Psilocybin and ketamine affect novel neuropeptides gene expression in the rat hypothalamus

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Abstract

Objective: Psychedelics are able to trigger highly intense and profound alterations in self-consciousness, perception, affective and cognitive processes. Indeed, recent studies show that ketamine and psilocybin could be used as fast-acting antidepressants. However the molecular and neurochemical mechanisms of these psychedelics and their actions at the level of diverse brain structures remains so far unclear. Hypothalamic neuropeptides are involved in a wide spectrum of neuronal activities being responsible for the central control of all fundamental autonomic functions.

Methods: The purpose of this exploratory pilot study was to assess the gene expression of both classical and novel neuropeptides, including nesfatin-1, phoenixin (PNX), spexin (SPX), neuromedin U (NMU), neuropeptide S (NPS), and their known receptors in the hypothalamus of male Wistar-Han rats subjected to single injections of psilocybin (dose 2 or 10 mg/kg) and ketamine (dose10 mg/kg). Total mRNA was isolated from homogenized tissue and Real-time PCR was used for estimation of related gene expression.

Results: It was found that a single administration of the higher dose of psilocybin increased the mRNA expression of most noncanonical neuropeptides examined in the study, with only the case of NMU was there a decrease in gene expression. Interestingly, psilocybin administration also increased mRNA expression of the serotonin receptors: 5-HT1A, 5-HT2A and 5-HT2B but not 5HT-2C. In contrast, the effect of ketamine on the expression of neuropeptides was much more limited compared to psilocybin, only increasing transcripts of NUCB2, GPR173 and POMC were demonstrated.

Conclusions: These results suggest for the first time that selected psychedelics may enhance the signaling of 5-HT2A receptors or inhibit NMDA receptor activity, affecting neuropeptide signaling and serotonin transmission in the rat hypothalamus, which may contribute to a better understanding of psychedelic action in the brain.

Key words;

Psilocybin; ketamine; hypothalamus; neuropeptides; brain

Introduction

Psychedelics belong to an intriguing family of drugs that affect brain neurotransmission as well as several intracellular signaling pathways. Even a single dose of a these psychoactive substances is able to profoundly alter the subjective human experience, highly modulate sensory perception and change affectivity (Vollenweider and Smallridge 2022). Classical psychedelics such as N,N-dimethyltryptamine (DMT) and psilocybin act as potent agonists of serotonin 2A receptors (5-HT2A) however they also affect a wide spectrum of brain receptors and intracellular signaling pathways (Holze et al. 2024, Hatzipantelis and Olson 2024). Both psilocybin and ketamine, a potent dissociative anesthetic, especially its isomeric form S-ketamine (esketamine), have recently been considered as safe and fast-acting antidepressants (Nutt et al. 2013, Kalfas et al. 2023, Seshadri et al. 2024, Krystal et al. 2023). Despite its fast and potent psychedelic activity ketamine does not represent the structure of classical serotonergic hallucinogen, being a glutamate NMDA receptor ionic channel blocker (Maeng and Zarate 2007).

The primary effects of the central action of psychedelics include strong distortions in the perception of reality, personality dissociation and peculiar entheogenic experiences which are related to their influence on neocortical centres (Swanson 2018). However, these substances may also, at least transiently, act at the level of hypothalamic signaling pathways, modulating key autonomic functions such as food intake, thermoregulation and cardiovascular physiology (Gouzoulis-Mayfrank et al. 1999, Erkizia-Santamaría et al. 2022, Ludwig et al. 2021, Patterson et al. 2017, Maqueda et al. 2016). The regulatory systems of the hypothalamus rely significantly on local neuropeptidergic circuits and their connections with limbic and brainstem centres. Neurons in this area, supplied by serotonergic fibres, express 5-HT2A receptors and may be a potential target of classic psychedelics (Martin-Gronert et al. 2016). On the other hand a large population of hypothalamic neurons that regulate core autonomic functions are rich in the NMDA receptor (Miracca et al. 2022, Lee and Stanley 2005), opening up the possibility of the potential action of ketamine and other highly psychoactive modulators on glutamatergic signaling.

