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# The effects of novel psychoactive substance, 25B-NBOMe on the central nervous system in comparison to psilocybin.

Praca doktorska wykonana w Zakładzie Farmakologii
Instytutu Farmakologii im. Jerzego Maja Polskiej Akademii Nauk

**Promotor:**

**Prof. dr hab. Krystyna Gołembiowska**

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**To those I have lost.**

*“Neque amicorum quisquam mihi gratificatus est,*
*neque inimicorum injuriam intulit, cui non par pari respondi”*
***-Lucius Cornelius Sulla Felix-***

*Badania opisane w niniejszej pracy doktorskiej zostały zrealizowanych w ramach funduszy pochodzących z :*

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# Streszczenie

Po trwającej ponad pół wieku ciszy, badania nad substancjami z grupy psychodelików przeżywają swoisty renesans. Przełom zapoczątkowany przez badania nad ketaminą sprawił, że kolejne substancje są badane pod kątem użycia ich w farmakoterapii zaburzeń afektywnych, lękowych, czy uzależnienień, tam gdzie "klasyczne" leki wykazują się niewielką skutecznością

Celem niniejszej pracy doktorskiej było scharakteryzowanie wpływu, jaki na ośrodkowy układ nerwowy posiada nowa substancja psychoaktywna „25B-NBOME", wysoce selektywny agonista receptora 5-HT2A, zarówno po pojedynczej, jak i wielokrotnej ekspozycji.. Następnie, w celu porównania jej do związku o dużo szerszym profilu receptorowym, scharakteryzowano wpływ psylocybiny na OUN, a ponadto jako związku referencyjnego użyto prototypowego szybko działającego leku przeciwdepresyjnego, tj. ketaminy. W przeprowadzonych badaniach wykorzystano mikrodializę u swobodnie poruszających się zwierząt, w celu określenia poziomu neuroprzekaźników, testy behawioralne określające wpływ na aktywność lokomotoryczną, lęk oraz nastrój a także metody histologiczne oraz molekularne do oceny potencjalnych uszkodzeń mózgu oraz ekspresji wybranych genów.

Uzyskane wyniki wskazują na znaczący wpływ 25B-NBOMe na neuroprzekaźnictwo glutaminianergiczne, dopaminergiczne, serotoninergiczne oraz cholinergiczne w korze, prążkowiu oraz jądrze półleżącym przegrody. Wzrosty w zewnątrzkomórkowych poziomach badanych neuroprzekaźników nie wykazały dawkozależności, a krzywa odpowiedzi na dawkę posiadała kształt odwróconej litery „U". Wynika to najprawdopodobniej z aktywacji receptora 5-HT2A, a następnie receptora 5-HT2C, wraz ze wzrostem stężenia substancji w osoczu. Efekt ten był również zaobserwowany w teście otrzepywania się (WDS), mierzącym halucynogenny potencjał badanej substancji, a także w pomiarze aktywności lokomotorycznej w teście wolnego pola.

Kolejnym etapem pracy była ocena wpływu chronicznego (1x dziennie/7 dni) podania 25B-NBOMe na ośrodkowy układ nerwowy. Wykazano gwałtowny wzrost tolerancji na działanie substancji począwszy od drugiego dnia podań chronicznych, mierzony w WDS, a także znaczące osłabienie wpływu 25B-NBOMe na zewnątrzkomórkowe poziomy badanych neuroprzekaźników w korze czołowej oraz prążkowiu szczura, po siedmiokrotnym podaniu. Co interesujące, 25B-NBOMe wciąż silnie wpływało na poziomy monoamin w jądrze

półleżącym przegrody, co może sugerować potencjał uzależniający. Ponadto, siedmiokrotna ekspozycja na 25B-NBOMe powodowała genotoksyczne uszkodzenie kory czołowej oraz hipokampa, oraz aktywację mikrogleju.

W następnej części badań oceniono wpływ jednokrotnego podania psylocybiny na neuroprzekaźnictwo dopaminergiczne, serotoninergiczne, glutaminianergiczne oraz GABAergiczne w korze czołowej szczura, porównując ten efekt do związku referencyjnego-ketaminy. Psylocybina w największym stopniu oddziaływała na zewnątrzkomórkowe poziomy badanych aminokwasów, natomiast efekt ketaminy był największy w przypadku monoamin. Ponadto potwierdzono postulowany efekt psylocybiny na filtrowanie wzgórkowo-korowe, gdyż psylocybina dawkozależnie zwiększała uwalnianie GABA w jądrze siateczkowatym wzgórza. Nie odnotowano wpływu badanych substancji na zachowanie szczura w teście wymuszonego pływania w 24h od podania, jednak test ten może nie być adekwatny do oceny przeciwdepresyjnego działania szybko działających leków przeciwdepresyjnych.

W ostatniej części badań oceniono wpływ psylocybiny oraz ketaminy na neuroprzekaźnictwo w strukturach układu limbicznego. Wykazano znaczący wpływ badanych substancji na zewnątrzkomórkowe poziomy badanych neuroprzekaźników we w jądrze półleżącym przegrody oraz hipokampie, lecz brak efektu w jądrze migdałowatym. Zaobserwowano także przeciwlękowy efekt psylocybiny, zarówno w godzinę oraz po 24 godzinach od podania leku, co sugeruje trwały potencjał przeciwlękowy. Efekt ten może być wynikiem zaobserwowanego nasilenia transmisji GABAergicznej.

Podsumowując, uzyskane wyniki sugerują, że selektywny agonista receptora 5-HT2A, 25B-NBOMe, wywiera istotny wpływ na neuroprzekaźnictwo oraz zachowanie zwierząt, powodując także oksydacyjne uszkodzenie DNA. Efekty wywołane przez psylocybinę są słabsze, sugerując szersze okno terapeutyczne tej substancji. Wynika to najprawdopodobniej z Polireceptorowego profilu tego związku i prawdopodobnej modulującej roli receptora 5-HT1A.

# Abstract

After a half-century-long hiatus, studies concerning psychedelic drugs are relieving their renaissance. The breakthrough initiated with ketamine allowed other substances to be tested as a therapy for affective, anxiety, and addictive disorders, areas where classical treatment shows little effectiveness.

This dissertation aimed to characterize the effect of selective 5-HT2A agonist, 25B-NBOMe, on the central nervous system after acute and repeated administration. The next step was to compare it with the effects of a more promiscuous drug, psilocybin, with the use of a prototypical fast-acting antidepressant drug, ketamine, as a reference substance. To accomplish these goals, microdialysis in freely moving animals was performed to assess the extracellular levels of selected neurotransmitters; a battery of behavioral tests to evaluate locomotor activity, anxiety levels, and behavioral despair; molecular and histological techniques to investigate potential damage to the brain tissue and expression of chosen genes.

The gathered data shows a significant effect of 25B-NBOMe on glutamatergic, dopaminergic, serotonergic, and cholinergic neurotransmission in the frontal cortex, striatum, and nucleus accumbens. The increases in extracellular levels of studied neurotransmitters were not dose-dependent and exhibited a hormetic response. This is most likely a result of the stimulation of the 5-HT2A receptor and subsequent activation of the 5-HT2C receptor as the plasma levels of the drug increase. This effect was also observed in the wet dog shake (WDS) test, used to assess the hallucinogenic potential of the investigated compound, and also in the locomotor activity measured in the open field test.

The next step was to evaluate the effects of chronic administration of 25B-NBOMe on the CNS. A rapid growth of tolerance, starting from the second day of treatment, was observed in the WDS test. The increases in extracellular levels of neurotransmitters were nearly all attenuated by the seventh day of treatment. What is interesting is that the increased levels of monoamines were still observed in the nucleus accumbens, suggesting the addictive properties of 25B-NBOMe. What is more, the chronic treatment with 25B-NBOMe leads to genotoxicity in the cortex and hippocampus and activation of microglia.

The next study investigated the effects of acute treatment with psilocybin on the dopaminergic, serotonergic, glutamatergic, and GABAergic transmission in the rat frontal cortex in comparison to a reference drug – ketamine. Psilocybin affected mainly the investigated amino-acidic neurotransmitters, while ketamine exerted a more robust effect on the monoamines. What is more, we have proven the hypothesized effect of psilocybin on cortico-thalamic gating, as psilocybin dose-dependently increased the release of GABA in the reticular nucleus of the thalamus. No effects of studied drugs were observed in the forced swim test 24h after the administration, but this assay may not be suitable for testing rapid-acting antidepressant drugs.

The last study investigated the influence of psilocybin and ketamine on the limbic neurotransmission. Significant changes were observed in extracellular levels of neurotransmitters in the nucleus accumbens and hippocampus but not in the amygdala. Psilocybin exhibited anxiolytic properties both 1 and 24h after its administration, which suggests a lasting anxiolytic effect. This may be a result of the observed intensification of GABAergic neurotransmission.

In summary, the gathered data indicates that selective 5-HT2A agonist 25B-NBOMe exerts a significant effect on rats' neurotransmission and behavior while also inducing oxidative DNA damage. The effects induced by psilocybin are more subtle, suggesting a broader therapeutic index of this drug. This is most likely due to its wider receptor profile and possible modulation via the 5-HT1A receptor.

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# Spis artykułów stanowiących rozprawę doktorską

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# Abbreviations

**25B-NBOMe** 2-(4-bromo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine

**25C-NBOMe** 2-(4-Chloro-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethan-1-amine

**25D-NBOMe** 2-(2,5-dimethoxy-4-methylphenyl)-N-(2-methoxybenzyl)ethanamine

**25H-NBOMe** 2-(2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine

**25I-NBOMe** 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine

**25N-NBOMe** 2-(2,5-dimethoxy-4-nitrophenyl)-N-[(2-methoxyphenyl)methyl]ethan-1-amine

**2C-B** 2-(4-bromo-2,5-dimethoxyphenyl)ethan-1-amine

**2C-H** 2-(2,5-dimethoxyphenyl)ethan-1-amine

**2C-I** 2-(4-iodo-2,5-dimethoxyphenyl)ethan-1-amine

**5-HIAA** 5-hydroxyindoleacetic acid

**5-HT** serotonin

**5-HT1A** serotonin receptor subtype 1A

**5-HT1B** serotonin receptor subtype 1B

**5-HT1D** serotonin receptor subtype 1D

**5-HT1E** serotonin receptor subtype 1E

**5-HT2** serotonin receptor subfamily 2

**5-HT2A** serotonin receptor subtype 2A

**5-HT2B** serotonin receptor subtype 2B

**5-HT2C** serotonin receptor subtype 2C

**5-HT5A** serotonin receptor subtype 5A

**5-HT6** serotonin receptor subtype 6

**5-HT7** serotonin receptor subtype 7

**5-OH-DMT** bufotenin

**ASCs** Altered States of Consciousness scale

**CPP** conditioned place preference

**D1** dopamine receptor subfamily 1

**D2** dopamine receptor subfamily 2

**DA** dopamine

**DAT** dopamine transporter

**DMT** N,N-dimethyltryptamine

**DOB** 2,5-dimethoxy-4-bromoamphetamine

**DOC** 2,5-dimethoxy-4-chloroamphetamine

**DOI** 2,5-dimethoxy-4-iodoamphetamine

**DOM** 2,5-dimethoxy-4-methylamphetamine

**FCX** frontal cortex

**FST** forced swim test

**GABA** γ-Aminobutyric acid

**GLU** glutamate

**GPCR** G-protein coupled receptor

**HP** hippocampus

**HTR** head-twitch response

**LD50** median lethal dose

**LSD** lysergic acid diethylamide

**MAO** monoamine oxidase

**MDMA** 3,4-Methylenedioxymethamphetamine

**mTORC1** mammalian target of rapamycin complex 1

# 1.Introduction

## 1.1 History of psychedelics

Along with ethanol and cannabis, serotonergic hallucinogens might be the oldest psychoactive substances used by humanity. These substances, known for inducing profound changes in perception, mood, and cognitive processes, are often associated with therapeutic, religious, and recreational applications (Schultes et al., 1998). It is due to those properties that Humphrey Osmond coined their widely-used name "psychedelic", originating from the Greek words *psukhḗ,* "mind," and dêlos "to reveal".

The modern era of psychedelic research began with the XX century, with discovery of mescaline, the active ingredient of hallucinogenic Peyote cacti (Nichols and Walter, 2021). The next chapter opened in 1938 with synthesis and 5 years later the discovery of psychoactive properties of lysergic acid diethylamide (LSD) (Hofman, 1979). Due to its extreme potency LSD quickly became the most intensely researched psychedelic compound, with more than 1000 published articles by the end of the 1960s (Nichols, 2016). It was studied as a possible aid in psychotherapy, or otherwise explored as potential treatment for substance abuse disorders, anxiety and mood disorders (Liechti, 2017). Unfortunately, alongside medical use, recreational use of these substances swept across the globe, resulting in the passage of the “Controlled Substances Act “ in 1970 and psychedelics being classified as “drugs with no currently accepted medical use and a high potential for abuse”. These circumstances made it difficult to continue research concerning psychedelic drugs and nearly all studies (with only a few exceptions) came to an abrupt end, followed by several decades of hiatus in psychedelic research.

## 1.2 Classification of psychedelics

All psychedelic compounds originate from the same structure, which consists of an aromatic group that is connected by a two-carbon chain with an amine group (Kwan et al., 2022). Basing on the type of the aromatic group (either a phenyl ring, or C3-substituted indole), they can be divided by their structure into two main categories: phenylalkylamines, e.g., mescaline or DOI (2,5-dimethoxy-4-iodoamphetamine) (Kwan et al., 2022) and indoleamines, e.g., DMT (N,N-dimethyltryptamine) or LSD. Both of them bear a resemblance to endogenous compounds – either phenethylamine or serotonin. While the former bind mainly to the 5-HT2 receptor family (Pierce and Peroutka, 1989; Titeler et al., 1988), the latter demonstrate affinity to several types of receptors and nearly all 5-HT receptors (Halberstadt and Geyer, 2011).

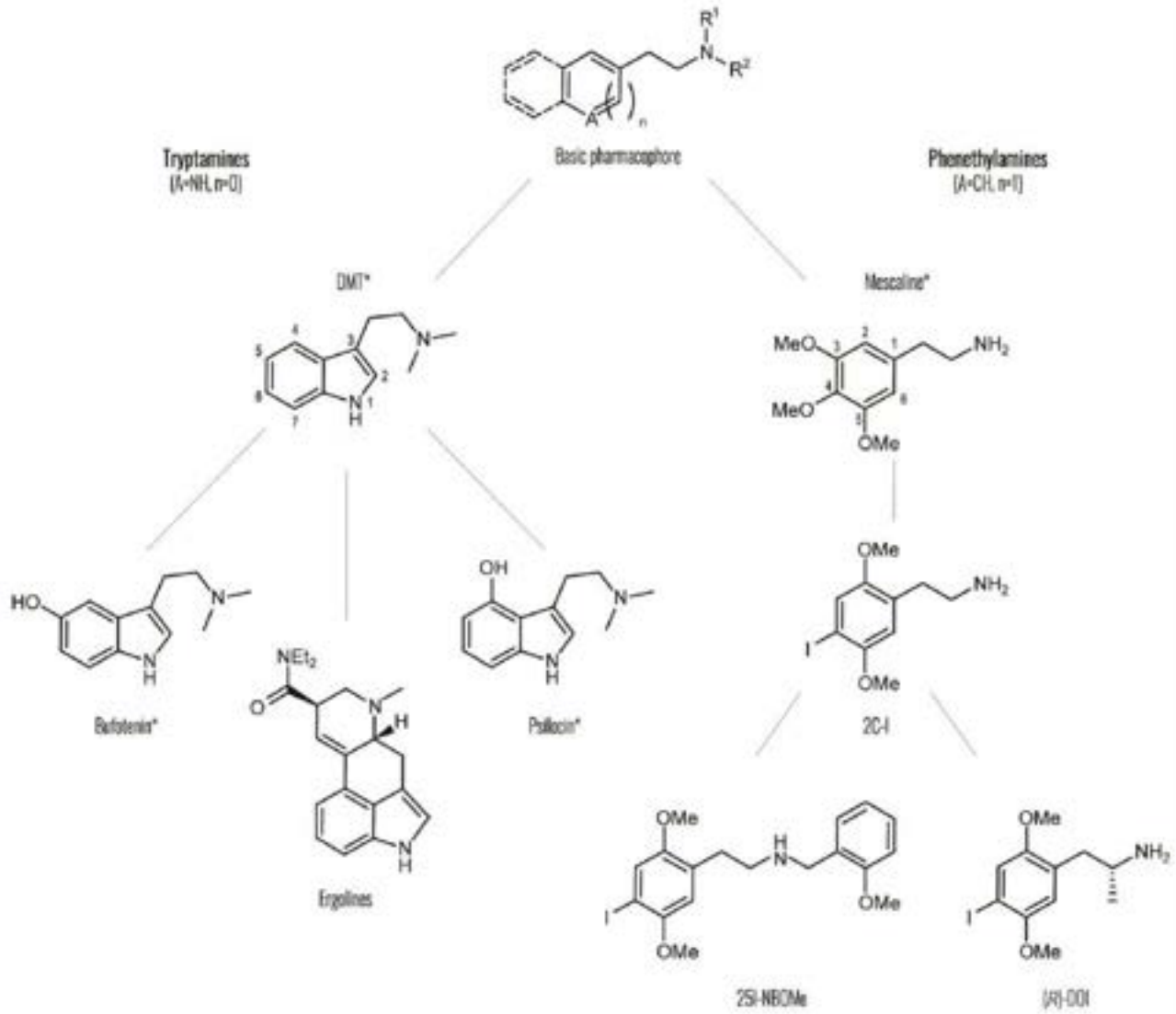


**Fig.1.** Structure of selected psychedelic drugs. Based on Kwan et al. (2022).

*Phenylalkylamines*

Phenylalkylamines can be further divided into two classes: phenethylamines and phenylisopropylamines (often called "hallucinogenic amphetamines"). While the difference between them lies only in the presence (or absence) of the methyl group attached to the atom of the α-carbon (Fig. 1), it may profoundly change the binding profile of the molecule, e.g., in the case of mescaline and 3,4,5-trimethoxyamphetamine (TMA), which differ from each other only by the presence of the aforementioned methyl group, the affinity to the 5-HT2A changes from micromolar to nanomolar (Rickli et al., 2016; Titeler et al., 1988).

The phenylalkylamines bind to the 5-HT2 receptor family, most potently to the 5-HT2A and 5-HT2C receptors. The phenethylamines act primarily as partial or weak agonists (although

there are some exceptions, which will be discussed later), while the phenylisopropylamines act as partial or full agonists (Moya et al., 2007; Nichols, 1981).

The only hallucinogenic phenethylamine that exists in nature is mescaline (3,4,5-trimethoxyphenethylamine), the active compound of *Peyote* and *Echinopsis* cacti (Nichols, 2012), while the biggest group being the "2C" series, explored by Shulgin and Shulgin (1991). In low doses (<10 mg in humans), they act primarily as stimulants, though in higher doses they exert hallucinogenic effects (Hill and Thomas, 2011).

Hallucinogenic amphetamines cannot be found in nature and have to be obtained through synthesis. Their best-known representatives are the 4-substituted-2,5-dimethoxyamphetamines, with the most important 2,5-dimethoxy-4-iodoamphetamine (DOI); 2,5-dimethoxy-4-methylamphetamine (DOM); 2,5-dimethoxy-4-bromoamphetamine (DOB) and (DOC) (Shulgin and Shulgin, 1991). Due to their high affinity (nanomolar or even subnanomolar) to 5-HT2A receptor, they have been used as radioligands to map the distribution of the aforementioned receptor in the brain (Glennon et al., 1984; Titeler et al., 1988).

*Indoleamines*

Quite like phenethylamines, indoleamines can also be further differentiated into two subclasses: simple tryptamines like DMT or psilocin and ergolines (lysergamides) which are tryptamine derivatives with more rigid conformation; they primarily consist of LSD and its derivatives (Fig. 1).

Tryptamines are quite often found in nature. DMT can be found in *Ayahuasca*¸ a brew made from the leaves of *Psychotria viridis*, bufotenine (5-OH-DMT) is secreted by glands of *Bufo alvarius*, an American species of toad, and the most common ones are psilocybin and its primary active metabolite psilocin, compounds found in *Psilocybe* fungi which can be found all around the globe (Araújo et al., 2015; Schultes et al., 1998). Due to the possible sites of substitution in the aromatic ring and on the nitrogen and carbon atoms of the side chain, a vast number of synthetic derivatives of tryptamine has been reported up to this day (Gibbons, 2012; Nichols, 2012; Fantegrossi et al., 2008). Users administer tryptamines via several routes: orally, by insufflation, inhalation, and by intravenous or intramuscular injections (Arunotayanun and Gibbons, 2012; Corkery et al., 2012; Hill & Thomas, 2011). Receptor affinities vary within the group, but generally, tryptamines show affinity to a large number of 5-HT receptors. This includes 5-HT1A with the affinity to this receptor being sometimes almost as high as for 5-HT2A, 5-HT1B, 5-HT1D, 5-HT1E, 5-HT2A, 5-HT2B, 5-HT2C, 5-HT5A, 5-HT6 and 5-HT7 (Halberstadt and Geyer, 2011). In high concentrations, they also bind to α-adrenergic receptors,

dopaminergic receptors, and serotonin transporter (SERT) (Halberstadt and Geyer, 2011). Nevertheless, these compounds induce their hallucinogenic effects via activation of the 5-HT2A receptor.

Finally, ergolines are derivatives of alkaloids secreted by the *ergot* fungi. Due to their tetracyclic structure they happen to be more rigid than the usual "simple" tryptamines (Halberstadt et al., 2018). Their history is inseparably bound with their most famous representative - LSD. It was due to the discovery of its molecule by A. Hofman in the 1943 (Nichols, 2004). It is, up to this day, one of the most potent psychedelics (Nichols, 2004, 2012). Similarly to tryptamines, ergolines exhibit high affinity to 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, 5-HT2A, 5-HT2B, 5-HT2C, 5-HT5A, 5-HT5B, 5-HT6 and 5-HT7 receptors (Ray, 2010). What is more unique to them, is that they also bind to the dopaminergic D1 and D2 receptors and adrenergic α1 and α2 receptors (Giacomelli et al., 1998; Watts et al., 1995; Ray, 2010).

### 1.3 The importance of 5-HT2A receptor

*5-HT2A receptor as a primary target for psychedelics*

Hallucinogen use leads to rapid development of tolerance to their effects (Abramson et al., 1956; Angrist et al., 1974). Moreover, indoleamines and phenylalkylamines exhibit a cross-tolerance phenomenon, which further suggests that they have a common mechanism of action (Wolbach et al., 1962) Holistic data gathered from many studies indicate that hallucinogens exert their psychoactive effects by acting as agonists for cortical 5-HT2A receptor (Aghajanian and Marek, 1996; Glennon et al., 1984; Sipes and Geyer, 1995; Wing et al., 1990). The 5-HT2AR belongs to the G-protein coupled receptors (GPCRs) family. It is coupled with the Gq/11 protein, and its activation leads to phosphoinositide hydrolysis resulting in the formation of diacylglycerol and inositol triphosphate, leading to the mobilization of intracellular calcium and subsequent membrane depolarization (Nichols, 2016). What is more, the intensity of the psychedelic experience in humans is correlated with the occupancy of the 5-HT2A receptor, mainly in the prefrontal cortex (PFC) (Madsen et al., 2019). This activation of 5-HT2ARs in the PFC launches a downstream cascade of changes in connectivity and alterations in blood flow across multiple regions of the brain, e.g. cingulate cortex, inferior parietal lobule, lateral temporal cortex, hippocampus (HP), thalamus, amygdala and claustrum (Vollenweider et al., 2022; Barrett et al. 2020; Kraehenmann et al. 2015; Preller et al. 2018). Those structures are

involved in cognition, emotional processing, sensory perception, or even self-recognition and theory of mind processes (Carhart-Harris et al., 2014; Nichols, 2016).

What is interesting is that even though psychedelics activate 5-HT2A receptors on glutamatergic pyramidal neurons in the brain, this typically doesn't induce depolarization or generation of action potentials. Instead, there's an increase in glutamate release from depolarized neurons, resulting in recurrent activity (Aghajanian and Marek, 2000). Notably, Beique et al. (2007) identified a subset of large neurons in the cortex's deep layers that exhibited significant sensitivity to 5-HT. These neurons were exhibiting substantial membrane depolarizations, leading to spiking activity. Basing on this findings, Martin and Nichols (2016) isolated from rat brains a specific subset of psychedelic-activated neurons. They identified that psychedelics directly stimulate only a minor proportion of 5-HT2A receptor expressing excitatory neurons, especially in crucial brain areas such as the PFC and claustrum. Notably, the psychedelic-responsive neurons exhibit elevated gene expression for the 5-HT2A receptor, which likely underlies their heightened sensitivity to psychedelics compared to other neurons. The authors postulate that this distinct neuron group acts as a "trigger population." The activation of these neurons might instigate cellular processes, leading to recurrent activity, cortical network destabilization, and the array of perceptual and cognitive effects characteristic of psychedelic experiences (Martin and Nichols 2016).

*Impact of 5-HT2A receptor activation on behavior*

As the 5-HT2A receptor is the key player in inducing the psychedelic experience in humans, it’s activation is considered as a proxy of hallucinogenic effect in animal models (Nichols, 2016). The head twitch response/wet dog shakes (HTR/WDS) test is based on this mechanism, exhibiting significant construct validity. The assay quantifies rapid, rhythmic head movements observed in rodents post-administration of psychedelic 5-HT2A receptor agonists (Halberstadt and Geyer, 2013). While some false positives have been identified, such as fenfluramine, p-chloroamphetamine, and 5-hydroxytryptophan, the test predominantly exhibits specificity for 5-HT2A receptor agonists (Halberstadt and Geyer, 2018). What is more, the HTR assay seems to be highly sensitive to 5-HT2A receptor agonists known to produce psychedelic effects in humans, proving its predictive validity. This is evidenced by the fact that the non-psychedelic 5-HT2A receptor agonist, namely lisuride, does not evoke the head twitch phenomenon (Halberstadt and Geyer, 2018). Unfortunately, the face validity of the assay is poor, as humans do not exhibit head-twitching behavior after being administered with a psychedelic drug.

On the other hand, the drug discrimination test exhibits higher face validity, although it can produce some false positives; specifically, lisuride can substitute fully for LSD (Appel et al., 2004). The procedure assesses the "subjective" effects under the influence of a drug. The animals are trained to press a lever when given the reference compound, for example, a well-studied psychedelic drug, and another lever when being given the vehicle. During the evaluation phase, the investigated compound is administered, and the interactions with both levers are quantified. The more responses associated with the training-drug lever, the more investigated drug substitutes with the training compound, meaning their subjective effects are similar (Nichols, 2016). What is more, the coadministration of other drugs, for example, specific antagonists, can be used to examine the specific effects of non-selective compounds (Halberstadt, 2015).

### 1.4 NBOMes

*Origin of NBOMe compounds*

As mentioned before, modifications of the side chain usually do not lead to any increase in the potency of the phenethylamine. There is however, an exception, N-benzylation (Nichols, 2012) (Fig. 2). The presence of the N-benzyl group drastically enhances the binding properties to the 5-HT2A receptor (Braden et al., 2006).

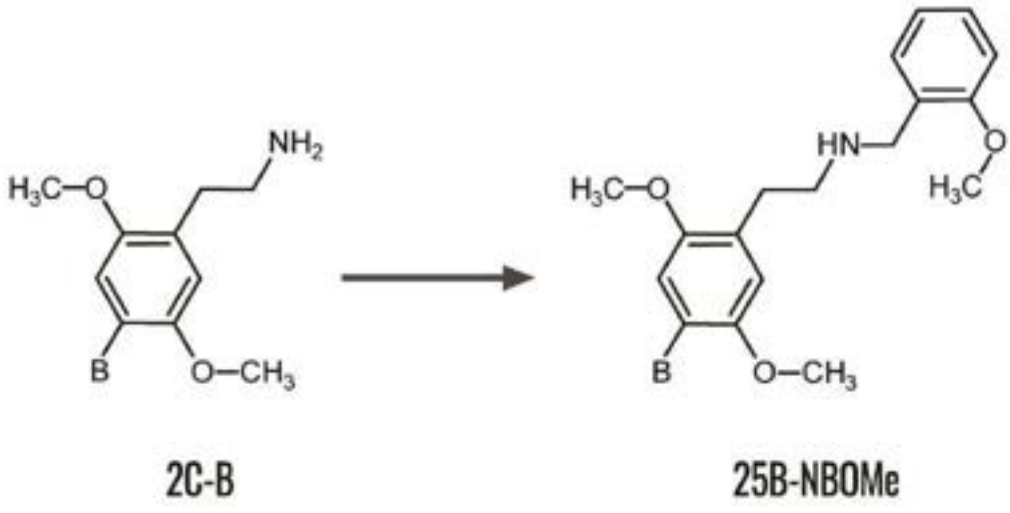


**Fig.2.** The process of N-benzylation.

The first compound that went through the N-benzylation was the 2C-I. In 2003, it was modified into 25I-NBOMe at the Free University of Berlin (Heim, 2003). Other members of the 2C class went through similar modification, resulting in a synthesis of 25B-NBOMe and many others (Halberstadt and Geyer, 2014). N-benzyl substitution is more efficient in compounds with lower 5-HT2A receptor affinity, e.g., modifying 2C-H into 25H-NBOMe increases its Ki from 227 nM to 0.067, while of 2C-I changes its Ki from 3.5 nM into 0.6 nM (Rickli et al., 2015). Besides the 5-HT2 receptor family, the NBOMes possess a relatively high affinity for the α-adrenergic receptors (Rickli et al., 2015)(Tab1.).

| receptor / Ki (nM) | 5-HT1A | 5-HT2A | 5-HT2C | α1A | α2A | SERT |
|---|---|---|---|---|---|---|
| 2C-I | 180 | 3.5 | 40 | 5100 | 1100 | 4900 |
| 2C-B | 240 | 8.6 | 47 | 8200 | 230 | 9700 |
| 25I-NBOMe | 1800 | 0.6 | 4,6 | 370 | 320 | 1000 |
| 25B-NBOMe | 3600 | 0.5 | 6,2 | 430 | 430 | 0840 |

**Tab.1.** Affinity of selected „2C" and „NBOMe" compounds, based on Rickli et al. (2015).

*Effects of NBOMes observed in animal studies*

A study conducted on C57BL/6J mice demonstrated that 25I-NBOMe, administered at doses of 0.1, 0.3, and 1 mg/kg was 14 times more potent that its' "parent" compound, 2C-I in inducing HTRs, and that this effect was fully reversible by volinserin, a selective 5-HT2A receptor antagonist (Halberstadt and Geyer, 2014). Escalating doses of 25B-NBOMe (0.1-10 mg/kg), caused a hormetic response in the number of head-twitch episodes in Wistar Han rats (Wojtas et al., 2021), an effect replicated in C57BL/6J mice, and reversed by 5-HT2A receptor antagonist, ketanserin (Custodio et al., 2020).

The psychoactive properties of NBOMe compounds have also been investigated in the drug discrimination test. When using DOM as a training drug, 25B and 25C-NBOMe fully substituted (≥80%), while 25I-NBOMe partially substituted (74%) for the DOM. While tested in 3,4-methylenedioxymethamphetamine (MDMA)-trained animals, only 25B-NBOMe demonstrated complete substitution, a phenomenon not unlike to its "parent" compound, 2C-B, which is often used as a replacement for MDMA (Papaseit et al., 2018).

A study by Gatch et al.(2017) showed that 25I-, 25B-, and 25C-NBOMe dose-dependently reduced animal locomotor activity. When administered subcutaneously, 25I-

NBOMe (0.03-3 mg/kg) produced a bell-shaped effect on C57BL/6J mice activity (Halberstadt, 2017). In agreement with this finding, the researchers have also shown that low (0.001-1 mg/kg) and moderate (0.1-1 mg/kg) doses of 25I-NBOMe increase mobility, while a high dose (10 mg/kg) causes a sharp decline in the spontaneous motor activity of male ICR mice (Tirri et al., 2022). Similarly, 25B-NBOMe inhibited the motor activity of Wistar Han rats measured in an open field (0.3–3 mg/kg) (Wojtas et al., 2021). This phenomenon is similar to another 5-HT2A receptor agonist, DOI, which modifies locomotor activity via a 5-HT2A/5HT2C dependent mechanism. Low and moderate doses activate primarily the 5-HT2A receptors located on pyramidal neurons, leading to neuronal excitation and stimulation of locomotor behavior. As the plasma levels of the drug increase, it leads to the activation of the 5-HT2C receptors located on GABAergic interneurons, which results in suppression of locomotor activity (Halberstadt et al., 2009).

Some studies suggest the potential addictive properties of the NBOMes. 25I-NBOMe (0.3mg/kg) induced place preference in the conditioned place preference (CPP) test and increased vocalization frequency, in a similar way to methamphetamine, in male C57BL/6J mice. On the other hand, those effects were not replicated in self-administration experiments in Sprague Dawley rats (0.03 mg/kg/infusion), this study reported weak addictive properties compared to place preference studies (Jeon et al., 2019). 25B-NBOMe at a dose of 1 mg/kg induced place preference in the CPP test in male C57BL/6J mice. Moreover, it produced statistically significant, but weaker than those reported for methamphetamine responses in the self-administration procedure at doses of 0.03-0.3 mg/kg/infusion (Custodio et al., 2020). 25N-NBOMe increased place preference in male C57BL/6J mice at a dose of 3 mg/kg and also produced self-administration in Sprague Dawley rats (0.1 mg/kg/infusion) (Seo et al., 2019). 25D-NBOMe (1 mg/kg) induced conditioned place preference in mice and was readily self-administered (0.03 mg/kg/infusion) in rats (Lee, et al. 2023).

The mechanism behind those behavioral symptoms seems to originate from a dopaminergic mechanism. 25B-NBOMe (Wojtas et al., 2021), 25I-NBOMe, (Herian et al., 2021), 25N-NBOMe (Seo et al., 2019), and 25D-NBOMe (Lee et al. 2023) increased extracellular dopamine (DA) levels in the striatum (STR) and the nucleus accumbens (Nacc), structures involved in the reward system. Furthermore, the administration of NBOMe compounds affected the protein levels of DA receptors, a hallmark of abuse potential. 25B-NBOMe elevated D1 receptor expression and decreased D2 receptor expression in mouse Nacc. 25N-NBOMe reduced the expression of D2 receptors both in Nacc and dorsal STR. It

also decreased the expression of DA transporter (DAT) in Nacc, while increasing its phosphorylation in Nacc and dorsal STR. Furthermore, the drug significantly reduced the expression of tyrosine hydroxylase (TH) in the nucleus accumbens. 25D-NBOMe increased the expression of dopamine receptor D1 while decreasing the expression of D2 receptor, and downregulated DAT (Lee et al., 2023). Taken together, these changes in dopaminergic activity, while not as potent as those induced by methamphetamine, may originate from a similar mechanism. The excessive DA levels in Nacc would stimulate the D2 autoreceptors, which are involved in regulation of DA release. Overstimulation of D2 receptors would then lead to their downregulation and reduction in activity of DAT and TH (Ford, 2014).

*Effects of NBOMes observed in humans*

The NBOMes became available to drug users when they first appeared on the drug market in 2010 (Poklis et al., 2015; Zuba et al., 2013). Similarly to LSD, they are most often sold on blotter paper; sometimes, they are even sold as LSD. They are administered either nasally or orally (either by swallowing or sublingually) in small, sub-milligram doses (Poklis et al., 2015). The duration of action varies for each route of administration (Halberstadt and Geyer, 2014). The effects of ingestion are usually an outcome of the activation of serotoninergic and adrenergic pathways and may include severe visual and auditory hallucinations, agitation, aggressiveness, long-lasting seizures, tachycardia, sweating, hypertension and hyperthermia and psychotic/paranoid behavior (Al-Imam and Abdul Majeed, 2017; Gee et al., 2016; Hill and Thomas, 2011; Nikolaou et al., 2015).

Since their initial appearance in 2010 NBOMes gave rise to multiple cases of significant intoxication, and a large portion of them resulted in users' death (Baumann et al., 2017; Shanks et al., 2015; Walterscheid et al., 2014). While mild effects of NBOMes ingestion consist of tachycardia, hallucinations, panic attacks, and other symptoms parallel to the use of other hallucinogens and can be treated relatively easily with benzodiazepines and antipsychotics, intoxication can result in more severe symptoms. In case of heavy poisoning seizures, renal failure, rhabdomyolysis, hyperthermia, and metabolic acidosis may occur (Andreasen et al., 2015; Kueppers & Cooke, 2015; Rose et al., 2013; Shanks et al., 2015; Walterscheid et al., 2014).

**1.5 Psilocybin**

Psilocybin-containing mushrooms belong to a diverse group within the *Basidiomycota* fungi, comprising over 200 species across genera, such *as Psilocybe, Gymnopilus, Panaeolus*, with the most prevalent genus being *Psilocybe*, with about 144 species (Guzmán, 2005).

Psychedelic mushrooms were consumed and revered as religious objects since prehistoric times by many cultures, with evidence of such practices being found in Europe, Africa and, most notably, Mesoamerica (Wasson, 1957; Carod-Artal, 2015; Johnson et al., 2018).

The modern history of psilocybin starts with Albert Hofmann, the chemist standing behind the discovery of the psychedelic properties of LSD. In 1957, he received a sample of *Psilocybe Mexicana* mushrooms from which he extracted two crystalline compounds. Through self-experimentation, he confirmed their psychoactive effects, and by 1958, he had identified these compounds as psilocybin and psilocin, which he synthesized in 1958 (Hofmann et al., 1958).

*Pharmacology of psilocybin*

After oral intake, psilocybin is quickly dephosphorylated in the stomach or by alkaline phosphatase in the intestine, kidney, and potentially blood, forming psilocin (fig. 3), which easily penetrates the blood-brain barrier (Horita and Weber, 1961a; Horita and Weber, 1961b; Hasler et al., 1997). Studies in rodents confirm almost complete conversion of psilocybin to psilocin before entering the bloodstream (Eivindvik et al., 1989). Both psilocybin and psilocin have similar psychotropic effects on humans at equimolar concentrations. Thus, psilocybin acts as a prodrug, with psilocin being the active agent responsible for its in *vivo* effects (Passie et al., 2002). Moreover, inhibition of alkaline phosphatase completely suppresses the psychoactive effects of psilocybin administration (Horita, 1963), further supporting the hypothesis that psilocin is the primary psychoactive agent in hallucinogenic mushrooms (Tyls et al., 2014).

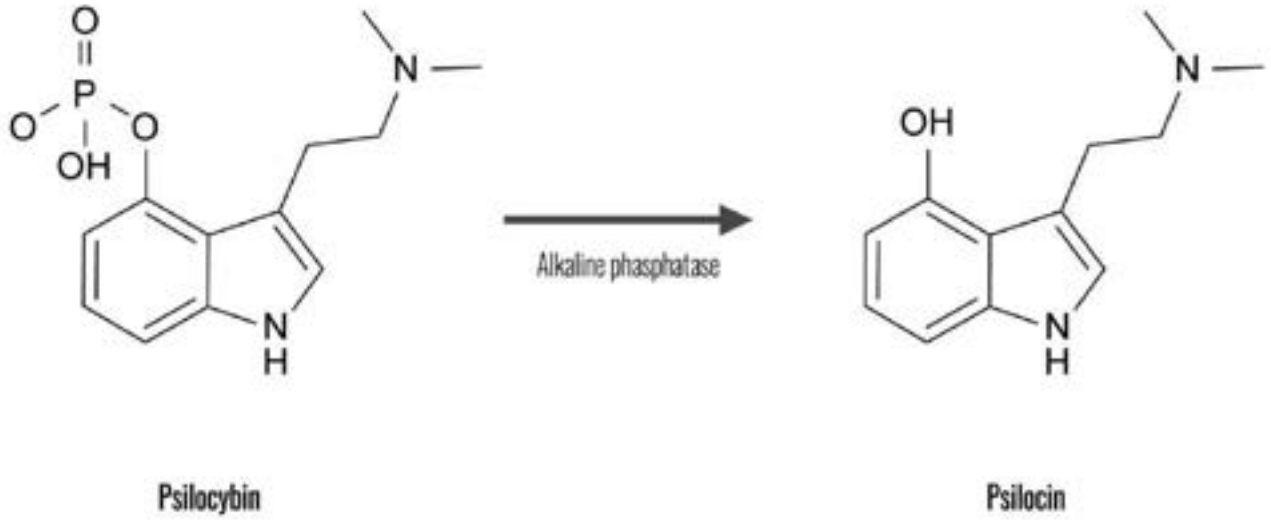


**Fig. 3.** Biotransformation of psilocybin into psilocin.

As psilocin is structurally akin to the neurotransmitter serotonin, it undergoes a similar metabolism. It is further degraded in the liver by monoamine oxidase (MAO) or aldehyde dehydrogenase, leading to various intermediates and end products (Kalberer et al., 1962; Lindenblatt et al.,1998). That is why MAO inhibitors are sometimes taken by users in an attempt to intensify psilocin's hallucinogenic effects. What is more, psilocin itself may competitively inhibit MAO, which may lead to elevated brain 5-HT levels and a decrease in 5-hydroxyindoleacetic acid (5-HIAA) levels (Dinis-Oliveira; 2017).

Like all psychedelics, psilocin has a strong affinity for 5-HT receptors in the brain, primarily acting as an agonist on the 5-HT2A, 5-HT2C, and 5-HT1A receptors. The psychedelic effects of psilocin are largely nullified by ketanserin, a 5-HT2A receptor antagonist, indicating the central role of the 5-HT2A receptor in its subjective effects (Preller et al., 2016, 2017; Vollenweider et al., 1998). The role of 5-HT1A receptors in the psychoactive effects of psilocin is yet to be examined. Furthermore, psilocin interacts with other serotonin receptors, including 5-HT1B, 5-HT1D, 5-HT2B, 5-HT5A, 5-HT6, and 5-HT7 (Tyls et al., 2014)(Tab.2).

| | 5-HT1A | 5-HT1B | 5-HT1D | 5-HT2A | 5-HT2B | 5-HT2C | 5-HT5 | 5-HT6 | 5-HT7 | α2A | SERT |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Ki (nM)* | 567.4 | 219.6 | 36.4 | 107.2 | 4.6 | 97.3 | 83.7 | 57.0 | 3.5 | 379 | 3801 |

**Tab. 2.** Affinity of psilocin to selected receptors. Based on Halberstadt and Geyer (2011).

Psilocybin's subjective and behavioral effects are influenced by interactions with non-serotonergic receptors. Research suggests that psilocybin indirectly elevates DA levels in the striatum, with such increases being associated with depersonalization and euphoria (Vollenweider et al., 1999). Nonetheless, the dopaminergic system plays only a partial role in psilocybin's psychological effects, as haloperidol, a nonselective DA receptor antagonist, reduces only about 30% of these symptoms (Vollenweider et al., 1998). Contrary to LSD, which binds to D2 receptors, psilocybin exhibits no affinity for dopamine D2 receptors (Marona-Lewicka et al., 2005; Vollenweider and Kometer, 2010). While there's evidence suggesting classical psychedelics can elevate dopaminergic transmission in human striatal regions, they don't significantly activate the Nacc in positron emission tomography (PET) imaging studies. This observation aligns with the absence of data linking classical psychedelics to substance use

disorder (Bogenschutz and Ross, 2018; Geyer and Vollenweider, 2008; Vollenweider et al., 1998, 1999).

*Effects of psilocybin observed in animal studies*

Both psilocybin and psilocin have been observed to evoke head twitch response and wet dog shakes in mice and rats, respectively (Corne and Pickering, 1967; Halberstadt et al., 2011a), and the effect was absent in 5-HT2A receptor knockout mice (Halberstadt et al., 2010). Psilocybin fully substituted for psilocin, DOM and LSD (Winter et al., 2007), and these effects were abolished by coadministration of volinserin, further confirming that the primary effects of psilocybin are mediated via the 5-HT2A receptor (Winter et al., 2007). Psilocin dose-dependently (1,25-5.0 mg/kg) reduced locomotor activity in rats (Halberstadt et al., 2011b) and mice (Halberstadt et al., 2011a). What is interesting is that treatment with selective 5-HT1A receptor antagonist, WAY-100635, completely reversed the effects of psilocin on mice locomotor activity. Furthermore, deletion of the 5-HT2A receptor gene did not affect the response induced by psilocin (Halberstadt et al., 2009). These findings suggest that the effect of psilocybin/psilocin on locomotor behavior may be primarily mediated via the 5-HT1A receptor, not the 5-HT2A receptor.

To date, there are no animal studies suggesting that psilocybin exhibits addictive properties. Fantegrossi et al. (2008) reported no significant differences between psilocybin and saline in self-administration in rhesus monkeys. A microdialysis study by Sakashita et al. (2015) demonstrated that psilocin elevates extracellular levels of DA in the nucleus accumbens in a slight but significant way while not affecting them in the ventral tegmental area (VTA), suggesting rather low reinforcing properties. What is more, psilocin inhibited methamphetamine-induced conditioned place preference formation during the acquisition phase (Wang et al., 2023). It also reduced intracranial self-stimulation (Sakloth et al., 2019), and this effect was only partially reduced by the administration of volinserin, suggesting that other receptors, besides the 5-HT2A receptor, may also contribute to this effect (Jaster et al., 2022). These experiments are consistent with the paradigm that classic psychedelics lack reinforcing properties (Nichols, 2016) and suggest a possible use of psilocybin in the treatment of addictive disorders.

Acute administration of psilocin has been reported either to increase anxiety, measured as the center avoidance in mice (Halberstadt et al., 2011a) or exerting no effect in this paradigm (Chen et al., 2023). The anxiogenic effect was also reported in rats, but it diminished with repeated exposure to the experimental arena, suggesting that the anxiety is a result of drug-

enhanced neophobia (Halberstadt and Geyer, 2018). A recent study by Hibicke et al. (2020) reported anxiolytic properties of psilocybin, if the animals are repeatedly exposed to the novel environment. Anxiety was not observed in rats 24 hours after psilocybin administration either in the open field or light/dark box (L/D) paradigm (Wojtas et al., 2022). What is more, psilocybin/psilocin facilitated fear extinction in rats (Catlow et al., 2013; Hagsäter et al., 2021).

Psilocybin tested acutely in Flinders Sensitive Line (Jefsen et al., 2019) and 24h after administration in naive controls (Wojtas et al., 2022) did not exerted antidepressant effect in the forced swim test (FST), but demonstrated significant antidepressant effect 5 weeks after its administration (Hibicke et al.,2020). What is more, it attenuated learned helplessness in mice 24 hours after the drug administration (Shao et al., 2021) and reversed anhedonia in chronically stressed mice (Hesselgrave et al., 2021), while not affecting the immobility time measured in the FST. These findings support the thesis that a) psilocybin/psilocin may exhibit antidepressant properties b) FST might not be suitable for evaluating the antidepressant qualities of psychedelics.

*Effects of psilocybin observed in humans*

Psilocybin has demonstrated minimal toxicity in animals, with an LD50 in rodents being 2,000 to 3,000 times a standard human dose on a mg/kg basis (Usdin and Efron,1972; Tyls et al., 2014). When assessing the acute toxicity, safety, and addictive potential of various psychoactive substances, psilocybin is consistently ranked at the lower harm end - in comparison, opioids, notably heroin, are at the higher harm end (Gable, 1993, 2004; Morgan et al., 2013; Nutt et al., 2007). Physiologically, psilocybin poses minimal risk to humans, showing no association with major organ damage, carcinogenicity, teratogenicity, enduring neuropsychological deficits, or overdose fatalities (Johnson et al., 2018).

Oral psilocybin doses range from 0.045–0.429 mg/kg, with the psychedelic observed with oral doses >15 mg (Hasler et al., 2004) or plasma psilocin levels of 4–6 ng/ml (Hasler et al., 1997). Doses exceeding 25 mg orally are considered high but not dangerous (Johnson et al., 2018). After oral intake, onset of action is 20-40 min, peaking at 60–90 min with effects lasting 4–6 h, and complete cessation by 24 h (Hasler et al., 2004; Vollenweider et al., 1998)(Fig.4).


**Fig. 4.** Time course of psilocybin's action in humans.

Psilocybin administration results in dose-dependent effects: low doses produce drowsiness and amplify pre-existing moods (Hasler et al., 2004); medium doses instigate a controllable altered state of consciousness (Passie et al., 2002); and high doses generate intense psychedelic experiences, characterized by altered perception, dream-like states, illusions, hallucinations, synesthesia and alterations in perception of time and space (Geyer and Vollenweider, 2008; Hasler et al., 2004; Hollister, 1961).

According to the Altered States of Consciousness scale (ASCs) (Dittrich, 1998), when juxtaposed with ketamine, psilocybin exhibits pronounced visual hallucinatory effects, whereas ketamine more markedly disrupts physical integrity (Studerus et al., 2011; Vollenweider and Geyer, 2001; Vollenweider and Kometer, 2010). Pharmacological interventions have provided insights into psilocybin's mechanism of action. Both ketanserin (5-HT2A/C antagonist) and risperidone (mixed 5-HT2A/C and D2 antagonist) have been observed to normalize psilocybin-induced alterations. Conversely, haloperidol, a D2 receptor antagonist, only modulates euphoria, derealization, and depersonalization but fails to influence visual hallucinations (Vollenweider et al., 1998).

Historical accounts, such as the Good Friday Experiment, underscore the spiritual and mystical experiences linked to psilocybin (Pahnke, 1963). Positive transformations in life attitudes persisted over prolonged periods post-administration (Doblin, 1991). Contemporary double-blind placebo-controlled studies reaffirm these findings. Many naive participants attributed substantial personal and spiritual significance to their psilocybin sessions, reporting lasting positive attitudinal and behavioral changes (Griffiths et al., 2006). While there is a common belief, that use of psychedelic drugs (including psilocybin) may increase the risk of mental disorders, especially psychosis and attempts of self-harm, the currently available data

contradict that notion, finding no correlation between the use of classic psychedelics and psychiatric disorders (Hendricks et al., 2015; Johansen and Krebs, 2015).

What is more, besides being a non-toxic and well-tolerated drug, psilocybin has been thoroughly examined as a potential fast-acting antidepressant drug during the last decade. Carhart-Harris et al. (2016) reported that acute administration of psilocybin produced significant effects in 67% of patients with treatment-resistant depression in the first week after the treatment, with 47% of treated individuals staying in remission in a 3-month and 6-month (Carhart-Harris et al., 2018). A study by Gukasyan et al. (2022) reported long-lasting antidepressant effects of psilocybin administration, with significant response to long treatment maintained in 75% of participants and a remission rate of 58% in a 12-month follow-up. The drug also withstands comparison to classical antidepressant treatment, with double administration of psilocybin (separated by a 3-week interval) being as efficient as a 6-week chronic treatment with escitalopram (Carhart-Harris et al., 2021; Barba et al., 2022).

The qualities mentioned above make psilocybin partly similar to the prototypical fast-acting antidepressant drug - ketamine. The drug, which is an N-Methyl-D-aspartic acid (NMDA) receptor antagonist, was found to be effective in animal models of depression during the 1990s by Skolnick and colleagues (Skolnick et al., 2009). Most importantly, ketamine exerted its antidepressant effect nearly instantly, contrary to the classical antidepressant drugs, which required chronic administration. These findings were then replicated in human patients, proving the rapid onset of this antidepressant effect, even in treatment-resistant individuals (Berman et al., 2000; Zarate et al., 2006; Marcantoni et al., 2020; Price et al., 2022). Its administration initiates a cascade of events resulting in an influx of glutamate and stimulation of the mammalian target of rapamycin complex 1 (mTORC1) pathway, which leads to restoration of synaptic plasticity, a feature disrupted in depressed individuals (Li et al., 2010).

However promising, ketamine's action is far from perfect. The main disadvantage is its psychotomimetic effects, including hallucinations, derealization, and disorientation, which may be distressing for patients. Moreover, it’s not free of somatic adverse effects, including nausea, cardiovascular effects, and possible toxicity (Rodrigues et al., 2020). Last but not least, the antidepressant effect, while rapidly induced, diminishes rather quickly, about two weeks after the treatment, creating the need for recurrent drug administration. This fact is especially concerning since there are not many studies examining the long-term effects of ketamine administration, and some of them point to potential neurotoxicity (Ionescu et al., 2018; Molero et al., 2018; Sanacora et al., 2017).

# 2. Research aims

This thesis has the overall aim to explore the pharmacological differences between a highly selective 5-HT2A receptor agonist from the phenethylamine group, 25B-NBOMe, and a far less selective agonist from the tryptamine group, psilocybin. Specifically, the focus was put on the discrepancies in the evoked neurotransmitter release and damage induced by either a selective or a promiscuous ligand, while additionally examining the effects on animals' behavior.

**Article 1 (A1)**

*"Neurochemical and Behavioral Effects of a New Hallucinogenic Compound 25B-NBOMe in Rats"*

To examine *in vivo* the effects induced by the acute 25B-NBOMe administration on rats' brain neurotransmission, behavior, and its possible genotoxicity.

**Article 2 (A2)**

***"****Hallucinogenic activity, neurotransmitters release, anxiolytic and neurotoxic effects in Rat's brain following repeated administration of novel psychoactive compound 25B-NBOMe"*

To examine *in vivo* the effects induced by the chronic 25B-NBOMe administration on rats' brain neurotransmission, behavior, and its possible toxicity in neural and glial cells.

**Article 3 (A3)**

*"Effect of Psilocybin and Ketamine on Brain Neurotransmitters, Glutamate Receptors, DNA and Rat Behavior"*

To examine *in vivo* the effects induced by the acute psilocybin administration on rat cortical neurotransmission, expression of selected receptor proteins, behavior and its possible genotoxicity, compared to ketamine.

**Article 4 (A4)**

*"Limbic system response to psilocybin and ketamine administration in rats: A neurochemical and behavioral study"*

To examine *in vivo* the effects induced by the acute psilocybin administration on rat limbic neurotransmission, expression of selected receptor proteins, and behavior compared to ketamine.

# 3. Discussion

After a half-century hiatus, psychedelic compounds are experiencing a renaissance, being considered a means to treat various mental disorders. The results of the first studies appear promising, yet the exact mechanisms standing behind the therapeutic effect are only partially understood. Given the significance of the issue at both the individual and societal levels, the pharmacological properties of psychedelics need to be thoroughly studied if psychedelics are to become widely used in the pharmacotherapy of mental disorders. While some of them appear to be physiologically safe, certain groups are reported as a severe threat to public health. To address this issue, the safety of each compound, differences in effects exhibited between subclasses, and precise mechanisms of action should be vigorously investigated.

### 3.1 Effects exerted by a single exposure to 25B-NBOMe.

While there is scientific consensus that the primary mechanism behind the psychedelic effect is the increase in glutamatergic neurotransmission in the frontal cortex (FCX) (Muschamp et al., 2004; Noworyta-Sokołowska et al., 2016; Herian et al., 2019; Nichols, 2016), the effects exerted on other neurotransmitters are not so consistent and vary from drug to drug. The NBOMe class is reported to be extremally potent (Poulie et al., 2019; Zawilska et al., 2020); both Miliano et al. (2019) and Herian et al.(2019) reported significant elevation of extracellular levels of DA, 5-HT, and glutamate (GLU) after acute administration of 25I-NBOMe, while also observing numerous WDS episodes.

Our results observed after administration of 25B-NBOMe are consistent with these findings; we have observed an increase in cortical extracellular glutamate levels (Wojtas et al., 2021), and this effect was more robust than the one exerted by 25I-NBOMe (Herian et al., 2019) **(A1; Fig. 1)**. What is more, 25B-NBOMe induced similar number of WDS to 25I-NBOMe but with a three-time smaller dose (0.3 mg/kg) (Wojtas et al., 2021) **(A1; Fig. 5)**. Combined, these findings confirm the stronger affinity and potency of 25B-NBOMe at the 5HT2A receptor, reported *in vitro* by Rickli et al. (2015). Like 25I-NBOMe (Herian et al., 2019), 25B-NBOMe significantly increased the extracellular 5-HT levels (Wojtas et al., 2021) **(A1; Fig. 1,2,3)**; this phenomenon may be the reason for the serotonin syndrome reported in humans after in ingestion of 25B-NBOMe (Gee et al., 2016). What is more, it increased the volume of dopaminergic neurotransmission akin to 25I-NBOMe; those changes observed in Nacc and STR **(A1; Fig. 1,2,3)** suggest that it might exhibit reinforcing properties.

