Escitalopram alters local expression of noncanonical stress-related neuropeptides in the rat brain via NPS receptor signaling

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Abstract

Background: Neuropeptide S (NPS) is a multifunctional regulatory factor that exhibits a potent anxiolytic activity in animal models. However, there are no reports dealing with the potential molecular relationships between the anxiolytic activity of selective serotonin reuptake inhibitors (SSRIs) and NPS signaling, especially in the context of novel stress-related neuropeptides action. The present work therefore focused on gene expression of novel stress neuropeptides in the rat brain after acute treatment with escitalopram and in combination with neuropeptide S receptor (NPSR) blockade. **Methods:** Studies were carried out on adult, male Sprague-Dawley rats that were divided into 5 groups: animals injected with saline (control) and experimental rats treated with escitalopram (at single dose 10mg/kg daily), escitalopram and SHA-68, a selective NPSR antagonist (at single dose 40mg/kg), SHA-68 alone and corresponding vehicle (solvent SHA-68) control. To measure anxiety-like behavior and locomotor activity the open field test (OFT) was performed. All individuals were sacrificed under anaesthesia and the whole brain excised. Total mRNA was isolated from homogenized samples of amygdala, hippocampus, hypothalamus, thalamus, cerebellum and brainstem. Real time-PCR was used for estimation of related NPS, NPSR, neuromedin U (NMU), NMU receptor 2 (NMUR2) and nesfatin-1 precursor nucleobindin-2 (NUCB2) gene expression. **Results:** Acute escitalopram administration affects the local expression of the examined neuropeptides mRNA in a varied manner depending on brain location. An increase of NPSR and NUCB2 mRNA expression in the hypothalamus and brainstem was abolished by SHA-68 coadministration, while NMU mRNA expression was upregulated after NPSR blockade in the hippocampus and cerebellum. **Conclusions:** Pharmacological effects of escitalopram may be connected with local NPSR-related alterations in NPS/NMU/NMUR2 and nesfatin-1 gene expression at the level of selected rat brain regions. A novel alternative mode of SSRI action can be therefore cautiously proposed.

Keywords: escitalopram, neuropeptide S, neuromedin U, NPSR, NMUR2, anxiety

Introduction

Pharmacology of anxiolytic drugs is an important area of contemporary neuroscience and clinical psychiatry. The receptor and molecular mechanisms of their actions, as well as the theory explaining their pharmacological effects are relatively well documented. These findings concur with wide ranging studies on the pathogenesis of anxiety and complement classical GABA-ergic and serotoninergic models. Clinically used anxiolytics may exhibit numerous side effects caused by their diverse influence on particular brain structures. Dynamic approaches of neurochemical studies have resulted in several reports that suggest a distinct involvement of neuropeptides in the origin of the anxiety responses. Hence, a hypothesis, assuming that pharmacological effects of some anxiolytics may be triggered alternatively via modulation of peptidergic signaling seems to be reasonable. An experimental model designed to verify this assumption may also help to understand endogenous mechanisms of anxiety and to outline potential perspectives in the field of novel therapeutic strategies aimed at modulation of neuropeptide action in the brain.

Stress may be defined as an assembly of unspecific physiological responses against exo- and endogenous stimuli that disturb the state of physiological homeostasis. In the standard model, brain sensory systems received all stressors and send neural information to the limbic systems that controls the hypothalamic-pituitary-adrenal (HPA) axis, a regulatory loop responsible for the generation of peripheral autonomic effects. Neuropeptides are widely involved in the mechanisms of stress responses and anxiety, being able to modulate synaptic release of classical neurotransmitters [1,2]. Some of them such as neuropeptide Y (NPY), orexins and oxytocin are considered endogenous anxiolytic factors, others e.g. corticotropin releasing factor (CRH), substance P, vasopressin and cholecystokinin (CCK) exhibit anxiogenic activity [3,4].

Recently, due to advancement in neuromolecular and bioinformatic techniques several novel stress and anxiety-related neuropeptides have been discovered in the brain. Almost all of them exposed both the unique properties and multidirectional activities at the level of numerous neural pathways. Against this background, newly

identified peptidergic modulators of stress responses: neuromedin U (NMU) neuropeptide S (NPS) and nesfatin-1 seems to be specifically worth investigating.

