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Escitalopram affects spexin expression in the rat hypothalamus, hippocampus and striatum

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Abstract

Background: Spexin (SPX) is a recently discovered neuropeptide that exhibits a large spectrum of central and peripheral regulatory activity, especially when considered as a potent anorexigenic factor. It has already been proven that antidepressants, including selective serotonin reuptake inhibitors (SSRI), can modulate peptidergic signaling in various brain structures. Despite these findings, there is so far no information regarding the influence of treatment with the SSRI antidepressant escitalopram on brain SPX expression.

Methods: In this current study we measured SPX mRNA and protein expression in the selected brain structures (hypothalamus, hippocampus and striatum) of rats chronically treated with a 10mg/kg dose of escitalopram using quantitative Real-Time PCR and immunohistochemistry.

Results: Strikingly, long-term (4 week) drug treatment led to the downregulation of SPX expression in the rat hypothalamus. This supports the hypothesis that SPX may be involved in the hypothalamic serotonin-dependent actions of SSRI antidepressants and possibly also in the central mechanism of body mass increase. Conversely, SPX expression increased in the hippocampus and striatum.

Conclusions: This is the first report of the effects of a neuropsychiatric medication on SPX expression in animal brain. Our findings shed a new light on the pharmacology of antidepressants and may contribute to a better understanding of the alternative mechanisms responsible for antidepressant action.

Key words: spexin, escitalopram, SSRI, neuropeptides, brain

Introduction

Neuropeptides regulate a wide pool of diverse brain functions. Biochemical and molecular analysis of cellular interplay within brain centres has recently improved together with precise description of local neuronal networks. This coupled with the novel discoveries of new multifunctional neuropeptides such as nesfatin-1 and phoenixin [1-3] has led to a new era of understanding of neuropeptide-brain interactions.

Spexin (SPX) is one such intriguing novel neuropeptide, a product of the *Ch12orf39* gene which was found thanks to advanced bioinformatics methods [4]. The function of this regulatory factor is currently under examination with SPX demonstrating no molecular structure similarities to known neuropeptides. Rat SPX differs from the human and mouse molecules by only one amino acid at the C-terminal domain [5]. In the brain, many SPX immunopositive neural populations have been described, while neuroglia are usually negative, the highest reaction was detected in the hypothalamic paraventricular and supraoptic nuclei. Hippocampal neurons show moderate immunoreactivity as well as cerebellar Purkinje cells and brainstem neurons [6].

SPX seems to have multiple physiological functions with studies in goldfish revealing the involvement of SPX in reproduction and food-intake regulation. Treatment of animals with SPX decreased the secretion of luteinizing hormone and also suppressed appetite. Brain injection of goldfish with SPX inhibited both basal and NPY- or orexin-dependent consumatory behaviour and food intake [7]. Similarly, a new finding also reports, that in another fish; orange-spotted grouper (*Epinephelus coioides*) central SPX administration decreased orexin but increased POMC mRNA expression in the hypothalamus [8]. Intriguing recent results published by Walewski et al. [9] showed that SPX may be a strongly anorexigenic factor involved in weight regulation, with novel potential for obesity therapy. SPX may also be a fat-expressed satiety factor [10]. Recent findings also demonstrates a role for SPX in the control of cardiovascular/renal function and nociception [5,11]. Intracerebroventricular SPX injection decreased the heart rate of rats without modulation of the blood pressure, but with a high elevation of urine production [11]. It also reported that SPX stimulated basal aldosterone secretion from freshly isolated adrenal endocrine cells.

Extended exposure of this culture to SPX resulted in a limited increase in corticosterone secretion and a significant decrease of cell proliferation [12]. In terms of mechanistic function, a ligand-receptor interaction study suggested that SPX may be a natural ligand for the GALR2/3 receptors. Moreover, SPX exhibits even higher potency toward GALR3 than galanin [13]. Interestingly an artificial SPX-based human GALR2 receptor agonist exerts an anxiolytic effect in mice [14]. On the other hand, SPX does stimulate intestinal peristaltic movement through GALR2-dependent activation of L-type calcium VDCC channels in the murine smooth muscle cells [15].

