

Long-term treatment with haloperidol affects neuropeptide S and NPSR mRNA levels in the rat brain.

Running title: Haloperidol affects NPS/NPSR mRNA levels in the rat brain

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Abstract

The brainstem-derived neuropeptide S (NPS) has a multidirectional regulatory activity, especially as a potent anxiolytic factor. Accumulating data suggests that neuroleptics affect peptidergic signaling in various brain structures. However, there is no information regarding the influence of haloperidol on NPS and NPS receptor (NPSR) expression. We assessed NPS and NPSR mRNA levels in brains of rats treated with haloperidol using quantitative Real-Time PCR. Chronic haloperidol treatment (4 weeks) led to a striking upregulation of NPS and NPSR expression in the rat brainstem. Conversely, the NPSR mRNA expression was decreased in the hippocampus and striatum. This stark increase of NPS in response to haloperidol treatment supports the hypothesis that this neuropeptide is involved in the dopamine-dependent anxiolytic actions of neuroleptics and possibly also in the pathophysiology of mental disorders. Furthermore, our findings underline the complex nature of potential interactions between dopamine receptors and brain peptidergic pathways, which has potential clinical applications.

Key words: neuropeptide S, NPSR, haloperidol, neuroleptics, brainstem

Significant outcomes

- For the first time, we assessed the influence of haloperidol on NPS/NPSR mRNA expression in the rat brain
- We proved a significant upregulation of NPS mRNA level in the rat brainstem after haloperidol administration

Limitations:

- We assessed only mRNA expression, protein level was not measured
- We did not determine receptor-ligand interactions nor downstream signaling pathways

Introduction

Neuropeptide S (NPS) is a selective 20-amino acid ligand for the NPS receptor (NPSR), a Gs and Gq – coupled receptor formerly identified as orphan receptor GPR 154 (1). NPS is an endogenous modulator of a wide spectrum of physiological activities in the brain. It exerts anxiolytic effects, stabilizes arousal state, regulates food intake as well as plays a role in the pathomechanism of fear modulation and addiction (2-8). In the rat brain, expression of NPS is shown almost exclusively within the locus coeruleus, principal trigeminal nucleus and lateral parabrachial nucleus, whereas in mouse NPS expression in the Kölliker-Fuse nucleus has also been reported (9). In contrast to the specific expression of NPS, NPSR mRNA is abundant within the brain and has been detected in the olfactory bulb, piriform cortex, amygdala, hypothalamus, thalamus and brainstem (10). Receptor stimulation leads both to a release of Ca²⁺ reserves to the neuroplasm and to an increase of cAMP levels (11,1). From a neuropsychiatric viewpoint, the most important role of NPS seems to be its activity at the level of neuronal pathways related to the neurobiology of fear (12). A number of behavioral tests prove that central or even intranasal NPS administration has a strong anxiolytic effect in rats, which may be related to an increase of dopamine release in the medial prefrontal cortex (13,14). The stimulatory effect of NPS on dopaminergic signaling suggests that this neuropeptide might increase dopamine synthesis and/or its turnover within the synaptic cleft (14). Moreover, NPS application to organotypic hippocampal slices activated synaptic plasticity in CA1 and CA3 areas, whereas targeted NPS microinjection directly to the ventral CA1 was enough to reduce fear related behaviour in mice (15). Furthermore, under *in vitro* conditions NPS, acting through its receptor, weakens the neural activity stream from the dentate gyrus to the CA1 area (15). NPS peptide administration into the mouse amygdala results in conditioned fear elimination, but it has no effect before the conditioning process