Neuropeptide S (NPS), as a product of 89-amino acid propeptide conversion, is a ligand of the G-coupled receptor (NPSR) formerly known as GPR 154 [5]. NPSR stimulation causes calcium release to the neuroplasm, increase cAMP levels and probably phosphorylates protein kinase MAPK resulting in the activation of neurons [6,7]. Structural homology between human and rat NPSR is around 90% [8]. NPS precursor mRNA exhibits coexpression with galanin, vesicular glutamate transporter (vGAT), choline acetyltransferase (ChAT) and corticotropin-releasing (CRH) hormone, suggesting possible functional connections between aminergic/peptidergic neurotransmission and NPS signaling in the brain [9]. In rodents, a limited population of NPS-expressing glutamatergic neurons is located in the vicinity of locus coeruleus including the nucleus of trigeminal nerve, lateral parabrachial and Kölliker-Fuse nuclei [10]. A few dispersed hypothalamic and amygdalar neurons exhibit NPS mRNA expression [11] with NPSR mRNA in turn widely distributed in the rat brain, especially in the hypothalamus, amygdala, olfactory bulb as well as in some thalamic and cortical regions [9,10]. NPS is a neuromodulator with a wide spectrum of regulatory activity in the brain, it exposes anxiolytic action, stabilizes wakefulness, regulates food intake and plays a role in the mechanisms of addiction [5,11-14]. From the neuropsychiatric viewpoint an anchoring of NPS in the fear-related neural pathways seems to be particularly important. Several behavioural studies have proven, that central NPS administration causes potent anxiolytic effect in rats [15-18] connected with elevated dopamine release in the prefrontal cortex but not with modulation of serotoninergic transmission [19]. Interestingly, several polymorphisms in the human NPSR gene may potentially increase a risk of panic anxiety episodes [20,21]. NPS secretion within particular brain structures is probably triggered by exposition to stress [22]. For instance, targeted NPS microinjection into the amygdala of rats exposed to acoustic stress resulted in a distinct reduction of anxiety-like behaviour [23]. To date, very little is known about the neurochemistry of NPS signalling in the human brain, with a population of NPS mRNA-expressing neurons so far only being found in the pontine gray matter [24].

Neuromedin U (NMU) is an anorexigenic peptide involved in the regulation of numerous neurophysiological processes [25,26]. In humans 25-amino acid molecule

(NMU-25) has been identified while in rats a shorter (NMU-23) form occurs [27]. In the rat brain, the highest, albeit dispersed, level of NMU immunoreactivity is found in the nucleus accumbens, hypothalamus, septum, amygdala, globus pallidus and brainstem [28]. It should be emphasized that, NMU-positive perikarya are localized in a few brain regions such as hypothalamic arcuate (ARC) and suprachiasmatic (SCN) nuclei medulla oblongata, cingulate gyrus, and medial frontal gyrus [29]. Interestingly, adrenocorticotropin (ACTH)-releasing endocrine cells of adenohypophysis show abundant NMU expression [30]. In the human brain NMU precursor protein was identified in the hypothalamus, nucleus accumbens, thalamus, locus coeruleus (LC), cingulate and medial frontal gyri [31]. In comparison, NMU mRNA expression was detected in the rat hypothalamus, especially in the ARC and ventromedial nuclei (VMH) and median eminence as well as in the brainstem centres: solitary nucleus, area postrema, dorsal trigeminal nucleus and olivary nucleus [32]. Two types of metabotropic NMU receptors are currently known: NMUR1 and NMUR2, coupled with G_q and $G_{i/0}$ proteins respectively [33]. NMUR1 is expressed mainly in the peripheral tissues–primarily in the gastrointestinal tract while in contrast, NMUR2 is expressed predominantly in the CNS. NMUR2 mRNA expression is restricted to the brain, including in the hypothalamus, hippocampus, substantia nigra, medulla oblongata, pontine reticular formation, spinal cord, thalamus, and cerebral cortex [34]. *In situ* hybridization studies revealed that NMUR2 mRNA was detected in the ARC and DMH, surrounding the VMH, the ependymal layer of the third ventricle and the paraventricular nucleus (PVN) of the hypothalamus [35]. Stimulation of brain NMUR2 has been found to modulate anxiety-like behaviour and trigger stress-related molecular events by CRH exocytosis [36-41]. NMU-23 has been shown to have antidepressant-like behaviuoral effects in mice [38]. On the other hand, local NMU signaling circuit in nucleus accumbens acting via NMUR2 may also reduce reward responses induced by several psychoactive substances and ethanol [42-43].

Nesfatin-1, an active 81-amino acid, pleiotropic neuropeptide is formed after convertase-dependent cleavage of prohormone nucleobindin-2 (NUCB2) [44-46]. Nesfatin-1 has 85% sequence homology between human and rodent genes [47]. Nesfatin-1 is probably a ligand of a so far unidentified G-coupled receptor [48]. In the rat brain the biggest number of nesfatin-1 expressing neurons was perceived in the following hypothalamic nuclei: ARC, paraventricular (PVN), supraoptic (SON) and

lateral hypothalamus [49,50]. Nesfatin-1 producing serotoninergic, cholinergic and noradrenergic cells were also found in the raphe nuclei, Edinger-Westphal (EW) nucleus and LC respectively [51]. Nesfatin-1 is a potent anorexigenic factor, it stabilizes satiety and inhibits food intake in animals [50,52,53]. Nesfatin-1 is considered to be involved in the neural mechanisms of stress responses, anxiety and depression [54]. Both central and peripheral administration of nesfatin-1 causes anxiety-like behaviour in rodents [55,56]. Several findings suggest that acute restraint stress does activate nesfatin-1 neurons in the hypothalamus and brainstem [49,57]. Adrenalectomy resulted in the increase of NUCB2 mRNA expression in the rat hypothalamus (PVN neurons) but intracerebroventricular injection of nesfatin-1 elevated serum stress hormones (ACTH, corticosterone) levels [58] and stimulates hypothalamic melanocortin pathways to increases blood pressure [59,60]. Stress-related increase of nesfatin-1 expression was also detected in rat brainstem centers such as the raphe nuclei, EW and LC [57,61,62]. An activation of stress-sensitive serotoninergic and noradrenergic neurons by nesfatin-1 strongly stimulates CRF release in the hypothalamus that finally puts the HPV axis in motion [63].

