in Figure 3. There was a main effect of drug at the 3.5 h time point ($F_{2,40}$ = 33.83, $P<0.001$) and both active drugs increased ACTH plasma concentrations compared with placebo at 3.5 h (both $P<0.001$).

Peak endocrine effects D-amphetamine and lisdexamfetamine compared with other prototypical substances.

The peak endocrine effects of D-amphetamine, lisdexamfetamine, methylphenidate, MDMA and LSD are shown in Table 2. The drug effects are presented as within-subject changes from placebo. D-amphetamine, lisdexamfetamine, and methylphenidate produced comparable increases in cortisol. D-amphetamine increased cortisone and 11-dehydrocorticosterone to greater levels than methylphenidate. MDMA induced higher peak concentrations of cortisol, lower levels of cortisone, but still higher cortisol+cortisone levels than D-amphetamine. LSD produced much higher peak concentrations of cortisol and corticosterone than D-amphetamine but in contrast to MDMA did not reduce cortisone of 11-dehydrocorticosterone levels relative to D-amphetamine.

DISCUSSION

The main finding of the present study was that lisdexamfetamine produced identical HPA axis stimulation and steroid plasma concentration-time curves as classic immediate release D-amphetamine. This finding was not in agreement with the study hypothesis of a smaller and prolonged endocrine response to lisdexamfetamine compared with D-amphetamine. The reason for the identical endocrine responses of the two D-amphetamine formulations was the unexpected finding of similar amphetamine peak concentrations after administration of lisdexamfetamine or D-amphetamine. Lisdexamfetamine had a significantly longer onset and T_{max} but otherwise a very similar amphetamine plasma concentration-time curve shape to D-amphetamine. Administration of D-amphetamine 1 hour later would likely have produced a similar pharmacokinetic profile to lisdexamfetamine. The steroid concentration time-curves were right-shifted similar to the amphetamine plasma concentration-time curve after lisdexamfetamine compared with D-amphetamine but this effect did not reach significance for any of the T_{max} values. The similar C_{max} of amphetamine and peak steroid responses at doses of lisdexamfetamine and D-amphetamine producing equivalent AUCs is in contrast to previous preclinical [14] and clinical [11, 12] reports used to set up the present study hypotheses.

In the present study we statistically compared the endocrine effects of D-amphetamine with other psychoactive substances tested in previous separate studies in our laboratory under similar

conditions [16, 37]. In contrast to our hypothesis, D-amphetamine and lisdexamfetamine produced similar effects on plasma cortisol and corticosterone to methylphenidate. Although, the effects of methylphenidate on these active corticosteroids did not reach significance compared with placebo in our previous smaller study [16], the respective effects of D-amphetamine that were significant compared with placebo in the present study were not significantly greater than those of methylphenidate. However, D-amphetamine produced greater cortisone and 11-dehydrocorticosterone levels than methylphenidate. Nevertheless, the present study indicates that the overall effects on plasma steroids of D-amphetamine, lisdexamfetamine and methylphenidate at equivalent psychostimulant doses [33] are largely similar.

NE, DA, and serotonin (5-HT) have all been implicated in mediating HPA axis stimulation. However, the relative contribution of these monoamines to HPA axis stimulation by psychotropics in humans is unclear [39, 40]. D-amphetamine may release cortisol mainly via NE [34]. Specifically, D-amphetamine more potently interacts with the NE compared with the DA and 5-HT transporters and it has a very low potency at the 5-HT transporter [31, 41]. Additionally, the effects of D-amphetamine or methamphetamine on plasma corticosteroids were blocked by α-adrenergic receptor antagonists [42, 43] but not by DA receptor antagonists [44, 45]. On the other hand, purely or predominantly serotonergic substances strongly stimulate the HPA axis [29, 37, 46]. In the present study, we compared the effects of D-amphetamine with similar historic data on MDMA and LSD obtained in the same laboratory and with the same clinical and analytical methods [16, 37]. Compared with D-amphetamine and methylphenidate, which stimulate NE and DA, MDMA and LSD, which mainly stimulate 5-HT, produced greater increases in plasma concentrations of the biologically active glucocorticoids cortisol and corticosterone. This finding supports the view that 5-HT primarily or more strongly increases cortisol compared with DA or NE [16, 37, 46]. Notably, the MDMA-induced increases in cortisol and corticosterone were paralleled by relatively smaller changes (decreases compared with D-amphetamine) in the respective 11β-hydoxysteroid dehydrogenase 2 (11β-HSD2) - formed metabolite and precursor cortisone and 11-dehydrocorticosterone indicating 11β-HSD2 inhibition by MDMA. In contrast, the LSD-induced increase in cortisol was paralleled by a similar increase of its metabolite cortisone and both reached significantly greater levels compared with D-amphetamine. Both the 5-HT releaser MDMA and the 5-HT receptor agonist LSD [38] increased the sum of cortisol + cortisone more than D-amphetamine indicating greater glucocorticoid production.

This finding further supports the critical role of 5-HT in HPA axis stimulation by psychoactive substances [16, 37].

The HPA axis activation by amphetamines may be clinically relevant. The activation reflects a pharmacological stress response and has been shown to include increases in other endocrine markers of stress including copeptin, oxytocin, epinephrine, and norepinephrine in the case of MDMA [16, 18, 36, 47-49]. In recreational settings, MDMA increased plasma cortisol levels by up to 800% [50]. These marked endocrine responses induced by psychostimulants may affect mood, energy metabolism, sleep, and immune function [15, 49, 51]. For example, D-amphetamine, methylphenidate, or MDMA increase natural killer cells in plasma reflecting activated innate immune function [15, 23] and increases in plasma concentrations of epinephrine after administration of methylphenidate and MDMA were associated with acute increases in circulating natural killer cells [15]. Increases in plasma cortisol following MDMA administration correlated with its cardiovascular effects and subjective drug liking [52]. Steroids may contribute to the mood-enhancing effects of psychostimulants [49, 52, 53], enhance the rewarding and reinforcing drug effects [20, 21], and increase the risk of misuse.

The present study has limitations. We used only one relatively high dose of lisdexamfetamine and D-amphetamine. We cannot exclude possible differences in the pharmacokinetics and endocrine effects of lisdexamfetamine and D-amphetamine at lower or higher doses than those used in the present study. Furthermore, repeated lisdexamfetamine administration may result in tolerance to the endocrine effects, which has been reported for the subjective effects with chronic use [54-56].

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Conflict of interest

The authors declare no conflicts of interest.

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Authors' contributions

PS, PCD, MEL, and AO designed the study. PS, PCD, MP, PV, DVK collected data. PS, PCD, MP, DVK, MEL, and AO analyzed data and contributed to the writing of the manuscript.

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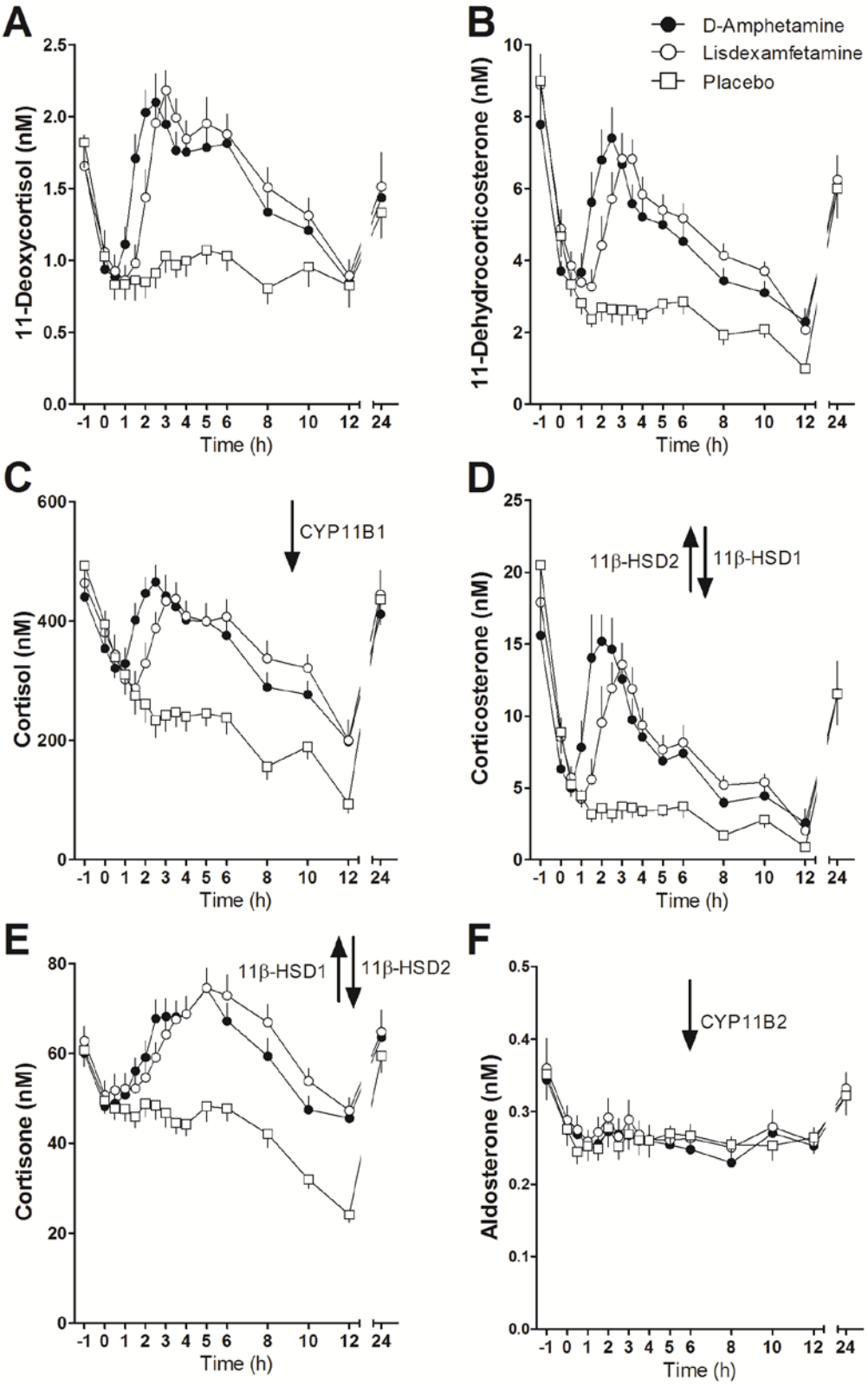

Figure 1. Plasma concentrations of glucocorticoids and mineralocorticoids (mean and SEM) following administration of D-amphetamine, lisdexamfetamine, and placebo in 23, 24, and 23 subjects, respectively. CYP = cytochrome P450; HSD = hydroxysteroid dehydrogenase. Lisdexamfetamine and D-amphetamine significantly increased the plasma concentrations of the glucocorticoids cortisol (C), cortisone (E), corticosterone (D), 11-dehydrocorticosterone (B), and 11-deoxycortisol (A) compared with placebo. The plasma concentration of aldosterone (F) was not altered after D-amphetamine and lisdexamfetamine administration compared with placebo.

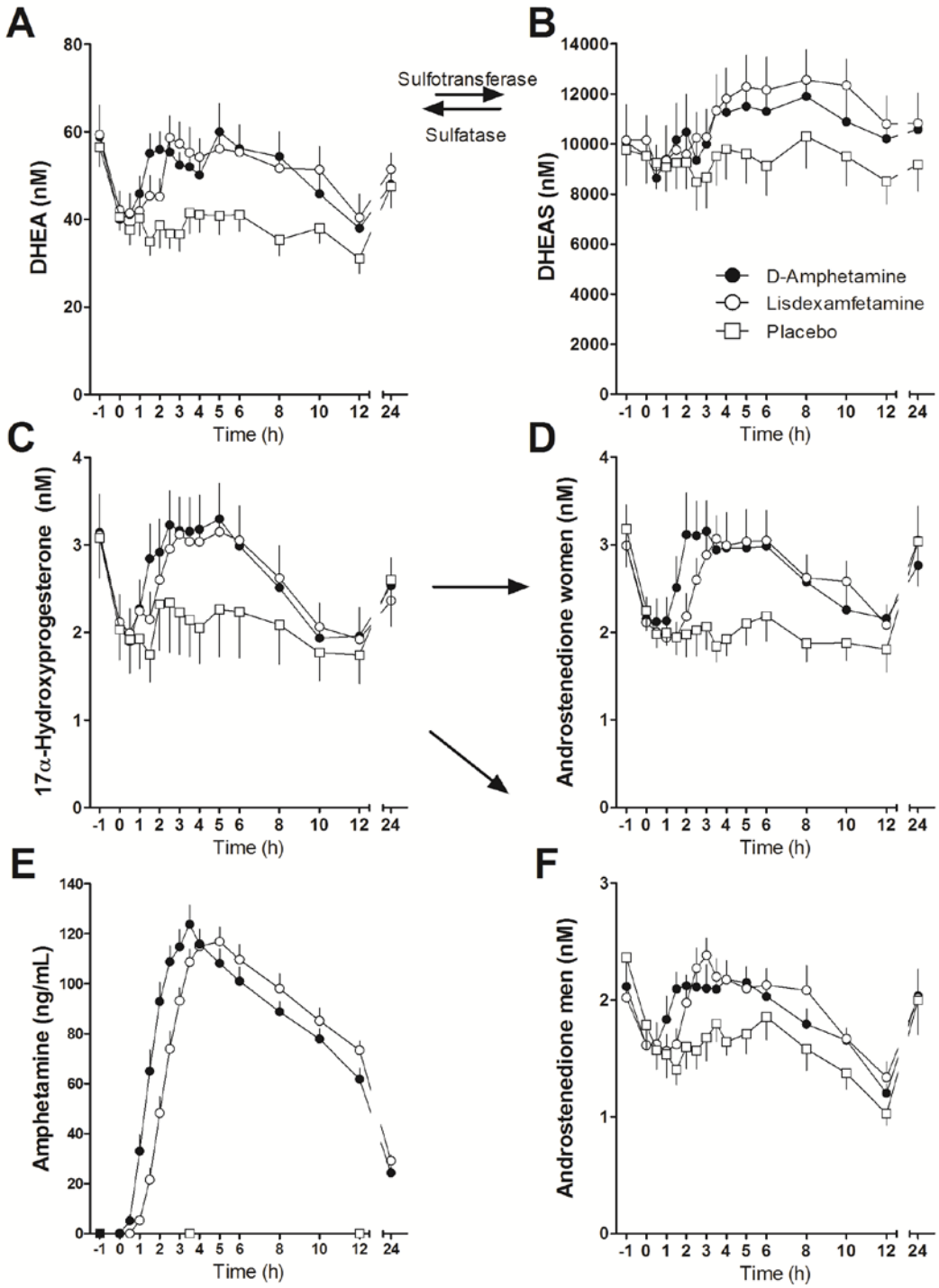

Figure 2. Plasma concentrations of androgens, one progestogen, and amphetamine (mean and SEM) following administration of D-amphetamine, lisdexamfetamine, and placebo in 23, 24, and 23 subjects, respectively. Data in men represent mean and SEM in 12 subjects. Data in women represent mean and SEM in 11, 12, 11 subjects following administration of D-amphetamine, lisdexamfetamine, and placebo, respectively. The plasma concentrations of dehydroepiandrosterone (DHEA) (A), dehydroepiandrosterone sulfate (DHEAS) (B), and androstenedione in women (D) and men (F) were significantly elevated following administration of D-amphetamine and lisdexamfetamine compared with placebo, whereas no effect was observed on 17α-hydroxyprogesterone (C). Plasma concentration-time curves of amphetamine (E) were similar after administration of lisdexamfetamine compared with D-amphetamine with the exception of a significantly later onset and therefore longer time to reach maximal concentrations. However, maximal concentrations of amphetamine and areas under the concentration-time curves were similar after the two treatments.

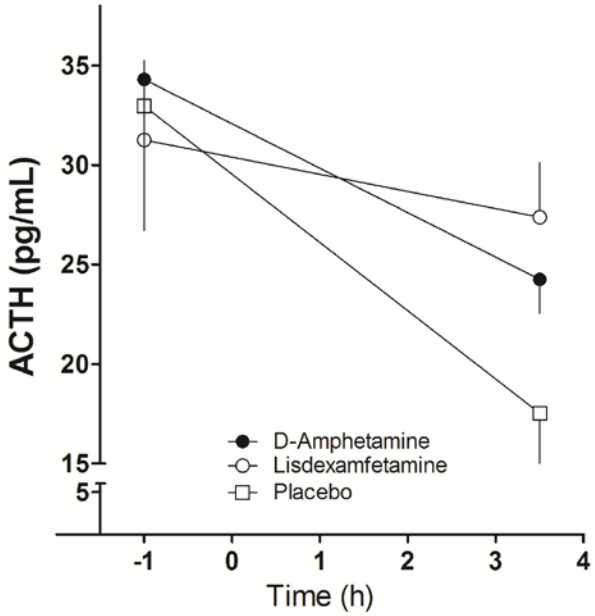

Figure 3. Plasma concentration of ACTH, measured 1 h before and 3.5 h after drug administration (mean and SEM). ACTH plasma concentrations were increased (P<0.001) at 3.5 h following administration of D-amphetamine and lisdexamfetamine compared with placebo.

Table 1. Kinetic parameters of plasma steroids and amphetamine after D-amphetamine, lisdexamfetamine, or placebo.

		D-Amphetamine	Lisdexamfetamine	Placebo	Main effect of drug ($^aF_{2,40}$)	P value
Amphetamine	T_{onset}	0.8±0.1	1.4±0.1$^{###}$			
	T_{max}	3.2±0.2	4.4±0.2$^{###}$			
	C_{max}	134±7	128±5			
	AUC_{12}	1014±47	983±42			
Glucocorticoids						
Cortisol	T_{max}	2.72 ± 0.29*	3.17 ± 0.41**	1.20 ± 0.41	6.89	<0.01
	C_{max}	534 ± 28.9***	519 ± 24.9***	417 ± 35.2	16.93	<0.001
	AUC_{12}	4116 ± 286***	4207 ± 287***	2621 ± 252	60.19	<0.001
Cortisone	T_{max}	4.39 ± 0.3*	4.67 ± 0.3**	2.80 ± 0.5	6.92	<0.01
	C_{max}	80.6 ± 4.2***	82.8 ± 4.3***	58.0 ± 3.0	42.47	<0.001
	AUC_{12}	707 ± 35.2***	739 ± 35.5***	503 ± 26.4	52.99	<0.001
Corticosterone	T_{max}	2.59 ± 0.47	3.0 ± 0.38	2.24 ± 0.5	1.71	NS
	C_{max}	22.6 ± 2.6***	20.1 ± 2.0***	11.6 ± 1.4	15.43	<0.001
	AUC_{12}	85.1 ± 6.2***	83.1 ± 6.0***	36.6 ± 3.2	59.28	<0.001
11-Dehydro-corticosterone	T_{max}	2.96 ± 0.48	2.88 ± 0.38	2.46 ± 0.55	0.66	NS
	C_{max}	8.93 ± 0.86***	8.82 ± 0.64***	5.43 ± 0.53	22.03	<0.001
	AUC_{12}	51.4 ± 4.0***	53.7 ± 3.2***	28.8 ± 2.2	63.36	<0.001
Cortisol + cortisone	T_{max}	2.93 ± 0.3*	3.65 ± 0.38***	1.20 ± 0.41	9.42	<0.001
	C_{max}	601 ± 31***	583 ± 26.5***	469 ± 36	19.44	<0.001
	AUC_{12}	4824 ± 299***	4945 ± 307***	3124 ± 266	66.47	<0.001
Ratio cortisol/cortisone	T_{max}	1.83 ± 0.44	3.10 ± 0.61	2.02 ± 0.63	2.57	NS
	C_{max}	9.42 ± 0.57	9.25 ± 0.49	8.81 ± 0.72	0.59	NS
	AUC_{12}	72.3 ± 5.1***	69.8 ± 4.0**	61.5 ± 5.2	10.50	<0.001
Corticosterone +	T_{max}	2.59 ± 0.47	3.0 ± 0.36	2.37 ± 0.54	1.34	NS

dehydro-corticosterone	C_{max}	31.2 ± 3.3***	28.5 ± 2.6***	16.9 ± 1.9	17.32	<0.001
	AUC_{12}	136 ± 9.5***	137.0 ± 8.6***	65.3 ± 5.0	70.78	<0.001
ratio corticosterone/ dehydrocorticosterone	T_{max}	2.59 ± 0.44	2.98 ± 0.43	2.39 ± 0.56	0.28	NS
	C_{max}	3.01 ± 0.24**	2.73 ± 0.14	2.24 ± 0.14	7.06	<0.01
	AUC_{12}	18.7 ± 1.1***	17.3 ± 0.93***	13.9 ± 1.03	17.27	<0.001
11-Deoxycortisol	T_{max}	3.02 ± 0.29	3.83 ± 0.32	4.22 ± 0.67	2.41	NS
	C_{max}	2.70 ± 0.17***	2.68 ± 0.17***	1.60 ± 0.14	34.33	<0.001
	AUC_{12}	17.7 ± 1.08***	18.1 ± 1.2***	11.0 ± 1.2	44.47	<0.001
Mineralocorticoids						
Aldosterone	T_{max}	4.11 ± 0.84	3.88 ± 0.73	3.43 ± 0.67	0.06	NS
	C_{max}	0.31 ± 0.03	0.34 ± 0.03	0.31 ± 0.03	1.23	NS
	AUC_{12}	3.06 ± 0.2	3.19 ± 0.21	3.12 ± 0.24	2.03	NS
11-Deoxy-corticosterone	T_{max}	3.43 ± 0.44	3.85 ± 0.48	2.98 ± 0.65	0.54	NS
	C_{max}	0.53 ± 0.08	0.57 ± 0.07	0.49 ± 0.07	2.42	NS
	AUC_{12}	5.84 ± 0.88	6.10 ± 0.87	5.45 ± 0.86	1.88	NS
Androgens						
DHEA	T_{max}	3.50 ± 0.53	4.88 ± 0.53	3.76 ± 0.7	1.45	NS
	C_{max}	80.9 ± 7.0**	78.5 ± 6.0**	57.1 ± 5.3	8.35	<0.001
	AUC_{12}	609 ± 41.1***	608 ± 43.0***	455 ± 36.9	22.01	<0.001
DHEAS	T_{max}	5.40 ± 0.68	5.80 ± 0.58	4.50 ± 0.64	1.33	NS
	C_{max}	13764 ± 1397**	14452 ± 1307***	11896 ± 1280	11.18	<0.001
	AUC_{12}	129057 ± 15047*	136822 ± 12643**	113005 ± 13334	7.10	<0.01
Androsterone	T_{max}	4.93 ± 0.89	5.08 ± 0.79	4.74 ± 0.76	0.15	NS
	C_{max}	6.93 ± 0.44	6.25 ± 0.46	6.42 ± 0.58	0.08	NS
	AUC_{12}	46.6 ± 3.6	44.1 ± 3.5	43.3 ± 4.5	0.26	NS
Androstenedione in women	T_{max}	3.36 ± 0.52	5.13 ± 0.58*	2.23 ± 0.81	5.02	<0.05
	C_{max}	3.63 ± 0.40***	3.46 ± 0.23**	2.59 ± 0.24	11.58	<0.001
	AUC_{12}	31.5 ± 3.4**	31.2 ± 2.1*	23.6 ± 2.1	8.07	<0.01

