Cerebrolysin protects PC12 cells from CoCl₂-induced hypoxia employing GSK3β signaling

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A B S T R A C T

Cerebrolysin (EVER Neuro Pharma GmbH, Austria) is a peptidergic drug indicated for clinical use in stroke, traumatic brain injury and dementia. The therapeutic effect of Cerebrolysin is thought to ensure from its neurotrophic activity, which shares some properties with naturally occurring neurotrophic factors. However, the exact mechanism of action of Cerebrolysin is yet to be fully deciphered. This study aimed to investigate the neuroprotective effect of Cerebrolysin in a widely used in vitro model of hypoxia-induced neuronal cytotoxicity, namely cobalt chloride (CoCl₂)-treatment of PC12 cells. CoCl₂-cytotoxicity was indicated by a reduced cell-diameter, cell shrinkage, increased pro-apoptotic Caspase-activities and a decreased metabolic activity. Cerebrolysin maintained the cell-diameter of CoCl₂-treated naïve PC12 cells, decreased the activation of Caspase 3/7 in CoCl₂-stressed naïve PC12 cells and restored the cells’ metabolic activity in CoCl₂-impaired naïve and differentiated PC12 cells. Cerebrolysin treatment also decreased the levels of superoxide observed after exposure to CoCl₂. Investigating the mechanism of action, we could demonstrate that Cerebrolysin application to CoCl₂-stressed PC12 cells increased the phosphorylation of GSK3β, resulting in the inhibition of GSK3β. This might become clinically relevant for Alzheimer’s disease, since GSK3β activity has been linked to the production of amyloid beta. Taken together, Cerebrolysin was found to have neuroprotective effects in CoCl₂-induced cytotoxicity in PC12 cells.

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1. Introduction

A central hallmark of neurodegenerative diseases and acute CNS lesions is neuronal cell death, which constitutes the prime cause of associated functional deficits such as cognitive and motor disabilities. In consequence, strategies aiming to protect neurons from cell death have been under development over the past decades. The identification of neurotrophic factors such as nerve growth factor (NGF, Cohen, 1960) and brain-derived neurotrophic factor (BDNF, Lindsay et al., 1985) triggered the search for druggable brain-derived neurotrophic activities (Longo and Massa, 2013). Cerebrolysin is produced by a standardized biochemical breakdown of brain lysates and consists of low molecular weight peptides and amino acids (EBEWEuroPharmaGmbH, 2009). The resulting drug displays neurotrophic activity (Mallory et al., 1999; Masliah and Diez-Tejedor, 2012), protects primary neurons from glutamate-induced excitotoxicity (Hutter-Paier et al., 1996), displays neuroprotective activity in animal models of neurodegeneration (Francis-Turner et al., 1996; Francis-Turner and Valuskova, 1996; Valuskova and Francis-Turner, 1996; Masliah et al., 1999; Weinbergs et al., 2000) or ischemic and traumatic CNS lesions (Zhang et al., 2013a,b) and modulates endogenous neurotrophin levels (Ubhi et al., 2013). Furthermore, Cerebrolysin is used in human to treat dementia, stroke and traumatic brain injuries. In this respect, Cerebrolysin administration was reported...
to improve cognitive functions and activities of daily living in patients suffering of Alzheimer's disease (Ruether et al., 1994, 2000; Alvarez et al., 2006, 2011). Despite the strong preclinical data and accumulating evidence of Cerebrosylin's clinical efficacy, little is known about the mechanisms of action. There is evidence for Cerebrosylin-promoted metabolic stabilization of neurons via protein synthesis modulation (Piswanger et al., 1990), prevention of lactate acidosis (Windisch and Pisswanger, 1985), and prevention of free radical formation (Sugita et al., 1993). Moreover, Cerebrosylin might also modulate neurotransmitter signaling such as adenosine (Xiong et al., 1995) or GABA (Wojtowicz et al., 1996). In the present study, we investigate the molecular mechanisms underlying Cerebrosylin neuroprotective activities following application of cobalt chloride (CoCl2) on naïve and differentiated PC12 rat pheochromocytoma cells. This well-established and widely-used model constitutes an in vitro paradigm that mimics a hypoxic condition and induces cell death. It involves the induction of mitochondrial DNA damage (Wang et al., 2000), the production of reactive oxygen species (ROS), activation of the pro-apoptotic gene APAF-1 (Zou et al., 2001), and activation of Caspase 3 and p38 mitogen-activated protein kinase (Zou et al., 2002) and suppression of mTOR signaling (Zhang et al., 2014).