Escitalopram is an S-enantiomer of citalopram, a well-known highly selective serotonin reuptake inhibitor (SSRI) and a very effective treatment for depression and anxiety disorders. It has more potent allosteric affinity to the neuronal serotonin transporter (SERT) molecule than pure R-enantiomer or even isomer mixtures and therefore its pharmacological effect is especially beneficial with a satisfactory tolerance profile [16]. Escitalopram does not bind to serotonin, dopamine (D1 and D2), muscarinic and histamine receptors, which minimize the range of its potential side effects. SSRI-related changes in energy homeostasis and weight gain are often clinically observed [17,18], however little is known about peptidergic neuronal pathways which could be additional targets for these medications. Serotonin neurons located in the midline raphe nuclei send numerous long afferents to almost all brain structures including the hypothalamus, thalamus, hippocampus and neocortex.

There are many suggestions that SSRI treatment may affect the hypothalamic corticotropin-releasing factor (CRF) pathway [19,20] and hypothalamo-pituitary adrenal (HPA) axis [21]. Interestingly, 2 hours after escitalopram administration the level of TRH-like peptides was increased in the rat nucleus accumbens, striatum, cerebellum and medulla oblongata, while TRH concentration was decreased in the nucleus accumbens. It should not be therefore excluded that SSRI action is mediated by modulation of TRH and TRH-like neuropeptides [22]. Potential direct or indirect action of SSRIs at the level of brain spexin signalling are so far completely unknown.

The present experimental paradigm aims to shed light on this area by determining if and how long-term treatment with escitalopram influences the expression of spexin in the rat brain. In the current study we evaluated for the first time the level of SPX mRNA and protein in the selected brain structures;

hippocampus, hypothalamus and striatum after drug administration. The results cautiously indicate that extended escitalopram administration may significantly modulate spexin regulatory activity.

2. Materials and methods

Animals

The studies were carried out on adult (2-3 months old, 185-220 g) male Sprague-Dawley rats housed at 22^0C with a regular 12/12 light-dark cycle with access to standard Murigran chow and water *ad libitum*. All experimental procedures were approved by the local bioethic committee (agreement no 36/2012).

Drug administration and tissue collection

Two groups of rats (n=4) received intraperitoneal injections with respectively physiological saline and escitalopram at dose 10mg/kg/day every day for 4 weeks (28 injections). The dose used at the experiment were taken from the publications where authors examine the influence of the escitalopram on the neuropeptide expression in the rat brain [19, 23]. 24 hours after the last drug administration, animals were anaesthetized with isoflurane and quickly sacrificed. Samples of hypothalamus, hippocampus and striatum were microsurgically excised from the brain halves for RNA isolation. Remaining tissues were fixed (with 4% paraformaldehyde in PBS, pH 7.2–7.4) for immunohistochemistry.

Real Time-PCR reaction

Total mRNA was extracted from the collected brain tissues via homogenization with an ultrasound homogenizer (Heildoph DIAX 900,Germany) in 1 ml of TRIzol® Reagent (Life Technologies). mRNA isolation was performed according to Chomczynski and Sacchi (1987) 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 ^{0}C for 60 min. using a DNA Thermal Cycler 480 (Perkin Elmer Inc., Waltham, MA). Initial mRNA solutions contained 5µg of RNA per 100 µl.

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 (B_2m) was chosen as a standard internal reference gene. Primer sequences; B_2m: Forward: 5'–CGAGACCGATGTATATGCTTGC–3', Reverse: 5'–GTCCAGATGATTCAGAGCTCCA-3'. cDNA was amplified using the TaqMan Gene Expression Assay Spexin (Rn01749065_m1, Applied Biosystems) and TaqMan Gene Expression Master mix (4369016, Applied Biosystems). Optimal hybridization temperature was established according to a gradient PCR and was 50 and 59^{0}C.