Escitalopram is an S-enantiomer of citalopram, a selective serotonin reuptake inhibitor (SSRI) with beneficial pharmacological properties and a satisfactory tolerance profile. Escitalopram has a minimal affinity to serotonin, dopamine, and cholinergic receptors, which highly reduce the range of its potential side effects. SSRI-related changes in energy homeostasis and weight gain are often clinically observed [64,65], however little is known about stress-related peptidergic neuronal pathways which could be additional targets for these medications. Although SSRIs often exhibit anxiolytic and sedative properties their potential direct or indirect effect on stress-related neuropeptides signalling are so far completely unknown.

The present experimental paradigm aims to shed light on this area by determining if and how acute treatment with escitalopram influences the expression of nesfatin-1, neuropeptide S (NPS), NPS receptor (NPSR), neuromedin U (NMU) and NMUR receptor 2 (NMUR2) genes in the rat brain. We hypothesize that anxiolytic properties of escitalopram may potentially be related to its stimulatory effect on NPS signalling. The second purpose of the study was therefore to investigate the possible effect of NPS transmission blockade with SHA-68, a selective NPSR antagonist, on escitalopram-related changes in neuropeptide gene expression and anxiety-like

behaviour in rats. SHA-68 is a polycyclic, fluorinated compound insoluble in aqueous solutions. A mixture of organic solvents based on Cremophor and dimethylsulfoxide (DMSO) should therefore be used to prepare a stable and homogenous SHA-68 solution [66]. However, number of publications report that DMSO itself is not a physiologically inert agent. For instance, its administration may increase functional expression of several receptors e.g. NMUR, bradykinin or opioid in cell cultures [67-69]. Moreover, DMSO is considered to affect the motor activity and to modulate the sleep architecture in rats [70-71]. Thus, in the present experiment, an additional control group of rats treated with DMSO-containing SHA-68 solvent was introduced.

2. Materials and methods:

2.1. Animals

The studies were carried out on adult (2-3 months old, 180-210 g) male Sprague-Dawley rats from Medical University of Silesia Experimental Centre housed at 22^0C with a regular 12/12 light-darkness cycle with access to standard Murigran chow and water *ad libitum*. All experimental procedures were approved by Local Bioethics Committee at the Medical University of Silesia (agreement no; 21/2019, dated 03.01.2019) and were conducted in a manner consistent with NIH Guidelines for Care and Use of Laboratory Animals.

2.2. Drug administration

Five groups of rats (n=5) have received respectively control vehicle (physiological salt, 0.25 ml), escitalopram (10 mg/kg), escitalopram (10 mg/kg)+SHA- 68 (40 mg/kg), SHA-68 (40 mg/kg) and SHA-68 vehicle solvent (0,25 ml) by a single intraperitoneal injection. Above mentioned non-toxic doses of drugs were established on the basis of pharmacological standards developed in preclinical studies. Escitalopram powder was dissolved in saline solution (NaCl 0.9%), crystalline SHA-68 in the PBS containing 10% Cremophor EL and, 10% DMSO and injected ip SHA-68 is a highly lipophilic derivative of diphenyltetrahydro-1H-oxazolo[3,4-a] pyrazine with selective antagonistic affinity for NPSR in nanomolar concentrations [66].

2.3. Behavioural tests

Open field test (OFT)

Typical testing method that allows evaluation of fear intensiveness is open field test, mostly due to the fact, that animals, such as rats, display a natural aversion to brightly lit open areas. Rats were moved to the testing room one day before the test for acclimatization. Lighting in the test room was diffused (12h light-dark cycle). Open field test was performed one hour after injection. Rats were placed in a square plastic cage (80 cm x80 cm, height 50cm) in the center of the cage, facing the same direction and left there for 10 minutes, allowed to explore unknown surroundings. Animal activity was recorded by camera and analyzed using Ethovison XT software (version 8.0; Noldus Information Technology B.V., Wageningen, The Netherlands). Measured parameters included frequency of entries to the central zone, time spent by the rat in the center zone (amount of time in seconds that the animal spent in central 40cmx40cm square), total distance travelled and velocity of movement.

2.4. Brain tissue collection

Four hours after the drug injection rats were anaesthetized with halothane sacrificed with CO_2 and their brains were quickly excised. Samples of hippocampus, amygdala, thalamus, hypothalamus, cerebellum and brainstem were microsurgically excised for immediate RNA isolation.

