

**Title: Harmine stimulates neurogenesis of human neural cells *in vitro***

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**Abstract**

Harmine is a  $\beta$ -carboline alkaloid present at highest concentration in the psychotropic plant decoction Ayahuasca. In rodents, classical antidepressants reverse the symptoms of depression by stimulating neuronal proliferation. It has been shown that Ayahuasca presents antidepressant effects in patients with depressive disorder. In the present study, we investigated the effects of harmine in cell cultures containing human neural progenitor cells (hNPCs, 97% nestin-positive) derived from pluripotent stem cells. After 4 days of treatment, the pool of proliferating hNPCs increased by 57%. Harmine has been reported as a potent inhibitor of the dual specificity tyrosine-phosphorylation-regulated kinase (DYRK1A), which regulates cell proliferation and brain development. We tested the effect of analogs of harmine, an inhibitor of DYRK1A (INDY) and an irreversible selective inhibitor of monoamine oxidase (MAO) but not DYRK1A (pargyline). INDY but not pargyline induced proliferation of hNPCs similarly to harmine, suggesting that inhibition of Dyrk1a is a possible mechanism to explain harmine effects upon the proliferation of hNPCs. Harmine also increased dendritic arborization, including total neurite length, number of segments, extremities and nodes in MAP2 positive neurons. Our findings show that harmine enhances neurogenesis of hNPCs *in vitro*, and suggest a biological activity associated with its antidepressant effects *in vivo*.

## Introduction

Throughout life, specific regions in the human adult brain continuously generate neural cells from a pool of neural progenitor cells (hNPCs). Many physiological and pathological events are able to control neurogenesis by modulating proliferation, differentiation, maturation and integration of newborn neurons into the existing circuitry <sup>1</sup>. This balance can be disrupted by chronic stress <sup>2</sup>, depression <sup>3</sup>, aging <sup>4</sup>, and neurodegenerative diseases <sup>5</sup>.

There are reports showing that classical antidepressants can reverse or block stress-induced hippocampal atrophy in rodents mostly by stimulating neuronal proliferation <sup>6</sup>. Fluoxetine, one of the most used selective serotonin reuptake inhibitors, induces proliferation of rat hypothalamic neural progenitors *in vitro* <sup>7,8</sup>. Unfortunately, treatment with classic antidepressants leads to full remission in only 50% of patients <sup>9</sup>, causes numerous side effects and the time required for achieving therapeutic response is usually measured in weeks. Thus, the demand for novel psychopharmacological agents able to reestablish neurogenesis remains significant.

Beta-carbolines, a large group of indole alkaloids, are widely distributed in plants. Two members of this group, harmine and harmaline, have been found in human plasma after ingestion of Ayahuasca <sup>10</sup>, a psychotropic beverage traditionally used in the Amazonian region of South America as part of local religious ceremonies.

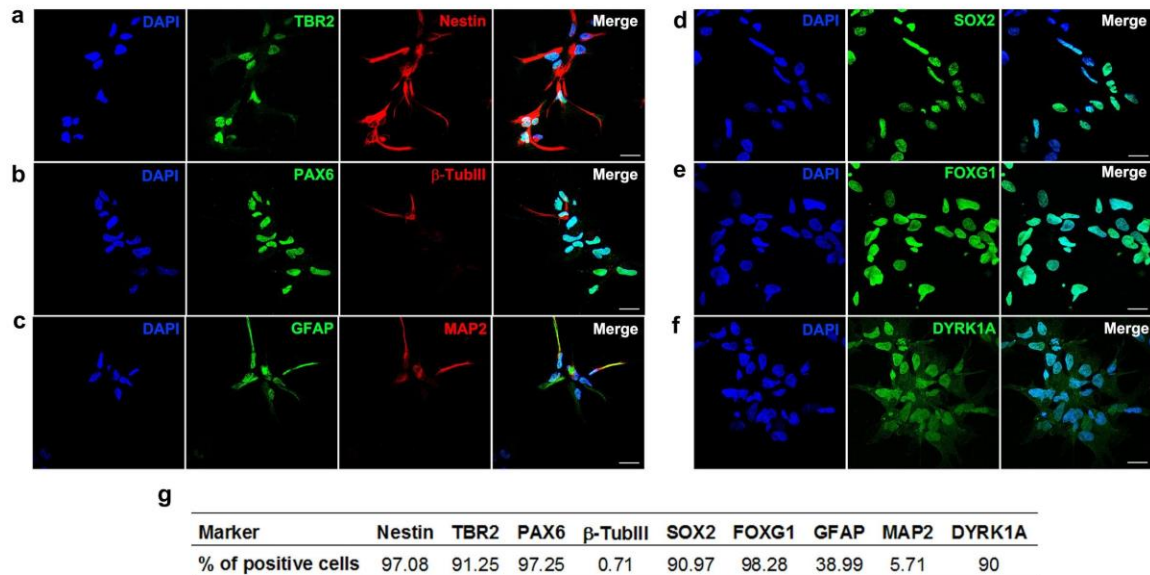
Evaluation of the effects of a single dose of Ayahuasca in six volunteers with a current depressive episode suggested that this plant decoction has fast-acting anxiolytic and antidepressant effects <sup>11</sup>. Moreover, the use of harmine in rodents leads to the reduction of symptoms associated with depression <sup>12</sup> and re-establishment of normal levels of hippocampal brain-derived neurotrophic factor (BDNF) <sup>13</sup>.

