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Short term changes in the proteome of human cerebral organoids induced by 5-MeO-DMT

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Dimethyltryptamines are entheogenic serotonin-like molecules present in traditional Amerindian medicine recently associated with cognitive gains, antidepressant effects, and changes in brain areas related to attention. Legal restrictions and the lack of adequate experimental models have limited the understanding of how such substances impact human brain metabolism. Here we used shotgun mass spectrometry to explore proteomic differences induced by 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT) on human cerebral organoids. Out of the 6,728 identified proteins, 934 were found differentially expressed in 5-MeO-DMT-treated cerebral organoids. *In silico* analysis reinforced previously reported anti-inflammatory actions of 5-MeO-DMT and revealed modulatory effects on proteins associated with long-term potentiation, the formation of dendritic spines, including those involved in cellular protrusion formation, microtubule dynamics, and cytoskeletal reorganization. Our data offer the first insight about molecular alterations caused by 5-MeO-DMT in human cerebral organoids.

Dimethyltryptamines are naturally-occurring molecules hypothesized to be involved in spontaneous altered states of consciousness such as dreams, free imagination, and insightful creativity^{1,2}. N,N-dimethyltryptamine (N,N-DMT) and bufotenine (5-HO-DMT) have been traditionally used as entheogens by Amerindians^{3,4} as major active ingredients of *Virola* snuff and a brew called *Ayahuasca*⁵. The popularity of *Ayahuasca* as part of religious ceremonies continues to spread in South America and other countries⁶, possibly motivated by its strong antidepressant effects^{7,8}. Chronic *Ayahuasca* ingestion has been associated with cognitive gains and structural brain changes in areas related to attention, self-referential thought, and internal mentation^{9,10}. Another member of this group of molecules is 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT), secreted in large amounts by *Incilius alvarius*^{11,12}. 5-MeO-DMT has been used more recently by the Seris, an indigenous group from the state of Sonora, in Mexico.

The search for the molecular mechanisms underlying the effects of dimethyltryptamines showed that N,N-DMT and 5-MeO-DMT, two closely related metabolic products, can act as systemic endogenous regulators of inflammation and immune homeostasis through both 5-hydroxytryptamine receptors (5-HTRs) and sigma-1 receptors (σ -1Rs)^{13,14}. Under severe hypoxia, N,N-DMT robustly increased the survival of *in vitro* cultured human cortical neurons, monocyte-derived macrophages, and dendritic cells acting through σ -1Rs¹⁵. The direct evidence of neuroimmune communication and neuroregenerative effects of N,N-DMT and 5-MeO-DMT greatly enhanced expectations for psychedelic research.

Our limited understanding of the physiological activity of dimethyltryptamines and other classic psychedelic substances is caused not only by legal restrictions on such research^{16,17} but also by the lack of adequate experimental models^{18–20}. In the past few years, considerable progress has been made regarding the neural differentiation of human pluripotent stem cells into mature neurons and cerebral organoids²¹. Human neural

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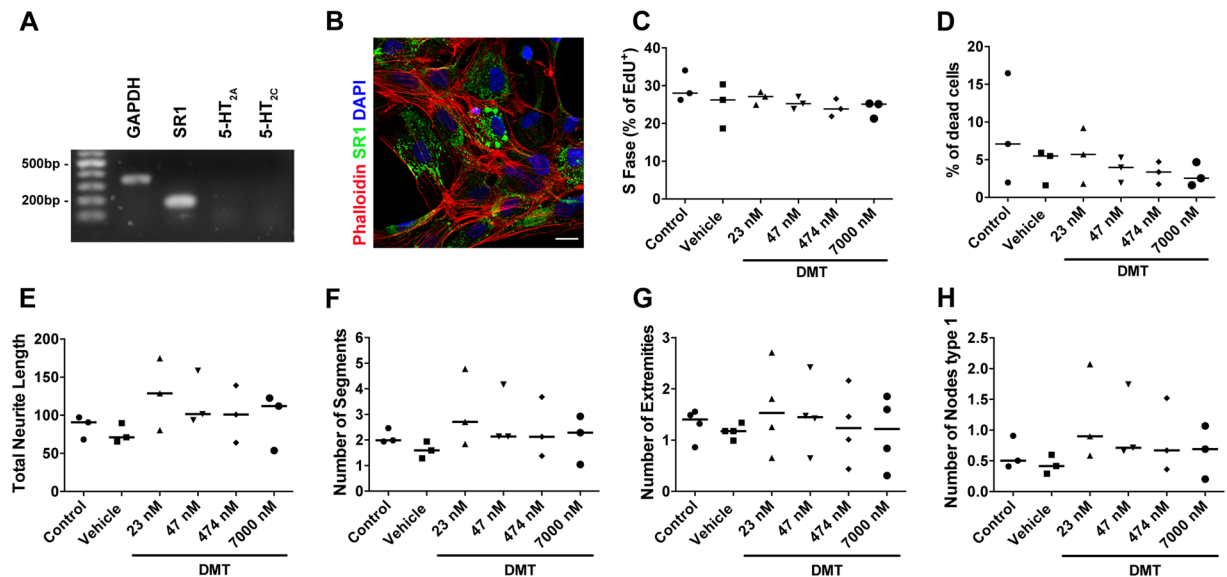


Figure 1. Effects of 5-MeO-DMT on hNPCs. (A) Expression of mRNA for internal control (GAPDH), SR1, 5-HT_{2A}, and 5-HT_{2C} in hNPCs. (B) Confirmation of σ -1R protein (green) expression by immunocytochemistry, phalloidin showing the cytoskeleton (red) and DAPI staining nuclei (blue), scale bar 20 μ m. (C) Quantification of cell proliferation based on EdU staining after treatment with 5-MeO-DMT. (D) Percentage of dead cells in hNPCs treated with 5-MeO-DMT. (E–H) Effects of 5-MeO-DMT on neuronal arborization by quantification of (E) total neurite length (sum of the length of all neurites attached to the cell), (F) number of segments, (G) number of extremities, and (H) number of nodes type 1. Bar represents median. Data were analyzed by one-way ANOVA with Tukey's multiple comparison test, and only p-values < 0.05 were considered significant. Here, all comparisons showed p-values > 0.05.

progenitor cells (hNPC) are useful cell systems for high-throughput screening due to their homogeneity, along with little complexity and limited differentiation potential. On the other hand, cerebral organoids are complex, three-dimensional (3D) culture systems composed of multiple cell types that self-organize into various brain regions similarly to those *in vivo*, including the cerebral cortex, ventral forebrain, midbrain–hindbrain boundary, and hippocampus^{22,23}. Combining different cell types in a complex 3D configuration can better simulate brain biology and function. As such, cerebral organoids can reproduce the function and architecture of the brain, especially regarding development and neuronal plasticity. A comparison of gene expression programs of human fetal neocortex and *in vitro* cortical development by single-cell RNA sequencing found remarkable similarities²⁴. Cerebral organoids may well recapitulate environmental effects on human nervous system, particularly related to plasticity and growth^{24–26}, and circumvent problems of discrepancies in metabolic pathways occurring in translational studies involving animal models. The development of such a model offers an exciting new range of opportunities to investigate the molecular responses of human neural tissue to psychoactive substances.