The dose-response curve observed in microdialysis studies had an inverted “U” shape, most likely resulting from the activation of the different subtypes of 5-HT receptors. The progressive elevation of extracellular levels of neurotransmitters (except acetylcholine (ACh)), starting from the lowest dose (0.1 mg/kg) and reaching the plateau at 0.3 mg/kg, is a result of the activation of 5-HT2A receptors located on pyramidal glutamatergic cells (Miner et al., 2003; Scruggs et al., 2003; Muschamp et al., 2004; Herian et al., 2019). As the plasma levels of the drug increase, subsequent activation of the 5-HT2C receptor located on cortical GABAergic interneurons happens, leading to an increase in GABA-ergic neurotransmission and attenuation of the observed effect (Fig. 5).

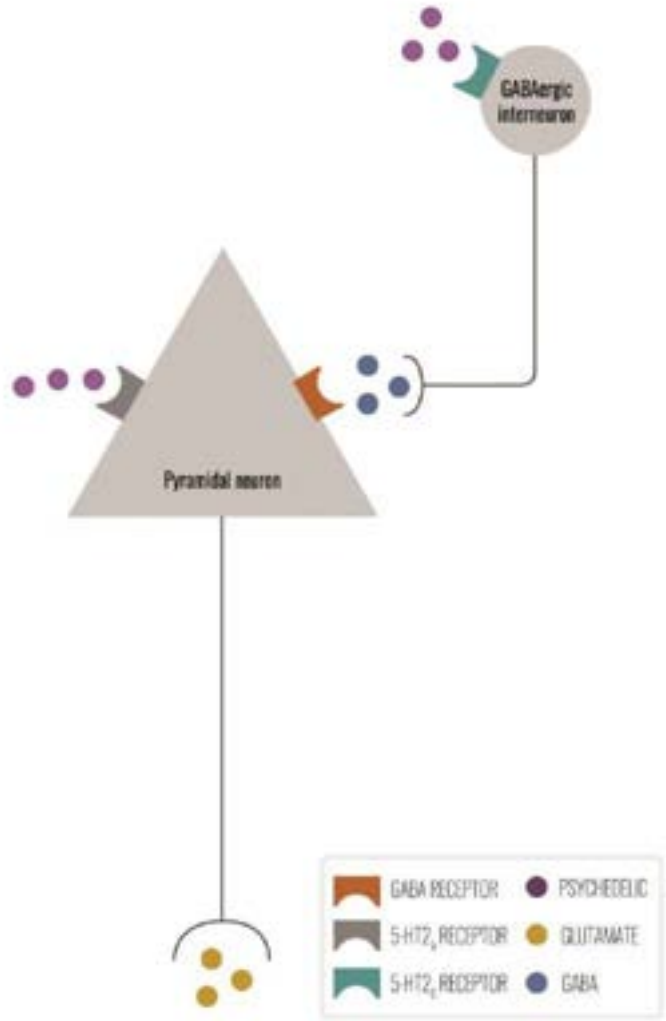


**Fig. 5.** Counteracting effects of 5-HT2A/C receptors on locomotor activity.

Both local (Abi-Saab et al., 1999) and systemic (Wojtas et al., 2022) administration of 5-HT2A/2C receptor agonists were reported to induce dose-dependent increase in extracellular levels of GABA. This effect was further replicated when assessing hallucinogenic potency, with the WDS response curve acquiring a similar shape, reaching the peak at the dose of 0.3 mg/kg (Wojtas et al., 2021) **(M1; Fig. 5)**, similar to 25I-NBOMe (Herian et al., 2019) and head twitches in mice (Custodio et al., 2020). What is more, 25B-NBOMe reduced locomotor activity and induced anxiety in animals **(M1; Fig. 7,8)**; these effects are also akin to those induced by 25I-NBOMe (Herian et al ., 2021) and other psychedelics (Halberstadt et al, 2009).Surprisingly, while being more active at the 5-HT2A receptor than 25I-NBOME and

inducing a greater increase in extracellular levels of neurotransmitters, 25B-NBOMe induced a very weak genotoxic effect, when compared both to the control group (Wojtas et al., 2021) **(A1; Fig. 9)** or when comparing it with the effect of 25I-NBOMe (Herian et al., 2022). As reported by Quirion et al.(1985), the 5-HT2A receptors are located on cortical cholinergic terminals, suggesting their role in the stimulation of ACh release. Nair and Gudelsky (2004) reported that activation of the 5-HT2A receptor by DOI or mescaline stimulates ACh release in the PFC, and this effect is abolished with a 5-HT2A/B/C antagonist. In contrast to these results, our study observed a decrease/no changes/increase in extracellular levels of cortical ACh after the administration of 25B-NBOMe, the effect varying on the dose **(A1; Fig. 1,2,3)**. These results are hard to explain due to a lack of data concerning the effects of psychedelics on cholinergic neurotransmission.

In summary, our results indicate that while being a powerful psychedelic, capable of inducing significant enhancement of neurotransmission, resulting in changes in animal behavior similar to those induced by other psychedelics, 25B-NBOMe might not be as toxic as other representatives of its class.

### 3.2 Consequences of repeated exposure to 25B-NBOMe.

As mentioned in section 4.1, 25B-NBOMe elevates extracellular levels of DA in Nacc and STR, which are elements of the reward system. Unlike other psychedelics, the NBOMes have been reported to exhibit rewarding properties, inducing CPP in mice and self-administration (SA) in rats (Seo et al., 2019; Jo et al., 2022; Lee et al., 2023). Most importantly, a study by Custodio et al. (2020) reported that 25-NBOMe also exhibit those properties in CPP and SA and those effects were neutralized when using D1 or D2 receptor antagonist, clearly suggesting the involvement of dopaminergic system. What is more, chronic administration of 25B-NBOMe induced changes in the expression of D1 and D2 receptors, upregulation of the former and downregulation of the latter, a common phenomenon induced by addictive compounds (Custodio et al., 2020).These findings encouraged us to investigate the consequences of chronic administration of 25B-NBOMe.

As stated before, frequent exposure to psychedelic drugs leads toward rapid induction of tolerance (Abramson et al., 1956; Angrist et al., 1974), originating from the downregulation of 5-HT2A receptor. This was also reported for 25I-NBOMe in rats (Herian et al., 2021) and 25B-NBOMe in mice (Custodio et al., 2020). Repeated administration of 25B-NBOMe completely suppressed its effect on cortical glutamatergic and dopaminergic neurotransmission

and significantly reduced the effect it induced on extracellular serotonin levels (Wojtas et al., 2023) **(A2; Fig. 1)**, this effect was more robust than the one induced by 25I-NBOMe (Herian et al., 2021). The influence of tolerance on the WDS response was similar to 25I-NBOMe, starting with a rapid decline of the number of WDS episodes on the second day of treatment **(A2; Fig. 4)**. This effect can be explained with the downregulation of 5-HT2A receptor, which diminishes enhancement of glutamatergic neurotransmission in the FCX, an essential event needed for HTR/WDS to occur (Nichols, 2016). The observed decrease in the locomotor activity **(A2; Fig. 6)** after chronic exposure to 25B-NBOMe may also be a result of tolerance. With the 5-HT2A receptor downregulated, the 5-HT2C receptor activation plays greater role in modulating the animal behavior, leading to suppression of locomotor activity, as suggested by Halberstadt et al.(2009).

The potency of 25B-NBOMe-induced increases in extracellular levels of examined neurotransmitters was also reduced in Nacc and STR **(A2; Fig. 2,3)**. It is important to notice that in the Nacc, the observed tolerance was much smaller (Wojtas et al., 2023). What is important, after 7-day treatment the compound still increased extracellular leveles of dopamine, which corresponds with its effect on DA receptors in Nacc reported in mice (Custodio et al., 2020) and increased Δ-fos b expression. Together these findings suggest, 25B-NBOMe might exhibit addictive properties, like other representatives of its family. Nevertheless, it's worth to notice that these effects while significant, ale much smaller than addictive properties of other classes of drugs of abuse, e.g. psychostimulants (Custodio et al., 2020)

While acute administration of 25B-NBOMe resulted only in minor genotoxic effect (Wojtas et al., 2021), it produced significant DNA damage in both FCX and HP after chronic treatment (Wojtas et al., 2023) **(A2; Fig.7)**, in as similar way to 25I-NBOMe (Herian et al., 2022). Repeated administration of 25B-NBOMe increased basal levels of GLU, DA and 5-HT **(A2; S1)** suggesting appearance of maladaptive changes in neurotransmission. These increases might lead to genotoxicity directly when elevated GLU levels overstimulate ionotropic glutamatergic receptors, resulting in DNA damage (Halliwel, 2006), and indirectly, when excess of monoamines leads to disturbances in their metabolism, leading to production of free radicals (Wrona and Dryhurst, 1998; Halliwell, 2006). Moreover, phenethylamines may directly produce oxidative stress, which was shown by Xu et al. (2019) in *in vitro* studies. Nevertheless, the observed genotoxic effect didn't translate into permanent loss of brain tissue, measured as the volume of cortical and hippocampal region **(A2; Fig. 8,9)**. Alongside with the previous study, these results suggest that while inducing some DNA damage, the toxicity of 25-NBOMe

can’t be explained by this phenomenon and is most likely a result of serotonin syndrome (Kawahara et al.,2017).

### 3.3 The effects of fast-acting antidepressants on mesocortical neurotransmission and related behavior.

While possessing distinct pharmacological targets, the 5-HT2A receptor for psychedelics and the NMDA receptor for ketamine, those drugs exhibit some overlapping properties. Either stimulation of cortical 5-HT2A receptors located on pyramidal cells by psychedelics or inhibition of cortical GABAergic interneurons by ketamine is supposed to result in a “glutamate surge,” which is a sudden elevation of cortical GLU levels (Savalia et al., 2021; Borsellino et al., 2023). Subsequently, this phenomenon leads to an increase in neuroplasticity, producing an antidepressant effect (de Gregorio et al., 2018; Duman et al., 2016). What is more, on the global level, the administration of either psychedelics (Carhart-Harris et al., 2012; Lebedev et al., 2015) or ketamine (Bonhomme et al., 2016) reduces the activity of DMN, which is hyperactivated in depressive disorders. As stated by Vollenweider and Kometer (2010), this translates into partially overlapping subjective experiences produced by psilocybin and ketamine. That is why we decided to investigate the influence of psilocybin and ketamine on neurotransmission and the resulting behavioral effects.

#### 3.3.1 Psilocybin

As reported in other works regarding psychedelics (Muschamp et al., 2004; Herian et al., 2019; Wojtas et al., 2021), psilocybin also elevated the extracellular glutamate levels in the FCX, providing further evidence for the “glutamate surge” hypothesis (Wojtas et al., 2022) **(A3; Fig. 1)**. What is more, it elevated cortical levels of DA only with a smaller dose, with the high dose being ineffective. This finding is in accordance with the work of Sakashita et al. (2015), who reported slight but significant decrease in cortical DA after administration of a similar dose (10 mg/kg) of psilocin. The authors explain this fact by the activation of 5-HT2C receptors, which is reported to decrease DA activity in the FCX (Millan et al., 2003). Psilocybin dose-dependently raised the extracellular levels of 5-HT in the FCX (Wojtas et al., 2022) **(A3; Fig. 1)**, the effect observed previously for psilocin by Sakashita et al. (2015). This effect might be mediated via the 5-HT2A receptor, as local administration of 5-HT2A agonist (DOI) into the PFC elevates extracellular levels of cortical 5-HT and this effect is neutralized with 5-HT2A receptor antagonist (volinserin) (Franberg et al., 2012). Furthermore, psilocybin dose-

dependently increased GABA in the PFC (Wojtas et al., 2022) **(A3; Fig. 1)**. What is important is that this also happens in humans after the administration of psilocybin, as stated by Mason et al. (2020). This could be explained by either the activation of the 5-HT2C (Santana et al., 2017) or 5-HT2A (Nichols, 2016; Zhou and Hablitz, 1999) receptor. Both are located on GABAergic interneurons in the PFC, and psilocin exhibits similar affinities to them (Halberstadt and Geyer, 2011).

As reported by Rodriguez et al. (2011), 5-HT2A receptors exhibit high expression in the Reticular Nucleus of the Thalamus (RNT), which is composed mainly of GABAergic interneurons. Vollenweider and Geyer (2001) proposed that it is responsible for filtering the thalamic input into the cortex and that psychedelics enhance its GABAergic activity, disrupting the negative-feedback (Fig. 6). As there is nearly no current data concerning this hypothesis, we decided to examine the effects of psilocybin on the amino-acidic neurotransmission in the RNT. Our observations seem to support this hypothesis, as we reported a dose-dependent increase in GABAergic neurotransmission while not observing any significant effect on glutamatergic neurotransmission (Wojtas et al., 2022) **(A3; Fig. 2)**.

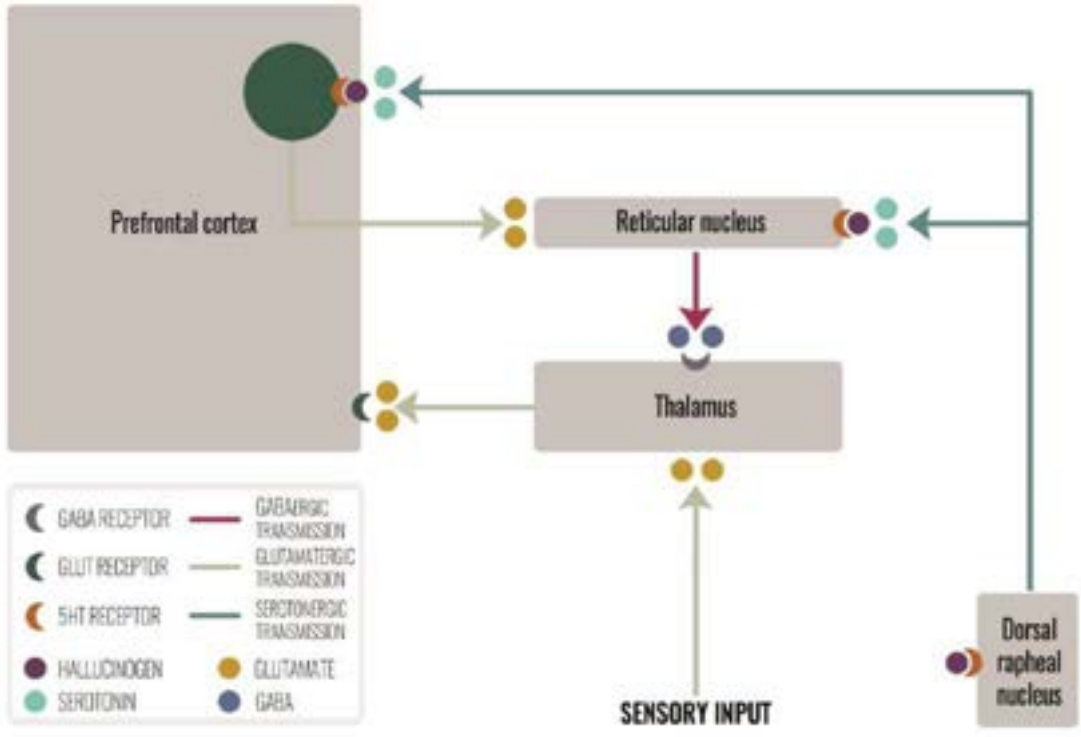


**Fig.6.** Psychedelics and thalamocortical gating. Based on Vollenweider and Smallridge, 2022.

Due to the release of glutamate induced by the administration of psilocybin, we examined the expression of GluN2A and GluN2B subunits of the NMDA receptor and GluA1 and GluA2 subunits of the AMPA receptor in the FCX 24 hours after administration of psilocybin. We observed no significant effect, except for the increase in expression of the GluN2A subunit when using the higher (10 mg/kg) dose of psilocybin) (Wojtas et al., 2022) **(A3; Fig. 3)**. These results are hard to explain, and perhaps it would be useful to replicate this experiment with different time points to evaluate if changes might happen earlier/later after the drug was administered.

While it's generally acclaimed that classical psychedelics are physiologically safe to use, we observed a significant increase in glutamatergic **(A3; Fig. 1)** activity in the FCX after high doses of psilocybin, which may result in genotoxicity. To examine this, we have assessed the oxidative DNA damage with the Comet Assay (Wojtas et al., 2022). The 10 mg/kg dose of psilocybin **(M3; Fig. 4)** significantly harmed the DNA in both the FCX and HP, suggesting caution when using high doses of this drug.

To investigate the long-lasting effect of psilocybin administration on rats' behavior, we conducted behavioral tests 24 hours after the drug administration to ensure that it would leave the system. We have observed no effect on the locomotor activity (Wojtas et al., 2022) measured in the open field test **(A3; Fig. 5)** and only a slight drop in the distance traveled in the L/D box test after administration of 2 mg/kg of psilocybin. This suggests that after 24 hours, the drug is washed out of the system and nearly no long-lasting effects are present, supporting the hypothesis about its safety. While it didn't produce an antidepressant effect in the FST **(A3; Fig. 6)**, it is worth noticing that this assay may not be suitable for testing fast-acting antidepressant drugs (Jefsen et al., 2019).

### 3.3.2. Ketamine

To verify the assumption of "glutamate surge" induced by the administration of ketamine, we have performed a microdialysis study in the FCX (Wojtas et al., 2022) and observed a significant increase in extracellular glutamate levels **(A3; Fig. 1)**. What is more, we reported elevated extracellular levels of GABA **(A3; Fig. 1)**. While at first look, this effect seems contradictory, as inhibition of GABAergic interneurons should lead to a decrease in GABAergic activity, it was also reported by other researchers in rats (Chowdhurry et al., 2012) and humans (Milak et al., 2016) and may originate from the enhancement of GLU/GABA cycling (Pham et al., 2019). The elevated levels of measured monoamines **(A3; Fig. 1)** might

be a result of two complementary mechanisms. In our study, we have used racemic ketamine, but the enantiomers inflict their effect in the FCX through different means; the (R)-Ketamine through AMPA-independent mechanism, the (S)-ketamine through AMPA-dependent mechanism (Ago et al., 2019).

As NMDA receptors are also expressed on the GABAergic interneurons in RNT (Troyano-Rodriguez et al., 2014), we expected to observe a suppression of GABAergic activity. Yet in our study, administration of ketamine did not affect extracellular levels of GLU and GABA in the RNT (Wojtas et al., 2022) **(A3, Fig. 2)**. This is hard to explain, and perhaps a wider range of doses should be applied to address this problem.

Despite increasing the extracellular glutamate levels and being a ligand for NMDA receptors, ketamine administration did not alter the expression of NMDA and AMPA receptor subunits **(A3; Fig. 3)**. The data concerning this phenomenon is scarce and not consistent, though it's hard to provide a convincing explanation.

The elevation of extracellular monoamine levels observed in the FCX after ketamine administration **(A3; Fig. 1)** prompted us to examine the oxidative stress damage, as it is a well-known fact that oxidation of DA and 5-HT results in the generation of free radicals (Halliwell, 2006). Surprisingly, we did not find any significant DNA damage in the FCX but did in the HP. The possible explanation might be that neurons in the cortex are more vulnerable to toxicity of glutamatergic origin. Ketamine, as an NMDA antagonist, reduces glutamate excitotoxicity (Chang et al., 2013).

After 24 h after ketamine administration, there was no effect on rats' anxiety **(A3; Fig. 5)** and only a slight but significant reduction of locomotor activity **(A3; Fig. 5).** Moreover, it did not affect immobility time measured in FST **(A3; Fig. 6)** (Wojtas et al., 2022); as suggested by Viktorov et al. (2022), this assay might not be suitable for examining NMDA antagonists on animals not subjugated to any model of depression.

### 3.4 The limbic response to fast-acting antidepressant drugs.

Alongside the PFC, the limbic system also exhibits maladaptive changes in depressive disorders (Pandya et al., 2012). Both the HP and amygdala undergo hypotrophy during depression, while Nacc exhibits dysfunctional hypertrophy (Pandya et al., 2012; Sheline et al., 1998; Abdallah et al., 2017). Recent studies reported that the administration of ketamine is able to reverse those changes in all aforementioned structures (Zhou et al., 2020; Abdallah et al., 2017), and these effects are correlated with antidepressant effect. As the data concerning the

effect of psychedelics on the limbic system is scarce, we decided to investigate the effect psilocybin exerts on the limbic system and limbic-related behavior and compare it with ketamine.

### 3.4.1 Psilocybin

As reported by Sakashita et al. (2015) psilocin dose-dependently increases extracellular DA levels in Nacc. Our studies confirm this effect when using psilocybin (Wojtas et al., 2023) **(A4; Fig. 1)**. This effect can be explained by the stimulation of 5-HT2A receptors, as local administration of 5-HT2A agonists enhances DA release in the Nacc (Guan et al., 1989; Parsons et al., 1993), while administration of 5-HT2A antagonists leads to inhibition of DA release in the Nacc (Parsons et al., 1993). Interestingly, while Sakashita and colleagues observed a slight but significant and dose-independent drop in the extracellular level of 5-HT in the Nacc after administration of psilocin, we have reported a dose-dependent increase **(A4; Fig. 1).** This effect might be explained by the stimulation of pyramidal cells, which send their projections to the RN, and subsequent stimulation of serotonergic neurons projecting into the Nacc. it's hard to explain the differences between our observations and those of Sakashita et al. (2015).

Interestingly, we have observed a decrease in accumbal glutamate levels **(A4; Fig. 2)**. Our previous studies with a more selective 5-HT2A agonist, 25B-NBOMe (Wojtas et al., 2021), reported increases in glutamatergic activity in the Nacc **(A1; Fig. 3).** This suggests the involvement of other receptor subtypes, as psilocin is a more promiscuous drug, with the most probable being the 5-HT1A receptors located on glutamatergic cells projecting into the Nacc (Celada et al., 2013). The observed increase in the extracellular level of GABA might result from the stimulation of 5-HT2A receptors located on GABAergic interneurons (Cornea-Hébert et al., 1999).

The opposing effect of lower and higher doses of psilocybin on extracellular levels of glutamate in the HP is hard to explain; the dose-dependent increase in GABA excludes the involvement of GABAergic interneurons **(A4; Fig. 2).** What is more, psilocybin increased extracellular levels of ACh in the HP **(A4; Fig. 3)**, with the lower dose being more potent. This might be a result of subsequent activation of the 5-HT2A receptor, which stimulates ACh release (Nair and Gudelsky, 2004), and then 5-HT1B receptors, which inhibits the ACh release in the rat HP (Izumi et al., 1994). Perhaps the inhibition of glutamatergic affinity by the lower dose of

psilocybin results from the elevated levels of acetylcholine acting at M4 muscarinic receptors located on hippocampal pyramidal cells (Dasari et al., 2011).

Psilocybin did not affect extracellular levels of GLU and GABA in the amygdala **(A4; Fig. 2)**. 5-HT2A receptors are highly expressed on pyramidal cells and both parvalbumin and somatostatin GABAergic interneurons in the amygdala, which suggests that the effect of their activation may be mutually suppressive (Bombardi et al., 2013).

Despite the fact that psilocin is a 5-HT2A receptor agonist and it elevated the extracellular levels of 5-HT in the Nacc **(A4; Fig. 1)** it did not affect the expression of 5-HT2A receptor **(A4; Fig. 7).** This may be due to their low density in the Nacc (Pompeiano et al., 1994). Changes were observed for the D2 receptor when using a higher dose of psilocybin. This may be a result of interplay between dopaminergic and serotonergic neurotransmission, as administration of haloperidol attenuates the psychomimtetic effects induced by psilocybin administration (Passie et al., 2002).

Both 5-HT2A and 5-HT1A receptors express high density in the HP (Palchaudhuri et al., 2005; Bombardi et al., 2013). The observed decrease in 5-HT1A receptor density **(A4; Fig. 6)** might be a result of their stimulation either by psilocybin or the release of serotonin, but there is a lack of data regarding the latter. The decrease in 5-HT2A receptor expression induced by the lower dose of psilocybin may be a result of rapid downregulation, while the increase caused by the higher dose might be a result of increase in synapto- and neurogenesis (Ly et al., 2018).

Psilocybin exhibited a dose-dependent anxiolytic effect measured as a decrease of center avoidance in the open field test **(A4; Fig. 8)** both 1 and 24 hours after the drug administration, this effect may resulting from intensification of GABAergic neurotransmission observed in limbic structures **(A4; Fig. 3)**.

### 3.4.2 Ketamine

A study by Del Arco and Mora (2008) reported that administration of phencyclidine increaseases extracellular DA levels in Nacc. This increase is explained by inhibition of cortical GABAergic interneurons via blockade of NMDA receptor and following disinhibition of glutamatergic neurons projecting into the VTA. Our results are in accordance with their work, as we reported increase in extracellular GABA in the FCX (Wojtas et al., 2022) **(A3; Fig. 1)** and elevated extracellular DA in the Nacc (Wojtas et al., 2023) **(A4; Fig. 1)**. What is more, it increased the extracellular 5-HT levels in the Nacc. This may also originate from disinhibition of the glutamatergic neurons, this time projecting to RN. Ketamine increases AMPA-related

synaptic transmission and glutamate release in raphe nuclei (RN) (Lopez-Gil et al., 2019; Llamosas et al., 2019) which may subsequently increase the release of accumbal 5-HT from neurons projecting from RN to Nacc. The observed increases in GLU reported in Nacc **(A4; Fig. 2)** might be resulting from inhibition of GABAergic interneurons-, this would disinhibit glutamate projections from cortex into the Nacc (Suska et al., 2013). The elevated GABA levels are harder to explain due to the lack of data.

Ketamine administration elevated extracellular GLU and GABA levels in rat HP **(A4; Fig. 2).** HP receives strong glutamatergic input from the entorhinal cortex (Basu et al., 2015) so disinhibition of glutamatergic neurons projecting into HP can explain this effect. The elevation of extracellular GABA is subsequent to the increase of glutamate. As stated by Zhang et al.(1990), administration of kainic acid results in increase in hippocampal GABA levels, suggesting that the increase in GABA observed in our study may result from the response of kainic receptors. The effect of ketamine administration on measured amino-acidic neurotransmitters in amygdala was statistically significant but transient and very weak.

Ketamine did not affected expression of D2 and 5-HT2A receptors in the Nacc in a significant way **(A4; Fig. 6**) but increased expression of both 5-HT1A and 5-HT2A receptors in HP **(A4; Fig. 5)**. The nature of this phenomenon is hard to explain, and perhaps results from the effect that ketamine exerts on 5-HT levels. As this effect was measured 7 days after the drug administration it is also possible that this effect might be a result of increased synapto- and neurogenesis (Ly et al., 2018).

Our study showed no effect on locomotor behavior 24 hours after ketamine administration and a significant increase 1 hour after the drug administration **(A4; Fig. 7)**. This acute effect is most likely a result from the enhancement of dopaminergic neurotransmission.

### 3.5.Summary

The data presented in this dissertation supports the hypothesis about the greater risk associated with the use of Novel Psychoactive Substances in comparison to naturally occurring psychedelics. Our studies allowed us to characterize the effects of a selective 5-HT2A/C receptor agonist on excitatory neurotransmitter systems and how those changes may translate into behavior and damage inflicted upon the central nervous system. A similar approach was then applied to a compound with a broader receptor profile (psilocybin), uncovering more subtle effects resulting from the interplay of 5-HT1A and 5-HT2A receptors, with possible beneficial

influence (i.e. lasting anxiolytic effect). Comparing the effects of psilocybin to ketamine allowed us to better understand the differences in mechanisms of action of possible fast-acting antidepressant drugs, with psilocybin's effect more potent on amino-acidic neurotransmission and ketamine on monoamines. The overlapping effects of ketamine and psilocybin are still not well studied, and further research could hopefully lead to an understanding of the mechanisms behind their therapeutic effect, leading to the successful development of novel, rapid-acting antidepressant drugs.

### 3.5.1 Main research achievements

**1. 25B-NBOMe significantly increases extracellular glutamate levels in the nucleus accumbens (Wojtas et al., 2021), while psilocybin induces opposing effects (Wojtas et al., 2023).** Maladaptive changes in glutamatergic neurotransmission occur during the development of addiction. Our findings support possible addictive properties of NBOMe compounds reported by other groups. The decrease in excitatory accumbal neurotransmission after the administration of psilocybin further reinforces its possible use in pharmacotherapy of addiction.

**2. Both 25B-NBOme and psilocybin elevate extracellular levels of DA in the nucleus accumbens after an acute treatment but the former also after chronic treatment.** This may result in development of addiction, due to the potential elevation of DA with each administered dose. On the other hand, psilocybin as a potential rapid-acting antidepressant drug is administered only once, avoiding the chance for addiction to develop.

**3. While repeated administration of 25B-NBOMe neutralizes its effect on cortical levels of glutamate and wet dog shake episodes, it still affects dopaminergic neurotransmission in the nucleus accumbens (Wojtas et al., 2023).** This may suggest that the rewarding properties of 25B-NBOMe are independent of its psychedelic effect.

**4. Chronic administration of 25B-NBOMe induces significant oxidative DNA damage in the FCX and HP (Wojtas et al., 2023).** Despite the rapid induction of tolerance, 25B-NBOMe produces a potent genotoxic effect, suggesting that the oxidative damage might be independent of its psychedelic effect.

**5. Psilocybin dose-dependently increases extracellular levels of GABA in the Reticular Nucleus of Thalamus (Wojtas et al., 2022).** Vollenweider et al. (2001) proposed that

psychedelics disrupt thalamocortical gating through the activation of interneurons in the Reticular Nucleus of Thalamus, which leads to GABAergic inhibition of the thalamus. Our study is the first one to support this hypothesis.

**6. 25B-NBOMe and psilocybin exert opposing effects on anxiety (Wojtas et al., 2021; Wojtas et al., 2023).** 25B-NBOMe produces anxiety in animals after acute administration, and this effect increases when it's given chronically, despite the rapid rise of tolerance. The anxiolytic effect of psilocybin was observed not only after acute treatment but also after the drug had left the system, suggesting long-lasting changes that result in decreased anxiety. This may be due to the increased GABAergic tone observed in nearly all investigated structures.

**7. Psilocybin administration elevates hippocampal levels of ACh (Wojtas et al., 2023).** Cholinergic projections from the basal forebrain projecting into the HP participate in the modulation of cognitive flexibility. Recent studies report that psilocybin enhances cognitive flexibility in rats; our findings may at least partially explain this phenomenon by the increases in cholinergic neurotransmission induced by psilocybin.

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# 5.Original articles

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ORIGINAL ARTICLE

# Neurochemical and Behavioral Effects of a New Hallucinogenic Compound 25B-NBOMe in Rats

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## Abstract

4-Bromo-2,5-dimethoxy-N-(2-methoxybenzyl)phenethylamine (25B-NBOMe) is a hallucinogen exhibiting high binding affinity for 5-$HT_{2A/C}$ serotonin receptors. In the present work, we investigated its effect on dopamine (DA), serotonin (5-HT), acetylcholine (ACh), and glutamate release in the rat frontal cortex, striatum, and nucleus accumbens. Hallucinogenic activity, impact on cognitive and motor functions, and anxiogenic/anxiolytic properties of this compound were also tested. The release of DA, 5-HT, ACh, and glutamate was studied using microdialysis in freely moving animals. Hallucinogenic activity was investigated using head and body twitch response (WDS), cognitive functions were examined with the novel object recognition test (NOR), locomotor activity was studied in the open field (OF), while anxiogenic/anxiolytic effect was tested using the light/dark box (LDB). Neurotoxicity was evaluated with the comet assay. 25B-NBOMe increased DA, 5-HT, and glutamate release in all studied brain regions, induced hallucinogenic activity, and lowered the recognition index (*Ri*) vs. control in the NOR test. It also decreased locomotor activity of rats in the OF test. The effect of 25B-NBOMe in the NOR test was inhibited by scopolamine. In the LDB test, the time spent in the dark zone was longer in comparison to control and was dose-dependent. In contrast to MDMA, 25B-NBOMe showed subtle genotoxic effect observed in the comet assay. Our findings indicate that 25B-NBOMe shows hallucinogenic activity in the wide range of doses. The changes in neurotransmitter levels may be related to 25B-NBOMe affinity for 5-$HT_{2A}$ receptor. Alterations in the NOR, OF, and LDB indicate that 25B-NBOMe impacts short-term memory, locomotion, and may be anxiogenic.

**Keywords** 25B-NBOMe · Hallucinogen · Neurotransmitters release · Behavior · Genotoxicity

## Introduction

It is without a doubt that humans have been using hallucinogens for ages; along with ethanol, they are the oldest psychoactive substances known to mankind (Nichols 2016; Schultes et al. 2001). They are known to induce powerful visual and auditory hallucinations, alter perception, and have profound effect on the users' mood (Nichols 2016).

Classical hallucinogens can be divided by their structure into 2 main categories: indoleamines, e.g., DMT (N,N-dimethyltryptamine) or LSD (lysergic acid diethylamide), and phenylalkylamines, e.g., mescaline or DOI (2,5-dimethoxy-4-iodoamphetamine) (Nichols 2012). While the former demonstrate affinity for nearly all subgroups of 5-HT receptors, the latter bind mainly to the 5-$HT_2$ receptor family (Pierce and Peroutka 1989; Titeler et al. 1988). Holistic data gathered from many studies indicate that hallucinogens exert their psychoactive effects via activation of the cortical 5-$HT_{2A}$ receptors (Glennon et al. 1984; Marek and Aghajanian 1996; Sipes and Geyer 1995; Wing et al. 1990). All hallucinogens produce head twitch response (HTR) in rodents, a phenomenon that is parallel to hallucinations in humans, as this effect can be a factor differentiating hallucinogenic from non-hallucinogenic 5-$HT_{2A}$ receptor agonists (González-Maeso et al. 2007; Halberstadt and Geyer 2013). Studies conducted in 5-$HT_{2A}$

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receptor knockout mice have proven that the activation of this receptor is obligatory for the induction of head twitches (González-Maeso et al. 2007; Halberstadt and Geyer 2013).

Even though the "classical" serotoninergic hallucinogens (known also as psychedelics) rarely induce any form of toxicity (Nichols 2016), recently, a new group of synthetic hallucinogens emerged, which are responsible for a plethora of cases of heavy poisoning and fatalities (Baumann et al. 2017; Shanks et al. 2015; Walterscheid et al. 2014). These drugs are the N-(2-methoxybenzyl)-2,5-dimethoxy-4-substituted phenethylamines (NBOMe). The NBOMe compounds are phenethylamine derivatives which undergo the process of N-benzylation, which greatly increases both their affinity and efficacy at the 5-$HT_{2A}$ receptor (Baumann et al. 2017). They have become available to drug users since they first appeared on the drug market around 2010.

One of the first representatives of this group 2-(4-bromo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25B-NBOMe) was synthesized by Ralf Heim in 2003 (Heim 2003). It is characterized by a very high, subnanomolar affinity for the 5-$HT_{2A}$ receptor ($Ki = 0.5$ nM) and activation potency ($EC_{50} = 40$ nM) (Rickli et al. 2015), which results in its strong psychoactive properties (Papoutsis et al. 2015). What is more, it seems to strongly affect dopaminergic system; it can induce conditioned place preference at certain doses in mice, and it induces self-administration in rats, which also suggests that it may exhibit reinforcing properties (Custodio et al. 2019; Miliano et al. 2019). What is more, it increases dopamine $D_1$ receptor levels in the nucleus accumbens, while decreasing dopamine $D_2$ receptor levels, and decreases dopamine transporter (DAT) levels in the ventral tegmental area (VTA) (Custodio et al. 2019).

Serotonin 5-$HT_{2A}$ receptors are widely distributed in the CNS, especially in the rat medial prefrontal cortex (mPFC) where they regulate functions of pyramidal projection neurons (Nocjar et al. 2015). In addition, the presence of 5-$HT_{2A}$ receptors was also evidenced in the basal ganglia, especially in the nucleus accumbens and caudate nucleus (Zhang and Stackman 2015). 5-$HT_{2A}$ receptors in cortico-striatal projection are involved in impairment of attention (Carli and Invernizzi 2014) and may play a role in restoration of motor function resulting from dopamine (DA) depletion (Ansah et al. 2011). Interestingly, clinical studies suggest that naturally occurring classical hallucinogens, such as DMT, psilocybin, and pharmacologically related LSD may be used to treat drug dependence, anxiety, and mood disorders (dos Santos et al. 2016).

Up to date, limited data have been gathered regarding NBOMes and their pharmacology. In our recent work, we have demonstrated that another NBOMe compound, 25I-NBOMe, having similar in vitro affinity for 5-$HT_{2A}$ receptor, affected DA, serotonin (5-HT), and glutamatergic neurotransmission and showed hallucinogenic activity (Herian et al. 2019).

The aim of this study was to assess in vivo 25B-NBOMe effect on brain neurotransmission and rat behavior. Microdialysis in the rat frontal cortex, striatum, and nucleus accumbens was performed to determine the influence of the 25B-NBOMe on DA, 5-HT, glutamate, and acetylcholine (ACh) levels. To assess the cognitive effects of 25B-NBOMe, the novel object recognition test (NOR) was performed. To estimate 25B-NBOMe hallucinogenic effect resulting from 5-$HT_{2A}$ receptor stimulation, head and body twitch response was observed. The effect of 25B-NBOMe on anxiety was measured using the light/dark box test (LDB). Additionally, locomotor activity was measured in the open field test (OF). Putative genotoxic effect was also tested using the comet assay.

## Materials and Methods

### Animals

Male Wistar-Han rats (Charles River, Sulzfeld, Germany) weighting from 280 to 350 g (age of 120–180 PND) were used in all performed experiments. The animals were initially acclimatized and housed (5 per cage) in environmentally controlled rooms under 12-h light/dark cycle (the light was switched on at 6 a.m.) at a temperature of 23 ± 1 °C and humidity of 55 ± 10%. Rats had free access to typical laboratory food and tap water (VRF 1, Special Diets Services, Witham, UK), enriched environment was not applied. The studies strictly conformed to European regulations for animal experimentation (EU Directive 2010/63/EU on the protection of animals used for scientific purposes). The experimental protocols were approved by the Local Ethics Commission for Experimentation on Animals (permit number: 186/2017, 188/2017, 189/2017). This article does not contain any studies with human participants by any of the authors.

### Drugs and Reagents

2-(4-Bromo-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)-ethanamine (25B-NBOMe) was purchased from Cayman Chemical Company (Michigan, USA), while MDMA from Toronto Research Chemicals Inc. (Canada). LSD and scopolamine came from Sigma-Aldrich (Poland). All necessary chemicals for analysis with the use of high-performance liquid chromatography (HPLC) were obtained from Merck (Warszawa, Poland) and were of the highest purity. O-phthalaldehyde (OPA) obtained from Sigma-Aldrich was used for derivatization of glutamate to

electroactive compound. The chemicals used for the comet assay were from Trevigen (Gaithersburg, MD, USA).

## Drug Administration

During the experiment animals received subcutaneous (*sc*) single injections of 25B-NBOMe dissolved in 0.9% NaCl at five doses of 0.1, 0.3, 1, 3, and 10 mg/kg. The subcutaneous injection of 25B-NBOMe was chosen because it seems to be a favorable way of administration in the case of this group of compounds, in comparison to intraperitoneal injection, as shown by Baumann et al. (2017). MDMA (10 mg/kg) was also dissolved in 0.9% NaCl and was injected intraperitoneally (*ip*). LSD was injected *ip* at a dose of 0.1 mg/kg. Scopolamine dissolved in 0.9% NaCl was injected *ip* at the dose of 1 mg/kg 5 min before administration of 25B-NBOMe. The control group was administered with 0.9% NaCl solution in the same way.

## Brain Microdialysis

Ketamine and xylazine solutions at doses of 75 mg/kg and 10 mg/kg, respectively, were used to anesthetize animals. Microdialysis probes (MAB 4.15.3Cu, MAB 4.15.4Cu, and MAB 4.15.2Cu AgnTho's AB, Lindingö, Sweden) were implanted into the rat frontal cortex, striatum, and nucleus accumbens using the following coordinates (mm): AP + 2.7, L + 0.8, V − 6.5, from the dura (frontal cortex); AP + 1.2, L + 0.8, V − 7.0 (striatum); and AP + 1.6, L + 1.0, V − 8.0 (n. accumbens) (Paxinos and Watson 1998). Each group contained six animals. On the next day, probe inlets were connected to a syringe pump (BAS, West Lafayette, IN, USA), delivering artificial cerebrospinal fluid composed of (mM) 147 NaCl, 2.7 KCl, 1.0 $MgCl_2$, 1.2 $CaCl_2$; pH 7.4 at a flow rate of 2 µL/min. After 2 h of washout period, five basal dialysate samples were collected every 20 min, then animals were injected subcutaneously with 25B-NBOMe as indicated in the figure captions and fraction collection continued for 240 min. At the end of the experiment, the rats were sacrificed and their brains were histologically verified for the proper probe placement.

## Extracellular Concentration of DA, 5-HT, and Glutamate

The DA and 5-HT concentrations in dialysate fractions were analyzed by HPLC with electrochemical detection. Chromatography was performed using an Ultimate 3000 System (Dionex, Sunnyvale, CA, USA), electrochemical detector Coulochem III (model 5300; ESA, Chelmsford, MA, USA) with a 5020 guard cell, a 5040 amperometric cell, and a Hypersil Gold C18 analytical column (3 µm, 100 × 3 mm; Thermo Fischer Scientific, Waltham, MA, USA). The mobile phase was composed of 0.1 M potassium phosphate buffer adjusted to pH 3.8, 0.5 mM $Na_2EDTA$, 100 mg/L 1-octanesulfonic acid sodium salt, and 2% methanol. The flow rate during analysis was set at 0.6 mL/min. The applied potential of a guard cell was 600 mV, while that of amperometric cell was 300 mV with a sensitivity set at 10 nA/V. The chromatographic data were processed by Chromeleon v.6.80 (Dionex) software package run on a personal computer. The limit of detection of DA and 5-HT in dialysates was 0.002 pg/10 µL for DA and 0.01 pg/10 µL for 5-HT.

Glutamate levels in the extracellular fluids were measured electrochemically after derivatization with OPA/sulfite reagent to form isoindole-sulfonate derivative (Rowley et al. 1995). Chromatography was performed using an Ultimate 3000 pump (Dionex), LC-4B amperometric detector with a cross-flow detector cell (BAS, IN, USA), and a HR-80 column (3 µm, 80 × 4.6 mm; ESA Inc, Chelmsford, MA, USA). The mobile phase consisted of 100 mM monosodium orthophosphate at pH 4.6 and 4% methanol. The flow rate was 1 mL/min, and the applied potential of a 3-mm glassy carbon electrode was set at + 600 mV at a sensitivity of 5 nA/V. Glutamate-derivative peak was compared with the respective standard, and the data were processed using Chromax 2005 (Pol-Lab, Warszawa, Poland) software on a personal computer. The limit of detection of glutamate in dialysates was 0.03 ng/10 µL.

## Extracellular Concentration of ACh

Extracellular levels of ACh were analyzed by UHPLC with electrochemical detection. The ACh analysis is based on ion-pairing HPLC separation, followed by online enzymatic conversion of ACh to hydrogen peroxide, and detection on a Pt working electrode (SenCell with 2 mm Pt working electrode) and HyREF reference electrode at the potential of 200 mV. Chromatography was performed using the ALEXYS Neurotransmitter Analyzer, a DECADE Elite electrochemical detector, AS 110 Autosampler, and LC 110 pump (Antec Leyden B. V., Zoeterwoude, The Netherlands). ACh as positively charged was separated on Acquity UPLC HSS T3 analytical column (1.8 µm, 1 × 50 mm; Waters, Milford, MA, USA). After separation, ACh passed through an immobilized enzyme reactor AChE/ChOx IMER (AC-ENZYM II, 1 × 4 mm, Eicom, Kyoto, Japan). The mobile phase was composed of 50 mM monosodium orthophosphate buffer adjusted to pH 7.8, 0.5 mM $Na_2EDTA$, 2.8 g/L 1-octanesulfonic acid sodium salt, and 0.5 mM tetramethylammonium chloride. The flow rate during analysis was set to 0.05 mL/min. The chromatographic data were processed by CLARITY v.6.2.0.208 (DataApex Ltd.) chromatography software run on a personal computer. The limit of detection of ACh in dialysates was 0.0037 pg/10 µL.

### Head and Body Shakes Test

The behavior defined as rapid shaking of the head, neck, and trunk from one side to the other, analogous to a wet dog shaking (WDS) to dry itself (Klein et al. 2018) was counted immediately after drug injection during 240-min observation period by an experienced observer who was blind to the treatments. Scores were summed and totaled from 12 observation periods. Results were expressed as an average of sum values of all episodes during the observation time.

### Novel Object Recognition Test

The procedure of the NOR test was adopted from Antunes and Biala (2012) and Orzelska-Gorka et al. (2016). Apparatus consisted of a wooden closed square arena with painted black walls (60 × 60 × 40 cm) illuminated with a bright white light (150 lx) focused on the center. Each animal was familiarized with the arena (pre-test, without any object) 24 h before the testing day. The animals were habituated to dimly lit experimental room at least 1 h before the procedure. The NOR test consisted of two sessions: introductory and recognition one (5 min each) with a 30 min inter-session interval. The introductory session was performed 20 min after injection of a drug with two identical objects (A1 and A2) situated in opposite corners, approximately 15 cm from the walls of the arena. In the recognition session, one object was replaced with a novel one (A = familiar, B = novel). The objects were a black metal can and a green vase, which were used interchangeably as a novel object in each experimental group. Location of a novel object in the recognition session was randomly assigned to each rat. The arena and the objects were cleaned after each session. Exploration of an object was defined as follows: licking, sniffing, or touching the object but not as sniffing, standing, sitting on the object or leaning against it. Exploration time was measured using a digital laboratory timer by two independent observers blind to the experimental design. Recognition index (*Ri*) was calculated using the equation:

$$Ri = \frac{\text{Time spent on novel object exploration} \times 100}{\text{Time spent on novel object exploration} + \text{time spent on familiar object exploration}},$$

for results obtained during recognition session. It is considered to be an index of exploration of a novel object relative to the total exploration of both objects. The "*Ri*" ratio over 50% was defined as a successful recognition.

### Open Field Test

The open field test was performed as a modification of the procedure described by Rogóż and Skuza (2011). A round black arena (1 m in diameter) was used, which was virtually divided into 8 sections of the wheel. The test was conducted in the dimly lit room, except the middle of the arena, which was illuminated by 75-W light bulb placed 75 cm above it. Rats were placed in the middle of the arena 30 min after 25B-NBOMe subcutaneous injection, and their behavior was recorded for 10 min. The exploration was quantified with the following parameters: time of walking, number of line crossings, episodes of peeping under the arena, number of events of grooming, and number of rearings.

### Light/Dark Box Test

The light/dark box (LDB) test was performed in 4 computer-controlled Seamless Open Field Arenas for rats (43 × 43 × 30 cm; Med Associates; St Albans, Vermont, USA) that have 16 infrared emitters and photodetectors on each side of the box. The procedure from Noworyta-Sokolowska et al. (2019) was adapted to the present experimental design. A dark insert was used to divide the chamber into two equally sized compartments: a light compartment and a dark compartment. A hole in the insert enabled rats to move freely between compartments. The rat was placed in the dark compartment 20 min after injection of a drug and was allowed to explore freely for 15 min. The measured parameters included immobility time, ambulatory distance, vertical activity time, stereotypical activity time, and the time spent in the dark and light compartment. The data were collected using Med State software (Activity monitor, Med Associates).

### Alkaline Comet Assay

The alkaline comet assay was performed with the use of CometAssay® Reagent Kit for Single Cell Gel Electrophoresis Assay (Trevigen, Inc., Gaithersburg, MD, USA). At 72 h after injection of 25B-NBOMe, animals were sacrificed by decapitation and the frontal cortex was dissected. Fresh tissue placed on ice was used to isolate a nuclear fraction. After homogenization and several purification and centrifugation stages (as described previously in Noworyta-Sokołowska et al. 2019), nuclear suspension was obtained using a sucrose gradient (2.8 M/2.6 M, bottom to top). The nuclear fraction was mixed with low melting point agarose and transferred immediately onto CometSlides™. The following steps were carried out in accordance with Trevigen CometAssay® protocol: membrane lysis, DNA unwinding, alkaline electrophoresis, and staining (SYBR® Gold). Stained slides were examined under a fluorescence microscope (NIKON Eclipse 80i, NIKON Instruments Inc., Melville, NY, USA). The data was analyzed using OpenComet software v.1.3, a plugin of ImageJ program v.1.47 (NIH, Bethesda, MD, USA). DNA damage was presented as a tail

moment. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected by the comet tail length) and the number of damaged pieces (represented by the intensity of DNA in the tail).

### Data Analysis

Drug effects on DA, 5-HT, ACh, and glutamate release in the brain regions were analyzed with repeated measures ANOVA followed by Tukey's post hoc test. All obtained data were presented as a percent of the basal level assumed to be 100%. The data from the wet dog shake test, the novel object recognition test, and locomotor behavior of rats in the open field were analyzed using one-way ANOVA followed by Tukey's post hoc test and the *t* test where appropriate. Data collected from the alkaline comet assay were analyzed with the *t* test, while the light/dark box test was analyzed using Mann–Whitney's test. The differences were considered significant if *p* value was smaller than 0.05. All statistical analyses were carried out using STATISTICA v.10 StatSoft Inc. 1984–2011 (San Francisco, CA, USA) and GraphPad Prism v.5.00 GraphPad Software Inc. (La Jolla, CA, USA).

## Results

### The Effect of 25B-NBOMe Administration on the Extracellular Levels of DA, 5-HT, ACh, and Glutamate in the Rat Frontal Cortex

25B-NBOMe significantly ($p < 0.0002$) increased DA levels in the rat frontal cortex. The dose of 0.3 mg/kg was the most potent while the doses of 1, 3, and 10 mg/kg were less potent, but their effect was still significant in comparison to control group. The dose of 0.1 mg/kg did not increase cortical extracellular DA level (Fig. 1a). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{5,30} = 266$, $p < 0.0001$), sampling period ($F_{11,330} = 130$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{55,330} = 20$, $p < 0.0001$). Tukey's post hoc tests showed significant difference in dialysate DA levels ($p < 0.001$) between doses of 0.1, 1, 3, and 10 mg/kg compared with the effect of 0.3 mg/kg 25B-NBOMe dose.

The extracellular 5-HT level was increased the most by the dose of 0.3 mg/kg, and the doses of 1, 3, and 10 mg/kg were less active but still significant ($p < 0.0002$). 25B-NBOMe at the dose of 0.1 mg/kg did not change extracellular 5-HT level (Fig. 1b). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{5,30} = 134$, $p < 0.0001$), sampling period ($F_{11,330} = 68$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{55,330} = 9.7$, $p < 0.0001$). Tukey's post hoc tests showed significant difference in dialysate 5-HT levels ($p < 0.001$) between doses of 0.1, 1, 3, and 10 mg/kg with respect to the effect of 0.3 mg/kg 25B-NBOMe dose.

The 25B-NBOMe dose of 0.3 mg/kg was also the strongest in increasing extracellular glutamate level. The effect of the remaining doses was less potent, yet still significant ($p < 0.0002$) (Fig. 1c). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{5,30} = 241$, $p < 0.0001$), sampling period ($F_{11,330} = 73$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{55,330} = 12.6$, $p < 0.0001$). Tukey's post hoc test showed significant difference in dialysate glutamate levels ($p < 0.001$) of doses 0.1, 1, 3, and 10 mg/kg with respect to effect of 0.3 mg/kg 25B-NBOMe dose.

The 25B-NBOMe doses of 0.1 and 10 mg/kg increased extracellular ACh level with similar potency ($p < 0.001$), the dose of 0.3 mg/kg significantly decreased ACh level ($p < 0.001$), while doses of 1 and 3 mg/kg were not effective (Fig. 1d). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{5,30} = 137$, $p < 0.0001$), sampling period ($F_{5,150} = 30$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{25,150} = 16$, $p < 0.0001$). Tukey's post hoc tests showed a significant difference in dialysate ACh levels ($p < 0.001$) of doses of 0.1, 1, 3, and 10 mg/kg with respect to the effect of 0.3 mg/kg 25B-NBOMe dose.

The total effect of 25B-NBOMe on extracellular levels of DA, 5-HT, glutamate, and ACh in the rat frontal cortex calculated as an area under the curve (AUC) and expressed as the percent of each basal level is presented in Fig. 4a.