Immunohistochemistry

Brain slices were dehydrated and embedded in paraffin, sectioned on a microtome (Leica Microsystems, Germany) in the coronal plane (−2.00 to−2.80 mm from bregma) at 7 µm thickness, according to Paxinos & Watson's The Rat Brain in Stereotaxic Coordinates (2007). After rehydratation, antigen retrieval (in a low pH antigen unmasking solution, Vector Laboratories) and blockage with 10% serum, brain sections were incubated overnight in 4 °C with a rabbit antibody against rat spexin (1:500, Phoenix Pharmaceuticals). Incubation with primary antibodies was followed by administration of biotynylated anti-rabbit secondary antibodies (1:200) for 30min, and then an avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit, Vector Labs) for another 30 min. Finally, 3,3′-diaminobenzidine (DAB) was used (Vectastain ABC kit, Vector Laboratories) for visualization of cells expressing particular neuropeptides. Sections (on the same slide) incubated with rabbit IgG instead of primary antibody were used as negative controls. Morphological analysis was performed with the use of Nikon Coolpix optic systems. Immunopositive cells were counted from three brain regions including hypothalamic arcuate, ventromedial, paraventricular and lateral nuclei, hippocampal CA1-CA3 areas with dentate gyrus and striatum using ImageJ 1.43u software.

Statistics

Statistical analyses were performed using Statistica (Systat Software). Data are presented as mean ± SEM. Mean differences between experimental groups were analyzed using one-way ANOVA followed by Tukey's *post-hoc* test. Differences were considered statistically significant at $p \leq 0.01$ (asterisks).

Results and Discussion

Recent reports suggest a multidirectional pattern of SPX action in the brain. The potential involvement of this novel neuropeptide in various autonomic processes, including the regulation food intake, blood pressure, renal function and hormone release, has also come into consideration [5]. Nevertheless, SPX physiology is still extremely poorly investigated, to date less than 20 research articles dealing with this regulatory factor have been published. There is also no literature concerning the potential relationships between SPX signaling and drug administration.

In this study we report for the first time that SPX mRNA and protein expressions are strongly downregulated in the hypothalamus after chronic escitalopram administration (45.13% and 23.06% of control respectively, $p = 0.00018$). A recent finding suggested that some SPX hypothalamic neurons take part in the central inhibition of food intake and energy homeostasis and the neuropeptide seems to be a potent anorexigenic factor in rats [9]. Furthermore, a previous microarray study reported that the SPX gene was distinctly depressed in adipose tissue of obese humans, and it is postulated that SPX is an adipokine, which would potentially impact food intake, energy expenditure as well as long chain fatty acid (LCFA) turnover [10]. Interestingly, a decreased serum SPX concentration was recently revealed in obese children compared with normal-weight controls without any correlation with adipokines and insulin levels [24]. Taking into account that escitalopram causes weight gain [25, 26], one can hypothesize cautiously that its action may be related to the inhibition of SPX expression in the hypothalamus. It should not be completely excluded that a decrease in SPX signalling may be an alternative unknown mechanism of SSRI-dependent weight-gain. However, this

suggestion is not yet justified by our results and it has to be verified by further studies including behavioural tests. Giving the potent affinity of SPX to GALR2/3 receptors [13] and suggested anorexigenic properties of galanin-like peptides (GALP) in mice [27, 28] but not in rats [28, 29] the potential changes in the hypothalamic GALRs expression after escilalopram administration requires further studies. They may be especially interesting in the context of suggestions that in mammals galanin affects food intake through GALR1 rather than GALR2/3 [13].

On the other hand, escitalopram has the ability to diminish the activation of hypothalamic neurons containing melanin concentrating hormone (MCH) and to suppress REM sleep rebound [30]. The potential interplay between SPX and MCH signalling is so far unknown, however it is worth noting that escitalopram may inhibit the action of another regulatory neuropeptide in the hypothalamus. Interestingly, a recent study reports that long-term treatment with escitalopram increased the serum level of other orexigenic factor; neuropeptide Y (NPY) in the patients with depression [31].