(16). Recent experiments on healthy individuals also suggest, that NPSR gene T-alleles could be connected with fear, excessive stress reaction and increased HPA axis stimulation (5). Moreover, *NPSR1* gene variation affects the glutamate/glutamine (Glx) levels in the rat cingulate cortex during induced panic attacks (17). Studies by Zhang et al. (18) prove that NPS expression in the locus coeruleus and a decrease of NPSR internalization level in the limbic system coexist with chronic pain and anxiety (24). On the other hand, NPS can stimulate inhibitory GABAergic transmission in the rat medial amygdala, reducing fear related responses (16). The NPS signaling may be also involved in the regulation of HPA axis acting as a part of negative feedback loop in the response to various stress stimuli (4). Another clinically intriguing report has associated NPSR polymorphisms with neuropsychiatric disorders including schizophrenia (19). Noteworthy, anxiety is considered as an important and relatively frequent symptom of schizophrenia, which is significantly connected with an elevated risk of severe consequences including suicidal behaviour (20). Post-traumatic stress disorder (PTSD) and obsessive compulsive disorder (OCD) are present in 38.3% of patients suffering from schizophrenia (21). Interestingly, some recent findings suggest a role of the NPS signaling in the regulation of motor functions at the level of basal ganglia (22,23).

Haloperidol, a classical but still widely administered D₂ receptor antagonist effectively reduces the psychosis suggesting impaired dopaminergic signaling as a key mechanism of positive schizophrenia symptoms (24). Noteworthy, haloperidol is a non-specific neuroleptic with affinity to numerous receptors, including dopamine D₂, serotonin 5-HT₂, α -adrenergic and σ -opioid receptors (25-27). Haloperidol shows either potentially beneficial anxiolytic and sedative properties or a wide spectrum of unfavorable side effects including tardive dyskinesia, neuroleptic malignant syndrome, akathisia and QT-interval prolongation (28-30). Nevertheless, the neurochemical mechanism of anxiolytic and dyskinetic actions of

neuroleptics, both classical and atypical, is so far poorly understood. Some reports show that atypical antipsychotics, but not haloperidol can upregulate the level of steroid GABA_A receptor stimulators in the rat brain (31). In turn, the blockage of serotonin 5-HT₂ receptors with haloperidol may be responsible for its anxiolytic effect (32). Hypothetically, the peptidergic signaling systems may play a significant role in the central regulation of anxiety-related events.

Aims of the Study

Accumulating but still limited findings suggest that neuroleptics may affect peptidergic regulatory pathways in various brain structures. For example, both chlorpromazine and clozapine affect corticotropin-releasing hormone (CRH) expression probably via activation of the PI3K/Akt signalling cascade, however PKC-related pathway may be also involved (33). Haloperidol stimulates CRH mRNA expression and also increases the gonadotropin-releasing hormone (GnRH) secretion in the rat hypothalamus (34). In turn, both quetiapine and olanzapine are able to inhibit the release of CRH from isolated rat hypothalami and hippocampi (35). Haloperidol upregulates neurotensin levels in the rat striatum, hippocampus and the frontal cortex, but risperidone decreases the peptide expression (36). Conversely, long-term treatment with haloperidol decreases NPY mRNA levels in the rat amygdala and hippocampus, while olanzapine and clozapine show the same effect in the striatum, nucleus accumbens and anterior cingulate cortex (37). Long-term treatment with risperidone also downregulates the NPY mRNA expression in the rat hypothalamus (38). In contrast, a stimulatory effect of subchronically administered olanzapine on NPY expression is also

suggested (39). Olanzapine increases calcitonin gene-related peptide (CGRP) immunoreactivity in the rat brain (40).

Despite these ongoing studies, almost no data exists regarding the interaction of neuroleptics with brain NPS signalling. We therefore examined the effect of administration of haloperidol, a classical butyrophenone type antipsychotic drug, on NPS and NPSR expression in the rat brain and showed a robust significant increase in brainstem NPS mRNA expression as a result of chronic drug administration.

Materials and methods

Animals

The experiments were performed on adult male Sprague-Dawley rats (2-3 months old, 180-210 g). We injected two groups of animals (n=4) with either control vehicle (saline) or haloperidol (2 mg/kg/day) every day for 4 weeks (28 injections per rat). All procedures were conducted in a manner consistent with NIH Guidelines for Care and Use of Laboratory Animals and approved by the Local Bioethical Committee at the Medical University of Silesia (agreement no. 36/2012).