Apart of these initial studies, there are no data available regarding the effects of

harmine in human models of neurogenesis. Here we examine the effects of harmine on proliferation, differentiation and neuronal arborization of neural cells derived from human pluripotent stem cells *in vitro*. We show that harmine increased the pool of neural progenitor cells and the complexity of processes of newborn neurons.

## Results

We generated hNPCs from human embryonic stem cells<sup>14</sup> using a protocol that recapitulates the early steps of nervous system development<sup>15</sup>. More than 90% of the hNPCs express characteristic neural progenitors markers like SOX2 (sex determining region Y-box 2), nestin (intermediate filament protein; neuroectodermal stem cell marker), PAX6 (paired box 6), FOXG1 (forkhead box G1; transcriptional repressor important for development of the brain and telencephalon), TBR2 (transcription factor Eomes; key regulator of neurogenesis in the subventricular zone) (Fig. 1a, b, d, e, g). Additionally, differentiated cells expressing  $\beta$ -Tubulin III (a class III member of the  $\beta$  tubulin protein family primarily expressed in neurons), GFAP (glial fibrillary acidic protein, marker of astrocytes), or MAP2 (marker of neuronal soma and dendrites) are also present (Fig. 1b, c, g). Ninety percent of cells express the dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) (Fig. 1f, g).

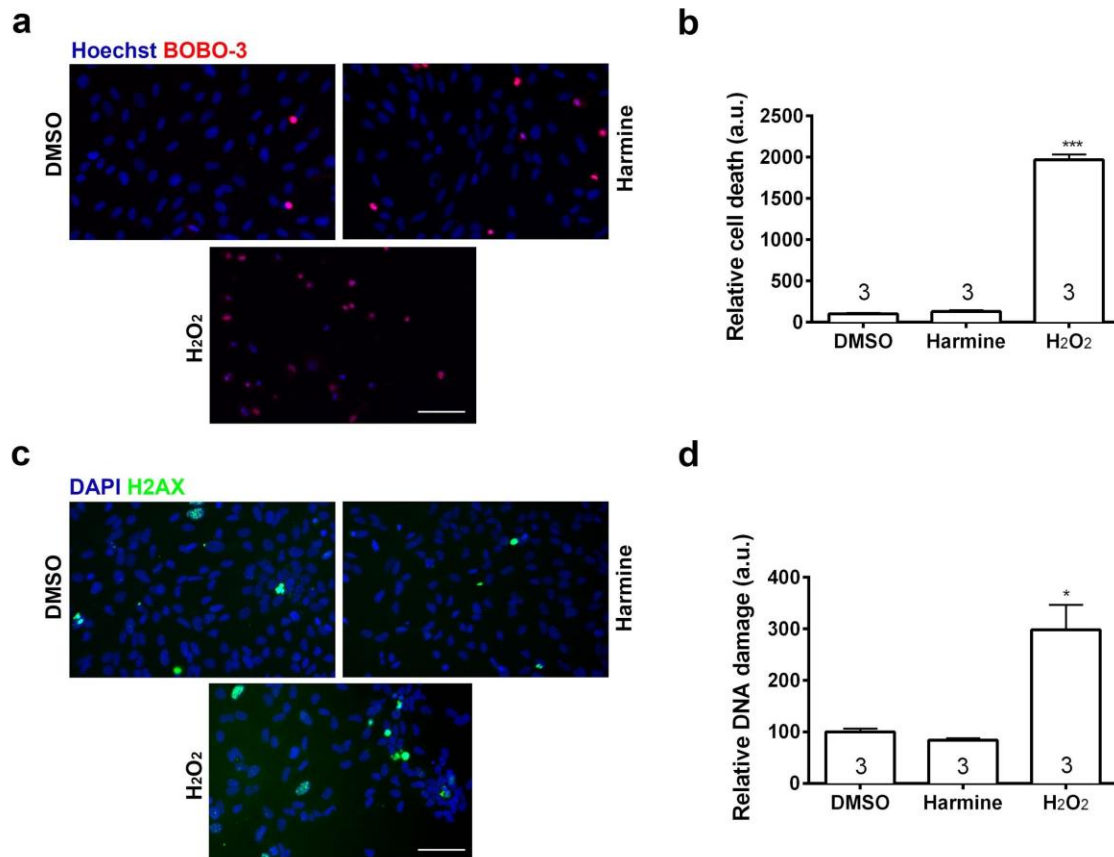


**Figure 1: Characterization of neural progenitor cells derived from human embryonic stem cells.** Representative images showing hNPCs stained for (a) TBR22 and Nestin, (b) PAX6 and  $\beta$ -Tubulin III, (c) GFAP and MAP2, (d) SOX2, (e) FOXG1, (f) DYRK1A. (g) Quantification of cell markers. A minimum of 10,000 hNPCs was counted per marker. Scale bar: 25  $\mu$ m.

