

The hallucinogen 2,5-dimethoxy-4-iodoamphetamine hydrochloride activates neurotrophin receptors in a neuronal cell line and promotes neurites extension

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Abstract Decreased neurotrophic factors expression and neurotrophin receptors signalling have repeatedly been reported in association with stress, depression, and neurodegenerative disorders. We have previously identified the hallucinogen 2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) as protective against trophic deprivation-induced cytotoxicity in human neuroblastoma SK-N-SH cells and established the dependence of this effect on the 5-HT_{2A} receptor, tyrosine kinases activity, and the extracellular signal-regulated kinase pathway. In the current study, we investigated the effect of DOI on tropomyosin-related kinase receptor A (TrkA) phosphorylation. Treatment with DOI increased TrkA tyrosine phosphorylation in SK-N-SH cells, determined by immunoprecipitation with TrkA antibody and immunoblotting with anti-phosphotyrosine- and TrkA-antibodies. Analysis of DOI's effect on individual TrkA residues in SK-N-SH cells showed that it increases TrkA Tyr490

phosphorylation ($177 \pm 23\%$ after 5 μM DOI for 30 min compared to vehicle). Furthermore, DOI treatment increased the percentage of SK-N-SH cells extending neurites in a TrkA-dependent manner ($17.2 \pm 2.2\%$ after 5 μM DOI treatment for 6 days compared to $5.6 \pm 1.7\%$ after vehicle). In a different cell model—lymphoblastoid cell lines—DOI treatment increased tropomyosin-related kinase receptor B (TrkB) phosphorylation, determined by immunoprecipitation with TrkB antibody and immunoblotting with anti-phosphotyrosine antibody and total Trk antibody. Our results identify the Trk receptors as a downstream target of the hallucinogen DOI. In light of the known involvement of Trk receptors in mental diseases, their participation in DOI-mediated effects warrants further investigation.

Keywords Hallucinogen · Tropomyosin-related kinase receptors · SK-N-SH neuroblastoma cells · Neurites

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Abbreviations

5-HT ₂	Serotonin 2
TrkA	Tropomyosin-related kinase receptor A
TrkB	Tropomyosin-related kinase receptor B
DOI	2,5-Dimethoxy-4-iodoamphetamine hydrochloride
BDNF	Brain-derived neurotrophic factor
ERK	Extracellular signal-regulated kinase
NGF	Nerve growth factor
NT-4/5	Neurotrophin-4/5
NT-3	Neurotrophin-3
MAPK	Mitogen-activated protein kinase
PI3 K	Phosphatidylinositol 3-kinase
Akt	Protein kinase B
PLC γ	Phospholipase C- γ
PKC	Protein kinase C
FBS	Fetal bovine serum

PBS	Phosphate-buffered saline
EBV	Epstein-Barr virus
BS3	Bis(sulfosuccinimidyl)suberate
ANOVA	Analysis of variance
GPCR	G protein-coupled receptors
LSD	Least statistical difference

Introduction

The tropomyosin-related kinase (Trk) family of receptor tyrosine kinases bind neurotrophins and are positively associated with cell survival and neuronal differentiation. The Trk receptor family includes three subtypes: TrkA, TrkB, and TrkC. TrkA is activated by the nerve growth factor (NGF), TrkB—by the brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5), and TrkC—by neurotrophin-3 (NT-3). Activation of TrkA and TrkB by NT-3 under some circumstances is also possible (Patapoutian and Reichardt 2001; Reichardt 2006). Trk receptor stimulation leads to receptor dimerization and initiation of second messenger pathways: Ras/Rap—mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)—protein kinase B (Akt), and phospholipase C- γ (PLC γ)—protein kinase C (PKC) cascades (Arévalo and Wu 2006). The activation of these pathways promotes cell survival, cellular differentiation, and neurites extension.

The principle of Trk activation in the absence of neurotrophins has been demonstrated initially for several compounds, including adenosine, the pituitary adenylate cyclase-activating polypeptide (PACAP), cyclic adenosine monophosphate, and extracellular zinc (Lee and Chao 2001; Lee et al. 2002; Piiper et al. 2002; Hwang et al. 2005). Adenosine and PACAP activate Trk receptors through their respective G protein-coupled receptors (GPCR). In neuronal cells, activation of Trk receptors by these compounds has mostly been associated with increased neuronal differentiation and neuroprotection.

2,5-Dimethoxy-4-iodoamphetamine hydrochloride (DOI) is a hallucinogen, which is an agonist of serotonin 2 (5-HT₂) receptor subtypes (Nichols 2004). It has the following rank of potency order on 5-HT₂ receptor subtypes: 5-HT_{2A} > 5-HT_{2B} > 5-HT_{2C} (Porter et al. 1999). Several reports have suggested that DOI can increase the expression of neurotrophic factors in cell lines in vitro (Meller et al. 2002; Tsuchioka et al. 2008). We have recently demonstrated that DOI protects a neuronal cell line (human neuroblastoma SK-N-SH cells) against serum deprivation-induced cytotoxicity. Its effect was blocked by pretreatment with the 5-HT_{2A} antagonist MDL11,939, implying cytoprotection was mediated through the 5-HT_{2A} receptor subtype. In our study, the cytoprotective effect of DOI was

dependent on tyrosine kinases activity, which prompted us to investigate its effect on tyrosine kinases phosphorylation levels, a marker for their activity. An antibody array suggested increase of the tropomyosin-related kinase A (TrkA) total tyrosine phosphorylation as one of the targets of DOI (Marinova et al. 2013).

The aim of the current study was to investigate the effect of DOI on TrkA and the related TrkB phosphorylation in two cell culture models (SK-N-SH neuroblastoma cell line and lymphoblastoid cells). The neuronal cell line SK-N-SH was selected, since it was the model used in our preceding study, characterizing the cytoprotective effect of DOI in vitro, and since it has previously been shown to express a number of neurotransmitter receptors/transporters (Klongpanichapak et al. 2008; Marinova et al. 2013; Seitz et al. 1998; Toyohira et al. 2010). Lymphoblastoid cell lines were chosen, since they allow studying molecular pathways activation in cell lines derived from individuals and may be very useful in the search for peripheral biomarkers.

