Modulatory effect of long-term treatment with escitalopram and clonazepam on the expression of anxiety-related neuropeptides: neuromedin U, neuropeptide S and their receptors in the rat brain

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Abstract:

Background: Newly identified multifunctional peptidergic modulators of stress responses: neuromedin U (NMU) and neuropeptide S (NPS) are involved in the wide spectrum of brain functions. However, there are no reports dealing with potential molecular relationships between the action of diverse anxiolytic or antidepressant drugs and NMU and NPS signaling in the brain. The present work was therefore focused on local expression of the aforementioned stress-related neuropeptides in the rat brain after long-term treatment with escitalopram and clonazepam. **Methods:** Studies were carried out on adult, male Sprague-Dawley rats that were divided into 3 groups: animals injected with saline (control) and experimental individuals treated with escitalopram (at single dose 5mg/kg daily), and clonazepam (at single dose 0.5 mg/kg). 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 method was used for estimation of related NPS, NPS receptor (NPSR), NMU, NMU and receptor 2 (NMUR2) mRNA expression. The whole brains were also sliced for general immunohistochemical assessment of the neuropeptides expression. **Results**. Chronic administration of clonazepam resulted in an increase of NMU mRNA expression and formation of NMU-expressing fibers in the amygdala, while escitalopram produced a significant decrease in NPSR mRNA level in hypothalamus. Long-term escitalopram administration affects the local expression of examined neuropeptides mRNA in a varied manner depending on the brain structure. **Conclusions:** Pharmacological effects of escitalopram may be connected with local at least partially NPSR-related alterations in the NPS/NMU/NMUR2 gene expression at the level selected rat brain regions. A novel alternative mode of SSRI action can be therefore cautiously proposed.

Keywords:

escitalopram; clonazepam; neuropeptide S; neuromedin U; neuropeptides

Introduction

A fast development of both neuromolecular and *in silico* methods results in several reports suggesting a far-reaching involvement of novel neuropeptides in the origin of anxiety responses and pathogenesis of depression. Hence, a hypothesis, assuming that pharmacological effects of some anxiolytics may be triggered alternatively via modulation of peptidergic signaling seems to be reasonable. According to this, two newly identified modulators of stress responses: neuromedin U (NMU) and neuropeptide S (NPS) are definitely worth investigating. An experimental model designed to verify this assumption should help to understand endogenous receptor and molecular mechanisms of anxiety and to outline some potential perspectives in the field of novel therapeutic strategies aimed at modulation of neuropeptide action in the brain.

Neuromedin U (NMU) is an anorexigenic peptide involved in the regulation of the wide spectrum of numerous neurophysiological processes. In the animal and human rat brain NMU-positive perikarya are localized in the nucleus accumbens, hypothalamus, septum, amygdala, globus pallidus and brainstem [1]. Two types of metabotropic NMU receptors are currently known: NMUR1 and NMUR2, coupled with Gq and Gi/0 proteins respectively [2]. NMUR1 is present mainly in the gastrointestinal tract while NMUR2 is expressed exclusively in the CNS. expecially in the hypothalamus, hippocampus and brainstem nuclei [3]. An activation of the brain NMUR2 has been found to modulate anxiety-like behaviour and trigger stress-related molecular events by CRH exocytosis [4]. On the other hand, local NMU signaling circuit in nucleus accumbens acting via NMUR2 may also reduce reward responses induced by several psychoactive agents [5].