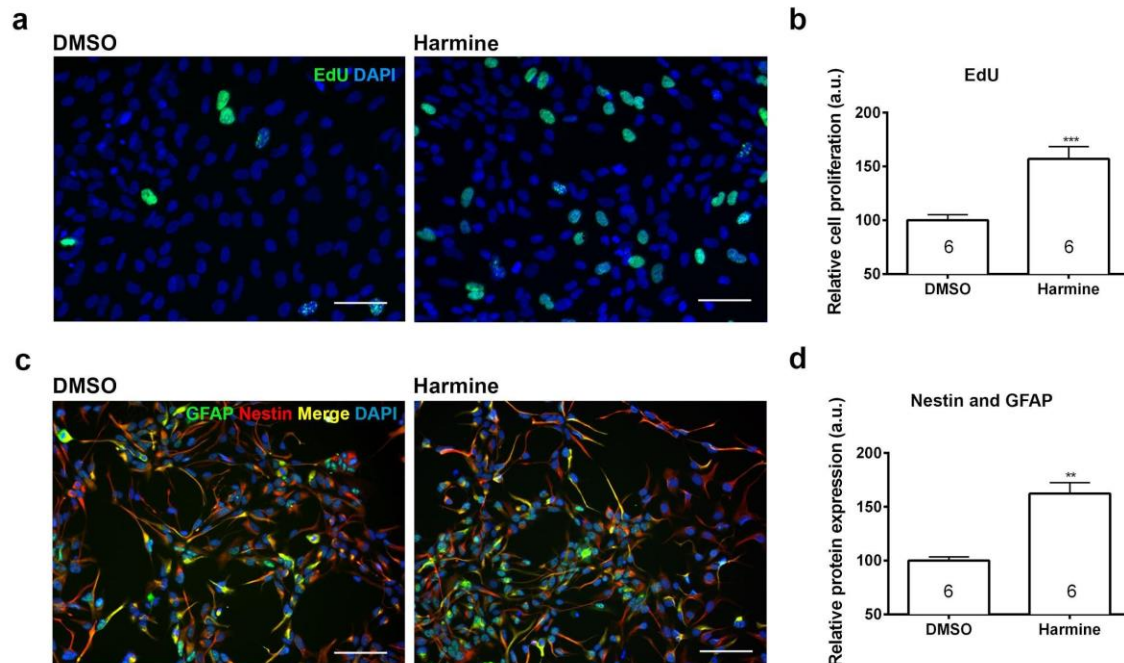
### Harmin increases proliferation of human neural progenitors: a possible role of DYRK1

In order to examine the effects of harmine upon neurogenesis, human neural cells were incubated for 96h with harmine ranging from 0.1 to 22.5  $\mu$ M (Supplementary Figs. 1 and 2). The 7.5  $\mu$ M dose of harmine was the most effective on proliferation of hNPCs (Supplementary Fig. 2), increasing the pool of proliferating cells by 57% (Fig. 3a, b). No evidence of cell death or DNA damage in response to harmine as measured by BOBO-3 (Fig. 2a, b) and H2AX (Fig. 2c, d) labelling was observed.

Treatment with harmine increased by 64.4% the pool of neural progenitors, which actively participates in adult neurogenesis (Nestin and GFAP labelled, Fig 3c, d)<sup>16</sup>. The number of cells positive for SOX2, MAP2 and FOXG1 was not altered (Supplementary Fig. 3).



**Figure 2: Percentage of cell death and DNA damage in neural cells treated with harmine.** (a) Representative immunostaining images of BOBO-3 (red) positive cells. Nuclei are stained with Hoechst (blue). (b) Cell death relative to DMSO control, measured as the percentage of cells stained with BOBO-3. (c) Representative images of cells positive for H2AX (green). Nuclei are stained with DAPI (blue). (d) Quantification of H2AX positive cells relative to DMSO control, as percentage of cells stained with H2AX. H<sub>2</sub>O<sub>2</sub> was used as positive control for both cell death and DNA damage. A minimum of 10,000 hNPCs was counted per condition/per experiment. Data was analysed by one-way ANOVA with Tukey's multiple comparison test, \*  $p < 0.05$ , \*\*\*  $< 0.0001$ . Values represent mean  $\pm$  s.e.m. The number inside the bar represents the number of experiments in each group. Scale bar: 100  $\mu$ m.