Methods

Cell culture

Human neuroblastoma SK-N-SH cells were purchased from LGC Standards (Wesel, Germany). They were cultured in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies, Zug, Switzerland) in 6-well plates (Thermo Scientific, Switzerland) or 4-well imaging chambers (Sigma-Aldrich, Buchs, Switzerland). SK-N-SH cell culture medium was replaced with medium without FBS (serum-free medium) for 2 h (h) before pharmacological treatments. Pharmacological substances for cell treatments included DOI, NGF, GW441756, and RS-127445 (Sigma-Aldrich) or vehicle control. Water and dimethyl sulfoxide were used for dissolving the drugs and as corresponding vehicle controls. A timeline for the treatments and analyses carried out is presented in Fig. 1.

Lymphoblastoid cell lines were established from lymphocytes isolated from three healthy control probands venipuncture blood samples (average age of the probands in years \pm SD was 21.3 \pm 7.2, all three probands were males). The protocol for blood withdrawal and lymphocytes isolation was approved by the Cantonal Ethic Commission of Zürich (Ref. Nr. EK: KEK-ZH-Nr. 2010-0340/3). For lymphocytes isolation, 9 ml blood was drawn through venipuncture in EDTA-coated tubes. 3 ml Ficoll-Paque PLUS reagent (GE Healthcare Life Sciences, Glatbrugg, Switzerland) was layered under the porous barrier of a 12 ml Leucosep tube (Greiner Bio-One—through Huberlab, Aesch, Switzerland) and 3 ml blood diluted with

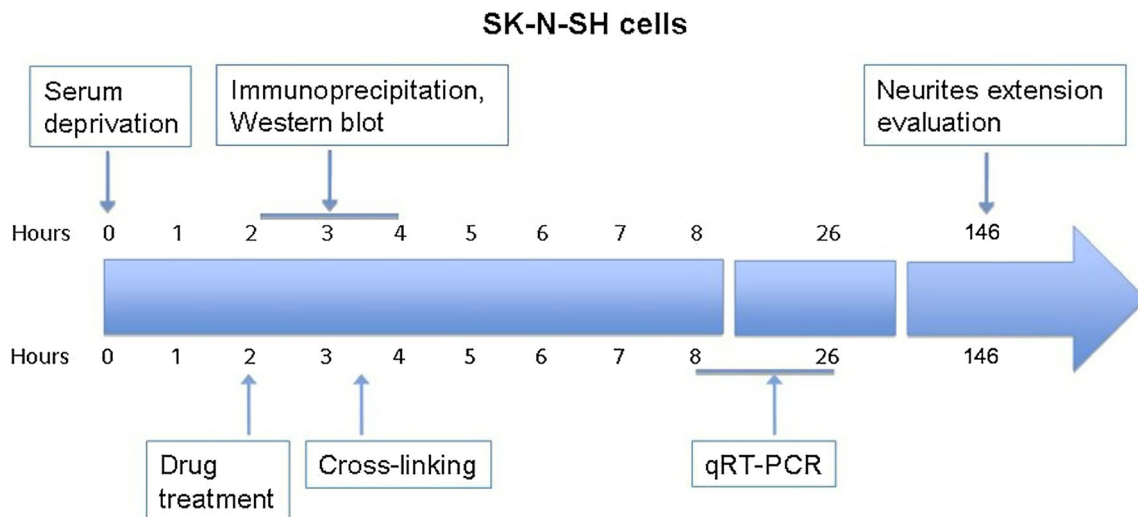


Fig. 1 Timeline of the experimental procedures performed in SK-N-SH cells

3 ml phosphate-buffered saline (PBS) was layered above the barrier. After centrifugation at $1100\times g$ for 10 min (min), a lymphocytes ring was formed. It was washed three times with PBS and lymphocytes were further processed for Epstein–Barr virus (EBV) transformation.

EBV transformation of lymphocytes was carried out according to authorization No A120564/2 approved by the Federal Office for the Environment (FOEN), Federal Coordination Centre for Biotechnology, Bern, Switzerland. For lymphoblastoid culture, lymphocytes were transformed with EBV (LGC standards) and cultured in RPMI medium, supplemented with 15% FBS and 2 mM glutamine (Life Technologies). Passages 5–9 lymphoblastoid cells were used in the presented experiments.

Western blot and immunoprecipitation

SK-N-SH cells were washed with ice-cold PBS and collected in radioimmunoprecipitation assay (RIPA) cell lysis buffer (Sigma–Aldrich), supplemented with protease and phosphatase inhibitors (Roche Applied Science). After centrifugation at $8000\times g$ for 10 min, supernatants were collected and protein concentration was determined using the Bradford method (Sigma–Aldrich) (Bradford 1976). Aliquots of lysates with equal protein content were mixed with sample loading buffer supplemented with reducing agent, loaded on 4–12% Nupage Bis–Tris gels, and subjected to electrophoresis on the XCell SureLock Mini blot module (all from Life Technologies). Gels were then transferred onto nitrocellulose membranes using the iBlot western blotting system (Life Technologies), blocked with 5% non-fat dry milk in PBS with 0.1% Tween 20 (PBST), incubated with primary antibody against phospho-TrkA (Tyr490)/TrkB (Tyr516), phospho-TrkA (Tyr785)/TrkB

(Tyr816), total Trk, TrkA, TrkB (all with dilution 1:100 in 5% BSA-PBST, all from Cell Signaling Technology–BioConcept, Allschwil, Switzerland) or β -actin (with dilution 1:10,000 in 5% non-fat dry milk-PBST, Santa Cruz Biotechnology–LabForce AG, Nunningen, Switzerland) at 4 °C overnight. After washing, membranes were incubated with the respective secondary HRP-labelled antibody (with dilution 1:2000 in 5% non-fat dry milk-PBST, Santa Cruz Biotechnology) for 1 h at room temperature. Two different protein standards were used for molecular weight estimation of the detected bands in western blot and immunoprecipitation experiments: all blue precision plus protein prestained standards (Bio-Rad) and SeeBlue prestained standard (Life Technologies). Bands were visualized with ECL Advance (GE Healthcare, Glattbrugg, Switzerland) on ChemiDoc XRS+ (Bio-Rad) and quantified using the ImageJ 1.47 software (NIH).

