doi: 10.4149/gpb_2022017

Inhibition of cytochrome P450 with proadifen alters the excitability of brain catecholamine-secreting neurons

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Abstract. The concentrations of circulating glucocorticoids are regulated by their synthesis and metabolism. Cytochrome P450 (CYP), primarily expressed in the liver, is one of the main metabolizers of glucocorticoids. Since glucocorticoids, as well as monoamines, are fundamental in stress, the link between hepatic glucocorticoid metabolism and central monoamine transmission might be important in pathophysiology of stress-related disorders. We had previously reported that CYP inhibition by proadifen (SKF525) led to the inhibition of central serotonin (5-HT) neurons. The aim of this study was to investigate the effect of SKF525 on the excitability of central catecholamine neurons. Adult male Wistar rats were administered SKF525 forty-eight, twenty-four, and one hour before electrophysiological assessments. Control animals were injected saline. Rats were anesthetized with chloral hydrate and glass electrodes were inserted into the locus coeruleus (LC) or ventral tegmental area (VTA). Noradrenaline neurons of the LC and dopamine of the VTA neurons were identified, and their firing activity was recorded. It was found that the SKF525 enhanced the excitability of noradrenaline and reduced the excitability of dopamine neurons. We suggest that corticosterone-induced inhibition of 5-HT neurons underlines, at least in part, the ability of SKF525 to stimulate noradrenaline neurons. The inhibitory effect of SKF525 on dopamine neurons might be in turn secondary to the stimulatory effect of this compound on noradrenaline neurons.

Key words: Noradrenaline — Dopamine — Locus coeruleus — Ventral tegmental area — *in vivo* electrophysiology

Introduction

Cytochrome-P450 (CYP) is a superfamily of microsomal and mitochondrial enzymes which catalyze oxidation of various biological molecules, such as arachidonic and fatty acids, catecholamines, lipid-soluble vitamins, various medications including antidepressant, antipsychotic, and mood stabilizing drugs, carcinogens, endogenous and exogenous toxins, and steroids, such as glucocorticoids and mineralocorticoids (Munro et al. 2018; Rendic 2002). Particularly, CYP irreversibly metabolizes corticosterone into 6β-corticosterone in rodents and cortisol into 6β-cortisol in humans (Peng et al. 2011). As a primary metabolizer of corticosteroids, CYP is an important regulator of glucocorticoid and mineralocorticoid signaling pathways, which are fundamental in stress and pathophysiology of stress-related disorders (Tseilikman et al. 2020).

CYP activity is reciprocally linked with central monoamine neurotransmission. Thus, the selective lesion of 5-HT neurons or inhibition of 5-HT synthesis led to a robust activation of the hepatic CYP (Kot and Daniel 2011). *Vice versa*, an injection of a 5-HT precursor 5-hydroxytryptophan into the lateral cerebral ventriculi increased brain 5-HT concentrations and diminished the activity of CYP

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(Rysz et al. 2016). On the other hand, specific lesions of noradrenaline neurons of the locus coeruleus (LC) led to increased hepatic CYP expression (Kot et al. 2015) and activity (Kot and Daniel 2011). Regarding dopamine-CYP interaction, the antagonists of D_2 receptors were reported to downregulate the expression of specific CYP isoforms in the liver (Daskalopoulos et al. 2012; Harkitis et al. 2015), suggesting an enhancing effect of dopamine system on the hepatic CYP, mediated *via* D_2 receptor. All these findings indicate that the expression and activity of the hepatic CYP is modulated by the central monoamine transmission (Tseilikman et al. 2020).