Here we analyzed the effect of 5-MeO-DMT on human neural cells and cerebral organoids. By employing mass spectrometry-based proteomics to analyze cerebral organoids, we managed to investigate effects on a large scale and in an unbiased manner, and also gained insight into its molecular mechanisms and biochemical pathways²⁷. To the best of our knowledge, our results are the first to show that 5-MeO-DMT modulates proteins involved in long-term potentiation (LTP), in addition to morphogenesis and maturation of dendritic spines, while inhibiting neurodegeneration and cell death.

Results

Human neural progenitor cells are unaffected by 5-MeO-DMT. First, we examined the effects of 5-MeO-DMT on hNPCs (detailed characterization in²⁸). hNPCs showed basal expression of σ -1Rs but not 5-HT_{2A} or 5-HT_{2C} receptors (Fig. 1A,B). Using a high-content screening analysis, we tested the effects of 5-MeO-DMT (23 nM to 7.11 μ M) on hNPC death, proliferation, and differentiation. There was no evidence of change in cell death or proliferation in response to 5-MeO-DMT (Fig. 1C,D). In addition, by quantifying some aspects of dendritic branch complexity, we measured neural arborization based on MAP2 staining of young neurons exposed to 5-MeO-DMT compared to an unexposed control. Despite a slight trend, there were no statistically significant differences in the measured parameters (Fig. 1E–H, all p > 0.05).

Human cerebral organoids express 5-MeO-DMT receptors. The lack of alterations in cell death, proliferation, and differentiation/arborization in hNPCs exposed to 5-MeO-DMT could be due to the low cellular diversity and lack of complex interactions between different cell types. Thusly, we tested human cerebral organoids, which better replicate the complexity and function of *in vivo* neural circuitry. In 45 day-old cerebral organoids, basal immunostaining was observed in AMPA (selective glutamate receptor 2) and NMDA (ionotropic glutamate receptor) – both characteristic of glutamatergic synapses – along with the neuronal marker MAP2

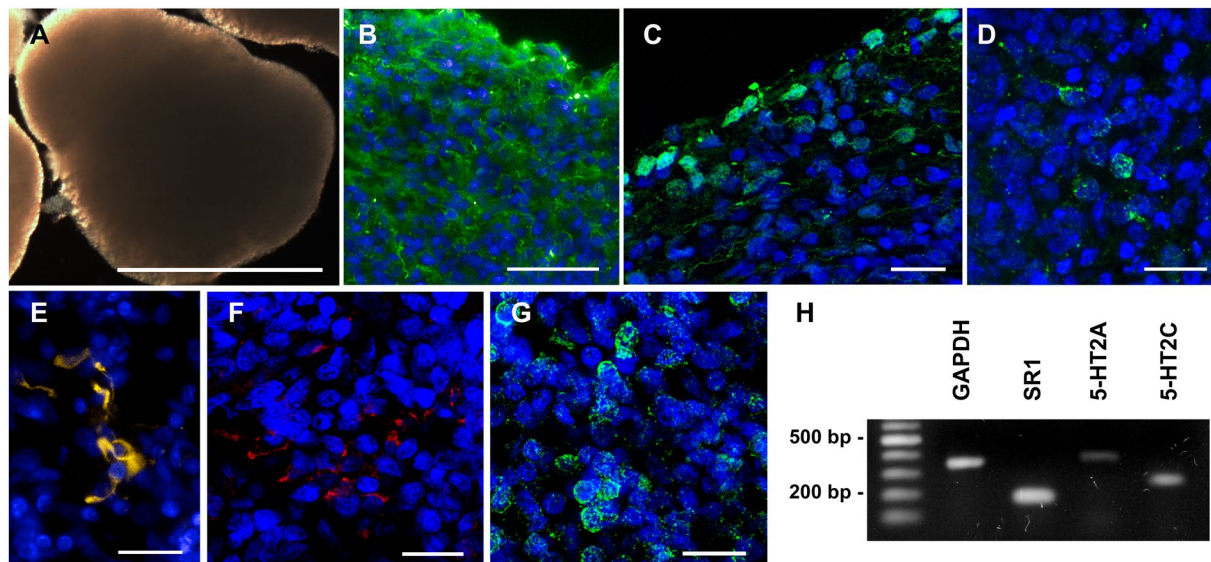


Figure 2. Cerebral organoids express 5-MeO-DMT receptors and different cell type markers. (A) Cerebral organoids presenting smooth texture and homogeneous coloring at 45 days of differentiation (scale bar 1000 μm). (B) Cerebral organoids are composed of several cell types, including mature neurons, as shown by MAP2 staining. (C) Cells expressing AMPAR1 are found at the organoid edge, while (D) cells expressing NMDAR1 and (E) GFAP are detected within the organoid. (F) Cells positive for 5-HT_{2A} receptor, and (G) σ -1R, the primary molecular targets for 5-MeO-DMT, are also found in the organoid. Scale bars: A = 1000 μm ; B = 50 μm ; C, D, E, F, and G = 20 μm . (H) The expression of molecular targets for 5-MeO-DMT was also confirmed by RT-PCR.

(Fig. 2A–D), as previously described²⁹. Glial cells (GFAP+) are also present in organoids, as shown in Fig. 2E. Interestingly, in contrast with hNPCs, we were able to detect the expression of the 5-HT_{2A} receptor via PCR and/or immunostaining, as well as of σ -1Rs, the primary pharmacological molecular targets for 5-MeO-DMT. As shown in Fig. 2F,G, cells expressing the 5-HT_{2A} receptor are present within the cerebral organoid, and the sigma-1 receptor was detected as well. RT-PCR confirmed the expression of 5-HT_{2A} and σ -1 receptors, and allowed the detection of serotonin 5-HT_{2C} receptors as well (Fig. 2H). Taken together, these data validate cerebral organoids as an appropriate platform to seek for the effects of 5-MeO-DMT in an amenable and realistic human neuronal network.

5-MeO-DMT alters the proteome of human cerebral organoids. Due to the complexity of the organoid system, we decided to cast a much wider net to detect potentially important 5-MeO-DMT effects. By analyzing the proteome of organoids with and without treatment, we were able to look for changes in the expression of a considerable number of proteins, in an unbiased approach. Thus, to resolve the proteome of human neural tissue under the effect of 5-MeO-DMT, we analyzed 45-day-old cerebral organoids after 24-hour treatment (Fig. 3A). A total of 144,700 peptides were identified at a false discovery rate (FDR) below 1%. These led to the identification of 6,728 unique proteins by, at least, two unique peptides present in no less than two out of three biological replicates analyzed. Notably, there was an overlap of 99% of identified proteins among all treatment groups (Fig. 3B), demonstrating the robustness of the method. From these commonly identified proteins, we found 934 differentially expressed (using a $-2 < \text{Log}_2 \text{ratio} > 2$ cut-off), comprising 360 downregulated and 574 upregulated proteins when comparing 5-MeO-DMT and vehicle groups (an overview of the proteomics results is presented in Supplementary Table 1). Functional enrichment for combined up- and downregulated proteins predicted the biological functions of those changes. Regarding diseases or functions, using prediction effect analysis ($-2 < z\text{-score} > 2.0$ is significant for inhibition/activation) (Fig. 3C), we observed a significant activation score for dendritic spine and cellular protrusion formation, microtubule and cytoskeletal organization, and also mild activation of T lymphocyte differentiation. On the other hand, biological functions such as neurodegeneration, cell death, and brain lesion were predicted to be inhibited.