For immunoprecipitation, 200 μ l cell lysates from SK-N-SH cells containing 200 μ g protein were precleared with 25 μ l protein G PLUS-agarose slurry (Santa Cruz Biotechnology) for 30 min. SK-N-SH lysates were incubated with 1 μ g TrkA antibody (clone 763, Santa Cruz Biotechnology) at 4 °C overnight. Samples were then incubated at 4 °C with 30 μ l protein G PLUS-agarose slurry for 1 h. The samples were washed three times with lysis buffer, mixed with sample loading buffer supplemented with reducing agent, and boiled for 4 min. Samples were further processed for immunoblotting as described above, using anti-phosphotyrosine antibody (clone PY350, Santa Cruz Biotechnology) or Trk A antibody (clone 763, Santa Cruz Biotechnology). Alternatively, protein A agarose beads (Cell Signaling–BioConcept) were used for immunoprecipitation in some experiments to confirm the data and similar results were obtained.

Enzyme-linked immunosorbent assay (ELISA)

Protein extracts from SK-N-SH cells were prepared and total protein concentration was determined with Bradford as described in the “[Western blot and immunoprecipitation](#)” section. Protein extracts were diluted to concentration 0.75 mg/ml. 100 μ l of SK-N-SH protein extracts or serially diluted NGF standards ranging in concentration between 5 and 0 ng/ml were added to the wells of a commercial beta-NGF human ELISA Kit (Abcam—Chemie Brunschwig, Basel, Switzerland). Anti-NGF biotinylated antibody, HRP-streptavidin solution, development and stop solution were subsequently added to the plate with intermittent incubation and washing steps, according to the manufacturer’s recommendation. Absorbance at 450 nm was recorded with a Mithras microplate multimode reader (Berthold Technologies, Bad Wildbad, Germany). Five-parameter logistic regression was used to calculate the concentrations of the samples after constructing the standard curve.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted from SK-N-SH cells with RNeasy plus mini kit (Qiagen, Hombrechtikon, Switzerland). 1 μ g RNA was used for reverse transcription with an iScript Select cDNA Synthesis kit (Bio-Rad, Reinach, Switzerland). cDNA was amplified by PCR with primers specific for the human *NGF* gene (primer assay number QT00001589, Qiagen), human *NTRK1* gene (QT00054110), human *NTRK2* gene (QT00082033), human *NTF3* gene (QT00204218), human *HTR2A* gene (QT00054306), human *HTR2B* gene (QT00060368), human *HTR2C* gene (QT01002680), human *ACTB* gene (QT01680476), human *ALAS1* gene (QT00073122), or human *RPL13A* gene (QT00089915), using a QuantiFast SYBR Green PCR kit (Qiagen). PCR efficiency was in the range 1.7–1.75 for the analyzed genes and was determined using LinReg (Ramakers et al. 2003). Normalization and quantification of the results were carried out with the “Biogazelle qBASE plus” software (Biogazelle NV, Zwijnaarde, Belgium)—a software program that automatically selects reference genes based on their stability for normalization and utilizes gene specific PCR efficiency correction and multiple reference gene normalization (Hellemans et al. 2007).

Chemical cross-linking

For chemical cross-linking, SK-N-SH cells were serum-starved for 2 h, treated pharmacologically as described, washed with ice-cold PBS, and then incubated with 1 mM of the cross-linker bis (sulfosuccinimidyl)

suberate (BS3) in PBS for 1 h at 4 °C. The cross-linking reaction was quenched by the addition of 20 mM Tris to the cells for 15 min. The cells were then washed with PBS and lysed in RIPA cell lysis buffer (Sigma–Aldrich), supplemented with protease and phosphatase inhibitors (Roche Applied Science). After centrifugation at 8000 \times g for 10 min, supernatants were collected and protein concentration was determined using the Bradford method. The protein lysates were then processed for western blot as described above.

Immunofluorescence staining for neurite outgrowth quantification

For neurite outgrowth quantification, SK-N-SH cells were cultured to about 30% density on 4-well imaging chambers and switched to serum-free medium. DOI was added to cells 2 h after medium change, in some cases, cells were pretreated with 2 μ M GW441756 for 30 min prior to DOI addition. Percentage of cells extending neurites was assessed after 6 days. For neurites extension assessment, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS, blocked with 5% normal goat serum/0.3% Triton X-100 for 1 h, incubated with anti- β tubulin antibody (dilution 1:100, Santa Cruz Biotechnology) overnight at 4 °C, washed with PBS, incubated with secondary Alexa 488-conjugated anti-rabbit antibody (dilution 1:2000, Life Technologies) for 2 h at room temperature and after washing mounted with medium containing 4',6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology). Immunofluorescence was detected in all cases with the Xcellence software on an Olympus fluorescent microscope (Olympus, Volketswil, Switzerland). For the neurite outgrowth quantification for each experiment and treatment condition, five fields were randomly selected and observed under a 20 \times objective. Slides from three independent cell cultures were analyzed. Cells with neurite length exceeding the length of the cell body were considered as cells with neurites.

Statistical analysis

All presented data are from at least three independent cell cultures with the exception of the ELISA experiment. Statistical analysis of the data was carried out with the SPSS version 18 software (IBM). Statistical significance of differences between the groups was analyzed with the one-way analysis of variance (ANOVA) followed by least statistical difference (LSD) post hoc test. Statistical significance was set at $p \leq 0.05$.

Results

The hallucinogen DOI increases TrkA tyrosine phosphorylation in SK-N-SH cells

We have previously shown that DOI in concentrations ranging from 25 nM to 5 μ M protected SK-N-SH cells against serum deprivation-induced cytotoxicity (Marinova et al. 2013). In the same study, we used a receptor tyrosine kinases antibody array to identify tyrosine kinases whose phosphorylation is affected by DOI treatment in SK-N-SH cells. The antibody array showed among others increased total tyrosine phosphorylation of TrkA after 15 min 1 μ M DOI treatment (Marinova et al. 2013). Since the antibody array is a screening method, the results from it need to be verified with an alternative method for protein levels determination.