Material collection and reverse transcription

24 hours after the last drug administration, animals were anaesthetized with isoflurane. Then, the brains were quickly removed and we performed microsurgical excision of samples from hypothalamus, hippocampus, striatum and brainstem. After that, all samples were homogenized with an ultrasound homogenizer (Heidolph DIAX 900, Germany) in 1 ml of

TRIzol® Reagent (Life Technologies) and total mRNA was extracted and dissolved in 50 µl of RNase-free water. Transcription of mRNA into cDNA was performed by incubation in buffered solution of reverse transcriptase MMLV-RT with RNAsin, oligo-dT and a mix of nucleotides at 42 °C for 60 min using a DNA Thermal Cycler 480 (Perkin Elmer Inc., Waltham, MA). Initial mRNA solutions contained 5 µg of RNA per 100 µl.

Quantitative real-time PCR

We performed quantitative Real-Time PCR reaction (qPCR) with the use of FastStart SYBR Green Master Mix (Roche) in a Light Cycler ® 96 (Roche) for 45 rounds. Expression levels of NPS and NPSR were compared with expression of housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH). cDNA amplification was performed using the following primers: for NPS; Forward: 5' - TTGGAGTTATCCGGTCCTCTCTT - 3', Reverse: 5'-TTGGAGTTATCCGGTCCTCTCTT-3', for NPSR; Forward: 5'-TGCAAGGTGCAAAGATCCCA-3', Reverse: 5'-AATCTGCATCTCATGCCTCTC-3', for GAPDH: Forward: 5'-GTGAACGGATTTGGCCGTATCG-3', Reverse: 5'-ATCACGCCACAGCTTTCCAGAGG-3'.

Statistics

Statistical analysis was done with Statistica (Systat Software). We presented data (on graphs) as mean ± SEM. Mean differences between groups of animals were analyzed using non-parametric Kruskal-Wallis test. Differences were considered statistically significant at $p \leq 0,05$.

Results

In the current study, rats treated chronically with haloperidol manifested strongly increased relative NPS mRNA expression in the brainstem; $128,75 \pm 6,48$ versus control: $1,08 \pm 0,33$ ($p = 0,000246$). Although, there is an unexpectedly massive (about 120-fold) increase of NPS mRNA level in this region we excluded the effect of haloperidol on GAPDH expression, as mean Cq values for this gene are very similar for control animals (15,65) and for haloperidol treated animals (15,48). We have also found a trend for decreased NPS mRNA level in the hypothalamus: $13,79 \pm 0,36$ versus control: $15,59 \pm 4,09$, nevertheless this change was not statistically significant (Fig.1). The NPS mRNA expression in the hippocampus and striatum was undetected. After long-term administration of haloperidol NPSR mRNA expression was downregulated in the hippocampus; $5,6 \pm 4,03$ versus control: $15,76 \pm 3,86$ and striatum: $9,78 \pm 2,42$ versus control: $21,19 \pm 8,67$, unchanged in the hypothalamus $20,17 \pm 0,81$ versus control: $24,01 \pm 2,65$ and highly increased in the brainstem: $60,36 \pm 10,48$ versus control: $24,86 \pm 4,59$ (Fig. 2).

Discussion

At present, there are relatively few reports concerning NPS expression changes related to antipsychotic drug activity. Studies on the influence of this group of pharmaceuticals on peptidergic regulatory pathways could be a source of hypothesis explaining alternative ways of their pharmacological effects. In our analysis we focused on the expression of the NPS and NPSR mRNA in selected brain structures of rats chronically treated with haloperidol. The

results indicate that chronic drug administration may modulate the expression of NPS and NPSR mRNAs in selected rat brain regions.