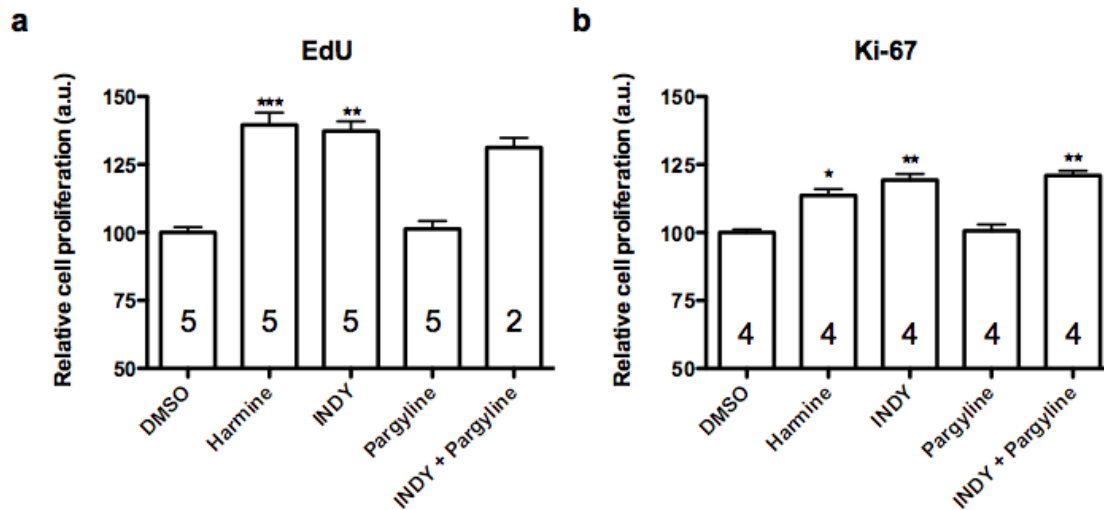


**Figure 3: Quantification of hNPCs proliferation and differentiation markers after harmine exposure.** (a) Representative images of EdU staining. (b) Cell proliferation (S phase) relative to DMSO control, measured by EdU incorporation. (c) Representative images of GFAP and Nestin staining. (d) Expression of Nestin and GFAP proteins relative to DMSO control. A minimum of 10,000 hNPCs was counted per condition/per experiment. Data was analysed by one-way ANOVA with Tukey's multiple comparison test, \*\*  $p < 0.001$ , \*\*\*  $< 0.0001$ . Values represent mean  $\pm$  s.e.m. The number inside the bar represents the number of experiments in each group. Scale bar: 100  $\mu$ m.

Harmine has been shown to be an inhibitor of DYRK1A<sup>17,18</sup>, other DYRK family members<sup>18</sup> and monoamine oxidase (MAO)<sup>19</sup>. To verify whether these two targets are involved in the harmine-dependent increase of neural proliferation, we examined the effect of two functional analogs of harmine in human neural cells: INDY, an inhibitor of DYRK1A; and pargyline, an irreversible selective inhibitor of MAO but not DYRK1A.

Harmine and INDY increased proliferation of hNPCs in comparison with control, analysed by two different methods, while pargyline did not induce the same effect. Concomitant inhibition of both DYRK1A and MAOs produced similar effect

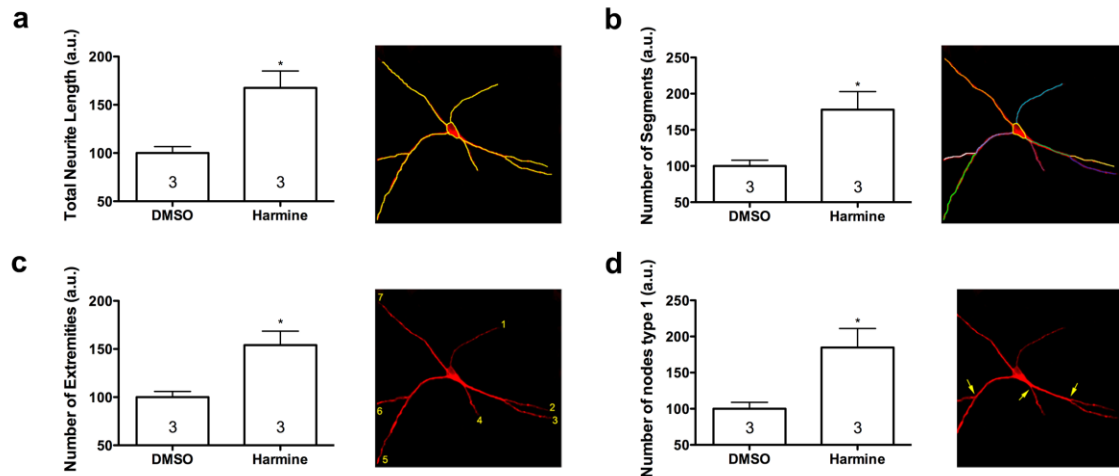
as harmine or INDY alone (Fig. 4).