5-MeO-DMT leads to inhibition of NF- κ B signaling pathway. Among the canonical pathways identified are nuclear factor of activated T-cells (NFAT) and nuclear factor kappa B (NF- κ B) signaling via toll-like receptor (TLR) and Gq-coupled receptors, which are all inhibited by 5-MeO-DMT treatment (Fig. 4). Interestingly, the direct targets of 5-MeO-DMT, receptors 5-HT_{2A} and 5-HT_{2C}, are Gq-coupled. Furthermore, NF- κ B is very well known to be the main transcriptional regulator of inflammatory, pro-inflammatory and anti-inflammatory cytokines and chemokines¹⁴.

Proteins associated with long-term potentiation are modulated by 5-MeO-DMT. We have also identified regulation of specific proteins that participate in LTP, one of the main properties of most excitatory synapses throughout the CNS³⁰. Proteins found upregulated are NMDAR, CaMK2 (Ca²⁺/calmodulin-dependent

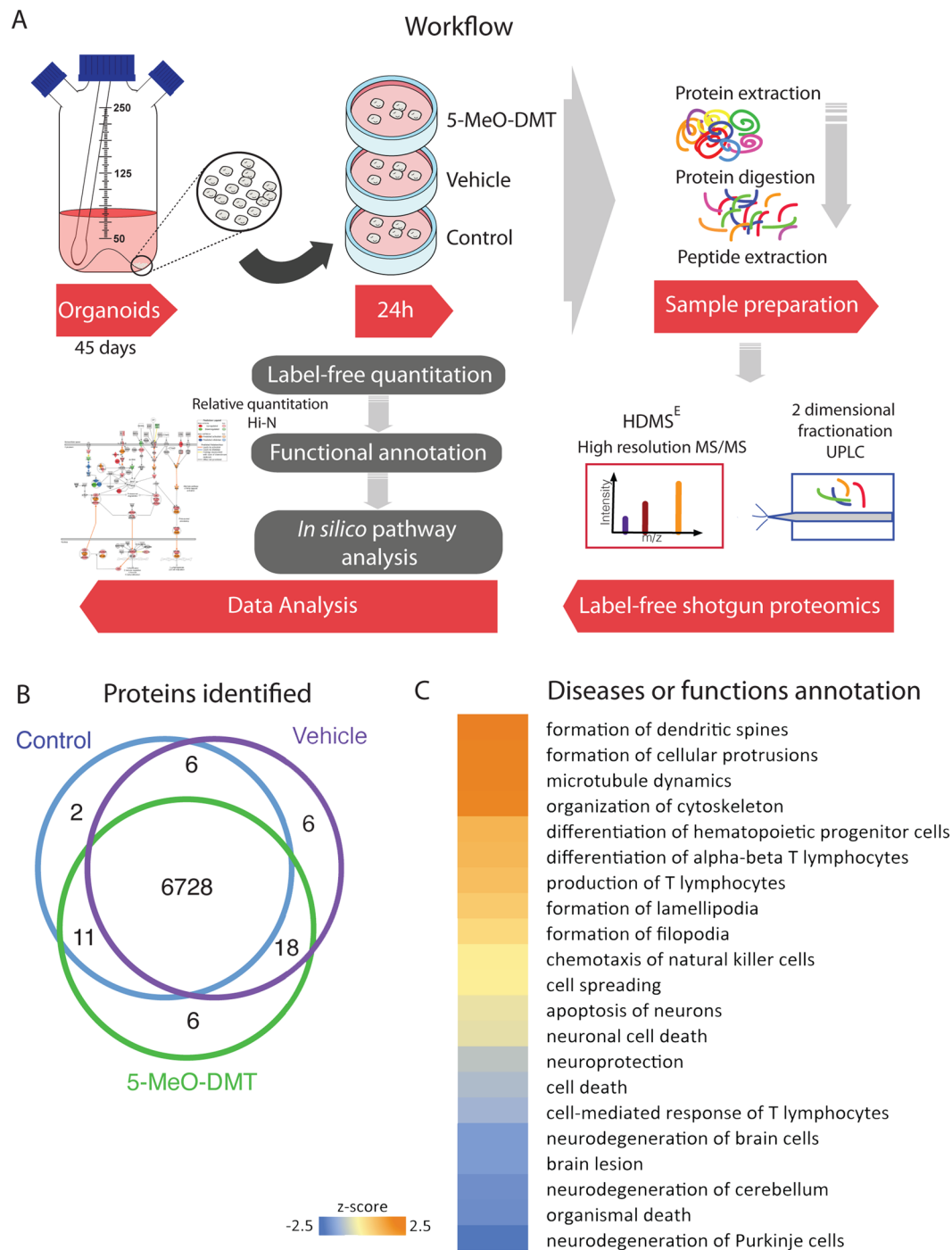


Figure 3. 5-MeO-DMT treatment effects on human cerebral organoid proteomics. **(A)** Experimental design workflow. 45-day-old cerebral organoids were treated with either 5-MeO-DMT, vehicle, or left untreated for 24 h. Samples were analyzed using label-free state-of-the-art quantitative proteomics, using two-dimensional fractionation and high-resolution mass spectrometry. Workflow art was modified from²⁹. **(B)** Venn diagram comparing the number of proteins identified by shotgun mass spectrometry in control human cerebral organoids, those treated with vehicle (EtOH), and 5-MeO-DMT. **(C)** Heat map showing significant functional enrichment between 5-MeO-DMT versus vehicle human cerebral organoids.

protein kinase), and CREB (cyclic AMP-responsive element-binding protein). The group of downregulated proteins included mGluR5 (metabotropic glutamate receptor 5), $G_{\alpha q}$ protein, protein kinase C (PKC), phospholipase c (PLC), calmodulin (CaM), AC1/8, inositol 1,4,5-trisphosphate receptor (IP3R), exchange factor directly activated by cAMP 1 (EPAC1) and PKA (protein kinase A). These changes in key components, and further regulation of several other proteins and secondary messengers suggest a complex regulation of this pathway. AMPAR, and the signaling cascade leading to c-Raf, mitogen-activated protein kinase kinase 1/2 (MEK1/2), and extracellular regulated kinase

