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The effect of chronic treatment with lurasidone on rat liver cytochrome P450 expression and activity in the chronic mild stress (CMS) model of depression

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Running title page

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Lurasidone effect on cytochrome P450 in the depression model

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d) ABBREVIATIONS: CYP, cytochrome P450; CREB, cAMP response element-binding protein; CMS, chronic mild stress; MD, early-life maternal deprivation; RS, repeated restraint stress

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ABSTRACT

Recent studies indicated an important role of the monoaminergic nervous systems (dopaminergic, noradrenergic and serotonergic systems) and stress in the regulation of cytochrome P450 (CYP) expression and activity in the liver. The aim of our present research was to determine the effect of the novel atypical neuroleptic drug with antidepressant properties lurasidone, on the expression (mRNA and protein level) and activity of liver CYP isoforms involved in the metabolism of drugs and endogenous steroids, in the chronic mild stress (CMS) model of depression. Male Wistar rats were subjected to CMS for 7 weeks. Lurasidone (3 mg/kg p.o./day) was administered to non-stressed or stressed animals for 5 weeks (weeks 3-7 of CMS). It has been found that 1) CMS moderately affects cytochrome P450 (CYP2B, CYP2C11 and CYP3A) and its effects are different from those observed after other kinds of psychological stress, such as repeated restraint stress (RS) or early-life maternal deprivation (MD); 2) chronic lurasidone influences the expression and/or activity of CYP2B, CYP2C11 and CYP3A isoforms; 3) CMS modifies the action of lurasidone on cytochrome P450 expression and function, leading to different effects of the neuroleptic in non-stressed and stressed rats. Based on the obtained results, it can be suggested that the metabolism of endogenous substrates (e.g. steroids) and drugs, catalyzed by the isoforms CYP2B, CYP2C11 or CYP3A may proceed at a different rate in the two groups of animals (non-stressed and stressed) in the rat CMS model.

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Introduction

Our earlier studies indicate an important role of the monoaminergic nervous systems (dopaminergic, noradrenergic and serotonergic systems) in the neuroendocrine regulation of cytochrome P450 (CYP) expression in the liver, involving growth hormone, corticosterone and thyroid hormones (Wójcikowski and Daniel, 2009; Kot and Daniel, 2011; Kot et al., 2012; Kot et al., 2013; Bromek et al., 2013; Sadakierska et al, 2013; Kot et al., 2015; Kot and Daujat-Chavanieu, 2016; Rysz et al. 2015; 2016 a, b; Kot, 2017). The above-mentioned hormones are the main physiological regulators of *CYP* genes in the liver (Gibson et al., 2002; Waxman and O'Connor, 2006, Monostory et al., 2009; Dvorak and Pavek, 2010; Monostory and Dvorak, 2011; Brtko and Dvorak, 2011).

Stress, depending on its nature, intensity and duration, produces multiple changes in the functioning of the monoaminergic systems and thus it may affect the central neuroendocrine and autonomic regulation of liver function and cytochrome P450 expression (Carrasco et al., 2003; Uyama et al., 2004; Kot et al., 2013; Chmielarz et al., 2015). Psychotropic drugs that act on monoaminergic receptors or transporters in the brain and periphery affect the endocrine and immune systems (Jaber et al., 1994; Rane et al., 1996; Raap et al., 1999; Drzyzga et al., 2006; Capuzzi et al., 2017; Köhler et al., 2017) and thus may also influence the liver function and cytochrome P450 regulation, independently of their direct action on the monoaminergic and other receptors present in the liver (Daniel, 2005; Ruddell et al., 2008; Zidek et al., 2009; Wójcikowski and Daniel, 2011; Konstandi, 2013; Kot and Daujat-Chavanieu, 2016).

Lurasidone is a novel atypical antipsychotic drug with a high affinity for dopamine D_2, serotonin 5-HT_{2A} and 5-HT_7 receptors. It has also antagonist activity at α_{2A} and α_{2C} adrenergic receptors and partial agonist activity at 5-HT_{1A} receptors (Ishibashi et al., 2010). It is effective in the therapy of patients with schizophrenia and shows antidepressant properties in patients with bipolar

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disorders (Meyer et al., 2009; Citrome, 2011; Fountoulakis et al., 2015). Lurasidone also shows antidepressant-like effects in the chronic mild stress (CMS) model of depression in the rat (Willner 1997; Luoni et al., 2015).

Lurasidone is an azapirone derivative with a benzisothiazol-piperazine side chain, metabolized predominantly by CYP3A4 in humans (Caccia et al., 2011; Chiu et al., 2014). The main metabolic pathways of lurasidone include oxidative N-dealkylation between the piperazine and cyclohexane rings, hydroxylation of the norbornane ring, and S-oxidation. Other metabolic pathways comprise hydroxylation of the cyclohexane ring and reductive cleavage of the isothiazole ring followed by S-methylation. The two major inactive metabolites are the N-dealkylation products (the acidic metabolites ID-20219 and ID-20220) and the two active metabolites are the norbornane hydroxylation products (ID-14283 and ID-14326).

In vitro study on human liver microsomes and cDNA-expressed CYP isoforms showed a modest inhibitory effect of lurasidone on the activity of CYP1A2, CYP2C19 and CYP3A4 isoforms (Wójcikowski et al., 2016; Greenberg and Citrome, 2017), but weak or no effect on other CYP enzymes, such as CYP2C9, CYP2B6 or CYP2D6. However, its possible effect on liver cytochrome P450 expression produced by chronic treatment, that is used to treat psychiatric disorders, has not been studied as yet, though the antipsychotic or antidepressant therapy lasts for months or years.

The liver enzymatic complex of cytochrome P450 is engaged in the metabolism of endogenous substrates (e.g. steroids) and drugs of different chemical structures and pharmacological groups including psychotropics (neuroleptics, antidepressants, anxiolytics). Since comorbidity and concomitant medications are common in psychiatric patients, pharmacokinetic interactions between psychotropic drugs at a level of cytochrome P450 may occur. Therefore, the aim of our present research was to investigate the effect of chronic treatment with lurasidone on the expression and activity of cytochrome P450 in the rat, in normal conditions and under chronic mild stress (CMS), an animal model of depression.

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Materials and Methods

Animals. Male Wistar Han rats (Charles River Laboratories, Sulzfeld, Germany), weighing 280–300 g, were-singly housed with food and water freely available, and were maintained on a 12-h light/dark cycle (lights on at 08.00 h) under conditions of constant temperature (22 ± 2°C) and humidity (50 ± 5%). All procedures used in this study were conducted in compliance with the rules and principles of the 86/609/EEC Directive, and were approved by the Bioethical Committee of the Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland.

Drugs and chemicals. Lurasidone (hydrochloride) was provided by TargetMol (Boston, MA, USA). NADP, NADPH, glucose-6-phosphate-dehydrogenase, glucose-6-phosphate, caffeine and its metabolites (theobromine, paraxanthine, theophylline, and 1,3,7-trimethyluric acid) were purchased from Sigma (St. Louis, MO, USA). Testosterone and its hydroxy-metabolites (2α-, 2β-, 6β-, 7α-, 16α- and 16β-hydroxytestosterone) were supplied by Steraloids (Newport, KY, USA). The polyclonal rabbit primary anti-rat CYP2C11 antibody was obtained from Abcam (Cambrige, UK), the anti-rat CYP3A23/3A1 and CYP3A2 antibodies came from Millipore (Temecula, USA). The polyclonal goat anti-rat CYP2B1 antibody was form Daiichi Pure Chemicals (Tokyo, Japan). The polyclonal anti-rat β-actin antibody was purchased from Santa Cruz (Dallas, TX, USA). The chemiluminescence reagents LumiGlo kit came from KPL (Gaithersburg, MD, USA). For RNA isolation, a mirVana kit purchased from Life Technologies (Carlsbad, CA, USA) was used. A Transcriptor High-Fidelity cDNA synthesis kit for reverse-transcription was supplied by Roche Diagnostics (Indianapolis, IN, USA). TaqMan assays and the TaqMan Gene Expression Master Mix were derived from Life Technologies (Carlsbad, CA, USA). RNA-free water was obtained from Sigma (St. Louis, MO, USA). All the organic solvents of HPLC purity were provided by Merck (Darmstadt, Germany).