On the other hand, hepatic CYP activity influences central monoamine transmission as well. Hence, Grinchii and co-authors (2018) reported that CYP inhibition by proadifen (SKF525) decreased the firing activity of 5-HT neurons of the dorsal raphe nucleus (DRN). SKF525 is non-selective CYP inhibitor; particularly, its inhibitory effect was demonstrated for the CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A, and, to the lesser amount, for the CYP1A2, CYP2A6, and CYP2E1 sub-types of the CYP (Franklin and Hathaway 2008). SKF525 was previously reported to increase plasma levels of corticosterone in rats (Magus et al. 1968). Since corticosterone enhances 5-HT_{1A} autoreceptor-induced inhibition of 5-HT neurons (Laaris et al. 1995), it was suggested that the high circulating corticosterone concentrations in SKF525-treated rats might have an inhibitory effect on the excitability of 5-HT neurons (Grinchii et al. 2018).

Monoamine neurotransmission is fundamental in the pathophysiology of schizophrenia, unipolar and bipolar depression, generalized anxiety, panic, and post-traumatic stress disorder (PTSD). Most of the drugs designated to treat these conditions primarily act on the central monoamine pathways (Grinchii and Dremencov 2020). On the other hand, many antidepressant, antipsychotic, and mood-stabilizing drugs are metabolized by the CYP and as such they are potent CYP inhibitors (Rendic 2002). Due to the functional interconnection between hepatic CYP activity and central monoamines, CYP-mediated effect of some central nervous system (CNS) drugs might interfere with their primary effect on central monoamine targets. This interference might be responsible, at least in part, to the limited efficiency of some CNS drugs (Tseilikman et al. 2020). Similarly, interaction between hepatic CYP and brain monoamines might be involved in the anxiogenic and depressogenic effect of various environmental and bacterial toxins (Brydges et al. 2021).

When the influence of central monoamine transmission on hepatic CYP expression and activity has been investigated in multiple studies, the reciprocal link between the CYP activity and the excitability of central monoamine-secreting neurons received lesser attention. In our previous study (Grinchii et al. 2018), we examined the effect of the CYP inhibition by SKF525 of the excitability of 5-HT neurons. The effect of the CYP inhibition on the excitability of catecholamine-secreting neurons, was not, however, yet directly examined. The aim of the present study was to investigate the effect of the CYP inhibition by SKF525 on the excitability of noradrenaline neurons of the LC and dopamine neurons of the ventral tegmental area (VTA).

Methods

Animals

Adult male Wistar rats (200–250 g) were ordered from the Breading Facility of the Institute of Experimental Pharmacology and Toxicology, Centre for Experimental Medicine, Slovak Academy of Sciences (Dobrá voda, Slovakia) and housed in a temperature-controlled room (22–24°C) with a 12:12 hours light-dark cycle and had *ad libitum* access to food and water. All experimental procedures were approved by the Animal Health and Animal Welfare Division of the State Veterinary and Food Administration of the Slovak Republic (Permit number Ro 3054/17-221/3) and conformed to the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes. Rats were allowed to acclimatize for one week after their arrival in our animal facility.

SKF525 administration

SKF525 was administrated using the protocol explained in our previous study (Grinchii et al. 2018). SKF525 was ordered from Abcam (Cambridge, UK) and dissolved in saline. To achieve the steady-state inhibition of the CYP, the rats received three intraperitoneal (i.p.) injections of SKF525 (25 mg/kg): forty-eight, twenty-four, and one hour before electrophysiological assessments. Control animals were injected saline using the same protocol.

Electrophysiological assessments

The assessment of excitability of catecholamine-secreting neurons was performed as explained in our previous studies (Dremencov et al. 2017; Koprdova et al. 2019; Csatlosova et al. 2021). One hour after the last saline or SKF525 injection, rats were anesthetized with chloral hydrate (Sigma-Aldrich spol. s.r.o., Bratislava, Slovakia, 0.4 g/kg, i.p.) and mounted into the stereotaxic frame (David Kopf Instruments, Tujunga, CA). Rat body temperature was maintained at 37°C with a heating pad (Gaymor Instruments, Orchard Park, NY, USA). The scalp was opened, and a 3 mm hole was drilled in the skull for insertion of electrodes. Glass-pipettes were