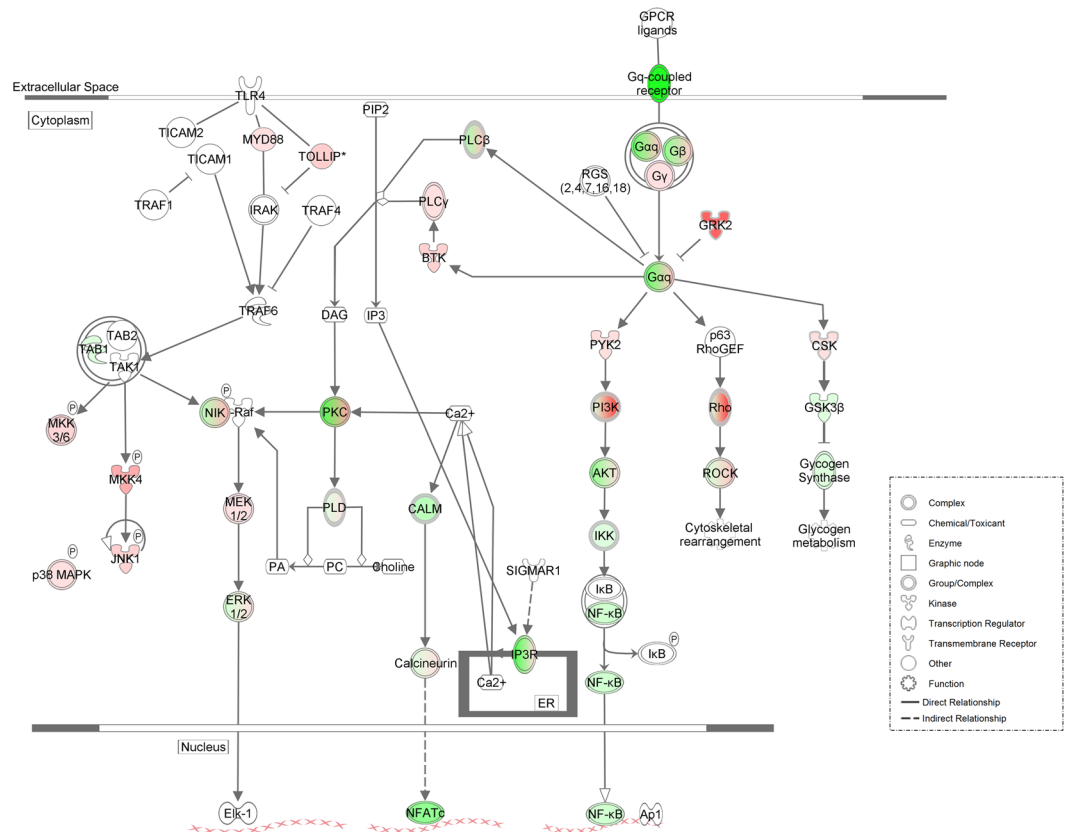


Figure 4. Schematic representation of the changes in protein expression of NFAT and NF- κ B pathways by 5-MeO-DMT. Canonical pathways showing upregulated (red) and downregulated proteins (green) after 5-MeO-DMT treatment.

1/2 (ERK1/2) are upregulated, suggesting pathway activation (Fig. 5). Based on the literature, activated ERK1/2 is transported to the nucleus and activates CREB, resulting in the expression of a large number of downstream genes³¹.

Cytoskeletal reorganization and dendritic spine morphogenesis proteins altered by 5-MeO-DMT.

Ephrin B was another canonical pathway upregulated, including both forward and reverse signaling, as shown by the analysis of differentially expressed proteins (see Fig. 6). Upregulation of ephrin-B2 causes activation of ephrin type-B receptor (EPHB) and, through intersectin, activates a cascade including CDC42 (cell division control protein 42 homolog), N-WASP (neural Wiskott-Aldrich syndrome protein), and ARP2/3 (actin-related protein 2/3). This upregulation additionally activates RAC1 (Ras-related C3 botulinum toxin substrate 1) through ELMO1. Together, these activated pathways trigger dynamic reorganization of the actin cytoskeleton and dendritic spine morphogenesis in forward signaling³². Meanwhile reverse signaling activates plexin, a protein that acts as a receptor for semaphorin through NCK adaptor protein 2 (GRB4) and focal adhesion kinase (FAK), causing axonal repulsion through paxillin (PXN).

Additionally, we found significant regulation of plexins, integrins, SLIT-ROBO Rho GTPase-activating protein (srGAP), Netrin receptor DCC, and metalloproteinase (Table 1) in 5-MeO-DMT-treated cerebral organoids, which corroborates actin regulation and orchestrates cytoskeletal reorganization.

Discussion

5-MeO-DMT is a structural analog of serotonin and melatonin and a functional analog of other psychedelic tryptamines such as N,N-DMT and 5-HO-DMT, a group of molecules about which little is known. The present results suggest that 5-MeO-DMT modulates the anti-inflammatory response, as well as the formation and maturation of new dendritic spines, via proteins implicated with cellular protrusion formation, microtubule dynamics, cytoskeletal reorganization, and LTP. These changes were observed in the organoids but not in monolayer cultures of neuronal cells, which suggests a more mature and complex 3D circuitry is necessary for the actions of 5-MeO-DMT.

Here we demonstrate anti-inflammatory effects of 5-MeO-DMT using human cerebral organoids. NFAT and NF- κ B signaling pathways were shown to be downregulated via Toll-like receptors (TLR) and Gq-coupled protein receptors, most probably 5-HT_{2A} and 5-HT_{2C}. Anti-inflammatory effects of 5-MeO-DMT were previously reported on human monocyte-derived dendritic cells, where inflammatory cytokine and chemokine release was shown to be blocked¹⁴. The immunomodulatory potential of other serotonergic psychedelics like lysergic acid diethylamide (LSD)^{33,34}, 3,4-methylenedioxy-methamphetamine (MDMA)^{35,36}, and