Androstenedione in men	T_{max}	3.46 ± 0.57	3.96 ± 0.51	2.83 ± 0.82	0.70	NS
	C_{max}	2.68 ± 0.18**	2.52 ± 0.18*	2.14 ± 0.19	8.42	<0.05
	AUC_{12}	22.3 ± 1.2**	23.0 ± 1.6**	18.7 ± 1.8	8.67	<0.01
Testosterone in women	T_{max}	3.32 ± 0.96	6.25 ± 0.83	3.64 ± 1.05	2.36	NS
	C_{max}	0.88 ± 0.1	0.88 ± 0.1	0.87 ± 0.14	0.15	NS
	AUC_{12}	9.25 ± 1.1	9.59 ± 1.2	8.14 ± 0.98	0.77	NS
Testosterone in men	T_{max}	4.21 ± 1.0	4.75 ± 0.66	3.50 ± 0.79	0.64	NS
	C_{max}	6.23 ± 0.40	6.17 ± 0.4	5.69 ± 0.37	1.46	NS
	AUC_{12}	62.0 ± 4.3	62.0 ± 3.8	57.0 ± 3.6	1.60	NS
Testosterone + androstenedione in women	T_{max}	3.41 ± 0.52	5.13 ± 0.58*	2.50 ± 0.78	4.14	<0.05
	C_{max}	4.45 ± 0.39**	4.30 ± 0.22*	3.40 ± 0.28	7.74	<0.01
	AUC_{12}	40.8 ± 3.5*	40.8 ± 2.2*	31.7 ± 2.4	6.83	<0.01
Testosterone + androstenedione in men	T_{max}	4.38 ± 0.96	4.13 ± 0.62	3.04 ± 0.69	0.79	NS
	C_{max}	8.50 ± 0.48	8.54 ± 0.48	7.62 ± 0.51	2.86	NS
	AUC_{12}	84.4 ± 4.7	85.0 ± 4.7	75.7 ± 4.7	3.20	NS
Progestins						
Progesterone in women	T_{max}	5.73 ± 1.3	6.54 ± 0.98	4.73 ± 1.4	0.12	NS
	C_{max}	3.38 ± 2.4	4.95 ± 3.2	1.94 ± 1.1	0.76	NS
	AUC_{12}	31.8 ± 24.4	41.6 ± 26.7	17.4 ± 11.7	0.57	NS
Progesterone in men	T_{max}	3.25 ± 0.40	3.42 ± 0.6	4.75 ± 0.94	1.53	NS
	C_{max}	0.52 ± 0.06***	0.50 ± 0.06**	0.39 ± 0.06	13.72	<0.001
	AUC_{12}	4.34 ± 0.64**	4.47 ± 0.64***	3.84 ± 0.68	13.88	<0.001
17a-Hydroxy-progesterone	T_{max}	4.20 ± 0.58	4.27 ± 0.35	4.24 ± 0.79	0.01	NS
	C_{max}	4.01 ± 0.47	3.72 ± 0.46	2.91 ± 0.58	2.80	NS
	AUC_{12}	31.2 ± 3.8	30.8 ± 4.0	24.5 ± 5.0	1.54	NS

Values are mean ± SEM in 23 and 24 subjects after administration of D-amphetamine and lisdexamfetamine. C_{max}, peak plasma concentration (nM); NS, not significant; T_{max}, time to reach C_{max} (h); AUC_{24}, area under the concentration–time curve to 24 h (ng×h/mL and nM×h and for amphetamine and steroids, respectively); DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulphate; [a]only women F (2;18) or only men F (2;22). *P<0.05, **P<0.01, ***P<0.001 compared with placebo. [###]significant difference compared with D-amphetamine. There were no significant differences in the steroide plasma concentrations between D-amphetamine and lisdexamfetamine.

Table 2. Peak effects of D-amphetamine, lisdexamfetamine, methylphenidate, MDMA, and LSD on plasma glucocorticoids.

	D-amphetamine 40 mg (N=23)	lisdexamfetamine 100 mg (N=24)	methylphenidate 60 mg (N=16)[a]	MDMA 125 mg (N=16)[a]	LSD 200 µg (N=16)[b]	$F_{4,88}$	P value
Cortisol	330.1 ± 27.4	306 ± 17.8	310.5 ± 52.2	523.7 ± 51.2**	713 ± 54.1***	17.84	<0.001
Cortisone	43.2 ± 3.9	40.7 ± 3.4	21.9 ± 2.9***	21.6 ± 2.5***	40.7 ± 3.5	10.27	<0.001
Cortisol + Cortisone	360.4 ± 30.7	333.8 ± 20	325.1 ± 51.8	532.6 ± 52.7*	743.7 ± 55.5***	16.52	<0.001
Cortisol / Cortisone	4.9 ± 0.4	4.6 ± 0.4	8.5 ± 1.6*	15.9 ± 1.5***	11.3 ± 0.9***	23.4	<0.001
Corticosterone	19.7 ± 2.7	16.5 ± 2	11.3 ± 2.3	27.2 ± 2.4	35 ± 3.7***	11.3	<0.001
11-Dehydrocorticosterone	6.5 ± 0.8	6.2 ± 0.6	2.0 ± 0.4***	4.3 ± 0.4	6.7 ± 0.8	8.43	<0.001
Corticosterone + 11-Dehydrocorticosterone	26 ± 3.4	22.1 ± 2.5	12.6 ± 2.7*	31.3 ± 2.5	41 ± 4.3**	9.85	<0.001
Corticosterone / 11-Dehydrocorticosterone	2 ± 0.3	1.6 ± 0.1	9.2 ± 3.6	17.6 ± 7.8*	3.9 ± 0.4	3.54	<0.01

Values are mean ± SEM of the peak differences from placebo. *P<0.05, **P<0.01, ***P<0.001 Tukey post hoc test compared with D-amphetamine. MDMA, 3,4-methylenedioxymethamphetamine or ecstasy; LSD, lysergic acid diethylamide. Data were adjusted from [a]Seibert et al. 2014 and [b]Strajhar et al. 2016.

5.4 Submitted manuscript: Pharmacokinetics and pharmacodynamics of lisdexamfetamine compared with D-amphetamine in healthy subjects

Original Research Article

Pharmacokinetics and pharmacodynamics of lisdexamfetamine compared with D-amphetamine in healthy subjects

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Running title: PK-PD of lisdexamfetamine and D-amphetamine

Conflict of interest: The authors declare no conflict of interest.

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Supplemental material online: Supplementary methods, Supplementary Tables S1 and S2, Supplementary Figures S1-S5

Abstract

Background and Objective Lisdexamfetamine is a prodrug of D-amphetamine, and both are used for the treatment of attention-deficit/hyperactivity disorder (ADHD). Lisdexamfetamine is thought to have a prolonged pharmacokinetic profile compared with oral D-amphetamine, possibly associated with lower euphoria, lower drug liking, and a lower risk of misuse. However, differences in the pharmacokinetics and pharmacodynamics of lisdexamfetamine and D-amphetamine have not been directly compared in healthy subjects.

Methods Equimolar doses of D-amphetamine and lisdexamfetamine, corresponding to 30 mg D-amphetamine base, and placebo were administered in 24 healthy subjects in a randomized, double-blind, placebo-controlled, cross-over study. Plasma concentrations of amphetamine**,** subjective effects, and vital signs were repeatedly assessed. The pharmacokinetic parameters were determined using compartmental modeling.

Results The increase in plasma concentrations of amphetamine had a 0.6 ± 0.6 h (mean ± SD) longer lag time and reached peak levels 1.1 ± 1.5 h later after lisdexamfetamine administration compared with D-amphetamine administration, but no differences in maximal concentrations or total exposure (area under the concentration-effect curve) were found between the two treatments. Consistent with the pharmacokinetics, the subjective and cardiovascular stimulant effects of lisdexamfetamine also occurred later compared with D-amphetamine. However, no differences in peak ratings of potentially abuse-related subjective drug effects (e.g., drug liking, drug high, stimulation, happy, well-being, and self-confidence) were observed after lisdexamfetamine administration compared with D-amphetamine administration. Lisdexamfetamine and D-amphetamine also produced similar peak increases in mean arterial blood pressure, heart rate, body temperature, pupil size, and adverse effects.

Conclusions The pharmacokinetics and pharmacodynamics of lisdexamfetamine are similar to D-amphetamine administered 1 h later. Lisdexamfetamine is likely associated with a similar risk of oral abuse as the classic stimulant D-amphetamine.

The study was registered at ClinicalTrials.gov (NCT02668926).

Key points

- The conversion of lisdexamfetamine to D-amphetamine occurs in the circulation and is thought to result in a more prolonged pharmacokinetic profile and attenuated subjective effects and abuse risk compared with D-amphetamine.
- The study compared pharmacokinetics of lisdexamfetamine and D-amphetamine directly within-subjects.
- Lisdexamfetamine and D-amphetamine produced similar peak plasma amphetamine concentrations and subjective and cardiovascular peak responses.
- The risk of oral misuse of lisdexamfetamine is likely similar to D-amphetamine.

Keywords: D-amphetamine, lisdexamfetamine, subjective effects, autonomic effects, healthy subjects

1. Introduction

Lisdexamfetamine is an inactive prodrug formulation of D-amphetamine [1-3] that is marketed for the treatment of attention-deficit/hyperactivity disorder (ADHD). D-amphetamine is a stimulant drug that is used as a second-line treatment for ADHD. D-amphetamine is similarly or even more effective than methylphenidate in the treatment of ADHD [4]. However, amphetamine is also misused recreationally to induce euphoria or enhance cognitive performance, with a lifetime use prevalence of 5.5-15% in adults [5, 6]. Lisdexamfetamine is thought to have lower abuse potential than D-amphetamine [7]. The conversion of lisdexamfetamine to its active metabolite D-amphetamine occurs in the circulation [8, 9]. When lisdexamfetamine is misused by intranasally or intravenously, the pharmacokinetics are similar to oral use [10, 11], and the subjective effects are not enhanced by parenteral administration. Intravenous lisdexamfetamine use also produced significantly lower increases in “drug liking” and “stimulant effects” compared with D-amphetamine in intravenous substance users [12]. After oral administration, the conversion of lisdexamfetamine to D-amphetamine is thought to occur gradually, reportedly resulting in a prolonged pharmacokinetic profile with low peak but sustained plasma amphetamine concentrations [12, 13]. Such a prolonged pharmacokinetic profile is considered to be associated with slower effects on dopamine release, lower euphoric effects, and a possibly lower risk of misuse [7, 12, 14]. This view is supported by animal studies. In rats, the peak plasma concentration (C_{max}) of amphetamine was lower after lisdexamfetamine, and it produced a gradual and sustained increase in dopamine efflux and much less locomotor activity compared with D-amphetamine [15]. Thus, in this rat study, lisdexamfetamine was shown to have markedly less stimulant effects than an equivalent dose of D-amphetamine [15]. However, differences in the pharmacokinetics of lisdexamfetamine and D-amphetamine after oral administration have not been studied. Additionally, we are aware of only one study that directly compared the acute pharmacodynamic effects of lisdexamfetamine and D-amphetamine within-subjects in humans [12]. In current stimulant users, 100 mg lisdexamfetamine produced significantly lower subjective “drug liking” than an equivalent dose of 40 mg D-amphetamine [12]. However, subjective drug effects were comparable on the morphine-benzedrine scale

(euphoria), amphetamine scale, and benzedrine (stimulation) scale of the Addiction Research Center Inventory (ARCI) [12]. Nonetheless, this previous study [12] did not assess the pharmacokinetics of amphetamine to demonstrate the equivalence of the doses used. Additionally, data from healthy non-stimulant-using subjects are lacking, and no industry-independent studies have been conducted. Therefore, in the present study, we directly compared both pharmacokinetic and pharmacodynamic differences between equimolar oral doses of lisdexamfetamine and D-amphetamine in healthy, non-stimulant-using subjects. Based on data from animal studies [15] and limited human data [12], we hypothesized that lisdexamfetamine would have (*i*) a longer time to C_{max} (T_{max}) than D-amphetamine, (*ii*) a lower C_{max} than D-amphetamine, (*iii*) an area under the amphetamine concentration-time curve that is identical to D-amphetamine, (*iv*) a longer T_{max}, (*v*) a smaller maximal effect (E_{max}) than D-amphetamine, and (*vi*) an area under the observed subjective drug effect-time curve that is identical to D-amphetamine.

2. Methods

2.1. Study design

The present study used a double-blind, placebo-controlled, cross-over design with three experimental test days (D-amphetamine, lisdexamfetamine, and placebo) in balanced order. The washout periods between sessions were at least 7 days. The study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization Guidelines in Good Clinical Practice and approved by the Ethics Committee northwest/central Switzerland (EKNZ) and Swiss Agency for Therapeutic Products (Swissmedic). All of the subjects provided written consent before participating in the study, and they were paid for their participation. The study was registered at ClinicalTrials.gov (NCT02668926).

2.2. Participants

Twenty-four healthy subjects (12 men, 12 women) with a mean ± SD age of 25.3 ± 3.0 years (range: 21-34 years) were recruited from the University of Basel. Inclusion criteria were

age 18-45 years, body mass index 18-27 kg/m^2, and birth control for women. Subjects with a personal or first-degree-relative history of psychiatric disorders or chronic or acute physical illness were excluded. Additional exclusion criteria were tobacco smoking (> 10 cigarettes/day), the consumption of alcoholic drinks (> 10/week), and a lifetime history of using illicit drugs more than five times, with the exception of occasional cannabis use in the past. Subjects who used any illicit drugs, including cannabis, within the past 2 months or during the study period were excluded. The subjects were asked to abstain from excessive alcohol consumption between test sessions and not to drink caffeine-containing liquids after midnight before the study day. We performed drug tests at screening and before each test session using TRIAGE 8 (Biosite, San Diego, CA, USA). Female subjects were investigated during the follicular phase of their menstrual cycle (day 2-14) to account for cyclic changes in the reactivity to D -amphetamine [16].

2.3. Study procedures

The study included a screening visit, three experimental sessions (test days), and an end-of-study visit. Experimental sessions began at 8:00 AM. An indwelling intravenous catheter was placed in an antecubital vein for blood sampling. A single oral dose of D-amphetamine, lisdexamfetamine, or placebo was administered at 9:00 AM. Autonomic and subjective drug effects were assessed repeatedly throughout the session. For the analysis of amphetamine concentrations in plasma, blood samples were collected in lithium heparin tubes 1 h before and 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, and 24 h after drug administration. The blood samples were immediately centrifuged, and the plasma was rapidly stored at -20°C and later at -80°C until analysis. During the test sessions, the subjects did not engage in any physical activity, were resting in hospital beds in a calm standard hospital room, and were served a standardized lunch and dinner at 11:30 AM and 6:30 PM, respectively. The test session ended at 9:00 PM. The subjects returned home and returned the following day at 9:00 AM for the final 24 h measurements and drawing of blood samples.

2.4. Study drugs

Gelatin capsules that contained either lisdexamfetamine dimesylate (100 mg salt; Opopharma, Rümlang, Switzerland) or D-amphetamine sulfate (40.3 mg salt; Hänseler, Herisau, Switzerland), both corresponding to an equivalent dose of 29.6 mg D-amphetamine, and placebo capsules (mannitol) were prepared by the pharmacy of the University Hospital Basel according to Good Manufacturing Practice. To induce greater subjective drug liking and mimic misuse, the selected dose of lisdexamfetamine was relatively high and above the upper recommended daily dose of 70 mg.

2.5. Measures

2.5.1. Quantification of amphetamine concentrations in blood plasma

Plasma concentrations of amphetamine were measured by ultra-high pressure liquid chromatography-mass spectrometry/mass spectrometry. The materials, procedures, and method validation are described in detail in the Supplementary Material. Lower limits of detection and quantification were 0.26 ng/ml and 0.78 ng/ml, respectively.

2.5.2. Subjective effects

Visual Analog Scales (VASs) were repeatedly used to assess subjective effects over time. The VASs included “any drug effect,” “good drug effect,” “bad drug effect,” “drug liking,” “drug high,” “stimulated,” “alertness,” “content,” “happy,” “closeness to others,” “talkative,” “open,” “concentration”, “trust”, and “want to be with others” and have previously been used [17, 18]. The VASs were presented as 100-mm horizontal lines (0 to +100), marked from “not at all” on the left to “extremely” on the right. The VASs for “happy,” “closeness to others,” “open,” “trust”, and “I want to be with others” were bidirectional (± 50), marked from “not at all” on the left (-50), to “normal” in the middle (0), to “extremely” on the right (+50). The VASs were administered 1 h before and 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, and 24 h after drug administration.

The 60-item Adjective Mood Rating Scale (AMRS) [19] was administered 1 h before and 2, 3, 4, 12, and 24 h after drug administration. The AMRS subscales for well-being, extroversion, emotional excitability, and self-confidence have previously been shown to be sensitive to the effects of psychostimulants [20, 21].

2.5.3. Autonomic effects

Blood pressure, heart rate, and tympanic body temperature were repeatedly measured 1 h before and 0, 0.5, 1, 1.5, 2.0, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 24 h after drug administration. Diastolic and systolic blood pressure and heart rate were measured using an automatic oscillometric device (OMRON Healthcare Europe NA, Hoofddorp, Netherlands). The measurements were performed in duplicate at an interval of 1 min and after a resting time of at least 10 min. The averages were calculated for analysis. The rate-pressure product was calculated as systolic blood pressure × heart rate. Core (tympanic) temperature was measured using an GENIUSTM 2 ear thermometer (Tyco Healthcare Group LP, Watertown, NY, USA). Pupillometry was performed 1 h before and 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, and 24 h after drug administration using a hand-held PRL 200 infrared pupillometer (NeurOptics, Irvine, CA). Pupil function was measured under standardized dark-light conditions and assessed by a Voltcraft MS-1300 luxmeter (Voltcraft, Hirschau, Germany) following a dark adaption time of 1 min as previously described [22].

2.5.4. Adverse effects

Adverse effects were assessed 1 h before and 12 h (acute) and 24 h (sub-acute) after drug administration using the 66-item List of Complaints [23]. The scale yields a total adverse effects score and reliably measures physical and general discomfort.

2.6. Pharmacokinetic analyses

All of the pharmacokinetic and pharmacodynamic analyses were performed using Phoenix WinNonlin 6.4 (Certara, Princeton, NJ, USA). Pharmacokinetic parameters were

estimated using compartmental modeling. A one-compartment model was used with first-order input, first-order elimination, and lag time. Initial estimates were derived from non-compartmental analyses. The model fit was assessed by visual inspection and Akaike information criteria. The model fit was impaired without lag time and not relevantly improved by a two-compartment model. A non-compartmental analysis was also performed prior to the modeling. Peak plasma concentration (C_{max}) and time to C_{max} (T_{max}) were obtained directly from the observed data. The terminal elimination rate constant (λ_z) was estimated by log-linear regression after semi-logarithmic transformation of the data using at least three data points of the terminal linear phase of the concentration-time curve. The area under the concentration-time curve (AUC) from 0 to 24 h after dosing (AUC_{24}) was calculated using the trapezoidal method. The AUC to infinity (AUC_{∞}) was determined by extrapolation of the AUC_{24} by using λ_z.

The lisdexamfetamine- and D-amphetamine-induced subjective and autonomic effects were determined as differences from placebo in the same subject at corresponding time points to control for circadian changes and placebo effects. Maximal effect (E_{max}) and the time to reach E_{max} (T_{max}) of the pharmacodynamic response were determined directly from the observed effect-time curves. The area under the observed effect-time curve (AUEC) was determined using the trapezoidal method. The onset of the response was determined using the effect-time curve, with 10% of the individual maximal response as the threshold. To assess the amphetamine exposure-effect relationship, the changes in pharmacodynamic effect after lisdexamfetamine and D-amphetamine administration for each time point were plotted against the respective plasma concentrations of amphetamine (hysteresis plots).

2.7. Statistical analyses

The data were analyzed using repeated-measures analysis of variance (ANOVA), with drug as the within-subjects factor. Repeated measures are expressed as E_{max} and AUEC values prior to the ANOVA. Tukey *post hoc* comparisons were performed based on significant main effects of drug. Plasma amphetamine concentrations after administration of lisdexamfetamine-

and D-amphetamine and differences from placebo were compared using paired *t*-tests. The criterion for significance was $p < 0.05$.

3. Results

3.1. Pharmacokinetics

The plasma amphetamine concentration-time curves after D-amphetamine and lisdexamfetamine administration are shown in Fig. 1. Individual plots are shown in Supplementary Fig. S1. The corresponding pharmacokinetic parameters that were derived from the compartmental and non-compartmental analyses are shown in Table 1 and Supplementary Table S1, respectively. As planned, the administration of equimolar doses of D-amphetamine and lisdexamfetamine resulted in similar AUC values. The increase in plasma amphetamine concentrations had a 0.6 ± 0.6 h (mean ± SD) longer lag time and reached peak levels 1.1 ± 1.5 h later after lisdexamfetamine administration compared with D-amphetamine administration (Fig. 1, Table 1, Supplementary Table S1). Both T_{lag} and T_{max} values were significantly different ($t = 2.87$, $p < 0.001$, and $t = 3.54$, $p < 0.001$, respectively; Table 1) between the two active drug conditions. However, the absorption constant, K_{01}, was only nonsignificantly greater after D-amphetamine administration compared with lisdexamfetamine administration ($t = 1.86$, $p = 0.07$). C_{max} values were similar (Table 1, Supplementary Table S1) after the administration of both drugs. Thus, in contrast to our hypothesis, a curve shift was observed, but no relevant difference in the shape or peak size of the two amphetamine concentration-time curves was found (Fig. 1).