pulled with a DMZ-Universal Puller (Zeitz-Instruments GmbH, Martinsried, Germany) to a fine tip approximately 1 μm in diameter and filled with 2 M NaCl solution. Electrode impedance ranged from 4 to 6 MΩ. The pipettes were inserted into the LC (8.0–8.3 mm posterior to bregma, 1.2–1.4 mm lateral to the midline, and 5.5–7.5 mm ventral to the brain surface) or VTA (4.5–5.5 mm posterior to bregma, 0.6–0.8 mm lateral to the midline, and 7.0–8.5 mm ventral to the brain surface) (Paxinos and Watson 2014) by hydraulic micro-positioner (David Kopf Instruments, Tujunga, CA). The signal from the electrodes was amplified ×1000 using the DP-311 Differential Amplifier (AD Instruments, Dunedin, New Zealand), filtered of the low-frequency (~50/60 Hz) harmonic with the VDL215EQ2 Graphic Equalizer (Velleman Group, Gavere, Belgium) and Hum Bag Noise Eliminator (Quest Scientific, Vancouver, BC, Canada) and fed to the Lenovo B50-35 PC using the Power Lab 4/35 Data Acquisition System (AD Instruments) with the sampling rate of 100 kHz. The bin size was set at 1 ms. The action potentials generated by monoamine-secreting neurons were recorded using the AD Instruments Extracellular Recording System (Dunedin, New Zealand). Noradrenergic neurons were recognized by action potentials with a long-duration rising phase, regular firing rate of 0.5–5.0 Hz, and a characteristic burst discharge in response to nociceptive pinch of the contralateral hind paw (Vandermaelen and Aghajanian 1983). Dopamine neurons were recognized by tri-phasic action potentials lasting between 3 and 5 ms with a rising phase lasting over 1.1 ms, inflection or "notch" during the rising phase, marked negative deflection, irregular firing-rate of 0.5–10 Hz, mixed single-spike and burst firing with characteristic decrease of the action potentials amplitude within the bursts (Grace and Bunney 1983). The same number of electrode descents *per* brain structure (four for the LC and five for the VTA) were made in saline- and SKF525-treated rats. All neurons in all groups of animals were recorded for two minutes.

Data analysis

Action potentials (spikes) of norepinephrine, and dopamine neurons were detected using the spike sorting algorithm, with the version 6.02 of Spike2 software (Cambridge Electronic Design, Cambridge, UK). The neuronal firing rate and burst activity characteristics were calculated using the burstiDAtor software (Oosterhof and Oosterhof 2013). The onset of a burst was signified by the occurrence of two spikes with inter-spike interval (ISI) < 0.08 s. The termination of a burst was defined as an ISI > 0.16 s (Grace and Bunney 1984; Dawe et al. 2001). All data were expressed as mean ± standard deviation (SD). Statistical assessments were performed using SigmaPlot 12.5 software (Systat Software Inc, Chicago, IL, USA). When the normal distribution of the experimental values was confirmed by Shapiro-Wilk test ($p > 0.05$), two-tailed Student's *t*-test was used to compare the firing rates and burst firing characteristics of noradrenaline and dopamine neurons in vehicle and SKF525-treated rats. Otherwise, non-parametric Mann-Whitney U test was used. The

Table 1. Characteristics of the firing activity of noradrenaline neurons of the locus coeruleus (LC) and dopamine neurons of the ventral regimental area (VTA) in control and SKF525-treated rats

	Group		*p* value	
	Control	SKF525	SW test	*t*- or U-test
Noradrenaline neurons of the LC				
Number of neurons *per* track	2.55 ± 1.93	2.83 ± 1.77	<0.05	0.47
% of neurons with bursts	88.67 ± 9.41	94.99 ± 7.21	>0.05	0.17
Firing rate (Hz)	2.53 ± 1.54	2.46 ± 1.72	<0.05	0.71
Bursts frequency (Hz)	0.26 ± 0.27	0.30 ± 0.30	<0.05	0.31
% of spikes in bursts	31.02 ± 24.78	41.04 ± 23.40	<0.05	0.02
Mean number of spikes in burst	3.84 ± 0.46	5.30 ± 11.95	<0.05	0.31
Dopamine neurons of the VTA				
Number of neurons *per* track	3.98 ± 3.00	3.59 ± 2.69	<0.05	0.53
% of neurons with bursts*	100.00 ± 0.00	100.00 ± 0.00	n.a.	n.a.
Firing rate (Hz)	3.87 ± 2.95	3.28 ± 2.50	<0.05	0.10
Bursts frequency (Hz)	0.48 ± 0.38	0.43 ± 0.38	<0.05	0.14
% of spikes in bursts	56.10 ± 26.03	50.70 ± 26.97	<0.05	0.07
Mean number of spikes in burst	5.41 ± 4.91	4.38 ± 3.95	<0.05	0.04