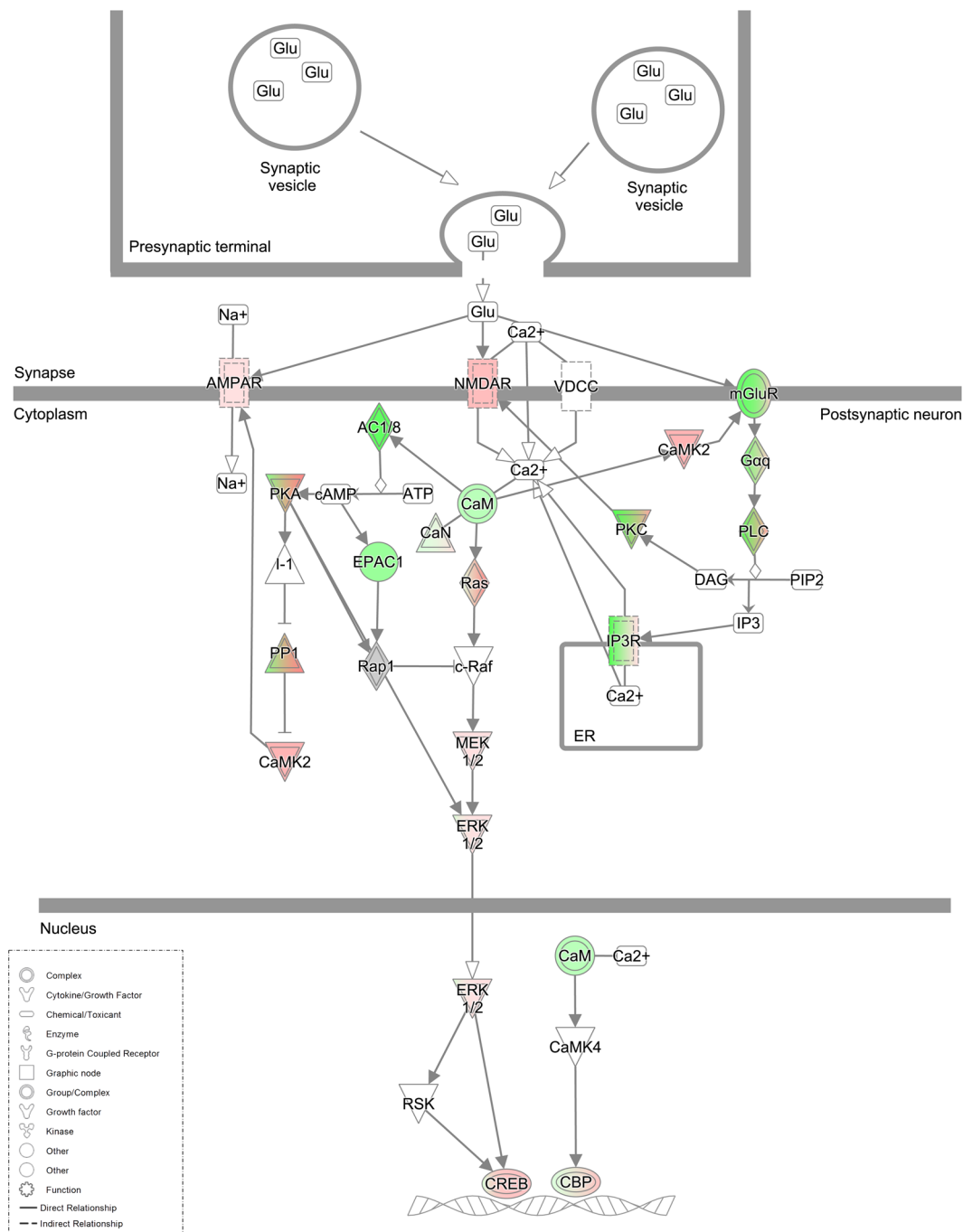


Figure 5. Schematic representation of long term potentiation modulation by 5-MeO-DMT treatment. Z-scores were calculated from an upstream shortest-path analysis and gave the probability that the interaction between the proteins and the common regulator is not occurring by chance. In red, upregulated proteins; in green, downregulated proteins after 5-MeO-DMT treatment. Glu, glutamate.

2,5-dimethoxy-4-iodoamphetamine (DOI)^{37,38} were also previously reported. It is hypothesized that there is cross-talk between TLR, serotonin receptors, and σ -1Rs³⁹.

Our work also suggests that a single, 24-hour-treatment with 5-MeO-DMT, i.e., a single dose, modulates specific signaling molecules identified as key players in LTP, a classic mechanism of learning and memory³⁰. Based on *in silico* predictions using proteomic data, modulation of these signaling molecules by 5-MeO-DMT would produce a complex regulation of LTP. One possibility is that LTP may be augmented in some cell types and inhibited in others, leading to a mixed profile, however; functional studies such as electrophysiology were not performed.

Additionally, we observed major downregulation of mGluR5 after treatment with 5-MeO-DMT. mGluR5 has a role in the rewarding effects for several drugs of abuse. It was shown that mice lacking the mGluR5 gene do not self-administer cocaine and show no cocaine-induced hyperactivity⁴⁰. They also have attenuated somatic signs

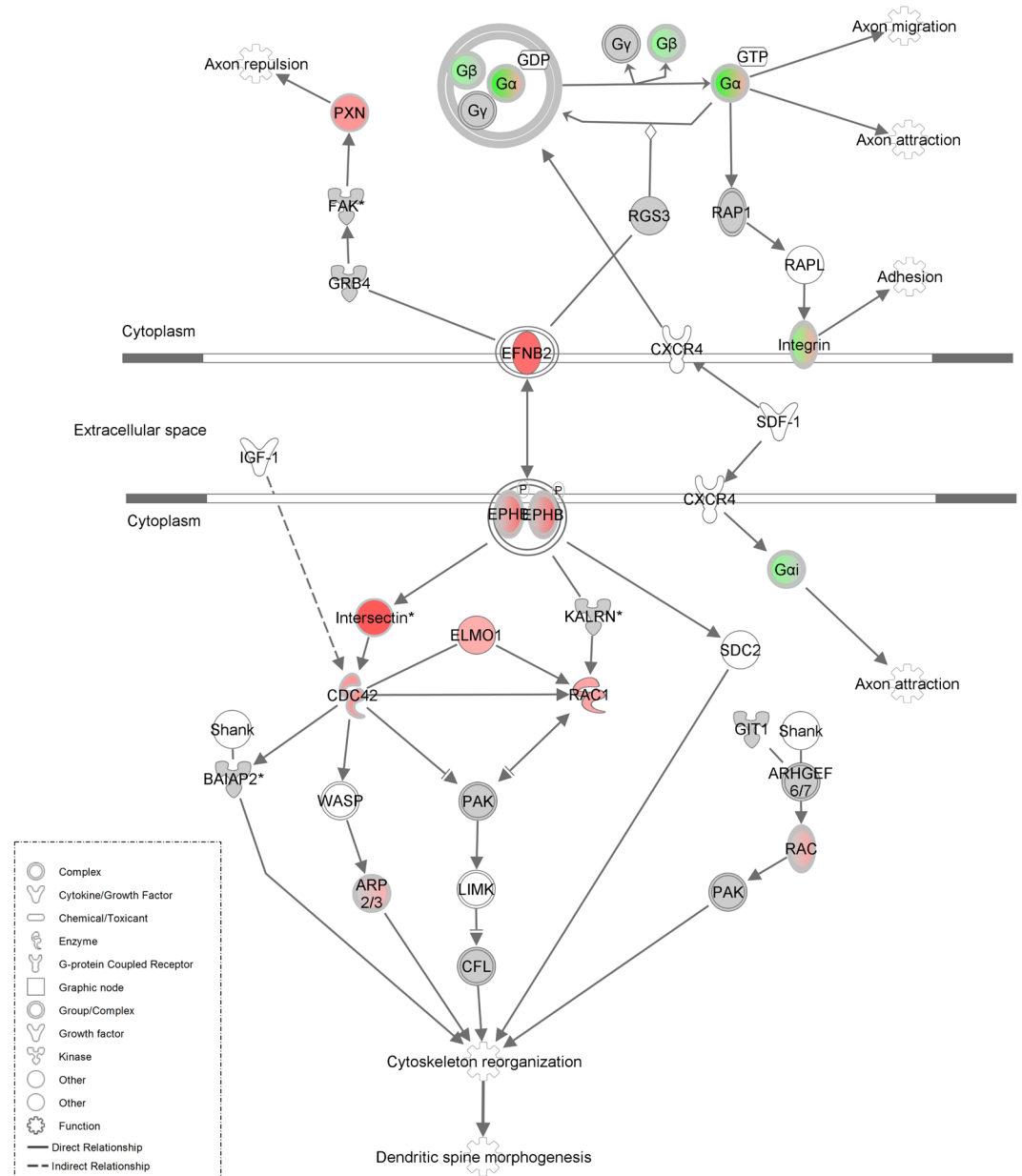


Figure 6. Pathway showing influence of 5-MeO-DMT on cytoskeletal reorganization and dendritic spine morphogenesis. Canonical pathway showing upregulated (red) and downregulated (green) proteins.