Several novel hypothalamic neuropeptides have recently been discovered which influence some behavioral pathways also modulated by the use of psychoactives opening up potential mechanisms of action. Nesfatin-1 and spexin (SPX) - pleiotropic, highly anorexigenic neuropeptides are considered to play an important role in several brain signaling processes including mechanisms underlying the generation of anxiety symptoms (Friedrich and Stengel 2023, Pałasz et al. 2018, Porzionato et al. 2010, Ma et al. 2018). Phoenixin (PNX), a novel modulator of the gonadoliberin (GnRH) releasing neurons, and ligand of GPR173 receptor (Treen et al. 2016), has also manifested potential anxiolytic properties (Jiang et al. 2015), and its multifaceted regulatory role in the brain has recently been suggested (Schalla and Stengel 2018). Another anorexigenic peptide neuromedin U (NMU) has been found to modulate anxiety-like behaviour acting via NMUR2 receptor (Tanaka and Telegdy, 2014). Highly anxiolytic neuropeptide S (NPS), a ligand of G-coupled receptor (NPSR, GPR154) is a neuromodulator with a wide spectrum of regulatory activity in the brain e.g. it stabilizes wakefulness and plays a role in the mechanisms of addiction (Reinsheid and Ruzza 2021, Tobinski and Rappeneau 2021). Orexigenic neuropeptide 26RFa (QRFP) acting via metabotropic receptor GPR103 stimulates food intake and regulates glucose homeostasis through the modulation of NPY/POMC neurons in the arcuate nucleus (Devere et al. 2024, El-Medi et al. 2020, Lectez et al. 2009).

So far, a number of basic studies have been conducted on the effects of diverse psychedelics on the level of expression of several neurotransmitters, their membrane receptors as well as some intracellular signaling factors. However, possible pharmacological action of psychedelics at the level of neuropeptidergic signaling in the hypothalamus are not yet revealed. The aim of this exploratory pilot study study was therefore to investigate for the first time the effect of short-term administration of two structurally and pharmacologically different psychedelic substances: psilocybin and ketamine on the gene expression of a relatively wide pool of both novel (nesfatin-1, PNX, SPX, NMU, NPS, 26RFa) and canonical (orexins, POMC, MCH) neuropeptides and their selected receptors in the rat hypothalamus. A common feature of all studied neuropeptides is their important role in the central regulation of food intake and energy homeostasis. The analysis of their hypothalamic expression seems to be important in the context of the potential use of psychedelics in the pharmacotherapy of eating disorders.

2. Materials and Methods

2.1 Animals

Adult male Wistar–Han rats (280–350 g; age: 2.0 to 2.5 month, Charles River, Göttingen, Germany) were were initially acclimatized and housed (5 per cage) in environmentally controlled rooms (ambient temperature 23 ± 1 °C, humidity 55 ± 10%, and 12:12 light:dark cycle). Rats were handled once daily before the beginning of the experiments; an enriched environment was not applied. The animals had free access to water and typical laboratory food (VRF 1, Special Diets Services, Witham, UK). All animal use procedures were conducted in strict accordance with European regulations for animal experimentation (EU Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes). The 2nd Local Institutional Animal Care and Use Committee (IACUC) in Kraków, Poland, approved the experimental protocols for Experimentation on Animals (permit numbers: 112/2021, app. 8 April 2021; 324/2021, app. 20 October 2020 and 79/2022, app. 10 March 2022).

2.2. Drugs and Reagents

Ketamine hydrochloride was purchased from Tocris/Bio-Techne (Warsaw, Poland) and psilocybin was synthesized at the Department of Medicinal Chemistry of the Maj Institute of Pharmacology using the method described by Shirota et al. (2003); both were dissolved in sterile water. All solutions were made fresh on the day of the experiment. The dose of ketamine (10 mg/kg) was based on a report by Popik et al. (2022), while doses of psilocybin (2 and 10 mg/kg) were based on work by Jefsen et al. (2019). Psilocybin was given subcutaneously while ketamine was given intraperitoneally in the volume of 2 mL/kg. The control group was treated with 0.9% NaCl solution (s.c. administration). Animals were injected with single doses.