### The Effect of 25B-NBOMe Administration on the Extracellular Levels of DA, 5-HT, ACh, and Glutamate in the Rat Striatum and Nucleus Accumbens

The doses of 0.3 mg/kg (more selective) and 3 mg/kg (less selective in activation of 5-$HT_{2A}$ receptors) of 25B-NBOMe were chosen for microdialysis experiments in the striatum and nucleus accumbens to minimize the number of animals. Both 25B-NBOMe doses increased extracellular levels of DA, 5-HT, glutamate, and ACh in the rat striatum (Fig. 2 a, b, c, and d). The dose of 0.3 mg/kg produced a larger increase in DA, 5-HT, and glutamate levels than the dose of 3 mg/kg, respectively. Repeated measures ANOVA showed an effect of treatment on DA levels ($F_{2,15} = 94$, $p < 0.0001$, time $F_{11,165} = 3.69$, $p < 0.0001$, time × treatment interaction $F_{22,165} = 3.74$, $p < 0.0001$). Tukey's post hoc tests showed a larger increase in dialysate DA in the striatum after 25B-NBOMe 0.3 and 3 mg/kg with respect to control values ($p < 0.0002$). Repeated measures ANOVA showed an effect of treatment on 5-HT levels ($F_{2,15} = 333$, $p < 0.0001$, time $F_{11,165} = 8.5$, $p < 0.0001$, time × treatment interaction $F_{22,165} = 3.03$, $p < 0.0003$). Tukey's post hoc tests showed a larger increase in dialysate 5-HT in

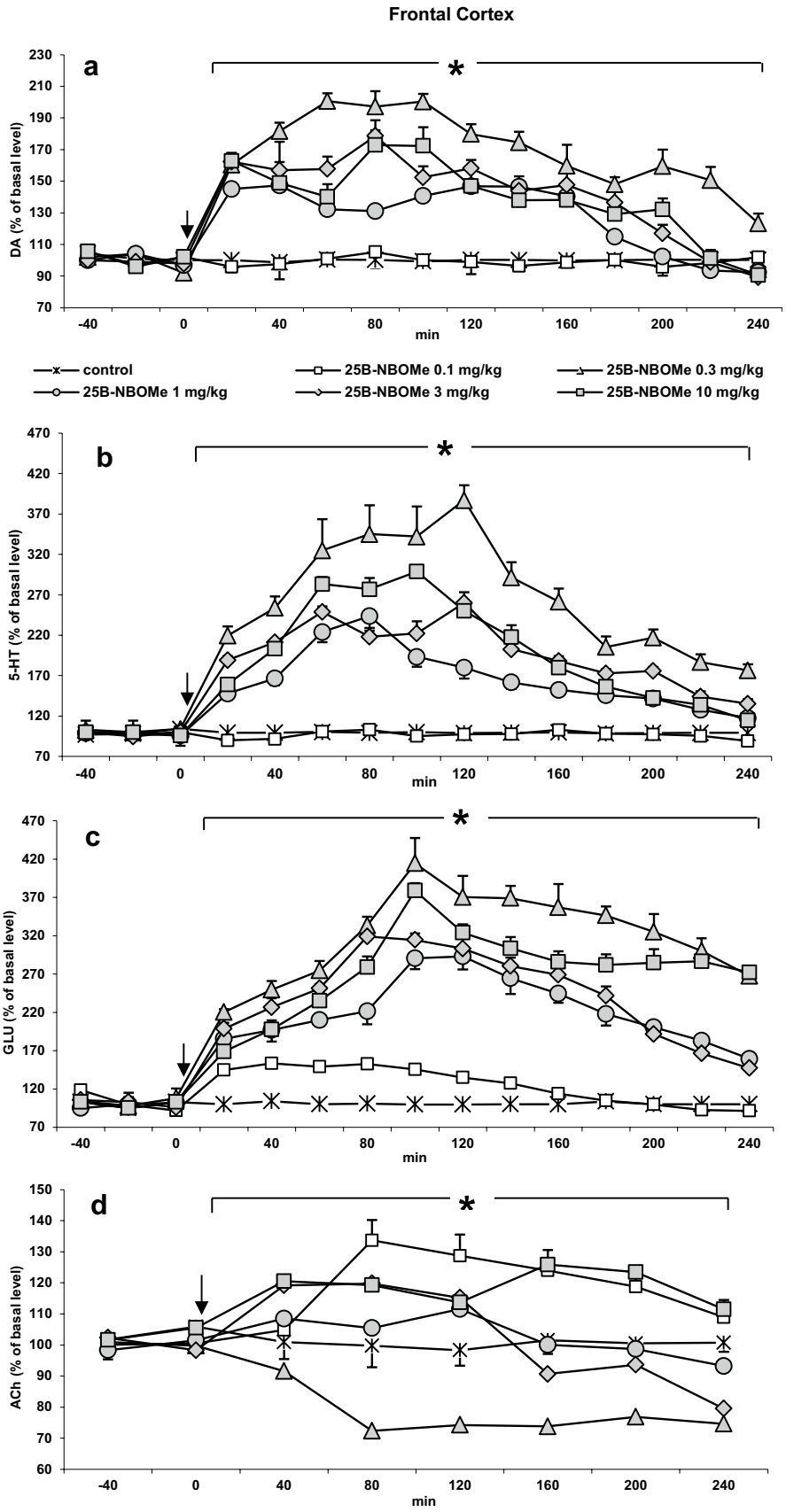


**Fig. 1** The time-course effect of 25B-NBOMe on extracellular levels of **a** dopamine (DA), **b** serotonin (5-HT), **c** glutamate (GLU), and **d** acetylcholine (ACh) in the rat frontal cortex. Values are the mean ± standard error of the mean (SEM), $n = 6$ per experimental group. For ACh measurements, two consecutive dialysate fractions were pooled. The time of drug injection is indicated by an arrow. The basal extracellular levels were as follows: for DA, 0.99 ± 0.06 nM, $n = 30$; for 5-HT, 0.19 ± 0.008 nM, $n = 30$; for ACh, 36.5 ± 3.93 nM, $n = 30$; for GLU, 1.76 ± 0.16 µM, $n = 30$; $^*p < 0.0002$ vs. control group (repeated measures ANOVA and Tukey's post hoc test)

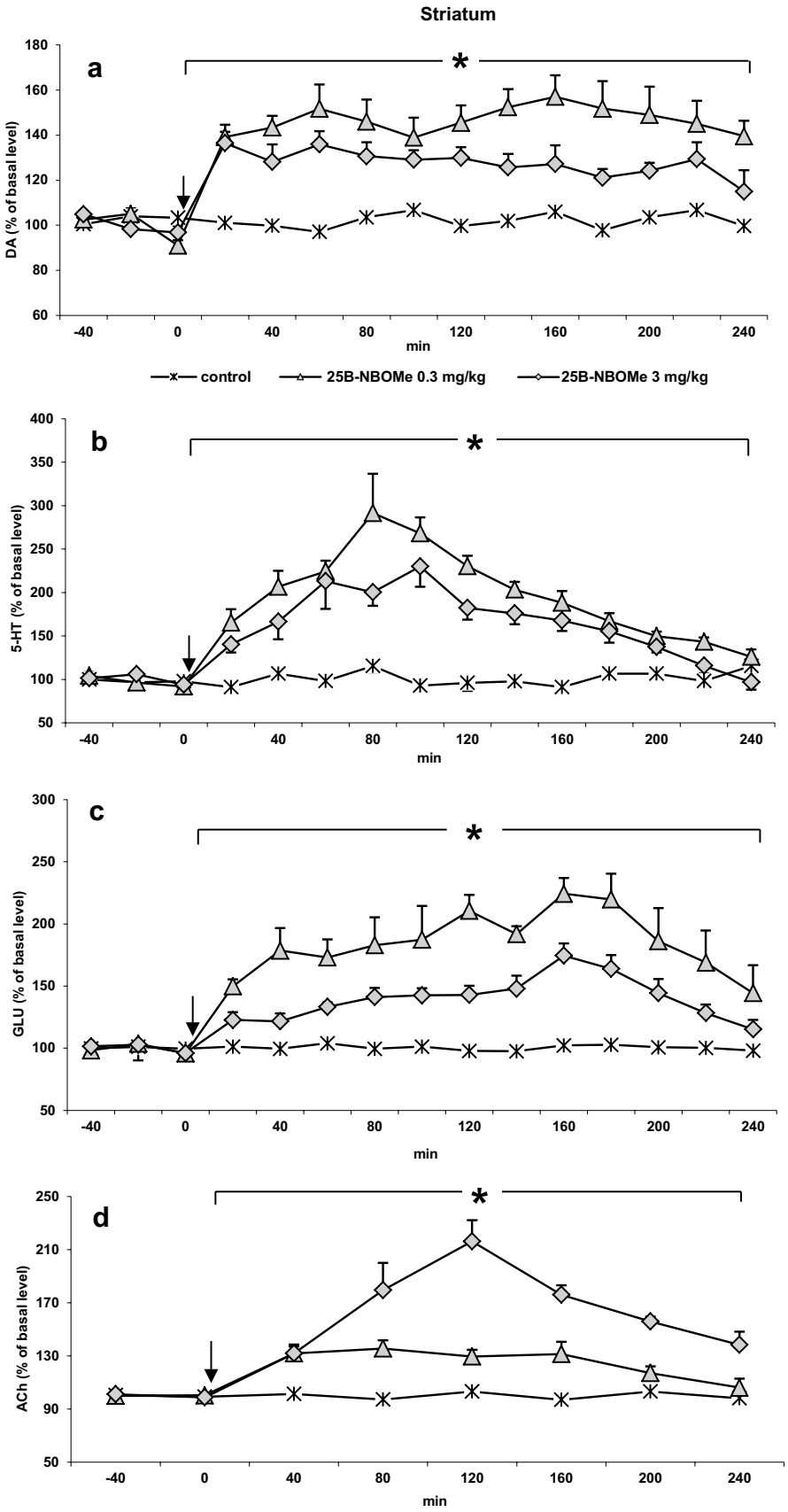


**Fig. 2** The time-course effect of 25B-NBOMe on extracellular levels of **a** dopamine (DA), **b** serotonin (5-HT), **c** glutamate (GLU), and **d** acetylcholine (ACh) in the rat striatum. Values are the mean ± standard error of the mean (SEM), $n = 6$ per experimental group. For ACh measurements, two consecutive dialysate fractions were pooled. The time of drug injection is indicated by an arrow. The basal extracellular levels were as follows: for DA, 5.17 ± 0.28 nM, $n = 30$; for 5-HT, 0.27 ± 0.014 nM, $n = 30$; for ACh, 48.9 ± 3.64 nM, $n = 30$; for GLU, 2.84 ± 0.22 µM, $n = 30$; $^*p < 0.0002$ vs. control group (repeated measures ANOVA and Tukey's post hoc test)

the striatum after 25B-NBOMe 0.3 and 3 mg/kg with respect to control values ($p < 0.0002$). Repeated measures ANOVA showed an effect of treatment on glutamate levels ($F_{2,15} = 339$, $p < 0.0001$, time $F_{11,165} = 21.65$, $p < 0.0001$, time × treatment interaction $F_{22,165} = 6.32$, $p < 0.0001$). Tukey's post hoc tests showed a larger increase in dialysate glutamate in the striatum after 25B-NBOMe 0.3 and 3 mg/kg with respect to control values ($p < 0.0002$). The dose of 3 mg/kg most potently affected the ACh levels. Repeated measures ANOVA showed an effect of treatment on ACh ($F_{2,15} = 58$, $p < 0.0001$, time $F_{5,75} = 12.30$, $p < 0.0001$, time × treatment interaction $F_{10,75} = 7.80$, $p < 0.0001$). Tukey's post hoc tests showed a larger increase in dialysate ACh in the striatum with respect to control values ($p < 0.0002$).

Similarly to the striatum, 25B-NBOMe at doses of 0.3 and 3 mg/kg significantly ($p < 0.0002$) increased extracellular levels of DA, 5-HT, glutamate, and ACh in the rat nucleus accumbens (Fig. 3 a, b, c, and d), but the effect was not dose-dependent since the lower dose of 25B-NBOMe was more effective in increasing the release of all neurotransmitters. Repeated measures ANOVA showed an effect of treatment on DA levels ($F_{2,15} = 337$, $p < 0.0001$, time $F_{11,165} = 49$, $p < 0.0001$, time × treatment interaction $F_{22,165} = 18$, $p < 0.0001$). Tukey's post hoc tests showed a larger increase in dialysate DA in the nucleus accumbens after 25B-NBOMe 0.3 and 3 mg/kg with respect to control values ($p < 0.0002$). Repeated measures ANOVA showed an effect of treatment on 5-HT levels ($F_{2,15} = 516$, $p < 0.0001$, time $F_{11,165} = 80$, $p < 0.0001$, time × treatment interaction $F_{22,165} = 28$, $p < 0.0001$). Tukey's post hoc tests showed a larger increase in dialysate 5-HT in the nucleus accumbens after 25B-NBOMe 0.3 and 3 mg/kg with respect to control values ($p < 0.0002$). Repeated measures ANOVA showed an effect of treatment on glutamate levels ($F_{2,15} = 81$, $p < 0.0001$, time $F_{11,165} = 67$, $p < 0.0001$, time × treatment interaction $F_{22,165} = 21$, $p < 0.0001$). Tukey's post hoc tests showed a larger increase in dialysate glutamate in the nucleus accumbens after 25B-NBOMe 0.3 and 3 mg/kg with respect to control values ($p < 0.0002$). The extracellular ACh level was increased more strongly by the lower dose of 25B-NBOMe. Repeated measures ANOVA showed an effect of treatment on ACh levels ($F_{2,15} = 201$, $p < 0.0001$, time $F_{5,75} = 31$, $p < 0.0001$, time × treatment interaction $F_{10,75} = 11$, $p < 0.0001$). Tukey's post hoc tests showed a larger increase in dialysate ACh in the nucleus accumbens after 25B-NBOMe 0.3 and 3 mg/kg with respect to control values ($p < 0.0002$).

The total effect of 25B-NBOMe on extracellular levels of DA, 5-HT, glutamate, and ACh in the rat striatum and nucleus accumbens calculated as an area under the curve (AUC) and expressed as the percent of each basal level is presented in Fig. 4 b and c.

## The Effect of 25B-NBOMe in the Wet Dog Shake Test

25B-NBOMe induced head and body twitches (WDS) in rats, which were observed immediately after the administration. The dose of 0.3 mg/kg produced the most potent effect (Fig. 5). The effect of 0.1, 1, 3, and 10 mg/kg doses was weaker but significant ($F_{6,49} = 127$, $p < 0.0001$). LSD at a dose of 0.1 mg/kg was nearly equally potent as the highest dose of 25B-NBOMe in inducing the hallucinogenic response.

## The Effect of 25B-NBOMe on Performance of Rats in the Novel Object Recognition Test

In this and the other behavioral experiments, we evaluated the effect of the two doses of 25B-NBOMe (0.3 and 3 mg/kg, sc). The results of this study showed an inverted "U"-shaped dose–response effect of 25B-NBOMe in increasing extracellular DA, 5-HT, and glutamate levels and in hallucinogenic activity in rats. The dose of 0.3 mg/kg was the most potent, while responses of higher doses were weaker. Therefore, for other behavioral tests, these two doses were chosen; the dose of 0.3 mg/kg representing more selective response resulting from activation of 5-$HT_{2A}$ receptors and less selective response to the dose of 3 mg/kg, possibly linked with activation of 5-$HT_{2C}$ or 5-$HT_{1A}$ receptors apart from 5-$HT_{2A}$ receptors.

The time of novel object exploration compared with familiar object exploration during the recognition session was observed to be increased in control group, and in the group treated with the lower but not the higher 25B-NBOMe dose (Fig. 6a). Statistical analysis of exploration time of the novel and familiar object during the recognition session showed a significant difference between control and both doses of 25B-NBOMe (Fig. 6a; $F_{2,23} = 278$, $p < 0.0001$). There was a significant difference in the *Ri* between animals treated with the higher dose of 25B-NBOMe and the control group (Fig. 6b; $F_{2,23} = 139$, $p < 0.0001$). Importantly, the *Ri* in the control and low-dose 25B-NBOMe-treated group reached ca. 72% and 68%, respectively, while in the 25B-NBOMe 3 mg/kg group, it was 45% (Fig. 6b).

Scopolamine (1 mg/kg, ip) increased the time of novel object exploration during the recognition session in comparison to control and to animals treated with 25B-NBOMe (3 mg/kg) (Fig. 7a). Statistical analysis of novel object exploration time in recognition session showed a significant difference between treatment groups ($F_{3,27} = 40$, $p < 0.0001$). There was also a reversal of 25B-NBOMe-induced decrease in *Ri* by scopolamine (Fig. 7b; $F_{3,27} = 18$, $p < 0.01$).

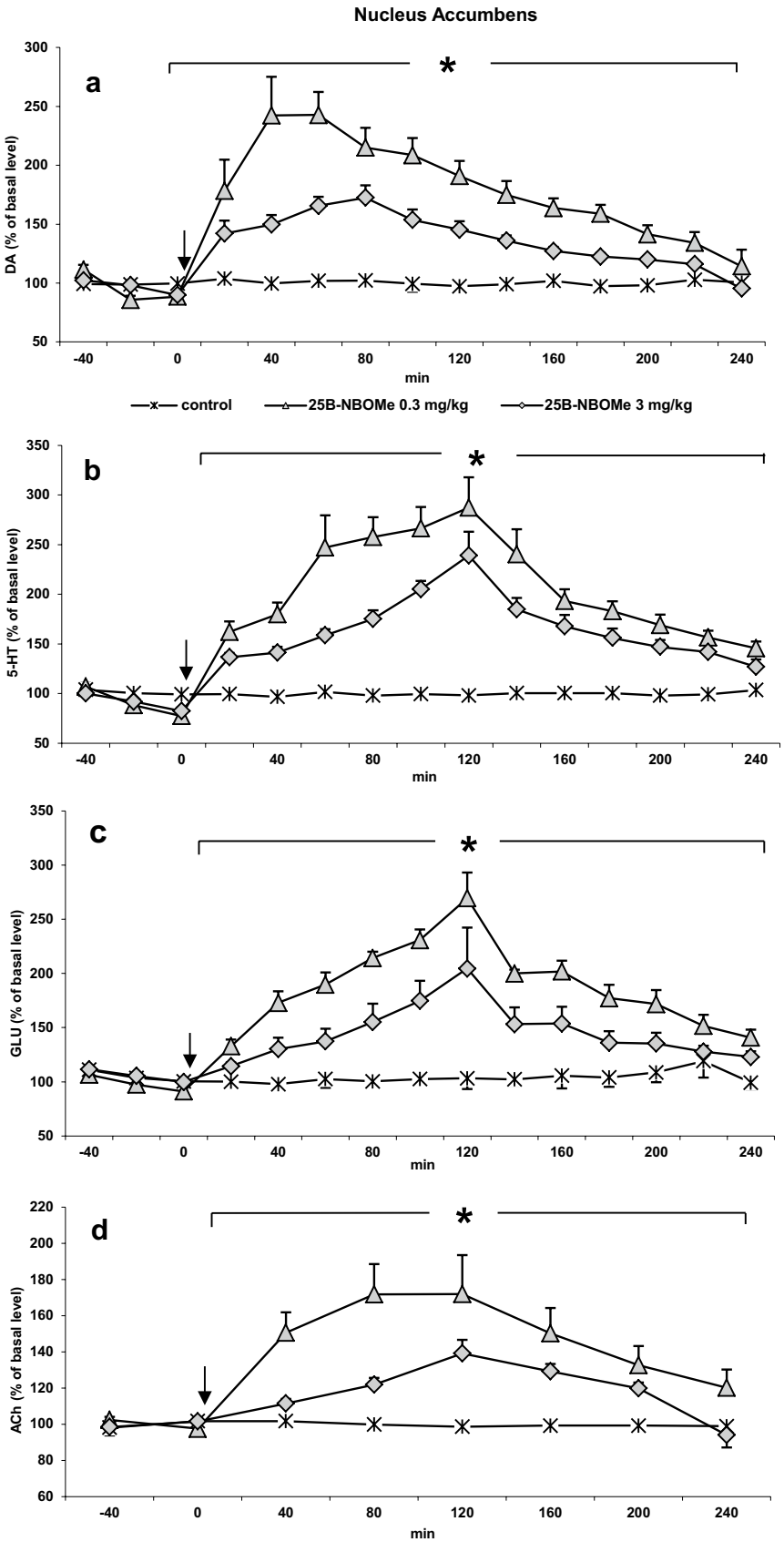


**Fig. 3** The time-course effect of 25B-NBOMe on extracellular levels of **a** dopamine (DA), **b** serotonin (5-HT), **c** glutamate (GLU), and **d** acetylcholine (ACh) in the rat nucleus accumbens. Values are the mean ± standard error of the mean (SEM), $n = 6$ per experimental group. For ACh measurements, two consecutive dialysate fractions were pooled. The time of drug injection is indicated with an arrow. The basal extracellular levels were as follows: for DA, 1.05 ± 0.059 nM, $n = 30$; for 5-HT, 0.199 ± 0.01 nM, $n = 30$; for ACh, 19.5 ± 1.34 nM, $n = 30$; for GLU, 2.10 ± 0.13 µM, $n = 30$; $^*p < 0.0002$ vs. control group (repeated measures ANOVA and Tukey's post hoc test)

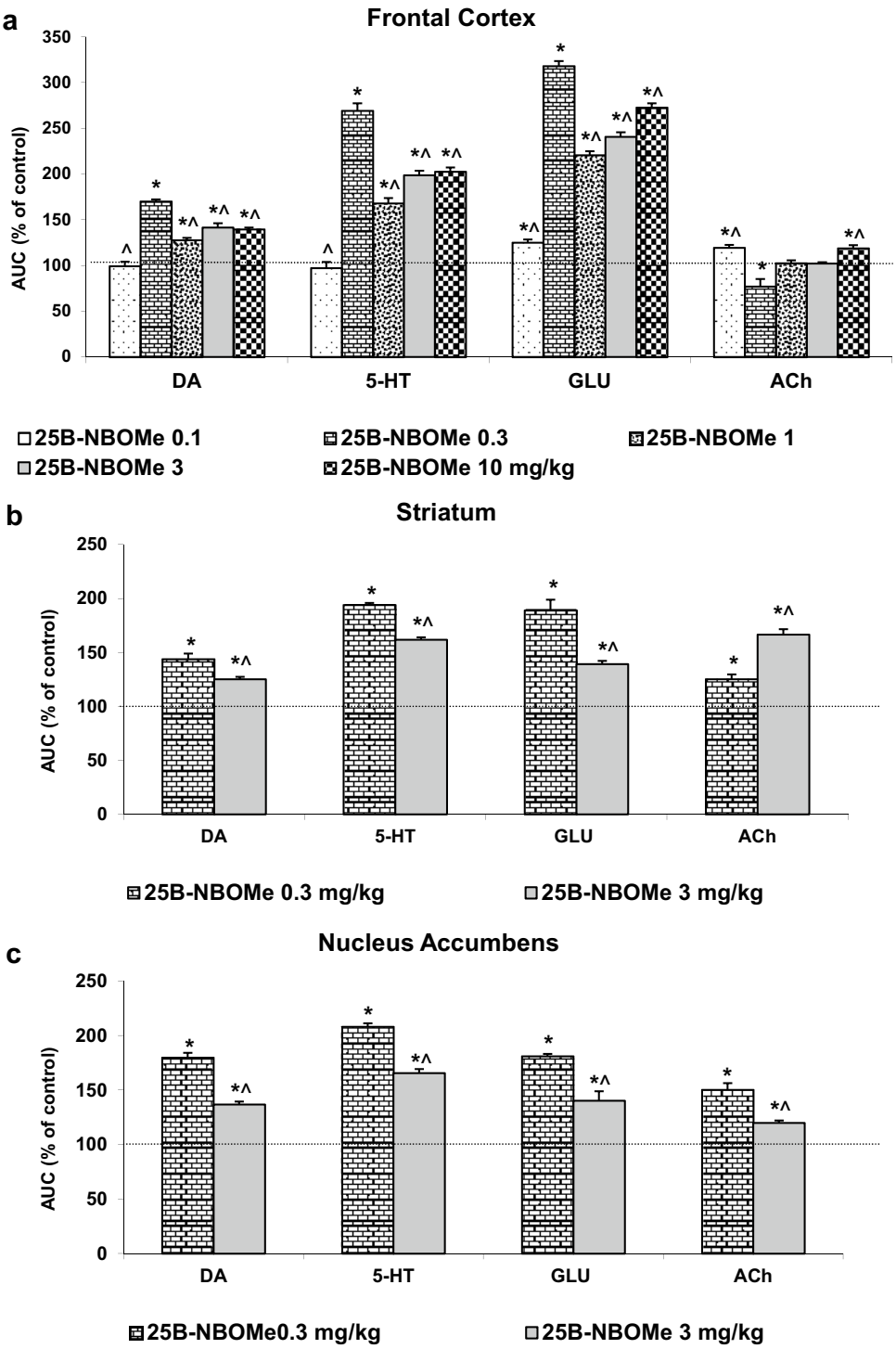


**Fig. 4** The total effect of 25B-NBOMe on extracellular levels of dopamine (DA), serotonin (5-HT), glutamate (GLU) and acetylcholine (ACh) in the rat frontal cortex (**a**), striatum (**b**), and nucleus accumbens (**c**) calculated as an area under the curve (AUC) and expressed as the percent of each basal level. Values are the mean ± standard error of the mean (SEM), $n = 6$ per experimental group. *$p < 0.0002$ vs. control group, ^$p < 0.05$ vs. 0.3 mg/kg (one-way ANOVA and Tukey's post hoc test)

## The Effect of 25B-NBOMe on Locomotor Activity of Rats in the Open Field Test

25B-NBOMe decreased the time of walking ($F_{2,19} = 134$, $p < 0.0001$) and the number of episodes of crossing ($F_{2,19} = 110$, $p < 0.0001$) and peeping ($F_{2,19} = 32$, $p < 0.0001$) in a dose-dependent manner (Fig. 6c). No episodes of rearing and grooming were observed in animals treated with both doses of 25B-NBOMe (data not shown).

Scopolamine (1 mg/kg) increased the time of walking and the number of episodes of crossing in comparison to control, but had no effect on episodes of peeping (Fig. 7c) and did not affect the decrease in these parameters induced by 25B-NBOMe (3 mg/kg). Statistical

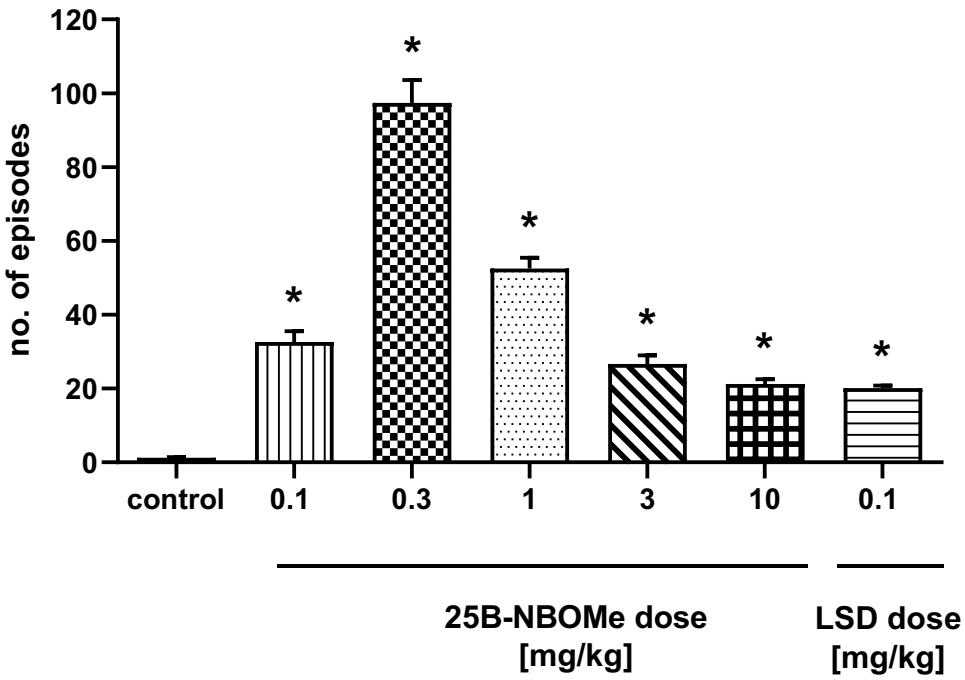


**Fig. 5** The effect of 25B-NBOMe and LSD on head and body twitches (WDS) in rats. The number of episodes counted for 240 min starting immediately after the injection is shown. Values are the mean ± standard error of the mean (SEM), $n = 8$ per experimental group. *$p < 0.0001$ vs. control group (one-way ANOVA and Tukey's post hoc test)

analysis showed a significant effect of treatment groups on walking ($F_{3,23} = 213$, $p < 0.0001$), crossing ($F_{3,23} = 164$, $p < 0.0001$) and peeping ($F_{3,23} = 68$, $p < 0.0001$). No rearing and grooming behavior was observed (data not shown).

### The Effect of 25B-NBOMe on Anxiolytic/Anxiogenic-Like Activity in Rats in the Light/Dark Box Test

The time spent in the dark compartment was longer than in the light zone for all groups of animals (control U = 11, $p < 0.001$; 0.3 mg/kg U = 6, $p < 0.001$; 3 mg/kg U = 0, $p < 0.001$) (Fig. 8a); however, the time was longer and statistically significant in the rats treated with 25B-NBOMe at the dose of 3 mg/kg as compared to control (U = 0, $p < 0.001$). Accordingly, the time spent by all animals in the light zone was shorter and was decreased in the 25B-NBOMe 3 mg/kg group in comparison to control (U = 0, $p < 0.001$). 25B-NBOMe at doses of 0.3 and 3 mg/kg significantly decreased exploration of the dark and light zones. Exploration of the dark zone expressed as ambulatory distance (dark zone: U = 0, $p < 0.001$, U = 0, $p < 0.001$, light zone: U = 2, p < 0.001, U = 0, $p < 0.001$, respectively), vertical (dark zone: U = 0, $p < 0.001$, U = 0, $p < 0.001$, light zone: U = 2, $p < 0.001$, U = 0, $p < 0.001$, respectively) and stereotypical activity (dark zone: U = 15, $p < 0.001$, U = 0, $p < 0.001$, light zone: U = 15, $p < 0.001$, U = 0, $p < 0.001$, respectively) were significantly decreased by both doses of 25B-NBOMe in comparison to control (Fig. 8b, c, d).

### The Effect of 25B-NBOMe on Oxidative DNA Damage in the Rat Frontal Cortex

In contrast to MDMA (10 mg/kg), 25B-NBOMe given at doses of 0.3 and 3 mg/kg produced a minor DNA damage in the rat frontal cortex, shown as a tail moment as measured at 72 h after administration (Fig. 9). The low dose of 25B-NBOMe of 0.3 mg/kg markedly and a dose of 3 mg/kg less potently but still significantly damaged DNA in the rat frontal cortex. The effect of 25B-NBOMe was inversely correlated with the dose, but it was much weaker than that produced by MDMA. We also observed that 72 h after the treatment with 25B-NBOMe in the dose of 0.3 mg/kg a vast amount of nuclear DNA was damaged (95%), like after the treatment with 10 mg/kg of MDMA (99%). However, the level of the 25B-NBOMe-induced damages was by ca. 85 times lower than after MDMA as presented in Fig. 9. Instead, the dose of 3 mg/kg induced small and less frequent (18%) damages in comparison to the 25B-NBOMe in a dose of 0.3 mg/kg.

## Discussion

### Hallucinogenic Activity

The present study demonstrates that 25B-NBOMe is an extremely potent 5-$HT_{2A}$ agonist which induces WDS in rats, although not in a dose-dependent manner. Our data are consistent with other studies concerning NBOMes where an inverted U-shaped dose–response curve for this

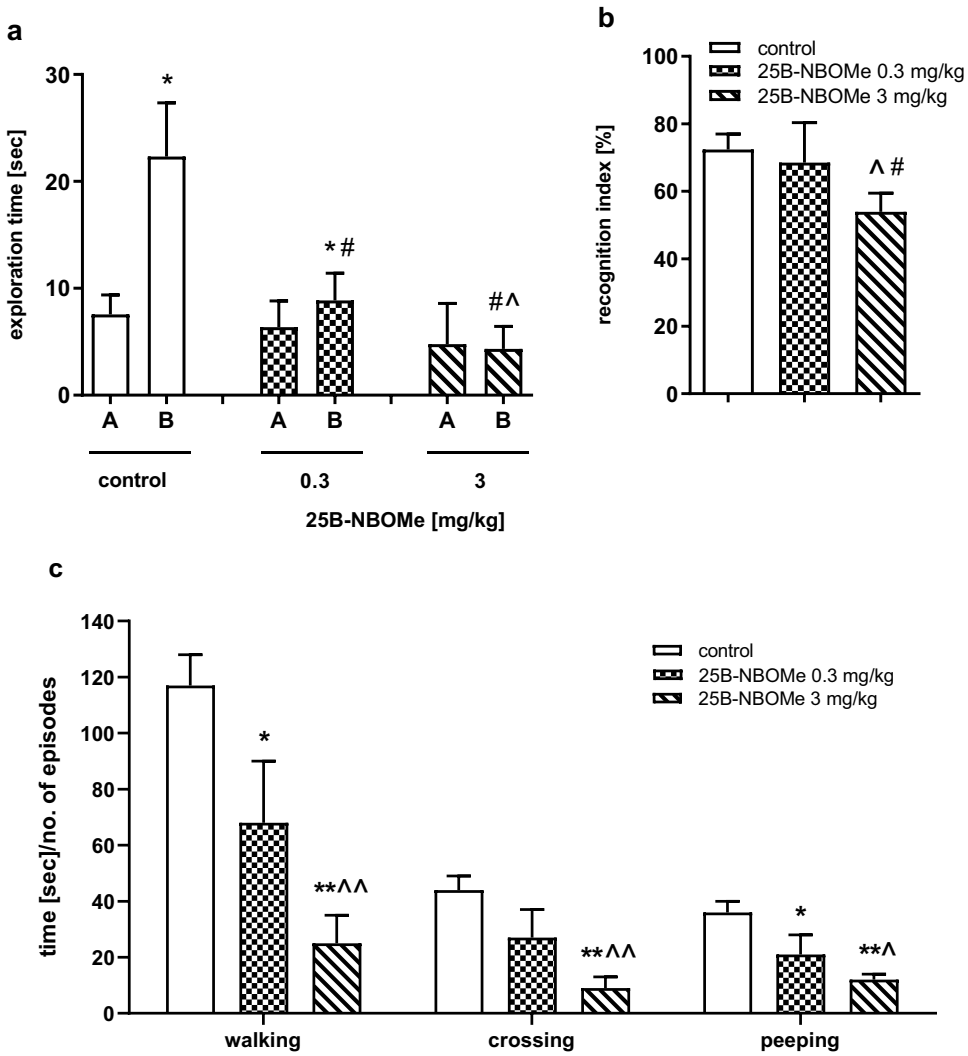


**Fig. 6** The effect of 25B-NBOMe (0.3 and 3 mg/kg) on performance of rats in the novel object recognition (**a**, **b**) test and on locomotor behavior of rats in the open field (**c**) test. **a** Exploration time in the recognition session for the familiar (A) and novel object (B). **b** *Ri* expressed as the time spent on novel object exploration in relation to the total exploration time of both novel and familiar objects. **c** The time spent on walking, the number of episodes of crossing and the number of episodes of peeping. Values are the mean ± standard error of the mean (SEM), $n = 6$–$10$ per experimental group. **a** $^{*}p < 0.05$, $^{**}p < 0.01$ novel vs. familiar object (*t* test); $^{\#}p < 0.01$ vs. control (one-way ANOVA and Tukey's post hoc test); **b** $^{\#}p < 0.01$ vs. control; $^{\wedge}p < 0.01$ vs. 25B-NBOMe 0.3 mg/kg (one-way ANOVA and Tukey's post hoc test); **c** $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control; $^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$ vs. 25B-NBOMe 0.3 mg/kg (one-way ANOVA and Tukey's post hoc test)

effect has been observed (Custodio et al. 2019; Elmore et al. 2018). It is possible that when plasma concentration of NBOMes increases, distinct 5-HT receptor subtypes become activated; it was evidenced by Fantegrossi et al. (2010), Vickers et al. (2001), and Klein et al. (2018) that 5-$HT_{2C}$ and 5-$HT_{1A}$ receptors activated by higher concentration of NBOMes modulated their effect on WDS. The data from our study suggest that 25B-NBOMe shares hallucinogenic activity with other classical hallucinogens, such as LSD, DOI, or mescaline. However, it has to be taken into account that many 5-$HT_{2A}$ agonists, such as lisuride, fenfluramine, p-chloromethamphetamine, and L-5-hydroxytryptophan, produce WDS in rats or head twitch response (HTR) in mice, but their effect on this behavior is classified as "false-positive response" (Halberstadt and Geyer 2018). The possible mechanism suggested to differentiate hallucinogenic properties from false-positive effect involves recruitment of different transduction signaling pathways by these groups of compounds (González-Maeso et al. 2007). However, the literature data are not fully conclusive in this respect. The specific effector mechanism responsible for WDS/HTR seems to rely on the $G_{q/11}$-PLCβ cascade resulting in phosphoinositide (PI) hydrolysis and mobilization of intracellular $Ca^{2+}$ ions (Halberstadt and Geyer 2018). However, this signaling cascade is activated by lisuride and other non-hallucinogenic 5-$HT_{2A}$ agonists stronger than by LSD. Furthermore, the study of Moreno et al. (2011) indicates that glutamate mGlu2 receptors and formation of mGlu2 and 5-$HT_{2A}$ receptor heterodimers are required for head twitch behavior. Therefore, other studies are necessary to determine whether WDS/HTR is a valid model of hallucinogenic activity. Regarding hallucinogenic activity of NBOMes, clinical observations and several case studies reported hallucinations after recreational use of 25I-NBOMe (Kyriakou et al. 2015).

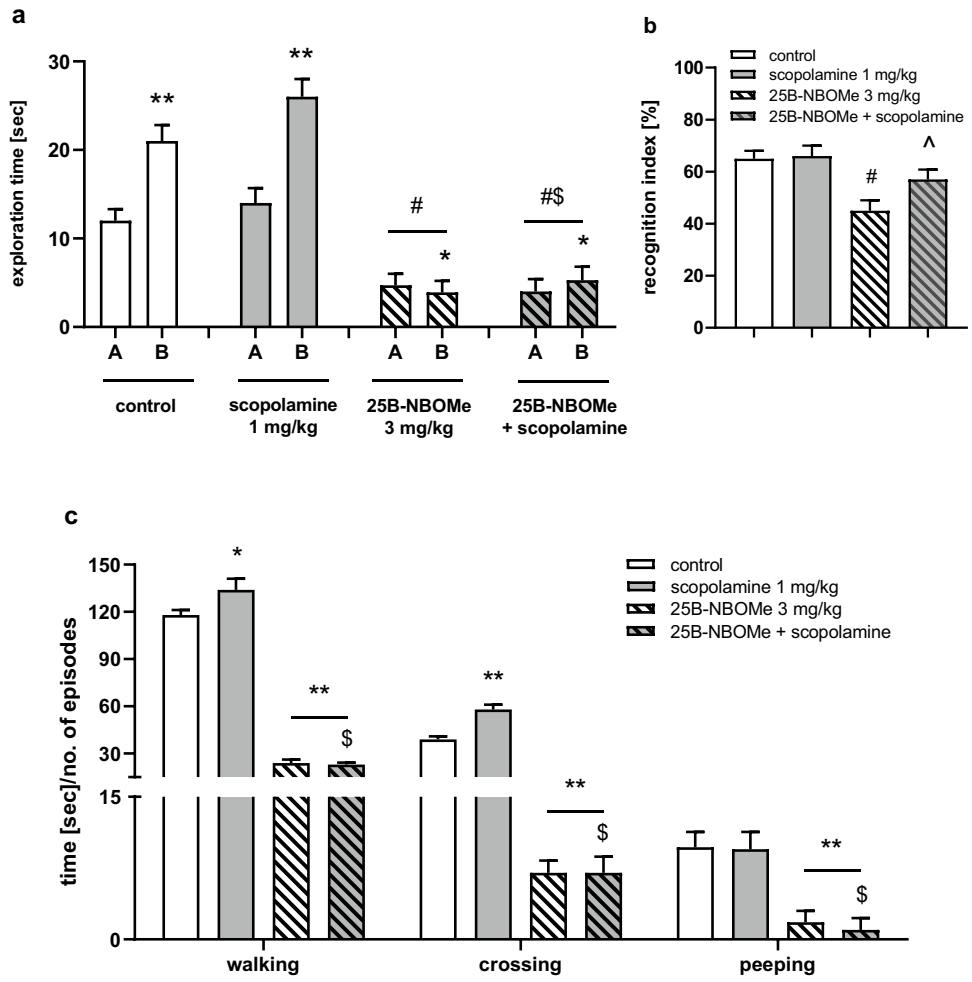


**Fig. 7** The effect of scopolamine (1 mg/kg) on 25B-NBOMe (3 mg/kg)-induced changes in rats' performance in the novel object recognition (**a**, **b**) test and locomotor activity in the open field (**c**) test. **a** Exploration time in the recognition session for the familiar (A) and novel object (B). **b** *Ri* expressed as the time spent on novel object exploration in relation to the total exploration time of both the novel and familiar objects. **c** The time spent on walking, the number of episodes of crossing, and the number of episodes of peeping. Values are the mean ± standard error of the mean (SEM), $n = 6$–12 per experimental group. **a** $^{*}p < 0.05$, $^{**}p < 0.01$ novel vs. familiar object (*t* test); $^{\#}p < 0.01$ vs. control; $^{\$}p < 0.01$ vs. scopolamine (one-way ANOVA and Tukey's post hoc test); **b** $^{\#}p < 0.01$ vs. control; $^{\wedge}p < 0.01$ vs. 25B-NBOMe 0.3 mg/kg (one-way ANOVA and Tukey's post hoc test); **c** $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control; $^{\$}p < 0.01$ vs. scopolamine (one-way ANOVA and Tukey's post hoc test)

## The Effect on Cortical Glutamate Level

Administration of 25B-NBOMe increased extracellular glutamate levels in all studied brain regions as already shown for LSD and DOI (Muschamp et al. 2004; Scruggs et al. 2003). It is consistent with the hypothesis that hallucinogens stimulate cortical glutamate release via 5-$HT_{2A}$ receptor, as shown earlier for DOI, and this effect was blocked by the selective antagonist of this receptor, MDL 100,907 (Scruggs et al. 2003). In our study, the dose of 0.3 mg/kg was the most effective in increasing glutamate release in the frontal cortex, while all the other doses were less potent, but their influence was still significant in comparison to control. Thus, 25B-NBOMe effect on WDS and glutamate release exhibits an inverted U-shaped dose–response curve, just as evidenced earlier for 25I-NBOMe (Herian et al. 2019). The weaker effect of 25I-NBOMe on WDS and glutamate release may be explained by its lower activation potency as shown by Rickli et al. (2015). The inverted U-shaped dose–response curve for inducing glutamate release may be related with negative contribution of 5-$HT_{2C}$ receptors. It is interesting to note that cortical GABA levels were increased by systemic DOI administration (Abi-Saab et al. 1999), which is consistent with the hypothesis that a subset of 5-$HT_{2C}$ receptors located on GABAergic interneurons may modulate the effect of 25B-NBOMe on glutamate release as demonstrated in our study.

## The Effect on Glutamate Level in the Nucleus Accumbens and Striatum

Glutamate release was increased by 0.3 and 3 mg/kg 25B-NBOMe doses in the striatum and nucleus accumbens. The effect was not dose-dependent as the higher dose was weaker in increasing glutamate release in both brain regions. 5-$HT_{2A}$ receptors are broadly expressed not only in the cerebral cortex but also in the nucleus accumbens and caudate nucleus. Receptor binding studies demonstrate relatively high levels of 5-$HT_{2A}$ receptors in the striatum, but its mRNA levels are very low (Bubser et al. 2001). Most 5-$HT_{2A}$ receptors seem to be localized on striatal afferents arising mainly from the cortex and globus pallidus but not in the substantia nigra. It has been suggested that 5-$HT_{2A}$ receptors localized on cortico-striatal

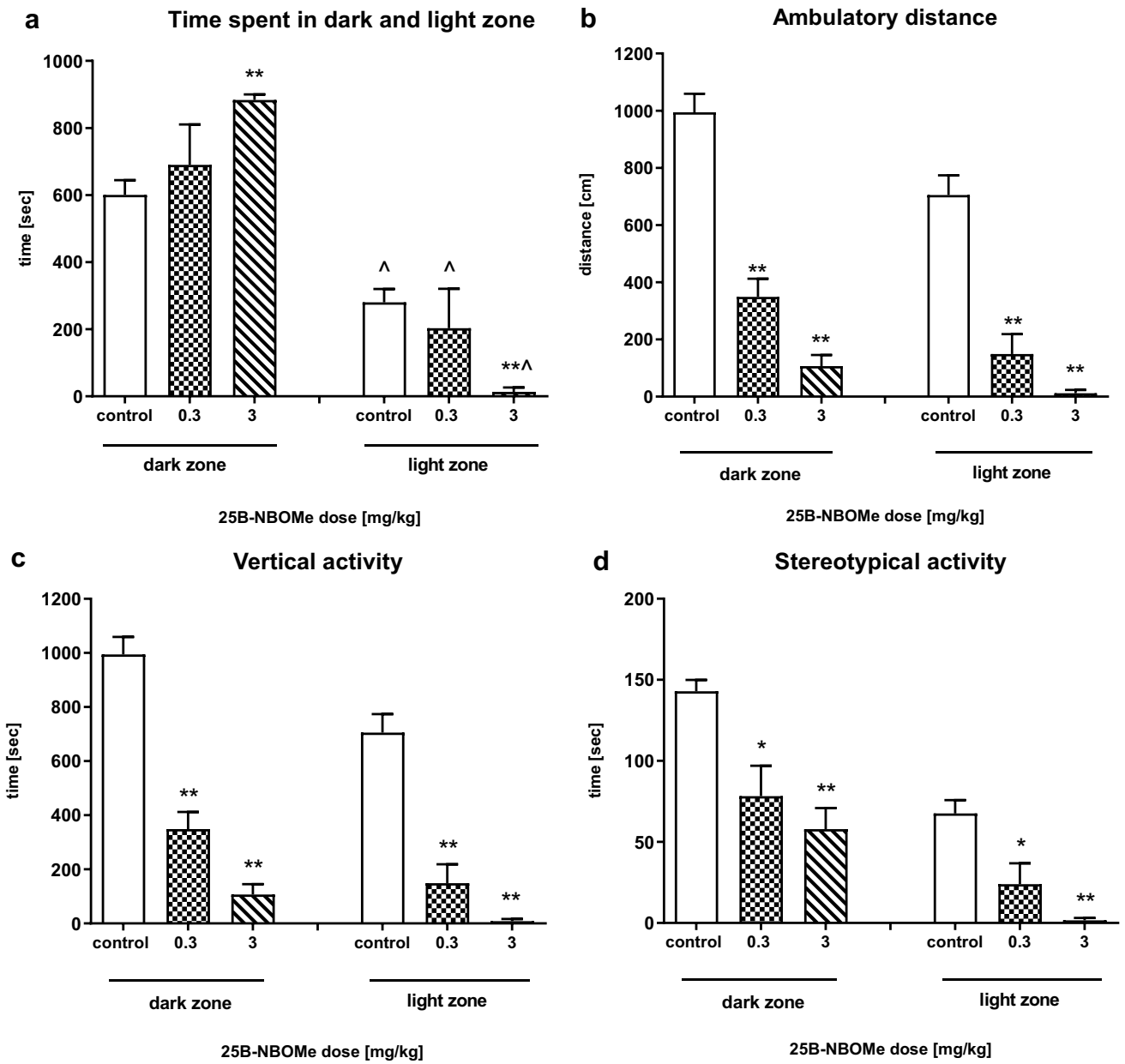


**Fig. 8** The effect of 25B-NBOMe (0.3 and 3 mg/kg) on activity of rats in the light/dark box test. **a** The time spent in the dark and light zone; **b–d** ambulatory distance, vertical, and stereotypical activity, respectively in the dark and light zone. Values are the mean ± standard error of the mean (SEM), $n = 8$ per experimental group. *$p < 0.05$, **$p < 0.01$ vs. control, ^$p < 0.001$ light vs. dark zone (Mann–Whitney's test)

axons can regulate glutamatergic activity in the striatum (Ansah et al. 2011). Similarly, 5-$HT_{2A}$ receptors localized on pyramidal cells projecting to the nucleus accumbens may be responsible for glutamate release in this region (Aghajanian and Marek 1997). Modulatory role of 5-$HT_{2C}$ receptors may contribute to a weaker effect of the higher 25B-NBOMe dose on glutamate level in the nucleus accumbens.

## The Effect on DA and 5-HT in the Frontal Cortex

The prefrontal cortex is reciprocally connected with the VTA by dopaminergic afferents and glutamatergic efferents. The observed increase in cortical extracellular DA levels may be mediated through long-distance axons of pyramidal neurons (Di Matteo et al. 2008; Pehek et al. 2006; Soiza-Reilly and Commons 2011) projecting to VTA cells. These

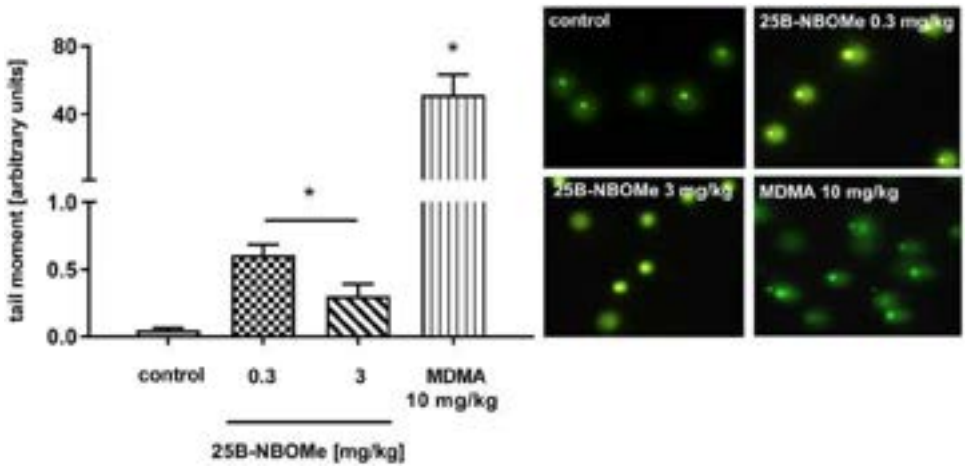


**Fig. 9** The effect of 25B-NBOMe (0.3, 3 mg/kg) and MDMA (10 mg/kg) on the oxidative damage of DNA in the nuclei from the rat frontal cortex. Data are the mean ± SEM ($n = 6$ animals per group) and represent tail moment shown as the product of the tail length and the fraction of total DNA in the tail. Typical microscopic images of nuclei from control, 25B-NBOMe- and MDMA-treated rats; $^{*}p < 0.01$ in comparison to control group (*t* test). DNA damage is presented in arbitrary units

axons, by forming synaptic contact with dopamine neurons, may increase DA release by cortical 5-$HT_{2A}$ receptors. This hypothesis was confirmed by studies which showed that 5-$HT_{2A}$ receptor-mediated stimulation of corticotegmental projections resulted in enhanced glutamate release in the VTA, which subsequently induced DA efflux in the prefrontal cortex (Kalivas et al. 1989; Kalivas 1993; Pehek et al. 2006). Pyramidal neurons may also participate in control of serotonergic activity. The activation of 5-$HT_{2A}$ receptors localized in layer V pyramidal neurons projecting to the dorsal raphe may be responsible for the hallucinogen-induced increase in cortical 5-HT release (Martín-Ruiz et al. 2001). On the other hand, 5-$HT_{2C}$ receptors expressed on GABAergic cells in the deep layers of the prefrontal cortex may exert inhibitory tone on pyramidal neurons by GABA release. This mechanism may account for a non-linear dose–response curve for DA and 5-HT releaset observed in our study (Nocjar et al. 2015).

## The Effect on DA and 5-HT in the Nucleus Accumbens and Striatum

Glutamatergic pathways from the prefrontal cortex modulate DA release in the nucleus accumbens by acting on dopamine cells in the VTA (Taber et al. 1995). However, this glutamate pathway does not seem to synapse directly on mesolimbic DA cells. Therefore, an indirect pathway was proposed involving cholinergic neurons located in the pedunculopontine tegmentum (PPT) and/or laterodorsal tegmentum (LDT), which would stimulate dopamine neurons in the VTA projecting to the nucleus accumbens (Semba and Fibiger 1992). Thus, activation through 5-$HT_{2A}$ receptors of both, direct and indirect glutamatergic pathways from the frontal cortex to the nucleus accumbens may be responsible for 25B-NBOMe-induced DA release in this region. A similar effect on DA neurons in the nucleus accumbens for another compound belonging to the same group of psychedelics, i.e., 25I-NBOMe was shown by Miliano et al. (2019). Few studies have evidenced a role of 5-$HT_{2A}$ receptors in nigrostriatal DA release. The 5-$HT_{2A}$ agonist DOI potentiated amphetamine-stimulated DA release in the striatum (Ichikawa and Meltzer 1995; Yamamoto et al. 1995). Furthermore, psilocybin, by binding to 5-$HT_{2A}$ receptors increased striatal DA release in humans (Vollenweider et al. 1999). Thus, this data are in line with our observations that the increase in striatal DA release induced by 25B-NBOMe may be mediated by similar mechanism. The 5-$HT_{2C}$ receptors localized on GABAergic interneurons in the VTA and substantia nigra (Alex and Pehek 2007) and activated by higher doses of 25B-NBOMe may tonically inhibit DA release in the nucleus accumbens and striatum. The cells in the raphe nuclei region are a target of descending glutamatergic pathways from the cortex (Martín-Ruiz et al. 2001). Thus, 25B-NBOMe acting at 5-$HT_{2A}$ receptors located on pyramidal cells increased 5-HT release from neuronal terminals in the nucleus accumbens and striatum. The inverted U-shaped dose–response curve observed in 25B-NBOMe effect on 5-HT release in both brain regions may be explained by inhibitory influence of GABA interneurons activated by higher 25B-NBOMe doses.

The DA striatal/accumbal transmission may also be regulated by ACh, the levels of which are increased in both brain regions. It was reported that cholinergic $M_5$ receptors on DA neuronal terminals enhanced DA release, while $M_2$/$M_4$ autoreceptors on cholinergic terminals inhibited ACh release and subsequent nicotinic nACh receptor-dependent DA release (Shin et al. 2015).

## The Effect on ACh Level in the Frontal Cortex

Basal forebrain projections comprise a majority of cholinergic innervation to the cortex (Lebois et al. 2018). It generally appears that 5-HT exerts a stimulatory influence on the release of ACh (Saito et al. 1996). The effect of stimulation

of 5-HT receptor subtypes in vivo remains unclear. In our study, the dose of 0.3 mg/kg of 25B-NBOMe decreased basal extracellular ACh level, but the doses of 0.1 and 10 mg/kg increased cortical ACh levels, while doses of 1 and 3 mg/kg of 25B-NBOMe were not effective. Authors of another study showed that the 5-$HT_{2A/2C}$ agonist DOI as well as mescaline enhanced ACh release in the rat mPFC (Nair and Gudelsky 2004). On the other hand, 5-HT has been shown to inhibit cholinergic neurons in the pedunculopontine and dorsolateral tegmental neurons which express 5-$HT_{2A}$ receptors (Koyama and Kayama 1993). Thus, the effect of 5-HT on the cholinergic system depends upon the receptor localization, making the regulation very complex.

## The Effect on ACh Level in the Nucleus Accumbens and Striatum

The striatum and nucleus accumbens contain numerous cholinergic interneurons (Meredith and Wouterlood 1990). The dose-dependent increase in ACh release in the striatum by 25B-NBOMe, observed in our study, is likely mediated by 5-$HT_{2A}$ receptors (Blomely and Bracci 2005; Bonsi et al. 2007). Moreover, there are DA and ACh relationships in the subcortical brain regions. DA inhibits ACh release acting at $D_2$ receptors expressed in cholinergic interneurons (Straub et al. 2014). The lower inhibitory effect of the higher 25B-NBOMe dose on DA striatal levels could be also responsible for a lesser inhibition of ACh release via $D_2$ receptors in the rat striatum. On the other hand, inhibition of ACh release by the higher 25B-NBOMe dose in the nucleus accumbens could result from weaker stimulation of glutamate inputs to the nucleus accumbens via cortical $D_2$ receptors.

## The Effect on Locomotor Activity in the OF Test

Apart from hallucinogenic activity, psychedelics affect other behaviors. 25B-NBOMe reduced locomotor activity of rats in a dose-dependent manner. The most apparent was a decrease in the number of episodes of walking, crossing, and peeping. This effect was not correlated with changes in concentration of DA, 5-HT, or glutamate as their levels were increased in all studied brain regions. On the other hand, we observed a dose-dependent increase in extracellular ACh levels in the striatum. The GABAergic medium spiny neurons are critical elements in striatal control of animal movement. DA acting through $D_2$ receptors modulates striatal cholinergic interneurons (Straub et al. 2014) leading to regulation of the direct and indirect GABAergic pathways (Gerfen and Surmeier 2011) the activity of which is controlled by muscarinic receptors (Threlfell and Cragg 2011). The results of our study may explain neurochemical mechanism underlying DA/ACh interaction in disruption of motor function by 25B-NBOMe.

## The Effect on Anxiolytic/Anxiogenic-like Activity in the LDB Test

Findings of other authors also showed that phenylethylamine and indoleamine hallucinogens such as mescaline, DOI, DOM or LSD, DMT, and psilocin reduced locomotor activity of rats in unfamiliar environment (Halberstadt and Geyer 2018). It has been suggested that this effect reflects the fear in novel settings and increased center avoidance induced by hallucinogens reminding agoraphobia observed in humans. Our results from the light/dark box test are in agreement with the above findings. In our study, 25B-NBOMe prolonged the time spent by animals in the dark zone, while it decreased the time spent in the light zone. These data suggest that acute 25B-NBOMe doses are likely to induce anxiety in animals. In addition, the decreased ambulatory distance, vertical and stereotypical activity time demonstrates that motor activity of rats was suppressed. This data confirm our findings in the open field test showing a decreasing effect of 25B-NBOMe on exploration of animals.

Several neurotransmitters may be involved in the anxiogenic effect of 25B-NBOMe, which are released into the synaptic cleft after stimulation of serotonin 5-$HT_{2A}$ receptors. Among the most important neurotransmitters which play the role in anxiety, there are GABA, glutamate, 5-HT, and ACh. GABA system regulates neuronal excitability and attenuation of GABAergic system results in anxiety (Nemeroff 2003). Glutamate, as a main excitatory neurotransmitter in the CNS has been shown to play an important role in different brain functions, *inter alia* stress and anxiety (Meldrum 2000). In particular, glutamate in limbic system plays a pivotal role in the pathogenesis of anxiety disorders (Bergink et al. 2004). 5-HT is another important player in the development of anxiety disorders, and an increase in 5-HT concentration in the brain also increases anxiety (Graeff 2002). A role of 5-HT in anxiety is supported by its modulating effect on the locus coeruleus (LC), while fear and stress activate serotonergic pathways (Akimova et al. 2009; Graeff 2002). Moreover, ACh which is mainly engaged in memory and learning processes can be modulated by stress (Deepak et al. 2012), and activation of cholinergic $M_1$ receptors induce anxiety through noradrenergic pathways (Mineur et al. 2013). In our study, we observed changes in the abovementioned neurotransmitters, important in generating the anxiety. 25B-NBOMe being a very potent 5-$HT_{2A}$ receptor agonist, by excitation of cortical pyramidal cells increases glutamate release not only in the frontal cortex, but indirectly, through descending neuronal pathways, in the striatum and nucleus accumbens. Thus, the rise in glutamate release seems to be the main cause of anxiogenic effect of

25B-NBOMe in the LDB test. Furthermore, the increase in 5-HT and ACh release from neuronal terminals in the studied brain regions may be strongly responsible for anxiogenic behavior observed in LDB test. The modulatory impact of GABA resulting from activation by 25B-NBOMe of 5-$HT_{2C}$ receptors located on GABAergic interneurons seems to be less effective in this test; therefore, linear dose–response is observed in this effect.