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In vivo experiment and liver sample preparation. CMS experiments were performed according to the method described previously (Papp et al., 2014). Briefly, stressed rats were subjected to the CMS procedure for a total of 7 weeks. The stress protocol consisted of: two periods of food or water deprivation, two periods of 45 degree cage tilt, two periods of intermittent illumination (lights on and off every 2 h), two periods of soiled cage (250 ml water in sawdust bedding), one period of paired housing, two periods of low intensity stroboscopic illumination (150 flashes/min), and three periods of no stress. All stressors were 10 – 14 h of duration and were applied individually and continuously, day and night. Control animals were housed in separate rooms and had no contact with the stressed animals. Following 2 weeks of initial stress, both control and stressed animals were divided into subgroups and for the next 5 weeks received once-daily per os (p.o.) either vehicle (1% hydroxyethylcellulose, 1 ml/kg) or lurasidone (3 mg/kg). After five weeks, the treatments were terminated and 24 h later all animals were sacrificed by decapitation and their livers were quickly removed, frozen using dry ice, and stored at -80°C. Microsomes were prepared from individual rat livers by differential centrifugation (11 000 g and 2 x 100 000 g) in a 20 mM Tris/KCl buffer (pH 7.4), including washing with 0.15M KCl, as described previously (Kot et al. 2012). The above procedure deprives microsomes of the presence of drug administered in vivo.

Determination of CYP isoform activity in the liver. The activity of CYP isoforms was studied in the livers of control rats (non-stressed and stressed) and lurasidone-treated animals (non-stressed and stressed), in the absence and presence of lurasidone added *in vitro* to liver microsomes. *In vitro* studies into isoform-specific metabolism of caffeine, warfarin, bufuralol and testosterone in liver microsomes were carried out at the linear dependence of product formation on time, protein and substrate concentration, in the previously optimized conditions (Haduch et al., 2006; Daniel et al., 2006; Kot and Daniel, 2008). Incubations were conducted in a system containing the liver

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microsomes (ca. 1 mg of protein/ml). The activity of CYP1A2 was determined by measuring the rate of caffeine metabolism, C-8-hydroxylation (catalyzed by CYP1A2 in the rat) and 1-N-, 3-N- and 7-N-demethylation (catalyzed by CYP1A2 and other CYP isoforms) at a substrate concentration of 100 μM as described previously (Kot and Daniel, 2008 a; Kot and Daniel, 2008 b) with a minor modification, i.e., the NADPH generating system was replaced with 1.5 mM NADPH (Kot et al, 2012). The final incubation volume was 1 ml and incubation time was 50 min. Caffeine and its metabolites were analyzed by HPLC with UV detection (Kot and Daniel, 2008). The activity of CYP2C6 was studied by measuring the rate of warfarin 7-hydroxylation at a substrate concentration of 60 μM as described previously (Daniel et al., 2006). The final incubation volume was 0.5 ml and incubation time was 15 min. Warfarin and its metabolite were analyzed by HPLC with fluorescence detection. The activity of CYP2D was estimated by measuring the rate of bufuralol 1'-hydroxylation at a substrate concentration of 10 μM as described previously (Hiroi et al., 1998; Bromek et al., 2010). The final incubation volume was 0.4 ml and incubation time was 10 min. Bufuralol and its metabolite were analyzed by HPLC with fluorescence detection. The activities of CYP2A, CYP2B, CYP2C11 and CYP3A were studied by measuring the rate of P450-specific reactions: the 7α-, 16β-, 2α- and 16α-, 2β- and 6β-hydroxylation of testosterone, respectively, at a substrate concentration of 100 μM and incubation time 15 min, as described previously (Haduch et al., 2006; Haduch et al., 2008; Wójcikowski et al., 2013). The final incubation volume was 1 ml. Testosterone and its metabolites were analyzed by HPLC with UV detection. In a separate experiment, the activities of CYP2A, CYP2B, CYP2C11 and CYP3A were determined in the absence or presence of lurasidone added *in vitro* to pooled liver microsomes (n=5) derived from non-stressed or stressed control rats, at a pharmacological/therapeutic concentration of 0.25 or 1 μM (Lee et al., 2011; Chiu et al., 2014), without or with preincubation with the neuroleptic for 30 min. Then the substrate (testosterone) was added and the incubation proceeded as above.

An analysis of CYP proteins in the liver. The protein levels of CYP2C11, CYP2B and

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CYP3A isoforms in the liver microsomes of control and lurasidone-treated rats were estimated using Western immunoblot analyses as described previously (Kot and Daniel, 2011; Rysz et al., 2016 a). Briefly, microsomal proteins (10 μg per each sample), were separated using an SDS polyacrylamide gel electrophoresis on a 12% separating gel and then the proteins were transferred to nitrocellulose membranes (Sigma–Aldrich). The following primary antibodies for liver microsomal CYPs were used: a polyclonal rabbit anti-rat antibody raised against CYP2C11 (Abcam, Cambrige, UK; catalog number ab3571; Lot number GR33348-6; dilution 1:1150) (Clarke et al., 2014), a polyclonal goat anti-rat antibody raised against CYP2B1 which also recognized the CYP2B2 form (Daiichi Pure Chemicals, Tokyo, Japan; catalog number 423550; Lot number C83104W; dilution 1:1600) (Zhang et al., 2000), a polyclonal rabbit anti-rat CYP3A23/3A1 (Millipore, Temecula, USA; catalog number AB1253; Lot number 2435027; dilution 1:1600) (Debri et al., 1995) and anti-rat CYP3A2 antibodies (Millipore, Temecula, USA; catalog number AB1276; Lot number 242677; dilution 1:1600) (Debri et al., 1995). After incubation with a primary antibody, the blots were incubated with a secondary antibody, e.g. an appropriate species-specific horseradish peroxidase-conjugated anti-IgG. Rat cDNA-expressed CYP2B1, CYP2C11 (5 μg), CYP3A23/3A1 and CYP3A2 (1 μg) isoforms (Supersomes) were used as standards. Bands on the nitrocellulose membrane were quantified with the Luminescent Image analyzer LAS-1000 using the Image Reader LAS-1000 and Image Gauge 3.11 programs (Fuji Film, Japan).

Isolation of liver RNA, cDNA synthesis and real-time analysis of the expression of genes encoding cytochrome P450 isoforms. The total RNA was isolated from the frozen liver tissue using a RNeassy® Plus Mini Kit (Qiagen) following the manufacturer's instructions. The quantity and the quality of isolated RNA were verified with a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was stored at –80°C until use. The first strand cDNA products were generated using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics) according to the manufacturer's instructions. Briefly, a reverse transcription was

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performed using 2 μg of the total RNA and oligo(dT) primers at a total volume of 20 μl. cDNA synthesis was carried out at 55°C for 30 min, and at 85°C for 5min to inactivate the enzyme. Following the reverse transcription, samples were diluted with 20 μl of an RNase-free water and stored at 20°C until the next step of analysis. The expression of genes coding for the cytochrome P450 isoform *CYP2B1* (Rn01457880), *CYP2B2* (Rn02786833), *CYP2C11* (Rn01502203), *CYP3A23/3A1* (Rn03062228), *CYP3A2* (Rn00756461) and of the reference gene *β-actin* (Rn00667869) was detected by a real-time PCR using a commercially available TaqMan Gene Expression Master Mix and species-specific TaqMan type probes and primers (TaqMan gene expression assay, Life Technologies). The reaction mixture (10 μl), consisted of 4.5 μl of cDNA, 5 μl of a TaqMan Gene Expression Master Mix and 0.5 μl of a TaqMan assay (Life Technologies). Negative control samples were processed in a similar way, but the template was omitted. Real-time PCR runs were performed using Bio-Rad CFX96 PCR system (Bio-Rad, Hercules, CA, USA), and standard thermal cycling conditions were used (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 and of 60°C for 1 min). The PCR reaction of the above-mentioned target and reference genes was run in duplicate. The level of *CYP* transcripts was normalized to the *β-actin* expression and relative quantification was obtained using the comparative delta-delta Ct method ($2^{-\Delta\Delta Ct}$). The relative amount of the target transcript was expressed as a fold change in the expression level relative to the calibrator (i.e. an average ΔCt of the control group).

Statistical analysis of data. All of the data are reported as the means (±S.E.M.). The results were analysed using a multivariate analysis of variance (ANOVA) followed by a post hoc Duncan test. The results were considered as statistically significant when $p < 0.05$.