2. Materials and methods

2.1. PC12 cell cultures and CoCl2-induction of cell death

For cell propagation, PC12 rat pheochromocytoma cells (ATCC CRL-1721TM) were grown in RPMI 1640-medium (Gibco), supplemented with 10% heat inactivated horse serum (Gibco), 5% heat inactivated fetectal bovine serum (Gibco), 100 U/mL penicillin/100 μg/mL streptomycin (Gibco) and 2 mM l-glutamine (Gibco) in a humidified incubator with 5% CO2 at 37 °C. The cells were grown as adherent cultures on poly-τ-ornithine-hydrobromide coated (final concentration 100 μg/mL H2O, Sigma Aldrich) T-25 flasks to a confluence of approximately 80%. For assay performance, the cells were harvested from the flasks using a 0.05% trypsin/EDTA 1 x-solution (Gibco), were counted using a CASY cell counter (Roche) and were seeded after resuspension in cell culture medium at a defined concentration. For Caspase-Glo® 3/7 Assay Kit 1 x 10⁶ cells per well, for AlphaScreen® Sure Fire® Assay Kits 5 x 10⁴ cells per well, for CellTiter96® Aqueous One Solution Cell Proliferation Assay 5 x 10⁴ cells per well (naïve PC12 assay) or 1 x 10⁴ cells per well (PC12 differentiation assay) were seeded in coated 96-well-plate. For FACS, cell count and diameter analysis 1 x 10⁶ cells per 10 mL were seeded in coated T-25 flasks. The next day (cells x 12 h), the cells were either treated with Cerebrosylin, NFG or Prionex ± CoCl2. The CoCl2-solution (Sigma Aldrich) [stock: 10 mM-solution in sterile water stored at 4°C] diluted with cell culture medium was added to a final concentration of 150 μM CoCl2 for naïve PC12 cells and 300 μM CoCl2 for differentiated PC12 cells. For medium control conditions, CoCl2 was replaced by the corresponding volume of cell culture medium. Cerebrosylin (CR) was added at various final concentrations of 0.1%, 0.5%, 1%, 2%, 3% and 5%. The following batches were used: #237077, #201070 and #136086. Prionex® protein stabilizer solution from porcine collagen (1:10 diluted in H2O, Sigma Aldrich) at final concentrations of 1%, 2% and 5% served as an unspecific peptide control (UP). Nerve growth factor (NGF) 2.5S Native Mouse Protein (Invitrogen) in concentrations of 20, 50 and 100 ng/mL was used as a positive control for neurotrophic activity. Cells were incubated in these various conditions for 22 ± 2 h in the incubator (37 °C, 5% CO2). After the incubation they were further analyzed for caspase activation, metabolic activity/cell proliferation, cell diameter, cell count, reactive oxygen species (ROS), superoxide production, and for phosphorylation of Akt and GSK3β.

2.2. Differentiation of PC12 cells

1 x 10⁴ PC12 cells per well were seeded at 96-well-plate and then differentiated by NFG treatment for 6 days (day 0: seeding, day 1-5: 50 ng/mL and day 6: 100 ng/mL NFG); adapted from Das et al. (2004) (see also Fig. 3A). At the end of the treatment, the NFG medium solution was removed and 100 μL well fresh medium was added to the cells before the specific treatment followed (procedure see previous chapter).