The statistically significant elevation of the brainstem NPS mRNA level, after long-term haloperidol administration stays in agreement with our recent results showing that short and chronic treatment with the neuroleptics chlorpromazine and olanzapine increased the NPS mRNA expression in the rat hypothalamus (41). In addition, haloperidol stimulates the secretion of gonadotropin-releasing hormone (GnRH) (34) and increases corticotrophin-releasing hormone (CRF) mRNA expression in the rat hypothalamus (42). On the other hand, haloperidol, but not risperidone, elevates neurotensin levels in the rat striatum, hippocampus and frontal cortex. Conversely, in the occipital cortex, risperidone, but not haloperidol, reduces neurotensin expression (36). In contrast, long-term haloperidol administration decreases NPY mRNA expression in the rat amygdala and hippocampus while olanzapine and clozapine show the same effect in the nucleus accumbens, striatum and anterior cingulate cortex (18.). Similarly, chronically administered risperidone decreases the NPY mRNA level in the rat hypothalamus (38). Only olanzapine significantly decreases the NPY mRNA level in the lateral septal nucleus (37). A decrease in the hypothalamic NUCB2 and nesfatin-1 expression after chronic haloperidol administration was also recently reported (43). In our experiment, the NPS mRNA expression remains unchanged in hypothalamus after drug treatment. This result is in line with the study showing that hypothalamic NPY mRNA level did not change after long-term neuroleptic administration to rats (43).

Furthermore, it is suggested, that haloperidol can induce disturbances in the oxidative-antioxidative balance. It should be noted that the elevation of NPS expression observed in our study may be a part of neuronal protective mechanism against an extended treatment with this drug. Moreover, it was shown, that NPS can weaken lipid peroxidation processes in the

mouse brain cortex, which confirms its neuroprotective activity during the occurrence of oxidative stress (44).

Single dose treatment with haloperidol and other typical antipsychotics may increase the number of spontaneously active dopaminergic cells in the rat brainstem (areas A9 and A10) whereas olanzapine and atypical neuroleptics stimulate cell populations in A10, but not A9 (45). The long-term treatment with neuroleptics proved that multiple haloperidol administration can lead to a decrease of the number of spontaneously active cells in A9 and A10 areas of the brainstem (46) whereas Stockton and Rasmussen (47) showed that chronic exposition to olanzapine results in a decreased number of active neurons in A10, but not in A9 area.

Interestingly, after long-term treatment with haloperidol, the NPSR mRNA levels were downregulated both in the hippocampus and striatum. This result is consistent with our recent finding that NPSR mRNA levels in the same structures were decreased after chronic chlorpromazine and olanzapine administration. Additionally, both short and long-term exposition to chlorpromazine decreased the NPS mRNA level in the hypothalamus (41). Presented phenomena are rather difficult to interpret without specific analysis of potential interactions between NPSR mode of action and dopaminergic signaling. Possibly, haloperidol may modulate the NPS-related synaptic plasticity in the hippocampus mediating any kinds of fear responses (15). Probably, the high elevation of NPS mRNA level in brainstem neurons may suggest an increased neuropeptide synthesis and release to the hippocampal formation and striatum. Thus, it should be taken into consideration that a decrease in NPSR mRNA expression could be a compensatory response to increased NPS concentration in these structures. In contrast, the NPSR mRNA level in brainstem was distinctly elevated after chronic haloperidol administration. Theoretically, this neuroleptic could increase the sensitivity and/or activity of NPS signaling in the brainstem and in consequence facilitate

anxiolytic mechanisms. Importantly, the brainstem contains a distinct population of aminergic NPSR-expressing neurons (10) that play numerous central roles such as modulation of cortical glutamatergic transmission and maintaining the arousal state. We therefore can hypothesize that haloperidol may affect them indirectly via stimulation of NPS pathway. Chronic administration of haloperidol evokes several considerable side effects including prolonged sedation and tardive dyskinesia besides other motor disabilities.