3.2. Subjective effects

Subjective drug effects over time are shown in Fig. 2. Lisdexamfetamine and D-amphetamine produced similar increases in VAS and AMRS scores compared with placebo (Fig. 2 and Supplementary Fig. S2, respectively; Table 1, Supplementary Table S2). The subjective drug effect-time curves were shifted to the right because of significantly longer T_{onset} and T_{max} values after lisdexamfetamine administration compared with D-amphetamine

administration, consistent with the pharmacokinetics of the two drugs. However, no differences in E_{max} or AUEC values were found between lisdexamfetamine and D-amphetamine. After both lisdexamfetamine and D-amphetamine administration, the subjective drug effect-concentration curves revealed similar clockwise hysteresis, indicating similar extents of acute pharmacological tolerance to lisdexamfetamine and D-amphetamine (Supplementary Fig. S3).

3.3. Autonomic effects

Vital signs over time are shown in Fig. 3. Lisdexamfetamine and D-amphetamine produced similar increases in blood pressure, heart rate, body temperature, and pupil size (Fig. 3, Supplementary Fig. S4, Table 2, Supplementary Table S2). The blood pressure-time curves were shifted to the right because of significantly longer T_{onset} values after lisdexamfetamine administration compared with D-amphetamine administration (Fig. 3, Supplementary Table S2). Diastolic blood pressure reached significantly higher values after D-amphetamine administration compared with lisdexamfetamine administration (Table 2). No differences were found in the placebo-adjusted increases in diastolic blood pressure (Supplementary Table S2), mean arterial pressure, or rate-pressure product, indicating similar overall cardiovascular stimulant effects after the two treatments (Table 2). After both lisdexamfetamine and D-amphetamine administration, the blood pressure responses returned to baseline faster than the plasma levels of amphetamine (Fig. 1, Fig. 3), whereas the heart rate responses increased more slowly and remained high up to 24 h. The blood pressure-concentration plot presented clockwise hysteresis, similar to the subjective drug effect-concentration plots, indicating acute pharmacological tolerance (Supplementary Fig. S5). In contrast, the heart rate responses presented counterclockwise hysteresis in the effect-concentration plots, indicating that the responses lagged behind the changes in plasma concentration, with no tolerance (Supplementary Fig. S5) to the effects of either lisdexamfetamine or D-amphetamine. These results indicate that there were no differences in the effect-concentration relationships between lisdexamfetamine and D-amphetamine.

3.4. Adverse effects

Both lisdexamfetamine and D-amphetamine increased acute and subacute adverse effect ratings compared with placebo (Table 2). Acute adverse effects mainly included a lack of appetite and dry mouth in most of the subjects. Subacute adverse effects mainly included insomnia in most of the subjects after both treatments.

4. Discussion

The present study compared the pharmacokinetics and pharmacodynamics of lisdexamfetamine and D-amphetamine within-subjects in healthy volunteers. In contrast to our hypothesis, no differences were found in the peak plasma concentrations of amphetamine and the associated subjective and cardiovascular peak effects between lisdexamfetamine and D-amphetamine. Increases in the plasma concentrations of amphetamine occurred an average of 0.6 h later and reached peak levels 1.1 h later after lisdexamfetamine administration compared with D-amphetamine administration, but the amphetamine concentration-time and drug effect-time curves were otherwise comparable between treatments. Thus, the pharmacokinetics and pharmacodynamics of a high dose of the newly marketed medication lisdexamfetamine were practically identical to an equimolar dose of the classic immediate-release D-amphetamine administered 1 h later. The present data indicate that the conversion of the prodrug lisdexamfetamine to D-amphetamine slightly delays the onset of the increase in amphetamine concentrations in the body without causing relevant alterations in the slope or maximal concentrations.

Pharmacokinetic factors, such as rapid drug delivery to the brain, are important predictors of abuse liability [24-26]. Substances with a slow absorption rate are less likely to be abused than drugs with a rapid absorption rate [24, 25, 27]. A slow rise of simulant blood concentration, which is usually observed with extended-release formulations, is associated with lower subjective effects and possibly lower abuse potential [26, 28]. Lisdexamfetamine was reportedly developed with the goal of providing a long duration of action and lower abuse potential [12, 13]. Preliminary unpublished data that were reported in a previous study [12]

indicated a longer T_{max} and lower C_{max} of D-amphetamine from lisdexamfetamine than from D-amphetamine. However, the present study found no such difference in C_{max} values after lisdexamfetamine and D-amphetamine administration. A previous study compared the pharmacodynamics (but not pharmacokinetics) of lisdexamfetamine and D-amphetamine and found lower peak ratings of drug liking in current stimulant users after lisdexamfetamine administration compared with D-amphetamine administration using the same doses as in the present study [12]. However, ratings of drug liking in the stimulant users reached mean peak levels that were only 17% of the scale maximum after administration of 40 mg D-amphetamine [12]. In the present study, mean ratings reached 51% and 48% of peak scale levels in the healthy and mostly stimulant-naive subjects after D-amphetamine and lisdexamfetamine administration, respectively. Additionally, lisdexamfetamine and D-amphetamine produced similar peak euphoria and amphetamine effects on the ARCI and cardiovascular effects and were reported by stimulant users to have similar abuse-related monetary street value [12]. These latter findings in stimulant users are consistent with our results, in which we found no relevant differences between the pharmacokinetics and pharmacodynamics of lisdexamfetamine and D-amphetamine. A study in adults with ADHD also reported comparable cardiovascular stimulation after administration of 50 mg lisdexamfetamine and 20 mg of mixed immediate-release amphetamine salts [29]. An analysis of exposures that were reported to poison centers reported overall similar clinical effects of lisdexamfetamine and D-amphetamine, including agitation, tachycardia, and hypertension [30]. A marked increase in reported lisdexamfetamine misuse cases was reported to poison centers between 2007 and 2012, resulting in more cases associated with lisdexamfetamine than immediate-release D-amphetamine [30].

Intranasal and intravenous lisdexamfetamine use has been shown to result in delayed and reduced subjective effects [10, 11]. In contrast, the minimal changes in the oral pharmacokinetics and pharmacodynamics of lisdexamfetamine compared with D-amphetamine that were observed in the present study did not relevantly slow the rise of amphetamine concentrations or subjective effects and thus were not sufficient to reduce the abuse potential of oral lisdexamfetamine use. In contrast, clear differences were found between the kinetics of

extended-release and immediate-release formulations [28, 31] and possibly also between extended-release formulations and lisdexamfetamine [32, 33].

The present study has limitations. We used only one relatively high dose of lisdexamfetamine and D-amphetamine. We cannot exclude possible differences in the pharmacokinetics and pharmacodynamics of lisdexamfetamine and D-amphetamine at lower or higher doses than those used in the present study. Additional studies that administer 50 and 150 mg lisdexamfetamine and 20 and 60 mg D-amphetamine, respectively, would be needed to further validate the present findings. The recommended doses of lisdexamfetamine for the treatment of ADHD are 30-70 mg/day, with an initial dose of 30 mg. Thus, the present study used a higher single dose (100 mg) in non-treated subjects to mimic the misuse of lisdexamfetamine rather than therapeutic steady-state conditions. Additionally, our subjects were fasted when the drugs were administered. T_{max} values have been reported to be prolonged by approximately 1 h in the fed state compared with the fasted state [34]. Furthermore, repeated lisdexamfetamine administration results in tolerance to the pronounced subjective and cardiostimulant effects, which has been reported with chronic use [13, 35, 36]. Similarly, acute insomnia was observed in the majority of the subjects after the single high-dose administration of lisdexamfetamine in the present study, but lisdexamfetamine was not associated with sleep disturbances when used chronically [37-39].

We are unaware of published direct comparisons of the pharmacokinetics of lisdexamfetamine and D-amphetamine. The present pharmacokinetic data for lisdexamfetamine and D-amphetamine are consistent with previous investigations of either formulation alone [2, 13, 40-43]. The present study showed that plasma amphetamine concentrations remained high after both lisdexamfetamine and D-amphetamine administration, with similarly long plasma elimination half-lives, consistent with previous studies [2, 40, 44]. However, the present study illustrates that acute tolerance develops to the subjective drug effects, which were similar for both formulations. This means that the subjective stimulant drug effect lasts only up to 8 h, but plasma concentrations of amphetamine remain high. Interestingly, in contrast to the subjective response, the cardiovascular effects did not present acute tolerance and remained high in

parallel with the plasma drug concentrations. The blood pressure response to both formulations presented clockwise hysteresis, but this effect was offset by counterclockwise hysteresis for heart rate that resulted from a net effect of no tolerance to the rate-pressure product. Similarly, previous studies showed that D-amphetamine rapidly increased blood pressure to peak values within 3 h after drug administration but produced only a very moderate and slow increase in heart rate [40, 44, 45]. Altogether, the findings indicate that D-amphetamine primarily induced a blood pressure response, whereas the heart rate response was reduced, likely via the baroreceptor reflex. As a result, the rate-pressure product better reflected the cardiovascular stimulation that was induced by D-amphetamine than the blood pressure or heart rate response alone, and the rate-pressure product also better reflected the plasma concentrations of amphetamine. In contrast to D-amphetamine, methylphenidate induced rapid and parallel elevations in both blood pressure and heart rate [17, 20, 45]. The mechanisms of the differential effects of D-amphetamine and methylphenidate on blood pressure and heart rate are unclear. Additionally, lisdexamfetamine and D-amphetamine significantly increased pupil size similarly to the amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA) [22], but comparable mydriatic effects were not induced by methylphenidate [20]. Thus, the autonomic effects of D-amphetamine and methylphenidate are different, despite their common stimulant action on the catecholamine systems.

Similar to the present study, previous studies reported the development of acute tolerance to the subjective but not cardiostimulant effects of D-amphetamine in healthy volunteers [40, 44]. No tolerance to the cardiostimulant effects of 60 mg methylphenidate was found [20]. However, in contrast to the present study, no tolerance to the subjective effects of methylphenidate was observed [20]. Another amphetamine derivative and serotonin and norepinephrine releaser, MDMA, also presented marked acute pharmacological tolerance to both subjective and cardiovascular effects [20, 46]. Tolerance was also observed after repeated daily oral administration of 10 mg methamphetamine [47]. The present observations of differential tolerance to the subjective and cardiovascular effects of D-amphetamine may have clinical implications. First, it indicates that pharmacokinetic properties may not necessarily

predict subjective stimulant effects. The clinical therapeutic response may be subject to similar tolerance, although the behavioral and motor activity responses in children have been reported to be related to the time course of plasma amphetamine levels [41]. Second, the mechanisms that underlie acute tolerance need to be further studied. For example, no tolerance was found to the subjective and cardiovascular effects of a direct serotonin receptor agonist [48], but tolerance was found to the effects of an indirect serotonin receptor agonist that releases serotonin from presynaptic terminals via the serotonin transporter [46]. D-amphetamine acts as an indirect dopamine and norepinephrine agonist and releases these catecholamines via their respective monoamine transporters [49]. In contrast, methylphenidate acts only as an inhibitor of the dopamine and norepinephrine transporter, without inducing their release [50]. Both stimulants show no relevant effects on monoamine receptors beyond their interaction with their transporters [49, 50]. Thus, monoamine depletion through release could potentially explain the phenomenon of acute tolerance to the subjective effects of D-amphetamine, in contrast to pure uptake inhibition by methylphenidate. However, this assumption is speculative and needs further study.

In rats, counterclockwise hysteresis was observed between the plasma concentration of amphetamine and locomotor activity after administration of lisdexamfetamine, but no such hysteresis was observed after D-amphetamine administration [15]. Additionally, counterclockwise hysteresis was observed between dopamine concentrations in the striatum and locomotor activity after lisdexamfetamine administration, but clockwise hysteresis was observed after D-amphetamine administration [15]. In contrast to these preclinical data, no differences were found in the hysteresis curves between lisdexamfetamine and D-amphetamine in the present study, further supporting the similarity of the two formulations.

In conclusion, the single oral dose pharmacokinetics and pharmacodynamics of lisdexamfetamine were similar to immediate-release D-amphetamine, although lisdexamfetamine had a longer lag time for the increase in plasma amphetamine concentration and subjective response. The risk of oral misuse of lisdexamfetamine is likely similar to D-amphetamine.

Compliance with ethical standards

The study was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee. All of the subjects provided written consent before participating in the study, and they were paid for their participation.

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Conflict of interest

The authors declare no conflicts of interest.

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Author contributions

Patrick Dolder: designed the research, performed the research, analyzed the data. Petra Strajhar: designed the research, performed the research, analyzed the data. Patrick Vizeli: performed the research, analyzed the data. Felix Hammann: performed the research. Alex Odermatt: designed the research. Matthias E. Liechti: designed the research, analyzed the data, wrote the manuscript.

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Figure Legends

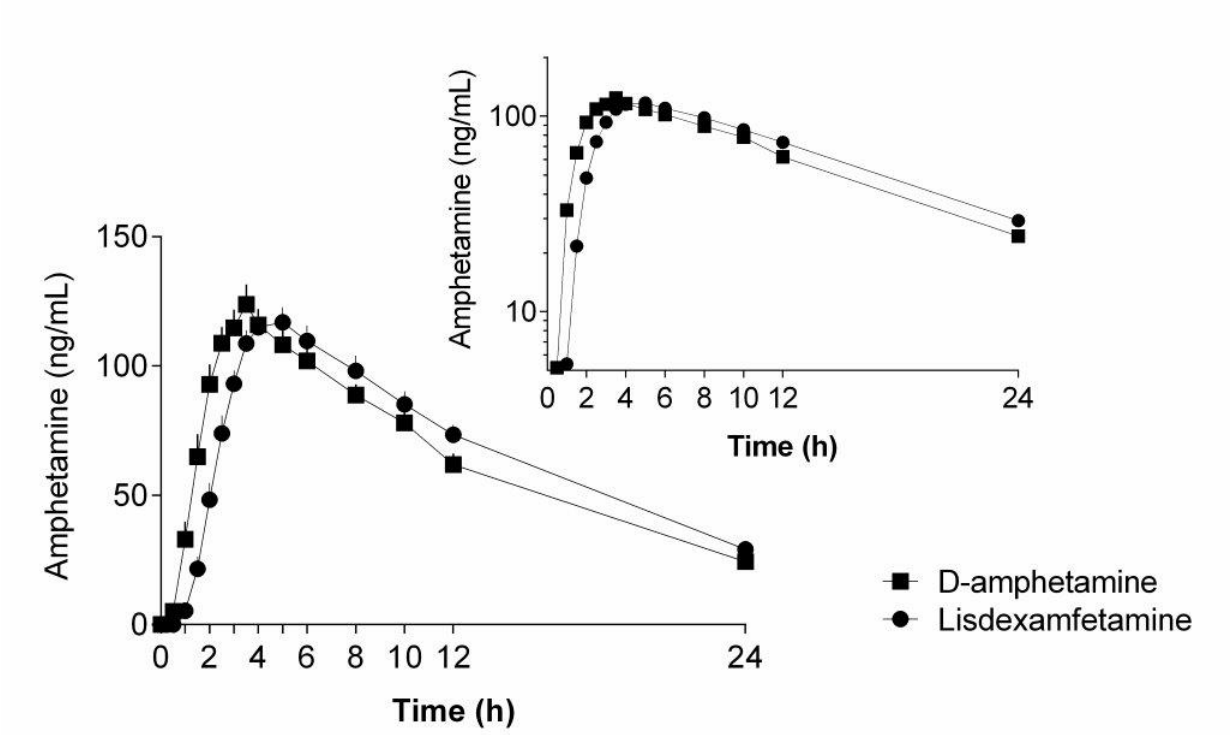


Figure 1. Amphetamine concentration-time curves (mean ± SEM) in 24 and 23 subjects after administration of lisdexamfetamine and D-amphetamine, respectively. The onset and peak times of the amphetamine concentration-time curve were longer after lisdexamfetamine administration compared with D-amphetamine administration, but no differences were found in the maximal concentrations, areas under the concentration-time curves, or absorption or elimination constants between the two treatments. The inset shows the semilogarithmic plot. The amphetamine concentration-time curves were shifted to the right after lisdexamfetamine administration compared with D-amphetamine administration but were otherwise almost identical. The drugs were administered at t = 0. The corresponding pharmacokinetic parameters were derived from compartmental and non-compartmental analyses and are shown in Table 1 and Supplementary Table S1, respectively.

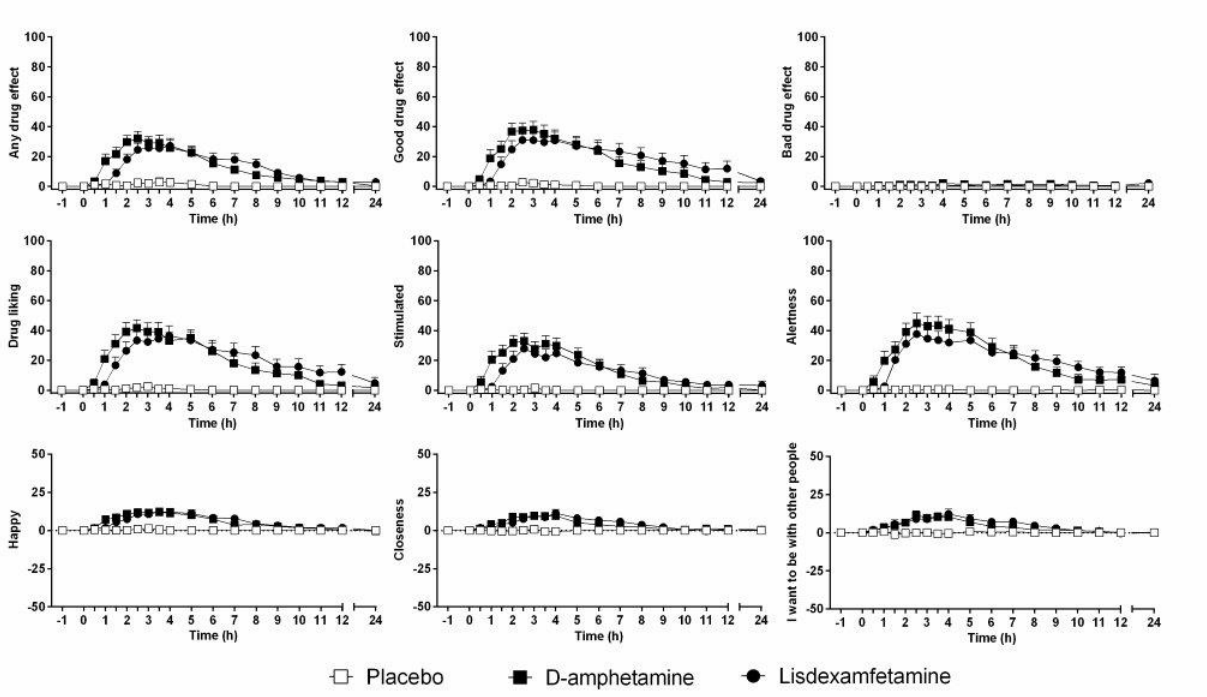


Figure 2. Lisdexamfetamine and D-amphetamine produced similar subjective responses compared with placebo. The effect onset and maximal response were nonsignificantly delayed after lisdexamfetamine administration compared with D-amphetamine administration, but the maximal effects and curve shapes were similar. The data are expressed as the mean ± SEM in 24 subjects.

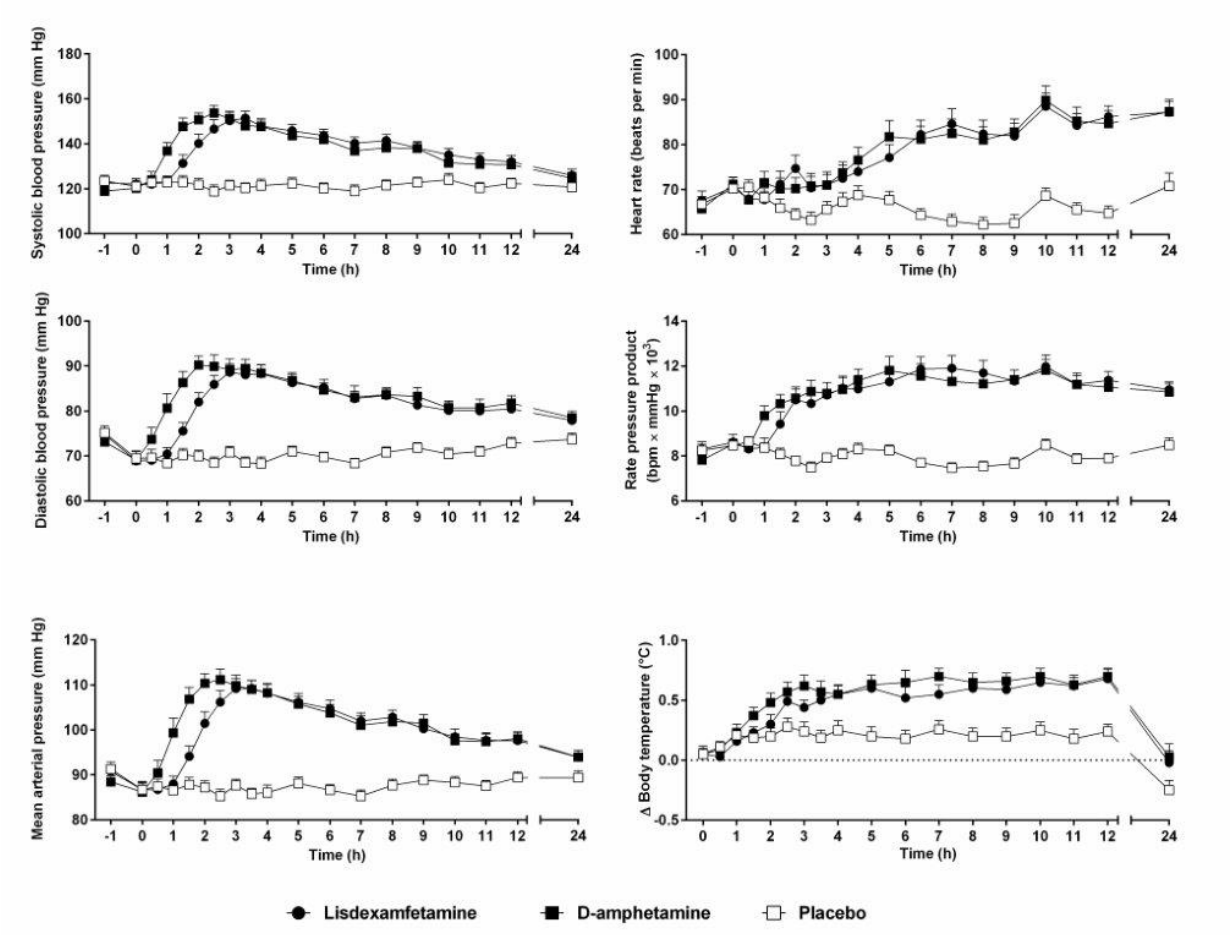


Figure 3. Lisdexamfetamine and D-amphetamine produced similar cardiostimulant responses compared with placebo. The blood pressure response onset was delayed and the diastolic pressure response was reduced after lisdexamfetamine administration compared with D-amphetamine administration. However, the rate-pressure product, reflecting the overall cardiovascular response, similarly increased after both active treatments compared with placebo. The data are expressed as the mean ± SEM in 24 subjects.