**Figure 4: Quantification of EdU and Ki-67 labelling in human neural progenitor cells in response to harmine and its functional analogs.** (a) Cell proliferation relative to DMSO control, measured by EdU incorporation. (b) Cell proliferation relative to DMSO control, measured by Ki-67. Values represent mean  $\pm$  s.e.m. A minimum of 10,000 hNPCs was counted per condition/per experiment. Data was analysed by one-way ANOVA with Tukey's multiple comparison test, \*  $p < 0.05$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.0001$ . Values represent mean  $\pm$  s.e.m. The number inside the bar represents the number of experiments in each group.

### Harmine increases neuronal arborization

We also analysed neurite formation in neurons exposed to harmine (see Supplementary Figure 1). After neural induction, the percentage of MAP2 positive cells increases to approximately 48% (data not shown). Harmine increased the dendritic branch complexity (Fig. 5), total neurite length (by 71.9%), number of segments (by 78%), number of extremities (by 54%) and number of nodes type I (by 84.8%). These findings indicate that the ability to initiate branch points and extend dendrites is altered by harmine in neural cells.



**Figure 5: The effects of harmine on neuronal arborization.** (a) Quantification of total neurite length (sum of the length of all neurites attached to the cell) relative to DMSO control. (b) Quantification of number of segments relative to DMSO control. (c) Quantification of number of extremities relative to DMSO control. (d) Quantification of number of nodes type 1 relative to DMSO control. Values represent mean  $\pm$  s.e.m. A minimum of 10,000 hNPCs was counted per condition/per experiment. Data was analysed by one-way ANOVA with Tukey's multiple comparison test, \*  $p < 0.05$ . The number inside the bar represents the number of experiments in each group. The image on the right of each graph illustrates the parameter quantified.

## Discussion

The use of *in vitro* models can potentially clarify mechanisms related to proliferation and differentiation as well as arborization of newly differentiated neurons<sup>20</sup>, processes that happen massively during embryogenesis but also later in specific brain regions. Numerous studies have shown a close relationship between disturbed adult neurogenesis and depression, highlighting the need for more efficacious and faster-acting treatments. Thus, the aim of this study was to investigate the effects of harmine, a compound with potential antidepressant properties, on human neural cells derived from pluripotent stem cells. Here we show that harmine increases proliferation of hNPCs and the dendritic arborization of young neurons *in vitro*.



In our cultures, with more than 90% of hNPCs, we described that treatment with harmine increased proliferation without DNA damage or cell death. These results are consistent with the findings published by Hämmerle and others <sup>21</sup>, where harmine exposure to chick embryos resulted in a strong increase in BrdU incorporation and number of mitotic cells in the spinal cord.

Interestingly, harmine treatment also increased the number of early progenitors cells, stained with both GFAP and Nestin. At first, this result suggests that harmine drives the differentiation of hNPCs into radial glial cells, which are the major source of neuronal and glial progenitors in the developing brain <sup>22</sup>. Similarly, in the adult hippocampus of rodents, these neural precursors are responsible for late neurogenesis and gliogenesis <sup>1</sup>. In this context, levels of GFAP tend to increase on cells that shifted to a glial fate. On the other hand, despite being GFAP+ cells, at this developmental stage, these progenitors can also shift to a phenotype PSA-NCAM+, which can give rise to neurons <sup>16</sup>. In the light of these evidences, we suggest that harmine could potentiate proliferation of radial glia-like cells (GFAP+/Nestin+) derived from hNPCs, which are capable to generate both neurons and astrocytes. Further experiments should be done in order to clarify the terminal fate of harmine-treated hNPCs.

Harmine has been described as an inhibitor of MAO and DYRK1A <sup>18,19</sup>. MAO inhibition increases serotonergic neurotransmission in the adult brain, which is a key component of the classical antidepressant action. The main outcome of such treatment is upregulation of cell proliferation and neurogenesis in the hippocampus <sup>23,24</sup>. Surprisingly, we did not detect changes in proliferation induced by pargyline, a specific MAO inhibitor. On the other hand, treatment with INDY, an inhibitor of DYRK1, induced proliferation similarly to the observed with harmine. These results are consistent with the findings of Wang and collaborators, where harmine-dependent DYRK1A inhibition increases the proliferation of pancreatic beta cells <sup>25</sup>. Since both harmine and INDY have multiple targets on DYRK family, including regulators of cell cycle <sup>17</sup>, we should consider other potential players of INDY- and harmine-mediated increase in

proliferation. DYRK1A closely related kinases; INDY can also inhibit DYRK1B and DYRK2. In cancer studies DYRK1B and DYRK2 have been described as modulators of proliferation<sup>25,26</sup>. In fact, harmine also inhibits DYRK1B and DYRK2 but the efficiency of this inhibition is, respectively, 5- and 50-fold lower in comparison to DYRK1A<sup>18,27,28</sup>. Thus, DYRK1A emerges as the major candidate on mediating the increase in proliferation seen in this study. Further studies are needed to reveal other aspects of cell proliferation stimulated by harmine.