Recent data suggested the role of NPS in locomotion and related these actions with dopaminergic signaling (44). It was shown that intracerebroventricular infusion of NPS to the striatum and substantia nigra increase the locomotor activity in rats. This effect is counteracted both by SHA 68 (a selective antagonist of NSPR) and antalarmin (a CRF-1 receptor blocker) suggesting that CRF plays a significant role in the NPS-related control of locomotion activity (23). Similarly, central injection of NPS abolished motor impairments evoked by dopaminergic neurotoxin 6-OHDA (22). Probably, NPS may stimulate dopamine release via selective activation of NPSR receptors in the extrapyramidal system (48). The observed downregulation of striatal NPSR mRNA expression suggests that pharmacological activity of haloperidol may also manifest at the level of NPS transmission in the basal ganglia, which may be one of the alternative ways of triggering dyskinetic side effects by this medication. Intriguingly, a recent study reports that haloperidol can also decrease the functional connectivity between substantia nigra and cortical motor regions, which may reflect motor disabilities (24).

Taken together, it is possible that dopamine may inhibit the expression of genes encoding some neuropeptides including NPS in certain brain regions. Thus, the blockage of dopamine receptors performed by haloperidol can result in compensational expression increase of the aforementioned proteins. Mechanisms of anxiolytic side effects of antipsychotics are not fully clarified, however it is suggested that the blockage of both

dopamine D₂ and serotonin 5-HT₂ receptors may be responsible for the haloperidol anxiolytic action (32). Conversely, some reports show that atypical neuroleptics clozapine and olanzapine, but not haloperidol can increase the level of endogenous allopregnanolone and allotetrahydrodeoxycorticosterone (THDOC), two positive GABA_A receptor modulators in the rat brain (49,31,50).

In conclusion, the fact that haloperidol highly affected the level of NPS and NPSR mRNA expression in the rat brain supports the hypothesis that NPS plays a role in the anxiolytic actions of neuroleptics and possibly also in the pathophysiology of mental disorders; for example in the control of negative schizophrenia. It was also shown recently, that synthetic NPS is a potent anxiolytic agent, even in rodents with an innate predisposition to high anxiety. Thus, it is strongly suggested that NPS may be a promising, potentially beneficial medication in the treatment of anxiety disorders especially in patients with the high-risk variant (8). Our intriguing initial data requires further basic pharmacological and behavioural studies on the wide spectrum of antipsychotic drugs, but nonetheless highlights the complex nature of potential interactions between dopamine receptors and brain peptidergic pathways and opens up an array of future potential clinical applications.

Authors Contributions

Artur Palasz – article conception and design, drafting the manuscript, analysis and interpretation of data, Ewa Rojczyk, Milosz Golyszny, Lukasz Filipczyk -acquisition of data, analysis and interpretation of results, John J. Worthington -drafting the article and revising it critically for important intellectual content, Ryszard Wiaderkiewicz - final review of the version to be published.

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Statement of interest

None

Animal Welfare

Experiments were conducted in a manner consistent with NIH Guidelines for Care and Use of Laboratory Animals.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals

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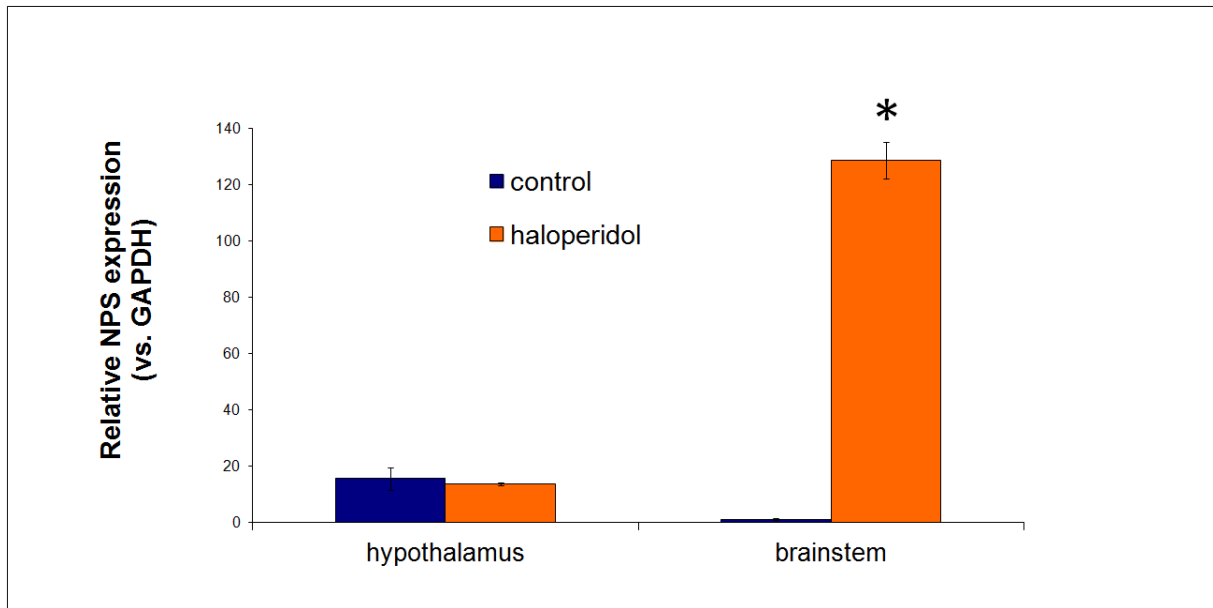