Neuropeptide S (NPS), as a product of 89-aminoacid propeptide conversion, is a ligand of G-coupled receptor (NPSR) formerly known as GPR 154 [6]. NPSR stimulation increases cAMP level and probably phosphorylates protein kinase MAPK that result in the activation of nerve cell [7]. A very small population of NPS-expressing glutamatergic neurons is located in the rodent brainstem [8] and limited number of hypothalamic neurons exhibit NPS mRNA expression [9]. Conversely, NPSR mRNA is widely distributed in the whole rat brain [8]. In the human brain, not numerous NPS mRNA-expressing neurons were only found in the pons [10]. NPS is a neuromodulator with a wide spectrum of regulatory activity in the brain, it exposes anxiolytic action, stabilizes wakefulness, regulates food intake, plays a role in the mechanisms of addiction [11]. An anchoring of NPS in the fear-related neural pathways seems to be particularly important from neuropharmacological point of view. Molecular and behavioural

studies have proven, that central NPS administration causes potent anxiolytic effect in rats [12] connected with elevated dopamine release in the prefrontal cortex but not with modulation of serotoninergic transmission [13]. Interestingly, several polymorphisms in the human NPSR gene may potentially increase a risk of panic anxiety episodes [14]. NPS secretion within particular brain structures is probably triggered by exposition to some stress-stimuli [15].

Escitalopram is a clinically important serotonin selective reuptake inhibitor (SSRI) with a minor affinity to neuronal aminergic receptors, which distinctly reduce the range of its potential side effects. The pharmacological effect of escitalopram in humans is associated with an increase of the serotonin within synaptic cleft. The downregulation of 5-HT_{2A} receptors results in the stimulation of postsynaptic 5-HT_1 receptors, which is responsible for the anxiolytic and antidepressant effects of the drug [16,17]. SSRI-related changes in energy homeostasis and weight gain are often clinically observed [18], however little is known about stress-related peptidergic neuronal pathways which could be additional targets for these medications.. Some reports suggest that SSRI treatment may modulate the hypothalamic corticotropin-releasing factor (CRF) pathway [19,20]. Escitalopram administration increased TRH-like peptides expression in the rat nucleus accumbens (NAc), striatum, cerebellum and brainstem, while TRH concentration was elevated in the NAc only [21]. Long-term treatment with escitalopram led to the downregulation of spexin expression in the rat hypothalamus [22].

Clonazepam is a benzodiazepine drug with anxiolytic and anticonvulsant properties. The mechanism of action of benzodiazepines is mainly based on the intensification of GABAergic neurotransmission by activation of the benzodiazepine receptor (BDZ), which is structurally related to the GABA-A receptor [23]. Activation of the GABA-A receptor causes the opening of chloride channels, hyperpolarization of the postsynaptic neuron and inhibition of its function. Clonazepam reduces also the serotonergic activity of neurons by acting as an antagonist of the postsynaptic 5-HT_{1A} receptors [24]. Thus, in terms of its action on 5-HT_{1A} receptors, clonazepam has an effect opposite to that of escitalopram. Although SSRIs and GABA-agonists often exhibit anxiolytic and sedative properties their potential direct or indirect effect on neuropeptide signalling are so far rather unknown.

The present experimental paradigm aims to shed light on this area by determining if and how long-term treatment with escitalopram and clonazepam influences the expression of neuromedin U (NMU), NMUR receptor 2 (NMUR2), neuropeptide S (NPS) and NPS receptor (NPSR), in the rat brain. We hypothesize that some anxiolytic properties of drugs examined may potentially be related to its stimulatory effect on NPS signalling. Escitalopram is commonly used to treat depression and clonazepam is approved for the treatment of panic disorder and anxiety. However, in our initial study we did not administer drugs in an animal model of anxiety to be focused on the neurochemical side of neuropeptide action in the normal rat brain

Nevertheless that kind of study including behavioural tests should be carried out in the near future.

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^{0}C 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 bioethic 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

Three groups of rats (n=5) have received respectively control vehicle (physiological salt, 0.5 ml), escitalopram (10 mg/kg), and clonazepam (0.5 mg/kg) by intraperitoneal injection for 4 weeks. Above mentioned non-toxic doses were taken from the previous preclinical studies where authors examine the effect of the antidepressant/anxiolytic medications on the neuropeptide expression and GABA-transmission in the rat brain [20, 25]. Both drugs in form of powder were dissolved in saline solution (NaCl 0.9%).