Results

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The effect of CMS and lurasidone on the activity of CYP isoforms in liver microsomes. Chronic mild stress (CMS) significantly decreased the CYP2B activity measured as the testosterone 16β-hydroxylation rate and the CYP3A activity measured as the testosterone 6β-hydroxylation rate (Fig. 1), not affecting significantly the activities of other CYP isoforms tested for testosterone transformation, such as CYP2C11 (testosterone 2α- and 16α-hydroxylation) and CYP2A (testosterone 7α-hydroxylation). The rate of caffeine metabolism representing the CYP1A2 activity (C-8-hydroxylation and N-demethylations) (Fig. 2), the rate of warfarin 7-hydroxylation corresponding to the CYP2C6 activity and the rate of bufuralol 1'-hydroxylation indicative of the CYP2D activity (Fig. 3) also remained unchanged. Lurasidone significantly increased the CYP2B and CYP3A activity in stressed rats, but decreased the CYP2C11 activity in both non-stressed and stressed animals (Fig. 1). The activities of other CYP isoform studied (CYP2A, CYP1A2, CYP2C6 and CYP2D) were not significantly affected by lurasidone (Fig. 1, 2, 3).

The effect of CMS and lurasidone on the protein level of CYP isoforms in liver microsomes. Searching for molecular mechanisms of the observed changes in the activities of CYP isoforms after CMS and/or lurasidone, the level of CYP protein was studied in liver microsomes. CMS significantly decreased the CYP2C11 and CYP3A2 protein level, but increased that of CYP3A23/3A1 (Fig. 4 A, B). Lurasidone significantly diminished the CYP2B protein level in non-stressed rats, but tended to enhance it in stressed animals. The CYP2B protein level after lurasidone treatment was significantly higher in stressed than non-stressed subjects. The CYP2C11 protein level was significantly reduced by lurasidone, but only in non-stressed animals. The neuroleptic significantly increased the CYP3A23/3A1 protein level (but not that of CYP3A2) in both non-stressed and stressed rats (Fig. 4 A, B). Consequently, the CYP3A1 protein level in lurasidone-treated rats was much higher in stressed than non-stressed animals.

The effect of CMS and lurasidone on the mRNA level of CYP isoforms in liver tissue. Searching further for genetic mechanisms of the observed changes in CYP isoform activities and

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protein levels, the effect of CMS and/or lurasidone on the mRNA level of *CYP* genes was investigated in liver tissue. CMS did not produce any significant changes in the mRNA levels of the tested genes *CYP2B1, CYP2B2, CYP2C11, CYP3A23/3A1* and *CYP3A2,* though a tendency towards increased mRNA level of *CYP2B1* and *CYP2B2* genes was noted (Fig. 5). Lurasidone significantly increased the *CYP3A23/3A1* and *CYP3A2* mRNA level in both non-stressed and stressed rats. The neuroleptic raised the *CYP2B1* mRNA level in non-stressed rats, but tended to decrease it in stressed animals (Fig. 5). The *CYP2B1* mRNA level after lurasidone treatment was significantly lower in stressed than non-stressed subjects. Lurasidone did not significantly affect the mRNA levels of the genes *CYP2B2* or *CYP2C11*.

The effect of lurasidone added in vitro to the control liver microsomes of non-stressed and stressed rats on the activity of CYP isoforms measured as the rate of testosterone metabolism. Lurasidone added to liver microsomes of non-stressed control rats at a concentration of 0.25 μM did not affect significantly the testosterone 2β- and 6β-hydroxylation rate, representing the CYP3A activity. However, at the higher concentration of 1 μM the neuroleptic moderately decreased the CYP3A activity (Fig. 6). After preincubation of the liver microsomes with lurasidone for 30 min the effect of the neuroleptic on the enzyme activity was seen also at its lower concentration of 0.25 μM. Similar effects were observed in the liver microsomes derived from stressed control animals, however, in this case the effect of lurasidone on the CYP3A activity was less pronounced, i.e., preincubation with 0.25 μM lurasidone was not effective in the inhibition of testosterone 2β-hydroxylation. Moreover, weak or moderate inhibitory effects of 0.25 μM and 1 μM lurasidone on the CYP2A activity (the rate of 7α-hydroxylation of testosterone), and 1 μM lurasidone on the CYP2B activity (the rate of 16β-hydroxylation of testosterone) were observed. The metabolic reactions of testosterone representing the CYP2C11 activity (2α- and 16α-hydroxylation) were least affected and only by the higher (1 μM) concentration of lurasidone.

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Discussion

The obtained results show significant effects of chronic mild stress (CMS) and chronic lurasidone treatment on cytochrome P450 expression and activity. They indicate that lurasidone differently influences cytochrome P450 in non-stressed and stressed animals (summarized in Table 1).

CMS, an animal model of depression, significantly decreased the CYP2B activity and tended to do so to the CYP2B protein, but it produced an opposite effect on mRNA level, i.e., a tendency to increase the *CYP2B1*and *CYP2B2* mRNA level was observed. The CYP2C11 protein level was decreased by CMS, without a change in the enzyme activity or mRNA level. In the case of the CYP3A subfamily, its activity and the protein level of CYP3A2 was decreased by CMS (at an increased CYP3A1 protein), but no change in the mRNA level of the two CYP3A23/3A1 and CYP3A2 isoforms was noted. The observed non-corresponding changes in the activity, protein and mRNA levels of the investigated CYP isoforms suggest some posttranscriptional or posttranslational modifications in CYP expression produced by CMS in the liver. The observed changes in cytochrome P450 expression and activity may be caused by the ability of CMS to produce alterations in the function of the brain nervous system (Willner, 2016), which leads to the release of glucocorticoids and catecholamines, and modifications in the immune system (Kvetnansky et al., 2009; Tank and Wong, 2015; Jiang et al., 2014; Konstandi et al., 2014; Faron-Górecka et al., 2016; Rossetti et al., 2016). The aforementioned CMS-evoked physio-pathological changes in the concentrations of peripheral glucocorticoids, catecholamines and cytokines affect hepatic signaling pathways mediating the regulation of cytochrome P450 (Konstandi, 2013). Glucocorticoids are known to have complex (direct and/or indirect), mostly positive effects on the expression/activity of rat hepatic CYP2B (Waxman et al., 1990), CYP2C11 (Iber et al., 1997) and CYP3A (Huss and Kasper, 2000). However, these effects may be modified by catecholamines (adrenaline,

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noradrenaline) acting positively *via* hepatocyte α_1 and $\beta_{1/2}$ adrenergic receptor through the CREB pathway, but negatively *via* pancreatic β_2 receptor through the insulin pathway (Konstandi, 2013) on cytochrome P450 expression (Konstandi et al., 2013). Moreover, CMS-induced immune disturbances that lead to elevation of plasma proinflammatory cytokines (IL-6, TNFα and TNFγ) may additionally diminish enzyme expression (Zidek et al., 2009; Jiang et al., 2014).

The obtained results indicate that the effect of CMS on liver cytochrome P450 is rather moderate and differs from the effects of other kinds of stress observed in rodents, such as repeated restraint stress (RS) or early-life maternal deprivation (MD) (Daskalopoulos et al., 2012 b). As compared to CMS, the CYP3A2 expression was increased both in RS and MD rats, which was not the case in the CMS-exposed animals in our experiment. The expression of liver CYP2C11 and CYP3A1 was increased in MD, but not in RS rats, while in the CMS animals a decrease and an increase of those CYP isoforms, respectively, was observed. In contrast, the CYP2D activity was increased in RS, but not in MD or CMS subjects. On the other hand, the CYP2B activity in RS rats was significantly suppressed (Konstandi et al., 2000) like in CMS animals in our study. The above-described stress-dependent differences in cytochrome P450 expression and function support an earlier assumption that the effect of psychological stress on cytochrome P450 is stress type-specific. The specificity of stress seems to be determined by the proportion of engagement of the brain stress-circuits and peripheral nervous systems and, in consequence, the contribution of the neuroendocrine and sympathetic nervous systems to the regulation of liver cytochrome P450 expression (Uyama et al., 2004; Kot et al., 2013; Tank and Wong, 2015, Chmielarz at al., 2015).