Fig. 1. Quantitative PCR results of relative neuropeptide S mRNA expression levels in the rat hypothalamus and brainstem (n=4). Data are presented as mean \pm SEM. Statistical analysis was performed using non-parametric Kruskal-Wallis test. Differences were considered significant at $p \leq 0,05$ (asterisk).

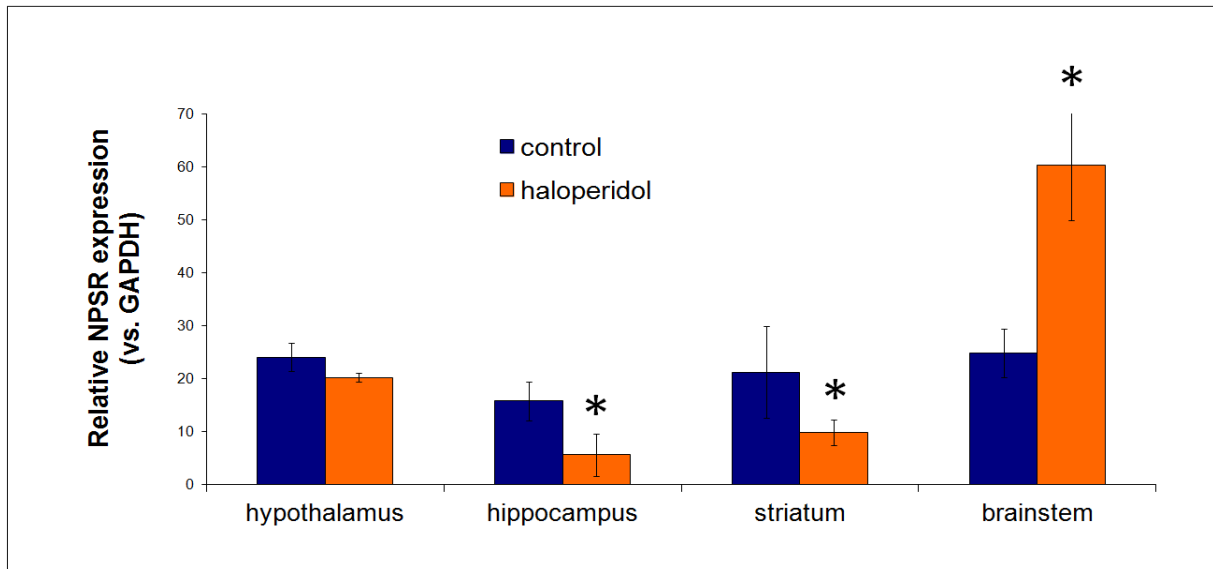


Fig. 2. Quantitative PCR results of relative NPSR mRNA expression levels in the selected rat brain regions (n=4). Data are presented as mean \pm SEM. Statistical analysis was performed using non-parametric Kruskal-Wallis test. Differences were considered significant at $p \leq 0,05$. (asterisks).