2.5. Real Time-PCR reaction

Total mRNA was extracted from the collected brain tissues via homogenization with an ultrasound homogenizer (Heildolph DIAX 900, Germany) in 1 ml of TRIzol® Reagent (Sigma-Aldrich). mRNA isolation was performed using chlorophorm/isopropanol and 75% ethanol with samples finally dissolved in 50 µl of RNAse-free water. Collected mRNA samples were transcribed into cDNA during

incubation in buffered solution of reverse transcriptase MMLV-RT with RNAsin, oligo-dT and mix of nucleotides at 42^{o}C for 60 min. using a thermal cycler Veriti 96 Well (Applied Biosystems). Initial mRNA solutions contained 1,5µl/ml. Quantitative Real-Time PCR reaction (qPCR) was performed by FastStart SYBR Green Master (Roche) in a Light Cycler ® 96 (Roche) thermal cycler for 40 rounds. Beta-2-microglobulin (B2M) was chosen as a standard internal reference gene. Primer sequences: B2M: Forward: 5'–CGAGACCGATGTATATGCTTGC–3', Reverse: 5'–GTCCAGATGA TTCAGAGCTCCA-3'. cDNA was amplified using the TaqMan Gene Expression Assay NMU (Rn00573761_m1 Applied Biosystems). NPS: Forward: 5`-TTGGAGTTATCCGGTCCTC, Reverse: 5`-GGGCAGGTACTCAGCAAAA-3', NPSR: Forward: 5'-TGCAAGGTGCAAAGATCCCA-3', Reverse: 5'-AATCTGCA TCTCATGCCTCTCG-3', NMUR2: Forward 5'-GCGAACAAA GTGGCTGTGAA-3', Reverse: 5'-GTCCAGCAGATGGCAAACAC-3', NUCB2: Forward 5'-CCATCCAAGCACGGTACTGTTTTC-3', Reverse: 5'-CCAGTGTCTTGAAGG GCATCC-3'. Optimal hybridization temperature was established according to a gradient PCR and was 59^{o}C for NPS, NSPR, NMUR2, NUCB2 and 64^{o}C for B2M. The analysis of the obtained results was performed on the basis of the 2^{-ddCt} algorithm, where the internal control was the reference gene B2M, however the reference point (internal calibrator) for all results reported was the saline control [72]. The primary metabolism gene B2M was used because its expression in the examined structures was the most stable.

Statistical analysis

Statistical analysis was performed using data analysis software system Statistica (TIBCO Software Inc. 2017, version 13). Due to heterogeneity of variances across groups (Levene's test $p<0.05$) or absence of normal distribution (Shapiro-Wilk test $p<0.05$), nonparametric Kruskal-Wallis test was used. Differences were considered statistically significant at $p < 0.05$ ($0.01 \leq p < 0.05$) with two confidence levels at $p < 0.01$.

3. Results:

Gene expressions

NPS

In the amygdala (H=4.49, N_{1-5}=5, p=0.34, Fig. 1a), and hippocampus (H=7.66, N_{1-5}=5, p=0.1, Fig. 1b) no significant changes in the NPS mRNA expression were noticed. In the hypothalamus (Fig.1c), a significantly reduced expression was found in animals receiving only SHA-68 compared to the to the rats treated with escitalopram (H=12.37, N_{1-5}=5, p=0.02). In the thalamus (Fig. 1d), the highest expression was noted after simultaneous administration of escitalopram and SHA-68, especially distinct when compared to the vehicle group (H=11.38, N_{1-5}=5, p=0.043). In the cerebellum (Fig. 1e), the escitalopram-treated animals appeared to exhibit lower expression when compared to the vehicle group, Kruskal-Wallis test showed no statistical significance between the groups (H=9.14, N_{1-5}=5, p=0.06). No differences were revealed in the brainstem (H=3.9, N_{1-5}=5, p=0.41, Fig. 1.f).

NPSR

In the amygdala (Fig. 2a), a slight reduction of NPSR mRNA expression in the SHA- 68 receiving groups was present. However, the non-parametric test on ranks shows no significant differences between the groups. (H=8.5, N_{1-5}=5, p=0.07). In the hippocampus, hypothalamus, and brainstem a distinctly increased NPSR mRNA expression in the escitalopram group was found. This effect appears to be abolished by the concomitant administration of an NPS receptor antagonist. For the hippocampus (Fig. 2b) the Kruskal-Wallis test showed significant differences between the Esc group and Esc+SHA68 group (H=15.2, N_{1-5}= 5, p=0.003). Similarly in the hypothalamus (Fig. 2c), an upregulation of NPSR mRNA expression after escitalopram injection was found when compared to the Esc+SHA68 (H=20.2, N_{1-5}=5, p=0.004). In the same brain region, a large increase in NPSR mRNA expression was observed not only in the Esc group, but also in the group of animals receiving solvent SHA-68 (H=20.2, N_{1-5}=5, p=0.002). In the thalamus (Fig. 2d), the NPSR mRNA levels were upregulation in

escitalopram group (H=9.8, N_{1-5}=5, p=0.04), but p-value for multiple comparisons showed no significant statistical differences. For the brainstem (Fig. 2f), the Kruskal-Wallis test showed significant differences between Esc, Esc+SHA68 groups (H=17, N_{1-5}=5, p=0.037), and control (H=17, N_{1-5}=5, p=0.048). No significant changes in NPSR gene expression were found in the cerebellum (H=4.85, N_{1-5}=5, p=0.3, Fig. 2e).