Due to possible cross-reactivity of antibodies detecting TrkA and TrkB phosphorylation, in the current study, we first confirmed TrkA and TrkB receptors expression in SK-N-SH cells. mRNA levels of the *NTRK1* gene coding for the TrkA receptor and the *NTRK2* gene coding for the TrkB receptor were measured using qRT-PCR. Both genes were expressed in SK-N-SH cells, but *NTRK1* expression greatly exceeded *NTRK2* expression (Fig. 2a). The expression of both genes was also tested on the protein level using western blot, and TrkA was detected in SK-N-SH cells, confirming the results obtained on the mRNA level (Fig. 2b). We investigated the effect of DOI on TrkA tyrosine phosphorylation by immunoprecipitation of SK-N-SH cells protein lysates with TrkA antibody, and western blot of the immunoprecipitates with antibodies against phosphotyrosine or TrkA. We tested the effect of treatment with 5 μ M DOI for 0–120 min on TrkA tyrosine phosphorylation and found statistically significant effect at 30 min (relative TrkA tyrosine phosphorylation (pTyr-TrkA) levels \pm SEM after 5 μ M DOI compared to vehicle $153 \pm 12\%$ at 30 min, $p < 0.05$) (Fig. 2c, d). We also determined the effect of 0–5 μ M DOI treatment for 30 min on TrkA tyrosine phosphorylation, and found statistically significant effect with 1, 2, and 5 μ M DOI treatment, with maximal effect at 5 μ M DOI treatment (relative pTyr-TrkA levels \pm SEM compared to vehicle were $125 \pm 2\%$ with 1 μ M DOI, $122 \pm 4\%$ with 2 μ M DOI and $145 \pm 3\%$ with 5 μ M DOI, $p < 0.05$ in all three cases) (Fig. 2e, f).

DOI increases TrkA Tyr490 phosphorylation in SK-N-SH cells

Several distinct TrkA tyrosine residues can be phosphorylated in a non-coordinated fashion following receptor activation, reflecting their separate functions in regulating

the catalytic and signalling activities of TrkA receptors (Segal et al. 1996). Therefore, we investigated by western blot the effect of DOI on the phosphorylation of individual TrkA tyrosine residues, associated with TrkA activation: TrkA Tyr490 and TrkA Tyr785. SK-N-SH cells were treated with 0–5 μ M DOI for 30 min (Fig. 3a–c) or with 5 μ M DOI for 0–120 min (Fig. 3d–f). Treatment with 5 μ M DOI for 30 min led to a statistically significant increase in the phosphorylation of TrkA Tyr490 (relative phosphoTyr490-TrkA levels \pm SEM compared to vehicle were $177 \pm 23\%$ with 5 μ M DOI for 30 min, $p < 0.05$) (Fig. 3a, b). Treatment with 5 μ M DOI for 120 min did not have a statistically significant effect on TrkA Tyr490 phosphorylation (Fig. 3d, e).

No statistically significant changes in phosphoTyr785-TrkA levels were detected after treatment with 1 or 5 μ M DOI for 30 min or 120 min (Fig. 3a, c, d, f). Total TrkA levels were used for normalization (Fig. 3a–f).

TrkA receptor dimerization and phosphorylation is considered critical for the receptor activation (Hartman et al. 1992); however, recent studies have shown that TrkA exists also as preformed, but inactive dimers in some cell types (Shen and Maruyama 2011). To determine whether TrkA dimers exist in SK-N-SH cells, we chemically cross-linked cells treated with vehicle, 5 μ M DOI for 30 min, or 100 ng/ml NGF for 5 min. Cross-linking led to detection of a clear band with molecular weight corresponding to a TrkA dimer. The band was already observable in vehicle-treated cross-linked cells, while absent in non-cross-linked cells, suggesting the presence of preformed TrkA dimers in SK-N-SH cells (Fig. 4).

Effect of DOI on NGF levels in SK-N-SH cells

The TrkA receptor ligand NGF is a powerful growth factor for neurons and a potential protective factor for neurodegenerative disorders (Aloe et al. 2012). We determined the effect of DOI on NGF protein and mRNA levels in SK-N-SH cells. Treatment with 0–5 μ M DOI for 30 min or with 5 μ M DOI for 0–120 min did not lead to significant change of NGF protein levels in SK-N-SH cells (Supplementary Figure S1). Endogenous NGF protein levels in SK-N-SH cells were very low—in the pg/ml range as measured using the ELISA assay. Treatment with 0–5 μ M DOI for 6 h or with 5 μ M DOI for 0–24 h did not significantly change NGF mRNA levels, even though numerically NGF mRNA levels were higher after DOI treatment (Supplementary Figure S2). We tested also the mRNA expression levels of *NTF3*, coding for NT-3, which can bind to the TrkA receptor with lower affinity compared to NGF. Low mRNA expression levels of *NTF3* were detected in SK-N-SH cells (data not shown), suggesting that it does not play a major