Chronic lurasidone, which exerts antidepressant action in the CMS model of depression (Luoni et al., 2015), has been shown to be active in the regulation of the expression of some CYP isoforms in our experiment. Notably, its effect was different in non-stressed and stressed animals, in particular in the case of the CYP2B subfamily (summarized in Table 1). Lurasidone produced an opposite effect on *CYP2B1* mRNA, CYP2B protein and activity in non-stressed rats (an increase, a

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decrease and a tendency to decrease, respectively) and stressed animals (a tendency to decrease, a tendency to increase, and an increase, respectively). Moreover, lurasidone decreased the activity of CYP2C11, the main CYP isoform in male rats, both in non-stressed and stressed rats, though it decreased the enzyme protein only in non-stressed animals, not affecting the levels of *CYP2C11* mRNA in both groups of rats (non-stressed and stressed). As concerns the CYP3A subfamily, the neuroleptic affected its expression in both non-stressed and stressed rats in a similar manner (an increase in *CYP3A23/3A1* mRNA and protein and in *CYP3A2* mRNA), however, the CYP3A activity was enhanced only in stressed animals.

The observed difference in the CYP3A activity between the two groups of rats (non-stressed and stressed) after lurasidone treatment may be caused by different concentrations of reactive lurasidone metabolites, a greater amount of which may be formed in non-stressed than stressed animals. This is because the results of our experiment show that CMS decreases the activity of CYP3A, i.e., the enzyme primarily responsible for the lurasidone metabolism (Caccia, 2011). As mentioned elsewhere, lurasidone is extensively metabolized *in vivo via* N-dealkylation, hydroxylation and S-oxidation, and reactive metabolites possibly generated during this process may inactivate the CYP3A protein and, in turn, mask the functional effect of increased enzyme expression by the neuroleptic in non-stressed animals. This kind of phenomenon was observed earlier for phenothiazine neuroleptics, tricyclic antidepressants and selective serotonin reuptake inhibitors, *in vitro* and *in vivo* (Murray and Field, 1992; Bensoussan et al., 1995; Murray and Murray, 2003; Daniel et al., 2005; Haduch et al., 2006). This suggestion finds some support in the results of our *in vitro* study showing that lurasidone added to control liver microsomes is more efficient in non-stressed rats than in CMS animals in the inhibition of CYP3A activity measured as the testosterone 2β-hydroxylation rate.

Finally, it should be mentioned that the observed effects of lurasidone on cytochrome P450 may be connected with the pharmacological action of the neuroleptic, in particular with its

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antagonist activity at dopaminergic D_2, serotonergic 5-HT_2 and adrenergic α_2 receptors in the brain and/or periphery, which affects the central (Wójcikowski and Daniel, 2009; Sadakierska et al., 2013; Rysz et al., 2015; Daniel et al., 2017) and peripheral (Daskalopoulos et al., 2012 a; Kot and Daujat-Chavanieu, 2016; Kot, 2017) endocrine regulation of liver cytochrome P450, and hepatic signaling pathways mediating enzyme expression. CMS affects the brain monoaminergic neurotransmission (Willner, 2016), which may modify the action of lurasidone on the regulation of some CYP isoforms in stressed animals. Combinations of atypical neuroleptics and antidepressants or antiepileptics are frequently used by clinicians, which may lead to pharmacodynamic and/or pharmacokinetic interactions (Kennedy et al., 2013; Spina and de Leon, 2014). Chronic lurasidone was found to modestly increase plasma concentration of the specific CYP3A4 substrate midazolam in patients with schizophrenia or schizoaffective disorders (Chiu et al., 2014). However, its possible effects on the pharmacokinetics of antidepressant drugs that are metabolized by CYP3A isoforms have been investigated neither in volunteers and depressive patients nor in an animal model of depression, as yet.

In summary, the present findings indicate that 1) CMS moderately affects cytochrome P450 (CYP2B, CYP2C11 and CYP3A) and its effects are different from those observed after other kinds of psychological stress, such as repeated restraint stress (RS) or early-life maternal deprivation (MD); 2) chronic lurasidone influences the expression and/or activity of CYP2B, CYP2C11 and CYP3A isoforms; 3) CMS modifies the action of lurasidone on cytochrome P450 expression and function, leading to the different effects of the neuroleptic in non-stressed and stressed rats. Future studies will focus on testing whether the metabolism of endogenous substrates (e.g. steroids) and drugs, catalyzed by the isoforms CYP2B CYP2C11 or CYP3A proceeds at a different rate in the two groups of animals (non-stressed and stressed) in the rat CMS model.

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Authorship Contributions:

Participated in research design: Daniel, Papp

Conducted experiment: Kot (biochemical study); Papp, Haduch (*in vivo* study)

Performed data analysis: Kot, Daniel

Wrote or contributed to the writing of the manuscript: Daniel, Kot, Papp

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Footnotes

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Legend for Figures

Fig. 1. The influence of a five-week treatment with lurasidone on CYP2B, CYP2C11, CYP3A and CYP2A activity measured as a rate of testosterone 16β-hydroxylation (**A**), 2α- and 16α-hydroxylation (**B, C**), 2β- and 6β-hydroxylation (**D, E**), and 7α-hydroxylation (**F**), respectively, in the liver microsomes from the chronic mild stress (CMS) model. Results are shown as the means (± S.E.M.) of data from 8-9 rats/group. The results were analyzed using a multivariate analysis of variance (ANOVA) followed by a post hoc Duncan test and statistical significance is shown as * $P < 0.05$, *** $P < 0.001$ compared to the non-stressed control and # $P < 0.05$, ### $P < 0.001$ compared to the stressed control. CON, control.

Fig. 2. The influence of a five-week treatment with lurasidone on CYP1A2 activity measured as a rate of caffeine metabolism in the liver microsomes from the chronic mild stress (CMS) model. **A.** C-8-hydroxylation, catalyzed by CYP1A2; **B.** 3-N-demethylation, catalyzed by CYP1A2 and CYP2C11; **C.** 1-N-demethylation, catalyzed by CYP1A2, CYP2C and CYP3A2; **D.** 7-N-demethylation, catalyzed by CYP2C, CYP1A2 and CYP3A2. Results are shown as the means (± S.E.M.) of data from 9 rats/group. The results were analyzed using a multivariate analysis of variance (ANOVA) followed by a post hoc Duncan test. CON, control.

Fig. 3 The influence of a five-week treatment with lurasidone on CYP2C6 and CYP2D activity measured as a rate of warfarin 7-hydroxylation (**A**) and bufuralol 1'-hydroxylation (**B**), respectively, in the liver microsomes from the chronic mild stress (CMS) model. Results are shown as the means (± S.E.M.) of data from 9 rats/group. The results were analyzed using a multivariate analysis of variance (ANOVA) followed by a post hoc Duncan test. CON, control.

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Fig. 4. The effect of a five-week treatment with lurasidone on the protein level of CYP2B, CYP2C11 CYP3A1 and CYP3A2 in rat liver microsomes from the chronic mild stress (CMS) model. **A.** Microsomal proteins, 10 µg, were subjected to the Western immunoblot analysis. Rat cDNA-expressed CYP2B1, CYP2C11, CYP3A1 and CYP3A2 isoforms (Supersomes) were used as standards. The original membranes of CYP2C11 and CYP3A23.3A1 were cut down to arrange protein bands according to the order of experimental groups presented in Fig. 4B. **B – D.** Results are shown as the means (± S.E.M.) of data from 6-8 rats/group. The results were analyzed using a multivariate analysis of variance (ANOVA) followed by a post hoc Duncan test and statistical significance is shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the non-stressed control and ## $P < 0.01$ compared to the stressed control or @ $P < 0.05$, @@ $P < 0.01$ compared to lurasidone non-stressed. CON, control.

Fig. 5. The effect of a five-week treatment with lurasidone on the mRNA expression level of *CYP2B1, CYP2B2, CYP2C11, CYP3A1* and *CYP3A2* genes (**A, B, C, D, E,** respectively) in the liver microsomes from the chronic mild stress (CMS) model. Results are shown as the means (± S.E.M.) of data from 7-10 rats/group. The results were analyzed using a multivariate analysis of variance (ANOVA) followed by a post hoc Duncan test and statistical significance is shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the non-stressed control and # $P < 0.05$ compared to the stressed control or @ $P < 0.05$ compared to lurasidone non-stressed. CON, control.

