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A single dose of psilocybin increases synaptic density and decreases 5-HT_{2A} receptor density in the pig brain

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Abstract: A single dose of psilocybin, a psychedelic and serotonin 2A receptor (5-HT_{2A}R) agonist, may be associated with antidepressant effects. The mechanism behind its antidepressive action is unknown but could be linked to increased synaptogenesis and down-regulation of cerebral 5-HT_{2A}R. Here, we investigate if a single psychedelic dose of psilocybin changes synaptic vesicle protein 2A (SV2A) and 5-HT_{2A}R density in the pig brain. Twenty-four awake pigs received either 0.08 mg/kg psilocybin or saline intravenously. Twelve pigs (n=6/intervention) were euthanized one day post-injection, while the remaining twelve pigs were euthanized seven days post-injection (n=6/intervention). We performed autoradiography on hippocampus and prefrontal cortex (PFC) sections with [³H]UCB-J (SV2A), [³H]MDL100907 (5-HT_{2A}R antagonist) and [³H]Cimbi-36 (5-HT_{2A}R agonist). One day post psilocybin injection, we observed 4.4% higher hippocampal SV2A density and lowered hippocampal and PFC 5-HT_{2A}R density (-15.21% to -50.19%). These differences were statistically significant in the hippocampus for all radioligands and in PFC for [³H]Cimbi-36 only. Seven days post-intervention, there was still significantly higher SV2A density in hippocampus (+9.24%) and PFC (+6.1%) whereas there were no longer any differences in 5-HT_{2A}R density. Our findings suggest that psilocybin's antidepressive actions are linked to increased persistent synaptogenesis and possibly also to an acute decrease in 5-HT_{2A}R density.

Keywords: Psilocybin, psychedelics, neuroplasticity, SV2A, 5-HT_{2A}, depression, autoradiography, functional-selectivity

1. Introduction

Serotonergic psychedelic drugs have for centuries been extensively used in religious practices and also recreationally [1]. The neurobiological and behavioral effects in mammals are mediated through stimulation of the brain serotonin 2A receptor (5-HT_{2A}R) as reviewed by Vollenweider et al. [2,3]. Upon ingestion of psilocybin, a tryptamine psychedelic [1], it quickly dephosphorylates to the active compound psilocin which has a high affinity to 5-HT_{2A}R, but also to other 5-HT receptors such as 5-HT_{1A}R and 5-HT_{2C}R [1,4,5].

Psychedelic stimulation of 5-HT_{2A}R, a G-protein-coupled receptor (GPCR), has recently shown potential as an anxiolytic and antidepressant therapy. Some clinical studies suggest that a single dose of psilocybin rapidly and effectively relieves symptoms in depression and anxiety, with effects that persist long after the psychedelic experience [6–9]. Research in rodents suggests that psilocybin, lysergic acid diethylamide (LSD), 2,5-dimethoxy-4-iodoamphetamine (DOI), N,N-dimethyltryptamine (DMT) and alkaloids like harmine, tetrahydroharmine, and harmaline (present

in ayahuasca) induce structural neuroplasticity and alter the expression of important proteins like VGLUT1, BDNF, and MAP2 [10–13]. The mechanism behind these synaptic changes is hypothesized to be exerted via the 5-HT_{2A}R pathway [10].

Changes in synaptic density in brain regions associated with emotional processing, i.e., hippocampus and prefrontal cortex (PFC), may play a vital role in the pathophysiology of mood disorders, e.g. major depressive disorder. Both post-mortem human brain [14,15] and in vivo [16] studies in depressed individuals have shown a loss of synapses through the down-regulation of synaptic proteins and genes. Hence, upregulation of presynaptic proteins and an increase in synaptic density may be associated with the potential antidepressive effects of psychedelics.

Synaptic vesicle protein 2A (SV2A) is an integral 12-transmembrane domain glycoprotein expressed in synaptic vesicles throughout the brain [17], and SV2A density is thought to reflect presynaptic density [18]. The levetiracetam derivative UCB-J, which binds selectively to SV2A, has in its radiolabeled form been shown to correspond to synaptic density as measured with the well-characterized presynaptic protein synaptophysin [19–21].

Classical receptor binding assay studies have demonstrated that 5-HT_{2A}R (and other GPCRs) exist in two affinity states, a high- and a low- affinity state [22–24]. The affinity states of the receptors are considered to represent different functional states of the receptor, high-affinity being functionally active (activation of G_{ai1}-protein pathway) in contrast to the low-affinity state (activation of canonical G_{aq/11}-protein pathway) [25]. Whereas 5-HT_{2A}R antagonists bind to the total pool of 5-HT_{2A}R, 5-HT_{2A}R agonists bind to the high-affinity state GPCRs [26]. Stimulation of 5-HT_{2A}R leads to rapid receptor internalization [27]. This endosomal internalization may lead to lysosomal degradation and down-regulation of 5-HT_{2A}R, as extensively reviewed by Gray J.A. and Roth B.L. [28].

In the present study, we hypothesize that a psychedelic dose of psilocybin increases presynaptic density as reflected in SV2A protein levels in the pig brain. We also test the hypothesis that the availability of 5-HT_{2A}R is decreased after agonist stimulation with psilocybin.

Using in vitro autoradiography, we measure SV2A and 5-HT_{2A}R protein levels one and seven days post-injection of a single dose of psilocybin, known to induce 5-HT_{2A}R associated behavioral changes corresponding to psychedelic effects, in healthy pigs [29]. We investigate brain effects one day after psilocybin administration because the days following a psychedelic experience may provide a therapeutic window to treat mood disorders [6,7]. A follow-up seven days after a psilocybin intervention is done because this is when depressive scores have been reported to be the lowest [6]. To investigate if potential psilocybin-induced reductions in 5-HT_{2A}R are due to changes in the total receptor pool or confined to functionally active 5-HT_{2A}Rs, we used both an antagonist ([³H]MDL100907) and an agonist radioligand ([³H]Cimbi-36) for autoradiography.