## The Effect on Cognitive Functions in the NOR Test

ACh is the major neurotransmitter involved in memory, and cholinergic systems in the cortex, striatum, and nucleus accumbens are implicated in cognitive functions (Lebois et al. 2018; Woolf and Butcher 2011). It is known that reduction in the central cholinergic system function occurs in dementia and Alzheimer's disease (Bartus et al. 1982). In our study, we observed different changes in cortical ACh release depending on the 25B-NBOMe dose. ACh release was decreased by the dose of 0.3 mg/kg, and no effect was seen after the dose of 3 mg/kg. The extracellular ACh levels were increased in the striatum and nucleus accumbens, but the response to 25B-NBOMe was dose-dependent only in the striatum. The higher dose of 25B-NBOMe was weaker in increasing ACh level than the lower one in the nucleus accumbens. Interestingly, the exploration of novel object in the NOR test was disturbed by the higher dose of 25B-NBOMe. This observation suggests that 25B-NBOMe may disturb memory processing linking this effect with the modulation of ACh levels in the nucleus accumbens.

In order to study further the role of ACh neurons in the NOR test, rats were co-injected with the non-selective muscarinic receptor antagonist scopolamine. Scopolamine increased exploration time and recognition index decreased by 25B-NBOMe, had no effect on motor activity in the OF test, reduced by 25B-NBOMe, but increased the time of walking alone when compared to control. These data strongly indicate that neuromodulatory ACh system is involved in memory and motor functions and that ACh modulates effects of serotonin receptors activated by 25B-NBOMe. The primary cholinergic input to the cerebral cortex which comes from the basal forebrain complex (Mesulam 1995) activates the pyramidal cells by $M_1$ ACh receptors or via $M_2$ receptors located on GABAergic interneurons (Picciotto et al. 2012). ACh also suppresses cortico-cortical transmission through $M_2$ receptors expressed in pyramidal cell axon terminals (Picciotto et al. 2012). The striatum and its ventral part, nucleus accumbens, contain cholinergic interneurons. The complex synaptic effects of ACh provide mechanism for the ability of ACh to modulate cognitive behaviors. Depending on which receptor is recruited, cortical ACh transmission may generate different responses. Scopolamine commonly used to induce cognitive deficit in animals, in our hands improved rats' behavior in the NOR test. This unexpected effect may be related with neuromodulatory role played by ACh in the CNS. ACh may diffuse within extracellular space through volume transmission on long distances to reach extra-synaptic receptors and may be also co-released with other neurotransmitters, e.g., glutamate or GABA (Colangelo et al. 2019). The observed changes in DA, glutamate, and 5-HT neurotransmission under influence of 25B-NBOMe can be modulated by ACh, and switching between different behavioral states in the presence of scopolamine may occur. The findings of Day et al. (1991) and Durkin et al. (1992) showed an increase in ACh release in the hippocampus, striatum, and frontal cortex of rats and increase in locomotor activity by scopolamine administration. On the other hand, the impaired novel object discrimination by scopolamine was reported by Ennaceur and Meliani (1992). The motor coordination in mice was decreased, and profound deficits in attention and memory were observed in mice treated with scopolamine (Falsafi et al. 2012). Furthermore, increased levels of $M_1$ and NMDA receptors co-localized in hippocampal pyramidal cells were observed in scopolamine treated animals. It is suggested that interaction between $M_1$ and NR1 subunit of NMDA receptor is essential for memory formation and is modified by scopolamine (Falsafi et al. 2012). It is proposed that the central cholinergic system modulates the excitatory transmission and that ACh stimulation of muscarinic receptors potentiates responses of NMDA. Our behavioral data suggest that scopolamine could modulate the response of cholinergic neurons to 25B-NBOMe treatment leading to improvement of cognitive deficit induced by this hallucinogen. However, the exact mechanism of this behavioral impairment needs further studies.

The alternative explanation of results in NOR and other behavioral tests is grounded on an alteration of preference of rats for novelty. Psychedelic drugs have profound effects on the response to novel stimuli. When rats are tested in novel environment, psychedelic drugs alter exploratory and investigatory behavior but have no effect in a familiar environment. The locomotor activity and investigatory behavior observed in the OF test and NOR test have been markedly attenuated by 25B-NBOMe and likely reflected potentiation of the neophobia exhibited by rats in novel settings (Adams and Geyer 1985; Mittman and Geyer 1991; Wing et al. 1990). Reduction of locomotor activity by hallucinogens in a novel environment, but no effect or increase in a familiar environment, has been reported by other researchers (Hillegaart et al. 1996; Ouagazzal et al. 2001; Tilson et al. 1975). In humans, LSD or other hallucinogens markedly enhance reactivity to unpleasant or threatening stimuli (Cohen 1960). Thus, the increased avoidance of novel and open areas observed in rats after administration of hallucinogens may be analogous to the enhanced reactivity to

environmental stimuli observed in humans. Notably, psychedelic drugs markedly enhance the ability of peripheral stimuli to activate the LC (Aghajanian 1980; Rasmussen and Aghajanian 1986), a brain region that functions as a novelty detector. Regional distribution of 5-$HT_{2A}$ and all subtypes of α1-adrenergic receptors are very similar in deep layers of the prefrontal cortex and mRNA of both receptors is abundant in pyramidal and GABAergic neurons (Nichols 2016). Systemic but not local administration of hallucinogens to anaesthetized rats decreased spontaneous activity of LC cells but enhanced the activity of LC neurons evoked by sensory stimuli (Aghajanian 1980; Rasmussen and Aghajanian 1986). As LC sends noradrenergic projections to the cortex, changes in LC firing would also affect pyramidal cells activity. LC reactivity may underlie the mechanism of hallucinogen-induced neophobia, reduction of locomotor activity and fear exhibited by rats in novel settings as observed in the OF and NOR test.

### Neurotoxicity

The observed increase in extracellular DA and glutamate levels may imply neurotoxic effect of NBOMe compounds. In fact, in vitro cytotoxic activity of 25B-NBOMe was demonstrated in primary rat cortical cultures (Zwartsen et al. 2019). However, 25B-NBOMe tested in the comet assay showed only minor damaging effect on DNA in the nuclear fraction from the rat frontal cortex. Interestingly, the 25B-NBOMe doses of 0.3 and 3 mg/kg produced some damage of nuclear DNA which was inversely correlated with the dose. In contrast, MDMA at a dose of 10 mg/kg used as the reference drug induced potent oxidative DNA damage. Oxidative stress and excitotoxicity represent mechanisms causing neuronal damage by MDMA (Cadet et al. 2001). MDMA at the dose of 10 mg/kg has been shown to augment DA and 5-HT release in several regions of rat brain, which may be a source of oxidative stress (Gołembiowska et al. 2016). DNA single- and double-strand breaks were observed in rat and mouse cortex after administration of MDMA (Frenzilli et al. 2007; Górska et al. 2018; Noworyta-Sokołowska et al. 2016). In the present study, the tissue contents of DA, its metabolites DOPAC and HVA and 5-HT and 5-HIAA were not affected by 25B-NBOMe (Table 1 in supplementary data). Similarly, any damaging effect was observed in our recent study with 25I-NBOMe (Herian et al. 2019). It is unclear why the drugs displaying so profound effects on several brain neurotransmitters, particularly on DA, 5-HT, and glutamate extracellular levels are slightly neurotoxic when tested in vivo. However, it is obvious that increased release of glutamate by 5-$HT_{2A}$ agonists from pyramidal cells is accompanied with GABA release from GABAergic interneurons. This effect balances excitation of pyramidal neurons driven by released glutamate (Beique et al. 2007). Our neurochemical data showing the dose of 0.3 mg/kg 25B-NBOMe as the most potent in enhancing glutamate, DA and 5-HT release stays in accordance with the pronounced damage of nuclear DNA caused by this dose. The lack or very weak neurotoxic effect of this class of hallucinogens is in contrast to indoleamine hallucinogens. In our earlier work, we have shown that tryptamine derivative, 5-MeO-DIPT, induced genotoxicity in the comet assay and affected caspase-3 activity and enzymatic defense system (Noworyta-Sokołowska et al. 2019). It is suggested that the underlying mechanism of tryptamine hallucinogen neurotoxicity involves oxidative stress generated by profound accumulation of DA and tryptamine oxidative products and excitotoxicity (Górska et al. 2018). Therefore, other methods need to be used to understand the gap between the in vitro cytotoxic action of NBOMe compounds and the lack or very weak damaging effect in vivo.

## Conclusions

In summary, administration of 25B-NBOMe, a potent agonist of 5-$HT_{2A/C}$ receptors, facilitated DA, 5-HT, glutamate, and ACh release in the rat frontal cortex, striatum, and nucleus accumbens. The enhancement of neurotransmitter levels seems to be regulated in an opposite manner by 5-$HT_{2A}$ and 5-$HT_{2C}$ receptors, which was reflected by a U-shaped dose–response curve. The increased cortical glutamate release may be responsible for hallucinogenic activity of 25B-NBOMe and increased cortico-striatal and cortico-accumbal neurotransmission. The impaired attention and motor activity may depend on changes in extracellular ACh levels in the nucleus accumbens and striatum, respectively. Prolongation of the time spent in the dark zone suggests anxiogenic effect of this compound. In spite of in vitro cytotoxic activity, the in vivo data do not indicate that 25B-NBOMe is evidently neurotoxic.

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### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethical Approval** The use of adult male rats for the experiments was approved by the Local Ethical Commission for Experimentations on Animals in Kraków. This article does not contain any studies with human participants.



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# Hallucinogenic activity, neurotransmitters release, anxiolytic and neurotoxic effects in Rat's brain following repeated administration of novel psychoactive compound 25B-NBOMe

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ABSTRACT

2-(4-Bromo-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)etanoamine (25B-NBOMe) is a highly selective 5-$HT_{2A}$ receptor agonist, exhibiting a potent hallucinogenic activity. In the present study, we investigated the effect of a 7-day treatment with 25B-NBOMe in a dose of 0.3 mg/kg on the following: the neurotransmitter release *in vivo* using microdialysis in freely moving animals, hallucinogenic activity measured in the Wet Dog Shake (WDS) test, anxiety level as measured in the light/dark box (LDB) and locomotor activity in the open field (OF) test, DNA damage with the comet assay, and on a number of neuronal and glial cells with immunohistochemistry. Repeated administration of 25B-NBOMe decreased the response to a challenge dose (0.3 mg/kg) on DA, 5-HT and glutamatergic neurons in the rats' frontal cortex, striatum, and nucleus accumbens. The WDS response dropped drastically after the second day of treatment, suggesting a rapid development of tolerance. LDB and OF tests showed that the effect of 25B-NBOMe on anxiety depends on the treatment and environmental settings. Results obtained with the comet assay indicate a genotoxic properties in the frontal cortex and hippocampus. An increase in immunopositive glial but not neuronal cells was observed in the cortical regions but not in the hippocampus. In conclusion, our study showed that a chronic administration of 25B-NBOMe produces the development of tolerance observed in the neurotransmitters release and hallucinogenic activity. The oxidative damage of cortical and hippocampal DNA implies the generation of free radicals by the drug, resulting in genotoxicity but rather not in neurotoxic tissue damage. Behavioral tests show that 25B-NBOMe exerts anxiogenic effect after single and repeated treatment.

## 1. Introduction

NBOMe compounds, N-2-methoxybenzylated derivatives of 2C hallucinogens, appeared on the illicit drug market in 2010 as a legal substitute for LSD (EMCDDA, 2018). They belong to the phenylalkylamine class of serotonergic hallucinogens and exert their psychoactive effects via activation of the cortical 5-$HT_{2A}$ receptors (Aghajanian and Marek, 1997; Glennon et al., 1984) with a special regard to those located on the apical dendrites of pyramidal cells in layer V and interneurons (Weber and Andrade, 2010). In addition, the presence of 5-$HT_{2A}$ receptors was also reported in the basal ganglia, such as the nucleus accumbens and caudate nucleus, and other subcortical regions like the hippocampus and the amygdala (Bombardi, 2012, 2014). Besides neurons, 5-$HT_{2A}$ receptor immunolabelling was also observed on glial cells: astrocytes (Xu and Pandey, 2000) and on microglia (Glebov et al., 2015). Several studies report that an increased glutamate release is a common mechanism of action for hallucinogens (Herian et al., 2019; Muschamp et al., 2004). Through activation of cortical 5-$HT_{2A}$ receptors, hallucinogens evoke head twitch response (HTR) in rodents, a behavioral marker corresponding with the hallucinogenic effect in humans (Glennon et al., 1984), with only a small number of exceptions (false-positives) (Jaster et al., 2022). HTR behavior in mice is referred to as wet dog shakes

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(WDS) in rats. At higher doses NBOMe compounds might also cause activation of 5-$HT_{2C}$ receptors mainly expressed on GABAergic interneurons which may exert inhibitory tone on pyramidal neurons by GABA release (Nocjar et al., 2015).

2-(4-Bromo-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanoamine (25B-NBOMe) is one of NBOMe derivatives with a high affinity for serotonin 5-$HT_{2A}$ (Ki = 0.05 nM) and 5-$HT_{2C}$ (Ki = 4.6 nM) receptors. High affinity and selectivity towards 5-$HT_{2A}$ receptors of 25B-NBOMe led to the development of [$^{11}$C]-CIMBI-36 used as a positron emission tomography tracer for imaging studies of the human 5-$HT_{2A}$ receptors (Ettrup et al., 2014). 25B-NBOMe also has moderate affinity for the serotonin transporter (SERT; Ki = 840 nM) and dopamine D2 receptors (Ki = 840 nM), but its affinity for dopamine D1 receptor and dopamine transporter (DAT) is low (Rickli et al., 2015). Our earlier study demonstrated that 25B-NBOMe induced a hallucinogenic activity in a wide range of doses, increasing the release of dopamine (DA), serotonin (5-HT), glutamate, and acetylcholine in several brain regions (Wojtas et al., 2021). It also impaired rats' attention and motor activity and was anxiogenic (Wojtas et al., 2021). There is evidence that hallucinogens may have therapeutic efficacy in treating post-traumatic stress disorder, alcohol and drug addiction, anxiety disorders (Bogenschutz and Ross, 2018; Grob et al., 2011) and drug-resistant depression (Carhart-Harris et al., 2017). Reinforcing and rewarding effects through change in dopaminergic system is suggested as 25B-NBOMe increased dopamine D1 receptor levels in the nucleus accumbens while decreasing dopamine D2 receptor and DAT levels in the ventral tegmental area (Custodio et al., 2019). Moreover, it induced conditioned place preference in mice and self-administration in rats (Custodio et al., 2019; Miliano et al., 2019). It has been shown that repeated administration of other hallucinogens such as LSD, DOM, and 25CN-NBOH produce tolerance by down-regulation of the 5-$HT_{2A}$ receptors (Buchborn et al., 2018; Buckholtz et al., 1990; Gresch et al., 2005). Our previous work demonstrated that repeated treatment with another congener of NBOMe series, 25I-NBOMe induced a decline in WDS response and loss of responsiveness of DA, 5-HT and glutamate neuronal pathways, but still hyperactive limbic systems may be underling mechanism of anxiogenic effect of this compound (Herian et al., 2021). However, it is not certain whether 25B-NBOMe compound is equally effective in its effects on neurotransmitter release and anxiety level.

Severe adverse effects of NBOMes and their toxicity (agitation, cardiotoxicity, seizures, serotonin syndrome) have raised public health concerns (Halberstadt et al., 2017; Kyriakou et al., 2015; Suzuki et al., 2015). Furthermore, another congener of NBOMe compounds, 25C-NBOMe, showed more potent than methamphetamine *in vitro* neurotoxicity in SH-SY5Y cells (Xu et al., 2019). The incubation of the rat primary cortical cultures with 25B-NBOMe decreased neuronal activity (Zwartsen et al., 2019), while 25I-NBOMe at single doses decreased neurogenesis (Catlow et al., 2013). Genetic damage resulting from reactive oxygen species (ROS) induction was observed in TK6 cells (Cocchi et al., 2020).

Here, we investigated the effect of chronic administration of 25B-NBOMe on neurotransmitter release in several brain regions using *in vivo* microdialysis and its influence on rats' behavior compared to acute treatment. Anxiogenic/anxiolytic effect was examined in the light/dark box while locomotor activity was examined with the open field test. Drug-elicited wet dog shake response as a marker of hallucinogenic activity was also evaluated. Genotoxic properties of 25B-NBOMe were evaluated using the comet assay. The possible damaging effect was examined by immunohistochemical assessment of cell numbers in the rat brain.

## 2. Methods

### 2.1. Animals

Adult male Wistar-Han rats (280–350 g; Charles River, Germany) were used in all the experiments. The animals were initially acclimatized and housed (6 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 tap water and typical laboratory food (VRF 1, Special Diets Services, UK). All animal use procedures were conducted in a strict accordance with the European regulations for animal experimentation (EU Directive, 2010/63/EU on the Protection of Animals Used for Scientific Purposes). The Local Ethics Commission approved the experimental protocols for Experimentation on Animals (permit numbers: 186/2017 and 188/2017).

### 2.2. Drugs and reagents

2-(4-Bromo-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25B-NBOMe) was purchased from Cayman Chemical Company (Michigan, USA), ketamine and xylazine hydrochlorides from Biowet Puławy (Puławy, Poland). All necessary chemicals of the highest purity used for analysis by high-performance liquid chromatography (HPLC) were obtained from Merck (Warszawa, Poland). O-phthalaldehyde (OPA) of Sigma-Aldrich (Poznań, Poland) was used for the derivatization of glutamate to an electroactive compound. The chemicals used for the alkaline comet assay were from Trevigen (Gaithersburg, MD, USA) and Merck (Warszawa, Poland). The reagents used in immunohistochemistry came from Sigma Aldrich (Poznań, Poland), Vector Laboratories (Burlingame, CA, USA), and Proteintech (Manchester, UK).

One group of animals received a once-daily subcutaneous (sc) injection of 25B-NBOMe at a dose of 0.3 mg/kg/day for seven days. The second group received 0.9% NaCl solution in the same manner, but on the seventh day the animals were injected with one dose of 0.3 mg/kg of 25B-NBOMe. The LDB and OF tests were conducted on separate groups of animals 20 min after the last injection. For WDS measurements, the response of rats to 25B-NBOMe injections was compared to the day "0" without injection. Other groups of animals were injected with 25B-NBOMe 0.3 mg/kg or saline for seven days and were implanted with microdialysis probes 6–8 h after the last injection. The next day, during the microdialysis experiments, rats received a challenge dose of 0.3 mg/kg of 25I-NBOMe.

25B-NBOMe was dissolved in 0.9% NaCl solution. The dose was chosen based on our previous studies (Wojtas et al., 2021); it affected the DA, 5-HT, and glutamate systems in the most potent way amongst all studied doses and brain regions. The subcutaneous injection of 25B-NBOMe was selected as it seemed to be a favorable manner of administration in the case of this group of compounds, compared to intraperitoneal injection, as shown by Halberstadt and Geyer (2018).

### 2.3. Brain microdialysis

75 mg/kg and 10 mg/kg of ketamine and xylazine, respectively, were injected intramuscularly to anesthetize animals. Microdialysis probes (MAB 4.15.3Cu, MAB 4.15.4Cu, MAB 4.15.2Cu, AgnTho's AB, Sweden) were implanted into the following brain structures using the determined coordinates (mm): frontal cortex AP +2.7, L +0.8, V −6.5, striatum AP +1.2, L +2.8, V −7.0, and nucleus accumbens AP +1.6, L +1.0, V −8.0; from the dura (Paxinos and Watson, 1998). The implantation of microdialysis probes in rats receiving multiple injections was performed after the last injection. The following day, probe inlets were connected to a syringe pump (BAS, West Lafayette, IN, USA) which delivered artificial cerebrospinal fluid composed of (mM): 147 NaCl, 4 KCl, 2.2 $CaCl_2 \cdot 2H_2O$, 1.0 $MgCl_2$ at a flow rate of 2 μL/min. Five baseline samples were collected every 20 min after the washout period of 2 h. A challenge dose (0.3 mg/kg) of 25B-NBOMe was administered, and dialysate fractions were collected for the next 240 min. As the experiment ended, the rats were sacrificed, and their brains underwent histological examination to validate the probe placement.

### 2.4. Extracellular concentration of DA, 5-HT and glutamate

Extracellular DA and 5-HT levels were analyzed using an Ultimate 3000 System (Dionex, USA), electrochemical detector Coulochem III (model 5300; ESA, USA) with a 5020 guard cell, a 5040 amperometric cell, and a Hypersil Gold C18 analytical column (3 μm, 100 × 3 mm; Thermo Fisher Scientific, USA). The details of the method were described elsewhere (Herian et al., 2021; Wojtas et al., 2021).

Glutamate levels in the extracellular fluid were measured by HPLC with electrochemical detection after derivatization of samples with OPA/sulfite reagent to form isoindole-sulfonate derivatives as described previously (Herian et al., 2021; Wojtas et al., 2021).

### 2.5. Wet dog shake test

The rats' behavior, defined as a rapid shaking of the head, neck, and trunk from one side to the other, resembling a wet dog shaking to dry itself, is called a Wet Dog Shake (WDS). The WDS test was carried out based on the procedure reported by Nagayama and Lu (Nagayama and Lu, 1996; Halberstadt and Geyer, 2018). Measurements of WDS were conducted for 80 min after each injection and were expressed as an average of sum values of all episodes during the observation time.

### 2.6. Open field test

The open field test was performed to modify the procedure described by Rogóż and Skuza (2011). A round black arena (1 m in diameter) was virtually divided into eight radiant sections formed by lines intersecting the center of the field. The test was conducted in the dimly lit room, except the middle of the arena, which was illuminated by a 75 W light bulb placed 75 cm above. Rats were placed in the middle of the arena 20 min after 25B-NBOMe subcutaneous injection. Their behavior was recorded for 10 min. The exploration was quantified with the following parameters: time of walking, number of line crossings reflecting ambulatory distance, episodes of looking under the edge of the field (peeping), number of grooming events, number of rearings as vertical activity and time spent in the central zone. The schematic presentation of the open field arena is shown in Fig. 6F.

### 2.7. Light/dark box test

The light/dark box (LDB) test was performed using four computer-controlled Seamless Open Field Arenas for rats (43 × 43 × 30 cm; Med Associates, USA) with 16 infrared emitters and photodetectors on each side of the box. The procedure of Noworyta-Sokołowska et al. (2019) was adapted to the experimental design. A dark insert with a hole divided the chamber into two equally sized compartments: light and dark. An additional light source (220 lm) was placed above the light compartment to be more anxiogenic. Animals were placed in the dark compartment and were allowed to explore the arena freely for 15 min. The measured parameters included: ambulatory distance, vertical and stereotypic activity time, and the time spent in the dark and light compartment. The data were collected using Med State software (Activity monitor, Med Associates).

### 2.8. Alkaline comet assay

The alkaline comet assay was performed with the use of CometAssay® Reagent Kit for Single Cell Gel Electrophoresis Assay. At 72 h after the acute or chronic treatment with 25B-NBOMe (0.3 mg/kg, sc × 7 days), animals were sacrificed by decapitation, and the frontal cortex that consists of the sum of the frontal (FC) and the medial prefrontal cortex (PFC) and hippocampus were dissected. After homogenization and several stages of purification and centrifugation (as described previously in Wojtas et al. (2021), the nuclear suspension was obtained using a sucrose gradient (2.8 M/2.6 M, bottom to top). The nuclear fraction was mixed with low melting point agarose and transferred immediately onto CometSlides™. The following steps, including membrane lysis, DNA unwinding, alkaline electrophoresis, and staining (SYBR® Gold), were carried out according to Trevigen CometAssay® protocol. Stained sections were acquired and analyzed under a fluorescence microscope (Nikon Eclipse50i, Japan) equipped with a camera and NIS Elements software. The data was analyzed using OpenComet software v.1.3, a plugin of ImageJ program v.1.47 (NIH, Bethesda, MD, USA). DNA damage was presented as a tail moment. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected by the comet tail length) and the number of damaged pieces (represented by the intensity of DNA in the tail).

### 2.9. Immunohistochemistry

Animals were deeply anesthetized and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). After 24 h of fixation in 4% PFA (4 °C), 300 μm sections were cut through the frontal cortex (FC) and medial prefrontal cortex (PFC) as well as hippocampus using a VT-1000S vibratome (Leica Microsystems, Heidelberg, Germany). Free-floating sections were processed for single staining of neurons with neuronal-specific nuclear protein (NeuN), glia with specific calcium-binding protein B (S100β), and glial fibrillary acidic protein (GFAP) microglia cells with ionized calcium-binding adaptor molecule 1 (IBA-1) antibodies. Subsequently, brain sections were rinsed and incubated for 1 h in a blocking buffer: 0.01 M PBS containing 0.3% Triton X-100 and 5% average horse, goat, or rabbit serum. After that, the sections were incubated for 48 h at 4 °C with one of the following primary antibodies: monoclonal anti-NeuN (1:1000), monoclonal anti-S100β-subunit (1:1000), polyclonal anti-GFAP (1:500) or polyclonal IBA-1 (1:500) diluted in 0.01 M PBS containing 0.3% Triton X-100 (PBST) and 3% average horse, goat or rabbit serum (depending on used primary antibody). Primary antibody binding was visualized with biotinylated secondary antibodies, the Avidin/Biotin Complex (Vectastain Elite ABC Kit) according to recommended by manufacturer concentration and 3,3′-diaminobenzidine tetrahydrochloride (DAB, 10 mg/50 mL and 0.025% $H_2O_2$) solution to give a brown color to NeuN, S100β and IBA-1-immunoreactive cells. In the case of GFAP, DAB-nickel solution (0.02% DAB + 0.03% $NiCl_2$ in 0.01 M PBS) was used, which resulted in a dark gray color of immunoreactive cells. For quantification digital images were captured using a digital camera CX 9000 (Bioscience Microbrightfield, Inc., German) attached to a Leica microscope (CTR 6000) with 2.5 and 5.0 dry or 63 × and 100× oil objectives (Leica) that was controlled by Stereo Investigator software (Bioscience Microbrightfield, Inc.). For data presentation, stained sections were imaged with an Aperio ScanScope slide scanner (Aperio UK). The final photomicrographs were composed using the Adobe Photoshop program. The numbers of NeuN-, S100β-, GFAP, and IBA-1-immunopositive cells in the analyzed brain regions were estimated using unbiased stereological methods (Maćkowiak et al., 2011; West et al., 1991). Briefly, every sixth (PFC and FC) or eighth (Hp) section from the systematic random sampling along the rostrocaudal axis was analyzed with a 63 × /1.4–0.7 lens using the Stereo Investigator stereology system software. The cells appearing in the upper focal plane were omitted to prevent counting cell caps (-5 μm of the topmost surface of the section). Immunopositive cells were marked within the optical dissectors, which comprised a focal plane of 1600 $\mu m^2$ × 15 μm. The total numbers of NeuN, S100β-, GFAP, and IBA-1-immunopositive cells in the FC, PFC, and hippocampus were automatically calculated by the Stereo Investigator software. In addition, the volume of the cortical regions and hippocampus was calculated using the Cavalieri Estimator option of the Stereo Investigator software.

### 2.10. Data analysis

Drug effects on DA, 5-HT, and glutamate release in the brain regions

were analyzed with repeated measures ANOVA on normalized responses followed by Tukey's post hoc test. All obtained data were presented as a percent of the basal level assumed to be 100%. The WDS test was analyzed using repeated-measures ANOVA followed by Tukey's post hoc test. The data collected from the LDB test was analyzed with the two-way ANOVA followed by Tukey's post-hoc test. The open-field test, immunohistochemical data, and results obtained in comet assay were analyzed with one-way ANOVA followed by Tukey's post hoc test. The differences were considered significant if $p < 0.05$. The detected outliers were removed from the data set using Grubb's test. All statistical analyses were carried out using STATISTICA v.10 StatSoft Inc. 1984–2011 (USA) and GraphPad Prism v.5.00 GraphPad Software Inc. (USA).

## 3. Results

### 3.1. The effect of acute and chronic administration of 25B-NBOMe on extracellular levels of DA, 5-HT, and glutamate in the rat frontal cortex

25B-NBOMe significantly increased extracellular levels of DA, 5-HT, and glutamate in the rat frontal cortex (Fig. 1 A, B, C). However, the response of DA, 5-HT, and glutamate systems to a challenge dose of 0.3 mg/kg in animals treated repeatedly for 7 days with 25B-NBOMe was weaker. Repeated measures ANOVA showed an effect of treatment ($F(2,15) = 873$, $p < 0.0001$) and time ($F(11,165) = 20$, $p < 0.0001$) on DA levels, and a time × treatment interaction ($F(22,165) = 16$, $p < 0.0001$). There was an effect of treatment ($F(2,25) = 668$, $p < 0.0001$) and time ($F(11,165) = 22$, $p < 0.0001$) on 5-HT levels, and a time × treatment interaction ($F(22,65) = 16$, $p < 0.0001$). Similarly, there was an effect of treatment ($F(2,15) = 1348$, $p < 0.0001$) and time ($F(11,165) = 63$, $p < 0.0001$) on glutamate levels, and a time × treatment interaction ($F(2,165) = 64$, $p < 0.0001$). There were significant differences in DA, 5-HT and glutamate extracellular levels for the acute group vs. control ($p < 0.001$). For the chronic group, there were significant differences in DA, 5-HT, but not in glutamate extracellular levels ($p < 0.001$). There were also significant differences ($p < 0.001$) in DA, 5-HT and glutamate extracellular levels between acute and chronic groups (Fig. 1 A, B, C).

### 3.2. The effect of acute and chronic administration of 25B-NBOMe on extracellular levels of DA, 5-HT, and glutamate in the rat striatum

The enhancement in DA, 5-HT and glutamate levels in response to challenge dose of 0.3 mg/kg was stronger in saline injected rats than in animals treated repeatedly with 25B-NBOMe (Fig. 2 A, B, C). Repeated measures ANOVA showed an effect of treatment ($F(2,15) = 70$, $p < 0.0001$) and time ($F(11,165) = 28$, $p < 0.0001$) on DA levels, and a time × treatment interaction ($F(22,165) = 16$, $p < 0.0001$). There was an effect of treatment ($F(2,15) = 459$, $p < 0.000$) and time ($F(1,165) = 82$, $p < 0.0001$) on 5-HT levels, and a time × treatment interaction ($F(22,165) = 40$, $p < 0.0001$). Similarly, there was an effect of treatment ($F(2,15) = 1484$, $p < 0.0001$), and time ($F(11,165) = 38$, $p < 0.0001$) on glutamate levels, and a time × treatment interaction ($F(22,165) = 44$, $p < 0.0001$). There were significant group differences between the acute and chronic groups for DA, 5-HT, and glutamate ($p < 0.001$). There were also significant differences between acute and control group ($p < 0.001$), but chronic group was significantly different vs. control ($p < 0.001$) only at some time points for DA, 5-HT, and glutamate

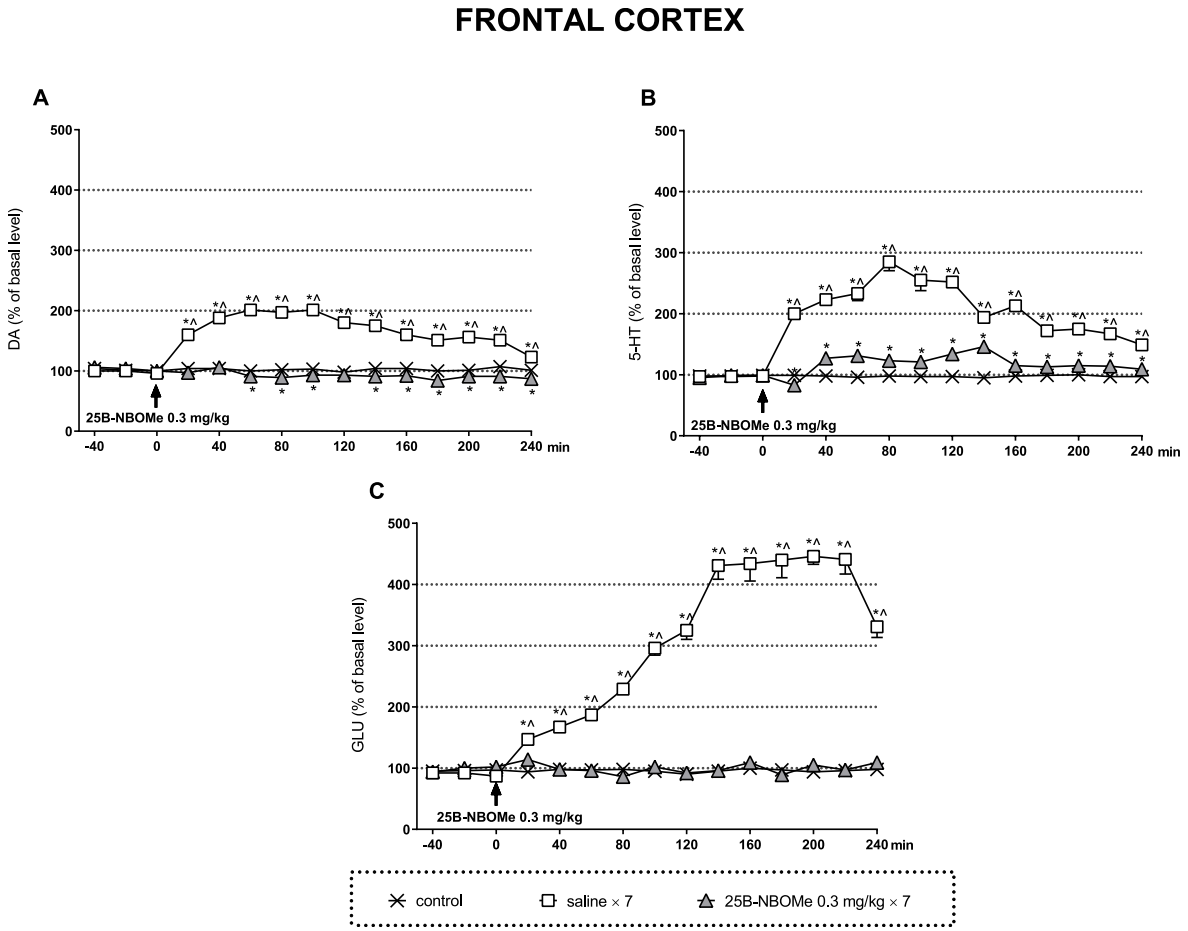


**Fig. 1.** The time-course effect of 25B-NBOMe on extracellular levels of dopamine (DA), serotonin (5-HT), and glutamate (GLU) in the rat frontal cortex (A, B, C), respectively. Values are the mean ± standard error of the mean (SEM), n = 6 per experimental group. The drug injection is indicated with an arrow. *$p < 0.001$ vs. control group; ^ $p < 0.001$ acute vs. chronic administration (repeated measures ANOVA and Tukey's post hoc test).

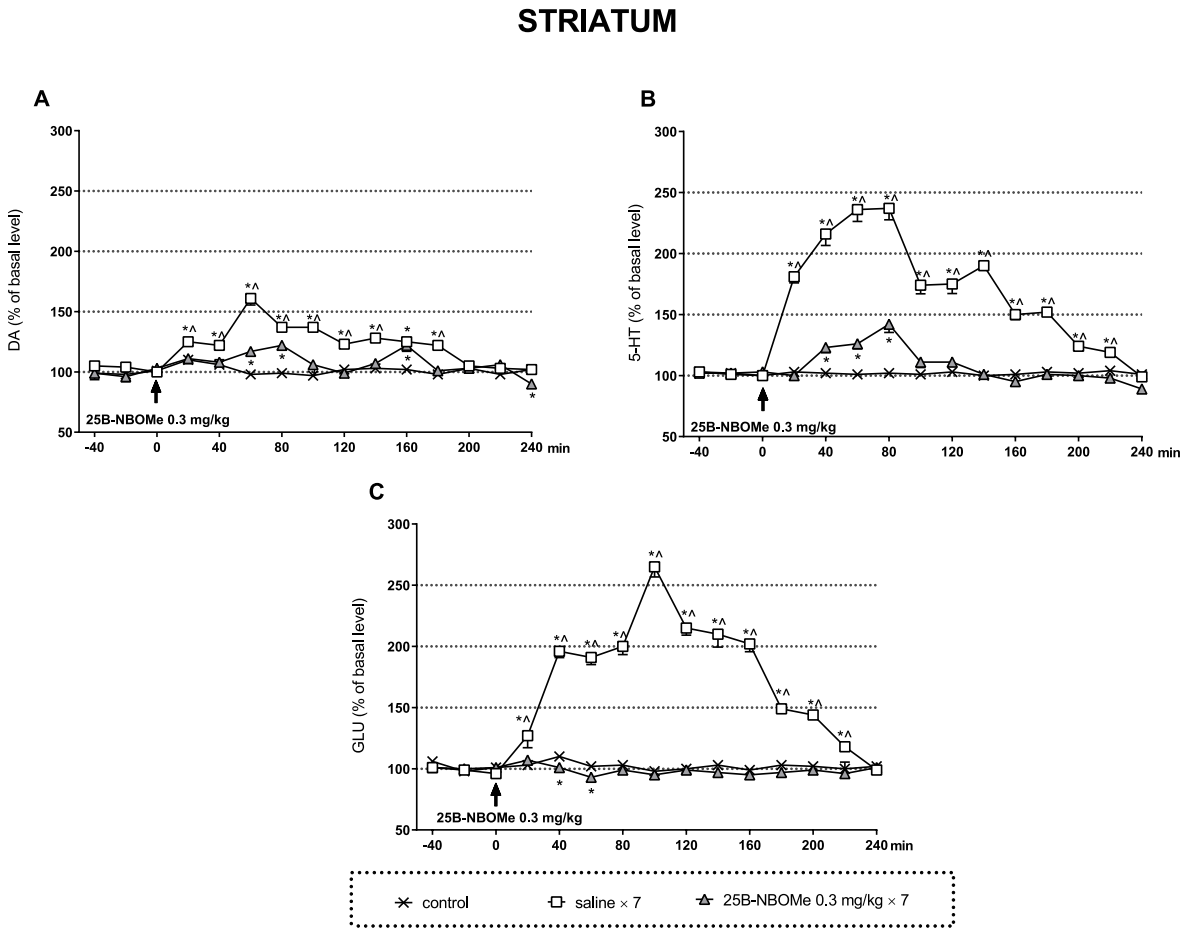


**Fig. 2.** The time-course effect of 25B-NBOMe on extracellular levels of dopamine (DA), serotonin (5-HT), and glutamate (GLU) in the rat striatum (A, B, C), respectively. Values are the mean ± standard error of the mean (SEM), n = 6 per experimental group. The drug injection is indicated with an arrow. *p < 0.001 vs. control group; ^ p < 0.001 acute vs. chronic administration (repeated measures ANOVA and Tukey's post hoc test).

extracellular levels (Fig. 2 A, B, C).

### 3.3. The effect of acute and chronic administration of 25B-NBOMe on extracellular levels of DA, 5-HT, and glutamate in the rat nucleus accumbens

The response of DA, 5-HT and glutamate systems to a challenge dose of 0.3 mg/kg in animals treated repeatedly with 25B-NBOMe was less potent than in saline injected rats (Fig. 3 A, B, C). Repeated measures ANOVA showed an effect of treatment ($F(2,15) = 669$, $p < 0.0001$) and time ($F(11,165) = 48$, $p < 0.0001$) on DA levels, and a time × treatment interaction ($F(22,165) = 19$, $p < 0.0001$). There was also an effect of treatment ($F(2,15) = 1316$, $p < 0.0001$) and time ($F(11,165) = 38$, $p < 0.0001$) on 5-HT levels, and a time × treatment interaction ($F(22,165) = 21$, $p < 0.0001$). The treatment effect on glutamate was significant ($F(2,15) = 33$, $p < 0.0001$), and there was also an effect of time ($F(11,165) = 4.5$, $p < 0.0001$), and time × treatment interaction ($F(22,165) = 8.6$, $p < 0.0001$). There were significant group differences for DA, 5-HT, and glutamate extracellular levels between the acute vs. control group ($p < 0.001$). However, there were significant ($p < 0.001$) group differences for DA, 5-HT but not glutamate extracellular levels (excluding three-time points) between the chronic vs. control group. Moreover, acute vs. chronic groups were significantly different ($p < 0.001$) for DA, 5-HT, and glutamate extracellular levels in nearly all time points (Fig. 3 A, B, C).

### 3.4. The effect of acute and chronic administration of 25B-NBOMe on rats wet dog shake behavior

25B-NBOMe induced the wet dog shake (WDS) response. The effect of repeated administration of 0.3 mg/kg 25B-NBOMe dose for seven days declined after day two and remained at a similar level until day seven (Fig. 4). Repeated measures ANOVA showed a significant effect of treatment ($F(1,42) = 21$, $p < 0.0001$) and time ($F(6,252) = 6.9$, $p < 0.0001$), and a time × treatment interaction ($F(6,252) = 6.2$, $p < 0.0001$).

### 3.5. Effect of acute and chronic administration of 25B-NBOMe on activity of rats in the light/dark (LDB) test

In the LDB test, the time spent in the dark compartment was longer than in the light compartment for control animals and rats administered with a single and chronic injection of 25B-NBOMe dose of 0.3 mg/kg (Fig. 5A). Two-way ANOVA did not reveal a major effect of treatment ($F(2,58) = 0.013$, $p < 0.987$), but showed significant effect of time spent in light and dark compartment ($F(1,58) = 406$, $p < 0.0001$) and significant interaction of both factors ($F(2,58) = 15.9$, $p < 0.0001$). Tukey's post hoc test showed that the time spent in the dark zone in groups treated with a single dose was similar to control but was slightly longer in the group treated with repeated doses of 0.3 mg/kg 25B-NBOMe than in control ($p < 0.002$). Moreover, the time spent in the light zone was significantly decreased in groups of rats administered repeated doses of 25B-NBOMe in comparison to control ($p < 0.004$) (Fig. 5A).

Exploration of the dark zone, expressed as the ambulatory distance,

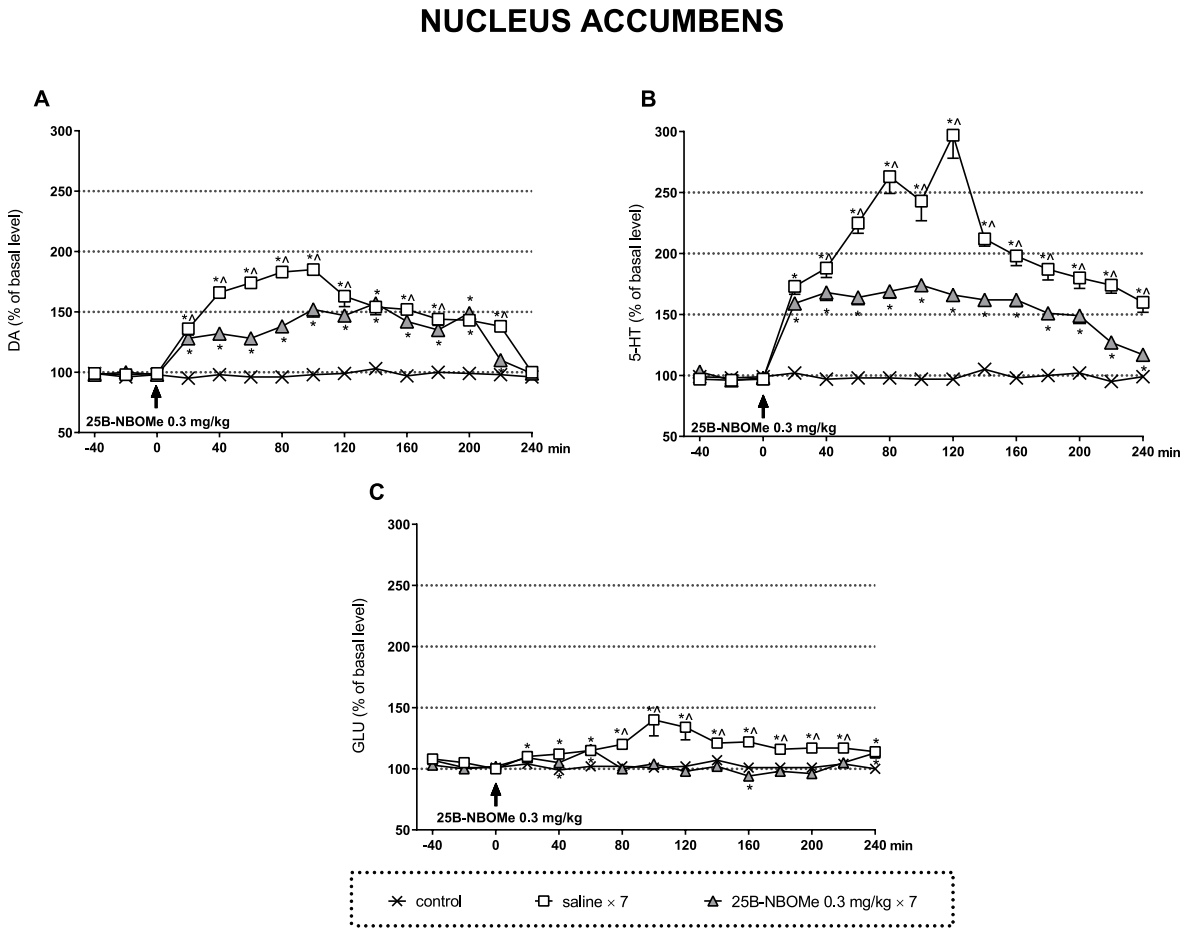


**Fig. 3.** The time-course effect of 25B-NBOMe on extracellular levels of dopamine (DA), serotonin (5-HT), and glutamate (GLU) in the rat nucleus accumbens (A, B, C), respectively. Values are the mean ± standard error of the mean (SEM), n = 6 per experimental group. The drug injection is indicated with an arrow. *p < 0.001 vs. control group; ^ p < 0.001 acute vs. chronic administration (repeated measures ANOVA and Tukey's post hoc test).

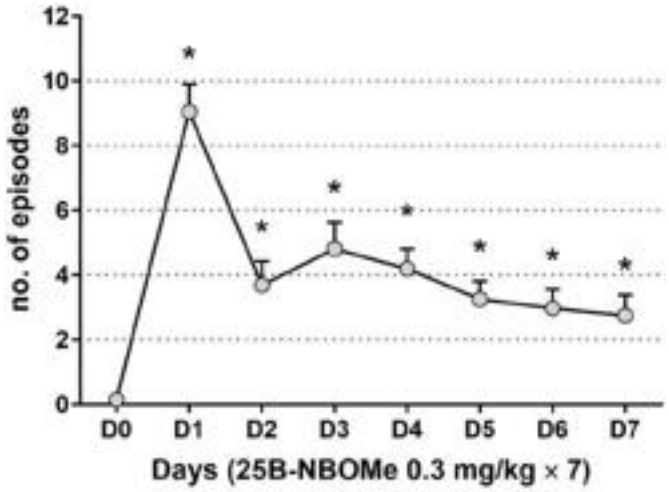


**Fig. 4.** The effect of 25B-NBOMe on wet dog shake response (WDS). The number of WDS episodes was counted for 80 min starting immediately after the injection. Values are the mean ± standard error of the mean (SEM), n = 9 for control and n = 32 in treatment group. *p < 0.0001 vs. control group (repeated measures ANOVA and Tukey's post hoc test).

was longer than in the light compartment in the control group and the rats administered with single and chronic injections of 25B-NBOMe dose of 0.3 mg/kg (Fig. 5B). Two-way ANOVA showed a major effect of treatment ($F(2,58) = 519$, $p < 0.0001$), significant effect of the distance traveled in light and dark compartment ($F(1,58) = 889$, $p < 0.0001$) and significant interaction of both factors ($F(2,58) = 139$, $p < 0.0001$). Tukey's post hoc test showed that the exploration of the dark zone significantly decreased after a single 25B-NBOMe dose of 0.3 mg/kg in comparison to control ($p < 0.001$), while in the group treated repeatedly with 0.3 mg/kg of 25B-NBOMe it significantly increased in comparison to control rats ($p < 0.001$). Exploration of the light zone significantly decreased by both single and repeated doses of 0.3 mg/kg of 25B-NBOMe in comparison to control rats ($p < 0.001$), but the decrease in animals repeatedly treated was weaker in comparison to single administration ($p < 0.001$).

Vertical activity time in the dark zone was significantly different from the light compartment in control and rats administered with chronic injections of 25B-NBOMe dose of 0.3 mg/kg (Fig. 5C). Two-way ANOVA showed a major effect of treatment ($F(2,58) = 1359$, $p < 0.0001$), significant effect of vertical activity in the light and dark compartment ($F(1,58) = 820$, $p < 0.0001$) and significant interaction of both factors ($F(2,58) = 1136$, $p < 0.0001$). Tukey's post hoc test showed that the vertical activity time in the dark zone significantly decreased with a single 25B-NBOMe dose of 0.3 mg/kg in comparison to control ($p < 0.001$), while in the groups repeatedly treated with 25B-NBOMe it significantly increased in comparison to control ($p < 0.001$) and to single administration ($p < 0.001$). In the light zone, vertical activity time was significantly decreased by both single and repeated doses of 25B-NBOMe in comparison to control rats ($p < 0.001$), but the decrease in animals treated repeatedly was weaker in comparison to single administration ($p < 0.001$).

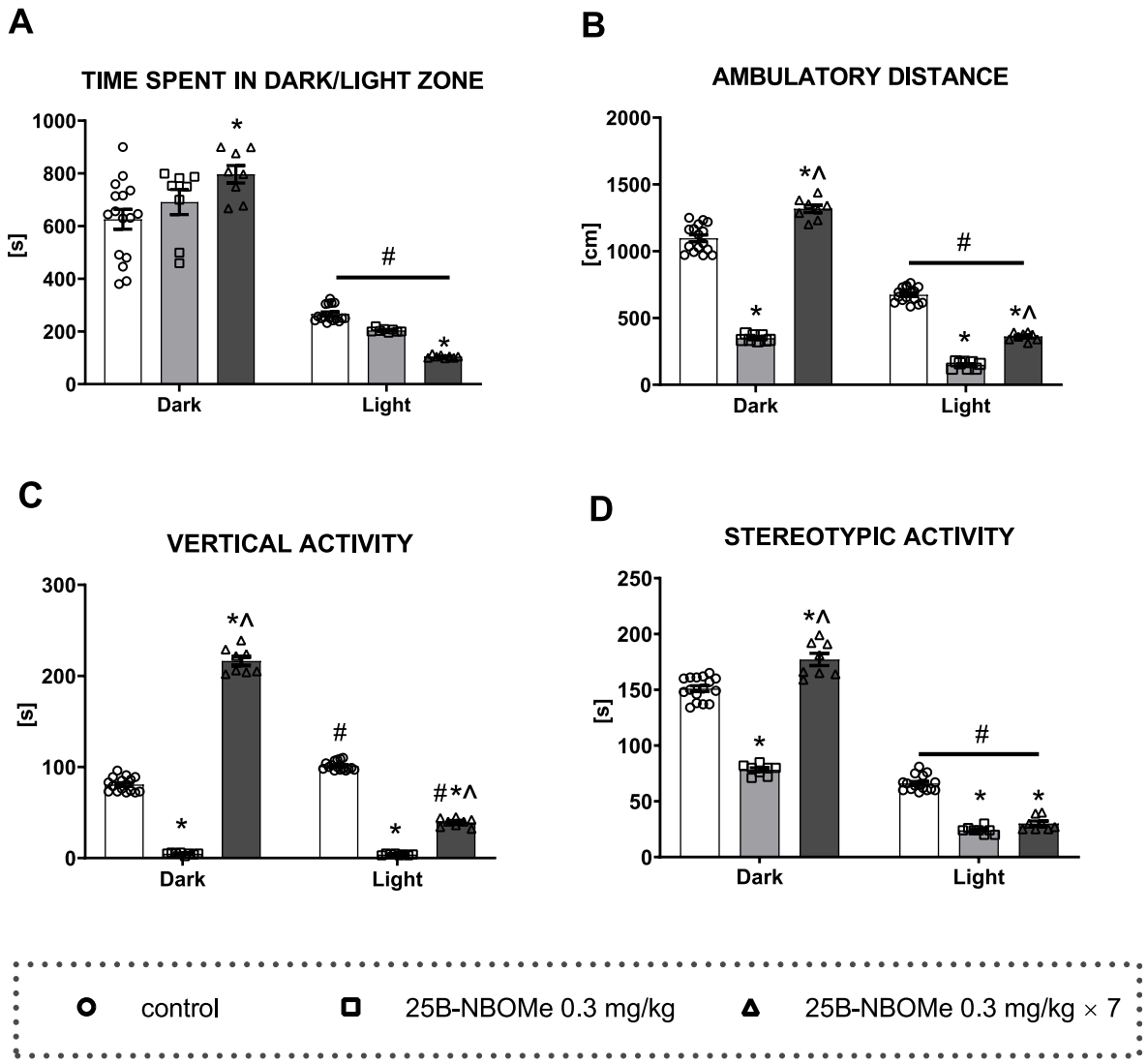


**Fig. 5.** The effect of 25B-NBOMe on the activity of rats in the light/dark box (LDB) test. The time spent in the dark and light zone (A). Ambulatory distance, vertical, and stereotypic activity, respectively (B–D) in the dark and light zone. Values are the mean ± standard error of the mean (SEM), n = 16 for the control group and n = 8 for both acute and chronic group. *p < 0.01 vs. control, ^ p < 0.001 acute vs. chronic; #p < 0.001 dark vs. light (two-way ANOVA and Tukey's post hoc test).

Stereotypic activity time in the light vs. dark zone significantly decreased for control animals and rats administered with a single and chronic injections of 25B-NBOMe dose of 0.3 mg/kg (Fig. 5D). Two-way ANOVA showed a major effect of treatment ($F(2,58) = 243$, $p < 0.0001$), significant effect of stereotypic activity in the light and dark compartment ($F(1,58) = 1695$, $p < 0.0001$) and significant interaction of both factors ($F(2,58) = 118$, $p < 0.0001$). Tukey's post hoc test showed that the stereotypic activity time in the dark zone was significantly decreased by a single 25B-NBOMe dose of 0.3 mg/kg, while in the groups treated repeatedly with 0.3 mg/kg of 25B-NBOMe was significantly increased in comparison to control rats ($p < 0.001$). Stereotypic activity time in the light zone significantly decreased by both single and repeated doses of 0.3 mg/kg of 25B-NBOMe compared to control rats ($p < 0.001$), and no significant differences were reported between both treatments.

### 3.6. The effect of acute and chronic administration of 25B-NBOMe on rats activity in the open field test

Chronic administration of 25B-NBOMe decreased the time of walking ($F(2,48) = 10.1$, $p < 0.001$) and the number of crossings reflecting ambulatory distance ($F(2,48) = 8.8$, $p < 0.001$). The number of episodes of peeping and rearing reflecting vertical activity was decreased by acute and chronic administration ($F(2,48) = 5.6$, $p < 0.01$ and $F(2,48) = 11.4$, $p < 0.001$, respectively). There were no significant differences between acute and chronic treatment in the number of episodes of peeping and rearing (Fig. 6 C, D, respectively). In contrast to single doses which showed nearly complete avoidance of central parts ($F(2,37) = 5.62$, $p < 0.007$), no difference between control and repeated 25B-NBOMe treatment was observed (Fig. 6E). No grooming episodes were observed in all experimental groups.

### 3.7. Effect of acute and chronic administration of 25B-NBOMe on DNA damage in the rat frontal cortex and hippocampus

Repeated but not single treatment with 0.3 mg/kg of 25B-NBOMe produced DNA damage by ROS in the rat frontal cortex ($F(2,21) = 7.99$, $p < 003$) and hippocampus ($F(2,21) = 113$, $p < 0.0001$), presented as a percent of tail moment at 72 h after the drug administration (Fig. 7A and B, respectively).