All values are mean ± standard deviation (SD); SW test, Shapiro-Wilk test; *t*-test, two-tailed Student's *t*-test; U-test, Mann-Whitney U test. * since all dopamine neurons exhibit burst firing, according to their identification criteria, statistical tests are non-available (n.a.).

significance in *t*- or U-tests was defined as $p < 0.05$. No corrections for multiplicity were performed.

Results

Noradrenaline neurons of the LC and dopamine neurons of the VTA exhibited mixed single-spike (tonic) and bursting-like (phasic) firing activity with average frequencies of 2.5 and 4 Hz, respectively. The characteristics of the firing activity of noradrenaline neurons of the LC and dopamine neurons of the VTA in control, and SKF525-treated rats, such as number of spontaneously active neurons *per* electrode descent, percent of neurons with burst firing, frequency of the action potentials are summarized, frequency of the bursts, percent of the action potentials occurring in bursts, and mean number of the action potentials in burst, are summarized in the Table 1.

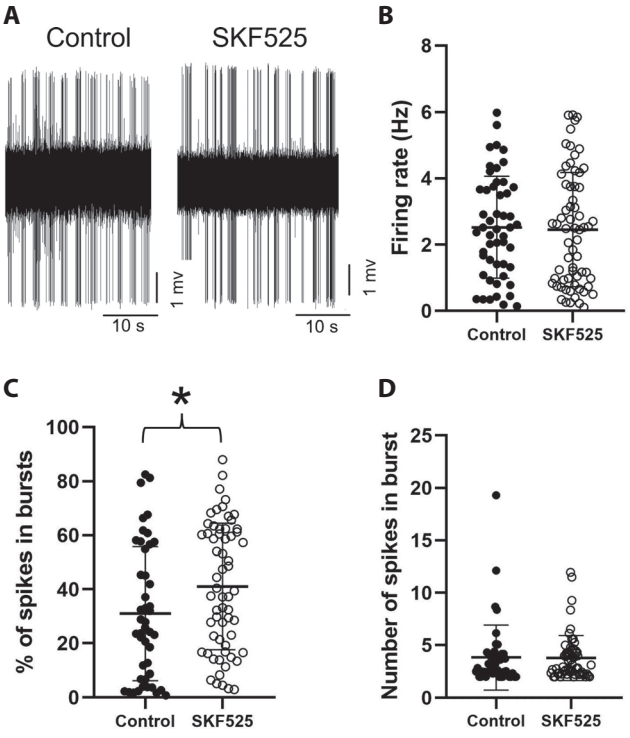

Figure 1. Effect of SKF525 on the excitability of noradrenaline neurons of the locus coeruleus (LC). **A**. Representative recordings from two noradrenaline neurons, from the LC of saline (Control)- and SKF525-treated rat. Mean firing rate (**B**), percentage of spikes occurring within the bursts (**C**) and mean number of spikes in burst (**D**) present summary effect calculated from 51 neurons from seven control rats and 65 neurons from eight SKF525-administered rats. Results are expressed as dot plots with each dot representing an individual recoding, with the mean ± standard deviation (SD) represented by horizontal lines. * $p < 0.05$, Mann-Whitney U test.