2.3. Brain tissue collection

Twenty four hours after the last drug administration rats (n=5) were anaesthetized with halothane, sacrificed with CO_2 and their brains were taken out immediately after fast skull opening. Samples of hippocampus, amygdala, hypothalamus, thalamus, cerebellum and brainstem were microsurgically excised for RNA isolation (Fig. 1.). Second group of animals (n=5) were anaesthetized and perfused with 4% paraformaldehyde PBS (pH 7.2-7.4). The brains were quickly excised, postfixed, dehydrated, embedded in paraffin and finally sectioned on the microtome (Leica Microsystems, Germany) in the coronal plane (-2.00 to -3.00 and -8.7 to -10.7 mm from bregma) at 7 μm slice thickness, according to Paxinos and Watson's The Rat Brain in Stereotaxic Coordinates (2007).

2.4. 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 °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'–GTCCAGATGATTCAGAGCTCCA-3'. cDNA was amplified using the TaqMan Gene Expression Assay NMU (Rn00573761_m1 Applied Biosystems). For NPS assay the following primers were used: Forward: 5`-TTGGAGTTATCCGGTCCTC, Reverse: 5`-GGGCAGGTACTCAGCAAAA-3', for NPSR: Forward: 5'-TGCAAGGTGCAAAGATCCCA-3', Reverse: 5'-AATCTGCATCTCATGCCTCTCG-3', for NMUR2: Forward 5'-GCGAACAAAGTGGCTGTGAA-3', Reverse: 5'-GTCCAGCAG ATGGCAAACAC-3'. Optimal hybridization temperature was established according to a gradient PCR and was 59 °C for NPS, NSPR, NMUR2, NUCB2 and 64 °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.

2.5. Immunohistochemistry and immunofluorescence

After rehydratation, antigen retrieval (in low pH citric acid buffer) and blockage with 10% serum (appropriate for primary antibody), brain sections were incubated overnight in 4ºC with polyclonal rabbit antibodies against the following rat antigens: neuropeptide S (1:500, Abcam, ab18252), NPSR (1:500, Bioss Antibodies, bs-11430R), neuromedin U (1:200, Boster, A01919-2) and NMUR2 (1: 500, Novus Biologicals, NBP1-02351). Overnight incubation with

primary antibodies were followed by administration of biotynylated anti-goat/anti-rabbit secondary antibodies (1:200), and then an avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit, Vector Labs). 3,3'-diaminobenzidine (DAB) was used to complete the reaction and visualize receptors expressing neurons. Alternatively, after incubation with primary antibody for NMUR2, several brain sections were kept in darkness with secondary antibody labeled with Alexa Fluor 555 (1:200, Abcam) and then, mounted on slides with the DAPI-containing medium. All images were captured with Nikon Coolpix optic systems and processed using Image ProPlus software (Media Cybernetics, USA). Histologically analogous serial sections were analyzed with use of ImageJ (v1.51j8).

2.6. Statistical analysis

Statistical analysis of mRNA expressions was performed using data analysis software system Statistica (TIBCO Software Inc. 2017, version 13). Data is presented as mean ± SD. Mean differences between groups were analyzed using nonparametric Kruskal-Wallis test followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$ with two confidence levels ($0.01 \leq p < 0.05$ and $p < 0.01$).

3. Results and discussion

In the current study we analyzed NPS, NPSR, NMU and NMUR2 expressions in the selected rat brain structures after long-term treatment with escitalopram and clonazepam using quantitative Real-Time PCR and immunohistochemistry. Our report is the first study to investigate changes in the gene expression of the aforementioned regulatory neuropeptides after pharmacomodulation which may enhance the understanding of the possible molecular interplay between the NPS/NMU signaling pathway and the anxiolytics/antidepressants action.