NMU

No significant changes in NMU mRNA expression were found in the amygdala (H=5.5, N_{1-5}=5, p=0.2, Fig. 3a), hypothalamus (H=5.1, N_{1-5}=5, p=0.3, Fig. 3c), thalamus (H=2.1, N_{1-5}=5, p=0.7, Fig. 3d) and the brainstem (H=6.6, N_{1-5}=5, p=0.1, fig. 3f). In the hippocampus (Fig. 3b), greater expression was noted in the group of rats receiving SHA-68 versus Esc+SHA6 group (H=9.8, N_{1-5} =5, p=0.045). In the cerebellum (Fig. 3e) Kruskal-Wallis test showed significant differences between the groups (H=10.6, N_{1-5}=5, p=0.03), but test for multiple comparisons of mean ranks show no statistical significance between SHA-68 versus control group (p=0.06).

NMUR2

The NMUR2 gene expression was not changed in the amygdala (H=4.8, N_{1-5}=5, p=0.3, Fig. 4a), hippocampus (H=5.4, N_{1-5}=5, p=0.25, Fig. 4b) and thalamus (H=8.9, N_{1-5}=5, p=0.06, Fig. 4d). In the hypothalamus (Fig. 4c), a significant reduction was found in the group of animals receiving SHA-68 compared to the group that additionally received escitalopram (H=14.71, N_{1-5}=5, p=0.007). Similar pattern of changes was present in the cerebellum (Fig. 4e), however in this structure, despite significant Kruskal-Wallis test (H=14.1, N_{1-5}=5, p=0.007), difference between SHA-68 and escitalopram groups was p=0.06. In the brainstem (Fig. 4f), reduction in NMUR2 mRNA expression was noted in vehicle group animals compared to controls (H = 11.19, N_{1-5} = 5, p=0.045).

NUCB2

No changes of the NUCB2 mRNA expression in the amygdala ($H=1.2$, $N_{1-5}= 5$, $p=0.86$, Fig. 5a), hippocampus ($H=4.74$, $N_{1-5}=5$, $p=0.3$, Fig. 5b), and thalamus ($H=1.68$, $N_{1-5}=5$, $p=0.8$, Fig. 5d) were found. In the remaining structures, a significant reduction in gene expressions was noted in animals treated with both escitalopram and the NPS receptor antagonist. In the hypothalamus (Fig. 5c) a decrease in NUCB2 mRNA expression was found in the Esc+SHA68 group versus control group ($H=11.15$, $N_{1-5}=5$, $p=0.03$). Analogous reduction of mRNA expression was detected in the cerebellum (Fig. 5e) versus Esc group ($H=16.2$, $N_{1-5}=5$, $p=0.02$) and vehicle group ($H=16.2$, $N_{1-5}=5$, $p=0.01$), in the brainstem (Fig. 5f) versus Esc group only ($H=13.8$, $N_{1-5}=5$, $p=0.02$).

Behavioural studies

In the open field test (Fig. 6), mean velocity of rats ($H=15.6$, $N_{1-5}=5$, $p=0.004$) and distance traveled ($H=15.6$, $N_{1-5}=5$, $p=0.004$) were increased after escitalopram injection as compared to controls while these parameters were slightly decreased in case of escitalopram and SHA-68 simultaneous administration (not statistically significant) (Fig.6. a,b). Of note, after a single dose of SHA- 68 or vehicle an elevated locomotor activity was detected ($p=0.04$). Escitalopram evoked a significant increase of time spent in the center square on open field ($H=14.8$, $N_{1-5}=5$, $p=0.02$), and number of entrances into the center zone ($H=10.6$, $N_{1-5}=5$, $p=0.03$) as compared to the control group (Fig.6. c,d). Administration of escitalopram with SHA-68 caused in turn a decrease of frequency and time spent in the center zone, but not statistically significant. Rats treated with SHA-68 alone as well as animals from Esc group remained significantly longer in center square than control group ($H=14.7$, $N_{1-5}=5$, $p=0.03$). Collectively, these results showed that the rats displayed a discrete decrease of anxious behavior after a single injection of escitalopram in the open field test, an effect which seems to be abolished by the addition of the selective NPSR antagonist SHA- 68.

Discussion

In the current study we examined NPS, NPSR, NMU, NMUR2 and NUCB2 mRNA expression using quantitative Real-Time PCR in selected rat brain structures following acute treatment with escitalopram and under condition of NPSR blockade with SHA-68. Our report is the first study to investigate NPSR-related changes in the gene expression of the aforementioned regulatory neuropeptides which may enhance the understanding of the possible molecular interplay between brain NPS signaling pathways and the NMU/nesfatin-1 system in anxiety.