2. Results

2.1. SV2A autoradiography

Figures 1(a) and 1(b) show the SV2A protein density as determined by [³H]UCB-J autoradiography in hippocampus and PFC. Compared to the saline-treated group, psilocybin treatment was associated with 4.4% higher SV2A in hippocampus ($p < 0.0001$) one day after psilocybin injection and 9.2% higher SV2A in hippocampus ($p = 0.024$) seven days after psilocybin (Figure 1(a) and Table 1). One day after psilocybin, there was no difference in PFC SV2A (Figure 2(b) and Table 1, 0.25%, $p = 1$), but seven days after psilocybin administration there was 6.1% higher SV2A in PFC ($p < 0.0001$).

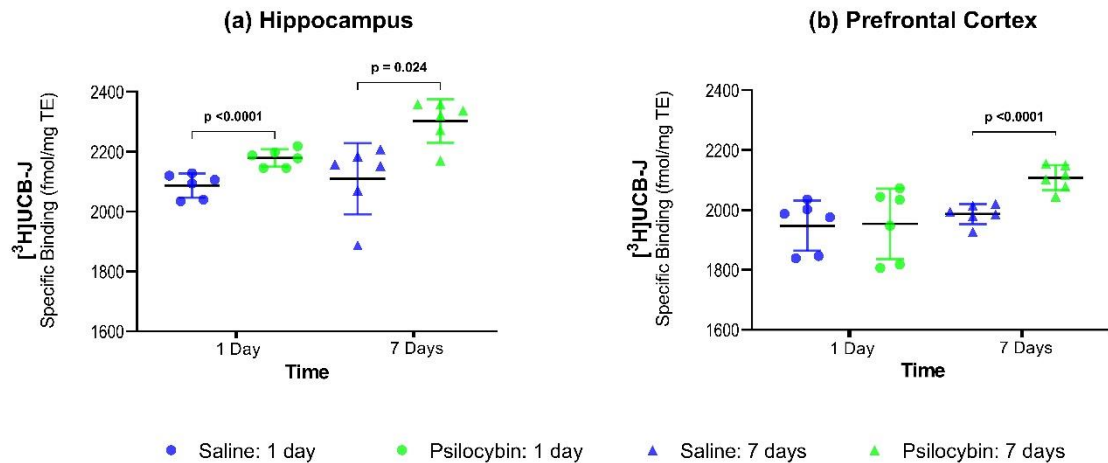


Figure 1. Group-wise comparisons of SV2A density (mean \pm SD) in hippocampus (a) and PFC (b) as measured with $[^3\text{H}]\text{UCB-J}$ autoradiography.

Table 1. Group-wise summary of statistical tests performed for each radioligand. All tests show the adjusted p-values using the Holm method. NA (not applicable) indicates test was not performed.

	Hippocampus		Prefrontal Cortex	
	1 day	7 days	1 day	7 days
$[^3\text{H}]\text{UCB-J}$				
(psilocybin vs. saline)	+4.42% ($p < 0.0001$)	+9.24% ($p = 0.024$)	+0.25% ($p = 1$)	+6.1% ($p < 0.0001$)
$[^3\text{H}]\text{MDL100907}$				
(psilocybin vs. saline)	-29.6% ($p < 0.0001$)	-3.58% ($p = 1$)	-15.21% ($p = 0.162$)	+1.32% ($p = 1$)
$[^3\text{H}]\text{Cimbi-36}$				
(psilocybin vs. saline)	-43.39% ($p = 0.013$)	+3.31% ($p = 1$)	-50.19% ($p < 0.0001$)	+2.23% ($p = 1$)
$[^3\text{H}]\text{MDL100907 vs.}$ $[^3\text{H}]\text{Cimbi-36}$	NA	NA	-41.26% ($p = 0.033$)	+0.90% ($p = 0.921$)

2.2 5-HT_{2A}R antagonist autoradiography:

Figures 2(a) and 2(b) show the 5-HT_{2A}R density as measured with the antagonist radioligand $[^3\text{H}]\text{MDL100907}$ in hippocampus and PFC. One day after the intervention, hippocampal 5-HT_{2A}R density (Figure 2(a) and Table 1) was 29.6% lower ($p < 0.0001$) and PFC 5-HT_{2A}R density was similar (-15.2%, $p = 0.162$, Figure 2(b) and Table 1) in the psilocybin-treated group than in the saline-treated group. Seven days after the psilocybin interventions, hippocampal and PFC 5-HT_{2A}R density was not significantly different from the saline-treated animals.

2.3 5-HT_{2A}R agonist autoradiography:

Figures 2(c) and 2(d) show the 5-HT_{2A}R density as measured with the agonist radioligand $[^3\text{H}]\text{Cimbi-36}$ in hippocampus and PFC. One day after psilocybin intervention, hippocampal 5-HT_{2A}R/5-HT_{2C}R density (Figure 2(c) and Table 1) was 43.3% lower ($p = 0.013$), and PFC 5-HT_{2A}R density (Figure 3(d) and Table 1) was 50.1% lower ($p < 0.0001$) in the psilocybin-treated group than in the saline-treated group. With $[^3\text{H}]\text{Cimbi-36}$, similar to $[^3\text{H}]\text{MDL100907}$, 5-HT_{2A}R density was not significantly different in the hippocampus and PFC seven days after the psilocybin intervention compared to saline.