Harmine also increased total neurite length, number of segments, number of extremities and number of nodes type I in neurons. In cultures of mouse embryonic hippocampal neurons, harmine reduced DYRK1A activity and, as a consequence, altered neuritogenesis<sup>18</sup>. However, the effect was different depending on the stage of differentiation. They showed that harmine increased the percentage of neurons with 2 or 3 neurites but decreased the percentage of neurons with 4 neurites, shifting the median number of neurites from four to three. Harmine had no effect in neurons that already had developed 5 or 6 neurites.

Since DYRK1A is located in the growing dendritic tree of mouse neurons<sup>29</sup>, it may have a role in the regulation of dendritic growth. In the present study, we showed increased arborization in young neurons differentiated from hNPCs when treated with harmine. Different results could be explained by transient (dynamic) expression of DYRK1 in neural progenitor cells before the onset of neurogenesis in vertebrate embryos<sup>30</sup>. The same authors have shown that expression of Dyrk1a changes during the transition from neural proliferation to neuronal differentiation<sup>31</sup>. This indicates that inhibition of DYRK1 may have distinct, stage-dependent functional role depending on the moment of development of the nervous system.

Taken together our results suggest that harmine may exert neurogenic effects in human neural cells *in vitro*, not only by stimulating proliferation but also by increasing arborization of early differentiated neurons. These results indicate that

harmine, as well as other beta-carbolines, should be considered as modulators of depression.

## Methods

### Chemicals

Harmine (286044), INDY (SML1011) and pargyline hydrochloride (P8013) were purchased from Sigma-Aldrich (St Louis, MO, USA) and diluted in DMSO. Subsequent dilutions were made in aqueous solution. Click-it EdU kit and BOBO™-3 were purchased from Thermo Fisher Scientific (Maltham, MA, USA). All controls received an amount of vehicle equivalent to drug treatment conditions and no significant difference was observed between controls with (DMSO) or without vehicle.

### Human pluripotent stem cells

Human embryonic stem cells<sup>14</sup> were cultured under feeder-free culture conditions on Matrigel-coated dishes (Corning, New York, USA) in Essential 8™ Medium (Thermo Fisher Scientific). Passaging was performed enzymatically using Accutase (Millipore, Bedford, MA, USA) by splitting colonies in clumps every 4-5 days and re-plating on Matrigel-coated dishes, having their medium changed every day. All cells were maintained at 37 °C in humidified air with 5% CO<sub>2</sub>.

### Human neural progenitor cells

To induce embryonic stem cells to direct neural differentiation, we performed an adaptation of Baharvand and coworkers protocol<sup>15,32</sup>. Briefly, 70% confluent BR1 culture was 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 replated on dishes coated with 10 µg/mL of Poly-L-ornithine and 2.5 µg/mL of laminin (Thermo Fisher Scientific). A population of hNPCs that migrated from neural tube-like

structures was tested for 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 B-27, 1% penicillin/streptomycin (Thermo Fisher Scientific). Cells were incubated at 37 °C and 5% CO<sub>2</sub>. Medium was replaced every other day. hNPCs were expanded for no more than 5 passages.

### High Content Screening

Cell proliferation, cell death, DNA damage and arborization experiments were performed in a High Content Screening (HCS) format. hNPCs (1500 cells) were plated per well on a multiwell 384 µClear plate (Greiner Bio-One, Kremsmünster, Austria) coated with 100 µg/mL Poly-L-ornithine and 10 µg/mL laminin (Thermo Fisher Scientific). After 24h, cells were treated for 4 days in quintuplicate (five wells per condition) with harmine, INDY and pargyline in N2B27 medium supplemented with bFGF and EGF (Supplementary Fig.1). On day 4 cells were labelled with 10 µM EdU for 2h (cell proliferation) or BOBO™-3 (cell death) for 30 minutes prior to fixation or image acquisition, respectively. For arborization experiments neural differentiation was induced 24h after plating by removing of bFGF and EGF from N2B27 medium. Treatment with harmine, INDY and pargyline 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 (Supplementary Fig.1).

### 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 manufacturer's instruction. Immunocytochemistry for Ki-67 was performed after EdU AF488 labelling. Total number of cells was calculated by nuclei stained with DAPI. S phase was determined by percentage of total cells labelled with EdU.

Whereas dividing cells in all phases of cell cycle, exempting G0, were measured by Ki-67 positive cells as percentage of total cells. Images were acquired with a 10x objective with high numerical aperture (NA).

For cell death analysis, cells were labelled with a fluorophore dye cocktail, containing the cell-permeant nuclear dye Hoechst and the cell-impermeant nuclear dye BOBO™-3 (Invitrogen) in fresh N2B27 medium for 30 minutes at 37 °C and 5% CO<sub>2</sub>. After incubation, the dye cocktail was replaced for new medium and live cell imaging was performed using temperature and CO<sub>2</sub> control option (TCO) of Operetta, set to 37 °C and 5% CO<sub>2</sub> at 10x magnification. For DNA damage analysis, immunocytochemistry was performed on fixed cells after 4 days of treatment using H2AX antibody, and images were acquired at 10x magnification. All quantification analysis were normalised to the number of cells in the well segmented by nucleus dyes. H<sub>2</sub>O<sub>2</sub> was used as positive control for both cell death and DNA damage.