Table 1. Pharmacokinetic parameters of amphetamine based on compartmental modeling

Dose	N=		k_{01} (1/h)	λ(1/h)	V_d (L)	C_{max} (ng/mL)	t_{lag}	t_{max} (h)	$t_{1/2}$ (h)	AUC_∞ (ng·h/mL)	CL/F (L/h)
D-amphetamine	23	geometric mean (95% CI)	1.3 (0.84-1.95)	0.088 (0.077-0.101)	195 (172-220)	120 (108-133)	0.8 (0.6-1.0)	3.3 (2.7-3.9)	7.9 (6.9-9.1)	1727 (1540-1935)	17 (15-19)
		range	0.41-17	0.046-0.162	113-375	77-181	0.3-2.0	0.9-5.9	4.3-15	1116-3463	9-27
Lisdexamfetamine	24	geometric mean (95% CI)	0.78 (0.63-0.98)	0.088 (0.078-0.098)	186 (166-209)	118 (108-128)	1.5 (1.3-1.7)***	4.6 (4.1-5.2)***	7.9 (7.1-8.9)	1817 (1637-2017)	16 (15-18)
		range	0.25-1.9	0.055-0.148	88-266	83-174	0.8-2.4	2.5-8.4	4.7-13	1087-3031	10-27

AUC_∞, area under the plasma concentration-time curve from time zero to infinity; C_{max}, estimated maximum plasma concentration; $t_{1/2}$, estimated plasma elimination half-life; t_{lag}, lag time or time; t_{max}, estimated time to reach $C_{max;}$ k_{01}, first-order absorption koefficient; λ, first order elimination coefficient; V_d, volume of distribution.
***P<0.001 compared with D-amphetamine.

Table 2. Comparison of the maximal pharmacodynamic effects of lisdexamfetamine and D-amphetamine

		Placebo (mean ± SEM)	Lisdexamfetamine (mean ± SEM)	D-amphetamine (mean ± SEM)	Main effect of drug $F_{2,46}$
Autonomic effects					
Systolic blood pressure (mmHg)	E_{max}	131±2.7	157±3.1***	158±2.8***	106.56
Diastolic blood pressure (mmHg)	E_{max}	79±1.1	93±1.7***	97±1.8***#	93.97
Mean arterial blood pressure (mmHg)	E_{max}	96±1.2	114±2.0***	116±1.8***	111.47
Heart rate (beats/min)	E_{max}	76±1.5	94±3.1***	94±3.4***	28.42
Rate pressure product (beats·mmHg/min)	E_{max}	9655±236	13083±561***	13245±603***	43.01
Body temperature (°C)	E_{max}	37.3±0.07	37.7±0.06***	37.7±0.07***	24.05
Pupil size (mm)	E_{max}	6.8±0.09	7.4±0.11***	7.4±0.10***	79.56
Pupil size after light stimulus (mm)	E_{max}	5.0±0.08	5.8±0.11***	5.7±0.11***	55.46
Constriction amplitude (mm)	E_{min}	1.7±0.04	1.5±0.08	1.6±0.05	5.03
Subjective effects					
Visual Analoge Scale (VAS, %max)					
Any drug effect	E_{max}	5.3±3.1	36±4.9***	39±4.8***	27.31

Good drug effect	E_{max}	4.0±2.5	42±6.5***	49±5.6***	36.65
Bad drug effect	E_{max}	0.04±0.04	5.1±1.9*	4.0±1.3	4.81
Drug liking	E_{max}	3.7±2.6	48±6.9***	51±5.8***	37.57
Drug high	E_{max}	3.3±2.6	29±6.3***	36±5.6***	16.98
Stimulated	E_{max}	2.4±1.7	38±6.9***	44±5.7***	24.97
Alertness	E_{max}	2.4±1.1	50±7.2***	56±6.4***	41.31
Content	E_{max}	1.1±0.63	19±3.0***	18±2.9***	24.93
Happy	E_{max}	1.7±1.3	18±2.9***	17±2.8***	21.77
Closeness to others	E_{max}	0.79±0.79	15±2.8***	15±2.5***	19.01
Talkative	E_{max}	1.3±1.0	23±2.7***	21±2.3***	39.33
Open	E_{max}	0.88±0.79	22±2.6***	22±2.7***	43.55
Concentration	E_{max}	0.38±0.27	21±3.1***	16±2.8***	26.97
Trust	E_{max}	0.96±0.96	15±2.6***	17±3.1***	21.18
I want to be with others	E_{max}	1.3±0.89	18±3.6***	16±3.1***	15.60
Adjective Mood Rating Scale (AMRS score)					
Well-being	ΔE_{max}	1.9±0.7	4.6±0.6**	5.6±0.6***	11.48
Extroversion	ΔE_{max}	1.3±0.4	3.4±0.4***	3.4±0.4***	13.61
Excitablility	ΔE_{max}	0±0.2	1.6±0.5**	2.4±0.4***	14.58
Self-confidence	ΔE_{max}	0.9±0.3	2.2±0.4*	2.8±0.5**	6.72
Adverse Effects					
Acute adverse effects	Δ12h	-0.3±0.3	6.6±1.0***	6.7±0.9***	29.25
Sub-acute adverse effects	Δ24h	-0.8±0.3	7.3±1.5***	6.9±1.2****	26.72

Values are mean±SEM in 24 subjects. *P<0.05, **P<0.01, and ***P<0.001 compared with placebo. $^{\#}$P<0.05 compared with lisdexamfetamine.

5.5 Achieved knowledge and future perspectives

We showed for the first time in humans that LSD induces changes in circulating steroids in a time course up to 24 h. The steroid plasma concentrations were determined after optimizing and newly validating a previously published ultra-high pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method [4]. The validated method is further described in the appendix "Supplementary data: Acute effects of lysergic acid diethylamide on circulating steroid levels in healthy subjects".

The major findings of this study were, LSD significantly increased the glucocorticoids cortisol, cortisone, corticosterone and 11-dehydrocorticosterone compared to placebo, indicating that total glucocorticoid production was enhanced. Furthermore, the plasma concentrations of androstenedione (only the area under the concentration-time curve from time 0 to 10 h (AUC_{10})) and dehydroepiandrosterone were significantly elevated by LSD. Although the adrenocorticotropic hormone (ACTH) plasma levels were not determined, the data suggests a disturbance of the HPA axis by LSD [91]. Previously, it has been shown, that LSD also increases prolactin plasma levels [94], which is an indicator of serotonin activity [120]. It is well established that serotonin stimulates the HPA axis [88]. We therefore postulate, that LSD may activate the HPA axis through serotonin receptor stimulation. This mechanism would be consistent with other reports on serotoninergic drugs, such as psilocybin [121], and MDMA [4, 122].

The other steroids such as the androgens androsterone, testosterone, DHEAS, and 5α-dihydrotestosterone, the glucocorticoid 11-deoxycortisol, the mineralocorticoids aldosterone and 11-deoxycorticosterone, and the progestins progesterone and 17a-hydroxyprogesterone were not affected by LSD. Furthermore, we wanted to study the relationship of active glucocorticoids cortisol and corticosterone with LSD plasma concentrations and the subjective "any drug effects". The pharmacokinetic and psychotropic data used in this study were previously published [94, 123]. We demonstrated a close relationship between LSD concentrations in the plasma and LSD induced changes in cortisol and corticosterone, where no acute pharmacological tolerance was observed. Nevertheless, our results cover only the steroid changes of a single dose of 200 µg LSD (pronounced psychotropic effects) in healthy volunteers. The LSD induced changes in steroid homeostasis in polydrug users, patients with comorbidities or chronical uses of LSD are still unknown.

Although 6%–8% of adults will abuse LSD in their lifetimes, there is currently a renewed interest of LSD in psychiatric research *e.g.* brain research [93, 124-126]. Furthermore, LSD has also been investigated for new medical indications, such as cluster headache [127], or anxiety associated with life-threatening diseases [94, 95]. However, since the 1970s only limited clinical research with LSD has been performed [93]. Therefore, our obtained results and findings lead to a better understanding of the pharmacology of LSD and support an appropriate benefit-risk assessment of LSD.

The worldwide prevalence of ADHD is estimated to be around 3.4% [128]. In 2010, the most prescribed pharmacological ADHD therapeutics in pediatrics patients were methylphenidate (38%), a mixture of D-amphetamine:L-amphetamine salts (22%), lisdexamfetamine (15%) and D-amphetamine (0.4%) [102].

Surprisingly, with the increasing prevalence of ADHD and the use of the amphetamine based drugs for its treatments, there is limited information as to the effects of this drug class on circulating steroids. Therefore, for the first time we investigated the effects of lisdexamfetamine and D-amphetamine on the steroid homeostasis in a study with healthy volunteers. Primarily, we measured plasma amphetamine concentrations after a single oral dose of lisdexamfetamine dimesylate (100 mg) or D-amphetamine sulfate (40.3 mg) by UHPLC-MS/MS. The validated method is presented in the appendix "Supplementary data: Pharmacokinetics and pharmacodynamics of lisdexamfetamine compared with D-amphetamine in healthy subjects". We showed that the pharmacokinetic parameters c_{max}, AUC_{24} and terminal plasma elimination half-life ($t_{1/2}$) of plasma amphetamine were identical after administration of lisdexamfetamine or immediate-release D-amphetamine at equimolar doses. However, lisdexamfetamine showed a significantly longer onset time (1.4 vs. 0.8 h) and t_{max} (4.4 vs. 3.2 h) compared with D-amphetamine, as a consequence of the required bioactivation of lisdexamfetamine by the erythrocytes [107, 108]. The only clinical data investigating the pharmacokinetics of lisdexamfetamine compared to immediate-release D-amphetamine at equimolar dose in healthy adults (*N* = 12) was sponsored by the New River Pharmaceuticals Inc. and reported in a poster presented at the 'US Psychiatric and Mental Health 19th Annual Congress' in 2006 [112], which has been referred to on numerous occasions in the literature [111-113, 129]. This abstract suggests [112] that lisdexamfetamine may be beneficial for ADHD patients due to its prolonged therapeutic duration and reduced abuse potential, however we did not observe a significant difference in c_{max} values (128 ng/ml vs. 134 ng/ml) for plasma amphetamine between lisdexamfetamine or D-amphetamine administration. The pharmacokinetics and pharmacodynamics of lisdexamfetamine and D-amphetamine in healthy volunteers are discussed in detail in the submitted manuscript "Pharmacokinetics and pharmacodynamics of lisdexamfetamine compared with D-amphetamine in healthy subjects".

Both lisdexamfetamine and D-amphetamine affects the circulating steroids compared to placebo. Consistently with previous studies [101, 114-119], we demonstrated, that D-amphetamine, as well as lisdexamfetamine significantly and similarly increased cortisol levels compared to placebo. Furthermore, the glucocorticoids cortisone, corticosterone, 11-dehydrocorticosterone, and 11-deoxycortisol, and the androgen precursors DHEA, DHEAS and androstenedione were significantly elevated after lisdexamfetamine and D-amphetamine administration. Corticosterone may have an eminent role in brain function, due to its ability to cross the blood-brain barrier more effectively than cortisol. This is reflected by a greater corticosterone to cortisol ratio in cerebrospinal fluid than in the plasma [130, 131]. In steroid concentration-time curves, the C_{max} and AUC_{12} were identical between lisdexamfetamine and D-amphetamine. However, the time to onset and consequently the time to maximal effects were delayed after lisdexamfetamine compared to D-amphetamine administration. The elevated glucocorticoid production, and increased levels of ACTH, DHEA and androstenedione after lisdexamfetamine or D-amphetamine suggest a stimulation of the HPA axis [132], which we hypothesize was mediated by adrenergic receptors [133, 134] rather than dopamine [135]. In contrast, lisdexamfetamine and D-amphetamine had no effect on the plasma concentrations of the mineralocorticoids; aldosterone and 11-deoxycorticosteone, the progestins; 17α-

hydroxyprogesterone and progesterone (except the male values), or the androgens; testosterone and androsterone. Progesterone, androgens and their precursors, such as dehydroepiandrosterone (DHEA), are associated with the magnitude of drug induced subjective response [136-138] and may be directly involved in the mechanisms of drug addiction [139-141]. In a next step, the drug-induced increase in glucocorticoids observed in the plasma samples, should be supported by determination of glucocorticoid metabolites in the 24-h urine samples, which were additionally collected during the clinical trial, by determining the urine steroid profile.

Phase plots of plasma amphetamine concentrations versus drug-induced changes in cortisol and corticosterone after lisdexamfetamine or D-amphetamine showed clockwise hysteresis, suggesting acute pharmacological tolerance. Previously, it was reported that a positive association exists between amphetamine-induced increases in cortisol levels and subjective drug effects [116, 142]. In addition, the HPA axis has a prominent role in the regulation of subjective drug effects [140]. In our study, we did not observe a correlation between plasma levels of amphetamine or cortisol and subjective “any drug effect” at the corresponding time points after lisdexamfetamine and D-amphetamine administration, which was analyzed using Spearman’s rank correlations (data not shown). Positive correlations between the MDMA-induced 11-deoxycorticosterone and aldosterone and systolic blood pressure have been demonstrated previously [4]. Therefore, it would be interesting to correlate in a next step the drug induced changes in steroid concentrations with the measured vital parameters such as systolic or diastolic blood pressure or heart rate after lisdexamfetamine and D-amphetamine administration.

However, the observed lisdexamfetamine or D-amphetamine induced HPA stimulation, resulting in a profound increase in circulating steroids, especially glucocorticoids, was solely demonstrated after a single acute supra-therapeutic dose in healthy adults. In contrast to the acute administration of psychostimulatory recreational drugs such as LSD, the treatments for ADHD symptoms involves chronic daily administration of lisdexamfetamine or D-amphetamine [105, 111, 143]. Therefore, the following questions still have to be addressed: first, how does chronic administration of lisdexamfetamine and D-amphetamine affect the HPA axis and steroid levels and second, does the HPA axis adapt over time. In rats, it was shown, that repeated administration of D-amphetamine down regulates the glucocorticoids receptors in the hippocampus [144]. Furthermore, they observed an increase in plasma ACTH and corticosterone levels compared to placebo. However, across the days, the ACTH and corticosterone responses to D-amphetamine decreased, suggesting partial desensitization of the HPA axis [145]. Humans given two repeated doses of MDMA (24 h apart), showed elevated cortisol levels (AUC) upon the first dose, which was further significantly increased following a second dose [146]. In another study, healthy volunteers exposed to repeated psychological stress (Trier Social Stress Test, which consists of a public speaking and mental arithmetic task in front of an audience), generally showed a significant elevation in cortisol levels in each examination. However, based on the magnitude in cortisol response after repeated mental stress, cortisol “high” and “low” responders could be distinguished [147]. Interestingly, following a discriminant analysis of psychological variables (personality questionnaires) of the healthy volunteers, the cortisol high responders showed results

similar to patients with depression or anxiety disorders [147]. It was shown, that depressed patients have an altered cortisol response to psychological stress [148].

Co-existing psychiatric problems such as anxiety (44%) and depression (32%) are common phenomena in ADHD patients, where greater than 85% of patients suffer from at least one co-existing psychiatric condition [149]. Therefore, we suspect that ADHD patients suffering such a comorbidity and/or receiving a pharmacotherapy, such as selective serotonin reuptake inhibitor citalopram [150] or the tricyclic antidepressant imipramine [151], which stimulate the HPA axis, may have an altered steroid response to lisdexamfetamine or D-amphetamine administration compared to ADHD patients alone. For example, it was demonstrated that children with ADHD and comorbid anxiety showed a greater increase in cortisol levels upon stress compared to ADHD children without anxiety [152]. However, there was no change in cortisol elevation upon mild psychosocial stress in ADHD patients without any medical or psychiatric comorbidities compared to healthy volunteers [153].

As previously detailed in the section “Acute effects of psychoactive drugs on steroids in healthy volunteers”, perturbations of HPA axis or circadian rhythm may have severe health related consequences such as learning and memory deficits, anxiety, depression, altered stress response and brain development, cardiovascular diseases [15, 17, 19, 20, 90, 91, 154-156]. The highest prevalence of ADHD is reported in 6–12 years-old children (11.4%), which decreases in adulthood (5.0%) [157]. Additionally, children due to their lower body size or ability to metabolize drugs, may have an increased drug exposure compared to adults. It was reported, that after lisdexamfetamine administration the c_{max} and AUC_{∞} of D-amphetamine were higher in children compared to adults for the same dose [111]. Taking together, and keeping in mind that children have ongoing brain development *e.g.* higher cognitive functions [158], the pediatric population is extremely vulnerable to drug induced changes in steroid homeostasis. This highlights once again the requirement for further research, especially in the pediatric population, to enhance the current knowledge of drug induced HPA stimulation, steroid disturbance, its recovery after the end of drug administration and the potential for adverse effects. This should allow a better benefit-risk assessment for the use of psychostimulants such as lisdexamfetamine and D-amphetamine for treatment of ADHD.

Subsequently we suggest, to conduct a randomized, double-blind, placebo-controlled, cross-over study, similar to our conducted study, but in a pediatric ADHD population and with a modified study procedure (dose, treatment duration) (Fig. 3). ADHD patients without any further pharmacotherapy, would be administered an oral once-daily maximal therapeutically dose of 70 mg of lisdexamfetamine, D-amphetamine at equimolar dose or placebo, following dose titration (Fig. 3). We suggest that each treatment should last for 1 week, since steady-state plasma concentrations of amphetamine after once-daily dosing of 70 mg lisdexamfetamine were achieved at day five [159]. Plasma samples should be collected over a 24 h period on selected days in all phases (dose titration, final study dose, washout) to obtain full steroid and ACTH profiles. This will allow us to monitor the drug induced changes in steroid homeostasis at a therapeutically dose, and additionally to observe how the steroids and the HPA axis adapt

during the drug titration phase. This setup, differed to our previous study, where drug-naive volunteers received one single dose of 100 mg lisdexamfetamine without dose escalation. Moreover, the washout period can be used to mimic the drug withdrawal. This will allow us to monitor how the steroid system "recovers" and the way in which it reacts upon further drug challenge. A study in rats revealed that D-amphetamine was able to induce sensitization of the HPA axis following drug challenge. After chronic administration of D-amphetamine (2 mg/kg for day 1-7, and 4 mg/kg for day 8-14), followed by a washout phase, the rats were challenged with a single D-amphetamine injection (2 mg/kg), which resulted in higher corticosterone and ACTH plasma levels compared to the drug challenged control group (chronic administration of vehicle) [160]. In another non-related study in rats, which followed a similar experimental design, but used a different D-amphetamine treatment scheme (2.5 mg/kg for 14 days), showed that D-amphetamine withdrawal potentiates restraint stress-induced corticosterone in the ventral hippocampus, but not in plasma compared to the control group [161].

study week	1	2	3	4	5	6	7	8	9	10	11	12
study phase	dose titration		final study dose	washout	dose titration		final study dose	washout	dose titration		final study dose	washout
study drug	dose titration schema[a]		70 mg lisdexamfetamine dimesylate or 28.2 mg d-amphetamine sulfate or placebo		dose titration schema[a]		70 mg lisdexamfetamine dimesylate or 28.2 mg d-amphetamine sulfate or placebo		dose titration schema[a]		70 mg lisdexamfetamine dimesylate or 28.2 mg d-amphetamine sulfate or placebo	

[a]The dose titration schema is dependent on the final study dose in the study week 3/7/11:
For the final study dose of 70 mg lisdexamfetamine dimesylate: first week 30 mg lisdexamfetamine dimesylate and second week 50 mg lisdexamfetamine dimesylate
For the final study dose of 28.2 mg d-amphetamine sulfate: first week 12.1 mg d-amphetamine sulfate and second week 20.2 mg d-amphetamine sulfate
For the final study dose placebo: 2 weeks of placebo

Fig. 3. Study procedure with study phases and drugs.

In general, clinical trials are costly and time consuming. Therefore, we suggest to measure the steroid concentration profiles in plasma samples from pre-executed trials. One such example would be the placebo-controlled, crossover study conducted by Biederman et al. 2007 [162], where each child diagnosed with ADHD received 1 week of placebo, 1 week of mixed amphetamine salts extended-release XR, and 1 week of lisdexamfetamine at equimolar dose.

Unfortunately, with both experimental approaches, we cannot investigate the long-term adverse effects of drug induced disturbances of steroid homeostasis. For this, we could conduct an observational study (*e.g.* case-control study) to investigate the association between ADHD pharmacotherapy lisdexamfetamine or D-amphetamine and the risk of developing diseases related to HPA axis disruption such as learning and memory deficits, impaired immune system and brain development, decreased intelligence quotient, cardiovascular diseases, metabolic syndrome in a pediatric ADHD population without comorbidities or any further pharmacotherapy. For this purpose, we could use the Clinical Practice Research Datalink (CPRD), which is an ongoing primary care database of anonymized medical records (diagnoses, drug prescriptions, demographics and personal characteristics *e.g.* body mass index (BMI)) of approximately 11.3 million patients from the United Kingdom (UK) [163, 164]. The current limitation of this approach would be the small sample size of patients receiving lisdexamfetamine dimesylate (Elvanse), since it was only recently launched in the UK in 2013 [165], whereas D-amphetamine was licensed in the UK in 2008 [166].