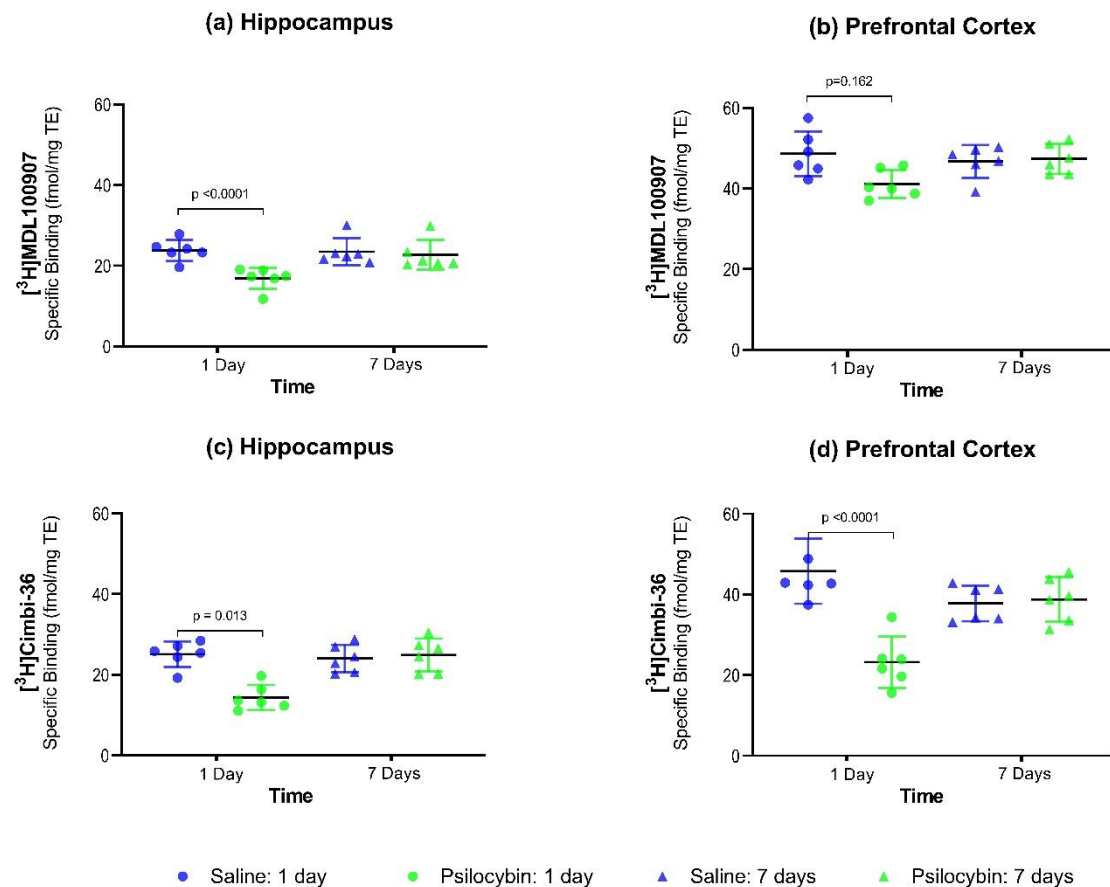


Figure 2. Group-wise comparison of 5-HT_{2A}R density (mean ±SD) as measured with [³H]MDL100907 and [³H]Cimbi-36 in the hippocampus (a and c) and PFC (b and d) using autoradiography.

2.4 Antagonist vs agonist radioligand for 5-HT_{2A}R density:

We found a more pronounced reduction of 5-HT_{2A}R density when measured with [³H]Cimbi-36 compared to [³H]MDL100907 one day after psilocybin intervention in the PFC. This difference of 41.26% was statistically significant at $p = 0.033$ (Table 1). We found no significant difference seven days after psilocybin intervention for either radioligand.

2.5 Plasma psilocin

Plasma psilocin levels at euthanasia one and seven days after the psilocybin intervention were all below the detection limit.

3. Discussion

To the best of our knowledge, this is the first large-animal study to investigate how a single dose of psilocybin changes the key proteins SV2A and 5-HT_{2A}R in brain regions involved in emotional processing. We find that a single dose of psilocybin increases the presynaptic marker, SV2A already after one day and that it remains high seven days after. We also show a transient reduction in the hippocampus and PFC 5-HT_{2A}R density; it is reduced one day after intervention but not seven days after.

The increase in synaptic marker SV2A may result from the stimulation of the 5-HT_{2A}R, TrkB and mTOR signalling pathways [10]. To understand the neurobiological basis of neuroplasticity and the implication of these changes, future proteomics studies must reveal which other proteins in these pathways are changed and the temporal evolvement of such changes. Our data support the notion of increased synaptogenesis following psychedelic exposure, which is hypothesized to underlie the

antidepressant effects observed in humans: We find higher SV2A density in hippocampus and PFC which also are regions where SV2A is reduced in patients with major depressive disorder [16]. Atypical antidepressants like ketamine are also associated with neuroplastic effects through proteins like cFos, pERK and BDNF in PFC and hippocampus in a social defect stress rodent model [30]. Together with other markers of neuroplasticity, increased levels of SV2A after intervention with a psychedelic drug adds to the scientific evidence that psychedelics enhance neuroplasticity which may be the mechanism of action of its antidepressant properties [31].

We have previously reported that 5-HT_{2A}R mRNA expression is unaltered in the brains of these pigs [29]. It is, however, well known that brain 5-HT_{2A}R mRNA expression does not correlate with 5-HT_{2A}R protein content [32]. Our finding of a transient decrease in 5-HT_{2A}R density, but not mRNA, one day after psilocybin is in line with results for the psychedelic substance DOI, where a significant difference in 5-HT_{2A}R protein expression was not accompanied by a change in mRNA gene expression [33].