2.3. Brain tissue collection and Real-Time-PCR reaction

Seven days after drug administration rats were sacrificed by decapitation, their brains were excised immediately and hypothalamus was microsurgically excised and frozen in dry ice prior to RNA isolation. Taking into account that psychedelics produce long-lasting epigenomic and transcriptomic alterations in the brain and these modifications lead to neuroplasticity (Vargas et al. 2023, de Vos et al. 2021) we decided to sacrifice animals a week after drug administration to correlate possible changes with other drugs' effects. 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 (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 of all studied neuropeptides and receptors as well as hybridization temperatures presented in the Table 1. The analysis of the obtained results was performed on the basis of the 2-$\Delta\Delta$Ct algorithm, where the internal control was the reference gene B2M.

2.4. Statistical analysis

Statistical analysis was performed using data analysis software system Statistica (TIBCO Software Inc. 2017, version 13). Mean differences between groups were analyzed using one-way ANOVA test followed by Dunnett’s post hoc test. The logarithmic transformation method was applied to conduct a one-factor analysis of variance for all genes. Results were presented as means ± SD ± 95% confidence intervals. Differences were considered statistically significant at $p < 0.05$ (Tab. 2.).

3. Results and Discussion

In the current study we analyzed neuropeptides mRNA expression in the rat hypothalamus after long-term exposure to psilocybin or ketamine using quantitative Real-Time PCR (Tab 2.). Our report is the first study to investigate changes in the gene expression of hypothalamic regulatory neuropeptides after psychedelic administration which may enhance the understanding of the possible molecular interplay between brain peptidergic signaling pathways and the psychomodulatory activity of psilocybin and ketamine. The effect of administering psilocybin at a higher dose (10 mg/kg) brought a statistically significant increase in the mRNA expression of the following neuropeptides: nucleobindin 2 (NUCB2), phoenixin (PNX) and receptors: GPR173, GPR103, NPSR, MC4R, MCHR (Fig. 1 and 2). In the case of NPS mRNA, the increase in expression occurred also with the use of a lower dose of psilocybin (2 mg/kg). It is worth emphasizing, the observed increase in gene expression of the examined neuropeptides was accompanied by a distinct up-regulation of 5-HT1A, 5-HT2A and 5-HT2B but not 5HT-2C mRNA levels (Fig. 3.). This may confirm some reports suggesting that 5-HT2A activation stimulates the synthesis and secretion of several neuropeptides such as CRF and oxytocin (Van der Kar et al. 2001) by hypothalamic neurons and also increases prolactin release from hypophyseal acidophilic cells (Bagdy et al. 1996). Moreover, it has been suggested that 5-HT2A-dependent signaling plays a role in the control of hypothalamic POMC neurons activity (Martin-Gronert et al. 2016). On the other hand, 5-HT2C receptors expressed on anorexigenic POMC neurons are considered the main regulator of food intake and energy homeostasis (Doslikova 2013), so the lack of effect of psilocybin on the 5-HT2C mRNA expression therefore suggests that the action of this psychedelic on hypothalamic peptidergic signaling is mediated by 5-HT1, 5-HT2A and 5-HT2B receptors (Fig. 3.). This corresponds with a report showing that hypothalamic responses to peripheral DOI administration are mediated by activation of 5-HT2A receptors in the paraventricular nucleus (Zhang et al. 2002). Interestingly, in the experiment performed, psilocybin and ketamine did not change POMC expression in the rat hypothalamus. Therefore, their potential impact on hypothalamic mechanisms of energy homeostasis is realized through regulatory circuits other than melanocortin pathway and may possibly be