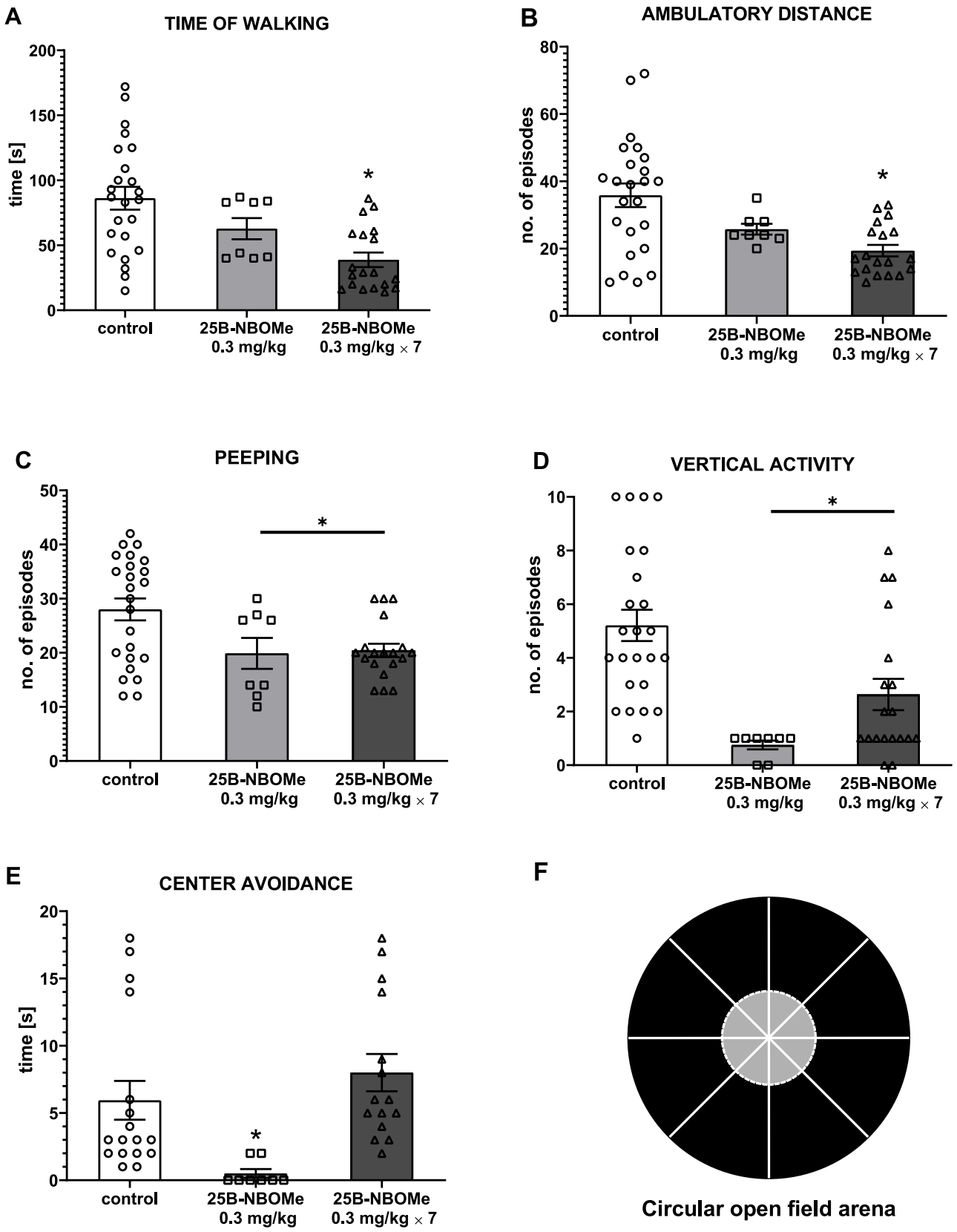


**Fig. 6.** The effect of acute and chronic 25B-NBOMe administration on time of walking (A), ambulatory distance (B), and number of peeping i.e. episodes of looking under the edge of the field (C), vertical activity (D), center avoidance (E) of rats in the open field test. Schematic presentation of the open field arena is shown in panel F. Values are the mean ± standard error of the mean (SEM), n = 24 for the control, n = 8 and 19 for the acute and chronically treated animals, respectively. *$p < 0.05$ vs. control (one-way ANOVA and Tukey's post hoc test).

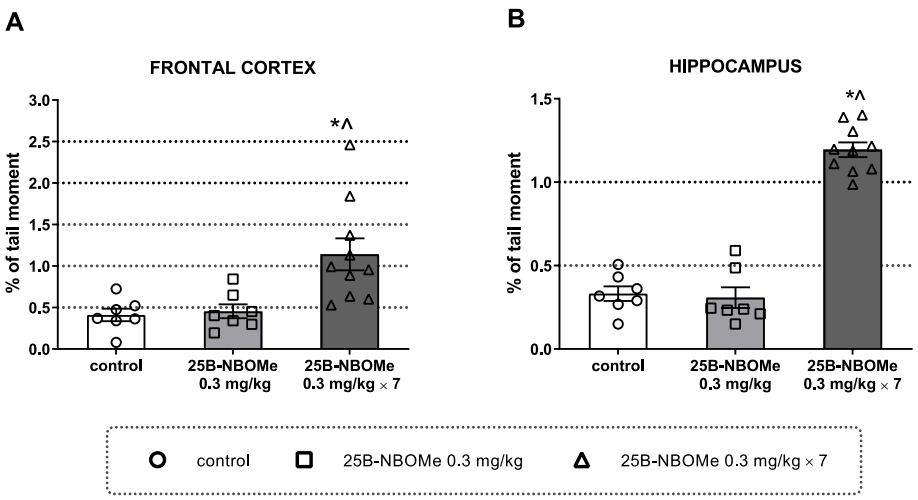


**Fig. 7.** The effect of 25B-NBOMe on the oxidative damage of DNA in the nuclei from rat frontal cortex (A) and hippocampus (B). Data are the mean ± standard error of the mean (SEM), n = 7 for the control and acute group, n = 10 for the chronic group and represent tail moment shown as the product of the tail length and the fraction of total DNA in the tail. *p < 0.01 in comparison to control group; ^ p < 0.01 acute vs chronic (one-way ANOVA and Tukey's post hoc test).

### *3.8. Cortical and hippocampal cells number after 25B-NBOMe administration*

The usage of antibodies specifically recognizing neurons (NeuN), astrocytes (S100, GFAP) and microglia (IBA-1) allowed assessment of the changes in the immunopositive cells number in the medial prefrontal cortex (PFC), frontal cortex (FC) and hippocampus (Figs. 8 and 9, respectively). The number of IBA-1- ($F(1,10) = 6.6$, $p < 0.03$), S100β- ($F(1,10) = 4.8$, $p < 0.05$) and GFAP-immunopositive cells ($F(1,10) = 81$, $p < 0.0001$) was significantly increased in PFC after repeated administration of 25B-NBOMe (Fig. 8 D, F, H). The IBA-1, S-100β and GFAP-immunopositive cells significant increase ($F(1,10) = 4.8$, $p < 0.05$; $F(1,10) = 32$, $p < 0.0002$; $F(1,10) = 9.5$, $p < 0.01$, respectively) was observed in FC of animals treated chronically with 25B-NBOMe (Fig. 8 D, F, H). We did not notice any significant change in the number of NeuN cells in the PFC ($F(1,10) = 0.76$, $p < 0.40$) and FC ($F(1,10) = 2.99$, $p < 0.11$) (Fig. 8B). On the other hand, immunohistochemical staining did not show any changes in the number of neuronal and glial cells in all analyzed hippocampal regions: CA1, CA3/CA2, and dentate gyrus (DG) (Fig. 9 B, D, F and H).

## 4. Discussion

In summary, in the current study we observed developing tolerance to the effect of repeated doses of 25B-NBOMe on neurotransmitters release in various brain regions and in hallucinogenic activity. Additional effects included anxiety symptoms, decrease in locomotor activity, genotoxicity and an increased number of glial cells.

### *4.1. The effect of repeated administration of 25B-NBOMe on DA, 5-HT, and glutamate levels in all studied brain regions*

The observed increase in extracellular glutamate levels after administration of a single dose of NBOMe compounds is most likely to be explained by the activation of cortical 5-$HT_{2A}$ receptors located on pyramidal neurons, a mechanism shared by all known psychedelic drugs (Aghajanian and Marek, 1997; Glennon et al., 1984; Weber and Andrade, 2010). The increase in DA release in all studied regions may result from activation of 5-$HT_{2A}$ receptors located in the ventral tegmental area and substantia nigra, which leads to activation of both mesocortical, mesolimbic, and nigrostriatal pathways (Nichols, 2016). It is also known that serotonergic dorsal raphe cells, which send projections to the prefrontal cortex, nucleus accumbens, and striatum (Di Matteo et al., 2008), are controlled by cortical pyramidal cells (Martin-Ruiz et al., 2001). It seems likely that the increased 5-HT levels in the frontal cortex, in the nucleus accumbens, and striatum most probably result from an indirect release mediated through elevated levels of glutamate (Frånberg et al., 2012; Martin-Ruiz et al., 2001; Sakashita et al., 2015; Vollenweider and Kometer, 2010).

Contrary to acute administration, the chronic treatment with 25B-NBOMe decreased the response of neuronal systems to a challenge dose of 0.3 mg/kg, a phenomenon most likely explained by the rapid development of tolerance due to the desensitization of 5-$HT_{2A}$ receptor (Nichols, 2016). The observed effect was very efficacious, as it practically brought the extracellular levels of DA, 5-HT, and glutamate to the baseline levels. However, it has to be noted that less tolerance was observed for DA and 5-HT neurons in the nucleus accumbens. The presence of elevated DA levels in the nucleus accumbens after a seven-day treatment corresponds to increased DA levels and D1 receptor expression, and decreased D2 receptor expression in mice (Custodio et al., 2019). This data supports the idea of slight reinforcing effects of 25B-NBOMe. Reinforcing effects on CPP test and rewarding effects on the self-administration test in mice were also evidenced for 25N-NBOMe by Seo et al. (2019). Authors suggest that the abuse potential of 25N-NBOMe may be due to changes in expression of D2 receptors, DAT and TH through increases in DA levels in the nucleus accumbens. As another representative of the NBOMe group, the 25I-NBOMe elicited similar effects on studied neurotransmitters (Herian et al., 2021) suggesting that this may be a common feature of all NBOMe compounds.

### *4.2. The effect of repeated administration of 25B-NBOMe in the wet dog shake test*

25B-NBOMe shares hallucinogenic activity with other classical hallucinogens such as LSD, DOI, and mescaline. Our former study (Wojtas et al., 2021) showed its' activity in the wet dog shake test in a wide range of doses. Several data indicate that hallucinogens exert their psychoactive effect, a phenomenon parallel to hallucinations in humans, via activation of cortical 5-$HT_{2A}$ receptors (Aghajanian and Marek, 1997; Glennon et al., 1984). HTR in mice induced by DOI completely blocked by a selective antagonist of 5-$HT_{2A}$ receptor M100907, but not by the highly selective 5-$HT_{2C}$ antagonist SB 242084, confirmed that the behavior is mediated by the 5-$HT_{2A}$ receptor and not by the 5-$HT_{2C}$ receptor (Vickers et al., 2001). Interestingly, WDS response to 25I-NBOMe in rats was reduced not only by M100907, but also by 5-$HT_{2C}$ antagonist SB242084 (Herian et al., 2020). Thus, 5-$HT_{2C}$

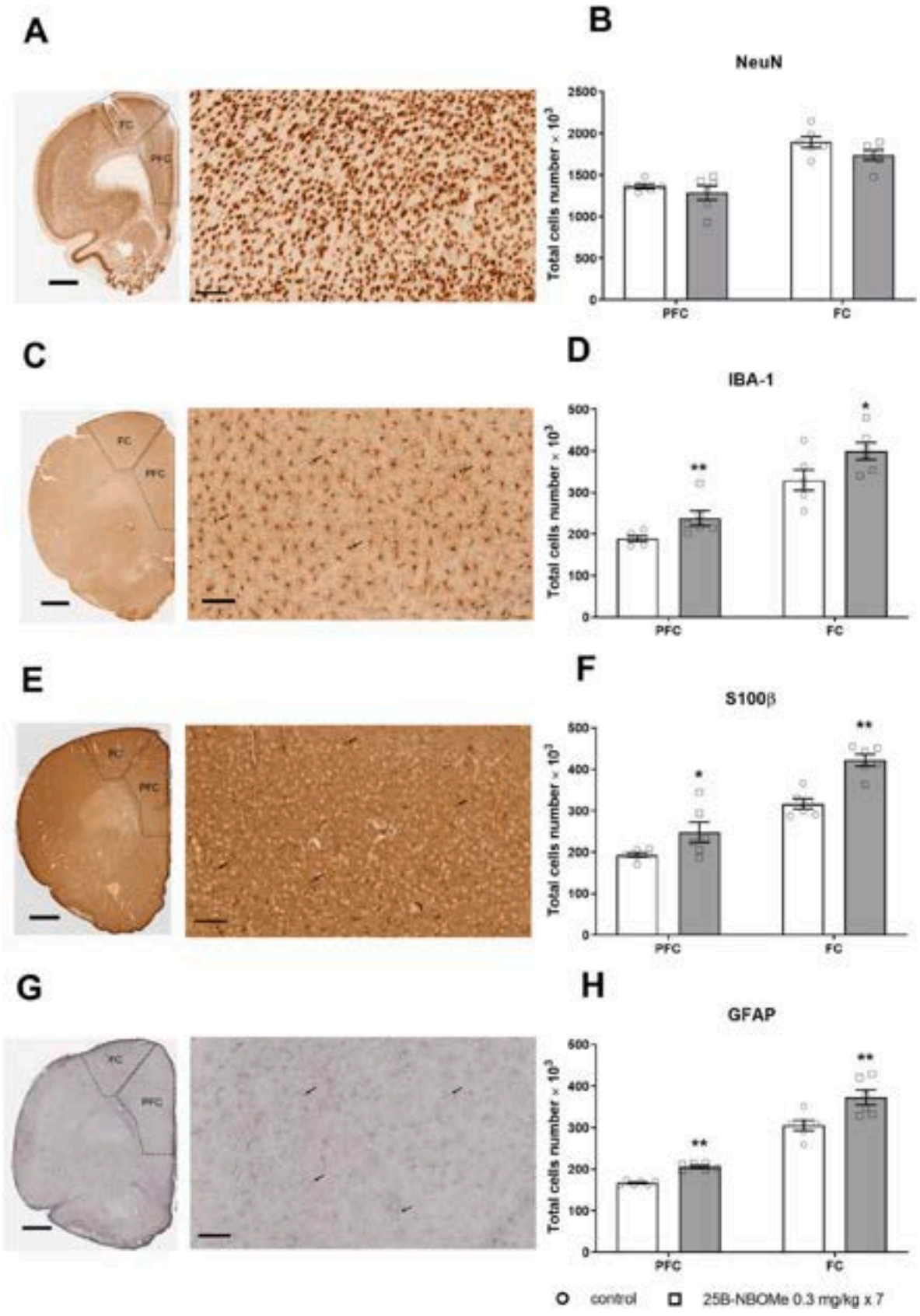


**Fig. 8.** The number of neurons and glial cells in the medial prefrontal cortex (PFC) and frontal cortex (FC). Light photomicrographs that were immunoprobed for NeuN (A), IBA-1 (C), S100β (E), and GFAP (G) and examined for the number of immunopositive neurons (B), microglia (D), and astrocytes (F and H). The graph bars indicate the mean ± standard error of the mean (SEM), n = 6 per experimental group. The scale bars represent 1 mm and 100 μm. The arrows show examples of cells immunopositive for NeuN, IBA-1, S100β and GFAP. *$p < 0.05$ vs control (one-way ANOVA and Tukey's post hoc test).

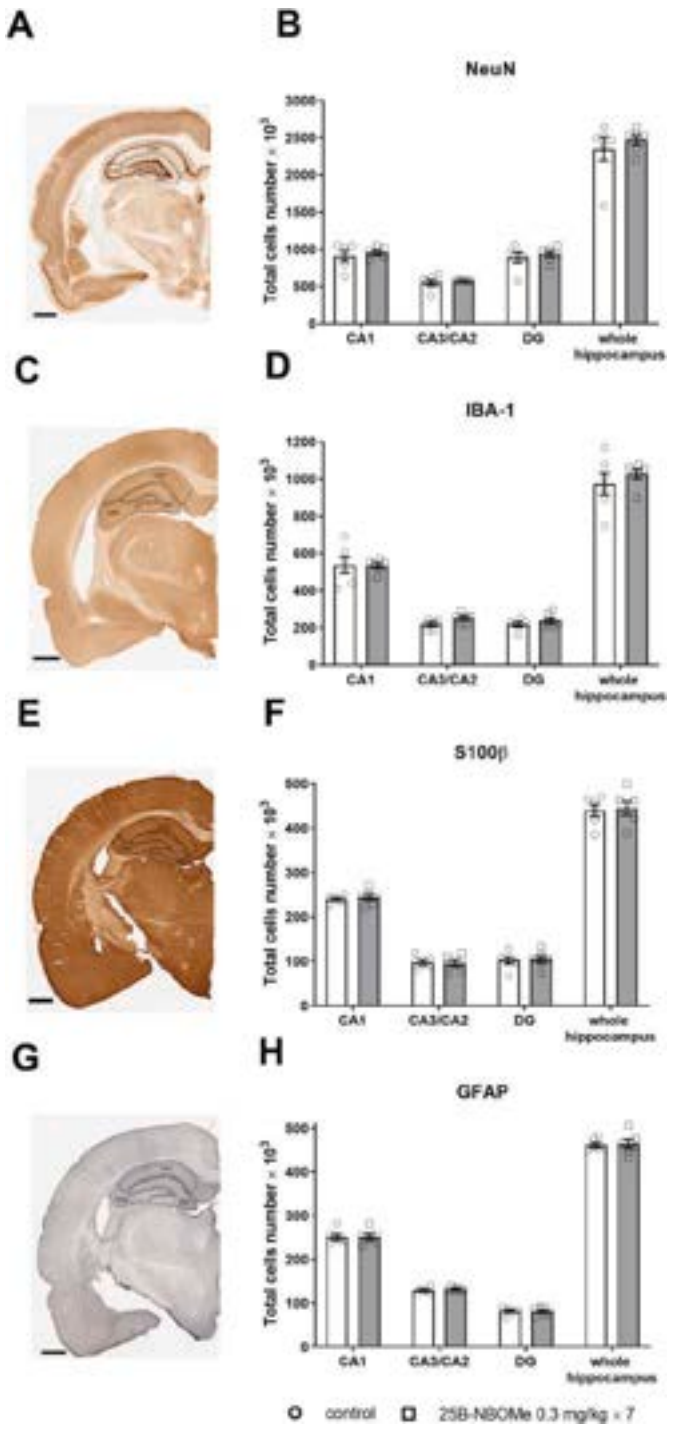


**Fig. 9.** The number of neurons and glial cells in the subregions and in the entire hippocampus. Light photomicrographs that were immunoprobed for NeuN (A), IBA-1 (C), S100β (E) and GFAP (G) and examined for the number of immunopositive neurons (B), microglia (D), and astrocytes (F and H). The graph bars indicate the mean ± standard error of the mean (SEM), n = 6 per experimental group. The scale bars represent 1 mm.

receptors activated with similar potency as 5-$HT_{2A}$ receptors by 25B-NBOMe (Rickli et al., 2015) seem to play a role in WDS response in rats. Although, many 5-$HT_{2A}$ agonists, such as lisuride, fenfluramine, p-chloromethamphetamine, and L-5-hydroxytryptophan, produce WDS in rats or head twitch response (HTR) in mice, and their effect on this behavior is classified as "false-positive response" (Halberstadt and Geyer, 2018), NBOMe compounds that induce mouse HTR or rat WDS normally also produce psychedelic effect in humans. Repeated administration of 25B-NBOMe drastically decreased the number of evoked wet dog shakes, supporting our observations derived from microdialysis experiments, indicating the rapid development of tolerance. Similar findings were found while investigating chronic administration of another congener from the NBOMe group, the 25I-NBOMe (Herian et al., 2021). Following chronic administration of DOI tolerance developed as indicated by ca. 40–50% decrease in the number of DOI-elicited HTR lasting for a 24 h. That tolerance disappeared over a 13-day period such that the number of HTR increased and returned to control levels (Darmani et al., 1990). This data is consistent with our findings especially since DOI has similar high affinity at 5-$HT_{2A/2C}$ receptors as 25B-NBOMe (Fitzgerald et al., 1999; Rickli et al., 2015).

### *4.3. The effect of repeated administration of 25B-NBOMe on rats anxiety level and locomotor activity in the LDB and open field test*

The data from the literature shows that phenethylamine and indoleamine hallucinogens induce fear in novel settings and increase avoidance of brightly lit areas which reflects agoraphobia observed in humans (Halberstadt and Geyer, 2018). In our earlier work, we demonstrated that 25B-NBOMe given in single doses (0.3–3 mg/kg) dose-dependently increased time spent in the dark zone while decreasing the time spent in the light zone; the locomotor activity of the animals was also suppressed (Wojtas et al., 2021). In the current study, repeated administration of 25B-NBOMe promoted anxiety measured as time spent in the dark zone; however, the time spent in the dark zone was only slightly, yet significantly longer than in control animals. Other behaviors measured in LDB test showed that single 25B-NBOMe doses significantly decreased ambulatory distance traveled, rats' vertical activity and stereotypic activity time in the dark zone. Decrease in exploratory behaviors is an indication of fear and correspond to suppressed ambulatory distance, peeping and vertical activity in the open field test. On the other hand, the repeated treatment increased ambulatory distance, vertical and stereotypic activity time in the dark zone, compared to control and single doses, thus observed reversal of normal rats' behavior is most likely due to tolerance of cortical 5-$HT_{2A}$ receptors. In contrast, time spent in the light zone and other behavioral measures were decreased by acute and chronic drug treatment which indicates an increase in anxiety level. This may be due to the design of the LDB box apparatus; animals staying in the dark zone are not exposed to the light (direct fearful stimuli) and explore the obscured area more willingly. Some differences between acute and chronic administration may reflect changes in 5-$HT_{2A}$ receptor sensitivity. Therefore, the finding in LDB test supports the conclusion that 25B-NBOMe induces anxiety given acutely and chronically.

The results obtained in the open field test showed that single and chronic injections of 25B-NBOMe decreased the time of walking, ambulatory distance measured as number of crossings, peeping and vertical activity expressed as rearing episodes, and a reduction in central space avoidance by single dose indicating occurrence of anxiogenic effect. A reversal of central space avoidance by repeated treatment more likely results rather from tolerant 5-$HT_{2A}$ receptor than anxiolytic effect. We have observed a similar phenomenon while investigating another hallucinogen from the NBOMe family, 25I-NBOMe (Herian et al., 2021). Suppressing effect of 25B-NBOMe on rats' general activity in the open field test cannot be also excluded, as was shown for this class of compounds by other studies (Gatch et al., 2017).

The discrepancies in the animal behavior may be a result of the different design of the open field arena, where animals are not able to hide themselves from the fearful stimuli (light source hanging above the arena) and limit their behavior to freezing.

Changes in neurotransmitter levels may be implicated in anxiety symptoms observed in both behavioral tests. As serotonin reuptake inhibitors and MDMA decrease anxiety (Bandelow et al., 2017; Wolfson et al., 2020), the decrease in 5-HT levels by 25B-NBOMe repeated

administration is likely to be responsible for some aspects of anxiety seen in the LDB and open field tests. In addition, elevated excitatory glutamatergic signaling is associated with panic, and this effect may be related with anxiety level observed in LDB and open field test after administration of single doses. However, this is not the case regarding repeated 25B-NBOMe administration as drugs that reduce glutamate availability are hypothesized to possess anxiolytic properties (Bergink et al., 2004). This issue needs further studies. In addition, the increases in extracellular DA levels observed in the nucleus accumbens after single and repeated 25B-NBOMe doses may contribute to anxiogenic effects, as local infusion of D1 receptor antagonist SCH23390 exert anxiogenic effect (Zarrindast et al., 2012). Thus, NBOMe compounds targeting primarily 5-$HT_{2A}$ receptors located at the apical dendrites of pyramidal cells in layer V seem to disrupt cortico-limbic circuits resulting in mechanism of 25B-NBOMe effect on anxiety.

### 4.4. The effect of 25B-NBOMe on genotoxicity and cell count

Our previous work showed only minor damaging effects on DNA in the nuclear fraction from the rat frontal cortex after a single administration of 25B-NBOMe (Wojtas et al., 2021). In the present study, DNA single and double-strand breaks were observed in the rat frontal cortex and hippocampus after repeated administration of 25B-NBOMe, but not after an acute one. It is accepted that the increase in DA, 5-HT, and glutamate extracellular levels is neurotoxic, leading to free radical generation and induction of oxidative stress (Halliwell, 2006). Our data indicate that the decreased levels of DA, 5-HT, and glutamate by 25B-NBOMe given repeatedly in response to the challenge dose cannot be the source of oxidative stress. However, the prolonged exposition to 25B-NBOMe caused an increase in basal glutamate extracellular levels in all studied brain regions (Table 1S, supplementary material). The increased basal glutamate concentration can result in the over-stimulation of ionotropic glutamate receptors, leading to oxidative damage of mitochondrial and nuclear DNA (Halliwell, 2006). We have also observed increased basal levels of DA and 5-HT in the frontal cortex; it is known that the oxidation of DA and 5-HT results in the production of free radicals and reactive quinones, which are harmful to the surrounding cells (Wrona and Dryhurst, 1998; Halliwell, 2006).

Moreover, the *in vitro* work of Cocchi et al., (2020) showed that phenethylamines might directly induce oxidative stress, particularly the brominated compounds with a halogen close to a double bond in the molecule (Cocchi et al., 2020). The cytotoxic effect of phenethylamine derivates was also shown *in vitro* in cortical cultures and in SH-SY5Y cells by Xu et al. (2019) and Zwartsen et al. (2019).

Repeated administration of 25B-NBOMe caused an increase in the number of astrocytes determined by the glial fibrillary protein (GFAP) level and specific calcium-binding protein B (S100β) in the PFC and FC. The increase in microglial cells labeled with ionized calcium-binding adaptor molecule 1 (IBA-1) was also observed, while no changes were found in the number of neurons labeled with neuronal-specific nuclear protein (NeuN). There is evidence that astrocytes play a crucial role in regulating the oxidative stress in the brain (Chen et al., 2020). Importantly, they are the primary cells to maintain the homeostasis of glutamate, which indirectly affects oxidative stress balance. It was evidenced that dysfunctional astrocytic glutamate transporters, GLAST and GLT-1, may be responsible for elevated extracellular glutamate and excitotoxicity, resulting in oxidative glutamate toxicity (Rothstein et al., 1996). Increased levels of GFAP and S100β observed in our study after repeated doses of 25B-NBOMe may indicate the astrocyte cells proliferation related to an increased basal level of glutamate. Under these conditions, astrocytes could be activated via stimulation from activated microglia, causing excessive secretion of free radicals and other molecules such as inflammatory factors, which leads to the aggravation of neurological damage. In turn, since it is extremely sensitive to changes in the cellular environment, microglia allows for rapid response to pathological factors. Following the CNS insult, microglia begins to proliferate and in the activated state may serve a variety of beneficial functions such as phagocyting damaged neurons, releasing anti-inflammatory factors (Zhang et al., 2010) or, on the other hand, be harmful by producing cytotoxic molecules such as superoxide, nitric oxide and several pro-inflammatory cytokines (Block et al., 2007).

Interestingly, we found DNA damage in the frontal cortex and hippocampus, but the number of glial cells was increased only in cortical fragments. The reason for this difference is difficult to explain based on presented results and needs further study.

Significantly, the volume of both cortical and hippocampal regions was not changed (Table 2S, supplementary material). That means that free radicals produced by 25B-NBOMe did not lead to permanent loss of brain tissue, yet caused DNA damage.

In conclusion, our study showed that chronic administration of 25B-NBOMe produces the development of tolerance observed at the level of neurotransmitters release and in hallucinogenic activity. Oxidative damage of cortical and hippocampal DNA implies the generation of free radicals by the drug, which may result in genotoxicity but not in neurotoxicity.

## CRediT authorship contribution statement

**Adam Wojtas:** Investigation, analysis of data, writing and editing. **Monika Herian:** Investigation, analysis of data, writing and editing. **Marzena Maćkowiak:** Investigation, revising, Formal analysis. **Anna Solarz:** Investigation, editing, Formal analysis. **Agnieszka Wawrzczak-Bargiela:** Investigation, editing, analysis of data. **Agnieszka Bysiek:** Investigation, editing, Formal analysis. **Karolina Noworyta:** revising, Formal analysis. **Krystyna Gołembiowska:** Conceptualization, and design of the study, Formal analysis, drafting and revising, Supervision.

## Declaration of competing interest

The authors declare no competing interests.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2023.109713.

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**Table 1S**. Basal levels of dopamine (DA), serotonin (5-HT), and glutamate (GLU) in the rat frontal cortex, striatum, and nucleus accumbens in saline and 25B-NBOMe (0.3 mg/kg × 7) treated rats.

| | (nM) | | (M) |
|---|---|---|---|
| | **DA** | **5-HT** | **GLU** |
| **saline** | | | |
| Frontal cortex | 1.17 ± 0.10 | 0.30 ± 0.02 | 2.53 ± 0.61 |
| Striatum | 7.49 ± 0.71 | 0.36 ± 0.03 | 4.20 ± 0.87 |
| N. accumbens | 1.38 ± 0.09 | 0.55 ± 0.04 | 3.63 ± 0.16 |
| **25B-NBOMe 0.3 mg/kg × 7** | | | |
| Frontal cortex | 1.84 ± 0.23** | 0.55 ± 0.06** | 3.78 ± 0.27* |
| Striatum | 4.96 ± 0.31** | 0.52 ± 0.04** | 7.10 ± 1.10* |
| N. accumbens | 1.08 ± 0.14* | 0.40 ± 0.03** | 7.52 ± 1.55** |

For each group n = 6; * $p < .05$; ** $p < .01$ saline vs. 25B-NBOMe 0.3 mg/kg × 7 (one-way ANOVA and Tukey's post hoc test)

**Table 2S.** The cortical regions and hippocampus volumes in saline and 25B-NBOMe (0.3 mg/kg × 7) treated rats.

| **Cortical regions** | **Volume ($mm^3$)** | | | |
|---|---|---|---|---|
| | **NeuN** | **IBA-1** | **S100** | **GFAP** |
| **saline** | | | | |
| Medial Prefrontal Cortex | 0.80 ± 0.03 | 0.77 ± 0.03 | 0.75 ± 0.03 | 0.70 ± 0.02 |
| Frontal Cortex | 1.42 ± 0.08 | 1.38 ± 0.07 | 1.21 ± 0.04 | 1.22 ± 0.03 |
| **25B-NBOMe 0.3 mg/kg × 7** | | | | |
| Medial Prefrontal cortex | 0.78 ± 0.03 | 0.78 ± 0.01 | 0.74 ± 0.02 | 0.77 ± 0.02 |
| Frontal Cortex | 1.25 ± 0.02 | 1.33 ± 0.05 | 1.29 ± 0.04 | 1.28 ± 0.03 |
| **Hippocampus** | **Volume ($mm^3$)** | | | |
| | **NeuN** | **IBA-1** | **S100** | **GFAP** |
| **saline** | | | | |
| CA1 | 1.82 ± 0.06 | 1.86 ± 0.06 | 1.87 ± 0.03 | 1.79 ± 0.03 |
| CA3/CA2 | 0.85 ± 0.04 | 0.78 ± 0.03 | 0.74 ± 0.03 | 0.76 ± 0.02 |
| DG | 0.83 ± 0.04 | 0.73 ± 0.03 | 0.80 ± 0.01 | 0.80 ± 0.02 |
| Whole Hippocampus | 3.50 ± 0.12 | 3.36 ± 0.08 | 3.41 ± 0.07 | 3.35 ± 0.05 |
| **25B-NBOMe 0.3 mg/kg × 7** | | | | |
| CA1 | 1.95 ± 0.03 | 2.03 ± 0.18 | 1.90 ± 0.06 | 1.76 ± 0.02 |
| CA3/CA2 | 0.81 ± 0.02 | 0.84 ± 0.03 | 0.85 ± 0.01 | 0.84 ± 0.01 |
| DG | 0.82 ± 0.02 | 0.76 ± 0.02 | 0.79 ± 0.03 | 0.79 ± 0.02 |
| Whole Hippocampus | 3.58 ± 0.04 | 3.63 ± 0.22 | 3.54 ± 0.07 | 3.39 ± 0.03 |

The values are the mean ± standard error of the mean (SEM); for each group n = 6 (one-way ANOVA and Tukey's post hoc test)

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# Effect of Psilocybin and Ketamine on Brain Neurotransmitters, Glutamate Receptors, DNA and Rat Behavior

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**Abstract:** Clinical studies provide evidence that ketamine and psilocybin could be used as fast-acting antidepressants, though their mechanisms and toxicity are still not fully understood. To address this issue, we have examined the effect of a single administration of ketamine and psilocybin on the extracellular levels of neurotransmitters in the rat frontal cortex and reticular nucleus of the thalamus using microdialysis. The genotoxic effect and density of glutamate receptor proteins was measured with comet assay and Western blot, respectively. An open field test, light–dark box test and forced swim test were conducted to examine rat behavior 24 h after drug administration. Ketamine (10 mg/kg) and psilocybin (2 and 10 mg/kg) increased dopamine, serotonin, glutamate and GABA extracellular levels in the frontal cortex, while psilocybin also increased GABA in the reticular nucleus of the thalamus. Oxidative DNA damage due to psilocybin was observed in the frontal cortex and from both drugs in the hippocampus. NR2A subunit levels were increased after psilocybin (10 mg/kg). Behavioral tests showed no antidepressant or anxiolytic effects, and only ketamine suppressed rat locomotor activity. The observed changes in neurotransmission might lead to genotoxicity and increased NR2A levels, while not markedly affecting animal behavior.

**Keywords:** dopamine; serotonin; glutamate; GABA; microdialysis; DNA damage; glutamate receptors; light–dark box test; open field test; forced swim test



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## 1. Introduction

Mood and anxiety disorders are among the leading causes of disability worldwide and place an enormous burden on society [1]. Major depressive disorder affects up to 5% of adults worldwide, leading to an increased susceptibility to a plethora of both mental and somatic diseases [2], and more importantly, an increased risk of suicide [3]. Currently used antidepressant drugs exert an effect after weeks or even months of treatment, and are not effective in so-called "treatment-resistant" depression [4]. Recently, it has been demonstrated that some dissociative drugs may alleviate the symptoms of major depressive disorder only hours after administration, and are also effective in treatment-resistant patients [5,6].

Studies conducted during the last 30 years show that ketamine exhibits strong antidepressant properties while working extremely fast after its administration [7,8]. Ketamine is a non-competitive NMDA receptor antagonist, used primarily as an anesthetic, but it can rapidly alleviate depressive symptoms after a single injection when used at subanesthetic doses [9]. Sadly, the effects only last for about 2 weeks; moreover, the drug has a plethora of side effects, and can be abused [7]. Ketamine's antidepressant effect results from a burst

of glutamate, which stimulates the mTOR pathway, leading to enhanced synaptic plasticity, which is disrupted in depressive patients [4]. This phenomenon translates into increased synaptic density in the frontal cortex and hippocampus [10,11].

Psychedelics might be some of the oldest psychoactive substances known to humankind [12]. They can be divided into two main categories by structure: indoleamines, e.g., DMT (N,N-dimethyltryptamine), psilocybin or LSD (Lysergic acid diethylamide), and phenylalkylamines, e.g., mescaline or DOI (2,5-Dimethoxy-4-iodoamphetamine) [13]. While the former demonstrate an affinity for several subtypes of 5-HT receptors [14], the latter bind mainly to the 5-HT2 receptor family [15,16]. Holistic data gathered from many studies indicate that psychedelics exert their psychoactive effects by activating cortical 5-HT2A receptors located on cortical pyramidal cells, leading to the release of glutamate [17–21], while possibly increasing thalamic GABA levels, resulting in increased sensory input to the cortex [22,23].

Similar to ketamine, psilocybin has been known to induce a rapid antidepressant effect that lasts for up to 6 months after a single administration, a level of effectiveness that is at least as high as "classic" antidepressants [6,24]. Those properties, such as in ketamine, are probably also mediated through elevated levels of glutamate stimulating the mTOR pathway [25]. Psychedelics enhance synaptic plasticity to a comparable or even greater degree than ketamine, promoting neuro- and synaptogenesis [25]. At the same time, they are non-addictive and seem to exhibit fewer side effects than ketamine [26].

The mechanisms underlying the antidepressant properties of both ketamine and psilocybin are still not fully understood. Detailed comparison studies should be conducted to evaluate their mutual properties. In addition, their safety and long-lasting effects should be carefully explored if they are to be used as a standard treatment for mood disorders, not only as a curiosity applied in experimental therapies. Psychedelics acting at 5-HT2A receptors in the reticular nucleus of thalamus can disrupt sensory input to the cortex and alter pyramidal cells' signaling [27]. Possible psilocybin-induced serotonin syndrome is of legitimate concern, as 5-HT2 receptor agonist DOI-treatment in wild mice resulted in oxidative stress and mitochondrial impairment, which led to cellular oxidative damage [28]. To address those questions, we have examined the effect of a single administration of both ketamine and psilocybin on the extracellular levels of dopamine (DA), serotonin (5-HT), glutamate and γ-aminobutyric acid (GABA) in the rat frontal cortex and reticular nucleus of the thalamus using in vivo microdialysis. The potential genotoxic effect in the frontal cortex and hippocampus was measured with the comet assay. MDMA was used in this test as a comparator as it was shown to augment DA and 5-HT release, which may be a source of oxidative stress [29]. Moreover, Western blot analysis was performed to measure the effect of the investigated drugs on selected protein levels in the rat frontal cortex. Finally, an open field test, light–dark box test and forced swim test were conducted to evaluate the effect on selected aspects of rat behavior. To distinguish long-lasting from acute, psychoactive effects, all the experiments (with the exception of microdialysis) were performed at least 24 h after administration of the chosen drug. The effect of psilocybin and ketamine on neurotransmitters' release was never studied in detail, but by a direct comparison of their impact on the neuronal network the mechanism of psychedelics in the brain may be explained. An identification of the possible undesirable side effects of psilocybin and ketamine, such as genotoxicity, behavioral disturbances and possible adaptive changes in glutamate receptors, is of great importance. Our findings may be helpful in the definition of the therapeutic capabilities of psychedelics.

## 2. Results

### *2.1. Effect of Psilocybin and Ketamine on Extracellular Levels of DA, 5-HT, Glutamate and GABA in the Rat Frontal Cortex*

Psilocybin at a dose of 2 mg/kg, but not at 10 mg/kg, significantly increased extracellular levels of DA up to ca. 200% of the baseline in the rat frontal cortex (Figure 1A). Ketamine (10 mg/kg) was very potent in increasing (up to ca. 500% of the baseline) the DA

extracellular level (Figure 1A). Repeated-measures ANOVA showed the significant effect of treatment groups ($F_{3,39} = 192$, $p < 0.0001$), sampling period ($F_{11,429} = 67$, $p < 0.0001$) and the interaction between treatment groups and sampling period ($F_{33,429} = 36$, $p < 0.0001$). Total effects expressed as AUC, shown in Figure 1B, were significantly increased for 2 mg/kg psilocybin and ketamine ($F_{3,39} = 192$, $p < 0.001$, one-way ANOVA).



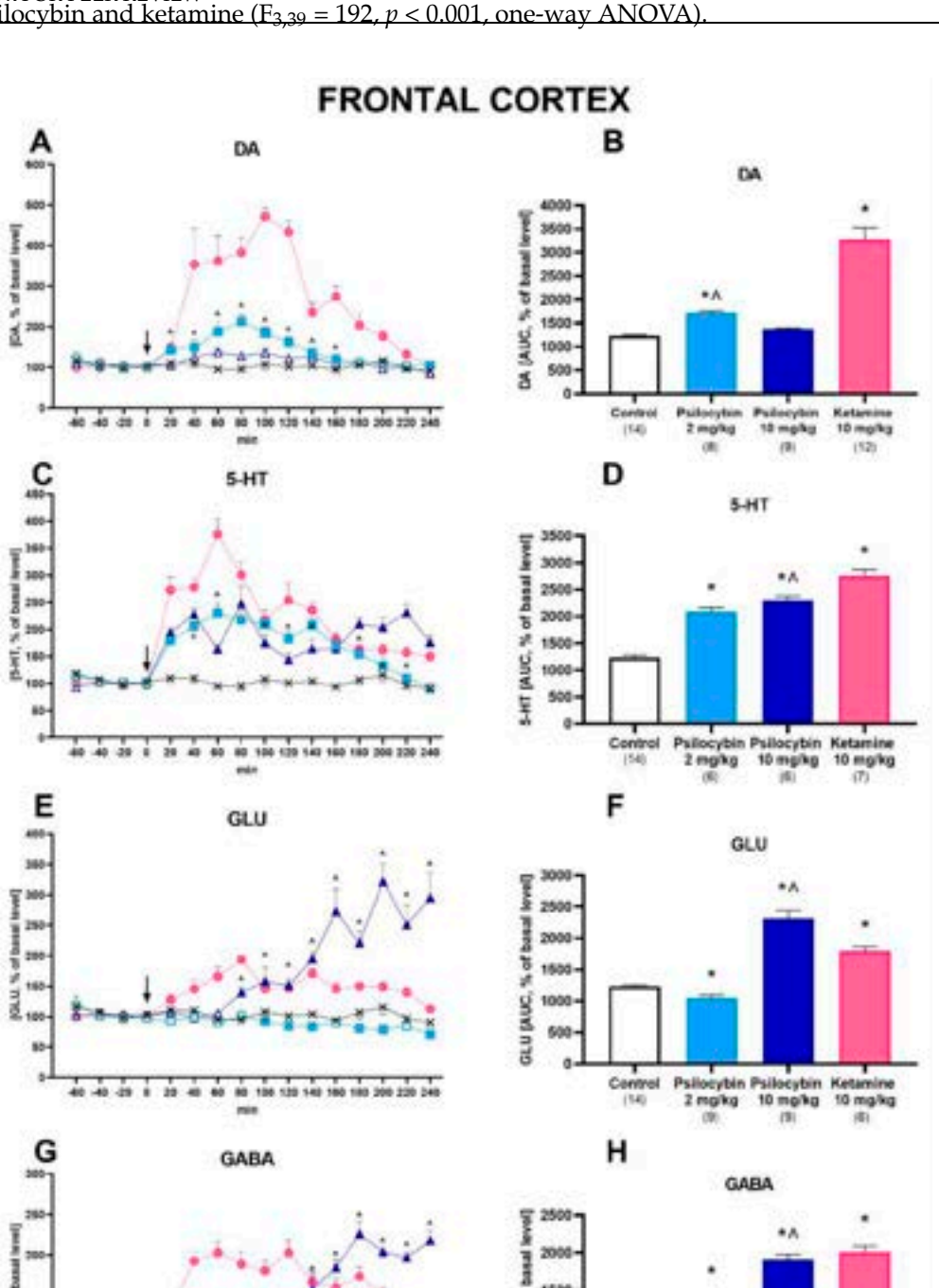


**Figure 1.** The time-course (**A**,**C**,**E**,**G**) and total (**B**,**D**,**F**,**H**) effect of psilocybin (2 and 10 mg/kg) and ketamine (10 mg/kg) on the dopamine (DA), serotonin (5-HT), glutamate (GLU) and γ- aminobutyric acid (GABA) extracellular levels in the rat frontal cortex. The total effect is calculated as an area under the concentration-time curve (AUC) and expressed as a percentage of the basal level. Values are the mean ± SEM (n is given under the name of the group). The drug injection is indicated by an arrow. Filled symbols or * show statistical differences ($p < 0.001$) between control and drug treatment groups; ^ $p < 0.001$ show differences between psilocybin 2 and 10 mg/kg groups as estimated by repeated measures ANOVA (time-course) or one-way ANOVA (total effect) followed by Tukey

acid (GABA) extracellular levels in the rat frontal cortex. The total effect is calculated as an area under the concentration-time curve (AUC) and expressed as a percentage of the basal level. Values are the mean ± SEM (n is given under the name of the group). The drug injection is indicated with an arrow. Filled symbols or * show statistical differences ($p < 0.001$) between control and drug treatment groups; ^ $p < 0.001$ show differences between psilocybin 2 and 10 mg/kg groups as estimated by repeated measures ANOVA (time-course) or one-way ANOVA (total effect) followed by Tukey's post hoc test.

The extracellular 5-HT level was increased in the rat frontal cortex by both doses of psilocybin (up to 200–250% of the baseline) and more potently by ketamine (up to 350% of the baseline) (Figure 1C). Repeated-measures ANOVA showed the significant effect of treatment groups ($F_{3,29} = 537$, $p < 0.0001$), sampling period ($F_{11,319} = 71$, $p < 0.0001$) and the interaction between treatment groups and sampling period ($F_{33,319} = 38$, $p < 0.0001$). The total effects expressed as AUC, shown in Figure 1D, were significantly increased for both psilocybin doses, 2 and 10 mg/kg, and for ketamine ($F_{3,29} = 537$, $p < 0.0001$, one-way ANOVA).

The extracellular glutamate level was slightly but significantly decreased by the lower psilocybin dose of 2 mg/kg (to ca. 80% of the baseline), and markedly increased by psilocybin at the dose of 10 mg/kg (up to ca. 300% of the baseline) as well as by ketamine (up to 150% of the baseline) (Figure 1E). Repeated-measures ANOVA showed the significant effect of treatment groups ($F_{3,33} = 326$, $p < 0.0001$), sampling period ($F_{11,3163} = 29$, $p < 0.0001$) and the interaction between treatment groups and sampling period ($F_{33,363} = 46$, $p < 0.0001$). Total effects expressed as AUC, shown in Figure 1F, were significantly decreased by 2 mg/kg psilocybin and significantly increased by psilocybin 10 mg/kg and ketamine ($F_{3,33} = 327$, $p < 0.0001$, one-way ANOVA) (Figure 1F).

The extracellular level of GABA was slightly but significantly increased by the psilocybin dose of 2 mg/kg (up to ca. 120% of the baseline), more potently by the higher dose of 10 mg/kg (up to ca. 200% of baseline) and by ketamine (up to 180% of baseline) (Figure 1G). Repeated measures ANOVA showed the significant effect of treatment groups ($F_{3,35} = 277$, $p < 0.0001$), sampling period ($F_{11,385} = 14$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{33,385} = 29$, $p < 0.0001$). Total effects expressed as AUC, shown in Figure 1H, were significantly increased by both psilocybin doses and ketamine ($F_{3,35} = 276$, $p < 0.0001$, one-way ANOVA).

Basal levels of DA, 5-HT, glutamate and GABA are shown in Supplementary Material S3.

### *2.2. Effect of Psilocybin and Ketamine on Extracellular Levels of Glutamate and GABA in the Rat Reticular Nucleus of the Thalamus*

Psilocybin and ketamine had no effect on the extracellular level of glutamate in the rat reticular thalamus nucleus (Figure 2A). Repeated-measures ANOVA did not show the significant effect of treatment groups ($F_{3,23} = 1{,}31$, $p < 0.29$), but there was a significant effect for sampling period ($F_{11,253} = 12$, $p < 0.0001$) and the interaction between treatment groups and sampling period ($F_{33,253} = 4.1$, $p < 0.0001$). Total effects expressed as AUC, shown in Figure 2B, were not changed by psilocybin doses or ketamine ($F_{3,23} = 1.29$, $p < 0.30$, one-way ANOVA).

The extracellular level of GABA was slightly but significantly increased by psilocybin at a low dose; however, it was potently increased by psilocybin at a dose of 10 mg/kg (up to ca. 170% of the baseline) (Figure 2C). Ketamine did not affect GABA extracellular level. Repeated-measures ANOVA showed the significant effect of treatment groups ($F_{3,22} = 22$, $p < 0.0001$). There was no significant effect for sampling period ($F_{11,242} = 1.73$, $p < 0.07$), but there was a significant interaction between treatment groups and sampling period ($F_{33,242} = 3.11$, $p < 0.0001$). Total effects expressed as AUC, shown in Figure 2D, were significantly increased by both doses of psilocybin but not by ketamine ($F_{3,22} = 33$, $p < 0.0001$, one-way ANOVA).

Basal levels of glutamate and GABA are shown in Supplementary Material S3.

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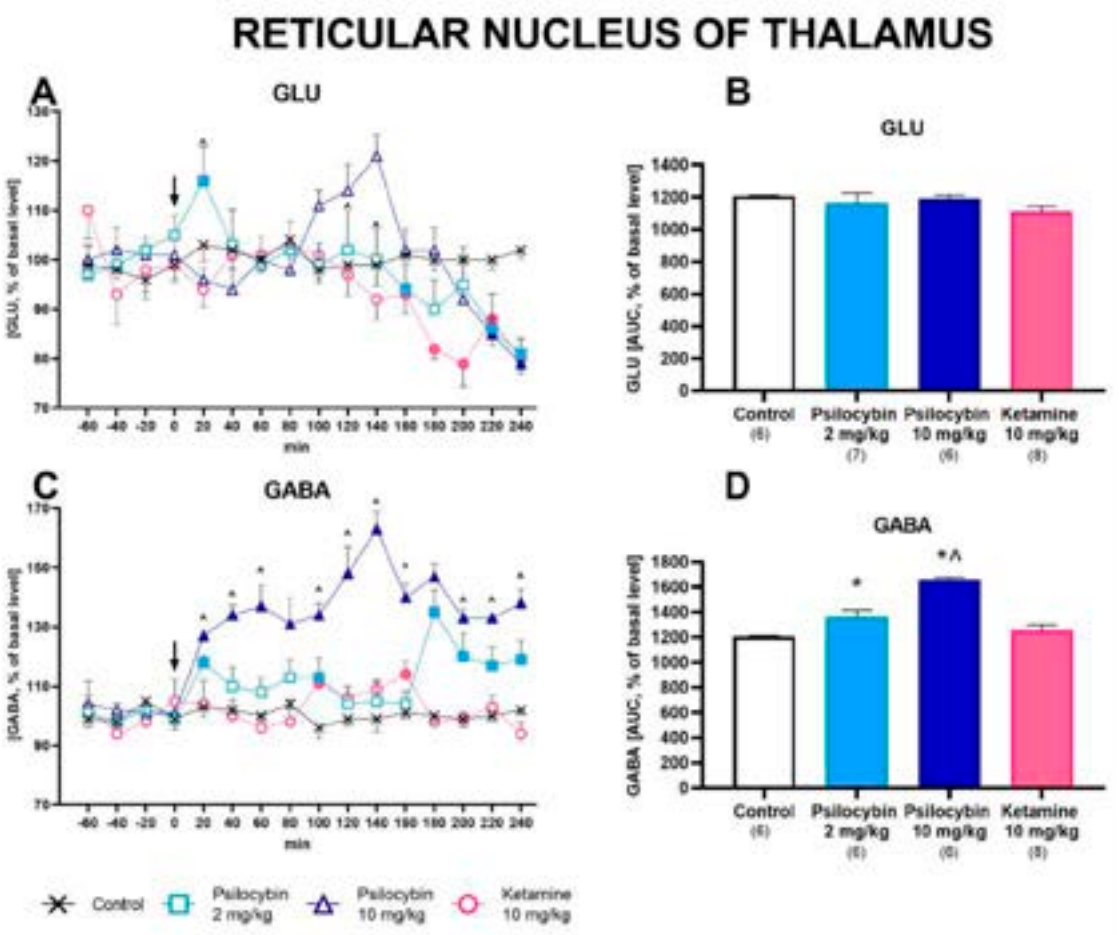


**Figure 2.** The time-course (**A**,**C**) and total (**B**,**D**) effect of psilocybin (2 and 10 mg/kg) and ketamine (10 mg/kg) on the glutamate (GLU) and γ-aminobutyric acid (GABA) extracellular levels in the rat reticular nucleus of the thalamus. The total effect is calculated as an area under the concentration-time curve (AUC) and expressed as a percentage of the basal level. Values are the mean ± SEM (n is given under the name of the group). The drug injection is indicated with an arrow. Filled symbols or * show statistical differences ($p < 0.03$–$0.001$) between control and drug treatment groups; ^ $p < 0.005$–$0.001$ show differences between psilocybin 2 and 10 mg/kg groups as estimated by repeated measures ANOVA (time-course) or one-way ANOVA (total effect) followed by Tukey's post hoc test.

### 2.3. The NMDA and AMPA Receptor Subunit Level in the Rat Frontal Cortex

The higher dose of psilocybin (10 mg/kg) significantly increased the GluN2A protein level by ca. 40% over the control, measured 24 h after administration ($F_{3,44}$ = 9.3, $p < 0.0001$, one-way ANOVA) (Figure 3A,B). In contrast, ketamine administration decreased the GluN2A protein level by ca. 30%, but this effect was not statistically significant ($p < 0.16$ vs. control group). GluN2B protein levels were not changed by psilocybin or ketamine administration ($F_{3,20}$ = 2.36, $p < 0.10$) (Figure 3A,C).

The protein levels of two AMPA receptor subunits, GluA1 and GluA2, measured 24 h after administration of psilocybin at doses of 2 and 10 mg/kg or ketamine at a dose of 10 mg/kg were not changed ($F_{3,20}$ = 0.22, $p < 0.88$ and $F_{3,20}$ = 0.85, $p < 0.48$, respectively; one-way ANOVA) (Figure 3D–F).

The uncropped, untouched, full original images of Western blots are shown in Supplementary Material S1.

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The uncropped, untouched, full original images of Western blots are shown in Supplementary Material S1.

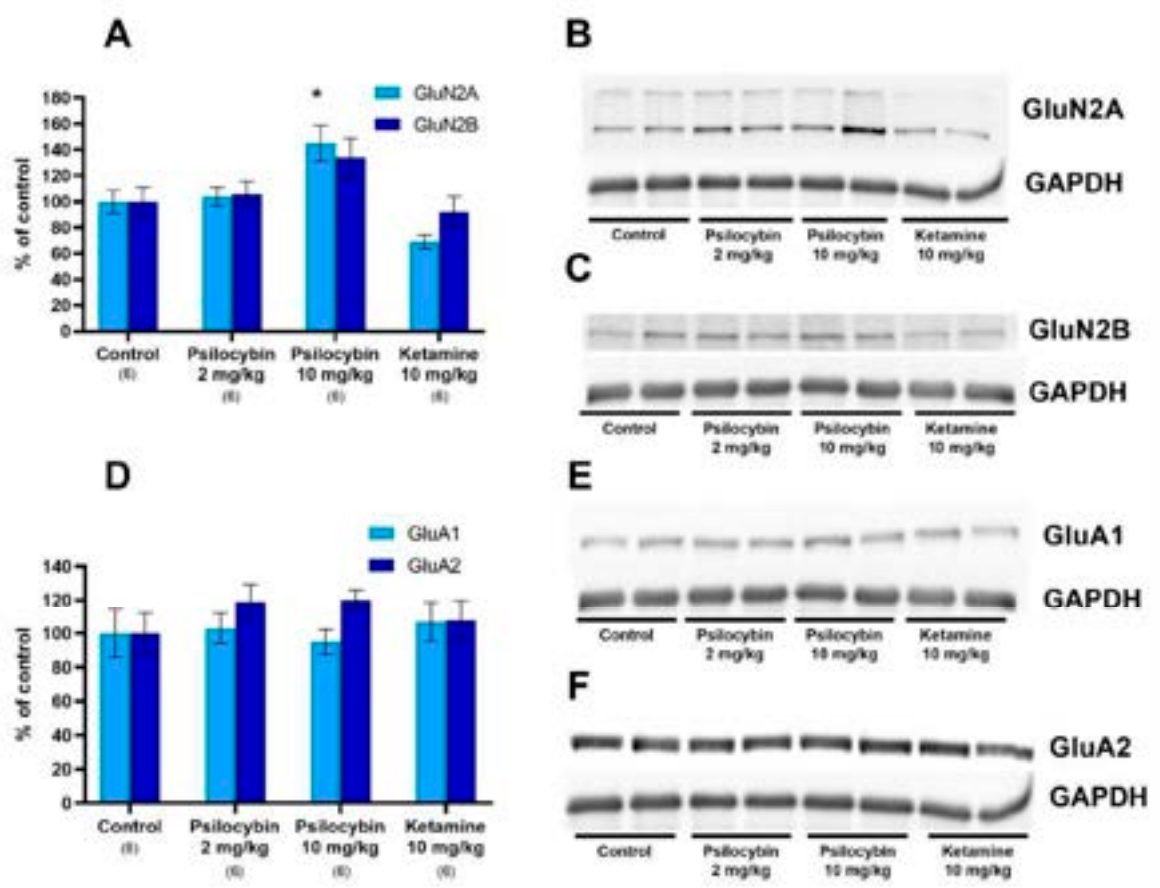


**Figure 3.** Levels of NMDA receptor subunits (GluN2A and GluN2B; (**A**) and AMPA receptor subunits (GluA1 and GluA2; (**D**) in the rat frontal cortex estimated 24 h after psilocybin (2 and 10 mg/kg) or ketamine (10 mg/kg) administration. The data are shown as percentages of the levels of the appropriate control groups. Each data point represents the mean ± SEM (given under the name of the group). Only in group GluN2A are data given in duplicates. * $p < 0.05$ vs. appropriate control group (one-way ANOVA followed by Tukey's post hoc test). Examples of photomicrographs of the immunoblots using GluN2A and GAPDH antibodies (**B**), GluN2B and GAPDH antibodies (**C**), GluA1 and GAPDH antibodies (**E**) and GluA2 and GAPDH antibodies (**F**).