Compared to other protein measuring techniques such as western blot and immunohistochemistry, autoradiography provides an added advantage of measuring receptors in the functionally active vs total receptor pool by the use of agonist or antagonist radioligands, respectively. The use of adjacent brain sections provides the ability to directly compare receptors. We find a statistically significant reduction in 5-HT_{2A}R density in the PFC one day after psilocybin administration when measuring with [³H]Cimbi-36 compared to [³H]MDL100907. The difference between the two radioligands offers circumstantial evidence of differential binding of antagonists versus agonists, at least when it comes to PFC. More caution should be exerted when comparing the radioligands in hippocampus because the hippocampus has high levels of 5-HT_{2C}R, with a density similar to 5-HT_{2A}R [34,35] and [³H]Cimbi-36 also has affinity to 5-HT_{2C}R [36,37]. That is, we cannot exclude that some of the observed reduction in [³H]Cimbi-36 in hippocampus could be due to a down-regulation of 5-HT_{2C}R. It could be a concern that the reduction in 5-HT_{2A}R one day after psilocybin was due to partial blocking by residual psilocin. However, plasma psilocin levels at euthanasia one day after was under the detection limit in all animals.

The fraction of functional 5-HT_{2A}R has to some extent been assessed in vivo in non-human primates [37] and humans [38] using [¹¹C]Cimbi-36 as an agonist and [¹¹C]MDL100907 or [¹⁸F]altanserin as antagonist radioligands, but a more precise estimate is difficult in vivo because of missing information about the free fraction of the radioligand and the radioligand affinity. A little unexpectedly, B_{max} did in our study not differ substantially between agonist and antagonist radioligands, but it should be kept in mind that uncertainties in the determination of the specific activity is reflected in the calculation of B_{max} . Functional receptors can also be measured with [³⁵S]guanosine triphosphate (GTP) γ S binding stimulation mediated with DOI followed by immunoprecipitation with specific antibodies, while the complex is captured with protein A-polyvinyl toluene scintillation proximity assay. When this approach is made on post-mortem brain tissue from patients with schizophrenia, the canonical $G_{\alpha q/11}$ -protein pathway of 5-HT_{2A}Rs is found to be unaltered in PFC, whereas the pro-hallucinogenic $G_{\alpha i1}$ -protein pathway is functionally overactive in PFC [39,40]. GTP γ S binding assay may be more sensitive to the measurement of functional receptors and could generate an outcome that was more straight-forward to interpret.

It is already well-known that a transient 5-HT_{2A}R down-regulation occurs upon agonist stimulation, followed by a return to baseline [41]. We have previously found that the 5-HT_{2A}R binding is normal seven days after healthy individuals take a single psychedelic dose of psilocybin [42]. To which extent the transient down-regulation of 5-HT_{2A}R is a prerequisite for boosting the formation of new synapses is intriguing and should be examined in future studies.

Some limitations of the study should also be mentioned. We chose to investigate only two time-points and selected a few highly relevant proteins in two relevant brain regions. It would be interesting to investigate whether the synaptic density increases further beyond one week, and for how long it is maintained. Although we cannot be certain that our findings translate to humans that consume a single dose of psilocybin, the SV2A density in hippocampus and PFC is in the same range as that reported in post-mortem human and non-human primates by Varnäs et al. [21]. The changes in SV2A and 5-HT_{2A}R were seen in healthy pigs; it might also be relevant to investigate changes in a

psychosocial chronic-stress pig model [43]. Further, to ensure that the pigs received a well-defined dose of psilocybin, we chose to administer the drug intravenously rather than peroral. This differs from the typical approach in patients. Despite the faster pharmacokinetics after intravenous administration, the dose and administration route result in the same 5-HT_{2A}R occupancy as in humans that take it perorally [29].

4. Materials and Methods

4.1 Animals and drug dosage.

The brain tissue was retrieved from pigs entering a previously published study [29] where more details are described. Briefly, female Danish slaughter pigs (Yorkshire x Duroc x Landrace) weighing around 20 kg (approximately nine weeks old) were used in the study. The animals were sourced from a local farm and allowed to acclimatize for at least one week before the start of the experiment. The animals were housed in individual pens with an enriched environment on a 12-hour light/dark cycle, with free access to water, and weight-adjusted food twice daily. The welfare of the animals was assessed daily. After arriving in the stables, animals were trained up to a week to allow for handling by humans.

Donovan et al. [29] identified which dose of psilocybin to give to make it comparable to a dose that elicits psychedelic effects in humans. This was based on behavioral response (headshakes, hindlimb scratches and rubbing against the pen wall) and on PET studies of the 5-HT_{2A}R occupancy using the agonist radioligand [¹¹C]Cimbi-36; 67% 5-HT_{2A}R occupancy will elicit psychedelic effects in humans [42]. Intravenous injection of psilocybin was given in an ear vein catheter to the awake pigs, and the animals were under no form of external stress during the experiment. At the time of euthanasia, blood was drawn for measurement of plasma psilocin levels which were measured by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry as previously described [42].

4.2 Ethical Statement:

All animal experiments conformed to the European Commission's Directive 2010/63/EU and the ARRIVE guidelines. The Danish Council of Animal Ethics had approved all procedures (Journal no. 2016-15-0201-01149).