In conclusion, we showed the pharmacokinetic profiles of amphetamine in the plasma following lisdexamfetamine and D-amphetamine administration to healthy volunteers are identical, except for a longer onset time and t_{max}. Furthermore, lisdexamfetamine and D-amphetamine, as well as LSD, had a profound effect on the circulating steroids, notably on the glucocorticoids, indicating HPA stimulation.

6. Appendix

6.1 Supplementary data: Steroid profiling in H295R cells to identify chemicals potentially disrupting the production of adrenal steroids

Supplementary information

Steroid profiling in H295R cells to identify chemicals potentially disrupting the production of corticosteroids and adrenal androgens

Petra Strajhar,[a,c] David Tonoli,[b,c] Fabienne Jeanneret,[b,c] Raphaella M. Imhof,[a] Vanessa Malagnino,[a] Melanie Patt,[a] Denise V. Kratschmar,[a] Julien Boccard,[b] Serge Rudaz,[b,c] Alex Odermatt[a,c,*]

Table S1: Sequences of oligonucleotide primers used for qPCR

Gene	Sense primer (sequence 5'-3')	Antisense primer (sequence 5'-3')
GAPDH	CAGCCTCAAGATCATCAGCA	TTCTAGACGGCAGGTCAGGT
STAR	GTCCCACCCTGCCTCTGAAG	CATACTCTAAACACGAACCCCACC
CYP11A1	GAGATGGCACGCAACCTGAAG	CTTAGTGTCTCCTTGATGCTGGC
HSD3B2	TGCCAGTCTTCATCTACACCAG	TTCCAGAGGCTCTTCTTCGTG
CYP17A1	AGCCGCACACCAACTATCAG	TCACCGATGCTGGAGTCAAC
CYP21A2	CGTGGTGCTGACCCGACTG	GGCTGCATCTTGAGGATGACAC
CYP11B1	GGTTTGCCAGGCTAAGC	CAAACTGCCCAGAGGACAG
CYP11B2	TCCAGGTGTGTTCAGTAGTTCC	GAAGCCATCTCTGAGGTCTGTG
CYP19A1	AGGTGCTATTGGTCATCTGCTC	TGGTGGAATCGGGTCTTTATGG
AKR1C3	GGATTTGGCACCTATGCACCTC	CTATATGGCGGAACCCAGCTTCTA
HSD17B1	GAAGGCTTATGCGAGAGT	GAAGGTGTGGATGTCCGT
HSD17B2	AAAGGGAGGCTGGTGAAT	GCAACTTTAATTCCCCAC
HSD17B3	TGCTTCCAAACCTTCTCCC	AGACCTTTCTGCCTTGATTCC

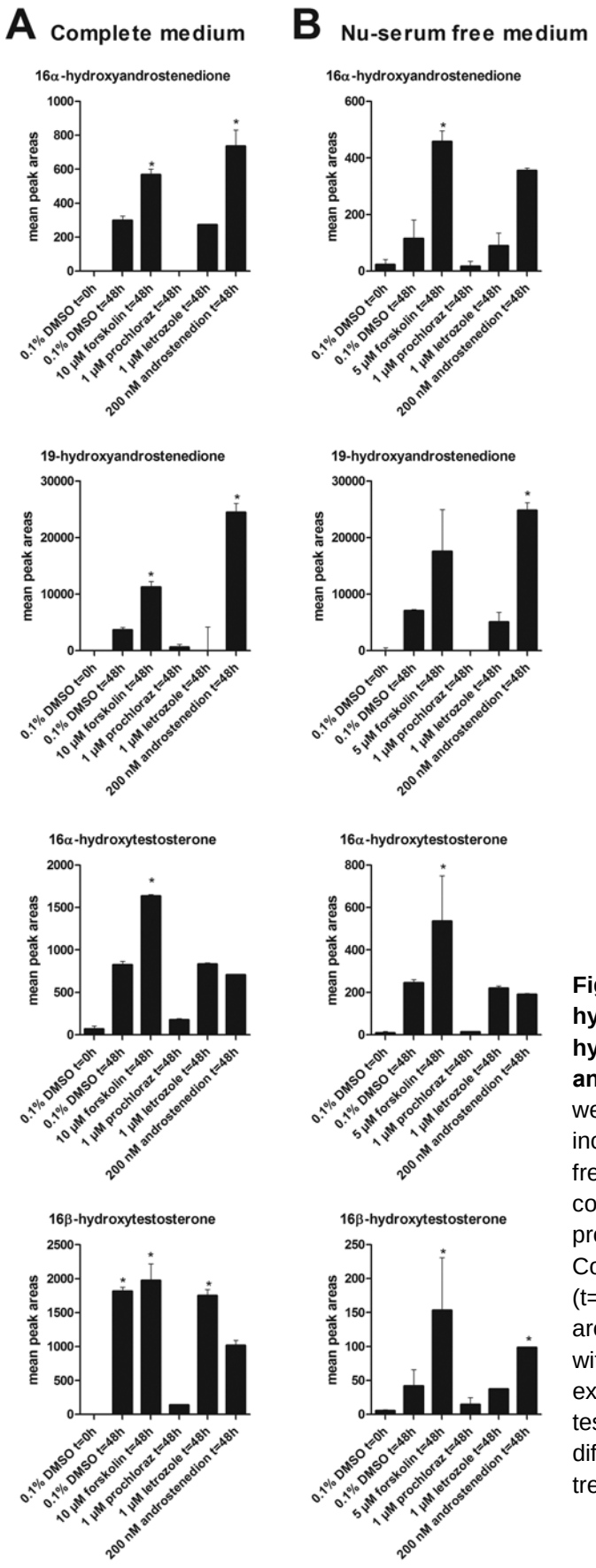


Fig. S1. *De novo* synthesis of 16α-hydroxyandrostenedione, 19-hydroxyandrostenedione, 16α-hydroxytestosterone and 16β-hydroxytestosterone by H295R cells. Steroids were quantified in culture supernatants of H295R cells incubated either in complete medium (A) or in Nu-serum-free medium (B) for 48 h with vehicle (0.1% DMSO solvent control), 10 µM (in A) or 5 µM (in B) forskolin, 1 µM prochloraz, 1 µM letrozole or 200 nM androstenedione. Controls for complete medium and Nu-serum-free medium (t=0 h) were included for comparison. Steroids (mean peak areas) were measured by LC-MS and represent median with range from one (out of three) representative experiment, performed in triplicate (n=3). Kruskal-Wallis test followed by Dunn's test was used to analyze significant difference ($p < 0.05$) of solvent control at t=0 to chemical treatment at t=48 h (*).

6.2 Supplementary data: Acute effects of lysergic acid diethylamide on circulating steroid levels in healthy subjects

Supplementary data

Acute effects of LSD on circulating steroid levels in healthy subjects

Petra Strajhar,[a, 1] Yasmin Schmid,[b, 1] Evangelia Liakoni,[b] Patrick C. Dolder,[b,c] Katharina M. Rentsch,[c] Denise V. Kratschmar,[a] Alex Odermatt,[a, 2] and Matthias E. Liechti[b, 2]

Supplemental methods:

1. Quantification of steroid hormones in human plasma samples

1.1. Chemicals and reagents

UPLC-grade purity methanol, acetonitrile and formic acid were purchased from Sigma-Aldrich (St. Louis, MO) or Biosolve (Dieuze, France). Distilled water was deionized using a MilliQ water purification system (Millipore, USA). Aldosterone, 11-deoxycorticosterone, corticosterone, dehydroepiandrosterone-3-sulfate, androstenedione, testosterone and [2,2,4,6,6,21,21-$^{2}H_{7}$]-aldosterone (98% isotopic purity) were purchased from Sigma-Aldrich (St. Louis, MO). 11-Dehydrocorticosterone, dehydroepiandrosterone (DHEA), 5α-dihydrotestosterone, androsterone, progesterone, 17α-hydroxyprogesterone, 11-deoxycortisol, cortisol, cortisone, [16,16-$^{2}H_{2}$]-androsterone (98% isotopic purity) and [16,16,17-$^{2}H_{3}$]-5α-dihydrotestosterone (98% isotopic purity) were purchased from Steraloids (Newport, RI). [1,2-$^{2}H_{2}$]-Testosterone (98% isotopic purity), [2,2,4,6,6,16,16-$^{2}H_{7}$]-4-androsten-3,17-Dione (98% isotopic purity) and [2,2,4,6,6,17α,21,21-$^{2}H_{8}$]-corticosterone (98% isotopic purity) were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Canada). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and of the highest grade available.

1.2. Instrumentation and analytical conditions

Analytical instruments: All analytes were measured simultaneously by ultra-pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using a Agilent 1290 UPLC instrument equipped with a binary solvent delivery system, an auto sampler (at 4 °C), and a column oven, coupled to an Agilent 6490 triple quadrupole mass spectrometer equipped with a jet stream electrospray ionization interface (AJS-ESI) (Agilent Technologies, Basel, Switzerland).

Liquid chromatography: The chromatographic separation of the analytes was achieved using a Waters ACQUITY UPLC BEH C18, 1.7 µm, 2.1×150 mm, column (Waters, Wexford, Ireland). The column temperature was maintained at 65 ºC. Steroids were separated using a mobile phase consisting of water-

acetonitrile-formic acid (A) (95/5/0.1; v/v/v) and (B) (5/95/0.1; v/v/v). The injection volume was 5 µL per sample. Methanol in water (50/50 v/v) was used as needle and needle-seat flushing solvent for 10 s after sample aspiration. Samples were stored until analysis in the auto sampler (maintained at 4 °C). *Method A*: Aldosterone, corticosterone, 11-dehydrocorticosterone, cortisol, cortisone, DHEA, DHEA-3-sulfate, progesterone, 17α-hydroxyprogesterone, 11-deoxycortisol, 11-deoxycorticosterone, aldosterone-d7, and corticosterone-d8 were eluted by the gradient 25 - 70% of mobile phase B during 0 - 10 min, and 100% of mobile phase B at 10.1 min onwards at a constant flow rate of 0.63 mL/min. The run was stopped after 12.0 min, followed by re-equilibration of the column for 2 min. *Method B*: Androsterone, testosterone, androstenedione, 5α-dihydrotestosterone, androsterone-d2, testosterone-d2, androstenedione-d7, and 5α-dihydrotestosterone-d3 were eluted by the gradient of 25 – 59% of mobile phase B during 0 - 20 min at a gradient flow rate from 0.65 mL/min to 0.36 mL/min, and 100% of mobile phase B at 22 min onwards at a constant flow rate of 0.65 mL/min. The run was stopped at 24 min, followed by re-equilibration of the column for 1 min.

Mass spectrometry: The AJS-ESI source was operated with nitrogen as drying and collision gas in the positive ion mode for all analytes, except for aldosterone and aldosterone-d7, which were analyzed using negative ionization. The ion source conditions were identified and optimized for all individual analytes using the source optimization software module (Agilent Technologies, California, USA, B.07.01) (Supplemental Table S1). Analytes were monitored by multiple reaction-monitoring (MRM) and characteristic precursor ions and corresponding transitions for quantifier- and qualifier-ions were automated defined by the use of the compound optimizer software module included within the Mass Hunter Workstation software (Agilent Technologies, California, USA) (Supplemental Table S2).

Data analysis: Data acquisition and subsequent data analysis was performed using Mass Hunter Workstation Acquisition software Version 07.01 SP1 and MassHunter Workstation Software Quantitative Analysis Version B.07.00 /Build 7.0457.0, respectively (Agilent Technologies, California, USA).

Sample extraction: Sample extraction was performed using a vacuum manifold (Agilent Technologies, California, USA) equipped with Oasis HBL SPE cartridges (Waters, Massachusetts; USA, Lot No. 116B32307A). Samples were evaporated to dryness using a Genevac EZ-2 plus centrifugal vacuum evaporator (Genevac, Suffolk, UK).

Standard solutions: Stock solutions of analytes and deuterium internal standards (I.S.) were prepared by weighing pure compounds on an analytical balance (Mettler-Toledo, Switzerland) and dissolving in methanol to obtain a concentration of 10 mM for analytes and I.S. The standard solutions of analytes and I.S. were freshly prepared in methanol by further diluting the corresponding stock solution to obtain a concentration of 100 µM. All stock solutions of standards and I.S. were stored at -20 ºC.

Sample preparation: For solid phase extraction each sample, calibrator or quality control (QC) (700 µL) was mixed with protein precipitation solution (100 µL, zinc sulfate 0.8 M in water/methanol (50/50 v/v)) containing I.S. and diluted to a final volume of 1 mL with water. The samples were incubated for 10 min in a

thermoshaker with thorough shaking (1300 rpm, 4 °C) and centrifuged (10 min, 16,000 × rcf, 4 °C). Supernatants (700 µL) were transferred to Oasis HBL SPE cartridges (preconditioned with methanol and water, 1 mL each). Following one wash with water (1 mL) and two washes with methanol/water (1 mL, 10/90 v/v), the samples were eluted with methanol (1 mL) and evaporated to dryness. The samples were reconstituted in 25 µL methanol (10 min, 1300 rpm, 4 °C, thermoshaker) and transferred into new glass vials.

Chromatographic performance: Ten point calibration curves were generated by a zero sample (charcoal treated human plasma/water mixture containing I.S.) and nine calibrators (Supplemental Table S3). To meet requirements of the FDA guidance for industry, the coefficient of determination (R^2) has to be higher than 0.96 and at least 75% of all calibrators have to be valid (Supplemental Table S3).

Specificity: Blank samples without the addition of analyte and I.S. were processed and injected into the UPLC–MS/MS within an analytical run. The peak areas evaluated in the blank samples were not allowed to exceed 20% of the mean LLOQ peak area.

Recovery: The absolute recovery was determined by comparing the mean peak areas for extracted with unextracted samples (100% recovery) at the concentrations of QC high, QC medium, and QC low (Supplemental Table S4).

Limit of detection (LLOD) and limit of quantification (LLOQ): Lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were determined by direct injection of decreasing amounts of analyte and were calculated as the concentration giving peaks with a signal-to-noise ratio of ≥ 3 and ≥ 5, respectively. The LLOQ was decided as the lowest concentration on the calibration curve which fulfilled the criteria of imprecision ±15%, and inaccuracy within ±15% (Supplemental Table S3).

Reproducibility: Five replicates of QCs at three concentration levels (QC high, QC medium, and QC low) were processed and injected into the UPLC–MS/MS. To ensure the reproducibility, these sets of QCs were tested within validation runs. In each run, intra-run imprecision (% coefficient of variation; CV%) of each QC series had to be below 15% (20% at the LLOQ) and intra-run inaccuracy (% relative error of measurement; RE%) had to be within ±15% of the nominal values (±20% at the LLOQ) (Supplemental Table S5).

Carry-over: To evaluate the carry-over of all analytes and I.S. in each analytical run blank samples were injected immediately after the highest calibrator upper limit of quantification (ULOQ). Mean carry-over in the blank sample following the ULOQ had not to exceed 20% of the signal of the LLOQ for analyte and 5% for I.S.

Supplemental Table S1 Optimized ion source conditions and analytical parameters.

Analyte	Gas temperature (°C)	Gas flow (l/min)	Nebulizer (psi)	Sheath gas temperature (°C)	Sheath gas flow (l/min)	Capillary voltage (V)	Nozzle voltage (V)	Positive high pressure RF	Positive low pressure RF	Negative high pressure RF	Negative low pressure RF
11-Dehydrocorticosterone	290	14	20	300	11	3000	1500	200	110	150	60
11-Deoxycorticosterone	290	14	20	300	11	3000	1500	200	110	150	60
11-Deoxycortisol	290	14	20	300	11	3000	1500	200	110	150	60
17α-Hydroxyprogesterone	290	14	20	300	11	3000	1500	200	110	150	60
5α-Dihydrotestosterone / 5α-Dihydrotestosterone-d3	290	20	15	350	11	3000	1500	120	90	150	60
Aldosterone / Aldosterone-d7	290	14	20	300	11	3000	1500	200	110	150	60
Androstendione / Androstendione-d7	290	20	15	350	11	2000	1500	170	90	150	60
Androsterone / Androsterone-d2	290	20	15	350	11	2000	1500	170	90	150	60
Corticosterone / Corticosterone-d8	290	14	20	300	11	3000	1500	200	110	150	60
Cortisol	290	14	20	300	11	3000	1500	200	110	150	60
Cortisone / Cortisone-d2	290	14	20	300	11	3000	1500	200	110	150	60
Dehydroepiandrosterone	290	14	20	300	11	3000	1500	200	110	150	60
Dehydroepiandrosterone-3-sulfate	290	14	20	300	11	3000	1500	200	110	150	60
Progesterone	290	14	20	300	11	3000	1500	200	110	150	60
Testosterone / Testosterone-d2	290	20	25	350	11	2500	1500	170	90	150	60

Supplemental Table S2 Multiple reaction monitoring (MRM) analyte transitions.

Analyte	Method	Precursor ion (*m/z*)	Quantifier ion (m/z)	Qualifier ion (m/z)	Collision energy (V)	Polarity	Dwell time (ms)	Internal Standard (I.S.)
11-Dehydrocorticosterone	A	345.2	121	54.9	21; 57	positive	100	Corticosterone-d8
11-Deoxycorticosterone	A	331.2	97.1	109	28; 25	positive	100	Corticosterone-d8
11-Deoxycortisol	A	347.2	97	109	32; 24	positive	100	Corticosterone-d8
17α-Hydroxyprogesterone	A	331.2	97	109	32; 28	positive	100	Corticosterone-d8
5α-Dihydrotestosterone	B	291.2	159	255	24; 12	positive	100	5α-Dihydrotestosterone-d3
5α-Dihydrotestosterone-d3	B	294.3	163	258	24; 12	positive	100	
Aldosterone	A	359.2	331	189	17; 24	negative	400	Aldosterone-d7
Aldosterone-d7	A	366.2	338	196	17; 24	negative	200	
Androstenedione	B	287.2	97.1	109	24; 20	positive	100	Androstenedione-d7
Androstenedione-d7	B	294.3	100	113	24; 28	positive	100	
Androsterone	B	273.2	147	255	24 ;12	positive	200	Androsterone-d2
Androsterone-d2	B	275.2	118	257	52; 8	positive	100	
Corticosterone	A	347.2	121	329	25; 9	positive	100	Corticosterone-d8
Corticosterone-d8	A	355.2	125	337	25; 12	positive	100	
Cortisol	A	363.2	121	105	36; 56	positive	100	Cortisone-d2
Cortisone	A	361.2	121	163	36; 24	positive	100	Cortisone-d2
Cortisone-d2	A	363.2	123	165	36; 24	positive	100	
Dehydroepiandrosterone	A	271.2	253	213	6; 12	positive	100	Androstenedione-d7
Dehydroepiandrosterone-3-sulfate	A	271.1	253	213	6; 10	positive	200	Corticosterone-d8
Progesterone	A	315.2	109	97.1	20; 24	positive	50	Corticosterone-d8
Testosterone	B	289.2	97.1	109	28; 32	positive	50	Testosterone-d2
Testosterone-d2	B	291.5	111	125	13; 24	positive	50	

Supplemental Table S3 Limit of detection (LLOD), lower limit of quantification (LLOQ), signal-to-noise ratio (S/N), retention time (RT), linearity, and calibration range.

Analyte	LLOD (nM)	LLOQ (nM)	(S/N)	RT (min)	Linearity (R^2)	Calibration range (nM)
11-Dehydrocorticosterone	0.49	0.98	10.5	3.1	0.998	0.98-250
11-Deoxycorticosterone	0.39	0.78	13.7	5.1	0.998	0.78-200
11-Deoxycortisol	0.39	0.78	9.9	3.73	0.992	0.78-200
17α-Hydroxyprogesterone	0.39	0.78	10.5	5.59	0.998	0.78-200
5α-Dihydrotestosterone	0.49	0.98	60.7	11.9	0.999	0.98-250
Aldosterone	0.10	0.2	12.0	1.72	0.999	0.2-50
Androstenedione	0.39	0.78	13.8	8.85	0.999	0.78-200
Androsterone	1.96	3.91	5.6	16.2	0.999	1.95-500
Corticosterone	0.49	0.98	54.1	3.56	0.996	0.98-250
Cortisol	0.98	1.95	5.7	2.29	0.991	3.91-1000
Cortisone	0.98	1.95	38.0	2.24	0.994	1.95-500
Dehydroepiandrosterone	1.96	3.91	7.3	5.37	0.999	1.95-500
Dehydroepiandrosterone 3-sulfate	9.77	19.53	9.1	3.55	0.993	19.53-5000
Progesterone	0.03	0.05	7.6	7.4	0.994	0.39-100
Testosterone	0.20	0.39	55.3	7.6	0.999	0.39-100

Supplemental Table S4 Recovery (QC high, QC medium, QC low).

	Nominal concentration (nM)			Recovery (%)			CV%		
	QC high	QC medium	QC low	QC high	QC medium	QC low	QC high	QC medium	QC low
11-Dehydrocorticosterone	125	31.3	2.0	114.0	103.2	95.9	3.7	2.1	2.2
11-Deoxycorticosterone	100	25.0	1.6	98.5	95.4	90.4	8.9	3.1	3.3
11-Deoxycortisol	100	25.0	1.6	106.7	91.8	95.0	6.7	2.9	3.4
17α-Hydroxyprogesterone	100	25.0	1.6	82.7	83.5	81.8	9.3	4.4	1.4
5α-Dihydrotestosterone	125	31.3	2.0	88.7	120.2	96.6	20.0	15.3	13.9
Aldosterone	25	6.3	0.4	99.2	99.1	98.3	5.2	4.6	10.3
Androstenedione	100	25.0	1.6	100.2	100.7	98.0	1.8	2.4	3.5
Androsterone	250	62.5	3.9	94.9	98.4	102.5	1.7	5.8	0.2
Corticosterone	125	31.3	2.0	111.4	98.9	95.1	5.6	2.0	2.2
Cortisol	250	62.5	3.9	108.0	102.9	97.4	3.5	4.5	2.2
Cortisone	250	62.5	3.9	100.2	104.5	98.5	4.9	5.1	1.6
Dehydroepiandrosterone	250	62.5	3.9	102.1	102.7	107.8	5.4	4.3	5.7
Dehydroepiandrosterone-3-sulfate	2500	625.0	39.1	88.9	103.8	96.8	5.6	4.1	2.6
Progesterone	50	12.5	0.8	80.9	92.4	98.0	11.4	6.0	0.8
Testosterone	50	12.5	0.8	101.7	103.9	100.5	3.9	1.5	2.2

Supplemental Table S5 Reproducibility: Nominal and measured concentration, standard deviation (S.D.), imprecision (CV%), and inaccuracy (RE%) of QC high, QC medium, and QC low.