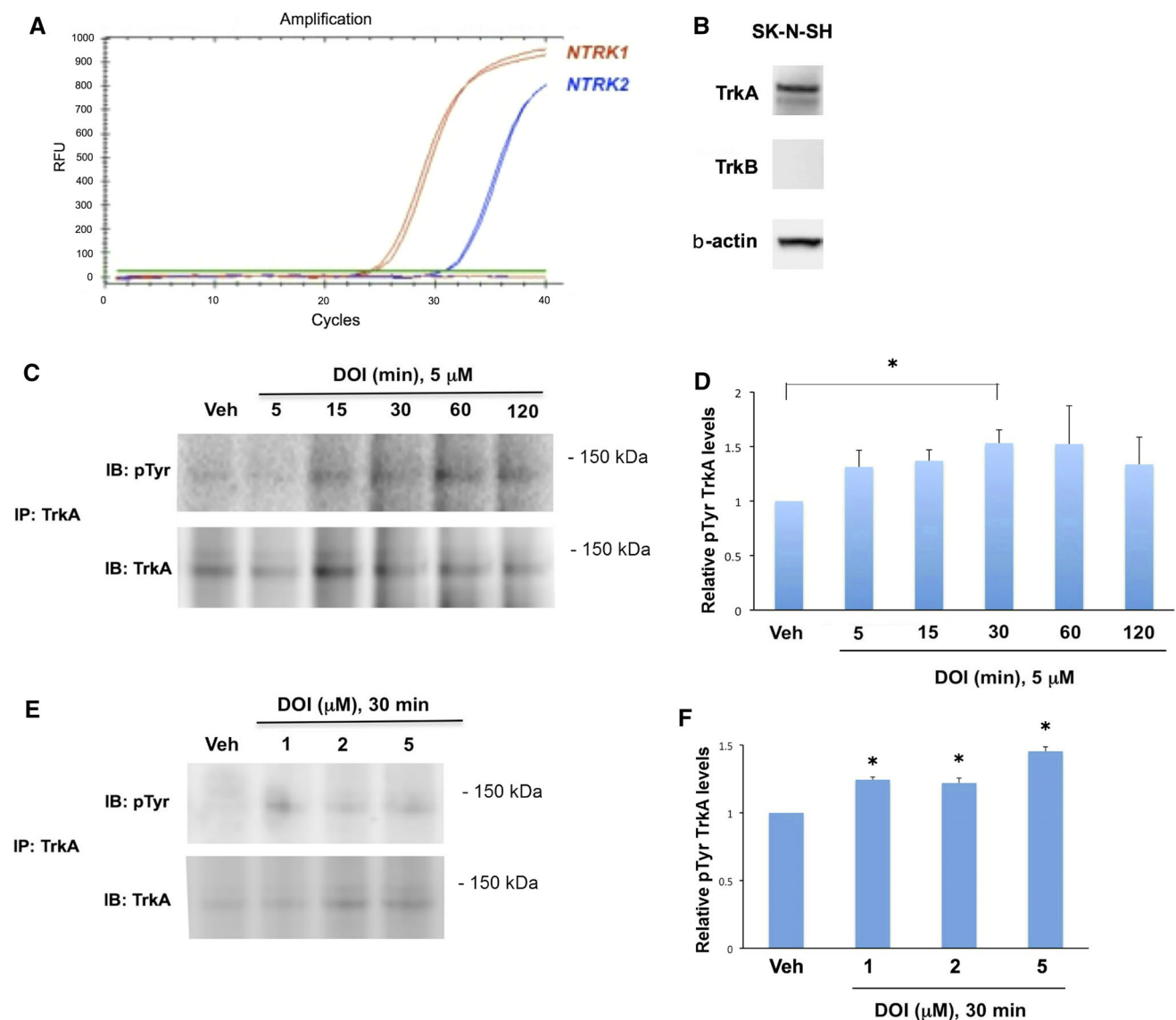


Fig. 2 Hallucinogen DOI increases TrkA tyrosine phosphorylation (pTyr-TrkA) in SK-N-SH cells. **a** Representative *NTRK1* and *NTRK2* amplification curves for their mRNA expression levels in SK-N-SH cells. **b** Representative western blot images for TrkA, TrkB and β -actin protein expression levels in SK-N-SH cells. **c** SK-N-SH cells were incubated with vehicle (Veh) or 5 μ M DOI for 5–120 min. Protein lysates were immunoprecipitated (IP) with anti-TrkA antibody and immunocomplexes were analyzed by immunoblotting (IB) with anti-phosphotyrosine antibody, or to confirm the immunoprecipitation with anti-TrkA antibody. **d** Quantification of relative phosphotyrosine-TrkA mean \pm SEM levels presented in C from three

independent cell cultures using one-way ANOVA followed by least statistical difference (LSD) post hoc test. $*p < 0.05$. **e** SK-N-SH cells were incubated with vehicle or 1–5 μ M DOI for 30 min. Protein lysates were immunoprecipitated with anti-TrkA antibody and immunocomplexes were analyzed by immunoblotting with anti-phosphotyrosine antibody, or to confirm the immunoprecipitation with anti-TrkA antibody. **f** Quantification of results for relative phosphotyrosine-TrkA mean \pm SEM levels presented in **e** from three independent cell cultures assessed by one-way ANOVA followed by LSD post hoc test. $*p < 0.05$ compared to vehicle control

role in the regulation of TrkA receptor activation compared to NGF in SK-N-SH cells.

Serotonin 2 receptor subtypes expression in SK-N-SH cells

DOI can activate 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptor subtypes (Porter et al. 1999); therefore, we assessed the

mRNA levels of the three 5-HT₂ receptor subtypes in SK-N-SH cells. *HTR2A* (coding for 5-HT_{2A}) and *HTR2B* (coding for 5-HT_{2B}) were expressed at comparable levels in SK-N-SH cells, while *HTR2C* (coding for 5-HT_{2C}) expression was much lower (Supplementary Figure S3A). mRNA levels of the three receptor subtypes were assessed also in postmortem human cortex sample used as a positive control (Supplementary Figure S3B).