4.3 Study design:

Figure 3 shows the overall design of the study. Twenty-four awake pigs were given an intravenous dose of either 0.08 mg/kg psilocybin (n = 12) or saline (n = 12) through an ear vein catheter. Half of the animals in each group were euthanized one day post-injection (n = 6/intervention). The remaining 12 animals were euthanized seven days post-injection (n = 6/intervention). That is, the animals were divided into four groups: *Saline: 1 day*, *Psilocybin: 1 day*, *Saline: 7 days* and *Psilocybin: 7 days* (Figure 3). The extracted brains were snap-frozen and stored at -80°C. From one hemisphere, 20 µm thick frozen sections were sliced on a cryostat (Leica CM1800, Leica Biosystems, USA) from the PFC and hippocampus and mounted on Superfrost Plus™ adhesion microscope slides. Sections were stored at -20°C for the remaining period of the study.

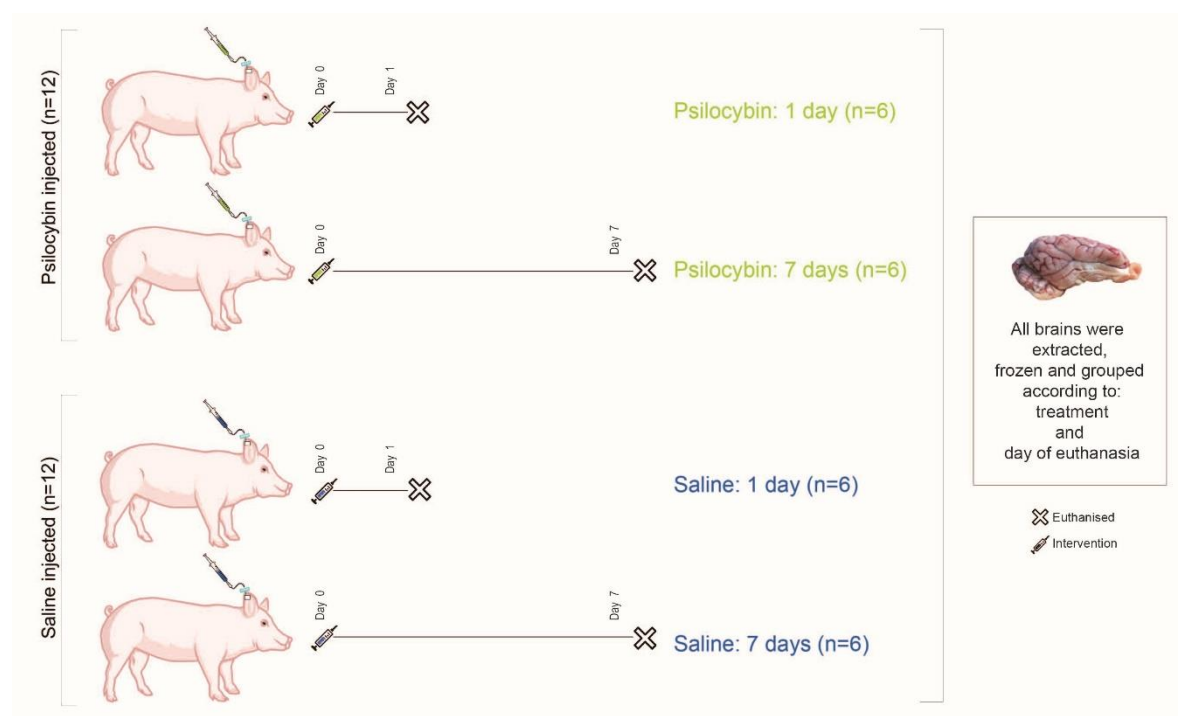


Figure 3. Study design. Twenty-four pigs received an intravenous dose of either 0.08 mg/kg psilocybin or saline. Six pigs from each type of intervention were euthanized one day or seven days post-injection. The pigs were divided into four groups, as depicted in the figure.

4.4 Autoradiography:

Radioligands used for autoradiography included SV2A imaging with [^3H]UCB-J (UCB Pharma, Belgium, specific activity 14 Ci/mmol or Pharmaron Ltd., UK, specific activity 28 Ci/mmol). [^3H]MDL100907 (ViTrax, USA, specific activity 56 Ci/mmol) was used as antagonist radioligand for 5-HT_{2A}R and [^3H]Cimbi-36 (kindly provided by Prof. Dr C. Halldin, Department of Neuroscience, Karolinska Institute, Sweden, specific activity 53 Ci/mmol,) as an agonist radioligand for 5-HT_{2A}R/5-HT_{2C}R. Radio-Thin-Layered-Chromatography (R-TLC) was performed for all radioligands, to measure the radiochemical purity (RCP) and integrity of the parent compound. The mobile phase for [^3H]UCB-J R-TLC was Acetonitrile:Ammonium formate [25:75] (0.1 M, with 0.5% AcOH, pH 4.2). The mobile phase for [^3H]MDL100907 R-TLC was Chloroform:Methanol:Ammonia solution [90:9:1]. The mobile phase for [^3H]Cimbi-36 R-TLC was Chloroform:Methanol:Triethylamine [94:5:1]. [^3H]UCB-J and [^3H]MDL100907 had high RCP (96-98%) while [^3H]Cimbi-36 had an RCP of 52-57%. Radioactivity was corrected for RCP of [^3H]Cimbi-36 after TLC.

Sections were thawed to room temperature for 30-45 minutes before prewashing twice for 10 minutes in 50 mM Tris-HCl pre-incubation buffer set to 7.4 pH containing 0.5% bovine serum albumin (BSA) for [^3H]UCB-J or 0.01% ascorbic acid, 4 nM CaCl₂ and 0.1% BSA for [^3H]MDL100907 and [^3H]Cimbi-36.