Fig. 6. The effect of lurasidone added *in vitro* to pooled liver microsomes of non-stressed and stressed (CMS) rats on the activity of CYP3A, CYP2B, CYP2C11 and CYP2A, measured as the rate of testosterone 2β- and 6β-hydroxylation (**A, B**), 16β-hydroxylation (**C**), 2α- and 16α-hydroxylation (**D, E**), and 7α-hydroxylation (**F**), respectively. All values are the mean ± S.E.M. (n=5). The results were analyzed statistically using a multivariate analysis of variance (ANOVA)

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followed by a post hoc Duncan test. Statistical significance is shown as * $P < 0.05$, *** $P < 0.001$ compared to the non-stressed control and # $P < 0.05$, ## $P < 0.01$ compared to the stressed control (withouot preincubation) or $ $P < 0.05$, $$ $P < 0.01$, $$$ $P < 0.001$ compared to the non-stressed control and & $P < 0.05$, &&& $P < 0.001$ compared to the stressed control (with preincubation). The control values (pmol/mg protein/min) for liver microsomes of non-stressed rats are as follows: without preincubation – 34.5±2.1, 466.7±2.3, 15.4±1.8, 556.0±21.4, 569.4±22.8, 219.4±4.0 (testosterone 2β- and 6β-, 16β-, 2α- and 16α-, and 7α-hydroxylation, respectively); with incubation – 34.3±1.2, 346.0±10.4, 11.2±0.7, 463.3±19.0, 511.5±11.3, 180.2±6.1 (testosterone 2β- and 6β-, 16β-, 2α- and 16α-, and 7α-hydroxylation, respectively). The control values (pmol/mg protein/min) for liver microsomes of stressed (CMS) rats are as follows: without preincubation – 25.7±0.5, 298.7±2.9, 14.9±0.3, 842.1±11.5, 938.5±9.1, 187.3±2.3 (testosterone 2β- and 6β-, 16β-, 2α- and 16α-, and 7α-hydroxylation, respectively); with incubation – 26.1±0.03, 231.0±2.7, 10.8±0.1, 738.7±6.7, 826.9±0.8, 171.1±3.3 (testosterone 2β- and 6β-, 16β-, 2α- and 16α-, and 7α-hydroxylation, respectively). L, lurasidone.

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TABLE 1. Summary of the effects of CMS and/or lurasidone on the activity, protein and mRNA levels of liver CYP isoforms.

<table>
<tr><th></th><th></th><th>CYP1A2</th><th>CYP2A</th><th>CYP2B1</th><th>CYP2B2</th><th>CYP2C6</th><th>CYP2C11</th><th>CYP2D</th><th>CYP3A1</th><th>CYP3A2</th></tr>
<tr><td rowspan="3">CMS</td><td>Activity</td><td>no change</td><td>(↓)</td><td colspan="2">↓</td><td>no change</td><td>no change</td><td>no change</td><td colspan="2">↓</td></tr>
<tr><td>Protein</td><td></td><td></td><td colspan="2">(↓)</td><td></td><td>↓</td><td></td><td>↑</td><td>↓</td></tr>
<tr><td>mRNA</td><td></td><td></td><td>(↑)</td><td>(↑)</td><td></td><td>no change</td><td></td><td>no change</td><td>no change</td></tr>
<tr><td rowspan="3">Lurasidone (vs. non-stressed control)</td><td>Activity</td><td>no change</td><td>no change</td><td colspan="2">(↓)</td><td>no change</td><td>↓</td><td>no change</td><td colspan="2">no change</td></tr>
<tr><td>Protein</td><td></td><td></td><td colspan="2">↓</td><td></td><td>↓</td><td></td><td>↑</td><td>no change</td></tr>
<tr><td>mRNA</td><td></td><td></td><td>↑</td><td>no change</td><td></td><td>no change</td><td></td><td>↑</td><td>↑</td></tr>
<tr><td rowspan="3">CMS+ Lurasidone (vs. stressed control)</td><td>Activity</td><td>(↑)</td><td>no change</td><td colspan="2">↑</td><td>no change</td><td>↓</td><td>no change</td><td colspan="2">↑</td></tr>
<tr><td>Protein</td><td></td><td></td><td colspan="2">(↑)</td><td></td><td>no change</td><td></td><td>↑</td><td>no change</td></tr>
<tr><td>mRNA</td><td></td><td></td><td>(↓)</td><td>no change</td><td></td><td>no change</td><td></td><td>↑</td><td>↑</td></tr>
</table>

↑,↓ increase or decrease, respectively; (↑) , (↓) a tendency to increase or decrease, respectively.