	Nominal concentration (nM)			Measured concentration (nM)			S.D.			CV%			RE%		
Analyte	QC high	QC med	QC low	QC high	QC med	QC low	QC high	QC med	QC low	QC high	QC med	QC low	QC high	QC med	QC low
11-Dehydrocorticosterone	125	31.3	2.0	120.7	28.6	1.8	9.9	1.0	0.1	8.2	3.4	7.0	-3.5	-8.5	-6.8
11-Deoxycorticosterone	100	25.0	1.6	101.6	25.3	1.7	5.3	0.6	0.1	5.2	2.2	8.2	1.6	1.1	5.8
11-Deoxycortisol	100	25.0	1.6	96.5	24.2	1.5	8.1	0.5	0.1	8.4	1.9	3.6	-3.5	-3.2	-1.8
17α-Hydroxyprogesterone	100	25.0	1.6	102.9	26.0	1.4	9.4	0.7	0.1	9.2	2.9	8.6	2.9	3.9	-12.4
5α-Dihydrotestosterone	125	31.3	2.0	123.8	30.2	1.8	2.9	0.5	0.1	2.4	1.7	3.7	-0.9	-3.4	-5.3
Aldosterone	25	6.3	0.4	23.5	5.5	0.3	1.9	0.5	0.0	8.0	8.4	10.7	-6.0	-11.6	-13.1
Androstenedione	100	25.0	1.6	98.9	24.3	1.4	1.7	0.9	0.0	1.7	3.7	1.0	-1.1	-2.8	-9.7
Androsterone	250	62.5	3.9	250.8	58.9	4.1	4.5	1.0	0.1	1.8	1.7	2.3	0.3	-5.8	4.5
Corticosterone	125	31.3	2.0	122.6	31.3	1.8	9.2	0.8	0.1	7.5	2.5	8.1	-1.9	0.0	-7.6
Cortisol	250	62.5	3.9	240.7	63.7	4.5	19.1	1.1	0.3	7.9	1.7	7.7	-3.7	2.0	13.9
Cortisone	250	62.5	3.9	243.5	63.9	3.4	11.5	2.3	0.3	4.7	3.5	9.4	-2.6	2.2	-13.5
Dehydroepiandrosterone	250	62.5	3.9	236.6	53.5	3.7	19.3	3.0	0.4	8.2	5.7	11.4	-5.4	-14.3	-5.9
Dehydroepiandrosterone-3-sulfate	2500	625.0	39.1	2643.0	685.2	42.0	5.8	38.7	6.1	0.2	5.7	14.6	5.7	9.6	7.4
Progesterone	50	12.5	0.8	49.2	11.2	0.7	3.2	0.2	0.0	6.5	2.1	4.2	-1.5	-10.7	-11.2
Testosterone	50	12.5	0.8	43.8	12.3	0.7	1.5	0.5	0.0	3.4	4.0	6.5	-12.3	-1.5	-13.4

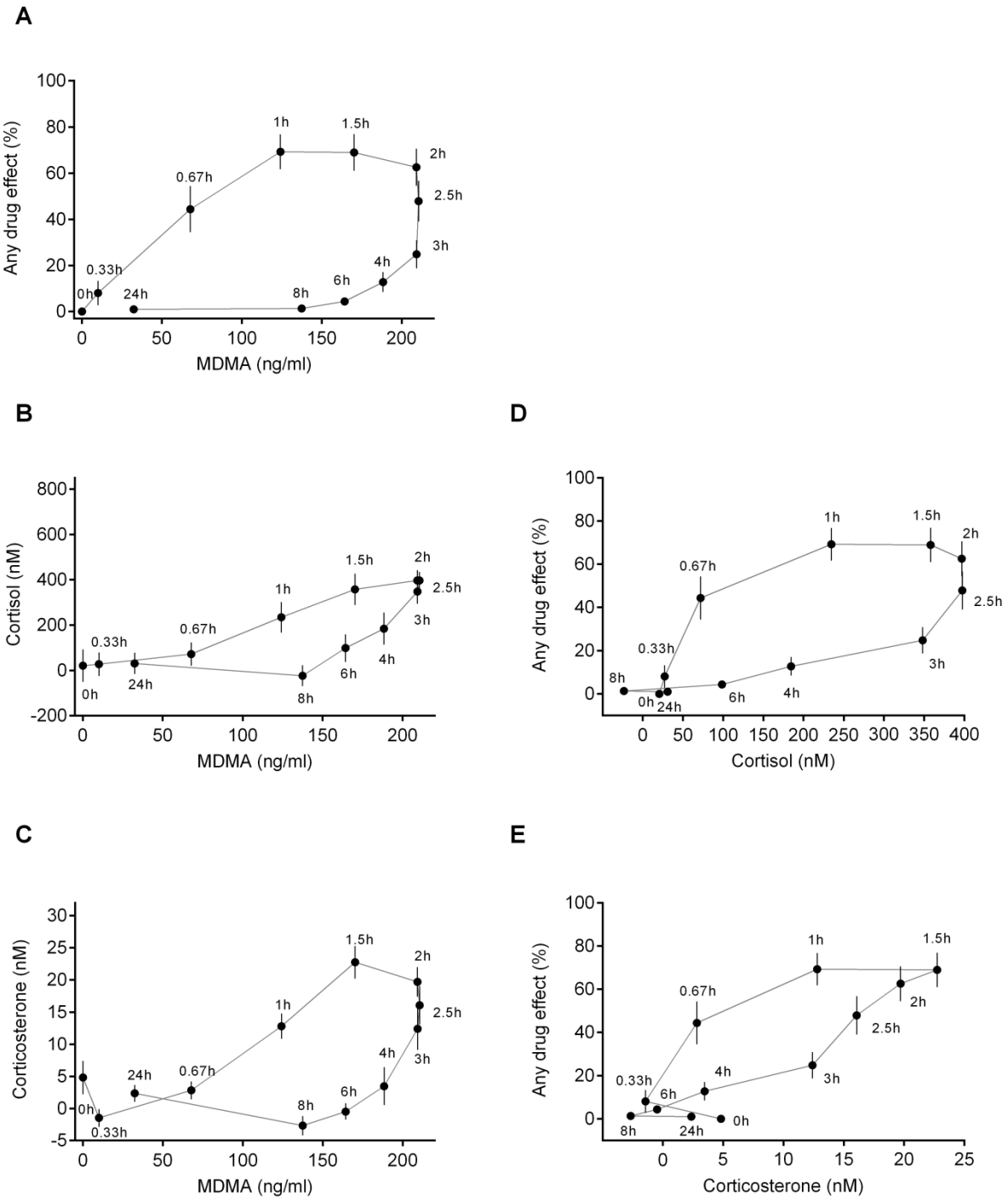
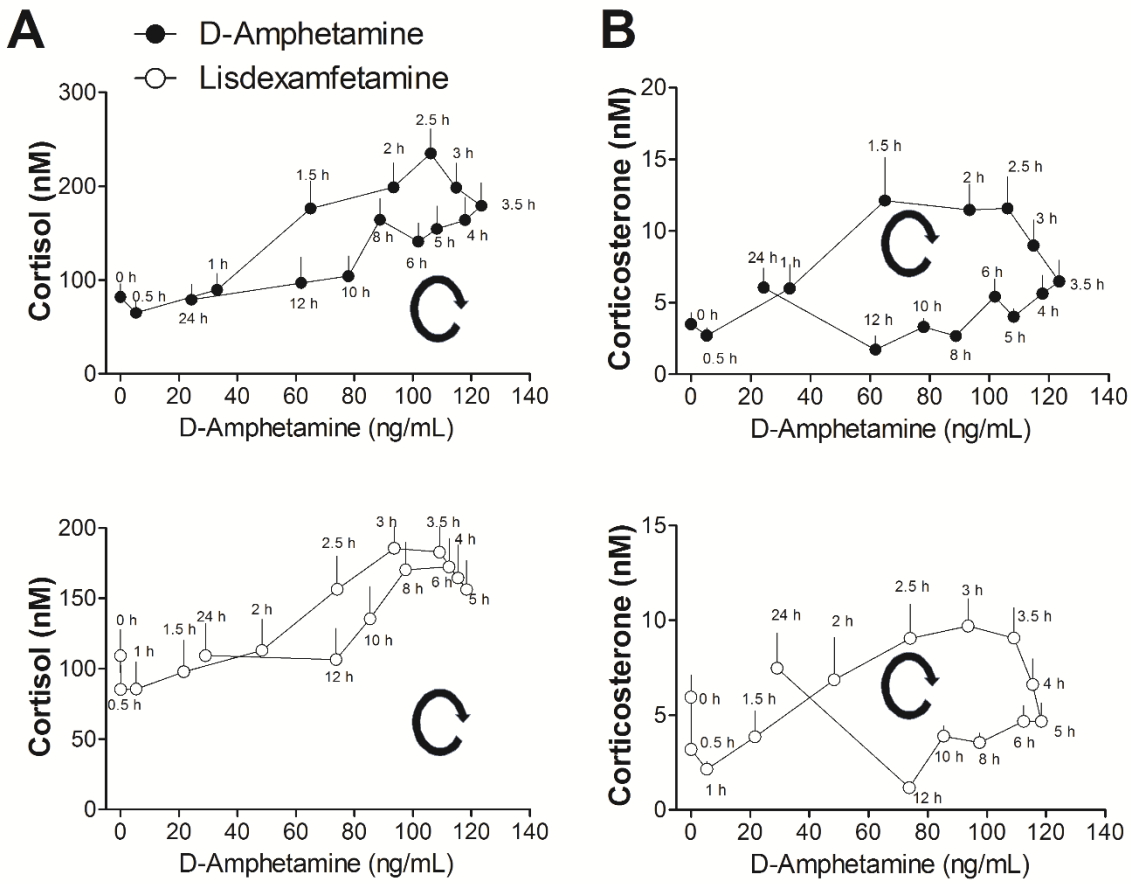

A
Any drug effect (%)
MDMA (ng/ml)
0h
0.33h
0.67h
1h
1.5h
2h
2.5h
3h
4h
6h
8h
24h
B
Cortisol (nM)
MDMA (ng/ml)
C
Corticosterone (nM)
MDMA (ng/ml)
D
Any drug effect (%)
Cortisol (nM)
E
Any drug effect (%)
Corticosterone (nM)

Figure S1

Supplemental Figure S1 MDMA exposure-response relationship. MDMA responses are shown as MDMA effect (any drug effect, cortisol or corticosterone concentration) minus the individual time-matched effect of placebo. MDMA or placebo was administered at t = 0 h. Subjective responses to MDMA (A) and MDMA-induced changes in cortisol (B) and corticosterone (C) concentrations (mean ± SEM) of 16 subjects are plotted against mean MDMA plasma concentrations (hysteresis curves). The time of sampling is noted next to each point (in hours after MDMA administration). Clockwise hysteresis was observed for any drug effects (A), cortisol (B), and corticosterone (C) consistent with acute tolerance to the effects of MDMA. After drug administration, the subjective drug effects increased faster and in particular decreased faster than the plasma levels of cortisol (D) or corticosterone (E) over time (clockwise hysteresis). Thus, over time MDMA-induced changes in glucocorticoids do not well reflect the psychotropic effects of the drug in contrast to LSD.

6.3 Supplementary data: Acute effects of D-amphetamine and lisdexamfetamine on plasma steroid concentrations in healthy subjects



Supplemental Figure S1. Drug-induced changes in plasma concentrations of cortisol (A) and corticosterone (B) plotted against D-amphetamine concentrations over time (hysteresis curves) after administration of lisdexamfetamine and D-amphetamine in 24 and 23 subjects, respectively. The endocrine response represents the difference from placebo calculated for each time point to account for circadian changes in hormone levels. Lisdexmfetamine and D-amphetamine were administered at t = 0. The time of sampling is noted next to each point. The clockwise hysteresis indicates acute pharmacological tolerance to the endocrine response of amphetamine which was comparable after administration of the two formulations. Data are mean ± SEM.

6.4 Supplementary data: Pharmacokinetics and pharmacodynamics of lisdexamfetamine compared with D-amphetamine in healthy subjects

Supplemental methods

Quantification of D-amphetamine in human plasma samples

Chemicals and reagents: HPLC-grade purity methanol and formic acid were purchased from Sigma-Aldrich (St. Louis, MO) or Biosolve (Dieuze, France). Distilled water was deionized using a MilliQ water purification system (Millipore, USA). Solutions of *d*-amphetamine hydrochloride and *d*-amphetamine-D_3 sulfate >99.9% were obtained from Lipomed (Arlesheim, Switzerland). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and of the highest grade available.

Instrumentation and analytical conditions

Analytical instruments: Ultra-High pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) using an Agilent 1290 UHPLC instrument equipped with a binary solvent delivery system, an auto sampler (at 4°C), and a column oven, coupled to an Agilent 6490 triple quadrupole mass spectrometer equipped with a jet stream electrospray ionization interface (AJS-ESI) (Agilent Technologies, Basel, Switzerland) was used to determine *d*-amphetamine and *d*-amphetamine-D_3.

Liquid chromatography: The chromatographic separation was performed on a Waters Acquity UPLC BEH C18, 1.7 µm, 2.1×150 mm, column (Waters, Wexford, Ireland) at column temperature of 65ºC. The mobile phase was water-methanol-formic acid (41/59/0.1; v/v/v) and the flow rate was set at 0.45 mL/min. The analysis time was 1.5 min. A methanol in water (75/25 v/v) mixture was used as needle and needle-seat flushing solvent for 10 s after sample aspiration. Samples were stored until analysis in the auto sampler (maintained at 4°C). The injection volume was 3 µL per sample. Under these conditions, *d*-amphetamine and *d*-amphetamine-D_3 showed a retention time of 0.8 min.

Mass spectrometry: Characteristic precursor ions and their corresponding product ions for multiple reaction monitoring (MRM) were defined by using the compound optimizer software module included within the Mass Hunter Workstation software (Agilent Technologies, California, USA). *D*-amphetamine and *d*-amphetamine-D_3 (internal standard) were quantified using the corresponding mass transitions (*d*-amphetamine *m/z* 136.1→91.0 (16 V, Dwell 100 ms), *m/z* 136.1→119 (12 V, Dwell 100 ms) and *d*-amphetamine-D_3 *m/z* 139.1→94.0 (16 V, Dwell 10 ms)). The AJS-ESI source conditions were optimized using the integrated source optimizer tool and set in the positive ion mode as following: Nitrogen gas temperature (290°C), gas flow (14 L/min), nebulizer (20 psi), sheath gas temperature (300 °C), sheath gas flow (11 L/min), capillary voltage (3000 V), and nozzle voltage (1500 V) (Agilent Technologies, California, USA, B.08.00/Build 8.0.8023.0).

Data analysis: The Mass Hunter Workstation Acquisition software Version B.08.00/Build 8.0.8023.0 and MassHunter Workstation Software Quantitative Analysis Version B.07.01 /Build 7.1.524.0, respectively (Agilent Technologies, California, USA) was used for data acquisition and subsequent data analysis.

Standard solutions: *D*-amphetamine hydrochloride (1 mg free base /1 mL methanol) and d-amphetamine-D_3 sulfate (0.1 mg free base /1 mL methanol) solutions were bought as reference standards. Stock solutions in methanol containing 10 µL/mL *d*-amphetamine or *d*-amphetamine-D_3 were prepared and stored at -20ºC.

Sample preparation: To 100 µL of sample, calibrator or quality control, 20 µL of a *d*-amphetamine-D_3 internal standard solution (0.25 µg/mL), and 500 µL ethyl acetate for liquid–liquid extraction was added. The samples were shortly vortexed, vigorously mixed on a rotating mixer for 5 min, and centrifuged for 10 min at 16,000 x g at 4°C. The upper ethyl acetate layer (350 µL) was transferred into fresh vials and evaporated to dryness under nitrogen. Afterwards the samples were reconstituted in 50 µL methanol (10 min, 1300 rpm, 4°C, thermoshaker) and transferred into new glass vials.

Chromatographic performance: Ten point calibration curves over the range of 0.78 to 200 ng/mL for *d*-amphetamine were generated by a zero sample and nine calibrators in human plasma. The coefficient of determination (R^2) was 0.99 and at least 75% of all calibrators have to be valid.

Specificity: Human plasma samples without the addition of *d*-amphetamine and *d*-amphetamine-D_3 were processed and injected into the UHPLC–MS/MS within an analytical run. The peak areas evaluated in the blank samples were not allowed to exceed 20% of the mean LLOQ peak area.

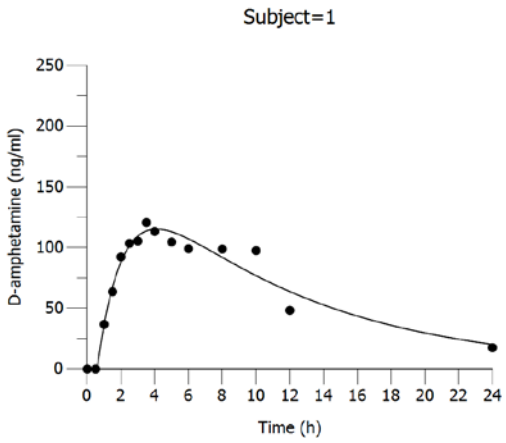
Recovery: By comparing the mean peak areas of extracted with those of unextracted samples (100% recovery) the absolute recovery was determined. The *d*-amphetamine recoveries were 101.7%, 102.2%, and 100.3% at concentrations of 1.66, 12.5, and 100 ng/mL.

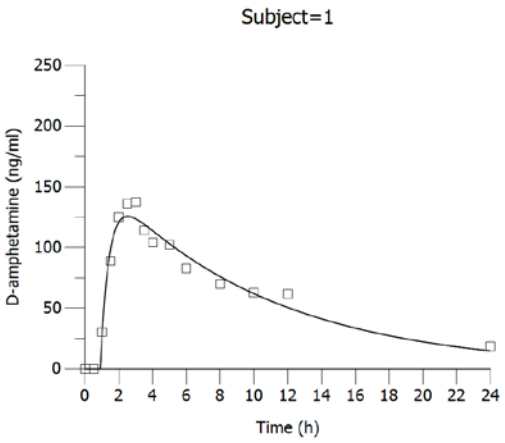
Limit of detection (LLOD) and limit of quantification (LLOQ): Lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were assessed by analyzing decreasing amounts of *d*-amphetamine in human plasma and were calculated as the concentration giving peaks with a signal-to-noise ratio of ≥ 5 and ≥ 10, respectively. The LLOQ was decided as the lowest concentration on the calibration curve which fulfilled the criteria of imprecision below 20%, and inaccuracy within ±20%. The method had a LLOD of 0.26 ng/mL, respectively a LLOQ of 0.78 ng/mL for *d*-amphetamine.

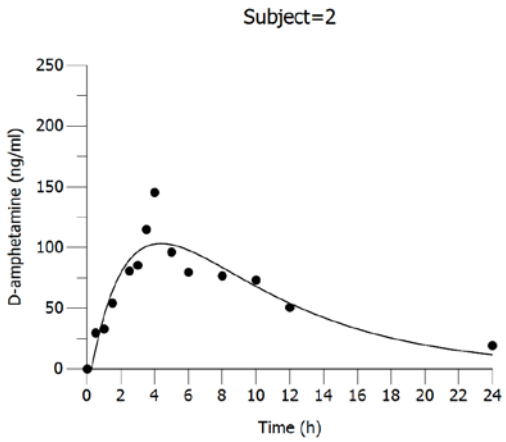
Reproducibility: Five replicates of quality controls (QCs) at the concentration of 1.66, 12.5, and 100 ng/mL were processed and injected into the UHPLC–MS/MS. To ensure the reproducibility, these sets of QCs were tested within validation runs. In each run, intra-run imprecision (% coefficient of variation; CV%) of each QC series had to be below 15% (20% at the LLOQ) and intra-run inaccuracy (% relative error of measurement; RE%) had to be within ±15% of the nominal values (±20% at the LLOQ). The intra-day precision was less than 8.8% and the accuracy ranged from −12.5 to 14.9% throughout all QC concentrations.

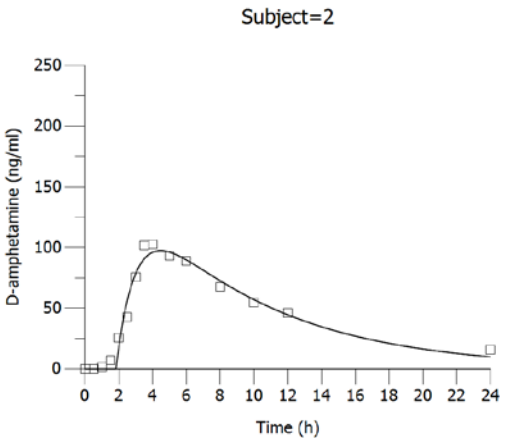
***Stability*:** The stability of *d*-amphetamine in human plasma was assessed using QC at the concentrations of 1.66, 12.5, and 100 ng/ml. The samples were reanalyzed after kept at different storage conditions. The determined auto sampler stability (QC stored at 4°C for 24 h), as well as the short-term stability (storage of QC samples at −20°C for 1-week) were within ±15% of the nominal values.