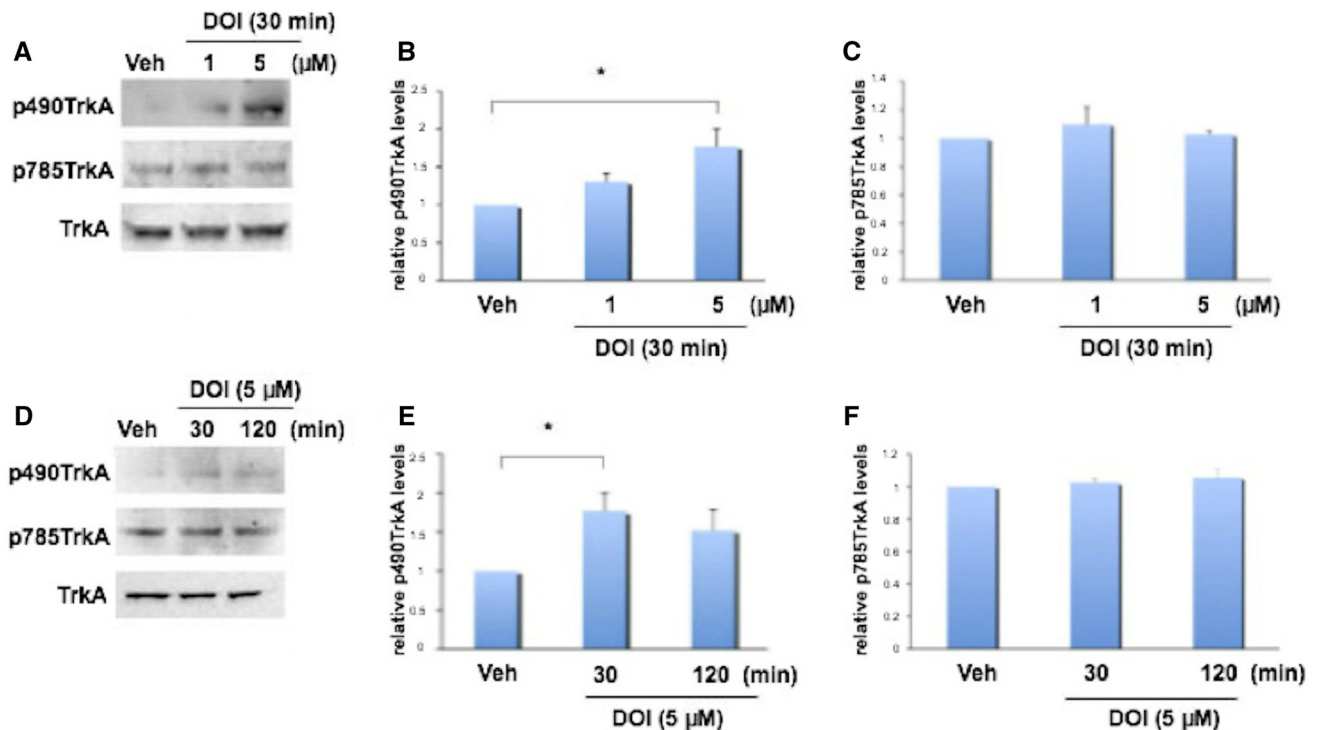


Fig. 3 Increase of TrkA Tyr490 phosphorylation by the 5-HT receptor agonist DOI in SK-N-SH cells. **a** SK-N-SH cells were incubated with vehicle (Veh), 1 or 5 μM DOI for 30 min. Protein lysates were collected from the cells and subjected to western blot analysis with antibodies for phosphoTyr490-TrkA, phosphoTyr785-TrkA and total TrkA. **b** Quantification of the results presented in **a** for relative phosphoTyr490-TrkA mean \pm SEM levels from three independent cell cultures with one-way ANOVA followed by least statistical difference (LSD) post hoc test. $*p < 0.05$. **c** Quantification of the results presented in **a** for relative phosphoTyr785-TrkA

mean \pm SEM levels from three independent cell cultures. **d** SK-N-SH cells were incubated with Veh or 5 μM DOI for 30 min or 120 min. Protein lysates were collected from the cells and subjected to western blot analysis with antibodies against phosphoTyr490-TrkA, phosphoTyr785-TrkA and total TrkA. **e** Quantification of the results presented in **d** for relative phosphoTyr490-TrkA mean \pm SEM levels from three independent cell cultures assessed by one-way ANOVA followed by LSD post hoc test. $*p < 0.05$. **f** Quantification of the results presented in **d** for relative phosphoTyr785-TrkA mean \pm SEM levels from three independent cell cultures

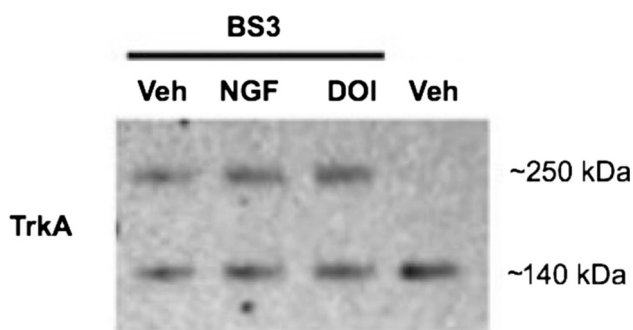


Fig. 4 SK-N-SH cells were treated with vehicle (Veh), 100 nM NGF for 5 min or 5 μM DOI for 30 min, and cross-linked with bis(sulfosuccinimidyl)suberate (BS3) for 1 h. Protein lysates were analyzed by western blot for the expression of TrkA

We next investigated the effect of 5-HT_{2A} and 5-HT_{2B} antagonists on pTyr-TrkA levels induction by DOI. Pretreatment with the 5-HT_{2A} receptor antagonist MDL 11,939 at 1 μM had no effect on pTyr-TrkA increase by DOI, while pretreatment with MDL 11,939 at 10 μM showed a trend to decrease of pTyr-TrkA induction

(Fig. 5a). Pretreatment with the 5-HT_{2B} antagonist RS-127445 at 100 nM or 500 nM also did not significantly block pTyr-TrkA induction by DOI (Fig. 5b).

We have previously assessed the toxicity of MDL 11,939 on SK-N-SH cells and found the tested doses not toxic (Marinova et al. 2013). To determine whether RS-127445 in the applied doses is toxic to SK-N-SH cells, we carried out an MTT assay after treatment with RS-127445 at 100 nM and 500 nM for 6 days. No toxicity of both doses was observed (Supplementary Figure S4).

Effect of DOI on neurites extension in SK-N-SH cells

Since TrkA receptor activation can be associated with neuronal differentiation and neurites extension, we tested the effect of DOI treatment on neurites extension in SK-N-SH cells. 5 μM DOI treatment for 6 days led to a statistically significant increase in the percentage of SK-N-SH cells with neurites (percentage of cells extending neurites \pm SEM were $17.2 \pm 2.2\%$ after 5 μM DOI treatment compared to $5.6 \pm 1.7\%$ after vehicle treatment,