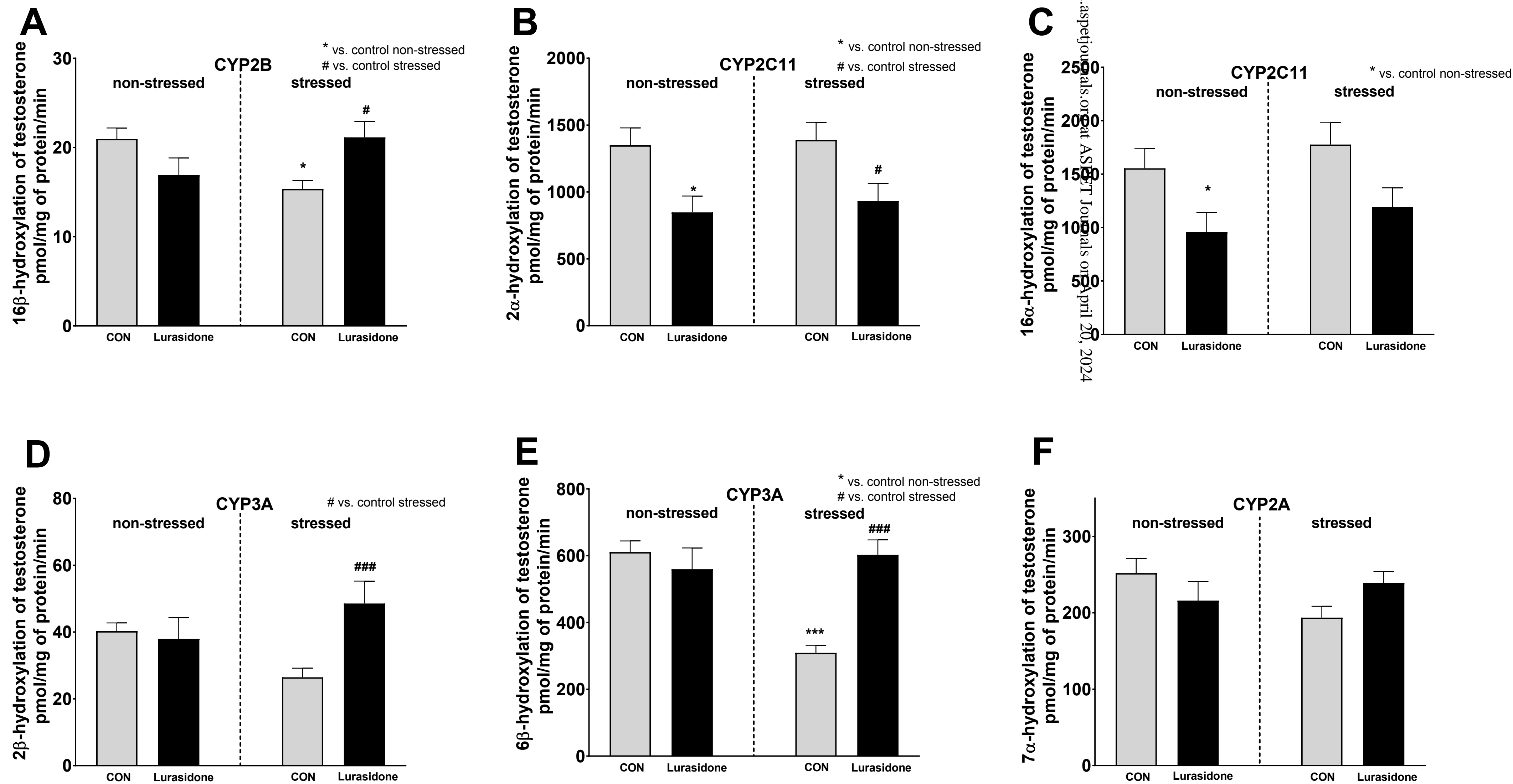

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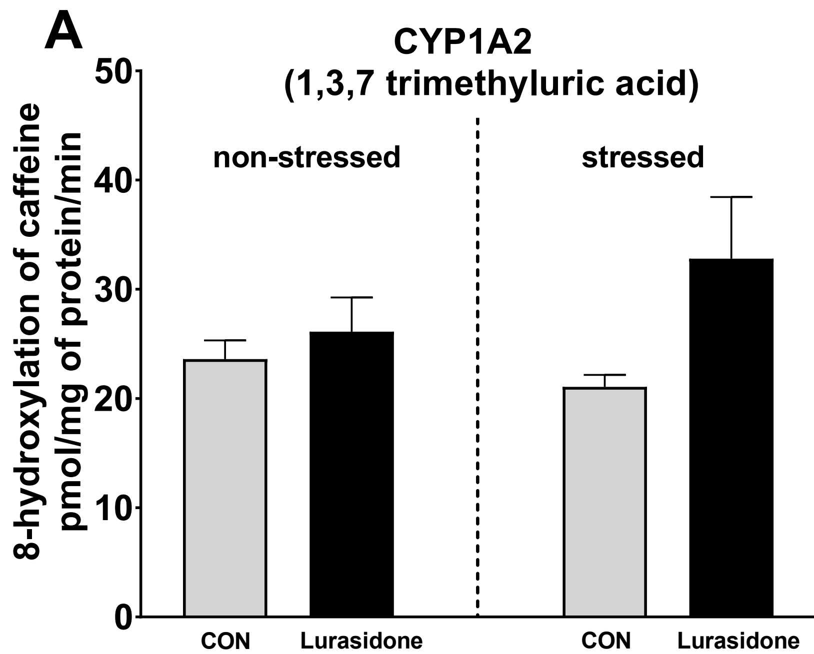
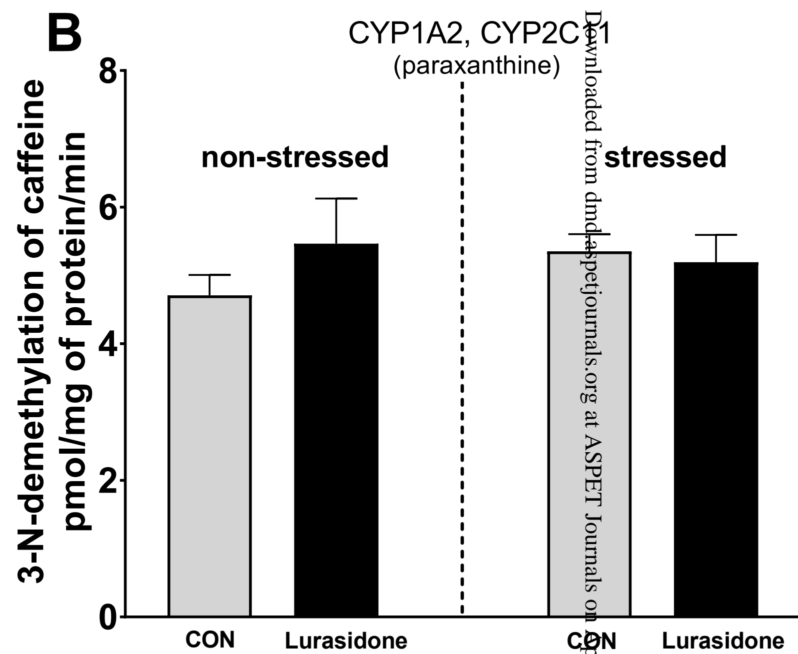
Fig. 1





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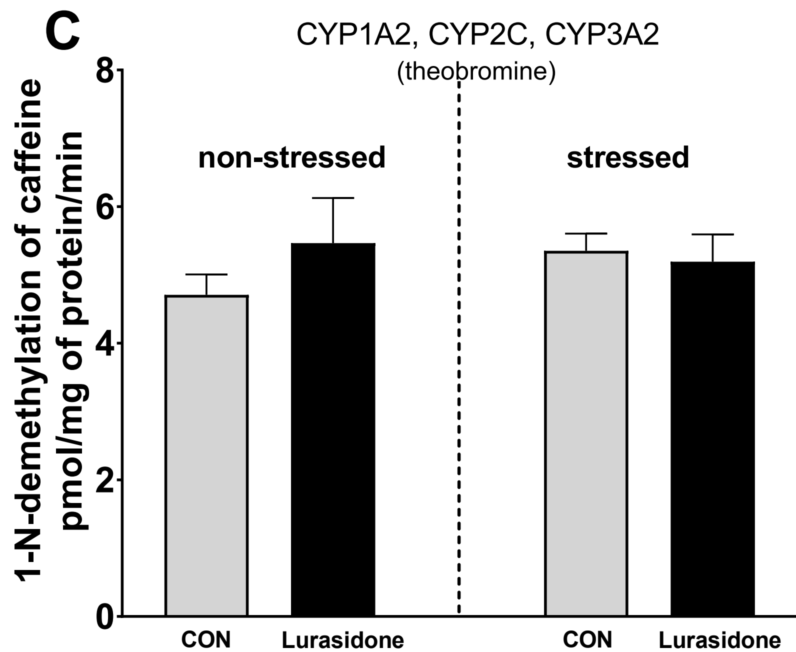