of nicotine withdrawal⁴¹, and reduced ethanol consumption behavior⁴², suggesting mGluR5 may be involved in addiction. The same effect for cocaine, nicotine, and ethanol in rats has been demonstrated with the use of mGluR5 receptor antagonists⁴¹. These effects of 5-MeO-DMT can possibly explain the therapeutic effect of dimethyl-tryptamines from *Ayahuasca* on substance dependence^{3,43–47}. Moreover, *Ayahuasca* seems to inhibit addictive behaviors in an animal model of alcohol dependence¹⁶. In humans, *Ayahuasca* administration to healthy subjects reduces rapid-eye-movement sleep (REM) and increases slow-wave sleep (SWS)⁴⁸, two major sleep stages respectively associated with increased and decreased LTP^{49–52}, while producing dream-like effects. Thus, the complex regulation of LTP detected here may also indicate that 5-MeO-DMT produces a mix of SWS and REM-like effects.

Changes in LTP are directly associated with increase in dendritic spine number and size; and conversely, changes in LTD are associated with a decrease in number and size⁵³. Spine morphogenesis relies on alterations of the actin cytoskeleton, but the molecular mechanisms that regulate this process are still not clear. 5-MeO-DMT caused significant upregulation of EFNB2, EPHB, and various secondary messengers involved in dendritic spine formation. Dendritic spine formation can be induced by direct stimulation of serotonergic receptors. Indeed the selective 5-HT_{2A} receptor agonist, DOI, has been shown to modulate spine morphology of mature cortical pyramidal neurons⁵⁴. In this study, a transient increase in spine size induced by DOI was kalirin-dependent, and it enhanced phosphorylation of PAK; whereas here we show upregulation of EFNB2, EPHB, intersectin, ELMO1, CDC42, and RAC1. Binding of ephrin-B to EphB receptors initiates bidirectional signaling, which by altering the

Accession Integrins	Gene Name	Peptide count	Description	Log ₂ Ratio
Q95965	ITGBL1	4 (2)	Integrin beta-like protein 1	3.69
P23229	ITGA6	21 (3)	Integrin alpha-6	3.18
P13612	ITGA4	10 (5)	Integrin alpha-4	2.57
P16144	ITGB4	42 (12)	Integrin beta-4	1.66
P11215	ITGAM	8 (3)	Integrin alpha-M	1.65
P20701	ITGAL	8 (3)	Integrin alpha-L	1.36
P05556	ITGB1	34 (15)	Integrin beta-1	1.26
P38570	ITGAE	16 (3)	Integrin alpha-E	1.10
P26006	ITGA3	22 (4)	Integrin alpha-3	0.69
Q9H0C8	ILKAP	13 (6)	Integrin-linked kinase-associated serine/threonine phosphatase 2 C	0.26
Q969R8	ITFG2	3 (2)	Integrin-alpha FG-GAP repeat-containing protein 2	-0.99
P20702	ITGAX	5 (1)	Integrin alpha-X	-1.53
P06756	ITGAV	16 (7)	Integrin alpha-V	-2.08
Q13683	ITGA7	10 (3)	Integrin alpha-7	-2.32
P17301	ITGA2	19 (2)	Integrin alpha-2	-2.64
Netrins and Plexins				
Q9ULL4	PLXNB3	19 (5)	Plexin-B3	0.71
O43157	PLXNB1	10 (2)	Plexin-B1	0.33
Q8NEU8	APPL2	37 (11)	DCC-interacting protein 13-beta	-0.30
Q9UKG1	APPL1	43 (21)	DCC-interacting protein 13-alpha	-0.41
O15031	PLXNB2	156 (45)	Plexin-B2	-2.14
P43146	DCC	50 (8)	Netrin receptor DCC	-4.51
Semaphorins				
Q13214	SEMA3B	14 (4)	Semaphorin-3B	2.11
Q13591	SEMA5A	16 (4)	Semaphorin-5A	-1.66
Q8NFY4	SEMA6D	8 (2)	Semaphorin-6D	-1.69
Q9NPR2	SEMA4B	15 (3)	Semaphorin-4B	-1.82

Table 1. Table of proteins showing regulation of integrins, netrins, plexins, and semaphorins by 5-MeO-DMT.

actin cytoskeleton, leads to changes in dendritic spine shape, size, and number⁵⁵. It was shown that EPHB2 interacts with intersectin and activates its GEF activity in cooperation with N-WASP, which in succession activates the Rho-family GTPase Cdc42 and spine morphogenesis⁵⁶. N-WASP is a critical regulator of Arp2/3-mediated actin polymerization⁵⁷. Henkemeyer and colleagues⁵⁸ demonstrated that triple EphB1,2,3-deficient hippocampal neurons have abnormal formation of actin clusters along dendrites, impairing normal dendritic spine formation *in vivo*. Meanwhile, *in vitro*, knockdown of EphB2 alone is sufficient to reduce synapse density⁵⁹. Postnatal re-expression of EphB2 in slice cultures from animals lacking EphB1–3 is sufficient to rescue dendritic spine defects⁵⁹. Although EphB signaling has a clear role in dendritic spine morphogenesis through kinase domain activity, it can also regulate activity-dependent synaptic plasticity interacting with both NMDA⁶⁰ and AMPA receptors⁵⁹. Literature shows that σ -1R also could contribute to the brain plasticity effects of 5-MeO-DMT. σ -1R is an endogenous regulator of dendritic spine morphology^{61,62} and neurite outgrowth⁶¹.

Typical psychological effects of psychedelics such as changes in perception and thought, renewed sensation of novelty, ineffability, and awe⁶³ may derive directly from the strong modulation of synaptic and cellular plasticity promoted by 5-MeO-DMT, and putatively compounds of other classical psychedelics. Given the pathways activated, the psychological effects of 5-MeO-DMT must also be tightly linked to the millisecond changes in sub-membrane calcium metabolism. To test these hypotheses, however, it would be necessary to go beyond *in vitro* studies to investigate in detail the links between the acute and chronic effects of 5-MeO-DMT.

Recently, organoid models have been used to study neural progenitor dysfunction and progenitor abnormalities resulting, for example, from Zika virus infections^{23,25}. The scientific community is still exploring the broader application of brain organoids. Beyond modeling early events of progenitor biology, brain organoids have the potential to model higher-order functions of the human brain, such as cellular interactions and neural circuit dysfunctions^{23,64}.

Apart from acting as a direct molecular mediator of plasticity, 5-MeO-DMT had effects on cell surface and extracellular proteins involved in regulating synaptic architecture, like plexins⁶⁵, DCC⁶⁶, metalloproteinase⁶⁷, and integrins^{68,69}. An upregulation of integrins, as we observed here in 5-MeO-DMT-treated organoids, was also found in major depressive disorder patients who responded well to antidepressants, suggesting the importance of this class of proteins in brain plasticity⁷⁰. One more protein significantly downregulated is srGAP, an intracellular

signaling molecule with a role in processes underlying synaptic plasticity, higher cognitive function, learning, and memory⁷¹.