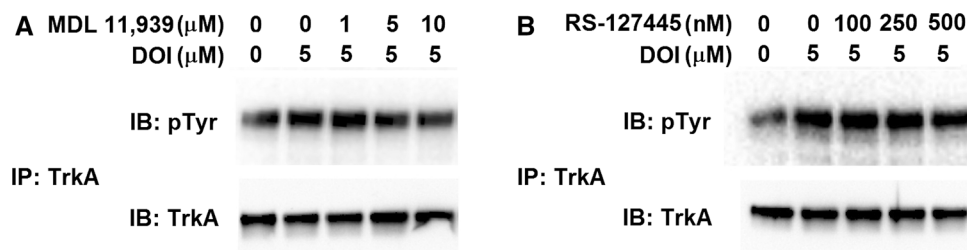


Fig. 5 Effect of pretreatment with the 5-HT_{2A} antagonist MDL 11,939 (a) and the 5-HT_{2B} antagonist RS-127445 (b) on TrkA tyrosine phosphorylation increase by DOI. Protein lysates were immunoprecipitated (IP) with anti-TrkA antibody and

immunocomplexes were analyzed by immunoblotting (IB) with anti-phosphotyrosine antibody, or to confirm the immunoprecipitation with anti-TrkA antibody

$p < 0.05$). DOI's effect on neurites extension was blocked by pretreatment with 2 μM of the TrkA inhibitor GW441756 (percentage of cells extending neurites \pm SEM were $8.8 \pm 2.5\%$ after 2 μM GW441756+5 μM DOI treatment compared to $17.2 \pm 2.2\%$ after 5 μM DOI treatment, $p < 0.05$) (Fig. 6).

DOI increases TrkB tyrosine phosphorylation in lymphoblastoid cell lines

TrkB as well as TrkA expression was determined also in lymphocytes isolated from three healthy control individuals and in lymphoblastoid cell lines derived from them through immortalization with EBV. The *NTRK1* gene and the *NTRK2* genes were both expressed in lymphocytes at relatively low levels (Supplementary Figure S5). In the established lymphoblastoid cell lines, *NTRK2* expression increased and was much higher than *NTRK1* (Supplementary Figure S6A). The expression of both genes was also tested on the protein level using western blot, and TrkB was shown to be the predominant form in lymphoblastoid cell lines (Supplementary Figure S6B). Treatment with 5 μM DOI for 30 min led to an increase in TrkB tyrosine phosphorylation, measured after immunoprecipitation with TrkB antibody and immunoblotting with antibodies against phosphotyrosine or total Trk (relative pTyr-TrkB levels \pm SEM compared to vehicle were $135 \pm 10\%$ after 5 μM DOI, $p < 0.05$) (Supplementary Figure S6C, D).

Discussion

In the current study we found that the hallucinogen DOI affects TrkA and TrkB signalling. DOI transiently increased tyrosine phosphorylation of the TrkA receptor in SK-N-SH cells, affecting particularly TrkA Tyr490 phosphorylation, and increased the percentage of cells with neurites in a TrkA-dependent manner. DOI increased also TrkB tyrosine phosphorylation in lymphoblastoid cells.

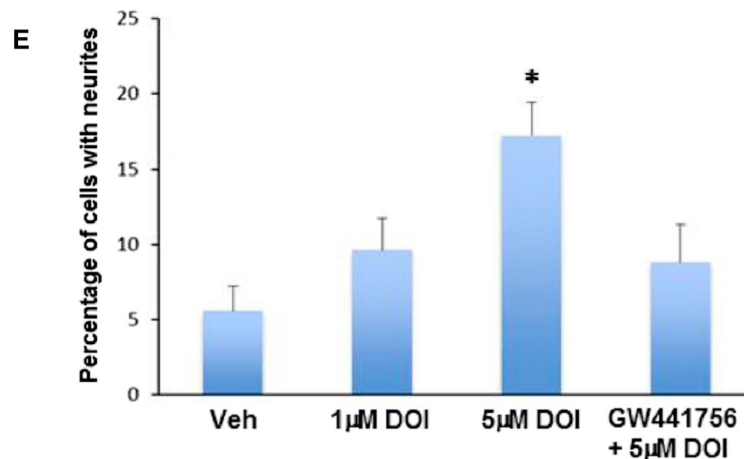
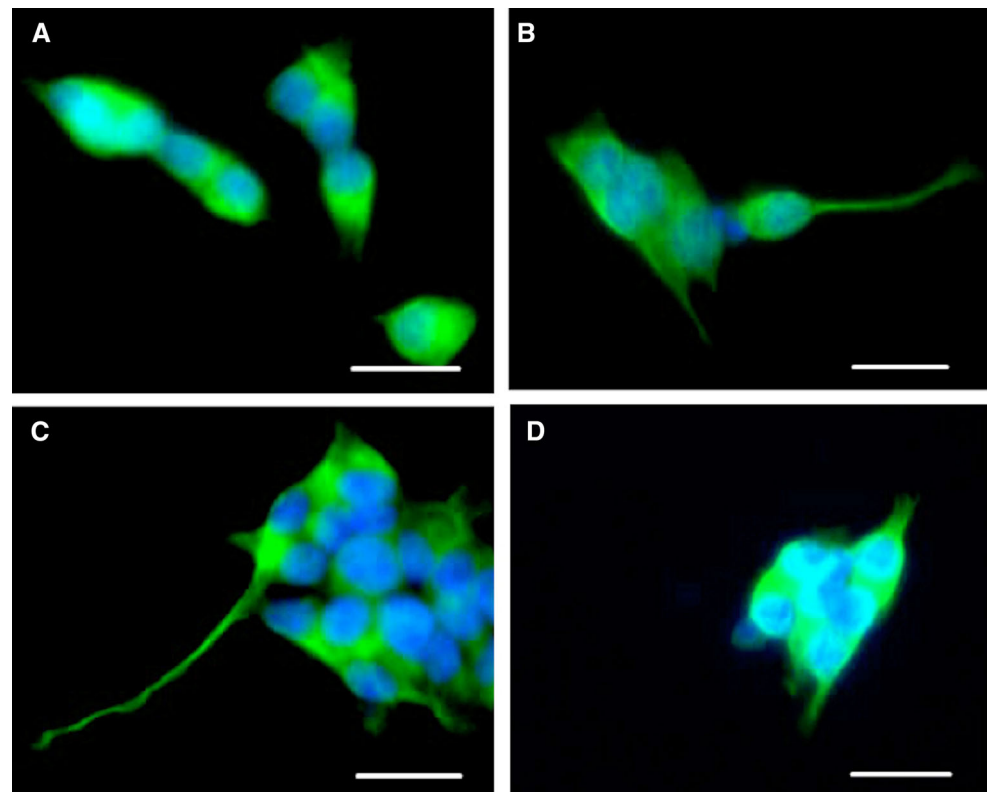
Trk receptor subtypes are expressed in serotonergic neurons, suggesting that both systems may interact (Sobreviela et al. 1994). However, data about potential effects of serotonergic related compounds on Trk signalling are scarce. Thus, serotonin did not affect TrkB phosphorylation in mouse neurons (Jang et al. 2010). On the other hand, *N*-acetylserotonin, a precursor of melatonin acetylated from serotonin by arylalkylamine *N*-acetyltransferase, and its derivatives specifically activated the TrkB receptor in a circadian rhythm in mouse neurons (Jang et al. 2010; Shen et al. 2012). Another recent study showed that serotonin can increase TrkB phosphorylation in human neuroblastoma SH-SY5Y cells in a reactive oxygen species-dependent manner (Kruk et al. 2013).