We found that rats treated chronically with clonazepam manifested increased NMU mRNA expression in the amygdala ($p=0.015$), but at the same time animals did show its decreased level in the brainstem ($p=0.02$). NMUR2 mRNA expressions were distinctly downregulated in the brainstem and cerebellum (Fig.2). Upon examining the whole brain of all studied animals, only single hypothalamic neurons exhibited a weak NMU immunoreactivity (Fig 3. n, arrow) but a dense network of NMU-expressing fibres was found in the amygdala (Fig 3.o). The observed formation of NMU-expressing fibers and increased NMU mRNA expression in amygdala may be related to the anxiolytic effect of clonazepam. Noteworthy, the

highest aggregation of intensively NMUR2 positive cells are observed in the lateral hypothalamus (Fig 3. e-f, i), perifornical area (Fig 3.j-k) and cerebellum (Purkinje cells, stellate cells and dentate nucleus neurons, Fig 3. l-m). Any significant drug treatment-related quantitative changes in the neuropeptide immunoexpression (e.g. number of cells) were not found. However, some Purkinje cell bodies of rats treated with escitalopram seemed to manifest more dense, perinuclear, ring-like immunostaining (Fig 3. m) when compared to controls (Fig 3. l). NMUR2-immunoreactive cells in the hypothalamus exhibited a distinct diversity of morphological forms (Fig. 3. a-k). Number of studies have reported a functional interplay between the NMU signaling and the brain reward system [1, 5, 26], therefore the revealed elevation of NMU mRNA expression after extended exposure to clonazepam seems to be especially interesting. For instance, some cocaine-induced c-fos-expressing neurons were co-localized with NMU-immunoreactive neurons in the NAc, the caudate putamen (CPu) and the basolateral amygdala (BLA), which are key brain regions associated with the brain reward system [26]. Not only in rodents, but also in humans, a single nucleotide polymorphism in NMUR2 was associated with alcohol abuse in a genome-wide allelic association study [27].

It should not be excluded that amygdalar NMU signaling as well as accumbal one regulates the reinforcing properties of addictive drugs in rodents. Mechanism of this action remain unknown, however there was shown that activation of NMUR2 expressed on GABAergic neurons projecting from dorsal raphe to NAc shell selectively decreases GABA release in response to NMU [28].

None of the drugs examined affected NPS gene expressions in all studied brain regions, but a significant decrease in NPSR mRNA level was found in the hypothalamus after long-term treatment with escitalopram ($p=0.029$) (Fig.2). This finding appears to be in keeping with our previous results showing elevated proopiomelanocortin (POMC) and kisspeptin receptor Kiss1 mRNA levels in the rat hypothalamus after chronic escitalopram administration (Pałasz et al. 2020). A distinct population of intensively NPS positive cells are observed almost exclusively within the brainstem (posterodorsal tegmental nucleus, locus coeruleus, parabrachial nucleus) and cerebellum (Purkinje cells and dentate nucleus). They displayed a relatively wide spectrum of perikaryal shapes and nerve cell bodies were often filled with neuromelanin deposits (locus coeruleus) (Fig. 4. e-f). Both clonazepam and escitalopram treatment did not significantly affect NPS and NPSR expression in the examined brain structures. Perikaryal intensity of dispersed NPS immunostaining was slightly lower in the locus coeruleus of rats treated with clonazepam (Fig. 4. g). Because several hypothalamic neurons exhibit NPS and NPSR expression [8], there was suggested that local NPS signaling circuit may be involved in the generation of anxiety-like responses in animals [30]. Importantly, NPS-releasing cells in the ventromedial hypothalamus (VMH) receive stimulatory input from the amygdala [31]. Therefore, it should not be excluded that some anxiolytic properties of

escitalopram can be related to its stimulatory effect on hypothalamic NPS signalling, however, the molecular mechanism of this alternative pharmacological action remains unknown. On the other hand, some NPS neurons express several types of 5-HT receptors that may be activated after escitalopram-dependent increase of serotonin concentration within synaptic cleft. Perhaps the activation of 5-HT_{1A} receptors associated with the long-term action of escitalopram is associated with a reduction in the number of NPSRs in hypothalamus, and thus a reduction in anxiety reactions. This may relate to the observed regulation of NPSR gene expression. In turn, the lack of this effect in the case of clonazepam may be due to the opposite effect to escitalopram on 5-HT_{1A} receptors.