Neuronal arborization was evaluated on fixed cells stained for MAP2 after 7 days of differentiation. The images were analysed 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 analyses 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).

### **Immunocytochemistry**

hNPCs were fixed in formaldehyde 3.7% for 15 minutes at RT and permeabilized in 0.2% Triton X-100 for 15 minutes. Primary antibodies were incubated overnight in 2% BSA at 4 °C, following 40 minutes of 2% BSA blockage. After

washing with PBS, secondary antibodies were incubated for 1h at RT in the dark. Cells were washed 3 times with PBS and nuclei were stained with DAPI. Coverslips were mounted on slides using Aqua-Poly Mount (Polysciences, Warrington, USA) whereas cells on the 384 well plates were covered with glycerol and sealed with AlumaSeal CS (Excel Scientific, Victorville, CA, USA) for image acquisition in confocal microscopy (Leica, Wetzlar, Germany) and Operetta (Perkin Elmer), respectively. Primary antibodies used: mouse anti-MAP2 (Sigma-Aldrich), mouse anti-Ki-67 (BD Biosciences, San Jose, CA, USA), rabbit anti-PAX6 (Santa Cruz Biotechnology), rabbit anti-GFAP (Dako, Glostrup, Denmark), rabbit anti- $\gamma$ -H2AX (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-FOXG1 (Abcam, Cambridge, United Kingdom), rabbit anti-DYRK1A (Sigma-Aldrich) rabbit anti-SOX2 (Millipore), mouse anti- $\beta$ -tubulin III (Millipore), mouse anti-nestin (Millipore), rabbit anti-TBR2 (Millipore). Secondary antibodies: goat anti-mouse Alexa Fluor 488, goat anti-rabbit Alexa Fluor 594, and goat anti-rat Alexa Fluor 647 (Thermo Fisher Scientific).

### **Statistical analysis**

All data are expressed as mean  $\pm$  s.e.m. Results were accepted as statistically significant at  $p < 0.05$ , as determined using one-way ANOVA with Tukey's multiple comparison test. A minimum of 10,000 hNPCs was counted per condition/per experiment.

### **Abbreviations**

hNPCs - human neural progenitors; DYRK - dual-specificity tyrosine phosphorylation-regulated kinase; MAO - monoamine oxidase; GFAP - Glial fibrillary acidic protein; MAP2 - Microtubule-Associated Protein 2;

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### Competing Interests

The authors declare there are no competing interests.

### Author Contributions

VD, RMM, SR designed the experiments; VD, RMM, HD performed the experiments; VD, RMM analyzed the data; VD, PT, SR wrote the paper; VD, RMM, JMN prepared figures; VD, RMM, HD, PT, JMN, SR reviewed drafts of the paper.