In our study, DOI increased TrkA receptor tyrosine phosphorylation (Fig. 2). Interestingly, the antidepressant amitriptyline has been shown to be a TrkA and TrkB receptor agonist, promoting TrkA/TrkB heterodimerization and with potent neurotrophic activity in vitro (Jang et al. 2009). Trk signalling has been implicated in the effect of a number of compounds with neurotrophic potential, including omega-3 fatty acids, whose deficiency was associated with decreased BDNF levels and signalling through TrkB receptors (Bhatia et al. 2011). In our study, DOI's effect on pTyr-TrkA induction required higher doses (5 μM) and a slight, non-significant decrease of TrkA tyrosine phosphorylation following incubation with 10 μM MDL 11,939 (a 5-HT_{2A} receptor antagonist) was found, but no effects of the 5-HT_{2B} receptor antagonist RS-127445. In addition, variability of 5-HT_{2A} or 5-HT_{2B} receptor antagonists effects between experiments was present. Together with the relatively higher dose required for DOI's effect on TrkA tyrosine phosphorylation, a specific role of serotonin receptors seems unlikely. A mechanism similar to amitriptyline's (binding to TrkA and TrkB receptors and induction of their phosphorylation and dimerization) may be relevant for DOI's effect.

We found that DOI transiently increased TrkA Tyr490 phosphorylation in SK-N-SH cells, while TrkA Tyr785 phosphorylation was not changed (Fig. 3). Since we

Fig. 6 Effect of DOI on neurites extension in SK-N-SH cells. SK-N-SH cells were treated with vehicle (a), 1 μ M DOI (b), 5 μ M DOI (c), or pretreated with 2 μ M GW441756 (30 min) and treated with 5 μ M DOI (d) for 6 days. Cells were stained with anti- β tubulin antibody and observed under a fluorescent microscope. 4',6-diamidino-2-phenylindole (DAPI) staining is presented in blue, and β -tubulin staining is presented in green. Scale bar 20 μ M.

e Quantification of results under treatment conditions presented in panels a–d from three independent cell cultures. Five randomly selected fields per each cell culture and treatment condition were analyzed by one-way ANOVA followed by least statistical difference (LSD) post hoc test. * $p < 0.05$



analyzed only short-term effects of DOI on TrkA phosphorylation, we cannot state with certainty if the observed increase is transient or if biphasic kinetics of the effect would be present. TrkA Tyr490 phosphorylation has previously been associated with complex formation between Trk and Src homology 2 domain-containing (SHC) adaptor protein, with the activation of PI3K and MAPK, as well as with neurite extension/neuronal differentiation (Baxter et al. 1995; Stephens et al. 1994). We did not find significant change in protein and mRNA levels of the TrkA agonist *NGF* in SK-N-SH cells after DOI treatment and endogenous NGF protein levels in SK-N-SH cells were low (pg/ml range) (Supplementary Figure S1).

DOI treatment led to an increase in the percentage of SK-N-SH cells extending neurites. The increase of neurites extension by DOI was blocked by pretreatment with the TrkA inhibitor GW441756, implying that the effect of DOI is mediated through the TrkA receptor (Fig. 6). Several other studies have investigated the effect of DOI on neurite outgrowth in different cell models. In primary cultures of fetal ventroposterior thalamic neurons DOI treatment led to increased primary neurites length and number of branching points (Persico et al. 2006). On the other hand, in organotypic culture DOI treatment decreased neurite density of serotonergic neurons (Dudok et al. 2009). Finally, DOI increased the growth cone periphery length in rat cortical

neurons (Ohtani et al. 2014). Together with our study, these results point towards differential effect of DOI on neurite extension in different neuronal cell types, possibly depending on their phenotype and culture conditions.

Finally, DOI treatment led to an increase in TrkB tyrosine phosphorylation in lymphoblastoid cell lines (Supplementary Figure S6). Neurotrophins and their receptors have been shown to play a role also outside of the central nervous system, including the immune system (Linker et al. 2009; Vega et al. 2003). Lymphocytes express neurotrophins and Trk receptors, and expression can depend on the degree of cell activation (Vega et al. 2003). Neurotrophins are hypothesized to serve as mediators in the cross-talk between neuronal and immune cells (Schulte-Herbrüggen et al. 2007). Alterations in neurotrophic factors or receptors levels in lymphocytes have been detected in a number of disorders affecting both the nervous and immune system (Schulte-Herbrüggen et al. 2007). The functional role of Trk phosphorylation by DOI in lymphoblastoid cells needs to be further elucidated. In addition, further studies need to assess whether DOI has an effect on the phosphorylation of TrkC in different cell models.

Limitations of the current study include the lack of confirmation of the findings in vivo and the fact that the potential role of Trk dimerization was not conclusively proven in Trk overexpression combined with cross-linking experiments.

We identified the hallucinogen DOI as a potential TrkA receptor activator through TrkA Tyr490 phosphorylation induction in SK-N-SH cells. In addition, we observed TrkA-dependent increase in neurites extension after DOI treatment. DOI also activated TrkB tyrosine phosphorylation in another cell model—lymphoblastoid cell lines. Changes in Trk receptors and their ligands have been shown in stress, depression, and neurodegenerative disorders. The potential involvement of Trk-related mechanisms in mediating DOI-dysregulated signalling in psychiatric or neurodegenerative disorders needs to be further investigated.

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