related to newly identified, noncanonical hypothalamic neuropeptides. This information seems to be particularly interesting in the context of a recent study be Peck *et al*. (2023) reporting the possibility of using a single dose of psilocybin (25 mg) in the safe treatment of female anorexia nervosa (AN). The molecular mechanism of the aforementioned pharmacological action of psilocybin is not yet known but it likely modulates aminergic and glutamatergic signaling in various brain structures. There are suggestions that some disturbances in 5-HT2A-dependent serotonergic signaling, such as abnormal receptor binding (Goethals et al. 2007, Bailer et al. 2004, Audenaert et al. 2003) or polymorphism of 5-HT2A gene (Calati et al. 2011, Gorwood et al. 2002), may play a role in the pathogenesis of AN and other eating disorders (Chen et al. 2015). It can therefore be assumed that the observed clinical improvement after treatment with psilocybin is related to its agonistic effect on 5-HT2A in various areas of the brain, including the hypothalamus. Activation of 5-HT2A could, under these conditions, increase the expression of food-intake promoting neuropeptides. However, in the presented study, we revealed an increase in the expression of both orexigenic (PNX, MCHR GPR173, GPR 103) and anorexigenic (NUCB2, MC4R, NPS, NPSR) hypothalamic regulatory factor genes (Fig.1 and 2). In contrast, there was a significant decrease in the mRNA expression of NMU, a neuropeptide that strongly inhibits eating (Teranishi and Hanada 2021). Therefore, the mechanism of action of psilocybin at the level of the hypothalamus is ambiguous and appears to be somewhat more complex. It is also unclear to what extent changes in gene expression of the studied neuropeptides translate into the level of protein synthesis and function in the hypothalamic neurons that regulate energy homeostasis. Moreover, it is difficult to clearly estimate which of the examined multifunctional neuropeptides are crucial in the pathogenesis of AN. It should also be emphasized that research on the neurochemical background of AN in animal models, such as activity based anorexia, is far from perfect and translating the obtained results to humans should be carried out with great caution and awareness of the limitations. In the course of AN, there is a persistent, emotionally charged disturbance of body image self-perception, a phenomenon that is likely not to occur in animals. Effects of psilocybin and other psychedelics on bodily self awareness may involve some populations of insular neurons which are responsible for self-recognition and interoceptive awareness (Craig 2009). It is suggested that some functions of insula are disturbed in the course of eating disorders including anorexia nervosa (Bulik et al. 2022). The affective and addictive aspects of

AN pathophysiology are related to the activity of the brain limbic and amygdalar pathways (Lipsman et al. 2015, Chowdhury et al. 2003, Joos et al. 2011, Burkert et al. 2019), in which 5-HT2A may also play an important role. Their stimulation by psilocybin could theoretically result in the release of so far promising pharmacological effects. However, at present these considerations are speculative and must be supported by further basic research.

Unlike psilocybin, the effect of ketamine on neuropeptide gene expressions was much more limited. In the case of this psychedelic agent, only an increase of NUCB2, GPR173, POMC and 5-HT1A mRNA level was observed (Fig. 1-3). Glutamate signaling via NMDA receptors plays an important role in the activity of hypothalamic nuclei involved in the regulation of energy homeostasis, sleep and cardiovascular functions (Miracca et al. 2022, Busnardo et al. 2016, Doane et al. 2007, Lee and Stanley 2005). The effects of ketamine on the gene expression of the aforementioned neuropeptides in several hypothalamic neurons may therefore occur via blockade of NMDAR activity. Recently, there have been suggestions regarding the possible use of ketamine in the treatment of anorexia nervosa and depression-related eating disturbances (Mitchel et al, 2023, Keeler et al. 2023). A single injection of ketamine (dose 30 mg/kg) increased food intake, attenuated hyperactivity and reduced anxiety-like behavior in rats (Chen et al. 2018). On the other hand, ketamine induced anaesthesia was associated with a long-term reduction in daily food intake in rhesus macaques (Springer et al. 2007). Nevertheless, the results of the presented experiment do not yet allow the conclusion that the orexigenic effects of ketamine are related to its impact on noncanonical neuropeptide signaling in the hypothalamus.

There was a simultaneous stimulation of the anorexigenic POMC, NUCB2 (a precursor of potent food-intake inhibiting factor – nesfatin-1) and GPR173 receptor gene expressions. The activation of GPR173 is attributed to orexigenic effects of PNX in animals (Schalla et al. 2017). It can therefore be assumed that the pharmacological effects of ketamine in the treatment of anorexia nervosa are probably related to its effect on dysfunctional neurotransmission in some brain regions other than hypothalamus such as insula or limbic structures (Mitchel et al 2023, Frank et al. 2019, Craig 2009). Interestingly, alterations in the NR2 subunits of NMDAR may be related to genetic predisposition to anorexia nervosa (Koronyo-Hamaoui et al. 2007).