For SV2A, sections were incubated in assay buffer containing 60 nM [^3H]UCB-J in 50 mM Tris-HCl buffer containing 5 mM MgCl₂, 2 mM EGTA and 0.5% BSA (pH 7.4) for 1 hour. Incubation was terminated by three 5-minutes washes with 4°C pre-incubation buffer followed by a rapid rinse in 4°C deionized H₂O (dH₂O). For 5-HT_{2A}R, sections were incubated in assay buffer containing 3 nM [^3H]MDL100907 or 1 nM [^3H]Cimbi-36 in 50 mM Tris-HCl containing 0.01% ascorbic acid, 4 nM CaCl₂ and 0.1% BSA (pH 7.4) for 1 hour. Incubation was terminated by two 10-minutes washes in ice-cold pre-incubation buffer followed by a rapid rinse in ice-cold dH₂O.

Assay buffer concentration of the respective radioligands was determined using 4-5 \times K_D values (Appendix A: Figure 1) to determine B_{max} values in the section. After washing, the slides were rapidly air-dried and fixated in a paraformaldehyde vapor chamber overnight in cold storage (4°C). The next day, the samples were moved to an excicator for 45-60 min to remove any excess moisture and then

placed in a cassette for autoradiography with tritium sensitive image plates (BAS-IP TR2040, Science Imaging Scandinavia AB, Nacka, Sweden) along with radioactive tritium standards (RPA510, Amersham Bioscience, GE Healthcare, USA) (Figure 4). Image plates were exposed for seven days. After the exposure, the image plates were read using a Fujifilm BAS 1000 scanner (Fujifilm Europe, GmbH, Germany). Calibration, quantification and data evaluation was done using ImageJ software (NIH Image, USA) [44]. Regions of interest were hand-drawn or using the wand tool and visually inspected after automated delineation, as shown in Figure 4. Four-parameter general curve fit (David Rodbard, NIH) of decay corrected tritium standards was used to convert mean pixel density (grayscale) to nCi/mg tissue equivalent (TE). Total binding was determined in the hippocampal and cortical grey matter while non-specific binding was determined in white matter on the same slides. Finally, the decay-corrected specific activity of the representative radioligand was used to convert nCi/mg TE to fmol/mg TE. Specific binding was calculated as the difference between total binding and non-specific binding. All experiments were performed in triplicates, and experimenters were blinded.

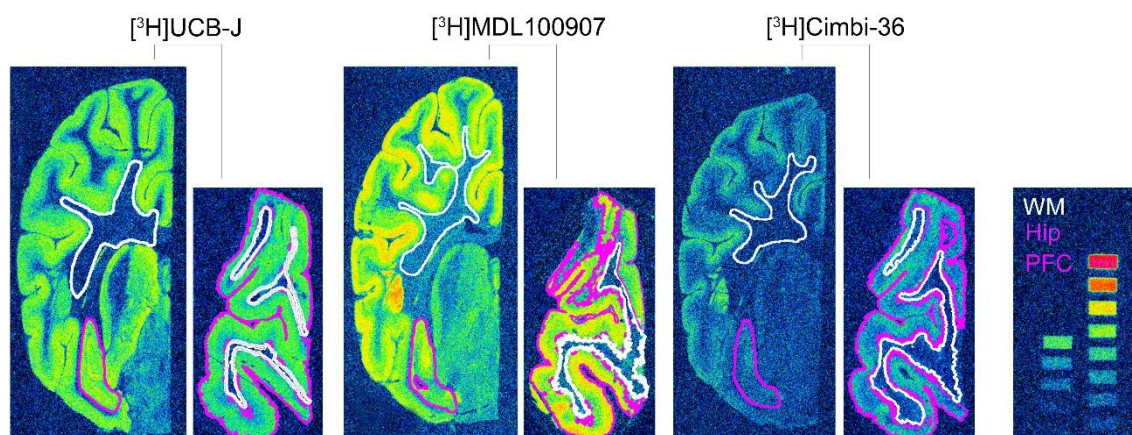


Figure 4. Representative autoradiograms of the radioligands used in this study. Half hemisphere sections of the hippocampus (larger coronal sections) and PFC (smaller coronal sections) from the same animal belonging to Saline: 1 day group. Color-coded lines show representations of the region of interest that is either hand-drawn or drawn using the wand tool in ImageJ and visually inspected. The figure also shows the radioactive standards used in the study; approximately 16 nCi/mg to 0.2 nCi/mg decay corrected to the time of experiment and day of exposure.

4.5 Statistical Analyses:

Data were analyzed using R (v. 4.0.3; R core team, Austria), while GraphPad Prism (v. 9.0.0; GraphPad Software, USA) was used for data visualization. Comparisons between group means (\bar{X}) for the respective radioligands (Eq:1 and Eq:2) were done using a permutation test (with 1000 permutations) on log-transformed values and adjusted for multiple comparisons (overtime, radioligand, and brain regions: 12 tests) using the Holm method. Comparison between the 5-HT_{2A}R radioligands, [3H]Cimbi-36 and [3H]MDL100907, was performed for the PFC using a permutation test (with 1000 permutations) on log-transformed values of treatment effect at one day and seven days (Eq: 3 and Eq:4).

$$\bar{X} \text{ (Psilocybin: 1 day)} - \bar{X} \text{ (Saline: 1 day)} \quad (\text{Eq.1})$$

$$\bar{X} \text{ (Psilocybin: 7 day)} - \bar{X} \text{ (Saline: 7 day)} \quad (\text{Eq.2})$$

$$[3\text{H}]\text{Cimbi-36} (\bar{X} \text{ (Psilocybin:1 day)} - \bar{X} \text{ (Saline: 1 day)}) - [3\text{H}]\text{MDL100907} (\bar{X} \text{ (Psilocybin:1 day)} - \bar{X} \text{ (Saline: 1 day)}) \quad (\text{Eq.3})$$

$$[^3\text{H}]\text{Cimbi-36}(\bar{X}(\text{Psilocybin:7 days}) - \bar{X}(\text{Saline: 7 days})) - [^3\text{H}]\text{MDL100907}(\bar{X}(\text{Psilocybin:7 days}) - \bar{X}(\text{Saline: 7 days})) \quad (\text{Eq.4})$$