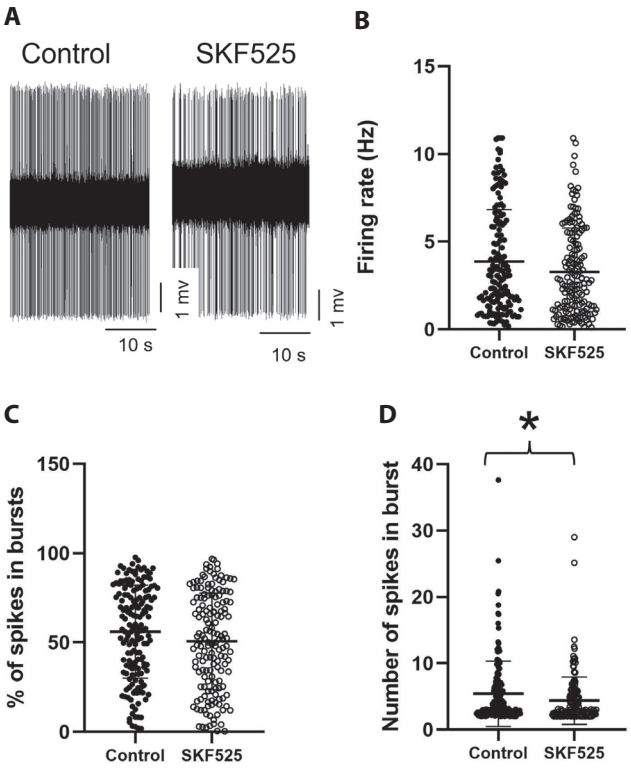

Figure 2. Effect of SKF525 on the excitability of dopamine neurons of the ventral tegmental area (VTA). **A**. Representative recordings from dopamine two neurons, from the VTA of saline (Control)- and SKF525-treated rat. Mean firing rate (**B**), percentage of spikes occurring within the bursts (**C**) and mean number of spikes in burst (**D**) present summary effect calculated from 163 neurons from ten control rats and 159 neurons from ten SKF525-administered rats. Results are expressed as dot plots with each dot representing an individual recoding, with the mean ± standard deviation (SD) represented by horizontal lines. * $p < 0.05$, Mann-Whitney U test.

We found that the CYP inhibition by SKF525 did not alter the mean spontaneous firing rate of noradrenaline neurons of the LC. It however significantly increased the percentage of action potentials which occurred within the bursts (* $p < 0.05$, Mann-Whitney U test; Fig. 1). Other characteristics of noradrenaline neuronal firing activity were not affected by SKF525 in a statistically significant way.

With regards to the dopamine neurons of the VTA, CYP inhibition by SKF525 led to the significant decrease in the mean number of action potentials in the burst (* $p < 0.05$, Mann-Whitney U test; Fig. 2). SKF also tended to decrease the mean firing rate ($p = 0.10$, Mann-Whitney U test) and the percentage of action potentials which occurred within the bursts ($p = 0.07$, Mann-Whitney U test). Other characteristics of dopamine neuronal firing activity were not affected by SKF525 in a statistically significant way.

Discussion

The characteristics of the firing activity of central catecholamine-secreting neurons, observed in the present study, were similar to those observed in our previous studies (Dremencov et al. 2017; Koprdova et al. 2019; Csatlosova et al. 2021). SKF525 increased the percentage of the action potentials generated by noradrenaline neurons which occurred within the bursts and decreased the average length of the bursts generated by dopamine neurons. Other characteristics of noradrenaline and dopamine neuronal firing activity were not affected by SKF525 in a statistically significant way.

CYP inhibition by SKF525 did not alter the mean firing rate of the spontaneously active noradrenaline neurons of the LC, but it increased the percentage of the action potentials which occurred in the bursts, and therefore decreased the fraction of the action potentials which were fired as single spikes. The burst mode of firing of noradrenaline neurons has a direct relevance to the efficacy of neurotransmitter release. Thus, same number of spikes, exhibited in a burst-like mode, results in higher amount of noradrenaline molecules being released from the nerve terminal than if the similar number of action potentials was fired in a single-spike mode (Florin-Lechner et al. 1996; Marzo et al. 2014). It is thus possible that SKF525 enhances central noradrenaline transmission in rats, even though it does not alter the mean firing rate of noradrenalin neurons.