To date, almost nothing is known about possible effects of classical and atypical psychedelics on food intake and energy homeostasis in animal models. However,

several studies show the impact of these substances on other forms of rat behaviour, especially anxiety and stress related responses. For instance, a marked anxiolytic effect of psilocybin in the acute phase and 24 h post-exposure was shown in the open field (OF) test. Interestingly, the increased exploration of the central zone of the OF persisted until 24 h and was no longer accompanied by suppression of rats' locomotion (Wojtas et al. 2023). A reduced anxiety-like behaviour in elevated plus maze (EPM) test was also evidenced in rats seven days after exposure to single doses of psylocybin and ketamine (Hibicke et al. 2020). Another study reported that typical psychedelic 25B-NBOMe shows distinct hallucinogenic activity in body twitch response (WDS) test. Moreover some alterations in the OF, the novel object recognition (NOR) and the light/dark box (LDB) tests suggest that 25B-NBOMe modulates locomotion, affects short-term memory and may produce anxiogenic effects (Wojtas et al. 2021).

Some limitations of this preliminary pilot study should definitely be noted. The main limitation is the reduced sample size in RT-PCR assay, more animals per group would have increased the statistical power. Secondly, the neuropeptide and receptor protein levels were not measured and immunohistochemistry will therefore urgently be examined in our ongoing research. In summary, we have exposed only part of the possible neuromolecular changes occurring following psilocybin and ketamine administration and our initial conclusions remain cautious. It should also be taken into account that any effects measured could well be different in animals which have experienced physical or social stressors. As such the discussion can be rather speculative and a bit over-stated when inferring anything about clinical observations with these drugs, particularly as no data is presented to validate protein changes or other mechanisms/functional consequences of the psychedelics exposure. Whether neuropeptide gene expressions after psilocybin and ketamine administration are directly related to drug pharmacological action or is a secondary effect, has to be investigated in the future. However, our results suggest for the first time that selected psychedelics enhance the signaling of 5-HT2A receptors or inhibit the action of NMDA receptors affecting neuropeptide signaling and serotonin transmission in the rat hypothalamus, which may contribute to a better understanding of the alternative ways of their psychomodulatory and autonomic action.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship and publication of this article.

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Figure captions

Fig. 1. Quantitative PCR mRNA expression levels of NUCB2, SPX, SMIM20, GPR173, NMU, CRH, NPS and NPSR in the rat hypothalamus. Results were normalized to beta-2-microglobulin reference gene and shown as $2^{-\Delta\Delta Cq}$ levels compared to control group. The use of logarithmic transformation made it possible to perform a one-factor analysis of variance for all genes. Plots show means ± SD (box) ± 95% confidence interval (whiskers). Differences were considered statistically significant at $p < 0.05$ (relative to controls). Abbreviations: Ket, ketamine at dose 10mg/kg; Psil2 and Psil10, psilocybin at doses 2mg/kg and 10mg/kg respectively.

Fig. 2. Quantitative PCR mRNA expression levels of 26RFa (QRFP), GPR103, MCH, MCHR, POMC, MC4R, PPOX, OX1R in the rat hypothalamus. Results were normalized to beta-2-microglobulin reference gene and shown as $2^{-\Delta\Delta Cq}$ levels compared to control group. The use of logarithmic transformation made it possible to perform a one-factor analysis of variance for all genes. Plots show means ± SD (box) ± 95% confidence interval (whiskers).Differences were considered statistically significant at $p < 0.05$ (relative to controls). Abbreviations: Ket, ketamine at dose 10mg/kg; Psil2 and Psil10, psilocybin at doses 2mg/kg and 10mg/kg respectively.

Fig. 3. Quantitative PCR mRNA expression levels of selected serotonin receptors in the rat hypothalamus. Results were normalized to beta-2-microglobulin reference gene and shown as $2^{-\Delta\Delta Cq}$ levels compared to control group. The use of logarithmic transformation made it possible to perform a one-factor analysis of variance for all genes. Plots show means ± SD (box) ± 95% confidence interval (whiskers). Differences were considered statistically significant at $p < 0.05$ (relative to controls). Abbreviations: Ket, ketamine at dose 10mg/kg; Psil2 and Psil10, psilocybin at doses 2mg/kg and 10mg/kg respectively.

Fig. 4. A summary table highlighting the pattern of changes in neuropeptides and receptors mRNA expression in the rat hypothalamus after psilocybin and ketamine administration (left). Heatmap shows statistically significant changes in gene expression presented as a percentage of control (right). Abbreviations: increase, (↑); decrease, (↓); no changes, (n).

Tab. 1. PCR primer sequences and reaction parameters.

Tab 2. A summary of one-way ANOVA effect sizes for all studied genes.