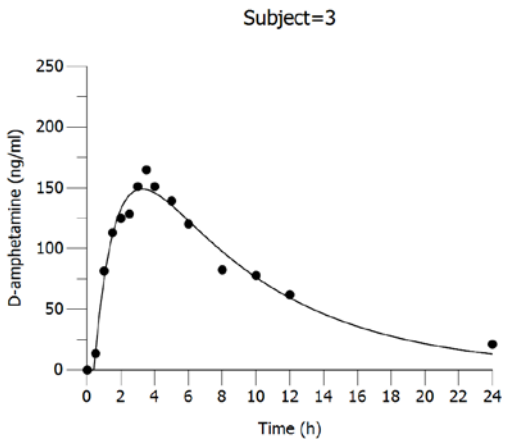
Supplementary Figures

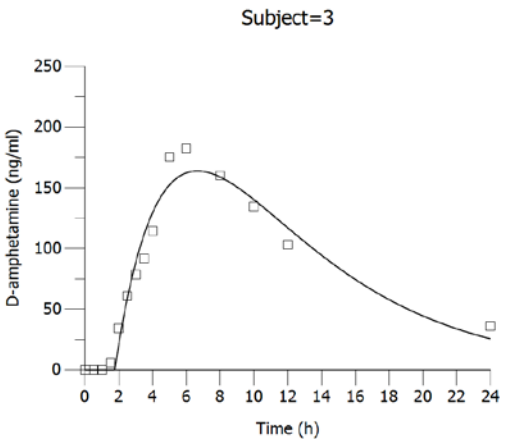


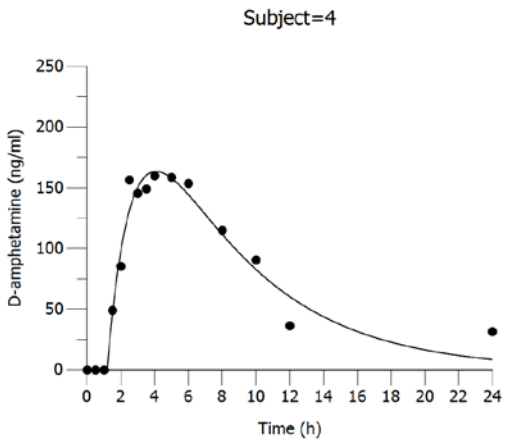


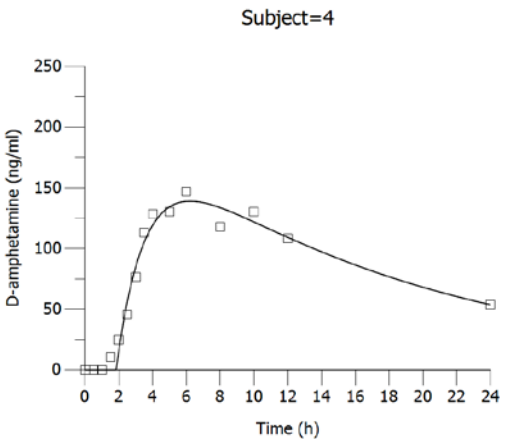












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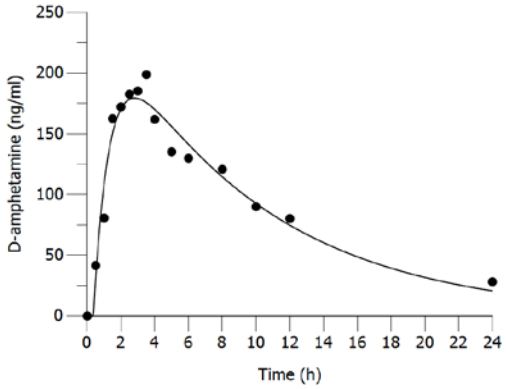

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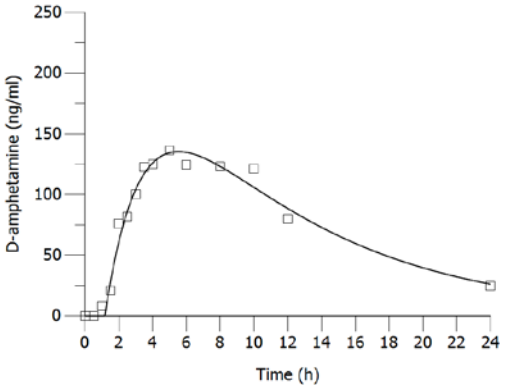

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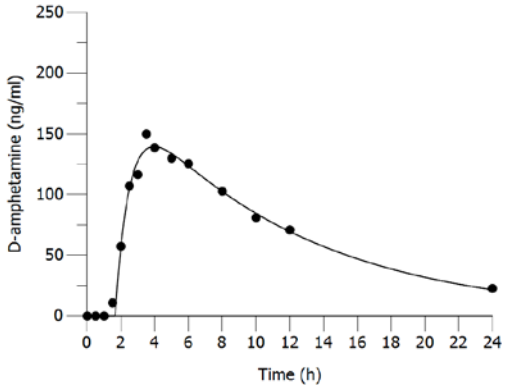

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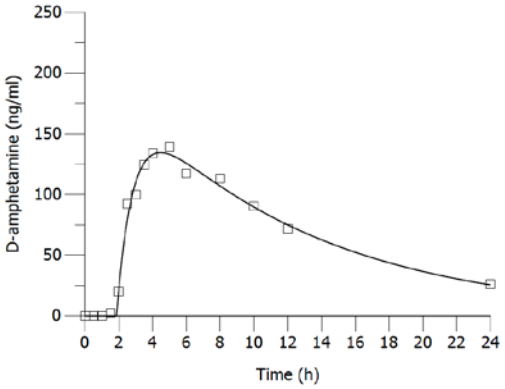

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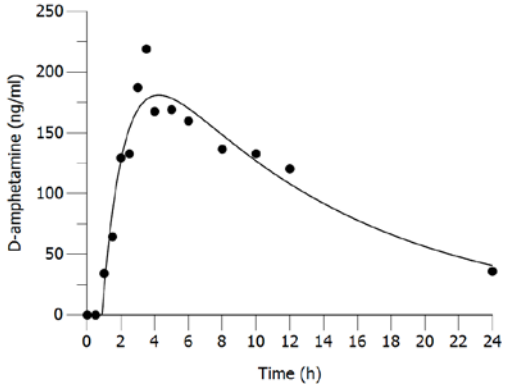

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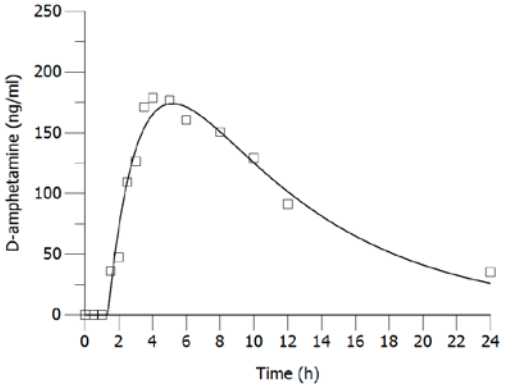

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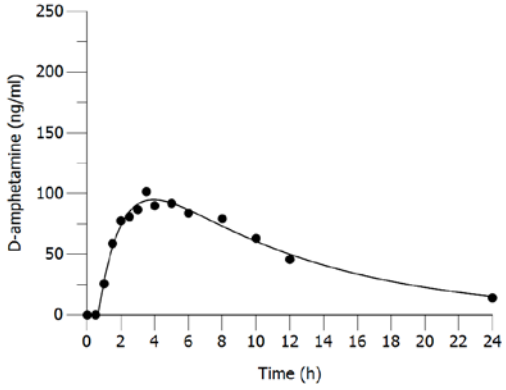

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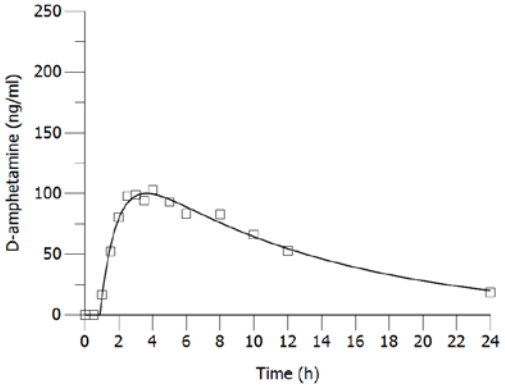

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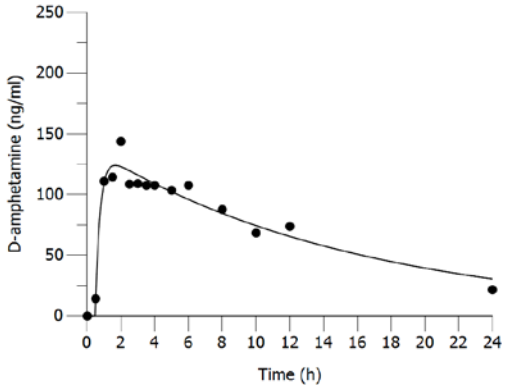

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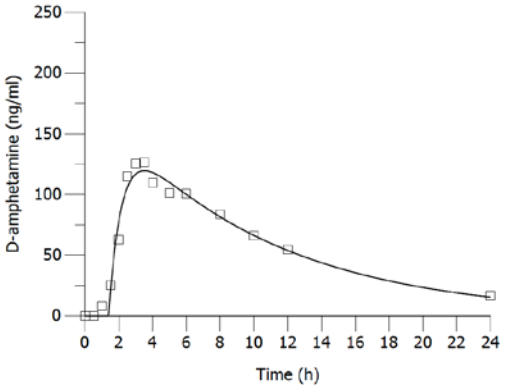

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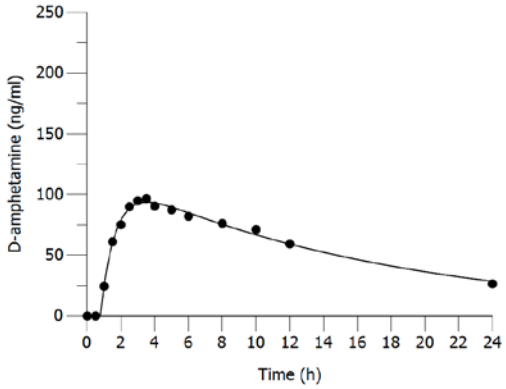

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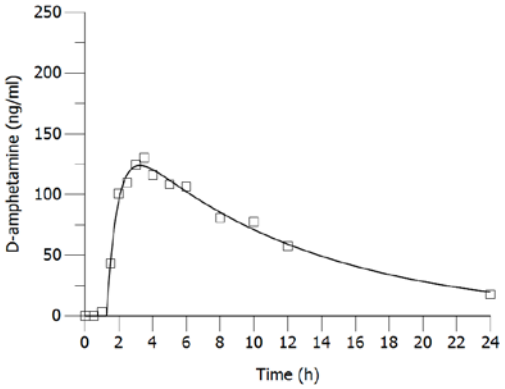

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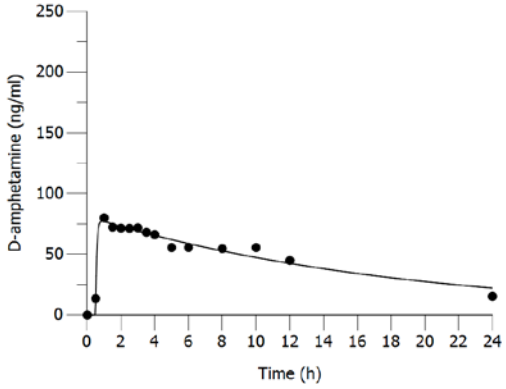

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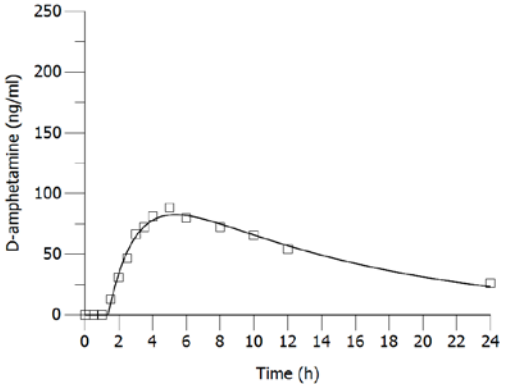

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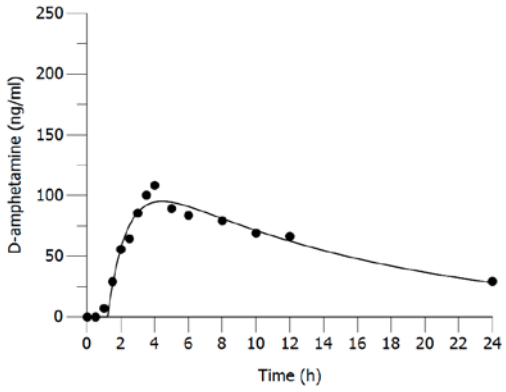

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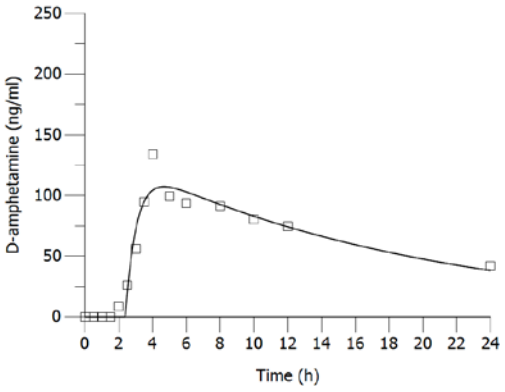

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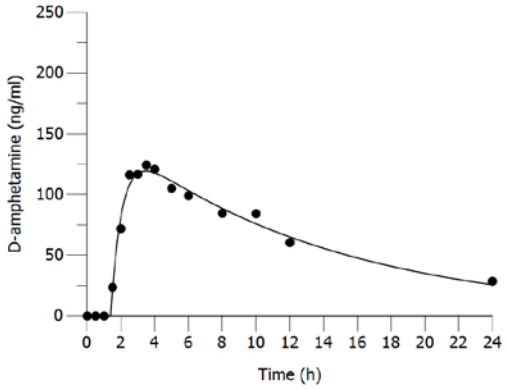

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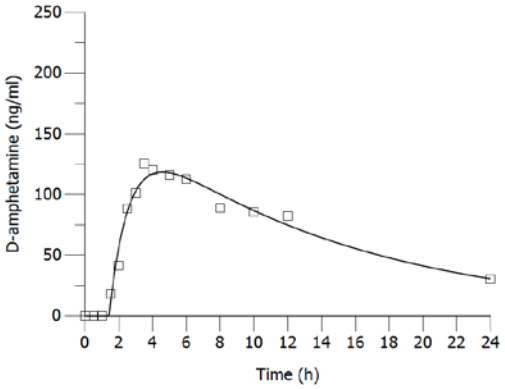

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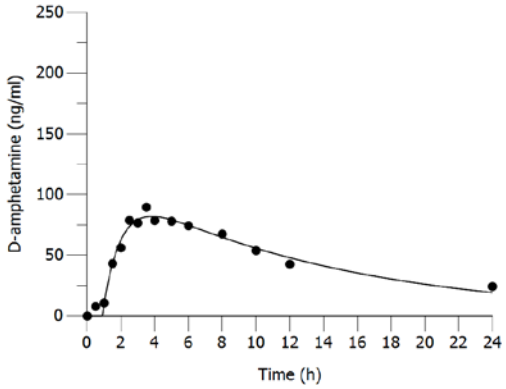

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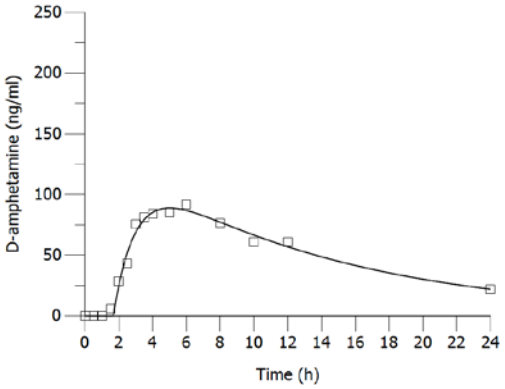

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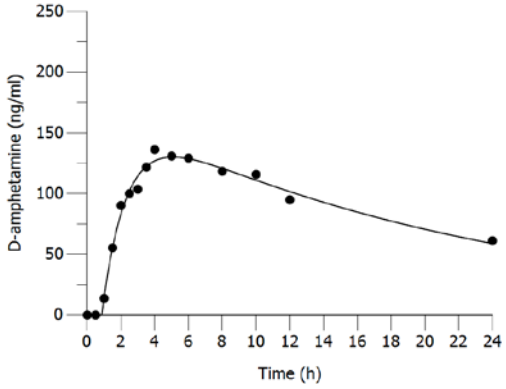

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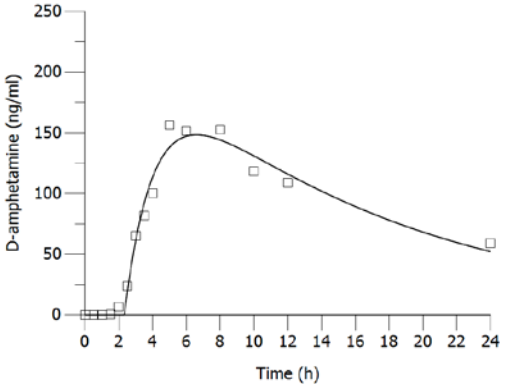

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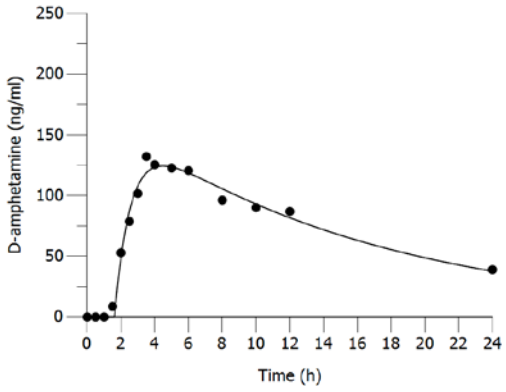

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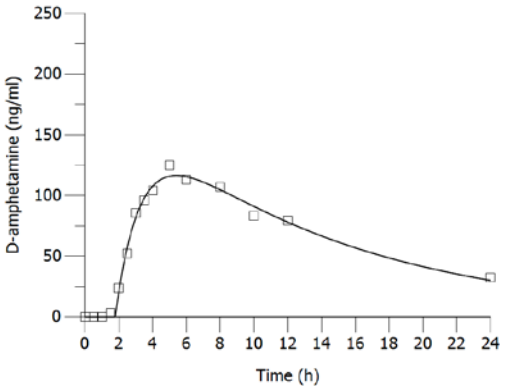

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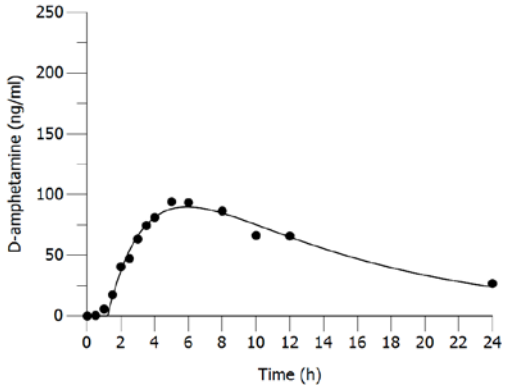

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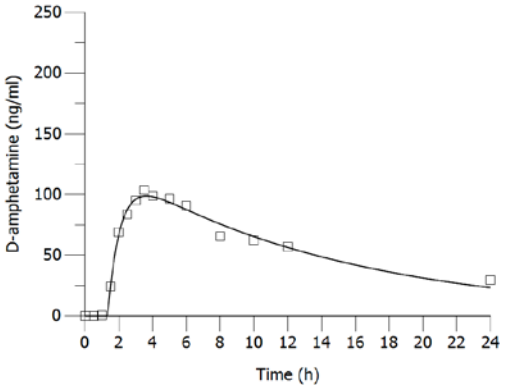

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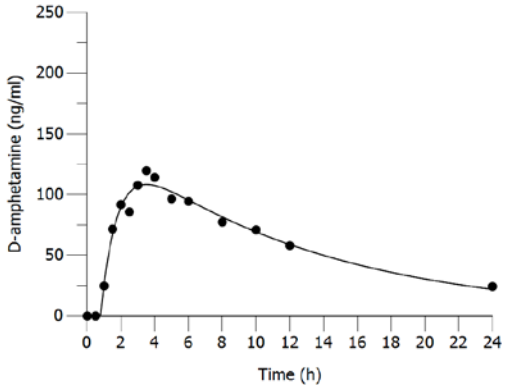

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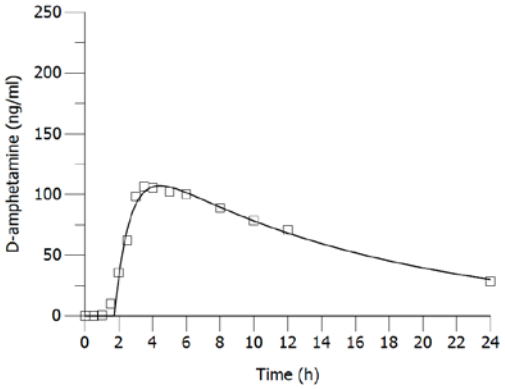

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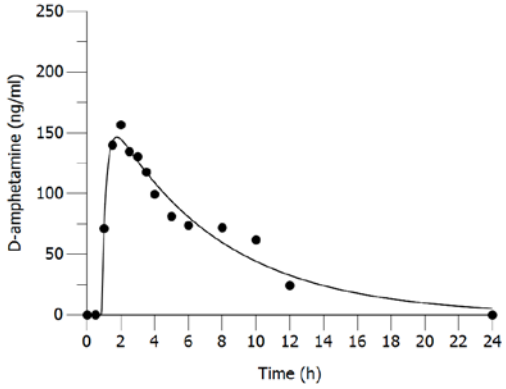

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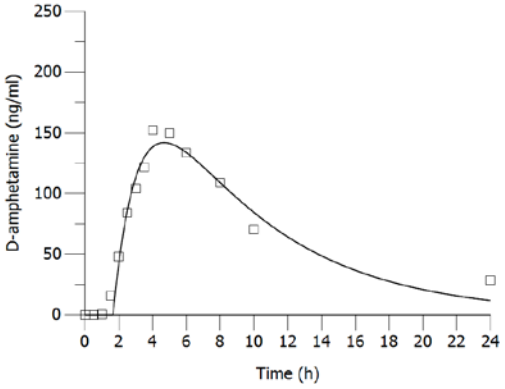