### 2.4. The Effect of Psilocybin and Ketamine on DNA Damage in the Rat Frontal Cortex and Hippocampus

Psilocybin at a dose of 2 mg/kg did not produce DNA damage by reactive oxygen species (ROS) in the frontal cortex and hippocampus, presented as a percentage of tail moment 7 days after administration. However, psilocybin at a higher dose of 10 mg/kg significantly increased DNA damage in both brain regions (Figure 4A,B). Ketamine (10 mg/kg) produced DNA damage only in the hippocampus, while MDMA, used as a comparative compound, caused potent damage of DNA in the frontal cortex and hippocampus. One-way ANOVA showed the significant effect of treatment in the frontal cortex ($F_{4,26} = 39$, $p < 0.0001$) and hippocampus ($F_{4,26} = 24$, $p < 0.0001$).

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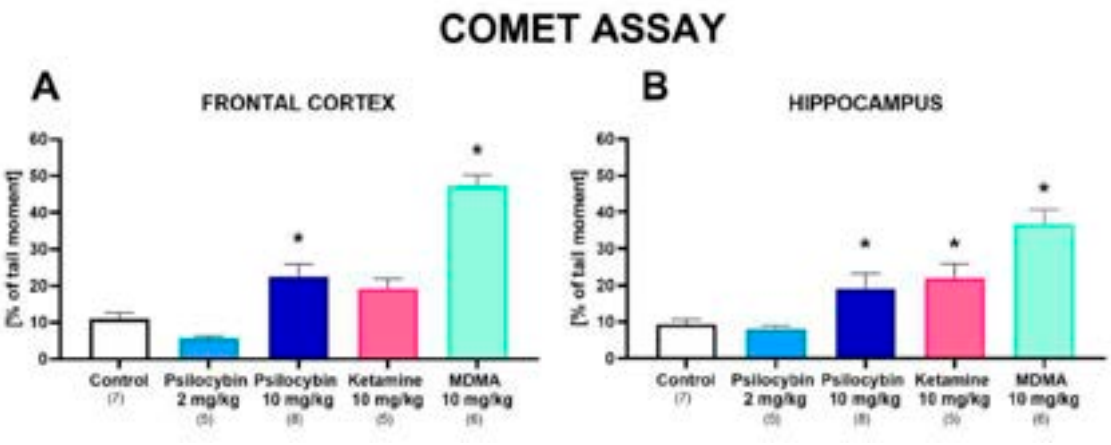


**Figure 4.** The effect of psilocybin (2 and 10 mg/kg), ketamine (10 mg/kg) and MDMA (10 mg/kg) on the oxidative damage of DNA in nuclei of the rat frontal cortex (**A**) and hippocampus (**B**) in the comet assay estimated 7 days after treatment. Data are the mean ± SEM (n is given under the name of the group) and represent tail moment shown as the product of the tail length and the fraction of total DNA in the tail. * $p < 0.05$ compared to the control (one-way ANOVA followed by Tukey's post hoc test).

### 2.5. The Effect of Psilocybin and Ketamine on Rat Behavior in the Open Field, Light–Dark Box and Forced Swimming Tests 24 h after Administration

Psilocybin did not affect time of walking and episodes of crossing in the open field test; however, ketamine decreased both parameters in this test ($F_{3,35}$ = 39, $p < 0.0001$, $F_{3,35}$ = 6.4, $p < 0.01$, respectively; one-way ANOVA) (Figure 5A,B).

The time spent in the dark compartment was longer than in the light zone for all groups of animals estimated in the light–dark test, but any difference was observed between treatments (Figure 5D). Two-way ANOVA showed no effect of treatment ($F_{3,72}$ = 0, $p < 1.0$), a significant difference between the dark and light zones ($F_{1,72}$ = 919, $p < 0.0001$) and no significant interaction between treatment and the light/dark zone ($F_{3,72}$ = 1.17, $p < 0.32$). Exploration of the dark zone expressed as ambulatory distance was significantly increased in comparison to exploration of the light zone for all experimental groups (Figure 5C). Psilocybin at a dose of 2 mg/kg and ketamine significantly decreased exploration of the dark and light zones. Two-way ANOVA showed the effect of treatment ($F_{3,72}$ = 17.4, $p < 0.0001$), a significant difference between the dark and light zone ($F_{1,72}$ = 412, $p < 0.0001$), and no significant interaction between treatment and the light/dark zone ($F_{3,72}$ = 1.73, $p < 0.16$).

Psilocybin at a dose of 2 mg/kg significantly increased immobility and swimming time (Figure 6A,C), while significantly decreasing climbing time (Figure 6B). Ketamine only significantly increased swimming time (Figure 6C). One-way ANOVA showed the significant effect of treatment on immobility time ($F_{3,76}$ = 8.12, $p < 0.0001$), climbing time ($F_{3,76}$ = 15, $p < 0.0001$) and swimming time ($F_{3,76}$ = 27, $p < 0.0001$).

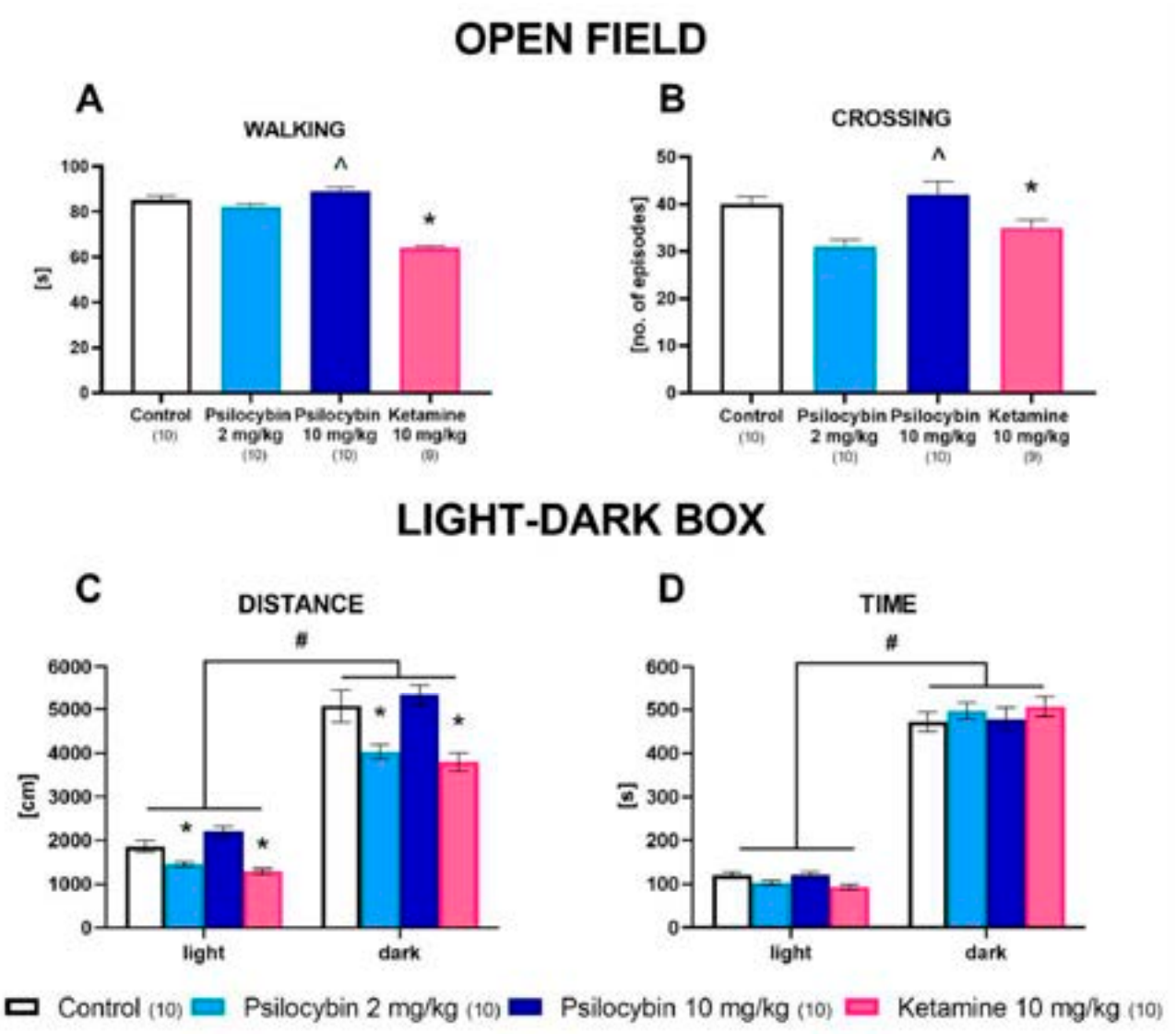


**Figure 5.** The effect of psilocybin (2 and 10 mg/kg) and ketamine (10 mg/kg) on locomotor behavior in the open field test (OF) and on activity of rats in the light–dark box (LDB) test 24 h after administration. The time spent on walking (**A**), the number of crossing episodes (**B**) in the OF test and ambulatory distance (**C**) and time spent (**D**) in the light and dark zone in the LDB test. Values are the mean ± SEM (n is given under or next to the name of the group). * $p < 0.001$–$0.05$ compared to the control; ^ $p < 0.05$ psilocybin 2 mg/kg compared to psilocybin 10 mg/kg; # $p < 0.001$ light vs. dark (one-way ANOVA or two-way ANOVA followed by Tukey's post hoc test).

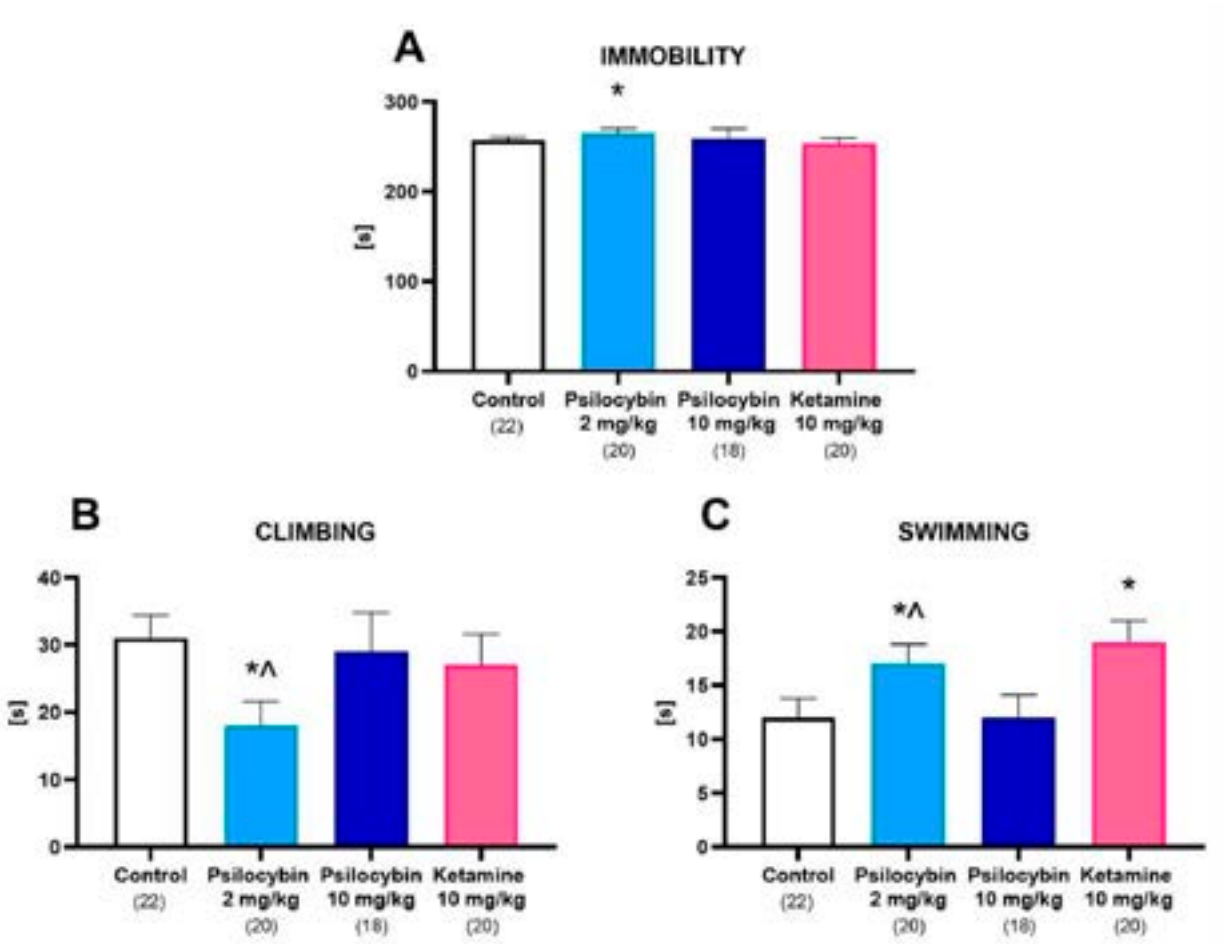


**Figure 6.** The effect of psilocybin (2 and 10 mg/kg) and ketamine (10 mg/kg) on the immobility (**A**), climbing (**B**) or swimming (**C**) time of rats in the forced swim test (FST), estimated 24 h after administration. Data are the mean ± SEM (n is given under the name of the group). * $p < 0.001$–$0.01$ compared to the control; ^ $p < 0.05$ psilocybin 2 mg/kg compared to psilocybin 10 mg/kg (one-way ANOVA followed by Tukey's post hoc test).

**Figure 6.** The effect of psilocybin (2 and 10 mg/kg) and ketamine (10 mg/kg) on the immobility (**A**), climbing (**B**) or swimming (**C**) time of rats in the forced swim test (FST), estimated 24 h after administration. Data are the mean ± SEM (n is given under the name of the group). * $p < 0.001$–$0.01$ compared to the control; ^ $p < 0.05$ psilocybin 2 mg/kg compared to psilocybin 10 mg/kg (one-way ANOVA followed by Tukey's post hoc test).

## 3. Discussion

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Current studies suggest that both ketamine and psychedelics share rapid antidepressant properties, indicating the existence of convergence between their mechanisms of action [25,30]. The gathered data strongly supports the important role of glutamate in the frontal cortex [4]. Ketamine blocks NMDA receptors located on cortical GABAergic interneurons, leading to the disinhibition of glutamatergic neurotransmission and resulting in increased glutamate release [31], while psychedelics activate 5-HT2A receptors located on pyramidal neurons, stimulating the release of glutamate [20].

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Our microdialysis experiments reinforce this hypothesis, showing a significant elevation of extracellular glutamate in rat frontal cortex levels after administration of either ketamine (10 mg/kg) or a high dose of psilocybin (10 mg/kg). Moreover, both drugs strongly affected DA, 5-HT and GABA levels, though the exact mechanisms responsible for this phenomenon may differ between the substances.

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The data suggest that low doses of ketamine used for clinical and preclinical studies can block NMDA receptors on subsets of GABA interneurons and reduce interneuron firing prior to increasing pyramidal neuron firing [32]. As cortical GABAergic interneurons seem to be the primary target involved in ketamine's antidepressant effect [33], a decrease in the extracellular levels of GABA should be expected. However, we observe an increase in GABA in parallel to an increase in the glutamate extracellular level in the frontal cortex. Our results are consistent with the findings of Pham et al., (2020) [34], who also found a concomitant increase in the release of both neurotransmitters in the medial prefrontal cor-

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a concomitant increase in the release of both neurotransmitters in the medial prefrontal cortex of mice by local ketamine injection, but the effect was observed 24 h after the injection. NMDA receptors are composed of tetraheteromeric assemblies containing various combinations of GluN1-3 subunits and it has been shown that the subunit composition of pre-, post- or perisynaptic locations is not the same [35]. GluN2D subunits, highly expressed in parvalbumin-expressing GABAergic interneurons, may be involved in the cortical disinhibition by ketamine to a greater extent, but other subsets of interneurons may be blocked less effectively by ketamine, thus resulting in increased extracellular GABA. Another hypothesis of Duman et al. (2019) [36] suggests that the enhancement of GABAergic neurotransmission is caused by the initial burst of glutamate. Ketamine disturbs tonic inhibition of pyramidal cells and increases the burst of glutamate, leading to the release of both neurotransmitters.

In our study, ketamine markedly increased the extracellular 5-HT level, indicating the association of serotonin transmission with glutamate. A microdialysis study in monkeys showed that subanesthetic doses of ketamine transiently increased 5-HT extracellular levels in the prefrontal cortex by inhibiting SERT activity [37]. In line, the depletion of 5-HT by para-chlorophenylalanine attenuated the acute antidepressant effect of ketamine, suggesting that endogenous 5-HT is partly involved in this effect [38]. In contrast, an increase in the extracellular 5-HT level by ketamine in the medial prefrontal cortex of mice was eliminated by the AMPA receptor antagonist NBQX given into dorsal raphe nuclei (DRN) [34]. Thus, ketamine seems to indirectly elevate 5-HT extracellular levels through the stimulation of AMPA receptors located in DRN. AMPA receptors might be also involved in regulating ketamine-induced DA release in the prefrontal cortex as the AMPA receptor antagonist NBQX blocks the effects of ketamine on DA release [39]. In our study, acute administration of ketamine potently increased the DA extracellular level in the frontal cortex of rats. The mechanism of ketamine's action on the DA system is not fully established. The medial prefrontal cortex sends projections to the ventral tegmental area (VTA) [40]. Exposure to stress disrupts the activity of a circuit formed by DA and glutamate projections that connect the medial prefrontal cortex and VTA, while ketamine restores these circuits [41]. The meta-analysis study by Kokkinou et al. (2018) [42] presents an elevation of frontal cortical DA levels shown in animal subjects and healthy humans after treatment with ketamine.

Serotonergic psychedelics acting primarily on serotonergic receptors mimic serotonergic input by presynaptic facilitation of glutamatergic neurons in the sensory, motor and limbic cortices. Psilocybin, after entering the body, is rapidly converted in the liver into psilocin [43]. Psilocin has an affinity for many 5-HT receptor subtypes, but mainly 5-HT2A and 5-HT1A receptors play a potential role in mediating the actions of serotonergic psychedelics [30]. 5-HT2A and 5-HT1A receptors showed 80% co-expression in the same pyramidal neurons in the rat frontal cortex [44]. 5-HT2A agonism leads to increased membrane excitability, while the effect of 5-HT1A agonism is a decrease in membrane excitability [30]. The subpopulation of pyramidal neurons that have high levels of 5-HT2A receptors may exhibit excitation, whereas other neurons with a high expression of 5-HT1A receptors display inhibition. Thus, opposing receptor actions correspond to different pharmacological responses. A minor fraction of 5-HT1A and 5-HT2A receptors reside in GABAergic interneurons; there are also 5-HT2A receptors in thalamocortical axons in the frontal cortex [45]. In our study, a low dose of psilocybin had a very weak effect on glutamate and GABA extracellular levels; however, a higher dose increased the release of both neurotransmitters, but this effect was significant in the second phase of the experiment. It seems likely that glutamate release by psilocybin may originate in a different neuronal location, i.e., glutamate from thalamocortical nerve terminals and GABA from cortical interneurons; both effects are mediated via 5-HT2A receptors. Support for our results can be found in the study by Mason et al. (2020) [46], who demonstrated an increase in glutamate and GABA levels due to psilocybin in the human medial prefrontal cortex.

The DA extracellular level was only elevated to a certain extent by the low psilocybin dose. It was also limited in time, and appeared unrelated to the glutamate and GABA extracellular level. This data is difficult to explain on the basis of glutamatergic or GABAergic projections into midbrain DA neurons. Psilocybin displays a high and comparable affinity for 5-HT2A and 5-HT1A serotonin receptors [14]. The inhibitory action of 5-HT1A receptors expressed by GABAergic neurons disinhibits pyramidal cells. Active pyramidal cells stimulate neurons in VTA, resulting in an increased DA release in the frontal cortex. Alternatively, psilocybin-activated 5-HT2A receptors located in pyramidal cells may be responsible for an increase in cortical DA release [47]. Thus, the stimulation of descending excitatory projection by 5-HT1A or 5-HT2A receptors could enhance glutamate release in VTA, subsequently stimulating glutamate receptors and increasing DA release in the frontal cortex. However, this process depends on the dose of psilocybin.

In our study, psilocybin at both doses increased the 5-HT extracellular level in the rat frontal cortex. The selective activation of 5-HT2A receptors enhances the release of glutamate, which acts on pyramidal AMPA receptors, stimulating a descending projection to DRN, thus resulting in increased 5-HT release in the frontal cortex. In turn, the activation of inhibitory 5-HT1A receptors on pyramidal neurons [44] counteracts the stimulatory effect of 5-HT2A receptors and decreases 5-HT release [48]. The same suppressing effect on 5-HT neurons can be mediated by 5-HT2A receptors located in GABAergic interneurons in DRN [49]. In our work, psilocybin's effect on the cortical 5-HT extracellular level may result from the activation of both subtypes of 5-HT receptors: the activation of 5-HT2A/5-HT1A receptors in the pyramidal location resulting in the stimulation of descending projection to DRN. A relatively small difference between the effects of both doses may be related to a difference in the activation of excitatory 5-HT2A or inhibitory 5-HT1A receptors by psilocybin. An inverted "U" shape dose–response effect on 5-HT release in the frontal cortex was demonstrated by us for 25I-NBOMe [50]. It should be noted that Sakashita et al. (2015) showed a slight but significant increase in 5-HT extracellular level in the medial prefrontal cortex due to psilocin (10 mg/kg) [51].

Due to the localization of both NMDA receptors [52] and 5-HT2A receptors [53] on the GABAergic interneurons in the reticular nucleus of the thalamus, it was of particular interest to evaluate the effects of both tested substances on the extracellular levels of amino acids. The reticular nucleus is made of GABAergic interneurons and is thought to be the center of the negative-feedback loop in the thalamus, as it is innervated by other thalamic nuclei while it projects inhibitory inputs back into the thalamus [54], regulating the amount of information sent into the cortex [22]. The NMDA antagonists seem to inhibit its activity, leading to the disinhibition of thalamic activity [52], while psychedelics are hypothesized to stimulate it, leading to the attenuation of thalamic gating [23,27,55]. Surprisingly, we observed only half of the expected outcome: while psilocybin dose-dependently increased extracellular GABA levels, ketamine did not affect either glutamatergic or GABAergic neurotransmission; this phenomenon is hard to explain, and further studies should be conducted to address this issue.

The main mechanism of ketamine action is a blockade of NMDA receptors localized on interneurons that leads to the disinhibition of glutamatergic neurons. Ketamine and serotonergic psychedelics acting at 5-HT2A receptors increase glutamate release, which stimulates mTOR signaling through the activation of AMPA receptors. As was shown further, the antidepressant effects of ketamine are mediated by the GluN2B subunit located on GABAergic interneurons, but not by GluN2A subunits in glutamatergic neurons [33]. Ketamine-like effects were demonstrated for selective NR2B antagonists in rodent models [56] and in a clinical study showing a rapid antidepressant response by a selective NR2B receptor antagonist, ifenprodil [57]. Knockdown of the GluN2A subunit in the medial prefrontal cortex in mice did not block the antidepressant effect of the positive allosteric modulator of the NMDA receptor rapastinel [58]. However, a significant increase in the GluN2A subunit was observed by us 24 h after a high dose of psilocybin administration in the rat frontal cortex. The GluN2B subunit level showed only a tendency to increase,

while neither subunit levels were significantly decreased by ketamine. A decrease in the expression level of the NR2B subunit was evidenced in the somatosensory cortex of mice at 24 and 72 h after administration of ketamine [59]. In contrast, the expression of *Nr2a* and *Nr2b* genes increased four weeks after cessation of treatment with LSD in the rat prefrontal cortex [60]. No effect was observed in our study 24 h after drug administration in the synaptic AMPA receptor subunits GluA1 and GluA2. It cannot be excluded that the increase in the level of NMDA receptor subunits 24 h after psilocybin administration might be related to the simultaneously observed enhancement in dendritic spine density induced by 5-HT2A receptor activation [61].

Compounds such as ketamine and serotonin psychedelics that are capable of promoting rapid plasticity have recently been defined as psychoplastogens. These substances are considered safe and not addictive. However, by producing an excessive glutamate release, they may induce excitotoxicity resulting in oxidative stress and neuronal atrophy. In our study, an increase in oxidative DNA damage was observed in the frontal cortex seven days after the administration of a single high dose of psilocybin but not ketamine. However, the appearance of double- and single-strand DNA breaks was observed in the hippocampus after treatment with a high dose of psilocybin and ketamine. These data suggest that potential risks may be associated with higher doses of psilocybin as well as ketamine. Importantly, in the recent study of Shin et al. (2021) [28], oxidative stress induced by amphetamine analog and 5-HT2A receptor agonist DOI contributes to DOI-induced serotonergic behaviors and cellular damage [28].

To discern between the acute, psychoactive effect induced by both substances and the persistent, antidepressant effect, our behavioral experiments were conducted 24 h post administration to limit high concentrations of circulating drugs and metabolites in the system. Ketamine negatively affected locomotor behavior in the open field test, while psilocybin had no effect. Moreover, ketamine and a low dose of psilocybin shortened the distance traveled in both the light and dark zone of the LDB apparatus, though a high dose of psilocybin had no effect; similarly, there was no effect on anxiety due to either substance. Acutely administered NMDA antagonists induce hyperlocomotion in rodents, while, for psychedelics having an inverted U-shaped response, low doses stimulate locomotor activity and high doses inhibit it [62]. We hypothesize that the observed changes in behavior might result from adaptive changes triggered to cope with the acutely induced effects of both drugs. Few studies have been conducted to assess the effect of either psilocybin or ketamine on behavior 24 h after injection and there is a clear lack of data. Surprisingly, none of the treatments reduced immobility time in the forced swim test. As suggested by Jefsen et al. (2019) [63], the forced swim test, while effective in evaluating the properties of more "traditional" compounds, might not be suitable for assessing the antidepressant effect of psychedelics. Moreover, our studies were conducted on naive animals; a recent article by Viktorov et al. (2022) [64] suggests that the effect of NMDA antagonists is limited when used on animals who are not subjected to any model of depression. On the other hand, our behavioral tests were performed 24 h after the administration of the drugs, and while that is enough to eliminate their acute effects, it might not be enough for the long-lasting effects to manifest fully, as Hibicke et al. (2020) [26] reported an antidepressant effect observed 7 days post psilocybin administration. In this context, a dissenting observation was reported in the latest study of Cao et al. 2022 [65], that the acute administration of LSD (30 min prior to the FST) significantly attenuated "depression-like" freezing behavior in the forced swimming test in naive mice. Taking into account the above studies, the time of test performance is critical for its outcome.

## 4. Materials and Methods

### *4.1. Animals*

Adult male Wistar Han rats (280–350 g; Charles River, Sulzfeld, Germany) were used in all the experiments. The animals were initially acclimatized and housed (6 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 tap 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, 324/2021 and 79/20226).

### 4.2. Drugs and Reagents

Ketamine hydrochloride was purchased from Tocris/Bio-Techne (Abingdon, UK) 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) [66]; both were dissolved in sterile water. All solutions were made fresh on the day of experiment. The dose of ketamine (10 mg/kg) was based on a report by Popik et al. (2022) [67], while doses of psilocybin (2 and 10 mg/kg) on work by Jefsen et al. (2019) [63]. MDMA purchased from Toronto Research Chemicals Inc. (Canada) was used as reference drug at the dose of 10 mg/kg. At this dose, it significantly and in a reliable manner induced oxidative DNA damage in the rat frontal cortex [29]. All drugs were given intraperitoneally (ip) in the volume of 2 mL/kg. Ketamine, xylazine hydrochlorides and sodium pentobarbital used for anesthetizing the animals came from Biowet Puławy (Puławy, Poland). All necessary chemicals of the highest purity used for analysis by high-performance liquid chromatography (HPLC) were obtained from Merck (Warszawa, Poland). O-phthalaldehyde (OPA), from Sigma-Aldrich (Poznań, Poland), was used for the derivatization of glutamate to an electroactive compound. The chemicals used for the alkaline comet assay were from Trevigen (Gaithersburg, MD, USA) and Merck (Warsaw, Poland). The reagents used in immunohistochemistry came from Sigma-Aldrich (Poznań, Poland), Vector Laboratories (Burlingame, CA, USA) and Proteintech (Manchester, UK). MDMA was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). The control group was treated with 0.9% NaCl solution in the same way.

### 4.3. Brain Microdialysis

Ketamine and xylazine (75 and 10 mg/kg, respectively) were injected intramuscularly to anesthetize the animals. Microdialysis probes (MAB 4.15.3Cu and MAB 4.15.2Cu, AgnTho's AB, Sweden) were implanted into the following brain structures using the determined coordinates (mm): frontal cortex AP +2.7, L +0.8, V −6.5, and reticular nucleus of thalamus AP −1.5, L +2.1, V −5.0 from the dura [68]. Seven days after implantation, probe inlets were connected to a syringe pump (BAS, West Lafayette, IN, USA) which delivered artificial cerebrospinal fluid composed of (mM) 147 NaCl, 4 KCl, 2.2 $CaCl_2$ and 1.0 $MgCl_2$ at a flow rate of 2 µL/min. The monitoring of extracellular levels of neurotransmitters has been performed in freely moving animals. Five baseline samples were collected every 20 min after the washout period of 2 h. The respective drugs were administered and dialysate fractions were collected for the next 240 min. As the experiment ended, the rats were terminated and their brains underwent histological examination to validate probe placement. Histological tracing of microdialysis probes in frontal cortex and thalamus are presented in Supplementary Material S2.

### 4.4. Extracellular Concentration of DA, 5-HT, Glutamate and GABA

Extracellular DA and 5-HT levels were analyzed using an Ultimate 3000 System (Dionex, Sunnyvale, CA, USA), electrochemical detector Coulochem III (model 5300; ESA, Chelmsford, MA, USA) with a 5020 guard cell, a 5040 amperometric cell and a Hypersil Gold C18 analytical column (3 µm, 100 × 3 mm; Thermo Fisher Scientific, Sunnyvale, CA, USA). The details of the method have been described elsewhere [29,69]. The chromatographic data were processed by the Chromeleon v.6.80 (Dionex, Sunnyvale, CA, USA) software

package run on a personal computer. The limit of detection of DA and 5-HT in dialysates was 0.002 pg/10 µL for DA and 0.01 pg/10 µL for 5-HT.

Glutamate and GABA levels in the extracellular fluid were measured by HPLC with electrochemical detection after the derivatization of samples with OPA/sulfite reagent to form isoindole-sulfonate derivatives, as previously described [29,69]. The data were processed using Chromax 2005 (Pol-Lab, Warszawa, Poland) software on a personal computer. The limit of detection of glutamate and GABA in dialysates was 0.03 ng/10 µL and 6.4 pg/10 µL, respectively.

### 4.5. Alkaline Comet Assay

The alkaline comet assay was performed with the use of a CometAssay® Reagent Kit for Single Cell Gel Electrophoresis. The animals were terminated by decapitation 7 days after drug injection and the frontal cortices and hippocampi were dissected. After homogenization and several stages of purification and centrifugation (as previously described in Wojtas et al. (2021)) [29], the nuclear suspension was obtained using a sucrose gradient (2.8 M/2.6 M, bottom to top). The nuclear fraction was mixed with low-melting point agarose and transferred immediately onto CometSlides™. The following steps, including membrane lysis, DNA unwinding, alkaline electrophoresis and staining (SYBR® Gold), were carried out according to the Trevigen CometAssay® protocol. Stained sections were acquired and analyzed under a fluorescence microscope (Nikon Eclipse50i, Nikon Corporation, Tokyo, Japan) equipped with a camera and NIS Elements software. The data was analyzed using OpenComet software v.1.3, a plugin of the ImageJ program v.1.47 (NIH, Bethesda, MD, USA). DNA damage was presented as a tail moment. The tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected by the comet tail length) and the number of damaged pieces (represented by the intensity of DNA in the tail).

### 4.6. Western Blotting

The animals were terminated by decapitation and their brains were quickly removed from the skull. The frontal cortex was dissected and rapidly frozen in liquid nitrogen and stored at −20 °C. The Western blot procedure was performed as previously described in Maćkowiak et al. [70], 2019 and Latusz and Maćkowiak, 2020 [71]. The tissue was homogenized (TissueLyser, Retsch, Munich, Germany) in lysis buffer (PathScan® Sandwich ELISA Lysis Buffer, Cell Signaling, Denver, CO, USA). Protein concentrations in the extracts were determined using a QuantiPro BCA Assay kit (Sigma-Aldrich, Poznań, Poland). The samples of equal protein content were adjusted to a final concentration of 10 mM Tris (pH 6.8) containing 2% SDS, 8% glycerol and 2% 2-mercaptoethanol with bromophenol blue as a marker and then boiled at 100 °C for 8 min. Protein extracts (10 µg of protein per lane, for GluN2A and GluA2 and 20 µg protein per lane for GluN2B and GluA1 analysis) were separated on 7.5% SDS-PAGE gel and transferred to nitrocellulose membranes using an electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, USA); then, the membranes were stained with Ponceau S to confirm gel transfer. The membranes were then cut into three parts: the lower portion was used for GAPDH protein and the proteins with molecular weights greater than 37 kDa were determined from the next portions of the membrane. The blots were washed, and non-specific binding sites were blocked with 5% albumin (Bovine Serum Albumin; Sigma-Aldrich, Poznań, Poland) and blocking reagent (Lumi Light Western Blotting kit, Roche, Basil, Switzerland) in Tris-buffered saline (TBS) for 1 h at room temperature. Then, the blots were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-GluN2A (1:500; AB1555P, Sigma-Aldrich, MercMillipore, Warszawa, Poland), rabbit anti-GluN2B (1:500; AB1557P, Sigma-Aldrich, MercMilipore, Warszawa, Poland), rabbit anti-GluA1 (1:1000; 04-855, Sigma-Aldrich, MercMilipore, Warszawa, Poland), rabbit anti-GluA2 (1:1000; AB1768-I, Sigma-Aldrich, MercMilipore, Warszawa, Poland) and rabbit anti-GAPDH (1:10,000; 14C10, 2118S Cell Signaling Technology, Denver, CO, USA). The peroxidase-conjugated secondary

anti-rabbit IgG antibody (1:1000, Roche, Basil, Switzerland) was used to detect immune complexes (incubation for 1 h at room temperature). Blots were visualized using enhanced chemiluminescence (ECL, Lumi-LightPlus Western Blotting Kit, Roche, Basil, Switzerland) and scanned using a luminescent image analyzer (LAS-4000, Fujifilm, Boston, MA, USA). The molecular weights of immunoreactive bands were calculated on the basis of the migration of molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA) using Multi Gauge V3.0 (Fujifilm, Boston, MA, USA) software. The levels of analyzed proteins were normalized for GAPDH protein.

### 4.7. Open Field (OF) Test

The open field test was performed to modify the procedure described by Rogóż and Skuza (2011) [72]. A round black arena (1 m in diameter) was virtually divided into eight sections, creating a wheel. The test was conducted in a dimly lit room, with the middle of the arena illuminated by a 75 W light bulb placed at a height of 75 cm. Rats were placed in the middle of the arena 20 min after drugs injection. Their behavior was recorded for 10 min. The exploration was quantified with the following parameters: time of walking, number of line crossings, episodes of peeping under the arena, number of grooming events, and number of rearings.

### 4.8. Light–Dark Box (LDB) Test

This experimental procedure was performed in the TSE Fear Conditioning System (TSE System, Germany). The light/dark exploration test was performed as previously described by Chocyk et al. (2015) [73] and Bilecki (2021) [74]. Briefly, each experimental cage included an arena (45 × 45 × 45 cm) with a light compartment made of clear acrylic and a dark compartment made of black acrylic. The black compartment covered 33% of the total cage area, and the black dividing wall was equipped with a central tunnel gate (11 × 8.4 cm). The light compartment was brightly illuminated (100 lx), whereas the dark compartment received no light at all. The animals were kept in total darkness for 1 h prior to the testing, and the entire experiment was conducted with the room lights off. The animals were individually tested in single 10 min trials. At the beginning of each testing session, a rat was placed in the center of the light compartment, facing away from the gate. The behavioral responses during the test session were recorded using Fear Conditioning software (TSE, Bad Homburg, Germany). Specifically, the number of transitions between the compartments, the time spent in each compartment and locomotor activity (the distance traveled) were measured.

### 4.9. Forced Swim Test (FST) in Rats

On the first day of the FST (pre-test), the rats were placed individually in a cylinder (50 cm high × 23 cm in diameter) filled to a 30 cm depth with water (25 ± 1 °C) for 15 min, then removed from the water, dried with towels, placed in a warmer enclosure for 15 min and finally returned back to their home cages, as previously described (Detke et al., 1995) [75]. The cylinders were emptied and cleaned between rats. Twenty-four hours following the first exposure to forced swimming, the rats were retested for five minutes under identical conditions. Retest sessions were evaluated by two observers who were unaware of the treatment condition and who measured the swimming, climbing and immobility time. A rat was rated to be immobile if it was only making the necessary movements to keep its head above water; swimming behavior was defined as actively making swimming movements that caused the rat to move within the center of the cylinder or swim below the surface of water (diving); climbing behavior was recorded if a rat was making forceful thrashing movements with its forelimbs against the walls of the cylinder.

### 4.10. Statistical Analysis

Drug effects on DA, 5-HT, glutamate and GABA release in the brain regions were analyzed with repeated measures ANOVA on normalized responses followed by Tukey's

post hoc test. All obtained data were presented as a percentage of the basal level, assumed to be 100%. The data collected from the LDB test were analyzed with the two-way ANOVA followed by Tukey's post hoc test. The results obtained in the open field test, forced swim test, Western blotting data and results obtained in the comet assay were analyzed with one-way ANOVA followed by Tukey's post hoc test. The differences were considered significant if $p < 0.05$. The detected outliers were removed from the dataset using Grubb's test. All statistical analyses were carried out using STATISTICA v.13.3 StatSoft Inc. 1984-2011 (TIBCO Software Inc., Palo Alto, CA, USA) and GraphPad Prism v.9.1.2 (GraphPad Software Inc., San Diego, CA, USA).

## 5. Conclusions

In conclusion, our results indicate that both psilocybin and ketamine exert a profound effect on thalamo-cortical neurotransmission. It seems likely that psilocybin and ketamine act on various molecular targets. Psilocybin activates 5-HT2A receptors; ketamine blocks subsets of NMDA receptors on GABA interneurons, which in turn disinhibits pyramidal cells. Both mechanisms result in the facilitation of glutamate release, which exerts stimulatory effect on dopaminergic VTA cells or serotonergic dorsal raphe neurons. As a consequence, this leads to an increase of DA and 5-HT levels. However, the modulatory role of 5-HT1A and 5-HT2C receptors in psilocybin's effect cannot be excluded. Our findings also add neurochemical evidence that GABA neurons in the reticular nucleus of thalamus underlie the mechanism regulating the sensory information provided to the cortex by psilocybin. The increase in glutamate extracellular level in the frontal cortex after acute doses of psilocybin seems to correspond with changes in the NMDA receptor subunit GluN2A level. The DNA damage produced by higher doses of psilocybin and ketamine may result from glutamate-induced excitotoxicity and oxidative stress. The changes observed in the level of neurotransmitters do not translate into rat behavior tested 24 h after administration. No effect on anxiety and the reduction of immobility may result from adaptative mechanisms triggered by acute doses of both drugs. Future studies in stress models of depression should be subjected to unravel the basis of the antidepressant effect of psychedelics.

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**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki and approved by the Local Ethical Commission for Experimentations on Animals in Kraków (permit no. 112/2021, 324/2021, 79/2022). The experimental procedures were conducted according to recommendations of the National Institutes of Health and were approved by the Ethical Committee of the 2nd Local Institutional Animal Care and Use Committee (IACUC) in Kraków, Poland in conformity with institutional guidelines and in compliance with national and international laws and policies (permit numbers: 112/2021, 324/2021 and 79/20226). According to the 3R policy, the number of animals was reduced to an essential minimum. All the procedures regarding the study design, animal experiments, statistical analysis, and data reporting fulfill the criteria of the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (https://www.nc3rs.org.uk/arrive-guidelines, access date: 14 May 2022).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data is contained within the article and Supplementary Material.

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# Limbic system response to psilocybin and ketamine administration in rats: A neurochemical and behavioral study

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*Article*

# Limbic System Response to Psilocybin and Ketamine Administration in Rats: A Neurochemical and Behavioral Study

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**Abstract:** Pathophysiology of depression is related with reduced volume of the hippocampus and amygdala and hypertrophy of the nucleus accumbens. The mechanism of these changes is not well understood, but clinical studies have shown that administration of the fast-acting antidepressant ketamine reversed the decrease in hippocampus and amygdala volume in depressed patients, and the magnitude of this effect correlated with the reduction of depressive symptoms. In the present study, we attempted to find out whether the psychedelic substance psilocybin affects neurotransmission in the limbic system in comparison to ketamine. Psilocybin and ketamine increased the release of dopamine (DA) and serotonin (5-HT) in the nucleus accumbens of naive rats as demonstrated using microdialysis. Both drugs influenced glutamate and GABA release in the nucleus accumbens, hippocampus and amygdala and increased ACh levels in the hippocampus. The changes in D2, 5-HT1A and 5-HT2A receptor density in the nucleus accumbens and hippocampus was observed as a long-lasting effect. A marked anxiolytic effect of psilocybin in acute phase and 24 h post-treatment was shown in the open field test. These data provide the neurobiological background for psilocybin effect on stress, anxiety and structural changes in the limbic system and translate into antidepressant effect of psilocybin in depressed patients.

**Keywords:** neurotransmitter release; dopamine D2 receptors; serotonin 5-HT1A; 5-HT2A receptors; limbic system

## 1. Introduction

As reported by the World Health Organization, major depressive disorder can affect up to 5% of adult population worldwide [1], while 10 to 15% of adults experience a depressive episode during their lifetime, though this statistics can be underestimated because many individuals suffering from affective disorders do not seek professional help [2]. Even though the pathophysiology of depression is still not clearly understood, a number of key factors relevant to this problem have been proposed. Firstly, numerous risk factors have been mentioned, with the most significant being either traumatic or chronic stress [3]. Moreover, depression-related changes have been observed in various brain structures, most importantly in the frontal cortex and the limbic system [4], though the direction of changes was opposite, namely hypoactivity was shown in the cortex while hyperactivity in the limbic structures [5]. The prefrontal cortex appears to be a center that integrates many sensory inputs from the structures like hippocampus, amygdala and nucleus accumbens, assigning them either rewarding or aversive properties [3].

The most important component of the limbic system that is affected in major depressive disorder is the hippocampus [4]. Like in the case of the prefrontal cortex, studies showed a clear correlation

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between atrophy of the hippocampus and severity and length of the disorder [3]. Patients with greater hippocampal volume seem to respond better to the treatment, while those with a smaller volume are more prone to being treatment resistant [4]. Those structural changes translate into functional ones, as the hippocampus plays a crucial role in processes of learning and memory [5], and is one of a few places where neurogenesis can occur in adults.

The amygdala plays a central role in processes associated with emotional states and fear processing. As with the hippocampus, its reduced volume correlates with the severity of the depression [6] moreover, its hyperactivity correlates with the severity of the disorder [5].

In contrast to the two aforementioned brain areas, the nucleus accumbens exhibits hypertrophy both in preclinical models and in individuals suffering from depression, which probably arises from the observed increase in the length of the dendrites and spine density [7]. Furthermore, according to DSM-V [8] anhedonia is one of the two core symptoms of depression, while inability to experience pleasure is a clear manifestation of disorders in the functioning of the reward system, where the nucleus accumbens plays a crucial role [9].

Recent studies showed that ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist proposed as a prototypical fast-acting antidepressant drug [10,11] exerts its beneficial effect not only in the frontal cortex, but also by targeting areas in the limbic system. Administration of ketamine promotes neural plasticity in the hippocampus in preclinical models [12] and increases hippocampal volume in depressed patients [13], while significantly improving their mood [13,14], which is an effect correlated with the volume of the hippocampus. Zhou et al. [15] observed that ketamine administration increased the amygdala volume in depressed patients, and the magnitude of the effect correlated with the reduction of depressive symptoms. Furthermore, ketamine treatment seems to reverse the depression-induced hypertrophy of the nucleus accumbens [7]. Altogether, the research cited above leads to the conclusion that ketamine affects not only the prefrontal cortex, but also the limbic structures, inducing a complex effect that normalizes its functioning and increases its connectivity with the prefrontal cortex [3].

Despite opening a new chapter in the treatment of affective disorders and becoming the prototypical fast-acting antidepressant drug, ketamine is far from perfect. Its effects fade around the second week after the drug administration, leading to the need of repeated dosing [16]. Furthermore, it possesses a plethora of adverse effects, of which drug abuse is the most significant one. Similarly to ketamine, psychedelics seem to induce synaptic plasticity and neurogenesis in preclinical models [16,17], while being devoid of the drawbacks of ketamine. This phenomenon translates further into antidepressant effects observed in preclinical models after administration of psychedelics [18,19]. Among serotonergic hallucinogens, psilocybin is currently the most studied compound, as it has proven to be at least as effective as common antidepressant drugs [20]. In contrast to ketamine, reports on the possible effects exerted by psychedelics on the limbic system are scarce, justifying the need for thorough studies.

To address the questions regarding the response of the limbic system to the administration of psilocybin and to compare it to ketamine, we have examined the effects of both drugs on neurotransmission in the hippocampus, amygdala and nucleus accumbens using microdialysis in freely-moving rats. Furthermore, Western blot analysis was performed to assess the long-lasting effects of the chosen drugs on protein levels of selected receptors. As the limbic system plays a key role in locomotion and fear response, the rat behavior was studied in the open field test both directly and 24h after administration of the drugs, to examine both acute and possible prolonged effects.

## 2. Materials and Methods

### *2.1. Animals*

Adult male Wistar-Han rats (280-350 g; Charles River, Germany) were used in all experiments. The animals were initially acclimatized and housed (6 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

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animals had free access to tap 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, 324/2021 and 79/2022).

*2.2. Drugs and reagents*

Ketamine hydrochloride was purchased from Tocris/Bio-Techne (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. [21]; both were dissolved in sterile water. All solutions were made fresh on the day of experiment. The dose of ketamine (10 mg/kg) was based on a report by Popik et al. [22], while doses of psilocybin (2 and 10 mg/kg) on work by Jefsen et al. [23]. Psilocybin was given subcutaneously (sc) while ketamine intraperitoneally (ip) in the volume of 2 ml/kg. The control group was treated with 0.9% NaCl solution in the same way. Ketamine, xylasine hydrochlorides and sodium pentobarbital used for anesthetizing the animals came from Biowet Puławy (Puławy, Poland). All necessary chemicals of the highest purity used for analysis by high-performance liquid chromatography (HPLC) were obtained from Merck (Warszawa, Poland). O-phthalaldehyde (OPA) from Sigma-Aldrich (Poznań, Poland) was used for the derivatization of glutamate and GABA to electroactive compounds. The reagents used in immunohistochemistry were purchased from Sigma-Aldrich (Poznań, Poland), Vector Laboratories (Burlingame, CA, USA), and Proteintech (Manchester, UK).

*2.3. Brain microdialysis*

Ketamine and xylazine (75 and 10 mg/kg, respectively) were injected intramuscularly to anesthetize the animals. Microdialysis probes (MAB 4.15.3Cu and MAB 4.15.2Cu, AgnTho's AB, Sweden) were implanted into the following brain structures using the determined coordinates (mm): nucleus accumbens AP +1.6, L +1.0, V -8.0, hippocampus AP -5.8, L 4.5, V -5.0 and amygdala AP -3.1, L +4.5, V -8.0 from the dura [24]. Seven days after implantation, probe inlets were connected to a syringe pump (BAS, West Lafayette, IN, USA) which delivered artificial cerebrospinal fluid composed of (mM): 147 NaCl, 4 KCl, 2.2 $CaCl_2 \cdot 2H_2O$, 1.0 $MgCl_2$ at a flow rate of 2 µL/min. Five baseline samples were collected every 20 minutes after the washout period of 2 hours. The respective drugs were administered, and dialysate fractions were collected for the next 240 minutes. As the experiment ended, the rats were terminated, and their brains underwent histological examination to validate probe placement.

*2.4. Extracellular concentration of DA, 5-HT, glutamate, GABA and acetylcholine*

Extracellular DA and 5-HT levels were analyzed using an Ultimate 3000 System (Dionex, USA), electrochemical detector Coulochem III (model 5300; ESA, USA) with a 5020 guard cell, a 5040 amperometric cell, and a Hypersil Gold C18 analytical column (3 µm, 100 × 3 mm; Thermo Fisher Scientific, USA). The details of the method have been described elsewhere [25,26]. The chromatographic data were processed by Chromeleon v.6.80 (Dionex, USA) software package run on a personal computer.

Glutamate and GABA levels in the extracellular fluid were measured by HPLC with electrochemical detection after derivatization of samples with OPA/sulfite reagent to form isoindole-sulfonate derivatives as previously described [25,26]. The data were processed using Chromax 2005 (Pol-Lab, Warszawa, Poland) software on a personal computer.

Extracellular levels of ACh were analyzed by UHPLC with electrochemical detection. The ACh analysis is based on ion-pairing HPLC separation, followed by on-line enzymatic conversion of ACh to hydrogen peroxide and detection on a Pt working electrode (SenCell with 2 mm Pt working electrode) and HyREF reference electrode at the potential of 200 mV. Chromatography was

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performed using the ALEXYS Neurotransmitter Analyzer, a DECADE Elite electrochemical detector, AS 110 Autosampler, and LC 110 pump (Antec Leyden B. V., Zoeterwoude, The Netherlands). ACh as positively charged was separated on Acquity UPLC HSS T3 analytical column (1.8 μm, 1 × 50 mm; Waters, Milford, MA, USA). After separation, ACh passed through an immobilized enzyme reactor AChE/ChOx IMER (AC-ENZYM II, 1 × 4 mm, Eicom, Kyoto, Japan). The mobile phase was composed of 50 mM monosodium orthophosphate buffer adjusted to pH 7.8, 0.5 mM $Na_2EDTA$, 2.8 g/L 1-octanesulfonic acid sodium salt and 0.5 mM tetramethylammonium chloride. The flow rate during analysis was set to 0.05 mL/min. The chromatographic data were processed by CLARITY v.6.2.0.208 (DataApex Ltd.) chromatography software run on a personal computer.

*2.5. Western blotting*

The Western blot procedure was performed as previously described in Wojtas et al. [27]. The hippocampus and nucleus accumbens were homogenized (TissueLyser, Retsch, Munich, Germany) in lysis buffer ( PathScan Sandwich ELISA Lysis Buffer, Cell Signaling, Denver, CO, USA). Bicinchoninic Acid Kit ( Sigma-Aldrich, Poznań, Poland) was used to determine protein concentrations. Protein extracts (20 μg of protein per lane for 5-HT1A, 40 μg protein per lane for 5-HT2A and 40 μg protein per lane for D2 analysis ) were separated on a 7.5% SDS-PAGE gel and transferred to nitrocellulose membranes using an electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were then stained with Ponceau S to confirm gel transfer and cut into three parts: the lower portion was used for GAPDH protein assessment, and the proteins with molecular weights greater than 37 kDa were determined from the next portions of the membrane. The blots were washed, and non-specific binding sites were blocked with 5% albumin (Bovine Serum Albumin; Sigma- Aldrich) and blocking reagent (Lumi Light Western Blotting kit, Roche, Basil Switzerland) in Tris-buffered saline (TBS) for 1 h at room temperature. Then the blots were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-5-HT1A (1:1000; ab85615, Abcam), rabbit anti-5-HT2A (1:500; ab216959, Abcam), rabbit anti-D2 (1:500; AB5084P, Sigma-Aldrich) and rabbit anti-GAPDH (1:10000; 14C10, 2118, Cell Signaling Technology). The peroxidase-conjugated secondary anti-rabbit IgG antibody (1:1000, Roche) was used to detect immune complexes (incubation for 1h at room temperature). Blots were visualized using enhanced chemiluminescence (ECL, Lumi-LightPlus Western Blotting Kit, Roche) and scanned using a luminescent image analyzer (LAS-4000, Fujifilm, Boston, MA, USA). The molecular weights of immunoreactive bands were calculated on the basis of the migration of molecular weight markers (Bio-Rad Laboratories) using Multi Gauge V3.0 (Fujifilm) software. The levels of analyzed proteins were normalized to GAPDH protein.

*2.6. Open Field Test*

The open field test was performed according to modification of the procedure described by Rogóż and Skuza [28]. A round black arena (1 m in diameter) was virtually divided into eight radiant sections formed by lines intersecting the center of the field. The test was conducted in a dimly lit room, except for the middle of the arena, which was illuminated by a 75 W light bulb placed 75 cm above. Rats were placed in the middle of the arena 60 minutes after drug injection. Their behavior was recorded for 5 minutes. The exploration was quantified with the following parameters: time of walking, number of line crossings reflecting ambulatory distance, episodes of looking under the edge of the field (peeping), number of grooming events, number of rearings as vertical activity and time spent in the central zone.

*2.7. Statistical analysis*

Drug effects on DA, 5-HT, ACh, GABA and glutamate release in the brain regions were analyzed with repeated measures ANOVA on normalized responses followed by Tukey's post hoc test. All obtained data were presented as a percentage of the basal level, assumed to be 100%. Total effects expressed as area under the curve (AUC) and GABA/GLU ratio were analyzed with one-way

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ANOVA followed by Tukey's post hoc test. The results obtained in the open field test and western blotting data were analyzed with one-way ANOVA followed by Tukey's post hoc test. The differences were considered significant if $p < 0.05$. The detected outliers were removed from the data set using Grubb's test. All statistical analyses were carried out using STATISTICA v.10 StatSoft Inc. 1984-2011 (USA) and GraphPad Prism v.5.00 GraphPad Software Inc. (USA).

## 3. Results

### *3.1. The effect of psilocybin and ketamine on extracellular levels of DA and 5-HT in the rat nucleus accumbens*

Psilocybin at doses of 2 and 10 mg/kg significantly increased extracellular levels of DA up to ca. 180% of baseline in the rat nucleus accumbens (Figure 1A). Ketamine (10 mg/kg) was more potent in increasing (up to ca. 250% of baseline) DA extracellular level (Figure 1A). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{3,23} = 123$, $p < 0.0001$), sampling period ($F_{11,253} = 15.2$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{33,253} = 15.9$, $p < 0.0001$). Total effects expressed as AUC shown in Figure 1B were significantly increased for psilocybin 2 and 10 mg/kg and ketamine ($F_{3,23} = 124$, $p < 0.001$, one-way ANOVA).