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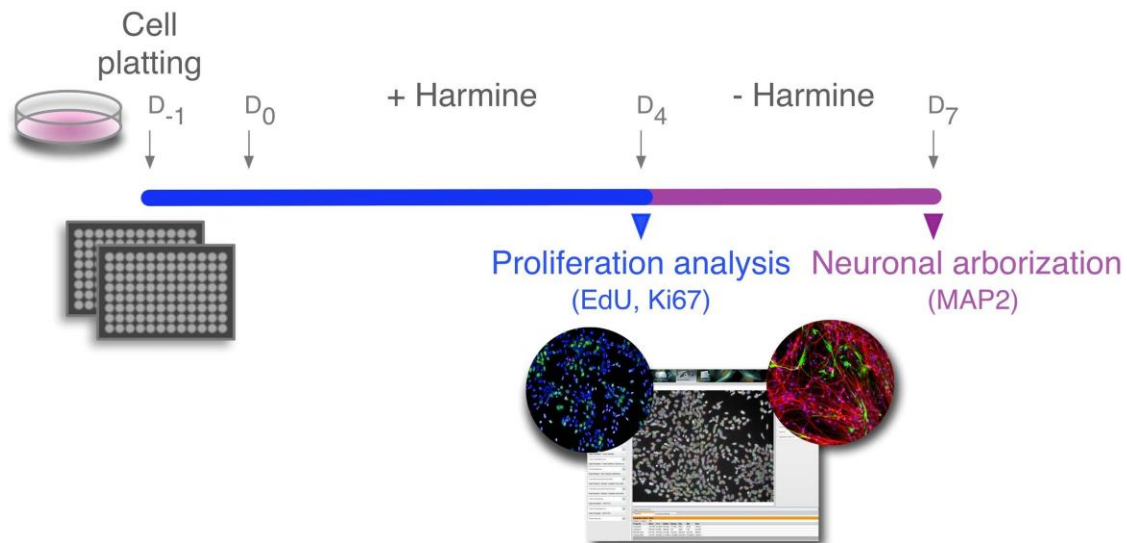
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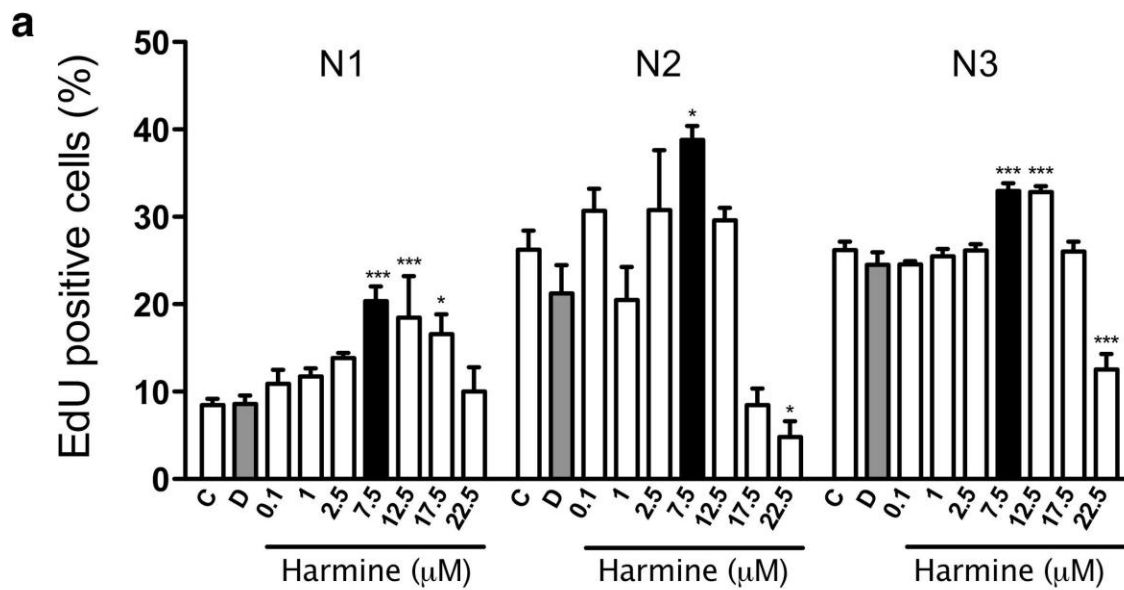
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### Supplementary information



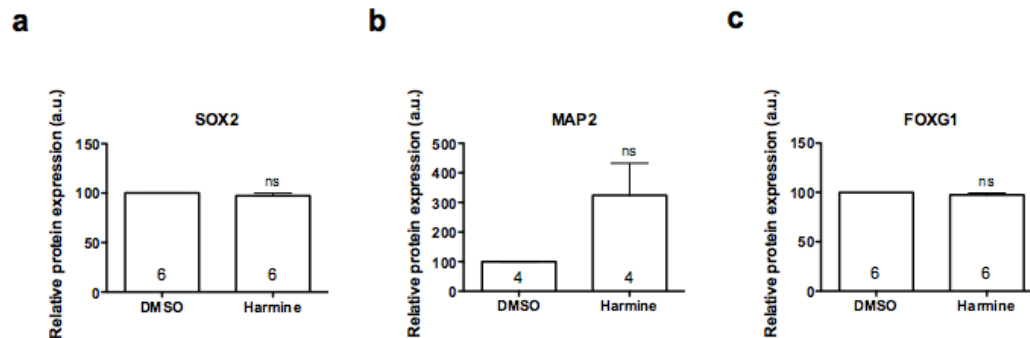
**Supplementary figure 1:** Experimental design scheme for proliferation and neuronal arborization experiments.



**b**

	Control DMSO	Harmine (7.5 $\mu\text{M}$ )	p
N1	8.60 $\pm$ 0.94	20.35 $\pm$ 1.69	0.0001 to 0.001
N2	21.24 $\pm$ 3.25	38.79 $\pm$ 1.61	0.01 to 0.05
N3	24.54 $\pm$ 1.4	32.97 $\pm$ 0.88	0.0001 to 0.001

**Supplementary figure 2: Effect of harmine on cell proliferation.** (a) Dose-response curve of harmine in 3 different lines of hNPC. (b) Table showing mean  $\pm$  s.e.m. and p value. A minimum of 10,000 hNPCs were counted per condition/per experiment. Data was analysed by one-way ANOVA with Tukey's multiple comparison test. C - control and D - Control DMSO.



**Supplementary figure 3: Quantification of differentiation markers with or without harmine exposure.** (a) Expression of SOX2 protein relative to DMSO control. (b) Expression of MAP2 protein relative to DMSO control. (c) Expression of MAP2 protein relative to DMSO control. A minimum of 10,000 hNPCs was counted per condition/per experiment. Data was analysed by one-way ANOVA with Tukey's multiple comparison test, ns =  $p > 0.1$ . Values represent mean  $\pm$  s.e.m. The number inside the bar represents the number of experiments in each group.