Author Contributions: Conceptualization, GMK, HDH, NRR, LLD and AJ; methodology, NRR, AJ, BO and NFR; software, NRR, and BO; validation, NRR, AJ, and NFR; formal analysis, NRR, AJ and BO.; investigation, NRR, NFR, AJ; resources, LLD.; data curation, NRR, AJ, and BO; writing—original draft preparation, NRR.; writing—review and editing, NRR, AJO, LLD, BO, HDH and GMK.; visualization, NRR; supervision, HDH and GMK.; funding acquisition, GMK. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: GMK: H. Lundbeck A/S (research collaboration), Novo Nordisk/Novozymes/Chr. Hansen (stockholder), Sage Therapeutics and Sanos (Advisor). GMK is currently the president of the European College of Neuropsychopharmacology. All other authors declare no conflicts of interest.

Abbreviations

5-HT	5-Hydroxytryptamine (serotonin)
5-HT _{1A} R	5-Hydroxytryptamine (serotonin) 1A Receptor
5-HT _{2A} R	5-Hydroxytryptamine (serotonin) 2A Receptor
5-HT _{2C} R	5-Hydroxytryptamine (serotonin) 2C Receptor
ARRIVE	Animal Research: Reporting of In Vivo Experiments
BDNF	Brain-Derived Neurotrophic Factor
B _{max}	Total density (concentration) of the receptor
BP _{ND}	Binding Potential (Non-displaceable)
BSA	Bovine Serum Albumin
DMT	N,N-dimethyltryptamine
DOI	2,5-dimethoxy-4-iodoamphetamine
GPCR	G-protein coupled receptor
K _D	Equilibrium dissociation Constant
LSD	Lysergic acid diethylamide
MAP2	Microtubule-associated protein 2
mTOR	Mammalian Target of Rapamycin
NSB	Non-specific Binding
PET	Positron Emission Tomography
pERK	Endoplasmic Reticulum Protein Kinase
PFC	Prefrontal Cortex
RCP	Radiochemical purity
R-TLC	Radio-Thin Layer Chromatography
SV2A	Synaptic Vesicular Protein 2A

TB	Total Binding
TE	Tissue Equivalent
TrkB	Tropomyosin Receptor Kinase B
VGLUT1	Vesicular Glutamate Transporter 1
\bar{X}	Mean

Appendix A

Saturation assays

Methods:

Two female Danish slaughter pig's brain that was not included in this study were used to perform a saturation assay. The extracted brains were snap-frozen and stored at -80°C. From one hemisphere, 20 µm thick frozen sections were sliced on a cryostat (Leica CM1800, Leica Biosystems, USA) from the prefrontal cortex (PFC) and mounted on Superfrost Plus™ adhesion microscope slides. Sections were stored at -80°C for the remaining period of the study.

All radioligands used for this autoradiography study were included for a saturation assay. Radioligands used are as follows: [³H]UCB-J for SV2A (UCB pharma, Belgium, specific activity 14 Ci/mmol), [³H]MDL100907 as an antagonist radioligand for 5-HT_{2A}R (ViTrax, USA, specific activity 56 Ci/mmol) and [³H]Cimbi-36 as an agonist radioligand for 5-HT_{2A}R/5-HT_{2c}R (kindly provided by Prof. Dr C. Halldin, Department of Neuroscience, Karolinska Institute, Sweden, specific activity 53 Ci/mmol) for 5-HT_{2A}/5-HT_{2c}. Saturation assay for [³H]Cimbi-36 was performed in 2012, when the tracer was produced, hence high radiochemical purity was assumed.

Experimental conditions previously described were also used for the saturation assay. Briefly, after sections were thawed to room temperature, they were prewashing twice for 10 minutes in 50 mM Tris-HCl pre-incubation buffer set to 7.4 pH containing 0.5% bovine serum albumin (BSA) for [³H]UCB-J or 0.01% ascorbic acid, 4 nM CaCl₂ and 0.1% BSA for [³H]MDL100907 and [³H]Cimbi-36.

For SV2A, sections (n=4) were incubated in assay buffer (50 mM Tris-HCl buffer containing 5 mM MgCl₂, 2 mM EGTA and 0.5% BSA (pH 7.4)) containing varying concentration (0 to 100 nM) of [³H]UCB-J for total binding (TB) and same varying concentration of [³H]UCB-J with 10 mM of levetiracetam (Keppra, UCB pharma, Belgium) for non-specific binding (NSB). Incubated for 60 mins. Incubation was terminated by three 5-minutes washes with 4°C pre-incubation buffer followed by a rapid rinse in 4°C deionized H₂O (dH₂O).

For 5-HT_{2A}R, sections (n=4 for [³H]MDL100907 and n=1 for [³H]Cimbi-36) were incubated in assay buffer (50 mM Tris-HCl containing 0.01% ascorbic acid, 4 nM CaCl₂ and 0.1% BSA (pH 7.4)) containing varying concentration of [³H]MDL100907 or [³H]Cimbi-36 (0 to 4 nM) for TB and same varying concentration of [³H]MDL100907 or [³H]Cimbi-36 with 10 mM ketanserin (Sigma-Aldrich, Denmark) for NSB. Incubated for 60 mins. Incubation was terminated by two 10-minutes washes in ice-cold pre-incubation buffer followed by a rapid rinse in ice-cold dH₂O.