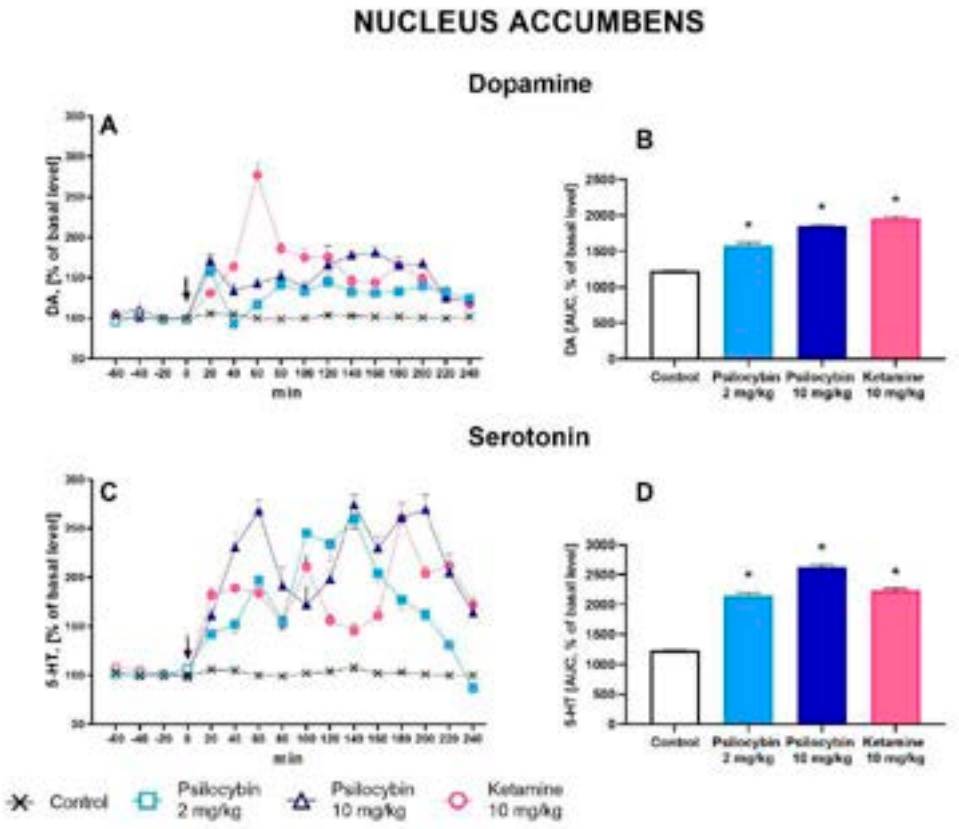


**Figure 1.** The time-course (**A**, **C**) and total (**B**, **D**) effect of psilocybin (2 and 10 mg/kg) and ketamine (10 mg/kg) on the dopamine (DA) and serotonin (5-HT) levels in the rat nucleus accumbens. The total effect was calculated as an area under the concentration-time curve (AUC) and is expressed as a percentage of the basal level. Values are the mean ± SEM (n = 6-8) for each neurotransmitter. The drug injection is indicated with an arrow. Filled symbols or * show statistically significant differences ($p < 0.001$) between control and drug treatment groups as estimated by repeated measures ANOVA (time-course) or one-way ANOVA (total effect) followed by Tukey's post hoc test.

The extracellular 5-HT level was increased in the rat nucleus accumbens by both doses of psilocybin (up to 200-250 % of baseline) and less potently by ketamine (up to 200 % of baseline) (Figure 1C). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{3,20} = 267$,

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$p < 0.0001$), sampling period ($F_{11,220} = 23$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{33,220} = 16.1$, $p < 0.0001$). Total effects expressed as AUC shown in Figure 1D were significantly increased for both psilocybin doses, 2 and 10 mg/kg, and for ketamine ($F_{3,20} = 268$, $p < 0.0001$, one-way ANOVA).

*3.2. The effect of psilocybin and ketamine on extracellular levels of glutamate and GABA in the rat nucleus accumbens, hippocampus and amygdala*

The extracellular glutamate (GLU) level in the nucleus accumbens was slightly but significantly decreased by both psilocybin doses (to ca. 80 % of baseline), but was markedly increased by ketamine (up to 160 % of baseline) (Figure 2A). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{3,26} = 99$, $p < 0.0001$), sampling period ($F_{11,286} = 32$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{33,286} = 8.7$, $p < 0.0001$). Total effects expressed as AUC shown in Figure 2B were significantly decreased by psilocybin 2 mg/kg and 10 mg/kg and increased by ketamine ($F_{3,26} = 99$, $p < 0.0001$, one-way ANOVA).

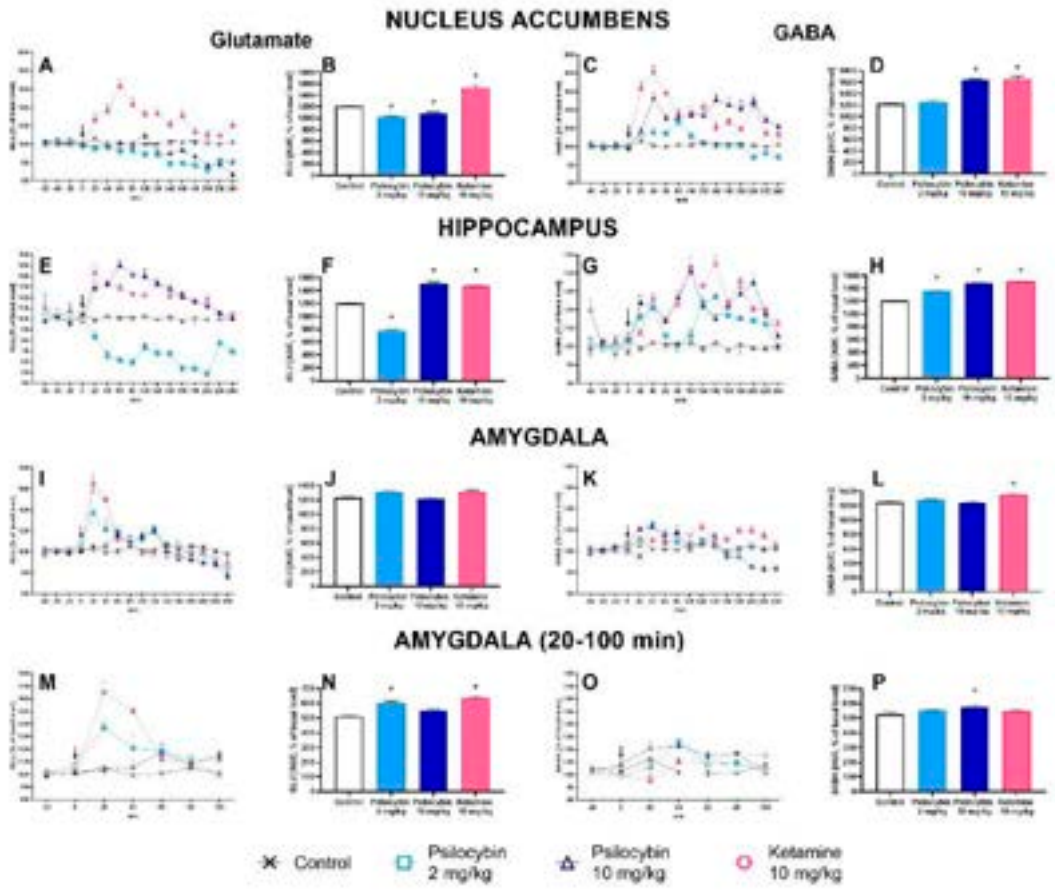


**Figure 2.** The time-course (**A, C, E, G, I, K, M, O**) and total (**B, D, F, H, J ,L, N, P**) effect of psilocybin (2 and 10 mg/kg) and ketamine (10 mg/kg) on the extracellular levels of glutamate (GLU) and γ-aminobutyric acid (GABA) in the rat nucleus accumbens, hippocampus and amygdala. The total effect was calculated as an area under the concentration-time curve (AUC) and is expressed as a percentage of the basal level. Values are the mean ± SEM (n = 6-9 in nucleus accumbens groups, n = 6-7 in hippocampus groups, n = 5-6 in amygdala groups) for each neurotransmitter. The drug injection is indicated with an arrow. Filled symbols or * ($p < 0.001$) show statistically significant differences between control and drug treatment groups as estimated by repeated measures ANOVA (time-course) or one-way ANOVA (total effect) followed by Tukey's post hoc test.

The extracellular level of GABA in the nucleus accumbens was slightly and not significantly increased by psilocybin dose of 2 mg/kg (up to ca. 120 % of baseline), more potently by the higher

dose of 10 mg/kg (up to ca. 150% of baseline) and by ketamine (up to 180 % of baseline) (Figure 2C). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{3,25} = 62$, $p < 0.0001$), sampling period ($F_{11,275} = 12.8$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{33,275} = 7.9$, $p < 0.0001$). Total effects expressed as AUC shown in Figure 2D were significantly increased by the higher dose of psilocybin and ketamine ($F_{3,35} = 276$, $p < 0.0001$, one-way ANOVA).

The extracellular glutamate (GLU) level in the hippocampus was significantly decreased by psilocybin dose of 2 mg/kg (up to ca. 50 % of baseline) but was markedly increased by the higher dose of 10 mg/kg (up to ca. 150% of baseline) and by ketamine (up to 140 % of baseline) (Figure 2E). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{3,22} = 365$, $p < 0.0001$), sampling period ($F_{11,242} = 19.4$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{33,242} = 8.7$, $p < 0.0001$). Total effects expressed as AUC shown in Figure 2F were significantly decreased for psilocybin 2 mg/kg and significantly increased for psilocybin 10 mg/kg and ketamine ($F_{3,22} = 366$, $p < 0.0001$, one-way ANOVA).

The extracellular level of GABA in the hippocampus was significantly increased by psilocybin dose of 2 mg/kg (up to ca. 120 % of baseline), more potently by the higher dose of 10 mg/kg (up to ca. 140% of baseline) and by ketamine (up to 140 % of baseline) (Figure 2G). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{3,22} = 223$, $p < 0.0001$), sampling period ($F_{11,242} = 16.8$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{33,242} = 8.5$, $p < 0.0001$). Total effects expressed as AUC shown in Figure 2H were significantly increased by both psilocybin doses and ketamine ($F_{3,22} = 224$, $p < 0.0001$, one-way ANOVA).

The extracellular glutamate (GLU) level in the amygdala was significantly increased by psilocybin dose of 2 mg/kg (up to ca. 140 % of baseline) and by ketamine (up to ca. 160% of baseline) between 20 to 100 min of collection period and was not affected by both psilocybin doses and ketamine for the rest of time (Figure 2I, M). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{3,18} = 20.5$, $p < 0.0001$), sampling period ($F_{4,12} = 15.4$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{4,72} = 11.6$, $p < 0.0001$). Total effects expressed as AUC between 20 – 100 min of collection period and shown in Figure 2N were significantly increased for psilocybin 2 mg/kg and ketamine and not changed for psilocybin 10 mg/kg ($F_{3,18} = 20.5$, $p < 0.0001$, one-way ANOVA). Total effects expressed as AUC for the whole collection period did not differ from the control group (Figure 2J).

The extracellular level of GABA in the amygdala was slightly but not significantly increased by psilocybin dose of 2 mg/kg and ketamine and more potently by higher psilocybin dose of 10 mg/kg (up to ca. 120% of baseline) (Figure 2O) between 20 to 100 min of collection period and was not affected by both psilocybin doses and ketamine for the rest of time (Figure 2K). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{3,18} = 3.2$, $p < 0.05$), sampling period ($F_{4,12} = 2.9$, $p < 0.03$), and the interaction between treatment groups and sampling period ($F_{4,72} = 4.9$, $p < 0.001$). Total effects expressed as AUC between 20 – 100 min of collection period and shown in Figure 2P were significantly increased for psilocybin 10 mg/kg and were not changed for psilocybin 2 mg/kg and ketamine ($F_{3,18} = 3.2$, $p < 0.05$, one-way ANOVA). Total effects expressed as AUC for the whole collection period did not differ from the control group in spite of ketamine ($F_{3,18} = 6.5$, $p < 0.004$, one-way ANOVA) (Figure 2L).

### *3.3. The effect of psilocybin and ketamine on GABA/glutamate ratio in the rat nucleus accumbens, hippocampus and amygdala*

To find out the net effect of psilocybin and ketamine on GABA and glutamate release in rat brain regions, the GABA/GLU ratio was calculated. The mean of GABA/GLU index of AUC values for each group is presented in Figure 3A. There was an increase in GABA/GLU ratio for the whole collection period for psilocybin 2 and 10 mg/kg but not for ketamine in the nucleus accumbens ($F_{3,25} = 31$, $p < 0.0001$, one-way ANOVA). The increase in GABA/GLU ratio of AUC values for psilocybin 2 mg/kg but not for its higher dose and ketamine was observed in the hippocampus (Figure 3A) ($F_{3,25} = 255$, $p < 0.0001$, one-way ANOVA). No change was found in GABA/GLU ratio of AUC values for the whole

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collection period in the amygdala (Figure 3A) ($F_{3,18} = 0.96$, $p < 0.43$, one-way ANOVA). However, the decrease in GABA/GLU ratio of AUC values for psilocybin 2 mg/kg and ketamine was observed between 20 – 100 min of collection period (Figure 3B) ($F_{3,18} = 8.3$, $p < 0.001$, one-way ANOVA).

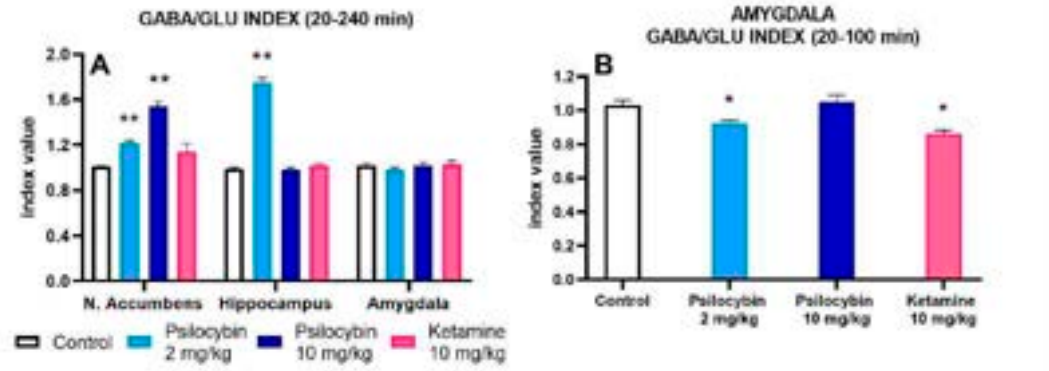


**Figure 3.** The AUC GABA/GLU ratio during 20-240 min of collection period after administration of psilocybin (2 and 10 mg/kg) and ketamine (10 mg/kg) in the rat nucleus accumbens, hippocampus and amygdala (**A**) and during 20-100 min of collection period in the amygdala (**B**). Values are the mean ± SEM (n = 6-9 in the nucleus accumbens groups, n= 6-7 in hippocampus groups and n=5-6 in amygdala groups) as estimated by one-way ANOVA followed by Tukey's post hoc test. * $p < 0.02$, * $p < 0.001$ indicate statistically significant differences between control and drug treatment groups.

*3.4. The effect of psilocybin and ketamine on extracellular levels of ACh in the rat hippocampus*

The extracellular ACh level was increased in the rat hippocampus by both doses of psilocybin and ketamine (up to 700 % , 200 % and 250 % of baseline, respectively) (Figure 4A). Repeated measures ANOVA showed a significant effect of treatment groups ($F_{3,18} = 74$, $p < 0.0001$), sampling period ($F_{5,90} = 92$, $p < 0.0001$), and the interaction between treatment groups and sampling period ($F_{15,90} = 35$, $p < 0.0001$). Total effects expressed as AUC shown in Figure 4B were significantly increased for both psilocybin doses, 2 and 10 mg/kg, and for ketamine ($F_{3,18} = 74$, $p < 0.0001$, one-way ANOVA).

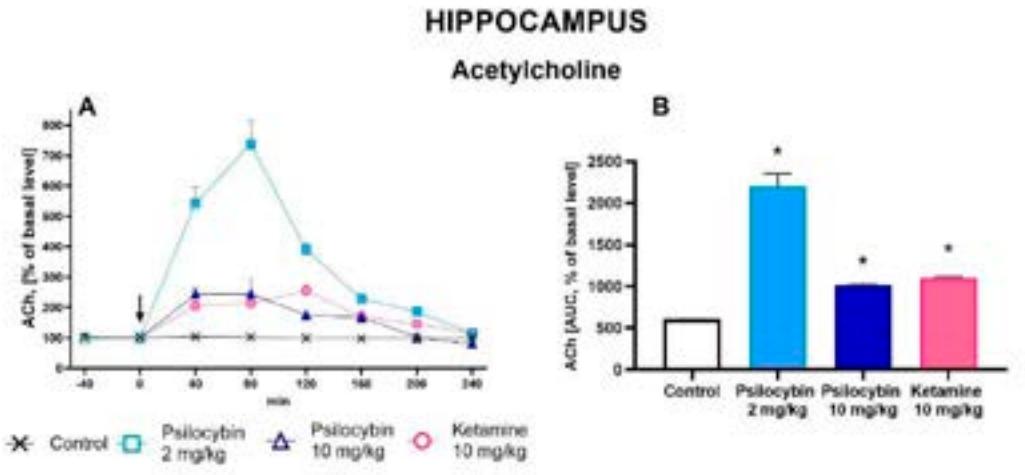


**Figure 4.** The time-course (**A**) and total (**B**) effect of psilocybin (2 and 10 mg/kg) and ketamine (10 mg/kg) on the extracellular acetylcholine (ACh) levels in the rat hippocampus. The total effect was calculated as an area under the concentration-time curve (AUC) and is expressed as a percentage of the basal level. Values are the mean ± SEM (n = 5-7). The drug injection is indicated with an arrow. Filled symbols or * show statistically significant differences ($p < 0.001$) between control and drug treatment groups as estimated by repeated measures ANOVA (time-course) or one-way ANOVA (total effect) followed by Tukey's post hoc test.

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*3.5. The effect of psilocybin and ketamine on 5-HT1A and 5-HT2A receptors level in the rat hippocampus*

Both doses of psilocybin significantly decreased the 5-HT1A protein level by ca. 12-13 % while ketamine significantly increased it by 18 % over control ($F_{3,28} = 60$, $p < 0.0001$, one-way ANOVA) as measured 7 days after drug administration (Figure 5A,B). 5-HT2A protein level was decreased by psilocybin 2 mg/kg dose, but was increased by the higher one and ketamine by 11 and 35 %, respectively in comparison to control ($F_{3,28} = 57$, $p < 0.001$, one-way ANOVA) (Figure 5C,D).

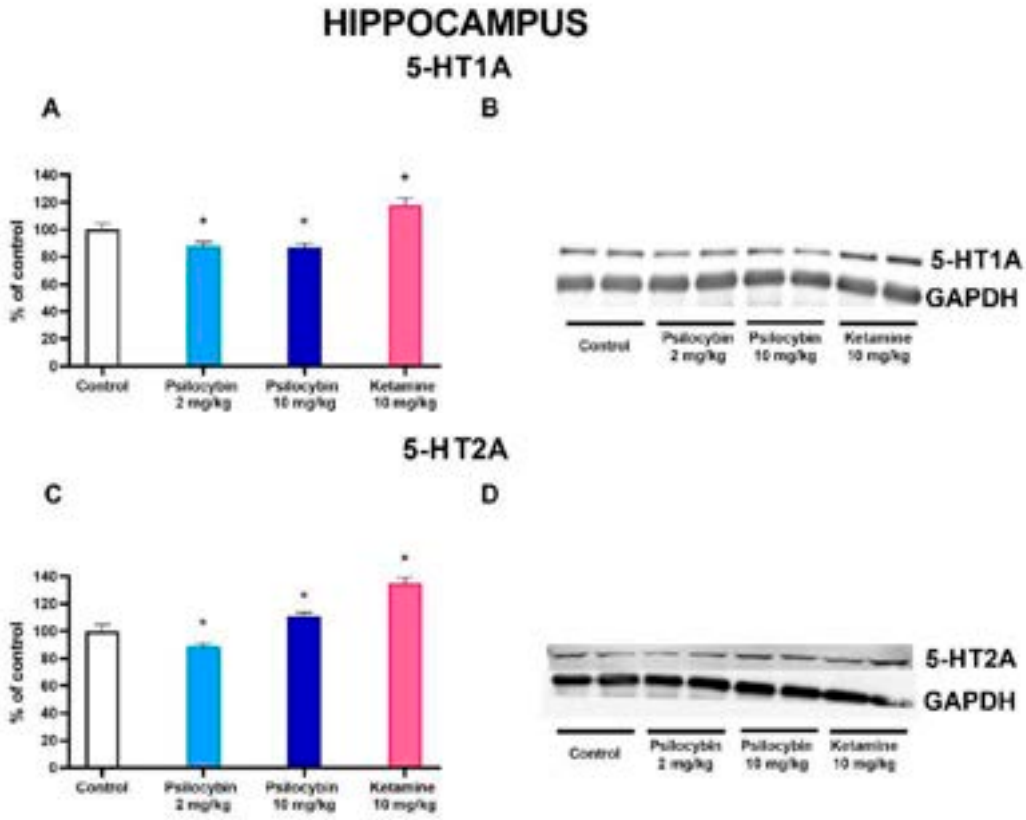


**Figure 5.** The levels of 5-HT1A receptor (**A**) and 5-HT2A receptor (**C**) in the rat hippocampus estimated 7 days after psilocybin (2 and 10 mg/kg) or ketamine (10 mg/kg) administration. The data are shown as percentages of the levels of the appropriate control groups. Each data point represents the mean ± SEM (n = 10). * $p < 0.001$ vs. appropriate control group (one-way ANOVA followed by Tukey's post hoc test). Representative examples of photomicrographs of the immunoblots using 5-anti-HT1A and anti-5-HT2A antibodies (**B** and **D**, respectively).

*3.6. The effect of psilocybin and ketamine on D2 and 5-HT2A receptors level in the rat nucleus accumbens*

In the nucleus accumbens, the dopamine D2 protein receptor level was significantly increased by the higher dose of psilocybin by ca. 30 %, but not by its lower dose or ketamine ($F_{3,28} = 11.6$, $p < 0.0001$, one-way ANOVA) as measured 7 days after drug administration (Figure 6C,D). 5-HT2A protein level was not affected by any treatment ($F_{3,28} = 0.24$, $p < 0.87$, one-way ANOVA)(Figure 6A,B).

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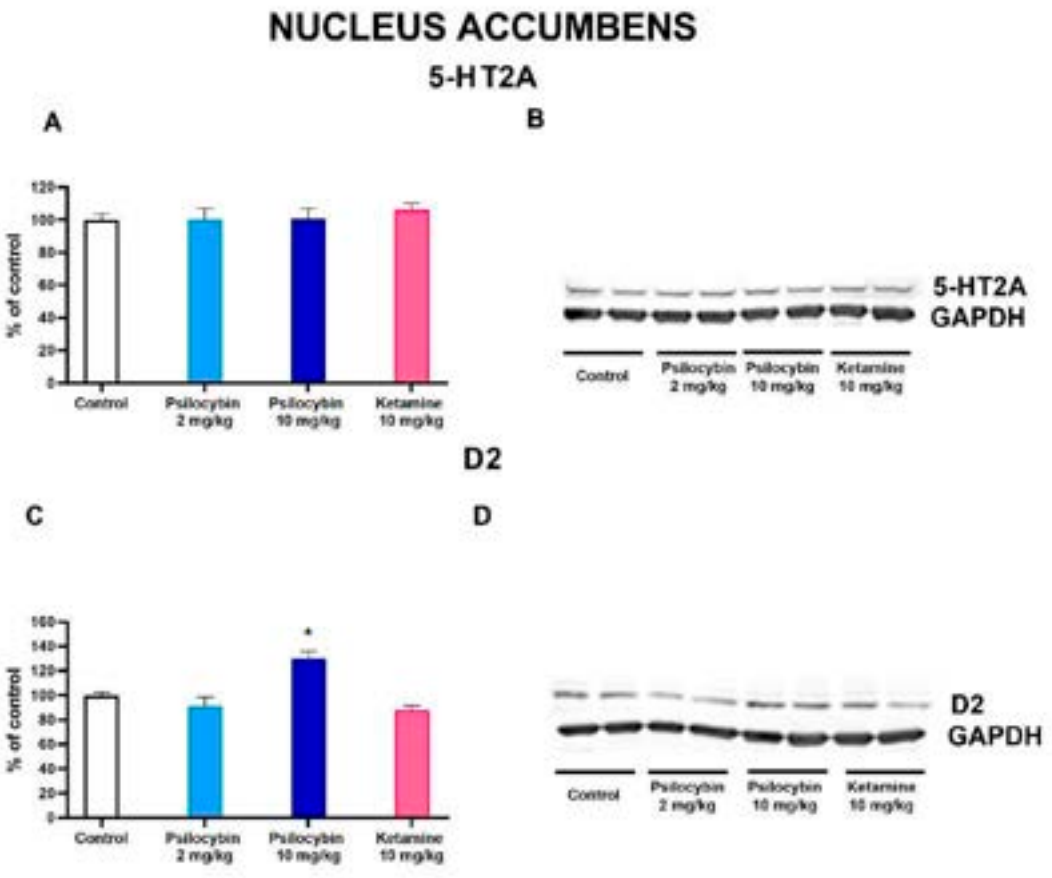


**Figure 6.** The levels of 5-HT2A receptor (**A**) and D2 receptor (**C**) in the rat nucleus accumbens estimated 7 days after psilocybin (2 and 10 mg/kg) or ketamine (10 mg/kg) administration. The data are shown as percentages of the levels of the appropriate control groups. Each data point represents the mean ± SEM (n = 10). * $p < 0.0001$ vs. appropriate control group (one-way ANOVA followed by Tukey's post hoc test). Representative examples of photomicrographs of the immunoblots using 5-anti-HT2A and anti-D2 antibodies (**B** and **D**, respectively).

*3.7. The effect of psilocybin and ketamine on activity of rats in the Open Field Test*

Psilocybin at doses of 2 and 10 mg/kg significantly decreased the time of walking while ketamine (10 mg/kg) increased it above the control level one hour after drug administration (Figure 7A) ($F_{3,36} = 47$, $p < 0.001$, one-way ANOVA). The number of crossings reflecting ambulatory distance was significantly decreased by both doses of psilocybin, but was not changed by ketamine (Figure 7A)($F_{3,36} = 44$, $p < 0.0001$, one-way ANOVA). The number of episodes of peeping and rearing reflecting vertical activity was decreased by both doses of psilocybin and not changed by ketamine one hour after administration (Figure 7B) ($F_{3,36} = 10.1$, $p < 0.001$; $F_{3,36} = 4.7$, $p < 0.01$, one-way ANOVA, respectively). The center exploration was significantly increased by both doses of psilocybin but no difference between control and ketamine was observed one hour after drug administration (Figure 7B)($F_{3,36} = 98$, $p < 0.0001$, one-way ANOVA).

The time of walking and the number of crossings reflecting ambulatory distance were not changed by both doses of psilocybin and ketamine 24 h after drug administration (Figure 7C) ($F_{3,36} = 0.49$, $p < 0.69$; $F_{3,36} = 0.02$, $p < 0.99$, one-way ANOVA, respectively). Similarly, the number of episodes of rearing was not changed by both doses of psilocybin and ketamine 24 h after drug administration (Figure 7D) ($F_{3,36} = 1.87$, $p < 0.15$, one-way ANOVA). However, the number of episodes of peeping was decreased by psilocybin dose of 2 mg/kg and ketamine 24 h after drug administration (Figure 7D)($F_{3,36} = 12.2$, $p < 0.0001$, one-way ANOVA). The center exploration was significantly increased by both doses of psilocybin and ketamine 24 h after drug administration (Figure 7D)($F_{3,36} = 29$, $p < 0.0001$, one-way ANOVA).

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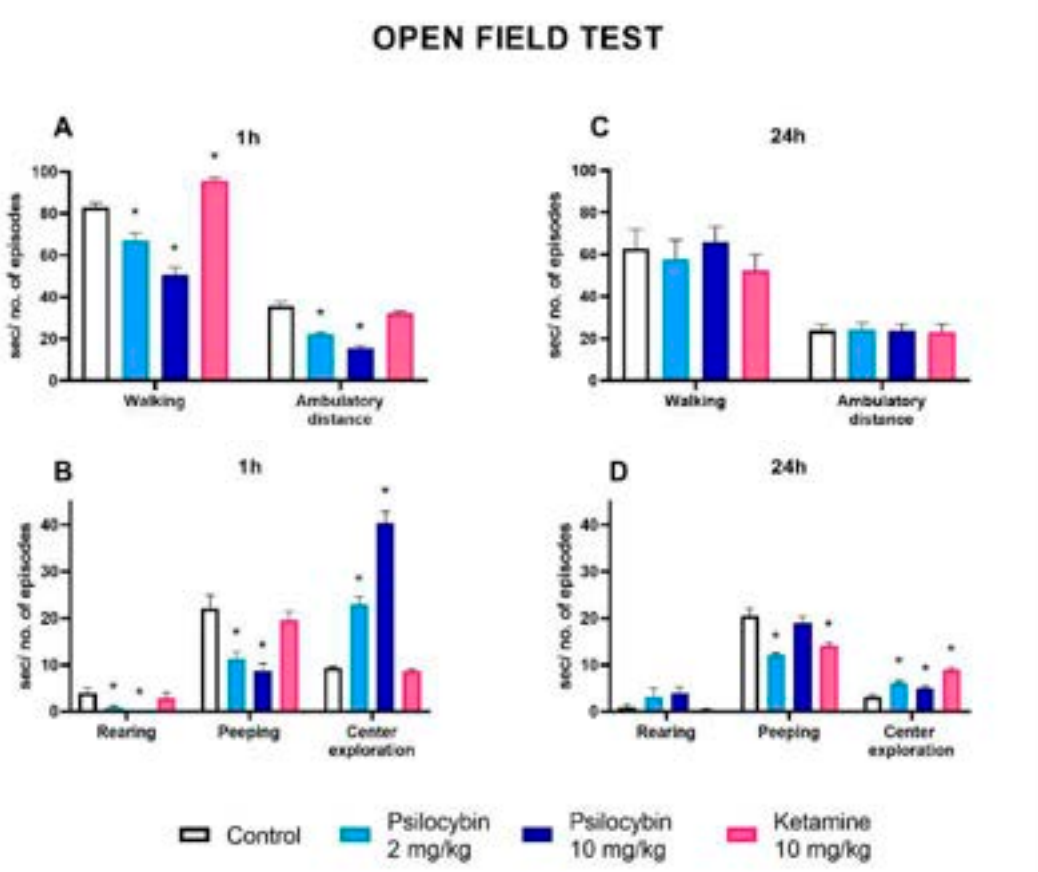


**Figure 7.** The effect of psilocybin (2 and 10 mg/kg) and ketamine (10 mg/kg) on rat behavior in the open field test. The time spent on walking and ambulatory distance at 1h (**A**) and 24 h (**C**) after administration, the number of rearing and peeping episodes and time spent in the center at 1 h (**B**) and 24 h (**D**), respectively. Values are the mean ± SEM (n = 10). * $p < 0.01$ compared to the control (one-way ANOVA followed by Tukey's post hoc test).

## 4. Discussion

The data from numerous empirical studies support the idea that fast-acting psychedelics enable signaling within anatomical networks essential for a range of cognitive and affective tasks. Antidepressant properties of psilocybin are mediated via modulation of the prefrontal and limbic regions [29]. The molecular target of psilocybin in the brain was identified as serotonin 5-HT2A receptor expressed in the subset of deep pyramidal cells in layer V of the prefrontal cortex [30]. The interaction of psilocybin with 5-HT2A receptors produces psychomimetic effects and rise in glutamate levels as confirmed in clinical studies and animal models [27,31]. Acute activation of glutamate neurotransmission was associated with upregulation of BDNF and subsequent synaptic plasticity [3,29]. Intriguingly, depression and chronic stress increase cortical and hippocampal extracellular glutamate and excitotoxicity, subsequently precipitating neuronal atrophy [32]. Thus, the question arises whether antidepressant effects of rapid-acting psychedelics are due to the inhibition of glutamate neurotransmission. Psilocybin also interacts with 5-HT1A receptors although with lower affinity [33,34]. Excitatory 5-HT2A and inhibitory 5-HT1A receptors colocalize in both cortical pyramidal neurons and GABAergic interneurons, thus the cellular response is determined by the summation of 5-HT1A inhibition and 5-HT2A excitation [35]. Through the modulation of cellular excitability, psilocybin may impact cortical projections to other brain circuits such as the nucleus accumbens, hippocampus and amygdala [36]. Thus, glutamatergic and GABAergic signaling would be implicated in cortico-limbic function.

Psilocybin exhibits no affinity for dopamine D2 receptors [29], but it interacts indirectly with mesolimbic dopaminergic pathways, which play a significant role in the brain reward system [37].

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This proposed indirect mechanism of action is suggested by a low addictive potential of psilocybin. Furthermore, there is a positive correlation between depression and dopamine deficiency [38].

In our work, we demonstrated a dose-dependent increase in extracellular levels of DA elicited by psilocybin. The regulation of mesocortical DA by psilocybin may involve cortical glutamatergic fibers expressing 5-HT2A receptors projecting to the nucleus accumbens or ventral tegmental area (VTA), thus indirectly increasing DA release. On the other hand, also 5-HT1A receptors seem indirectly alter DA release due to a low density of 5-HT1A receptors in the nucleus accumbens. However, 5-HT1A receptors may control DA release by reducing the 5-HT neuron activity as a consequence of the stimulation of 5-HT1A autoreceptors in the dorsal raphe nucleus or by reducing pyramidal cell activity projecting to VTA neurons [39]. A similar effect on DA release in the nucleus accumbens exerted by psilocin, an active metabolite of psilocybin, was demonstrated by Sakashita et al. [40].

Ketamine, a fast-acting antidepressant used in our study for comparison, also elevated DA release with a potency similar to the higher dose of psilocybin. However, the mechanism of ketamine action in the regulation DA release in the nucleus accumbens differs from that of psilocybin since it is mediated through disinhibition of glutamatergic fibers projecting to the VTA by blocking NMDA receptors located in cortical GABAergic interneurons [41].

The excitatory effect of psilocybin on cortical pyramidal neurons projecting to the dorsal raphe may be responsible for a dose-dependent increase in 5-HT release in the nucleus accumbens. However, 5-HT release from serotonin terminals may be also modulated by 5-HT2A receptors located in GABAergic interneurons in the dorsal raphe cells, but this mechanism seems to be less pronounced since the levels of 5-HT2A receptor mRNA in the dorsal raphe cells are low [42]. The weaker effect of ketamine on serotonin release as compared to psilocybin may involve NMDA receptors in GABAergic neurons disinhibiting glutamatergic innervation of dorsal raphe cells [35]. In addition, AMPA receptors might be also involved in the behavioral and neurochemical effects of ketamine [43]. 2016). What is more, ketamine might enhance serotonergic transmission by the inhibition of SERT activity [44].

Intriguingly, preclinical studies demonstrated hypertrophy of the nucleus accumbens in models of depression [32]. It is suggested that the stress-induced nucleus accumbens hypertrophy may be related to dopaminergic neurotransmission abnormalities in the VTA pathway to the nucleus accumbens [45]. Furthermore, stress and depression are believed to precipitate phasic activation of the VTA – nucleus accumbens pathway, leading to DA and BDNF release in the nucleus accumbens [46]. Subsequently, the stress-induced BDNF release results in nucleus accumbens hypertrophy and in depressive-like behavior [47]. Our experiments showing the stimulation of DA release in the nucleus accumbens by psilocybin do not seem to explain the abovementioned observations. However, the effect of psilocybin in naive rats may differ from its action in animals exposed to stress. Further studies are necessary to find out whether fast-acting antidepressants are able to normalize stress-induced abnormalities in the nucleus accumbens observed in rodents and depressive patients [7].

Our data show another pattern of psilocybin action on extracellular glutamate levels in limbic regions. Direct stimulation of 5-HT1A receptors located on pyramidal cells or 5-HT2A receptors on GABAergic interneurons by psilocybin, reducing the prefrontal cortex output to the nucleus accumbens [35,36], may be responsible for the decrease in glutamate release in the nucleus accumbens. A lack of dose-response linearity in this effect stems from differences in density of receptor subtypes in both locations [33,35]. Ketamine used in our study as a comparator significantly increased glutamate levels in the nucleus accumbens. The ketamine-induced enhancement of glutamate release is likely mediated via its blocking activity on NMDA receptors within GABAergic interneurons resulting in disinhibition of pyramidal cells projecting to the nucleus accumbens [48].

Moreover, psilocybin and ketamine increased GABA release in this region. GABAergic neurons are widely distributed in the shell and core of the nucleus accumbens. They bear 5-HT1A and 5-HT2A receptor subtypes although differ in their density [49]. Direct activation of inhibitory or excitatory subtypes may depend on the psilocybin dose. In addition, cortical projections to the nucleus

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accumbens may also influence excitability of GABAergic neurons regulating GABA levels via other receptor types [50]. The effect on cortical projection by NMDA receptor blockade may explain a possible mechanism of ketamine action on GABA release in the nucleus accumbens [48].

Diverse psilocybin effect on glutamate release was found in the hippocampus. Its low dose decreased while the higher one increased glutamate levels. In contrast, GABA release was dose-dependently enhanced by psilocybin. The observed changes in glutamate and GABA release depend on the stimulation of hippocampal 5-HT1A and 5-HT2A receptors by psilocybin. Both receptor subtypes have various distribution and density patterns in the hippocampus. 5-HT1A receptors are highly expressed on both principal glutamatergic cells and GABAergic interneurons [51]. Activation of 5-HT1A receptors primarily leads to the inhibition of hippocampal pyramidal cells [52]. This may be the cause of the decrease in glutamate release by low-dose psilocybin in our study. However, activation of 5-HT1A receptors expressed on GABAergic interneurons would disinhibit principal glutamatergic cells and thus would counteract the direct effect of 5-HT1A receptors expressed on principal neurons. Additionally, 5-HT2A receptors are expressed on both principal glutamatergic cells and on different subtypes of hippocampal interneurons, though in a lower density than 5-HT1A receptors [53]. Thus, since 5-HT2A receptors are stimulatory and are expressed on both principal cells and GABAergic interneurons, it could be expected that they would have mixed effects on the both cell types. This mechanism may underlie the stimulatory effect of the higher psilocybin dose on glutamate release and it's both doses on GABA release in the hippocampus. However, it has to be noted that the resultant effect of the lower psilocybin dose on amino acid neurotransmission is inhibitory. Activation of corticolimbic excitatory projections, either through selective antagonism of inhibitory interneurons or cortical disinhibition by ketamine is a probable mechanism of the increase in glutamate and GABA extracellular levels in the hippocampus [43]. Considering reduced hippocampal volume in depressed patients resulting from hippocampal stress-induced neuronal atrophy and low levels of GABA and glutamate demonstrated in depressed patients [7,32,54], normalization of amino acid neurotransmission and hippocampal volume by psilocybin may be expected.

Additional beneficial effect of psilocybin in depressed patients with cognitive impairments may be related to its stimulatory effect on ACh levels as found in our study. ACh appears to act as a neuromodulator in the brain and its role is to change neuronal excitability, alter presynaptic release of neurotransmitters and coordinate the firing of groups of neurons. ACh contributes also to synaptic plasticity [55]. The primary source of cholinergic innervation of the hippocampus derives from the basal forebrain cholinergic system [56]. Generally 5-HT exerts stimulatory influence on the release of ACh; however, the effect depends on mediation via particular serotonin receptor subtypes. 5-HT1A receptor subtype mediates stimulatory effect on ACh release in the hippocampus as shown in the hippocampal perfusate of conscious freely moving rats [57]. However, systemic administration of the 5-HT2A receptor agonist DOI in a high dose also increased ACh release in the hippocampus, but mescaline, a potent 5-HT2A agonist, did not affect ACh release[58]. This findings are in line with our data and support the idea that both receptor subtypes seem to be involved in the stimulatory effect of psilocybin on ACh release in the hippocampus. However, the lack of linearity in the dose-response effect needs further studies. The recent data of Pacheco et al. [59] showing enhancement of cognitive flexibility in rats by psilocybin may correlate with the psilocybin effect on ACh release in the hippocampus. The role of NMDA receptors in the regulation of ACh release cannot be excluded as ketamine also increased ACh release with the strength similar to the psilocybin higher dose.

Abnormally high amygdala reactivity to negative affective stimuli has been implicated in the pathophysiology of depression [60]. Negative affect and amygdala response was reduced by psilocybin suggesting that psilocybin may increase emotional and brain plasticity [61]. In our study, psilocybin and ketamine effects on extracellular glutamate and GABA levels were of short duration and the overall effect shown as GABA/glutamate index was stimulatory. Considering the fact that prolonged anxiety induced by chronic stress in mice causes dysfunction of basolateral amygdala projection neurons [62] and that reduced amygdala volume correlated with the severity of depression [6], our data indicate that facilitation of synaptic transmission from the prefrontal cortex to the

amygdala and especially predominant role of glutamatergic pathways may be the underlying mechanism of the beneficial effect of psilocybin and ketamine in treatment of anxiety and depressive states.

In order to assess the long-lasting effects of psilocybin, we examined the density level of several receptor subtypes in the limbic system. The increase in dopamine D2 receptor density in the nucleus accumbens by the higher psilocybin dose may be subsequent effect to the increased DA release in the nucleus accumbens and the regulatory mechanism restoring balance in dopaminergic nerve terminals. The 5-HT2A receptor mRNA levels were intermediate in the nucleus accumbens [63] and were mostly observed in spiny projecting neurons responsible principally for movement control [64]. Their density was not changed by acute administration either of psilocybin or ketamine in our study.

Instead, long term density changes were detected for 5-HT1A and 5-HT2A receptors in the hippocampus. The decreased density of 5-HT1A receptors induced by psilocybin may be beneficial due to sensitization of this receptors in depression, while the increased levels of 5-HT2A receptors after the higher dose of psilocybin may reduce functional deficit of this subtype observed in depression [65] and related to increased synaptogenesis found in the hippocampus of the pig brain [66]. Interestingly, decreased density of 5-HT2A receptors in the hippocampus and prefrontal cortex of the pig brain found by Raval et al. [66] supports our findings after the lower dose of psilocybin. Ketamine-induced increase in 5-HT1A and 5-HT2A receptor density in the hippocampus observed in our work as was also evidenced for other non-competitive antagonists NMDA receptor [67].

The changes in the levels of neurotransmitters in the limbic system provide the neurobiological background for psilocybin effect on stress and anxiety. In our study, psilocybin affected animal behavior in the open field test. The data demonstrate marked anxiolytic effect of psilocybin in acute phase and 24 h post-treatment as shown by increased center penetration and decreased exploration of peripheral zone of the open field. Instead, ketamine was not effective or its weak anxiolytic effect was observed only 24 h after treatment. Furthermore, in contrast to psilocybin, ketamine significantly affected exploration resulting probably from stronger as compared to psilocybin stimulation of DA levels in the nucleus accumbens. The psilocybin effect observed in the open field test linking behavioral symptoms with modest elevation of DA and 5-HT in the nucleus accumbens and predominance of GABAergic neurotransmission in other regions of the limbic system has therapeutic implication for treating anxiety and mood disorders.

## 5. Conclusions

In conclusion, the presented data have mechanistic significance and show the implications of the psilocybin impact on neurotransmitter levels for the therapy of depression and anxiolytic disorders. The increased dopaminergic and serotonergic neurotransmission in the nucleus accumbens and predominant GABAergic signaling in the studied brain regions link the current findings with limbic system abnormalities observed in clinical studies.

**Supplementary Materials:** Original Western blot details are presented in supplementary material 1, elevated plus maze data are presented in supplementary material 2 (Figure 1S), extracellular basal levels of neurotransmitters in respective animal groups are presented in supplementary material 3 (Table 1S).

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**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki and approved by the Local Ethical Commission for Experimentations on Animals in Kraków (permit no. 112/2021, 324/2021, 79/2022). The experimental procedures were conducted according to recommendations of the National Institutes of Health and were approved by the Ethical Committee of the 2nd Local Institutional

Animal Care and Use Committee (IACUC) in Kraków, Poland in conformity with institutional guidelines and in compliance with national and international laws and policies (permit numbers: 112/2021, 324/2021 and 79/2022). According to the 3R policy, the number of animals was reduced to an essential minimum. All the procedures regarding the study design, animal experiments, statistical analysis, and data reporting fulfill the criteria of the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (https://www.nc3rs.org.uk/arrive-guidelines, access date: 14 May 2022).

**Informed Consent Statement:** Not applicable.

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On each blot 2 animals (one cohort) were used from 8 different cohorts

**A blot**

| | |
|---|---|
| **Control** | **1, 2** |
| **Psilocybin 2mg/kg** | **1, 2** |
| **Psilocybin 10mg/kg** | **1, 2** |
| **Ketamine 10mg/kg** | **1, 2** |

**B blot**

| | |
|---|---|
| **Control** | **3, 4** |
| **Psilocybin 2mg/kg** | **3, 4** |
| **Psilocybin 10mg/kg** | **3, 4** |
| **Ketamine 10mg/kg** | **3, 4** |

**C blot**

| | |
|---|---|
| **Control** | **5, 6** |
| **Psilocybin 2mg/kg** | **5, 6** |
| **Psilocybin 10mg/kg** | **5, 6** |
| **Ketamine 10mg/kg** | **5, 6** |

**D blot**

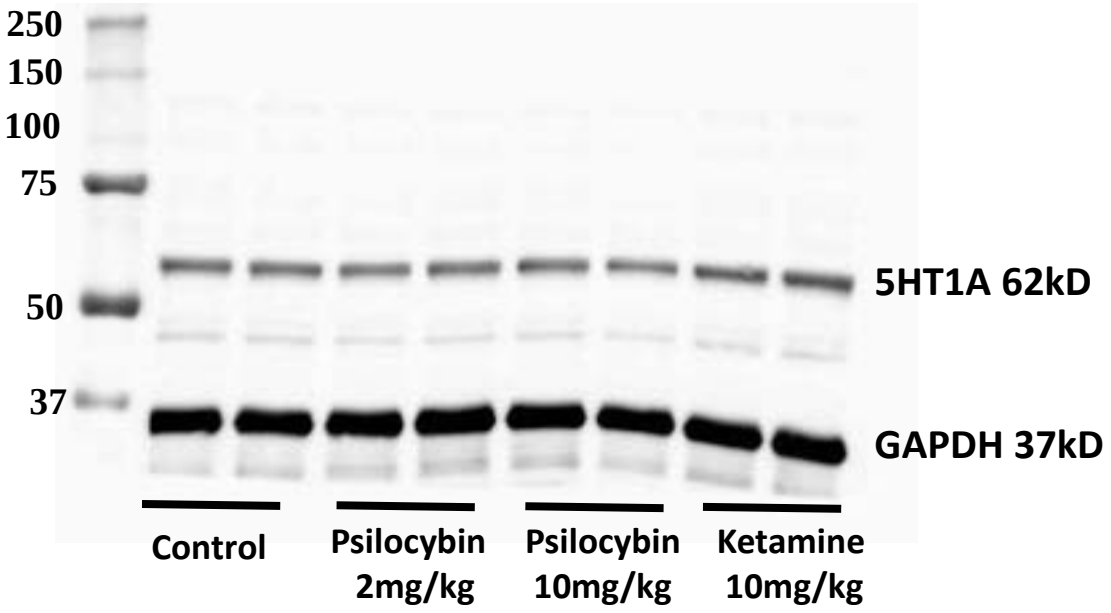
| | |
|---|---|
| **Control** | **7, 8** |
| **Psilocybin 2mg/kg** | **7, 8** |
| **Psilocybin 10mg/kg** | **7, 8** |
| **Ketamine 10mg/kg** | **7, 8** |