The mechanism underlying SKF525-induced stimulation of burst firing of noradrenalin neurons cannot yet be completely identified. We had previously reported that SKF525 decreased the firing rate of 5-HT neurons of the DRN, which might result in decreased 5-HT transmission (Grinchii et al. 2018). We had previously found that the increase in extracellular 5-HT, induced by the selective 5-HT reuptake inhibitor escitalopram, led to decrease in the firing rate and bursting activity of noradrenalin neurons (Dremencov et al. 2007a, 2007b; El Mansari et al. 2020). It is therefore possible that the enhancing effect of SKF525 on the bursting activity of noradrenalin neurons is secondary to the suppressing effect of this compound on the excitability of 5-HT neurons.

SKF525 decreased the average length of the bursts generated by dopamine neurons and tended to decrease their

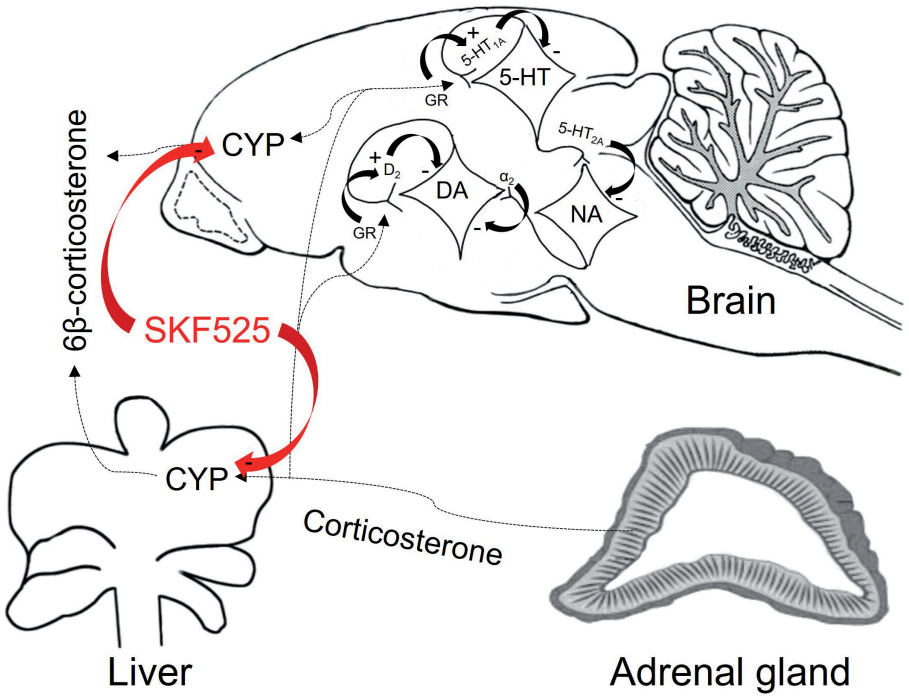

Figure 3. Putative mechanism underlying the effect of SKF525 (proadifen) on the excitability of catecholamine-secreting neurons. SKF525 inhibits the activity CYP (cytochrome P450) in the liver and in the brain, which leads to decreased corticosterone metabolism and increased circulating levels of this corticosteroid. Corticosterone activates glucocorticoid receptors (GR) in the dorsal raphe nucleus (DRN) and ventral tegmental area (VTA), which enhances the activity 5-HT_{1A} and D_2 autoreceptors and leads to decreased excitability of 5-HT (serotonin) and dopamine (DA) neurons. Decreased excitability of DRN 5-HT neurons leads to the increased excitability of NA (noradrenaline) neurons of the locus coeruleus (LC), *via* a mechanism putatively involving 5-HT_{2A} receptors. Finally, increased excitability of NA neurons of the LC contributes to the decreased excitability of DA neurons of the VTA (ventral tegmental area), *via* a mechanism putatively involving α_2 receptors. + stimulatory/increasing effect; – inhibitory/decreasing effect.