An injection of escitalopram led to the upregulation of NPSR mRNA expression in the hypothalamus, thalamus and brainstem. This effect seemed to be abolished by NSPR blockade with SHA-68. On the other hand, SHA-68 decreased the level of NPS mRNA in the hypothalamus. Because both hypothalamic and brainstem nuclei contain NPS and NPSR-expressing neurons [10,73], it has been suggested that local NPS signaling pathways in the hypothalamus, as well as in the amygdala-hypothalamus circuits, may be involved in the generation of anxiety-like responses in animals [74]. Importantly, amygdalar neurons send their stimulatory axonal processes to NPS-producing cells in the ventromedial hypothalamus (VMH), and to several brainstem centres e.g. periaqueductal gray responsible, for anxiety behaviour [75]. Therefore, it should not be excluded that some anxiolytic properties of escitalopram can be related to its stimulatory effect on hypothalamic and brainstem NPS systems. However, the molecular mechanism of this alternative antidepressant action remains unclear. Some NPS neurons express several types of 5-HT receptors that may be activated after escitalopram-dependent increase of serotonin concentration within the synaptic cleft. This may result in the stimulation of NPS/NPSR gene expression, subsequent protein synthesis, neuropeptide exocytosis, NPSR activation and finally triggering of anxiolytic NPS effects. Open field test results seem to be in line with this assumption as some slightly marked decrease in anxiety-like behaviour was found after a single dose of escitalopram. Moreover, this effect can be removed with a blockade of NPSR via SHA- 68. This may cautiously suggest that a single dose of escitalopram triggers anxiolytic responses in the brain and an elevation of NPSR mRNA level is reflected in the more intense synthesis of receptor protein that undergoes internalization at synaptic membranes. On the other hand, a potential depression of NPS signalling may

be a result of decreased NPS mRNA synthesis after SHA-68 administration. It suggests that NPS neuron activity may be autoregulated by their own presynaptic or somatic NPSR molecules. It should be emphasized, that pharmacological effects of the SSRI in patients suffering from depression and anxiety disorders are detected after about four weeks of drug administration, a longitudinal experiment with NPSR blockade would be therefore especially valuable. However, an interesting hypothesis does suggest that antidepressant drug action may occur after the first dose, by changing the way individuals process affective information [76,77]. Moreover, while SSRIs pharmacological response generally takes weeks before it is manifested clinically, an accumulating number of studies have examined the ability to predict the outcome of antidepressant therapy [78,79]. There was reported that there are some observable neurochemical changes that appear within hours of administering a single dose, suggesting that differential psychopharmacological effects can be present following acute rather than long-term SSRIs administration before detectable behavioural changes occur [76,80].

Alterations in NPS/NSPR mRNA levels in the hypothalamus after SSRI administration were not yet investigated, but previous report shows that both long and short-term olanzapine (a dopamine receptors antagonist) administration caused increased NPS expression in the rat hypothalamus, supporting a hypothesis that NPS signalling at the level of hypothalamic centres may contribute to pharmacological effects of atypical antipsychotic medications [81]. Importantly, an existence of physiological interplay between hypothalamic NPS signalling and orexin/CFR regulatory pathway has been found [82,83]. This regulatory pathway may play an important role in the mechanism of addiction. For instance, intra-amygdalar and central injection of NPS causes anxiolytic-like behaviour in animals, that may be reversed by SHA-68 administration [17], supporting the hypothesis that NPS release is evoked by stress stimuli. Possibly, an NPS mode of action in anxiety may be untypical and bidirectional: acute neuropeptide administration promotes anxiety-like behaviour, while long-term it appears to promote silencing of fear flashbacks. On the other hand, long-term treatment with escitalopram increased the kisspeptin mRNA expression in the rat amygdala. The level of kisspeptin 1 receptor (Kiss1R) mRNA in the hypothalamus, amygdala, hippocampus and cerebellum was also upregulated but POMC mRNA expression was in turn decreased in the aforementioned structures [84]. In general,

some SSRIs may modulate the expression of several neuropeptides in the rat brain. Escitalopram administration reduced thyreotropin releasing hormone (TRH) level in rat nucleus accumbens (NAc) but increased TRH-like peptide expression in the same structure as well as in the striatum, cerebellum and brainstem [85]. Risperidone downregulated the proopiomelanocortin (POMC) mRNA levels in the rat hypothalamus [86]. It was also recently suggested that excitatory effects of fluoxetine on hypothalamic POMC cells are dependent on the mTOR signaling pathway [87]. Long-term treatment with citalopram increased the number of RF-amide related peptide (RFRP) expressing neurons in the rat dorsomedial hypothalamus [88].

Alterations in the NMU and NMUR2 mRNA level after pharmacomodulation used in the current study were more ambiguous and weakly expressed when compared to NPR/NPSR. In the hippocampus the NMU mRNA level was decreased after injection of escitalopram+SHA-68, but SHA-68 only evoked the opposite effect both in this structure and the cerebellum. The role of NPS signalling in the hippocampal circuits still remains almost unknown. A recent report shows that activation of NMUR2 receptors with NMU increased the excitability of GABA-ergic neurons in the CA1 region of the rat hippocampus slices [89]. Interestingly, SHA-68 but not escitalopram decreased NMUR2 mRNA expression in the hypothalamus and cerebellum suggesting that NMUR signalling in these brain regions may be facilitated by NPSR activation. A small population of NMUR2-expressing neurons in the hypothalamus play a regulatory role in the control of food intake in rats, a stimulation of NMURs triggers anorexigenic effects [90,91]. A cluster of NMU-expressing perikarya in the lateral hypothalamus send their peptidergic axonal projections to PVN and dorsal raphe nuclei [92]. The knockdown of NMUR2 molecules in the PVN neurons by using small hairpin RNA caused increased consumption of a high fat diet [93]. It should not be excluded that the well proven anorexigenic effect of NPS [94,95] may be mediated by NPSR-expressing NMU neurons in the hypothalamus, a potential affinity of NPS to NMUR2 receptors should also be taken into account. To verify this hypothesis, further molecular studies as well as precise measurement of food intake after NMUR2 and NPSR blockade using special behavioural methods has to be provided. Hypothalamic NMU neurons are also involved in thermoregulation acting as stimulators of local prostaglandin E2 synthesis and ligands β3 adrenergic receptors [96]. A role of the NMUR2 in the central mechanisms of REM/nREM sleep transitions is also suggested

[97]. All these functions may theoretically be mediated by NMU signaling circuits within several hypothalamic nuclei.