Finally, we also found that pathways, associated with cell death, in brain organoids were inhibited by 5-MeO-DMT. These neurorestorative and cellular protective effects are expected upon activation of $\sigma 1R$ ^{15,72}. $\sigma 1R$ agonists exert neuroprotective effects by regulating intracellular calcium levels⁷³, preventing expression of pro-apoptotic genes⁷⁴, and protecting mRNA against anti-apoptotic genes such as Bcl-2.

Fast antidepressants also have strong effect on synaptic plasticity, reversing functional and structural synaptic deficits caused by stress. A typical example of this group is ketamine, a hallucinogenic, non-competitive NMDA glutamate receptor channel antagonist, which causes an improvement in mood ratings within hours, as opposed to weeks as seen in typical antidepressants⁷⁵. Ketamine increases mammalian target of rapamycin complex 1 (mTORC1) signaling, via activation of protein kinase B (PKB or Akt) and ERK. mTOR signaling then boosts synaptic protein synthesis and spine stability and function in the prefrontal cortex^{75–77}.

Whereas other dimethyltryptamines could have similar effects, our results do not support that this is common to actions of other dimethyltryptamines or non-psychoactive tryptamines, which should be examined independently. Taken together, our data offer insight about molecular changes induced by 5-MeO-DMT in human cerebral organoids. The proteomic profile, observed after exposure to 5-MeO-DMT, points to actions on synaptic plasticity and cell survival in human brain organoids.

Materials and Methods

Human embryonic stem cells. BR1 lineage of human embryonic stem cells (hESCs)⁷⁸ was cultured in mTeSR1 media (Stemcell Technologies) on Matrigel (BD Biosciences) - coated surface. The colonies were manually passaged every seven days and maintained at 37 °C in humidified air with 5% CO₂.

Human neural progenitor cells. To induce hESCs towards neural differentiation, we adapted Baharvand and coworkers' protocol^{28,79}. Briefly, 70% confluent human embryonic stem cells were differentiated to the neural lineage in defined adherent culture by retinoic acid and basic fibroblast growth factor (bFGF) within 18 days of culture. On the 18th day, neural tube-like structures were collected and plated on dishes coated with 10 μ g/mL of poly-L-ornithine and 2.5 μ g/mL of laminin (Thermo Fisher Scientific). The population of human neural progenitor cells (hNPCs) that migrated from neural tube-like structures was tested for the expression of neuronal markers and expanded. Expansion was done in N2B27 medium supplemented with 25 ng/mL bFGF and 20 ng/mL EGF (Thermo Fisher Scientific). N2B27 medium consisted of DMEM/F-12 supplemented with 1X N2, 1X B27, 1% penicillin/streptomycin (Thermo Fisher Scientific). Cells were incubated at 37 °C and 5% CO₂. Medium was replaced every other day. hNPCs were expanded for no more than 5 passages. Basic characterization of this culture was published in²⁸.

High Content Screening. Cell proliferation, cell death and arborization experiments were performed in a High Content Screening (HCS) format. hNPCs (1,500 cells per well) were plated on a 384-multiwell μ Clear plate (Greiner Bio-One, Kremsmünster, Austria) coated with 100 μ g/mL poly-L-ornithine and 10 μ g/mL laminin (Thermo Fisher Scientific). After 24h, cells were treated for 4 days in quintuplicate (five wells per condition) with 5-MeO-DMT (Sigma-Aldrich) in N2B27 medium supplemented with bFGF and EGF. Cells were labeled with 10 μ M EdU for 2h min prior to fixation and image acquisition. For the cell death assessment, cells were labeled with LIVE/DEAD[®] viability/cytotoxicity kit (Thermo Fisher Scientific). This kit contains two probes: calcein AM and ethidium homodimer (EthD-1). The former allows measuring of intracellular esterase activity and the latter, plasma membrane integrity. Mixing of probes was done in DMEM/F-12 (without phenol red, Life Technologies), together with the cell-permeant nuclear dye Hoechst. After incubation for 30 min at 37 °C and 5% CO₂, the dye cocktail was replaced by new medium and images were acquired. For arborization experiments, neural differentiation was induced 24h after plating by removal of bFGF and EGF from N2B27 medium. Treatment with 5-MeO-DMT was done concomitantly with neural differentiation. Medium was changed after 4 days of treatment and cells were allowed to differentiate for 3 more days. On day 7 cells were fixed for immunocytochemistry.

High Content Analysis. All images were acquired on Operetta high-content imaging system (Perkin Elmer, USA). For proliferation, incorporated EdU was detected with Alexa Fluor 488 using Click-iT EdU kit (C10351, Invitrogen, Carlsbad, USA) following the manufacturer's instructions. Total number of cells was calculated by nuclei stained with 1 mg/mL of DAPI (4',6-diamidino-2-phenylindole). S phase was determined by percentage of total cells labeled with EdU. Images were acquired with a 10x objective with high numerical aperture (NA).

Live cell imaging was performed with LIVE/DEAD[®] viability/cytotoxicity kit, using temperature and CO₂ control option (TCO) of Operetta, set to 37 °C and 5% CO₂ at 10x magnification. Quantification analyses were normalized to the number of cells in the well segmented by nucleus dyes.

Neuronal arborization was evaluated of fixed cells stained for MAP2 after 7 days of differentiation. The images were analyzed using the Neurite Outgrowth script of the Harmony software. Briefly, neurites were detected on MAP2 positive cells using the Find Neurite building block, which provides a dedicated algorithm for segmenting neurites. Morphological characteristics of neuronal arborization, such as total neurite length (sum of the length of all neurites attached to the cell), number of extremities, number of segments, and number of nodes (type I) were defined based on selected threshold parameters of the Find Neurite building block.

All analysis sequences were designated by combining segmentation steps with morphological- and fluorescence-based object characterizations using the image analysis software Harmony 3.5.1 (Perkin Elmer, Waltham, MA, USA).

Differentiation into cerebral organoids. Differentiation of hESCs into cerebral organoids was based on a previously described protocol^{26,29}. Briefly, hESC cells were inoculated into a spinner flask, and to enable embryoid body formation, after six days the medium was changed to neural induction media (DMEM/F12, 1X N2 supplement (Gibco), 2 mM Glutamax (Invitrogen), 1% MEM-NEAA, and 1 µg/mL heparin (Sigma)) and the aggregates were cultured for five more days. After being embedded in matrigel, differentiation media composed of 1:1 DMEM/F12:Neurobasal (Gibco), 0.5X N2, 1X B27 minus vitamin A (Gibco), 2 mM Glutamax, 0.5% MEM-NEAA, 0.2 µM 2-mercaptoethanol and 2.5 µg/mL insulin was used. After 4 days, cell aggregates were grown in neuronal differentiation media, composed as aforementioned except by replacing with 1X B27 containing vitamin A (Gibco). The medium was changed once per week. Cerebral organoids were grown for 45 days (30 days in neuronal differentiation media).