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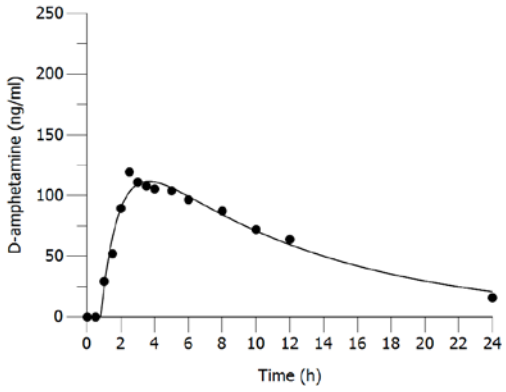

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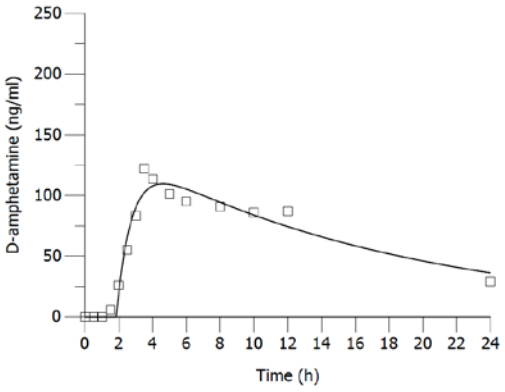

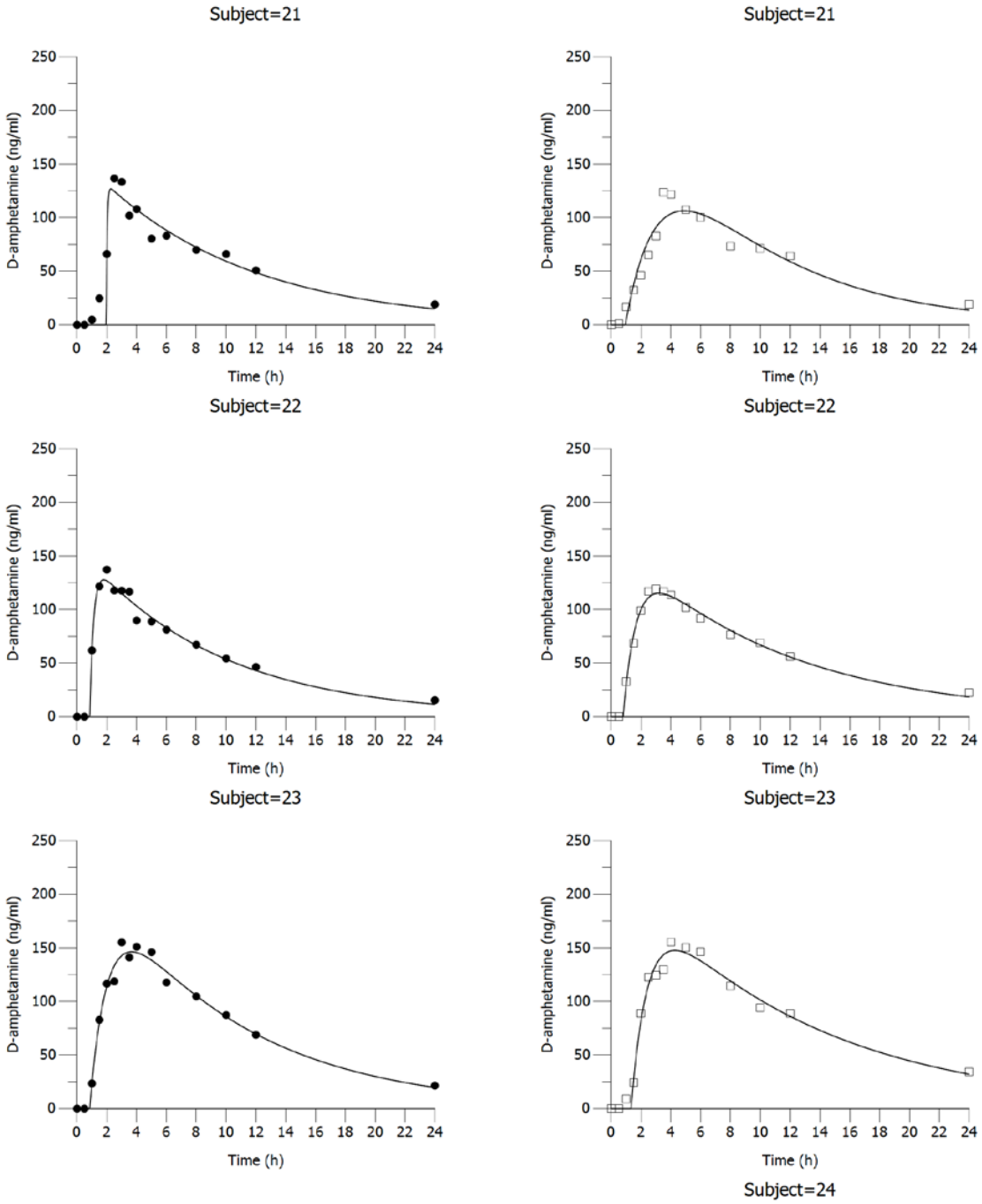

Figure S1. Individual plasma amphetamine concentration-time curves. D-amphetamine was orally administered at a dose of 40 mg, and lisdexamfetamine was administered at a dose of 100 mg at t = 0 in the same subjects. The data represent individual observed plasma amphetamine concentrations measured at different time points (●, D-amphetamine; □, lisdexamfetamine) and amphetamine concentrations predicted by the one-compartment pharmacokinetic model (black lines). Note the longer lag time in the lisdexamfetamine condition in most subjects but the otherwise similar curve shapes, including similarly steep rates of increasing amphetamine concentrations.

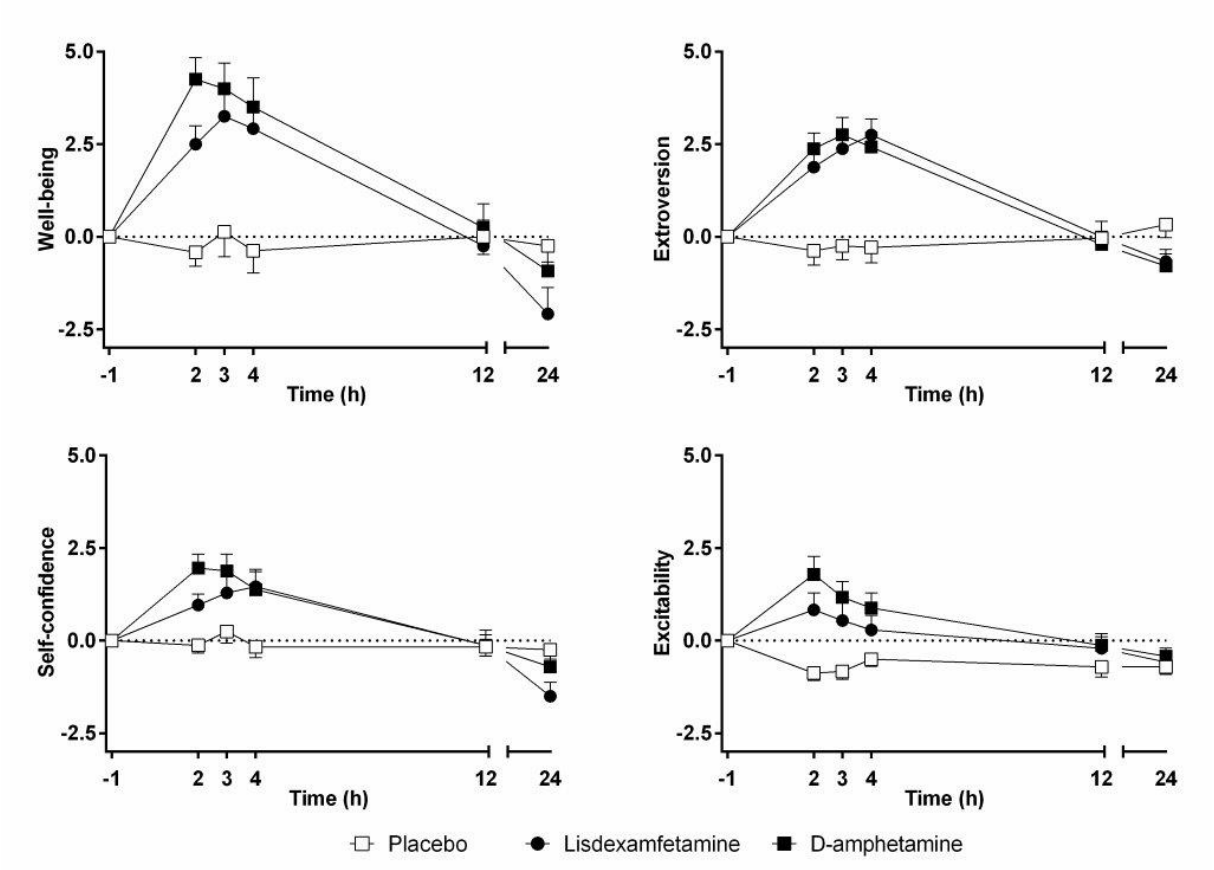

Figure S2. Lisdexamfetamine and D-amphetamine similarly increased subjective effects on the Adjective Mood Rating Scale compared with placebo. The data are expressed as the mean ± SEM in 24 subjects.

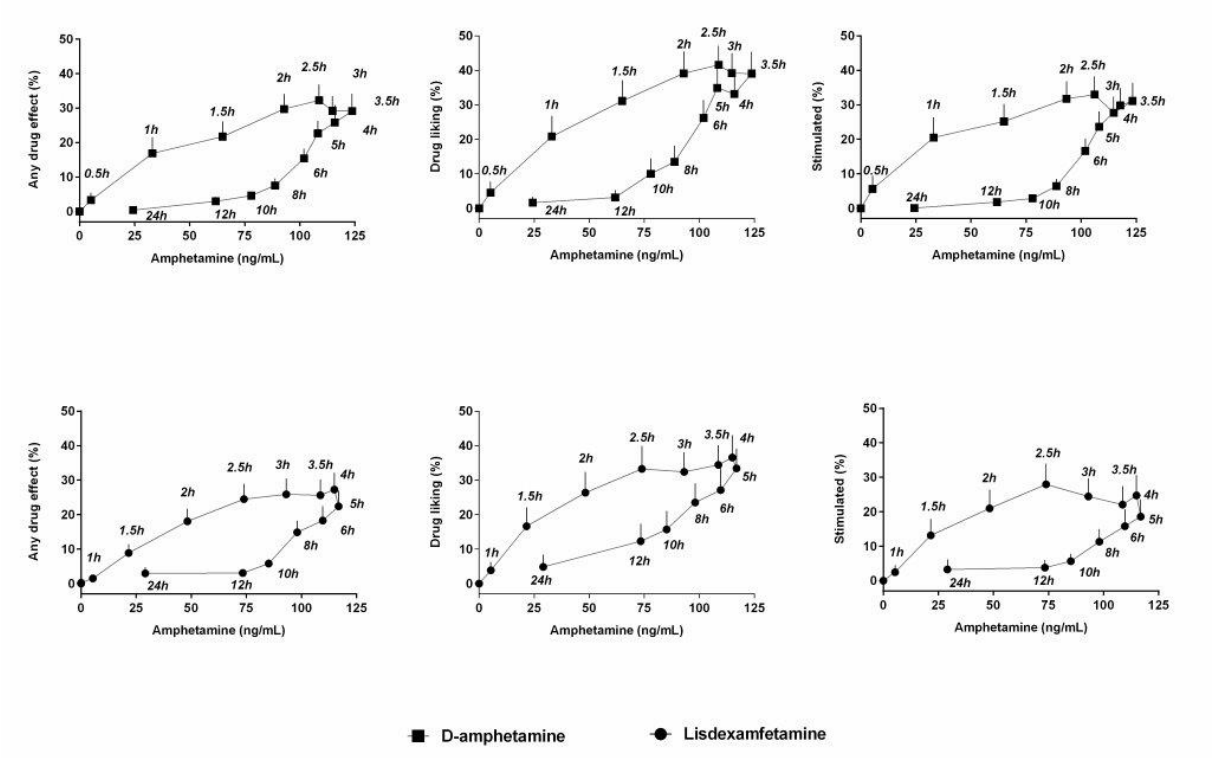

Figure S3. Amphetamine concentration-effect plots (hysteresis curves). The subjective effects presented similar clockwise hysteresis after administration of lisdexamfetamine and D-amphetamine, indicating comparable acute pharmacological tolerance. The data are expressed as mean ± SEM. The time of sampling is noted next to each point. The drugs were administered at t = 0.

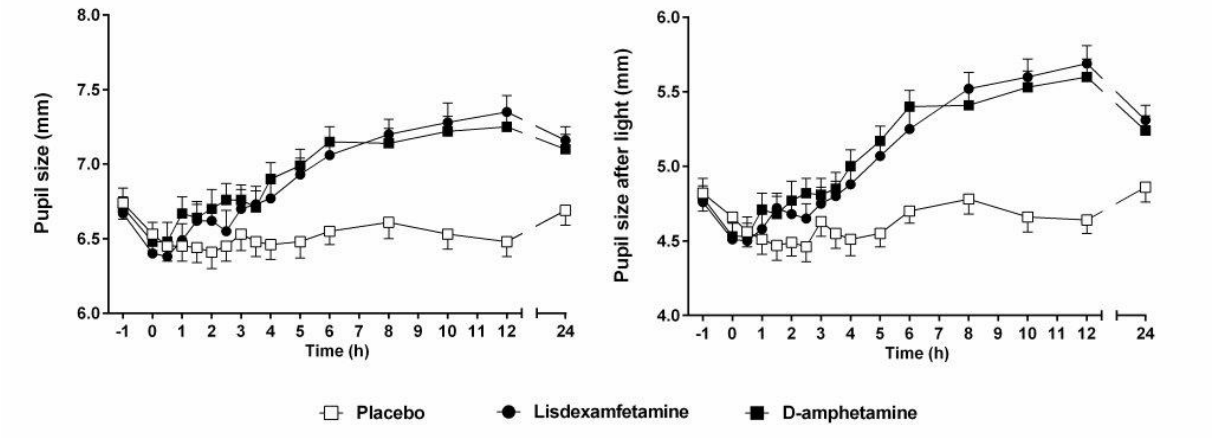

Figure S4. The pupil diameter at rest in the dark or after a light stimulus changed similarly over time after administration of lisdexamfetamine and D-amphetamine compared with placebo. The data are expressed as the mean ± SEM in 24 subjects.

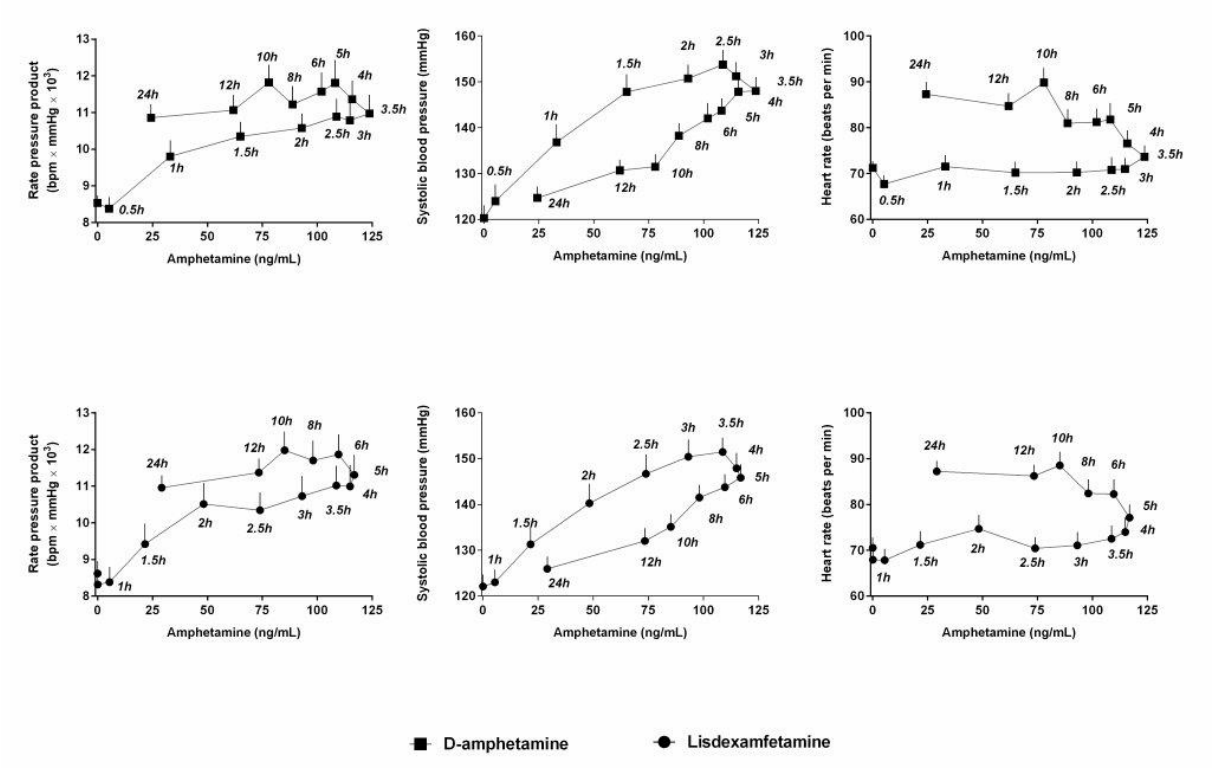

Figure S5. Amphetamine concentration-effect plots (hysteresis curves). The blood pressure response presented similar clockwise hysteresis after administration of lisdexamfetamine and D-amphetamine, indicating comparable acute pharmacological tolerance. The heart rate response presented similar counterclockwise hysteresis after administration of lisdexamfetamine and D-amphetamine, indicating a comparable lag and the absence of tolerance. The data are expressed as mean ± SEM. The time of sampling is noted next to each point. The drugs were administered at t = 0.

Supplementary Tables

Table S1. Pharmacokinetics of amphetamine after administration of D-amphetamine or lisdexamfetamie based on non-compartmental analysis

Drug condition	N=		C_{max} (ng/mL)	t_{max} (h)	t_{onset} (h)	$t_{1/2}$ (h)	AUC_{24} (ng·h/mL)	AUC_{∞} (ng·h/mL)
D-Amphetamine	23	geometric mean (95% CI)	130 (117-145)	3.1 (2.7-3.6)	0.73 (0.58-0.92)	8.5 (7.6-9.6)	1494 (1355-1646)	1800 (1601-2024)
		range	80-219	1-5	0.2-1.6	4.7-16.8	1040-2514	1098-3648
Lisdexamfetamine	24	geometric mean (95% CI)	126 (115-139)	4.3 (3.8-4.7)***	1.4 (1.2-1.6)***	9.2 (8.4-10)	1564 (1435-1705)	1955 (1759-2174)
		range	84-185	3-8	0.7-2.3	6.7-14.6	1096-2208	1277-3392

AUC_{∞}, area under the plasma concentration-time curve from time zero to infinity; AUC_{24}, area under the plasma concentration-time curve from time zero to 24 h; C_{max}, maximum plasma concentration; $t_{1/2}$, terminal plasma elimination half-life; t_{max}, time to reach C_{max}; t_{onset}, time to reach 10% of C_{max}; ***P < 0.001 significant difference compared with D-amphetamine (T=5,11 and 3,76 for t_{onset} and t_{max}, respectively).

Table S2. Pharmacodynamic effect parameters and statistics

	Lisdexamfetamine (mean±SEM)	D-amphetamine (mean±SEM)	Difference (T value)	P value
Subjective effects (Visual Analog Scales)				
Any drug effect				
T_{onset} (h)	1.5±0.13	0.8±0.08	4.63	<0.001
T_{max} (h)	4.3±0.38	3.0±0.32	2.58	<0.05
E_{max} (%)	36±4.9	39±4.8	0.44	0.66
AUEC (%·h)	203±35	194±30	0.19	0.85
Drug liking				
T_{onset} (h)	1.6±0.15	0.9±0.10	3.65	<0.001
T_{max} (h)	4.1±0.50	2.7±0.37	2.28	<0.05
E_{max} (%)	48±6.9	51±5.8	0.36	0.72
AUEC (%·h)	363±85	280±48	0.85	0.40
Stimulated				
T_{onset} (h)	1.5±0.14	1.0±0.13	2.63	<0.05
T_{max} (h)	3.5±0.34	2.3±0.21	2.94	<0.01
E_{max} (%)	38±6.8	44±5.7	0.66	0.51
AUEC (%·h)	194±47	189±32	0.08	0.94
Autonomic effects				
Systolic blood pressure				
T_{onset} (h)	1.3±0.14	0.7±0.09	3.64	<0.001
T_{max} (h)	3.8±0.45	3.3±0.36	0.91	0.37
E_{max} (mmHg)	37±2.5	40±2.2	0.91	0.37
AUEC (mmHg·h)	274±39	307±45	0.57	0.57
Diastolic blood pressure				
T_{onset} (h)	0.9±0.13	0.6±0.09	2.21	<0.05
T_{max} (h)	3.3±0.32	3.2±0.31	0.16	0.88
E_{max} (mmHg)	27±2.1	31±2.2	1.25	0.22
AUEC (mmHg·h)	225±37	257±37	0.61	0.54
Mean arterial pressure				
T_{onset} (h)	1.1±0.14	0.6±0.09	3.13	<0.01
T_{max} (h)	3.5±0.27	3.3±0.39	0.40	0.69
E_{max} (mmHg)	29±2.1	33±2.1	1.21	0.23
AUEC (mmHg·h)	241±34	274±36	0.65	0.52
Rate pressure product (beats·mmHg/min)				
T_{onset} (h)	1.3±0.12	1.0±0.15	1.80	0.08
T_{max} (h)	7.7±0.58	6.2±0.61	1.85	0.07
E_{max} (mmHg·BPM)	5399±429	5556±497	0.24	0.81
AUEC (mmHg·BPM·h)	68267±6281	68554±8250	0.03	0.98

Values are differences from placebo (mean±SEM) in 24 subjects.

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