Cerebellar and brainstem NUCB2 mRNA expression in rats that received escitalopram+SHA-68 was lower than in animals after escitalopram injections. Simultaneous escitalopram+SHA-68 administration also decreased NUCB mRNA in the hypothalamus and cerebellum compared with both the escitalopram and the vehicle groups. Neuroanatomical distribution of the changes perceived is in line with previous studies labeling hypothalamus and brainstem as the main sites of nesfatin-1 activity [57]. Although, these brain regions expose abundant NPSR expression, to date there are no reports dealing with possible functional interplay between nesfatin-1 and NPS regulatory systems in the brain. Our results may therefore suggest that local nesfatin-1 synthesis can at least partially be regulated by NPSR receptors. Given the aforementioned pleiotropic physiological activity of nesfatin-1 one can conclude that numerous autonomic effects of this peptide may be related to NPS signaling. Further molecular and behavioral studies are required to verify this possibility. Escitalopram does in turn not alter nesfatin-1 synthesis in the rat brain. This result stays in accordance with our recent observation showing that long-term treatment with olanzapine did not affect NUCB2 mRNA in the rat brainstem, while the gene expression of SMIM20/phoenixin and spexin was distinctly modulated [98]. Perhaps NUCB2 gene expression exposes relatively high stability in particular brain regions. It seems to be important taking into account that nesfatin-1 expression is modulated by diverse psychological and physical stressogenic stimuli in animal models and distinct NUCB2/nesfatin-1 impairment was detected in several neuropsychiatric dysfunctions [99].

Our present study focuses for the first time on analysis of NPS, NPSR, NMU, NMUR2 and NUCB2/nesfatin-1 gene expressions after treatment with an SSRI drug in combination of NPSR inhibition. Nevertheless, we have to point out some limitations of the study, including that neuropeptide protein levels were not measured but this will be provided in our ongoing research project. Secondly, the longitudinal study based on the same paradigm would add further detail to the mechanisms involved. In summary, in this initial report we have exposed only a part of possible neuromolecular/behavioural changes, our conclusions remain therefore rather cautious (Fig. 7.). Furthermore, it should be taken into account that translation from

mRNA to the active protein e.g. neuropeptide or its membrane receptor is stretched over a longer period of time (hours, days). Besides, the level of mRNA encoding peptides meant to undergo exocytosis from the cell usually do not provide information about amount of a given peptide ready to be released or actually secreted. The limitations of the statistical methods should also be taken into account. Two way ANOVA test is a highly recommended method for this study, however number of results did not exhibit Gaussian distribution after Shapiro-Wilk test or/and variance homogeneity after Levene test. In this case we have decided to use Kruskal-Wallis test, because there is no an appropriate nonparametric test comparable to very restrictive two way ANOVA test, that can be used in our investigation.

Whether NPS, NPSR, NMU, NMUR2 and NUCB2/nesfatin-1 gene expressions after SSRI pharmacomodulation are directly related to NPSR action or is a secondary effect, has to be investigated in the future. However, our results suggest an existence of possible functional interconnections between brain NPS signaling, novel stress-related neuropeptides expression and antidepressant action in animal models, which may contribute to a better understanding of the alternative ways of antidepressant action.

Author contributions

APN, AP, ADV: Conceptualization, Investigation, Data curation, Writing—original draft. APN, AP, ASS, : Methodology, Immunohistochemistry, Tissue acquisition. ADV, AG: Resources. AP, MK, JJW: Formal analysis, corrections.

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Compliance with ethical standards

Conflict of interest

All authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed

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Figure captions

Fig.1. Neuropeptide S (NPS) mRNA expression levels in the rat brain. Treatments (single ip injections): Control - saline (0.25 ml); Esc - escitalopram (10mg/kg), SHA-68 (40 mg/kg); Vehicle - SHA-68 solvent (0.25 ml). Results were normalized to beta-2-microglobulin reference gene and shown as 2^- ddCq levels compared to control group. Boxes show median, quartiles, minimum and maximum. Kruskal- Wallis test. N=5. ** $p \leq 0.01$; * $p \leq 0.05$

Fig.2. Neuropeptide S receptor (NPSR) mRNA expression levels in the rat brain. Treatments (single ip injections): Control - saline (0.25 ml); Esc - escitalopram (10mg/kg), SHA-68 (40 mg/kg); Vehicle - SHA-68 solvent (0.25 ml). Results were normalized to beta-2-microglobulin reference gene and shown as 2^- ddCq levels compared to control group. Boxes show median, quartiles, minimum and maximum. Kruskal- Wallis test. N=5. ** $p \leq 0.01$; * $p \leq 0.05$