After washing, the slides were rapidly air-dried and fixated in a paraformaldehyde vapour chamber overnight in cold storage (4°C). The next day, the samples were moved to an exicator for 45-60 min to remove any excess moisture and then placed in a cassette for autoradiography with tritium sensitive image plates (BAS-IP TR2040, Science Imaging Scandinavia AB, Nacka, Sweden) along with radioactive tritium standards (ART0123B, American Radiolabelled Chemical, Inc., USA and RPA510, Amersham Bioscience, GE Healthcare, USA) (Figure 4). Image plates were exposed for seven days. After the exposure, the image plates were read using a Fujifilm BAS 1000 scanner (Fujifilm Europe, GmbH, Germany). Calibration, quantification and data evaluation was done using ImageJ software (NIH Image, USA) [44]. Regions of interest were hand-drawn or using the wand tool and visually inspected after automated delineation, as shown in Figure 4 of the main manuscript. Four-parameter general curve fit (David Rodbard, NIH) of decay corrected tritium standards was used to convert mean pixel density (grayscale) to nCi/mg TE. TB was determined in cortical grey matter from TB slides while NSB was determined in the cortical grey matter of NSB slides, and white matter was defined on the same TB slides. Finally, the decay-corrected specific activity of the representative

radioligand was used to convert nCi/mg TE to fmol/mg TE. Specific binding was calculated as the difference between TB and NSB.

Data were analyzed using GraphPad Prism (v. 9.0.0; GraphPad Software, USA). Non-linear regression analysis (One site- Fit total and non-specific binding) was used for all radioligands. Curve fitting used for saturation assays was "One site- Fit total and non-specific binding". The fitting method used was the least squared regression with no weighting.

Results:

Saturation assay with [^3H]UCB-J generated a K_D of 11.47 ± 7.8 nM and B_{max} of 1090 ± 253 fmol/mg TE. [^3H]MDL100907 generated a K_D of 0.49 ± 0.1 nM and B_{max} of 44.58 ± 3.9 fmol/mg TE while [^3H]Cimbi-36 shows a K_D of 0.31 ± 0.1 nM and B_{max} of 56.07 ± 3.3 fmol/mg TE. [^3H]UCB-J white matter binding was lower (slope = 3.9) compared to NSB in the grey matter of levetiracetam blocked slides (slope = 5.6) but with less variation. On the other hand, white matter binding with [^3H]MDL100907 (slope = 8.8) and [^3H]Cimbi-36 (slope = 15.1) correlate (visually) with NSB in the grey matter of ketanserin blocked slides (slope = 8.2 and 16.4 respectively).

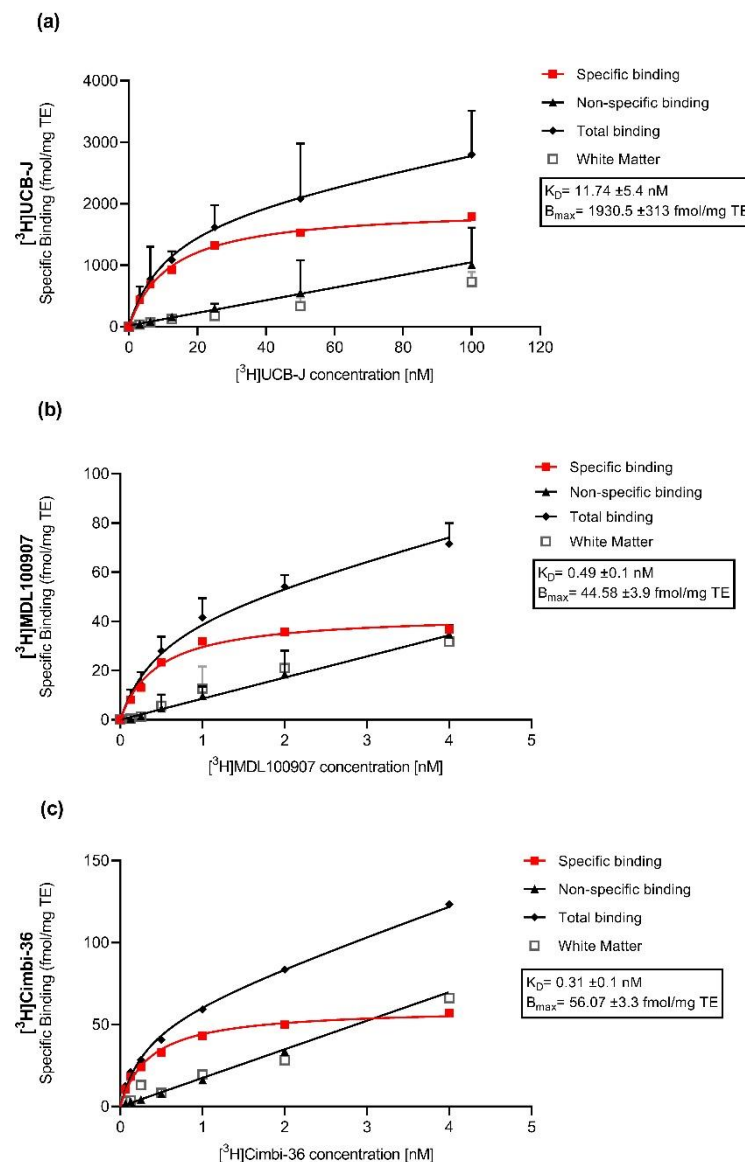


Figure 1. Saturation assay of [^3H]UCB- J (a), [^3H]MDL100907 (b) and [^3H]Cimbi-36 (c) in the grey matter of the pig brain.

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