Conversely, the neuropeptide gene expression level was increased in the hippocampus and striatum (262.91%, and 280.79% of control respectively p = 0.00017 and 0.0091). However, the number of SPX-expressing cells was significantly elevated only in the hippocampus, especially among the dentate granular neurons (540% of control). The increased number of SPX- immunopositive cells in the striatum was not statistically significant. These results are currently extremely difficult to interpret without molecular analysis of the potential relationship between SPX signalling and serotoninergic inputs in the studied brain regions. Perhaps, serotonin excess may promote the expression of the SPX gene in the hippocampus, thus escitalopram could increase the activity of SPX signalling in this structure through SERT inhibition.

Our results partly correlate to data suggesting that escitalopram treatment induced changes in various rat hippocampal proteins (not neuropeptides), except for those involved in energy expenditure and neuroprotection [32] but it had no effect on hippocampal volume [33]. Interestingly administration of escitalopram during adolescence could modify the alterations of increased choline-containing compounds and decreased glutamate, N-acetylaspartate and myoinositol relative to the stable neurometabolite creatine in rat hippocampus [33]. Probably, escitalopram modulates

some cellular populations during neuronal differentiation in the rat dentate gyrus and the behavioural changes in these animals may be the consequence of an escitalopram mediated increase in specific subtypes of immature neurons [34].

The antipsychotic potential of the combined treatment of escitalopram, in therapeutic relevant doses, and the 5-HT_1A receptor antagonist, WAY-100635, has been evaluated by assessment of conditioned avoidance (CAR) behaviour and the use of microdialysis in freely moving rats. The combined treatment was found to decrease both conditioned avoidance (CAR) behavior, without affecting the basal extracellular levels of dopamine in the nucleus accumbens (NAc) or in the striatum, suggesting an antipsychotic-like effect with mesolimbic selectivity. Furthermore, escitalopram, in combination with the partial 5-HT1A agonist, (-)-pindolol, decreased basal dopamine levels in the NAc [35].

Despite its novelty, there are several limitations of the current study, which has to be taken into account. Firstly, there was rather small group of animals (only 4 individuals) examined. No behavioural tests were performed, thus, while literature data support the use of escitalopram in the given dose, its antidepressant effects were unfortunately not validated. It should be emphasized that in the case of these medications, different classes may differently influence neuropeptide expressions, like in the case of fluoxetine [36] and venlafaxine on the galanin system [37]. The current study has not examined different time points. Antidepressant actions are chronically not necessarily related to the serotonergic system, other theories suggesting a role of diverse factors including neurotrophins have also been proposed [38] and serotonergic markers may remain unaltered after SSRI drugs at therapeutically relevant time points [39]. Thus, whether spexin expression is directly related to antidepressant actions or is a secondary consequence, has to be investigated in the future.

At present, as antidepressant-related changes in peptide expression are not yet clarified, comparing the actions of escitalopram on SPX mRNA expression, in the defined brain structures, to its effects on other neuropeptides is unfortunately merely speculative. Although satisfactory explanation of spexin functions requires numerous further studies, even at this early explorative stage, SPX can be considered a potentially important regulatory factor in the brain.

Conclusions

We have demonstrated for the first time, that extended treatment with an SSRI drug, modulates the expression of SPX mRNA in the rat brain. This suggests a so far unknown relationship between this novel regulatory factor and brain function, which has implications for food intake control, HPA axis modulation and probably additional brain functions.

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Figure captions

Fig. 1. Quantitative PCR results of relative spexin mRNA expression levels in the rat hypothalamus, hippocampus and striatum. (A). Obtained results were normalized to beta-2-microglobulin reference gene. The number of spexin immunopositive cells in the examined brain structures (B). Data are presented as multiples/decimals of control (1) ± SEM. Differences were considered statistically significant at $p \leq 0.01$ (asterisks).

Fig 2. Representative expression of spexin in the rat hypothalamus, hippocampus and striatum in controls and after long-term treatment with escitalopram. Scale bars: 50 μm.