Fig.3. Neuromedin U (NMU) mRNA expression levels in the rat brain. Treatments (single ip injections): Control - saline (0.25 ml); Esc - escitalopram (10mg/kg), SHA-68 (40 mg/kg); Vehicle - SHA-68 solvent (0.25 ml). Results were normalized to beta-2-microglobulin reference gene and shown as 2^- ddCq levels compared to control group. Boxes show median, quartiles, minimum and maximum. Kruskal- Wallis test. N=5. ** $p \leq 0.01$; * $p \leq 0.05$

Fig. 4. Neuromedin U receptor 2 (NMUR2) mRNA expression levels in the rat brain. Treatments (single ip injections): Control - saline (0.25 ml); Esc - escitalopram (10mg/kg), SHA-68 (40 mg/kg); Vehicle - SHA-68 solvent (0.25 ml). Results were normalized to beta-2-microglobulin reference gene and shown as 2^- ddCq levels compared to control group. Boxes show median, quartiles, minimum and maximum. Kruskal- Wallis test. N=5. ** $p \leq 0.01$; * $p \leq 0.05$

Fig. 5. Nucleobindin-2 (NUCB2) mRNA expression levels in the rat brain. Treatments (single ip injections): Control - saline (0.25 ml); Esc - escitalopram (10mg/kg), SHA-68 (40 mg/kg); Vehicle - SHA-68 solvent (0.25 ml). Results were normalized to beta-2-microglobulin reference gene and shown as 2^- ddCq levels compared to control group. Boxes show median, quartiles, minimum and maximum. Kruskal- Wallis test. N=5. ** $p \leq 0.01$; * $p \leq 0.05$

Fig. 6. Open field test results. Effects of a single, intraperitoneal injection on mean velocity of movement (a), cumulative distance travelled in 10 min (b), the time spent in central zone (c) and the number of entries in the central zone (d). Treatments (single ip injections): Control - saline (0.25 ml); Esc - escitalopram (10mg/kg), SHA-68 (40 mg/kg); Vehicle - SHA-68 solvent (0.25 ml). Boxes show median, quartiles, minimum and maximum. Kruskal- Wallis test. N=5. ** $p \leq 0.01$; * $p \leq 0.05$

Fig. 7. A model of possible molecular mechanism of escitalopram action at the level of NPS-related transmission in the context of NMU and nesfatin-1 signaling. Escitalopram directly increases serotonin level within synaptic cleft due to inhibition of serotonin transporter SERT. Activation of postsynaptic G-coupled 5-HT2 receptors triggers phospholipase Cβ (PLCβ) signaling pathway, increase inositol triphosphate (IP3) and diacylglycerol (DAG) synthesis and finally elevate the NPS precursor gene expression and neuropeptide synthesis in the brainstem neurons. Dopaminergic, noradrenergic and peptidergic neurons manifest NPSR expression. Activation of NPSR increases the adenylate cyclase (AD) activity and cAMP synthesis. SHA-68 does inhibit NPSRs action in both types of brainstem cells and possibly blocks presynaptic autoreceptors of NPS neurons. The SHA-68-dependent silencing of NPSRs function in the aminergic neurons with NMU/NMUR2 and nesfatin-1 coexpression causes alterations in the neuropeptide gene expressions. All those neurochemical events can be considered responsible for the generation of some anxiolytic effects of escitalopram. All those neurochemical events can be considered responsible for the generation of some anxiolytic effects of escitalopram. However, it should be strongly highlighted that

translation from mRNA to the active protein takes time. Besides, the level of mRNA encoding peptides meant to be secreted from the neuron usually do not provide information about amount of a given peptide ready to be released or actually released. Therefore, the results of the study do not fully justify the preliminary model presented above. Nevertheless, this may be perceived as noteworthy voice in the discussion about possible alternative SSRIs action.

List of abbreviations:

ACTH – adrenocorticotropic hormone

ARC – arcuate nucleus

B2M –beta-2-microglobulin

CNS – central nervous system

CRH/CRF – corticotrophin releasing hormone/ factor

DMH – dorsomedial hypothalamic nucleus

DMSO – dimethyl sulfoxide

Esc – escitalopram

EW –Edinger-Westphal nucleus

ip – intraperitoneal

LC – locus coeruleus

NAc - nucleus accumbens

NMU – neuromedin U

NMUR2 - neuromedin U receptor 2

NPS – neuropeptide S

NPSR - neuropeptide S receptor

NUCB2 – nucleobindin 2

OFT – open field test

POMC – proopiomelanocortin

PVN – paraventricular nucleus

SCN - suprachiasmatic nucleus

SHA-68 – N-[(4-Fluorophenyl)methyl]tetrahydro-3-oxo-1,1-diphenyl-3H-oxazolo[3,4-a]pyrazine-7(1H)-carboxamide, 3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide, neuropeptide S receptor antagonist

SON – supraoptic nucleus

SSRI - selective serotonin reuptake inhibitor

TRH - thyreotropin releasing hormone

VMH – ventromedial hypothalamus