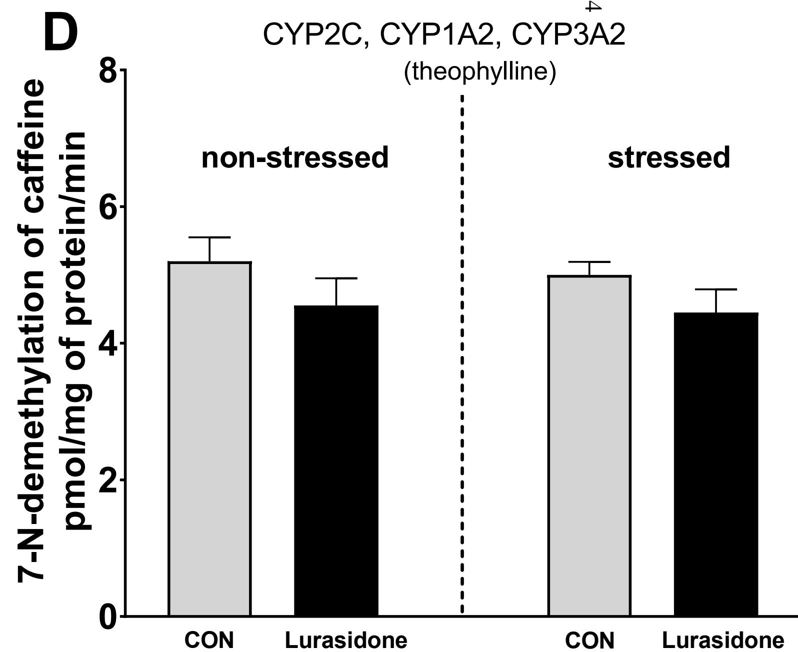

Fig. 2

A

CYP2C6

7-hydroxylation of warfarin pmol/mg of protein/min

non-stressed

stressed

15
10
5
0

CON Lurasidone CON Lurasidone

B

CYP2D

1'-hydroxylation of bufuralol pmol/mg of protein/min

non-stressed

stressed

150
100
50
0

CON Lurasidone CON Lurasidone

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Fig. 3

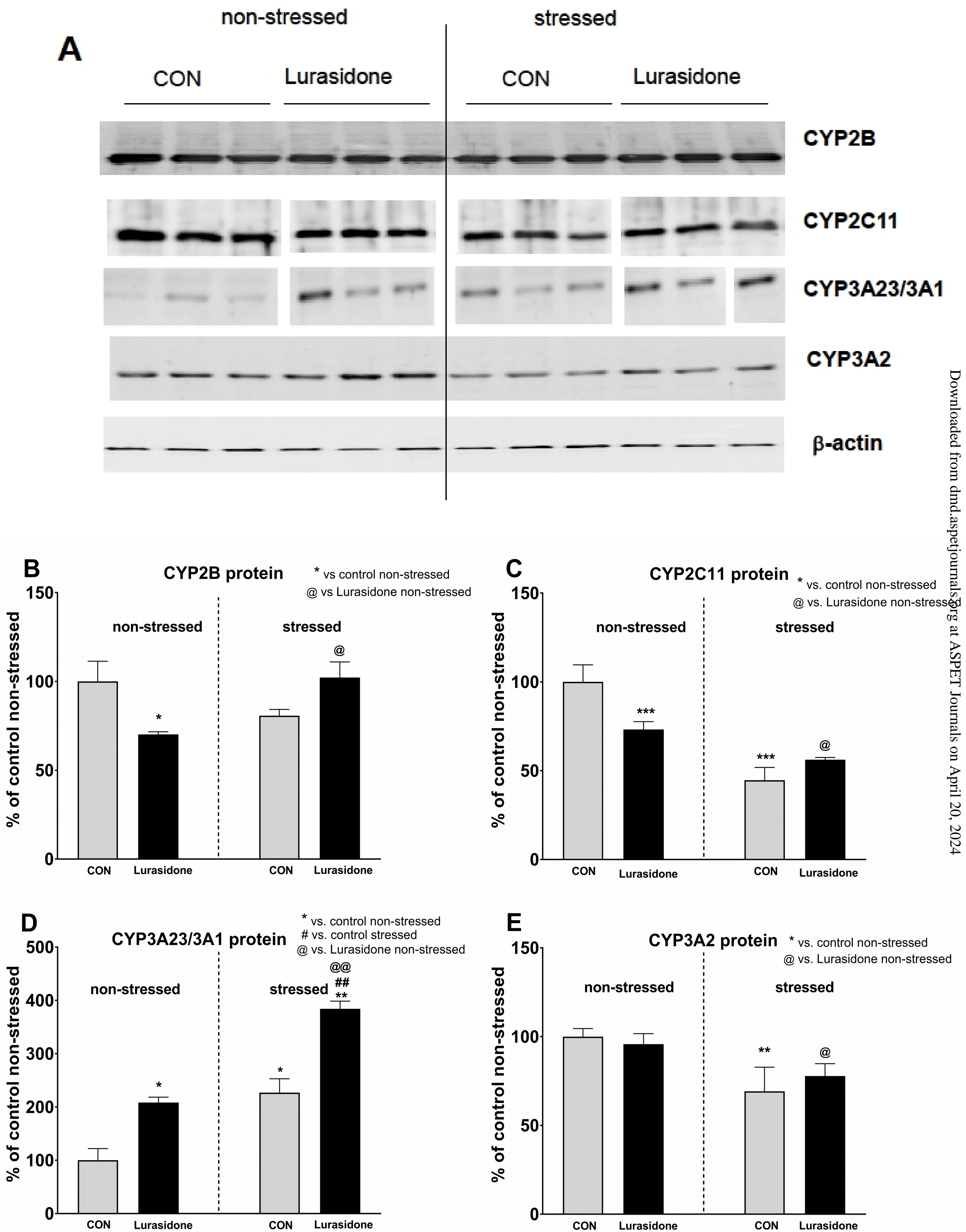

Fig. 4

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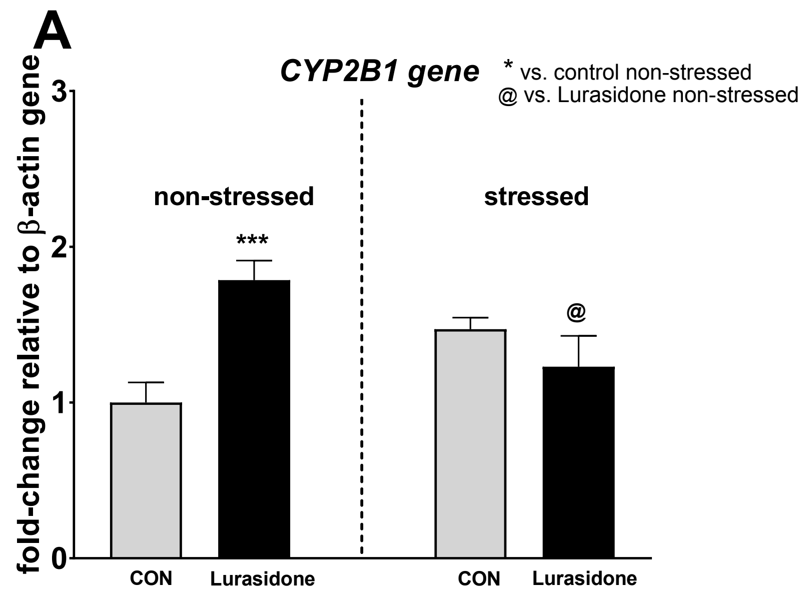
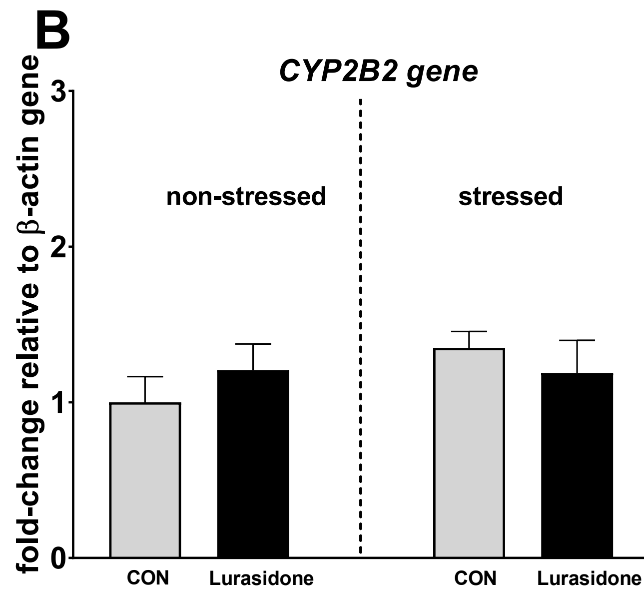
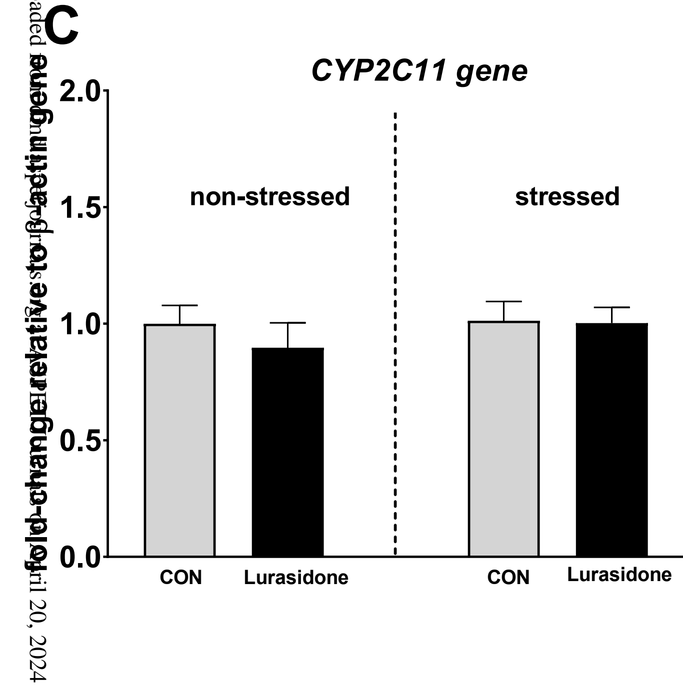
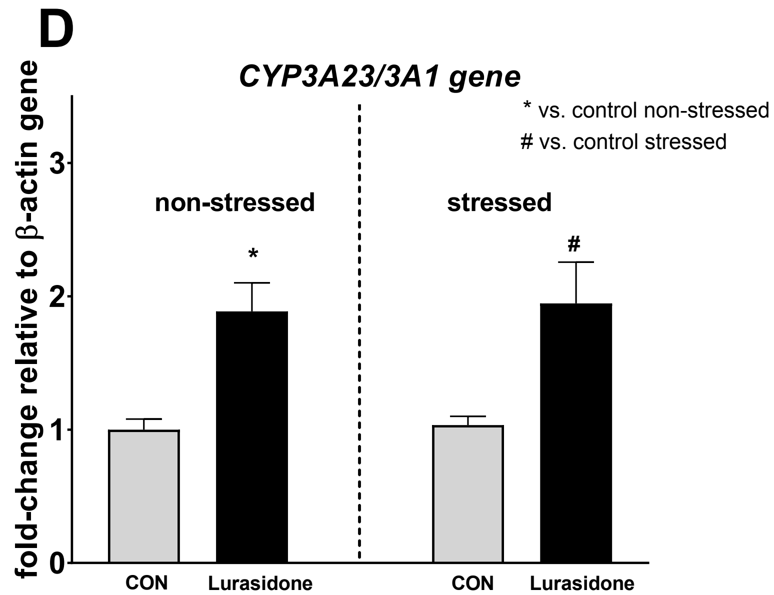