RNA isolation and PCR analysis. Total RNA was isolated using the GeneJET RNA Purification Kit (Thermo Scientific) and digested with DNase using DNase I (Invitrogen), following the manufacturer's instructions. Complementary DNA was generated from 1 µg total RNA using M-MLV Reverse Transcriptase (Invitrogen), according to the manufacturer's recommendations. PCR was performed using the following primer sequences: GFAP-For: 5'-TTC GAC AGT CAG CCG CAT C-3' GFAP-Rev: 5'-GAC TCC ACG ACG TAC TCA GC-3', Sigma receptor 1-For: 5'-AGT AGG ACC ATG CAC TCA CAC C-3', Sigma receptor 1-Rev: 5'-CCC CAT CCT TAA CTC TAG AAC C-3', 5-HT_{2A}-For: 5'-TTG GGC TAC AGG ACG ATT-3', 5-HT_{2A}-Rev: 5'-GAA GAA AGG GCA CCA CAT C-3', 5-HT_{2C}-For: 5'-TGT CCC TAG CCA TTG CTG ATA TGC-3', 5-HT_{2C}-Rev: 5'-GCA ATC TTC ATG ATG GCC TTA GTC-3'. Each PCR reaction was carried out for 40 cycles in a reaction mixture containing 0.25 U Taq DNA Polymerase (Invitrogen), 1x Taq DNA Polymerase Buffer containing 1.5 mM MgCl₂ (Invitrogen), 200 nM of each primer (forward and reverse), 200 µM dNTP mixture containing the four deoxyribonucleotides (dATP, dCTP, dTTP, dGTP), and 15 ng of cDNA.

Immunohistochemistry. On the 45th day of differentiation, cerebral organoids were fixed in 4% paraformaldehyde, incubated with sucrose solutions (10, 20, and 30%) in phosphate buffered saline (PBS), embedded in optimal cutting temperature compound (OCT), and frozen in liquid nitrogen. The organoids were sectioned with a cryostat into 20 µm thick sections. Immunofluorescence was performed using the primary antibodies: anti-MAP2 (M1406, Sigma-Aldrich), anti-AMPA1 (Abcam, ab86141), anti-NMDAR1 (Abcam, ab28669), anti-sigma receptor 1 (sc-137075, Santa Cruz), and anti-5-HT_{2A} receptor (RA24288, Neuromics). Secondary antibodies used were as follows: Alexa Fluor 488 goat anti-mouse (A11001, Invitrogen) and Alexa Fluor 594 goat anti-mouse (A-11008, Invitrogen). DAPI was used for nucleus staining. Images were acquired using an Operetta Imaging System (Perkin Elmer) and a Leica TCS SP8 confocal microscope, when specified.

Treatment of cerebral organoids with 5-MeO-DMT. On day 45 of differentiation, four to five organoids per group were transferred from the spinner flask to a non-adherent dish and treated with either 13 µM 5-MeO-DMT (Sigma-Aldrich), 0.3% ethanol (vehicle) or only medium (control), for 24 hours. After treatment, cerebral organoids were pelleted and homogenized in buffer containing 7 M Urea, 2 M thiourea, 4% CHAPS, 70 mM DTT, and Complete Protease Inhibitor Cocktail (Roche)⁸⁰. The homogenates were kept on ice for about 20 min and frozen at -80 °C until sample processing for mass spectrometry-based label-free shotgun proteomics. The experiment was repeated three times with the three different derivations of cerebral organoids.

Sample preparation. Sample lysates were thawed and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was collected and total protein was quantified by Qubit[®] 3.0 Fluorometer (Thermo Fisher Scientific). Each sample (50 µg) was subjected to a SDS-PAGE gel electrophoresis. Gel lanes were sliced and digested *in gel* overnight as previously described⁸⁰. Generated peptides were dried in a SpeedVac concentrator and stored at -80 °C prior to shotgun mass spectrometry analyses.

Liquid chromatography-mass spectrometry. Qualitative and quantitative proteomic analyses were performed on a 2D-LC-MS/MS system with ion-mobility-enhanced, data-independent acquisitions⁸¹. Peptides were injected for two-dimensional, reverse-phase liquid chromatography using an Acquity UPLC M-Class System (Waters Corporation, Milford, MA) coupled to a Synapt G2-Si mass spectrometer (Waters Corporation, Milford, MA).

In first-dimension chromatography, peptides (5 µg) were loaded into a M-Class BEH C18 Column (130 Å, 5 µm, 300 µm × 50 mm, Waters Corporation, Milford, MA). Fractionation was performed using discontinuous steps of acetonitrile (11%, 14%, 17%, 20%, and 50%). After each step, peptide loads continued to second-dimension separation, in a nanoACQUITY UPLC HSS T3 Column (100 Å, 1.8 µm, 75 µm × 150 mm, Waters Corporation, Milford, MA). Peptide elution was achieved using an acetonitrile gradient from 7% to 40% (v/v) for 54 min at a flow rate of 0.4 µL/min directly into a Synapt G2-Si. The mass spectrometer acquired in data-independent acquisition mode (DIA) with ion-mobility separation. This approach, called high-definition data-independent mass spectrometry (HDMS[®]), significantly enhances the proteome coverage⁸². MS/MS analyses were performed by nano-electrospray ionization in positive ion mode, nanoESI (+), and used a NanoLock Spray (Waters, Manchester, UK) ionization source. The lock mass channel was sampled every 30 s. The mass spectrometer was calibrated with an MS/MS spectrum of a [Glu1]-Fibrinopeptide B human (Glu-Fib) solution that was delivered through the reference sprayer of the NanoLock Spray source. Samples were all run in technical and biological triplicates, for a total of 9 replicates per sample.

Database search and quantification. Raw data was processed with Progenesis[®] QI version 2.1 (Waters) and proteins were identified. Quantitative data was processed using dedicated algorithms and searched against

the Uniprot human proteomics database (version 2015/09), with the default parameters for ion accounting and quantitation⁸³. The databases used were reversed “on the fly” during the database queries and appended to the original database to assess the false-positive identification rate. The following parameters were considered in identifying peptides: 1) Digestion by trypsin with at most one missed cleavage; 2) variable modifications by oxidation (M) and fixed modification by carbamidomethyl (C); and 3) false discovery rate (FDR) less than 1%. Identifications that did not satisfy these criteria were not considered.

In silico analysis. Protein networks and canonical pathways associated with differentially expressed proteins were identified using Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Qiagen, Redwood, CA, USA; www.ingenuity.com). This software uses curated connectivity information from literature to determine interaction networks among the differentially expressed proteins and canonical pathways in which they are involved. Here, we have considered information from nervous system tissues and cells, immune cells, and stem cells. The significant biological functions were based on Fisher’s exact test. Multiple correlation hypotheses were based on Benjamini-Hochberg (B-H) approach using a 1% FDR threshold; the significance of the IPA test was expressed as p-values.

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Author Contributions

V.D., J.M.N., S.K.R. designed the experiments; V.D., J.M.N., R.M.M., R.S. performed the experiments; D.M.S. supervised analysis and interpretation of the proteomics data; V.D., J.M.N., and R.M.M. analyzed the data; V.D., J.M.N., S.K.R. wrote the paper; V.D., J.M.N., R.M.M., R.S. prepared figures; V.D., J.M.N., R.M.M., R.S., D.A., S.R., D.M.S., S.K.R. reviewed drafts of the paper.

Additional Information

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