mean firing rate and percentage of action potentials which occurred in the bursts. As with noradrenalin neurons, the burst mode of firing of dopamine neurons is linked with more efficient stimulation of dopamine release (Cooper 2002; Wieland et al. 2014). The inhibitory effect of SKF525 on the burst-like firing of dopamine neurons might therefore result in decreased central dopamine transmission. The inhibitory effect of SKF525 on the excitability of dopamine neurons of the VTA is surprising, since a previous study showed a mineralocorticoid receptor-mediated enhancing effect of glucocorticoids on the excitability glutamatergic input to dopamine neurons (Overton et al. 1996). One the other hand, Peng and colleagues had recently reported that corticosterone diminishes the excitability of dopamine neurons of the VTA, *via* a mechanism involving D_2 receptors (Peng et al. 2021). Since escitalopram-induced increase in 5-HT tone led to the decreased firing activity of dopamine neurons (Dremencov et al. 2009; Hamati et al. 2020), the inhibition of 5-HT tone by SKF525 is expected to result in stimulation rather than to inhibition of dopamine neurons. However, since the selective loss of noradrenaline neurons of LC results in increased excitability of dopamine neurons of the VTA (Guiard et al. 2008), it is possible that the inhibition of burst-like firing of noradrenaline neurons contributes to the inhibitory effect of SKF525 on dopamine neurons. Further studies should be performed to examine the interactions between corticosteroids and excitability of dopamine neurons of the VTA.

Summarizing, the inhibition of liver and/or brain CYP by SKF525 has an enhancing effect on the excitability of central noradrenalin, and inhibitory – on the excitability of central dopamine neurons. The stimulatory effect of the CYP inhibition on noradrenaline neuronal firing activity might be triggered, at least in part, by corticosterone-induced inhibition of 5-HT transmission. The effect of CYP inhibition on dopamine neuronal firing activity might be in torn secondary the putative activation of central noradrenaline transmission (Fig. 3). It is possible that the inhibitory effect of SKF525 on dopamine neurons, as well as the inhibitory effect of this CYP inhibitor on 5-HT neurons, observed on our previous study (Maj et al. 1981; Grinchii et al. 2018), at least partially explains the ability of SKF525 to diminish the efficacy of imipramine, a non-selective tricyclic antidepressant acting as 5-HT, noradrenaline, and dopamine reuptake inhibitor (Maj et al. 1981). Notably, Maj and colleagues reported the lack of diminishing effect of SKF525 on the efficacy of desipramine, an antidepressant drug primarily acting on noradrenaline system. Since glucocorticoids, as well as central catecholamines, are fundamental in stress, the interactions between circulating glucocorticoids and the excitability of catecholamine-secreting neurons might be of particular importance in pathophysiology of stress-related disorders. Further studies are however required to test this hypothesis.

The main limitations of this study are the use of a non-selective CYP inhibitor and non-distinguishing between brain and hepatic CYP inhibition. The involvement of the inhibitory effect of SKF525 on the nitric oxide synthase (NOS; Sykes et al. 2016) cannot be excluded as well. In future studies, the effect of the selective inhibitors of the specific CYP subtypes, such as CYP3A1, CYP3A2, CYP3A4, and CYP3A5, which are fundamental in glucocorticoid metabolism (Peng et al. 2011), should be tested.

Acknowledgements. This study was funded by the Scientific Grant Agency of Ministry of Education of Slovak Republic and SAS (grant VEGA-2/0057/22) and Slovak Research and Development Agency (grant APVV-20-2020). The work of RNB was supported by the National Scholarship Programme of the Slovak Republic. The authors thank Katarína Hrivíková, PharmD, PhD, for the assistance with the figures' preparation and Viera Komínková, PhD, for critical proof-reading of this article.

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Received: February 21, 2022
Final version accepted: March 26, 2022