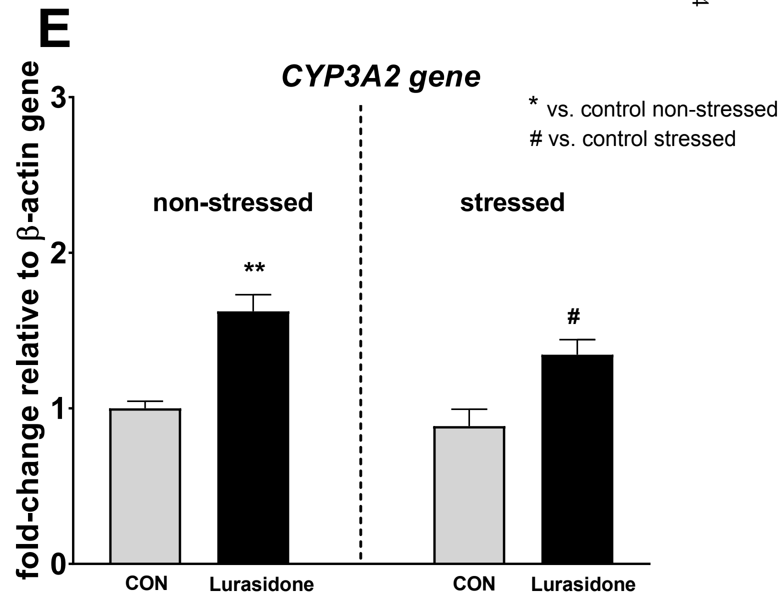
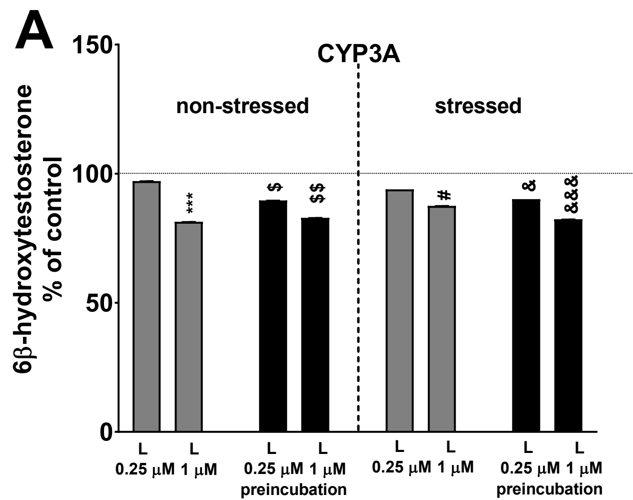
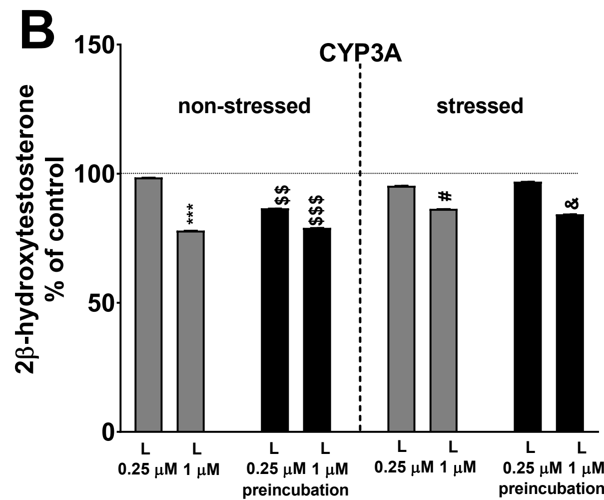
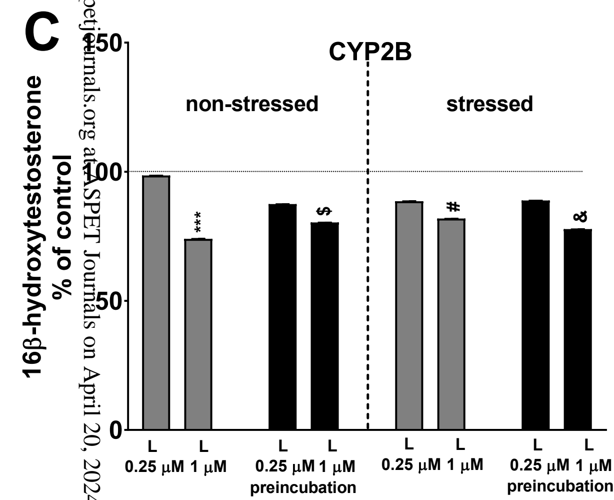


Fig. 5







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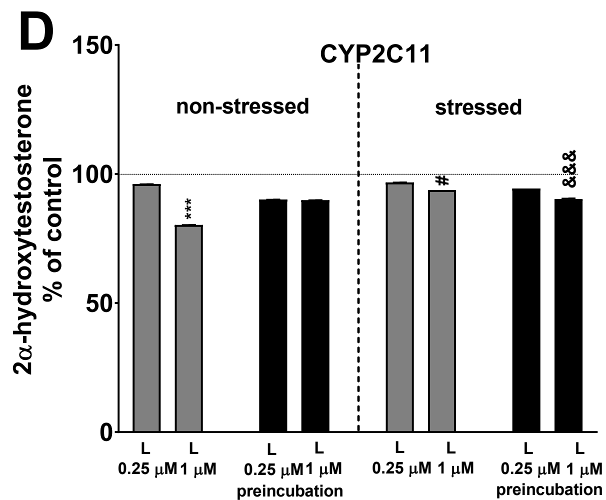

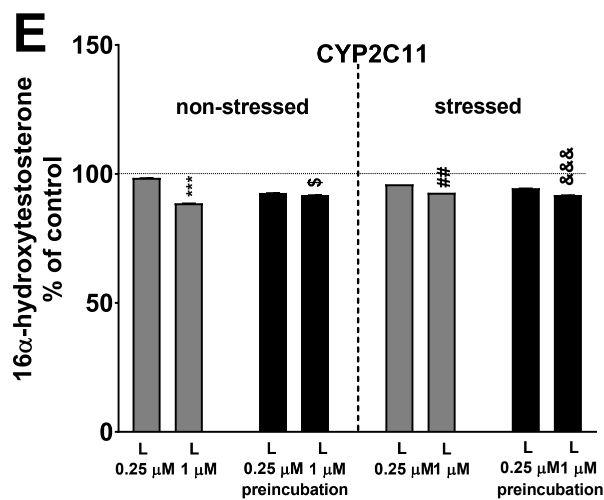

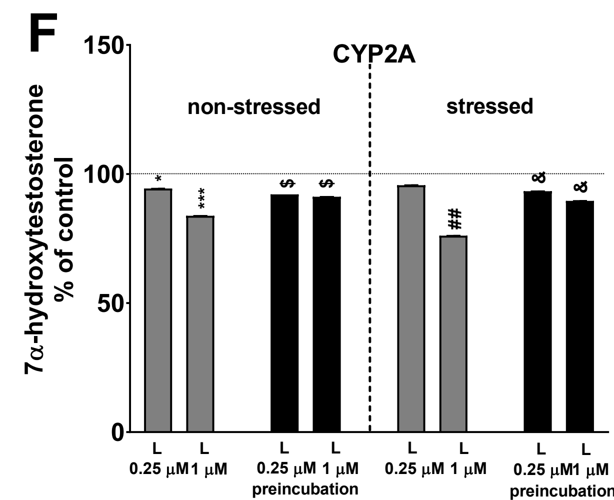

* vs. control non-stressed

\$ vs. control non-stressed with preincubation

vs. control stressed

& vs. control stressed with preincubation

Fig. 6