**N=8**

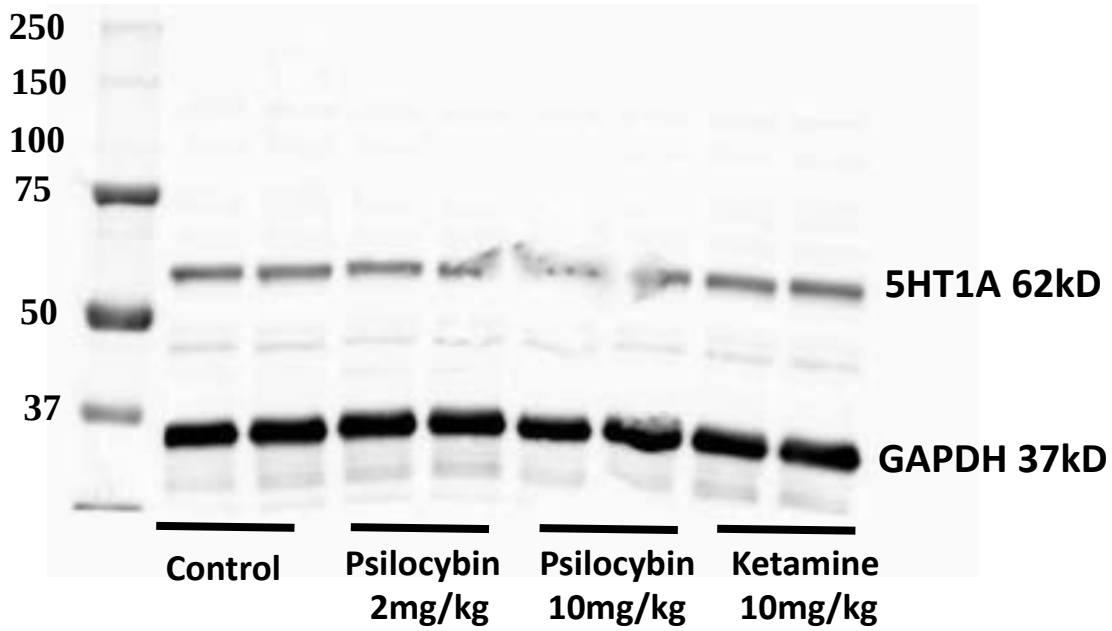
# HIPPOCAMPUS

## 5HT1A

### A blot (this blot in publication)



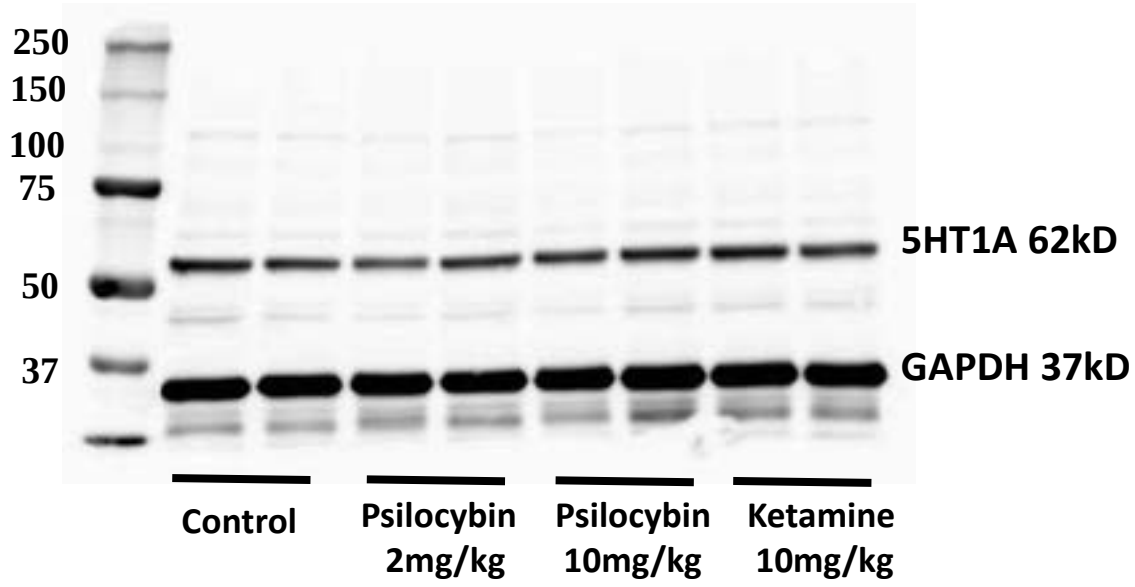

### B blot



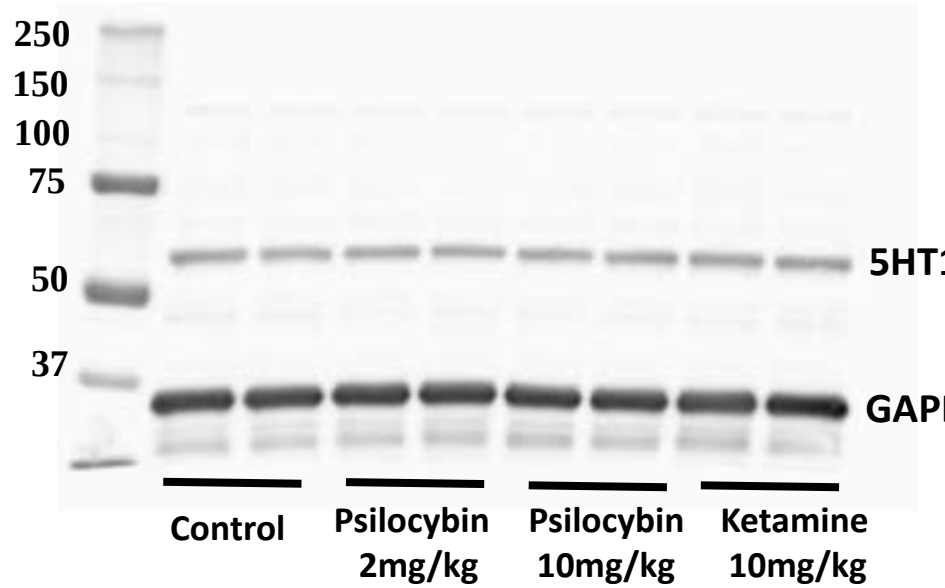
# HIPPOCAMPUS

## 5HT1A

### C blot



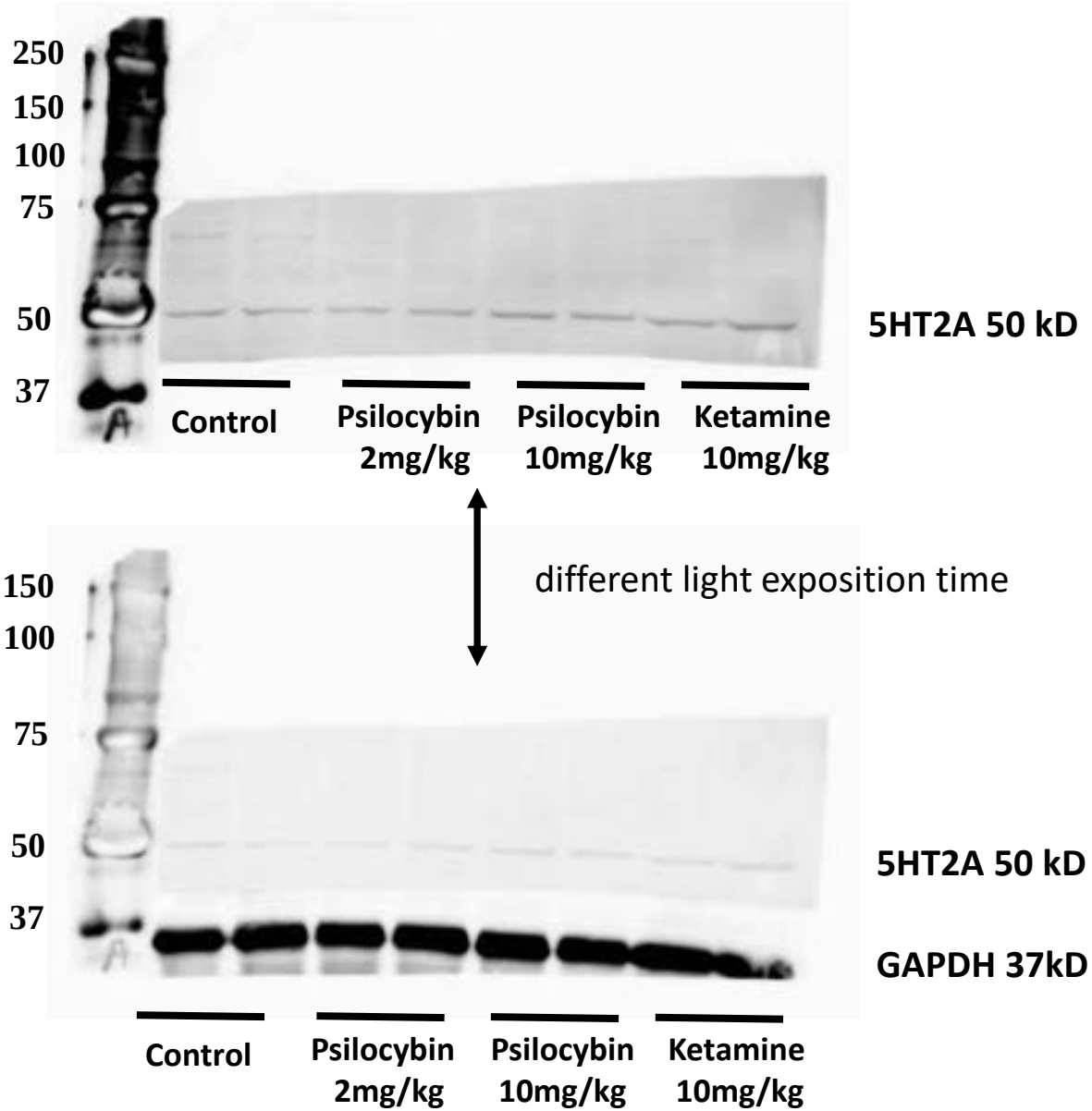

### D blot



# HIPPOCAMPUS

## 5HT2A

### A blot (this blot in publication)

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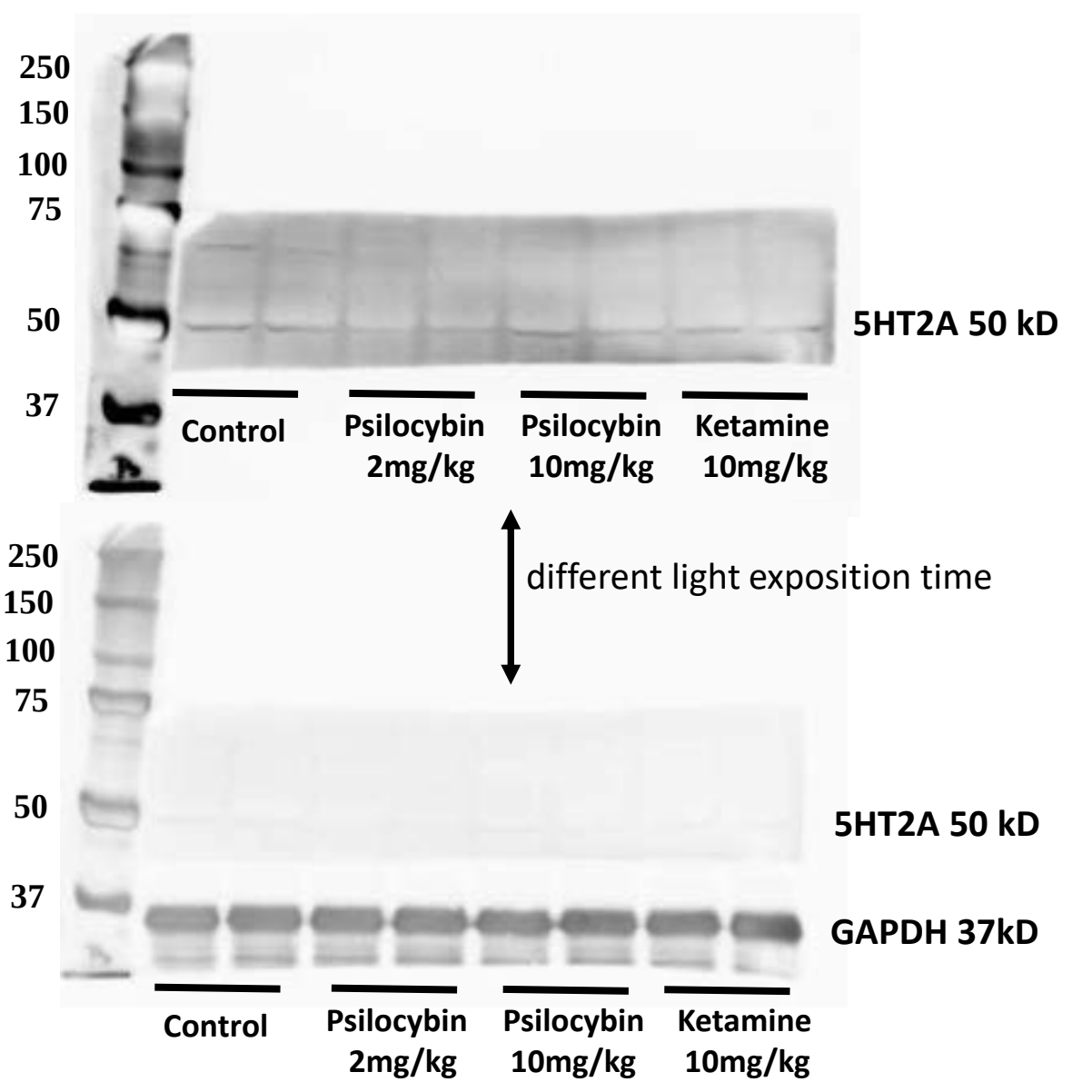
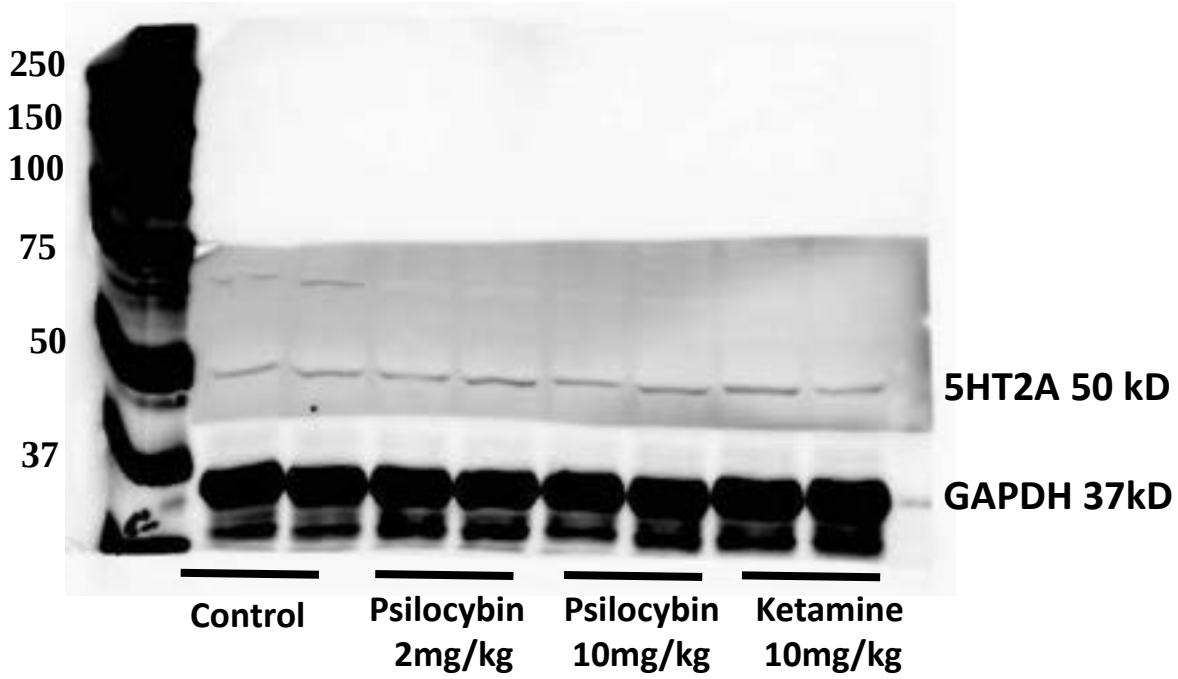
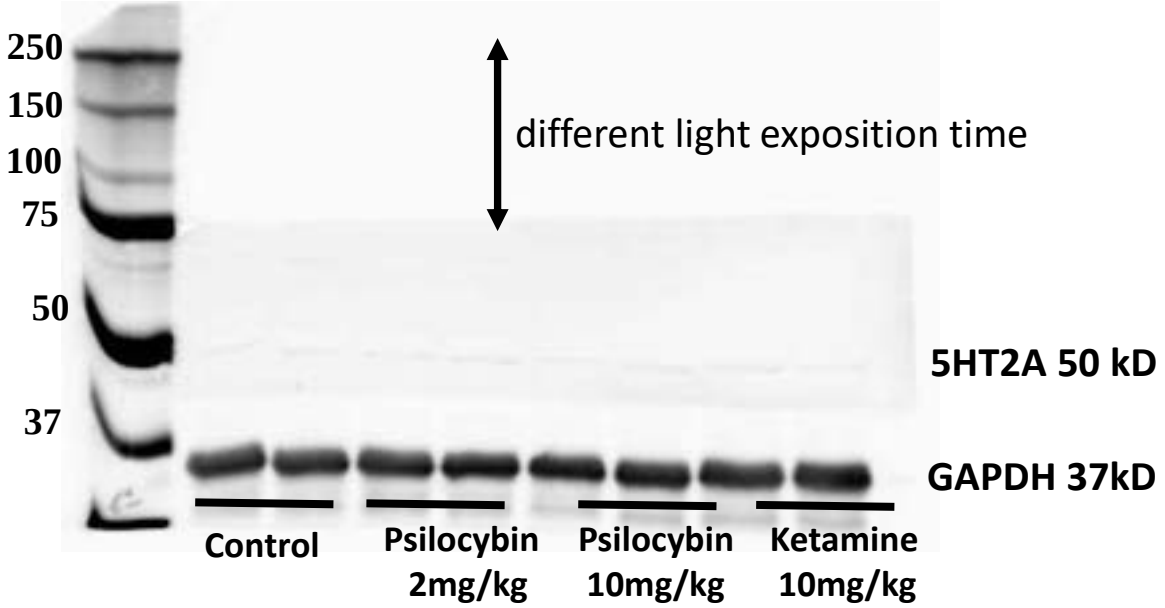


# 5HT2A

# HIPPOCAMPUS

## C blot

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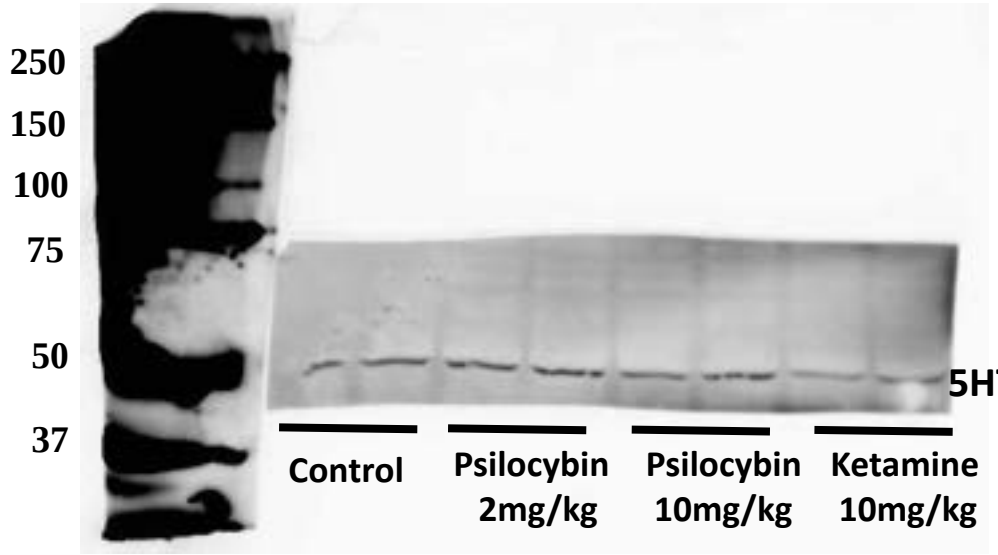
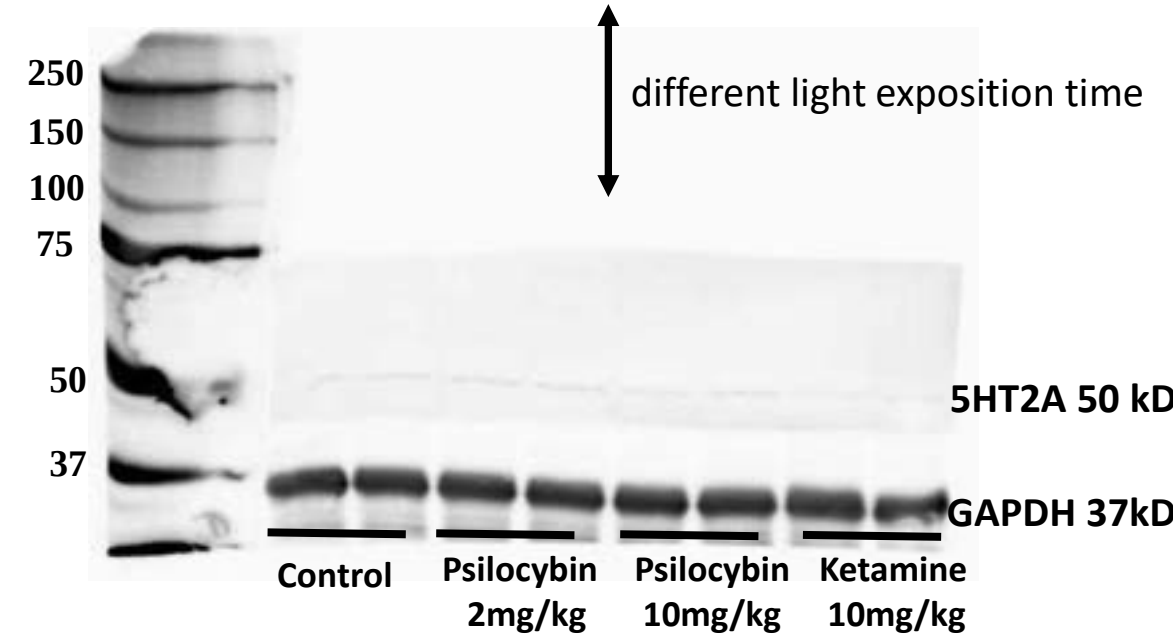
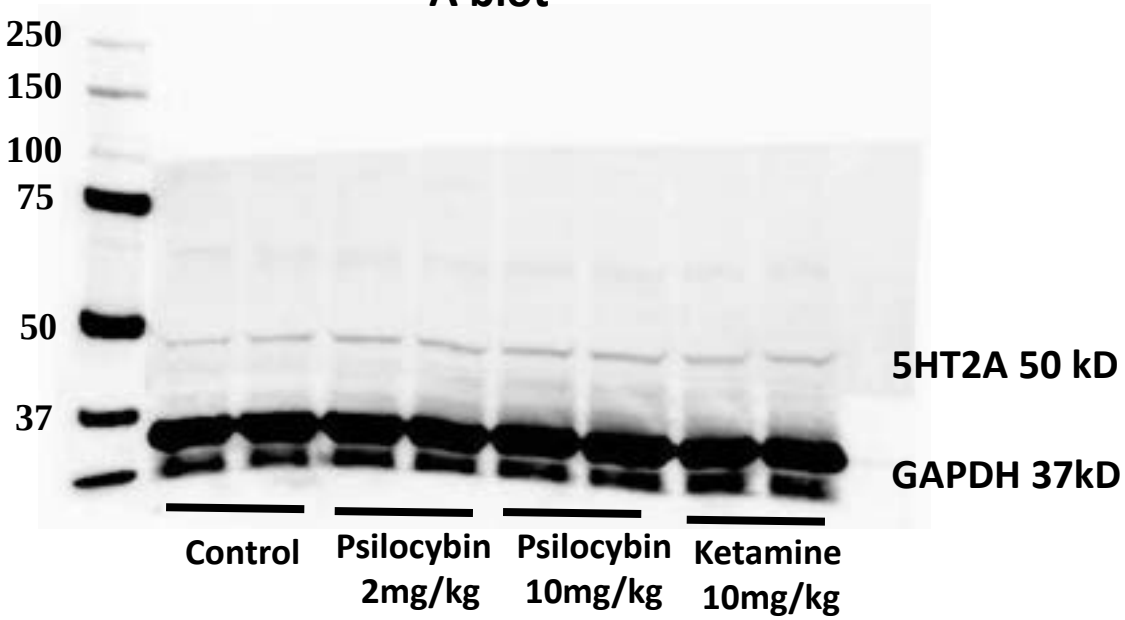


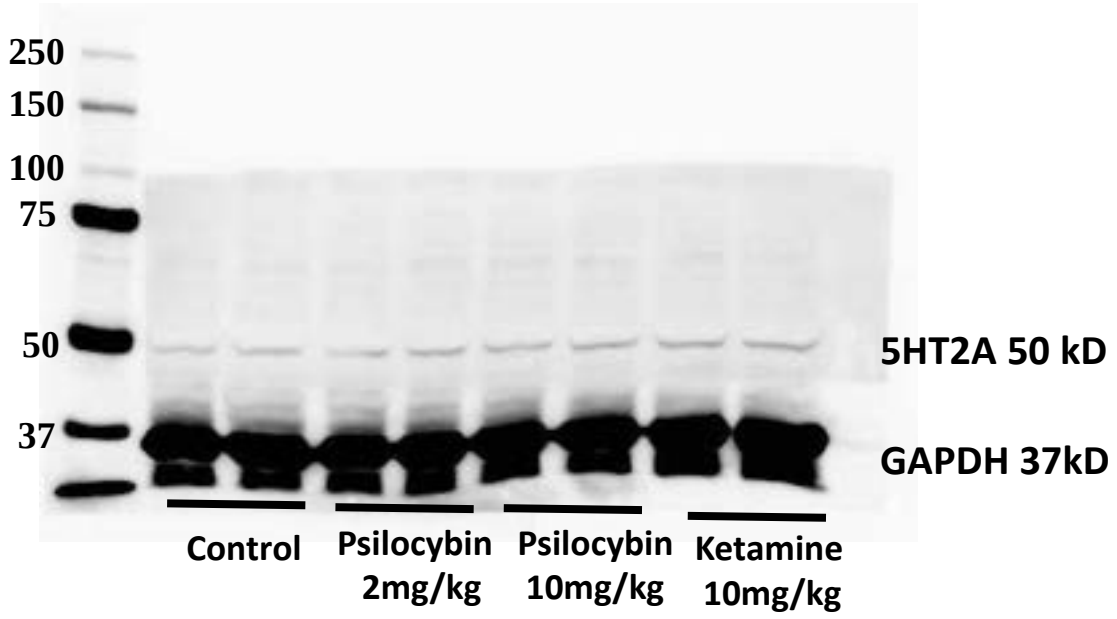
# Nucleus Accumbens

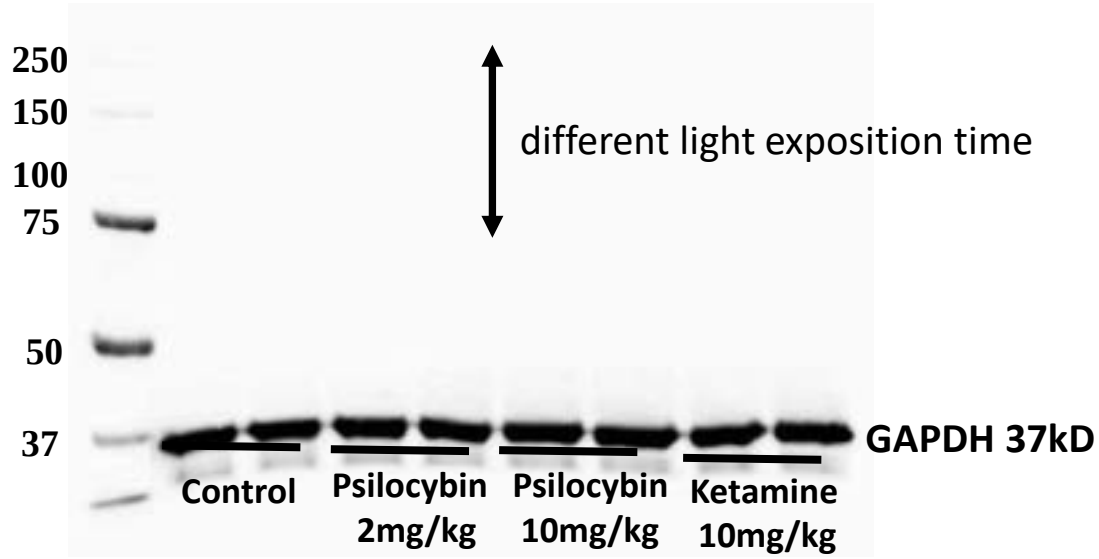
## 5HT2A

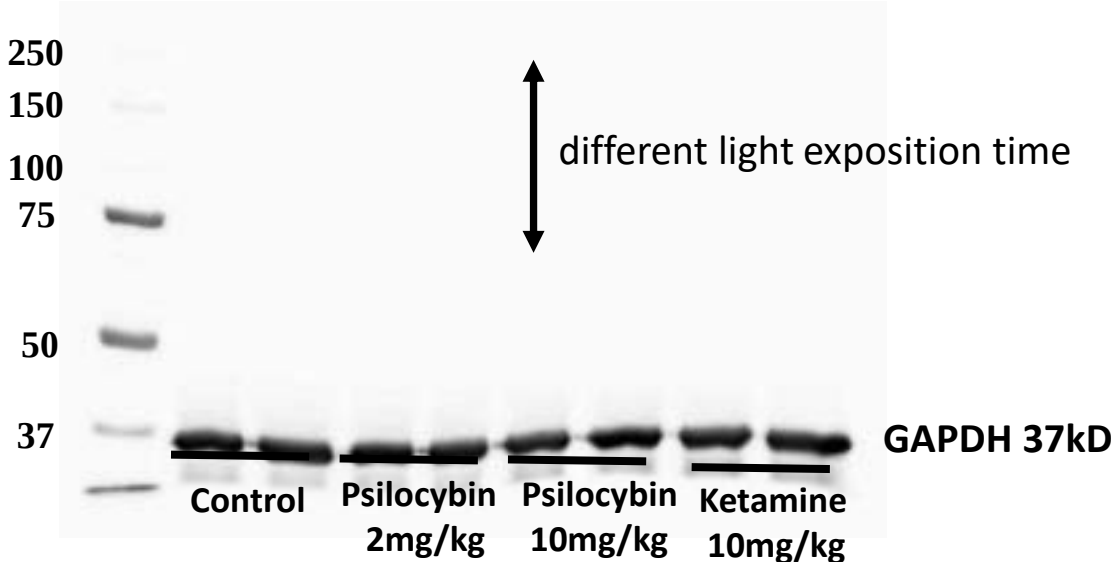
### A blot




### B blot



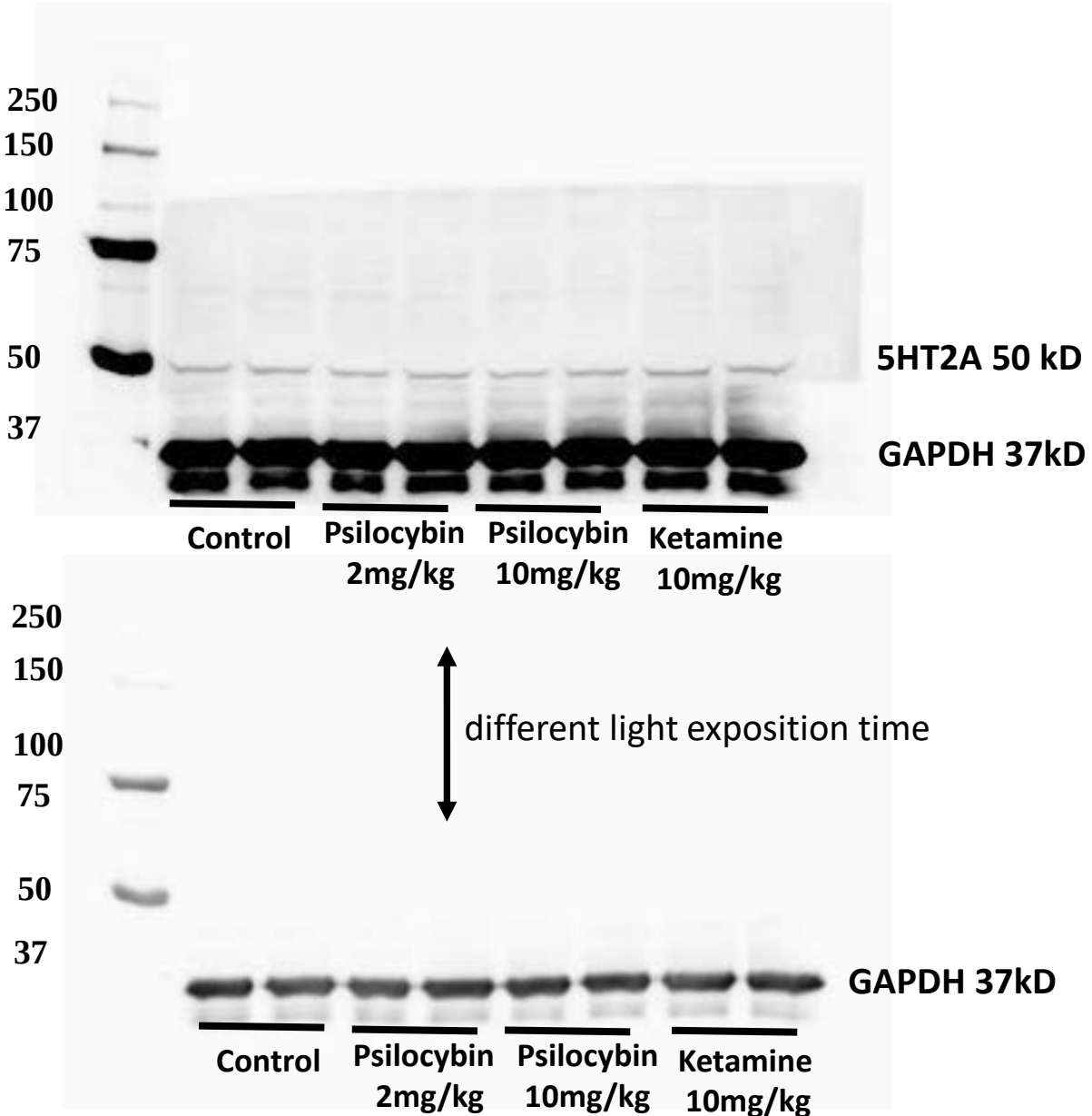



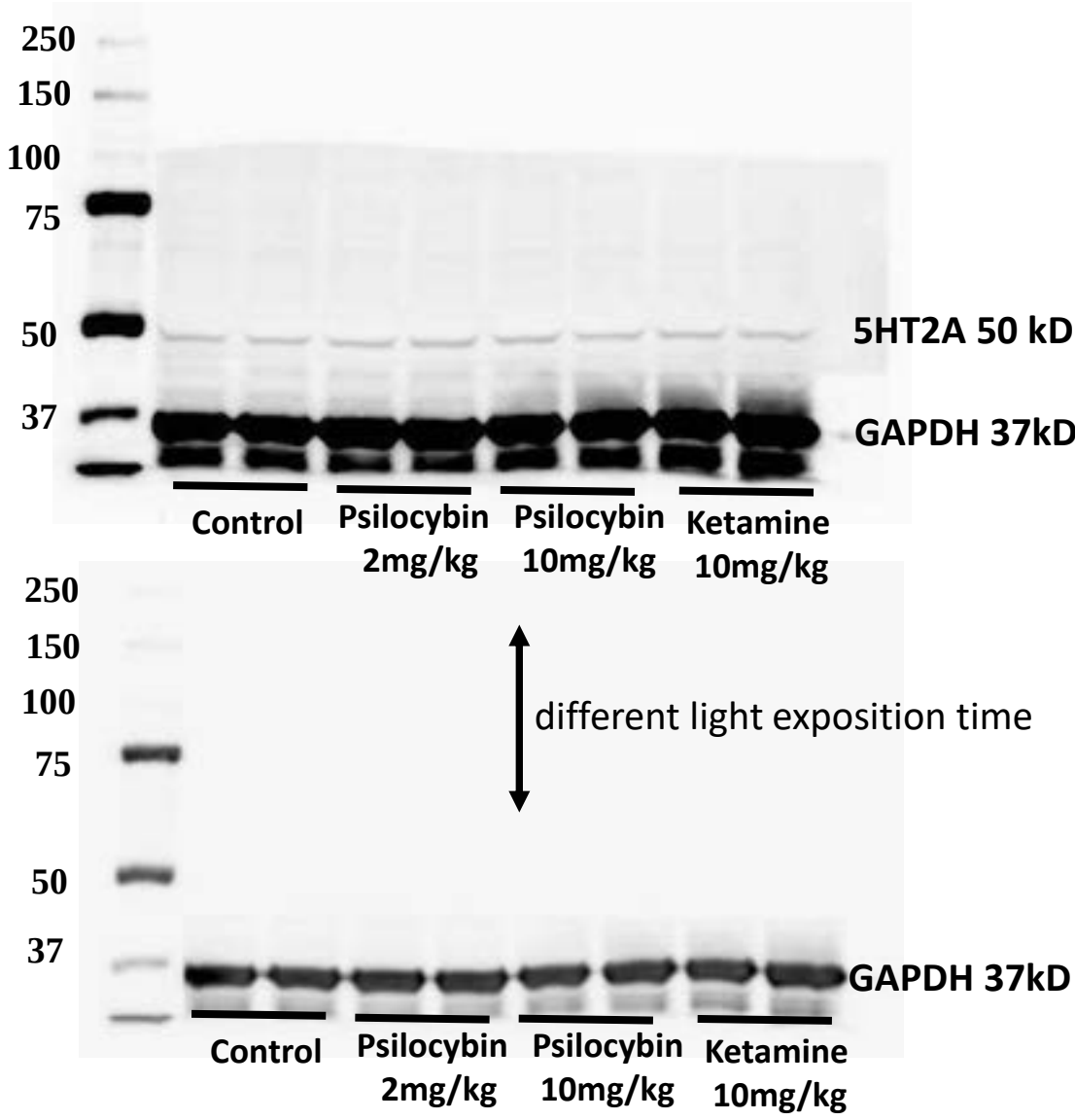
# Nucleus Accumbens

## 5HT2A

### C blot



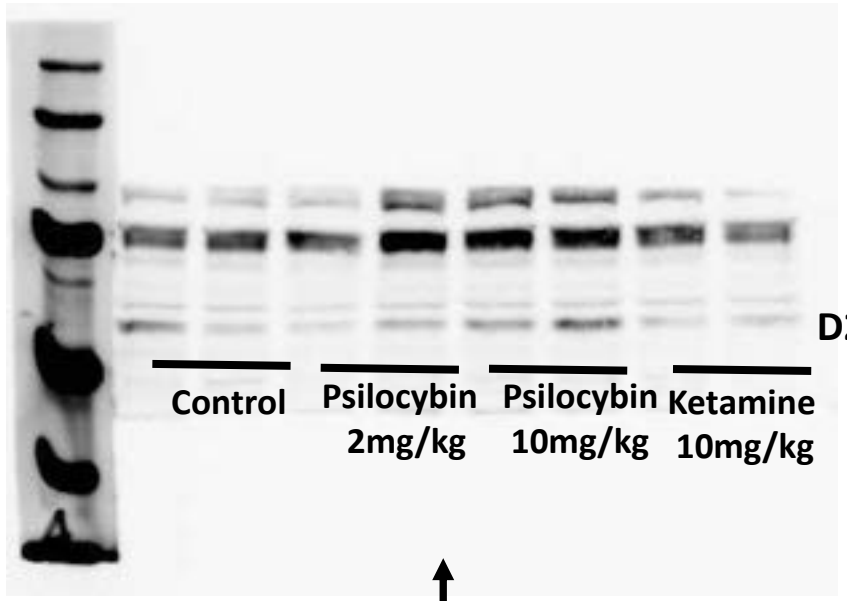

### D blot (this blot in publication)



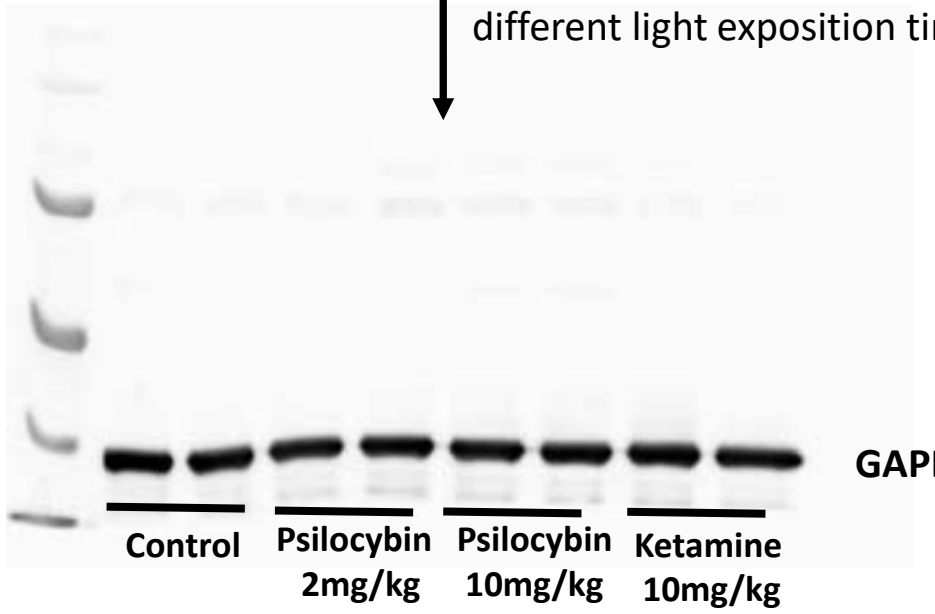
# D2

# Nucleus Accumbens

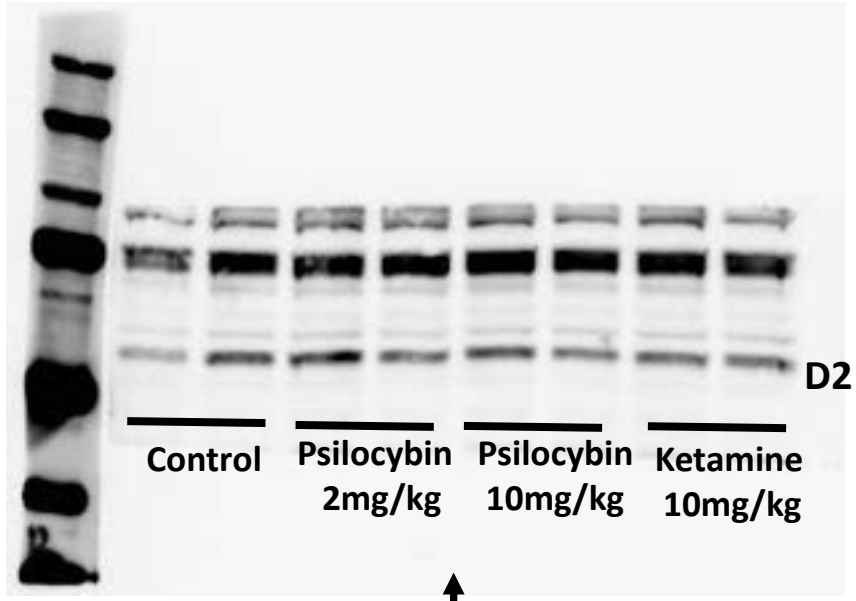
## A blot



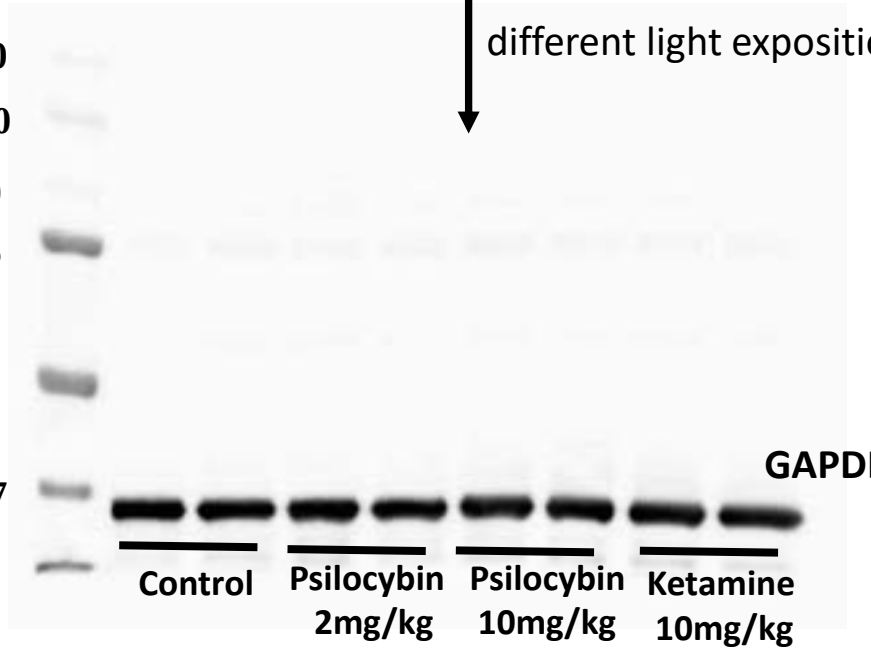

different light exposition time




## B blot

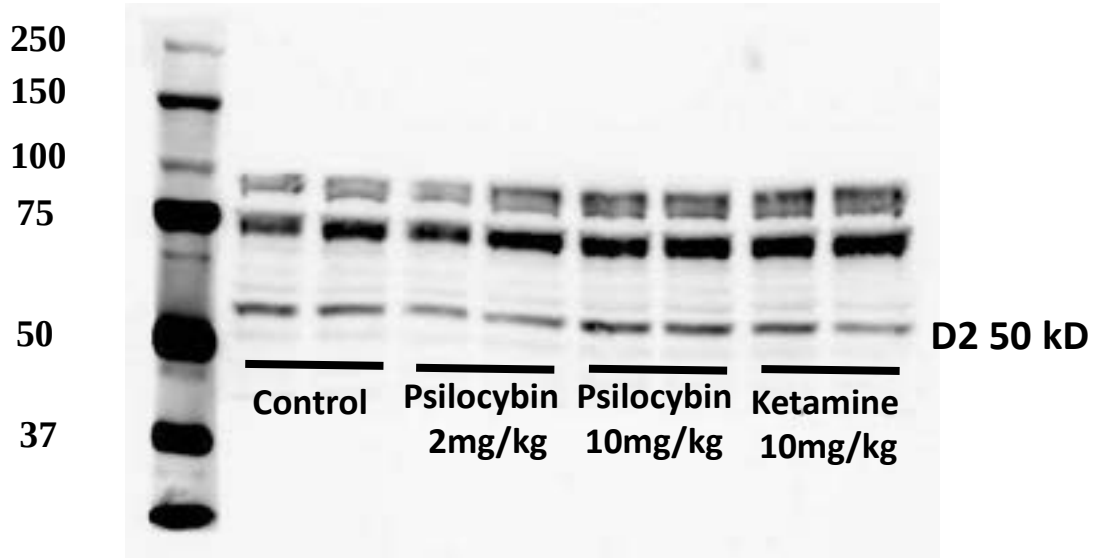



different light exposition time

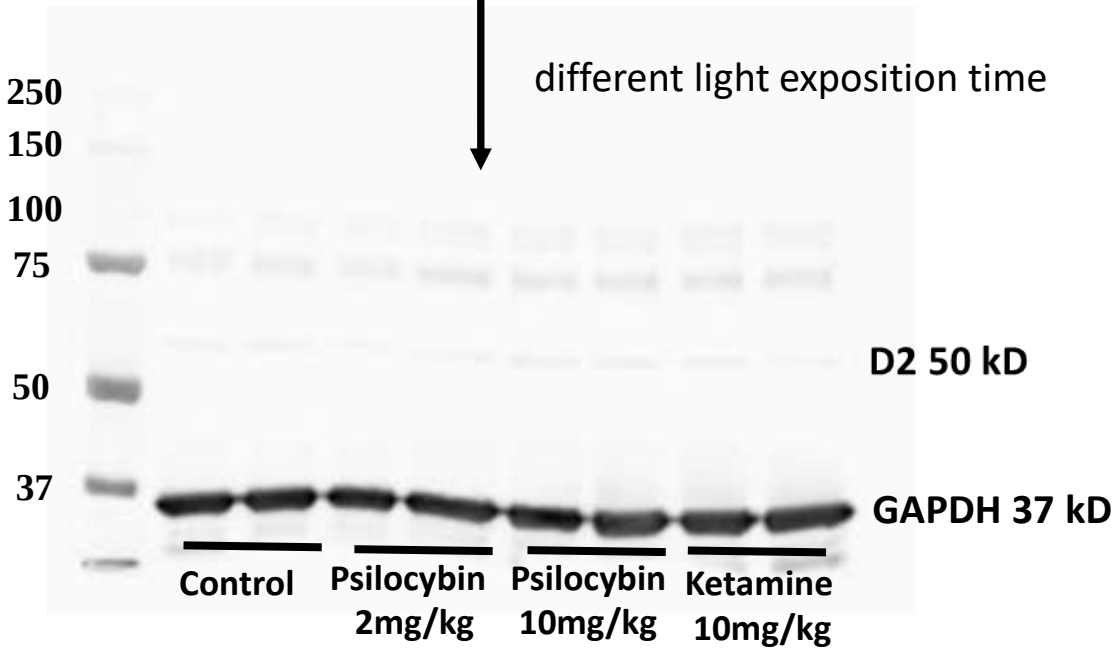


# Nucleus Accumbens

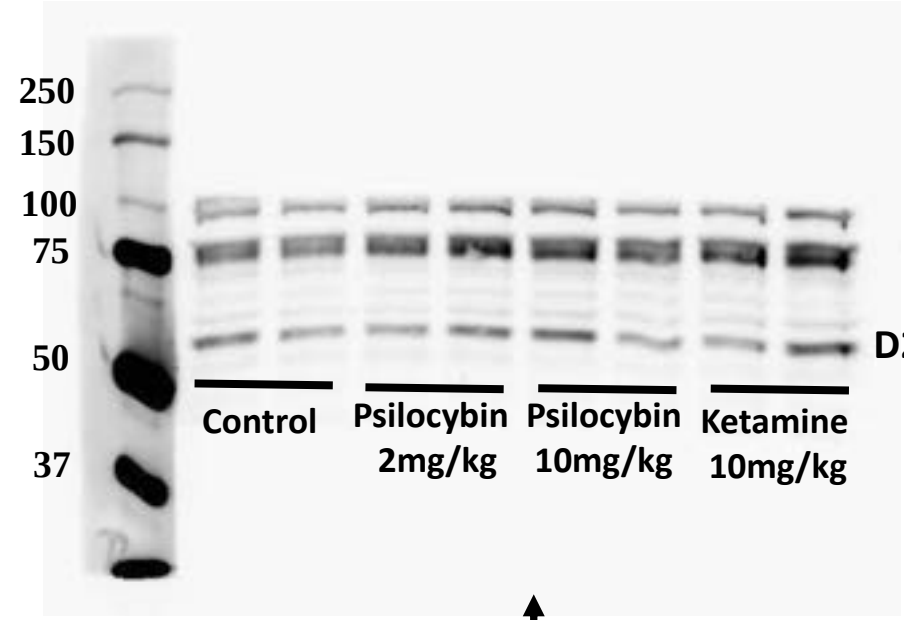
## C blot (this blot in publication)



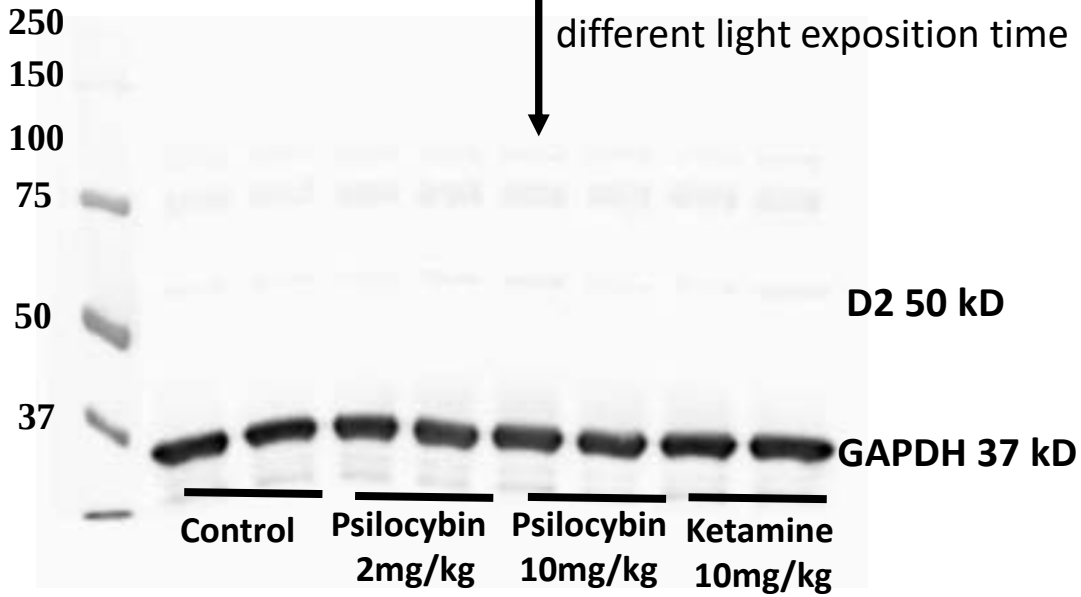

different light exposition time




## D blot




different light exposition time



**Supplementary material 2**

**Method**

Elevated plus maze. The apparatus comprised two open (50× 11 cm) and two non-transparent closed (50× 11 × 40 cm) arms elevated 50 cm above the floor and illuminated by two 25 W bulbs located beneath the open arms. The animals were placed individually in the center of the apparatus and the number of entries of all 4 feet into open and closed arms, and the time spent in each arm, were recorded manually in a 5-min test by an observer who was blinded to the treatments. The results are expressed as a percentage of entries/ time in the open arms: open/(open+closed)× 100.

**Results**

**The effect of psilocybin and ketamine on the activity of rats in the elevated plus maze**

There was no effect of both doses of psilocybin or ketamine on open arm entries and time spent in open arms at 2 h ($F_{3,36}$ = 2.03, p < 0.126, $F_{3,36}$ = 0.72, p < 0.55, respectively, one-way ANOVA) or 24 h after drugs administration ($F_{3,36}$ = 1.26, p < 0.30, $F_{3,36}$ = 1.86, p < 0.15, respectively, one-way ANOVA) (Fig. 1S).

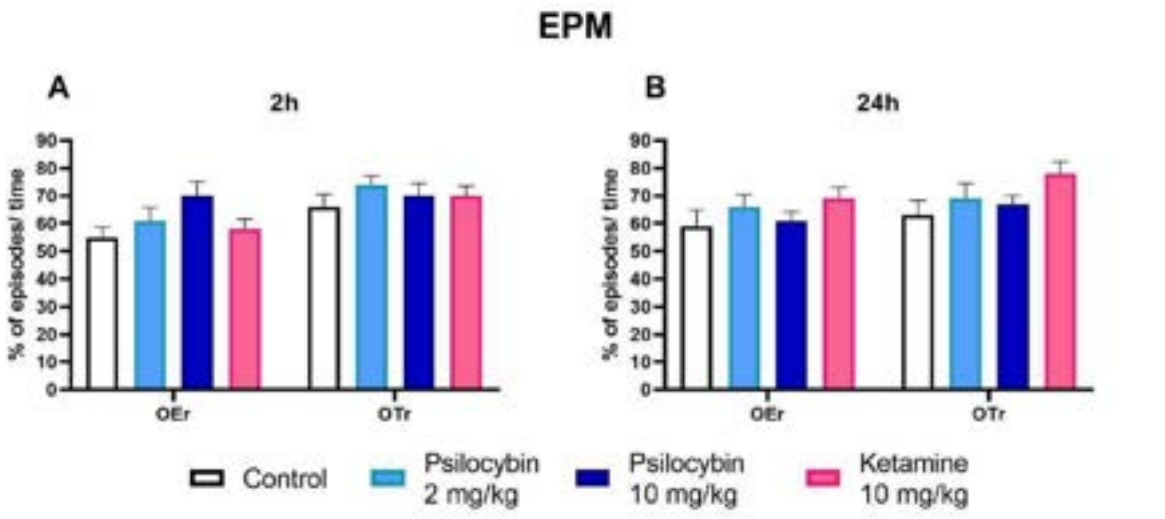



**Figure 1S.** Open arm entries (OEr) and open arm time (OTr) shown as a percentage of total open + closed entries/time) in the elevated plus maze for control (white bars), psilocybin (2 and 10 mg/kg) and ketamine (10 mg/kg) 2 h (**A**) and 24 h (**B**) after administration. Values are the mean ± SEM (n = 10). One-way ANOVA followed by Tukey's post hoc test.

**Supplementary materials 3**

Table 1S. Basal levels of neurotransmitters in treatment groups

| Treatment | DA (pg/10 µl) | 5-HT (pg/10 µl) | GLU (ng/10 µl) | GABA (pg/10 µl) | ACh (nM) |
|---|---|---|---|---|---|
| **Nucleus accumbens** | | | | | |
| control | 0.89 ± 0.05 | 0.26 ± 0.02 | 7.82 ± 0.83 | 43.36 ± 2.43 | nd |
| Psilocybin 2 mg/kg | 0.86 ±- 0.09 | 0.26 ± 0.02 | 7.72 ± 0.88 | 47.95 ± 4.99 | nd |
| Psilocybin 10 mg/kg | 0.85 ± 0.09 | 0.29 ± 0.03 | 7.70 ± 1.41 | 39.2 ± 2.26 | nd |
| Ketamine 10 mg/kg | 0.99 ± 0.04 | 0.22 ± 0.02 | 8.21 ± 0.81 | 44. 68 ± 5.33 | nd |
| **Hippocampus** | | | | | |
| control | nd | nd | 5.54 ± 0.41 | 42.97 ± 2.05 | 0.37 ± 0.06 |
| Psilocybin 2 mg/kg | nd | nd | 5.34 ± 0.62 | 35.89 ± 2.77 | 0.30 ± 0.09 |
| Psilocybin 10 mg/kg | nd | nd | 6.60 ± 0.49 | 49.72 ± 2.98 | 0.37 ± 0.16 |
| Ketamine 10 mg/kg | nd | nd | 4.77 ± 0.50 | 45.98 ± 3.79 | 0.43 ± 0.06 |
| **Amygdala** | | | | | |
| control | nd | nd | 4.38 ± 0.30 | 48 ± 2.82 | nd |
| Psilocybin 2 mg/kg | nd | nd | 4.77 ±3 0.51 | 44.5 ± 3.31 | nd |
| Psilocybin 10 mg/kg | nd | nd | 4.19 ± 0.48 | 49.0 ± 5.73 | nd |
| Ketamine 10 mg/kg | nd | nd | 4.08 ± 0.57 | 52.0 ± 4.9 | nd |

# 6.Author’s statements

20.09.2023

**Oświadczenie**

Oświadczam, że mój udział w niżej wymienionych publikacjach polegał na stworzeniu ogólnej koncepcji badań, wykonaniu części doświadczalnej, analizie uzyskanych wyników oraz przygotowaniu manuskryptu do druku

1.**Adam Wojtas**, Monika Herian, Mateusz Skawski, Małgorzata Sobocińska, Alejandro González-Marín, Karolina Noworyta-Sokołowska, Krystyna Gołembiowska. Neurochemical and Behavioral Effects of a New Hallucinogenic Compound 25B-NBOMe in Rats. Neurotoxicity Research, 2021. 39, 305-326. doi: 10.1007/s12640-020-00297-8.

2. **Adam Wojtas**, Monika Herian, Marzena Maćkowiak, Anna Solarz, Agnieszka Wawrzczak-Bargiela, Agnieszka Bysiek, Karolina Noworyta, Krystyna Gołembiowska. Hallucinogenic activity, neurotransmitters release, anxiolytic and neurotoxic effects in Rat's brain following repeated administration of novel psychoactive compound 25B-NBOMe. Neuropharmacology, 2023. 9, 240:109713. doi: 10.1016/j.neuropharm.2023.109713.

3. **Adam Wojtas**, Agnieszka Bysiek, Agnieszka Wawrzczak-Bargiela, Zuzanna Szych, Iwona Majcher-Maślanka, Monika Herian, Marzena Maćkowiak, Krystyna Gołembiowska. Effect of Psilocybin and Ketamine on Brain Neurotransmitters, Glutamate Receptors, DNA and Rat Behavior. International Journal of Molecular Sciences, 2022. 16, 23, 6713. doi: 10.3390/ijms23126713.

4. **Adam Wojtas**, Agnieszka Bysiek, Agnieszka Wawrzczak-Bargiela, Marzena Maćkowiak, Krystyna Gołembiowska. Limbie system response to psilocybin and ketamine administration in rats: A neurochemical and behavioral study. International Journal of Molecular Sciences, 2023. Manuscript submitted.

Adam Wojtas

20.09.2023

**Oświadczenie**

Oświadczam, że udział mgr Adama Wojtasa w niżej wymienionych publikacjach polegał na stworzeniu ogólnej koncepcji badań, wykonaniu części doświadczalnej, analizie uzyskanych wyników oraz przygotowaniu manuskryptu do druku. Żadna z poniższych publikacji nie stanowi oraz nie będzie stanowiła dla mnie podstaw do uzyskania stopni naukowych. Mój udział w powstaniu niżej wymienionych prac polegał na współuczestnictwie w wykonywaniu części doświadczeń.

1. Adam Wojtas, Monika Herian, Marzena Maćkowiak, Anna Solarz, **Agnieszka Wawrzczak-Bargiela**, Agnieszka Bysiek, Karolina Noworyta, Krystyna Gołembiowska. Hallucinogenic activity, neurotransmitters release, anxiolytic and neurotoxic effects in Rat's brain following repeated administration of novel psychoactive compound 25B-NBOMe. Neuropharmacology, 2023. 9, 240:109713. doi: 10.1016/j.neuropharm.2023.109713.

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Agnieszka Bargiela

20.09.2023

**Oświadczenie**

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20.09.2023

**Oświadczenie**

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Noworyta

20.09.2023

**Oświadczenie**

Oświadczam, że udział mgr Adama Wojtasa w niżej wymienionych publikacjach polegał na stworzeniu ogólnej koncepcji badań, wykonaniu części doświadczalnej, analizie uzyskanych wyników oraz przygotowaniu manuskryptu do druku. Żadna z poniższych publikacji nie stanowi oraz nie będzie stanowiła dla mnie podstaw do uzyskania stopni naukowych. Mój udział w powstaniu niżej wymienionych prac polegał na współuczestnictwie w wykonywaniu części doświadczeń.

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4. Adam Wojtas, Agnieszka Bysiek, Agnieszka Wawrzczak-Bargiela, Marzena Maćkowiak, **Krystyna Gołembiowska**. Limbic system response to psilocybin and ketamine administration in rats: A neurochemical and behavioral study. International Journal of Molecular Sciences, 2023. Manuscript submitted.

Krystyna Gołembiowska

20.09.2023

**Oświadczenie**

Oświadczam, że udział mgr Adama Wojtasa w niżej wymienionych publikacjach polegał na stworzeniu ogólnej koncepcji badań, wykonaniu części doświadczalnej, analizie uzyskanych wyników oraz przygotowaniu manuskryptu do druku. Żadna z poniższych publikacji nie stanowi oraz nie będzie stanowiła dla mnie podstaw do uzyskania stopni naukowych. Mój udział w powstaniu niżej wymienionych prac polegał na współuczestnictwie w wykonywaniu części doświadczeń.

1. Adam Wojtas, Monika Herian, **Marzena Maćkowiak**, Anna Solarz, Agnieszka Wawrzczak-Bargiela, Agnieszka Bysiek, Karolina Noworyta, Krystyna Gołembiowska. Hallucinogenic activity, neurotransmitters release, anxiolytic and neurotoxic effects in Rat's brain following repeated administration of novel psychoactive compound 25B-NBOMe. Neuropharmacology, 2023. 9, 240:109713. doi: 10.1016/j.neuropharm.2023.109713.

2. Adam Wojtas, Agnieszka Bysiek, Agnieszka Wawrzczak-Bargiela, Zuzanna Szych, Iwona Majcher-Maślanka, Monika Herian, **Marzena Maćkowiak**, Krystyna Gołembiowska. Effect of Psilocybin and Ketamine on Brain Neurotransmitters, Glutamate Receptors, DNA and Rat Behavior. International Journal of Molecular Sciences, 2022. 16, 23, 6713. doi: 10.3390/ijms23126713.

3. Adam Wojtas, Agnieszka Bysiek, Agnieszka Wawrzczak-Bargiela, **Marzena Maćkowiak**, Krystyna Gołembiowska. Limbic system response to psilocybin and ketamine administration in rats: A neurochemical and behavioral study. International Journal of Molecular Sciences, 2023. Manuscript submitted.

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Agnieszka Bysiek