Although NMU and NPS represent different families of regulatory neuropeptides, both of them expose anxiolytic activity in animal models of anxiety and depression [4, 12]. Neuroanatomical distribution of some changes perceived is in line with previous studies labeling hypothalamus and amygdala as the main sites of NMU expression and NMUR2-dependent signalling [1]. Although, these brain regions expose abundant NPSR expression, to date there are no reports dealing with possible functional interplay between NMU and NPS regulatory systems in the brain. Present study focuses for the first time on analysis of NPS, NPSR, NMU and NMUR2 expressions in the rat brain after treatment with SSRI and GABA agonist. Nevertheless, we have to point out several limitations of the study, which has to be taken into account. Firstly, the neuropeptide protein levels were not measured in a quantitative manner using Western blotting but this will be provided in our ongoing research project. Secondly, there was also relatively small number of rats, however we have applied a set of appropriate statistical methods. To sup up, in this first report we exposed only a part of possible neuromolecular/behavioural changes, our conclusions remains therefore rather cautious. Whether NPS, NPSR, NMU and NMUR2 expressions are directly related to clonazepam/escitalopram action or is a secondary effect, must be investigated in the future. However, our results suggest an existence of possible functional interconnections between brain NMU/NPS signaling, novel stress-related neuropeptides expression and anxiolytic drug treatment in the animal model.

Author contributions

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

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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. Schematic representation of the experimental method. After long-term treatment (28 days) with escitalopram and clonazepam, six rat brain regions were excised, total mRNA was isolated and the Real Time-PCR method was used for estimation of related NMU, NMUR2, NPS and NPSR mRNA expressions. Brain slices were also examined histochemically for general assessment of local NMU, NMUR2, NPS and NPSR immunoexpression.

Fig. 2. Relative mRNA expression of NMU, NMUR2, NPS and NPSR in the rat brain after long-term treatment with clonazepam and escitalopram. Number of animals per group (n=5). Beta-2-microglobulin (B2M) was used as a reference gene. Values are expressed as means ± SD. Differences between experimental groups were analyzed using Kruskal-Wallis test followed by Tukey's post-hoc test and they were considered significant at $p \leq 0.01$, versus control (black asterisks) and versus clonazepam group (blue asterisks).

Figure 3. Representative expression of NMUR2 in the rat hypothalamus and cerebellum. NMUR2 immunopositive neurons in the ventromedial hypothalamus (a,b,g), paraventricular nucleus (c,d,h), lateral hypothalamus (e,f, i) of control rats and in the lateral hypothalamus of animals treated with escitalopram (j,k) and clonazepam (l). NMUR2-expressing cells in In the cerebellar cortex, both Purkyne and stellate cells show abundant NMUR2 immunoreactivity both in control (l) and escitalopram treated-rats (m). An example of a single NMU expressing cell in the paraventricular nucleus (n, arrow) and NMU-positive fibres in the amygdala (o) of control rats. Scale bars: 100μm (a, c, e, g, k), 50 μm (j, l-o). Images captured with Nikon Eclipse Ti microscope.

Figure 4. Neuropeptide S (NPS) and NPSR expressing cells in the rat brainstem and cerebellum. NPS immunopositive neurons in the posterodorsal tegmental nucleus (PDTg) of controls (a,b) and after escitalopram administration (c), in the *locus coeruleus* of controls (e) and individuals treated with escitalopram (f) or clonazepam (g) - dark neuromelanin deposits are visible inside the multipolar perikarya. NPSR immunopositive cells in the PDTg (h,i) and cerebellar cortex (j,k), somata of Purkyne cells a s well as some stellate neurons exhibit a distinct NPSR immunoreactivity. Scale bars: 150μm (a,h), 100μm (b, d, i, j), 50 μm (c, e-g, k). Images captured with Nikon Eclipse Ti microscope.