2.3. Caspase activation assay

The Caspase-Glo® 3/7 Assay Kit (Promega) was prepared according to the manufacturer's instruction and added to the wells (dilution 1:1). Cells were further incubated for 1 h at room temperature in the dark. Then, luminescence (RLU) was measured using a Tecan Infinite® M200 platterreader. The background luminescence was excluded by performing blank corrections (subtraction of RLU values obtained for the same treatment without cells).

2.4. Metabolic activity/cell proliferation – MTS

MTS-solution (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega) was added to each well (dilution 1:6) and cells were further incubated for 2 h at 37 °C and 5% CO2, before absorbance (OD) was measured at 490nm using a Tecan Infinite® M200 platterreader. Background absorbance was excluded by performing blank corrections.

2.5. Measurement of cell count/diameter of PC12 cells

After treatment the cells were detached by 0.05% trypsin/EDTA 1 x-solution. Then, the diameter of the cells and the cell count were measured using the CASY cell counter (Roche).

2.6. FACS analysis of reactive oxygen species (ROS)/superoxide levels

For the analysis of the generation of ROS and superoxides the total ROS/Superoxide Detection Kit (Enzo) was performed according to the manufacturer’s instruction. Briefly, after treatment of the cells the medium was removed and cells were detached, washed with 1 x wash buffer and then treated with the ROS/Superoxide Detection Mix for 30 min at 37 °C. Positive control (Pyocyanin) was applied 30 min before treatment with the ROS/Superoxide Detection Mix.

2.7. Sure fire® analysis of phosphorylation of GSK3β (p-Ser9), Akt (p-Ser473) and Akt1 (p-Thr308)

After treatment the medium was removed, the cells lysed and then treated with the assay reagents according to the manufacturer’s instructions (AlphaScreen® Sure Fire® GSK3β (p-Ser9) Assay Kit, AlphaScreen® Sure Fire® Akt (p-Ser473) Assay Kit, AlphaScreen® Sure Fire® Akt1 (p-Thr308) Assay Kit, Perkin Elmer). The signal was detected using the Alpha Technology on Perkin Elmer, EnSparc® 2300 platerreader.

2.8. Statistics

Data were expressed as mean ± standard deviation (SD). All analyzed data result from at least three independent experiments. Multiple comparisons were analyzed by ANOVA (parametric) or Kruskal–Wallis One Way Analysis of Variance on Ranks (non-parametric) and specific Post hoc Tests, as Tukey Test, Student–Newman–Keuls Method, or Dunn’s Method. A p value of p < 0.05 was considered as statistically significant.

3. Results

3.1. Cerebrosylin and NFG protect PC12 cells from CoCl2-induced cell death

In the first set of experiments, the effects of CoCl2 treatment on cell viability and morphology of naïve PC12 cells and the protective activities of the neurotrophic compounds NFG and Cerebrosylin were studied. Treatment of PC12 cells with 150 μM of CoCl2 induced shrinkage of cells (medium control: 13.3 ± 0.2 μm vs. CoCl2: 11.9 ± 0.3 μm) and significantly decreased the viable cell number suggesting the onset of cell death (Fig. 1A and C). This effect of CoCl2 on PC12 cells was not influenced by the unspecific peptide mix Prionex (5%) or 100 ng/mL NFG. Furthermore, shrinkage of PC12 cells was observed as the cell diameter of CoCl2 and CoCl2 + Prionex treated cells (11.7 ± 0.4 μm) significantly decreased (Fig. 1B). As expected from previous reports (Das et al., 2004; Greene, 1978: Young et al., 1983), 100 ng/mL of NGF induced neurite outgrowth in naïve PC12 cells (Fig. 1C) which was confirmed by microscopy analysis as well as by a significant increase in cell diameter (14.6 ± 0.8 μm). Cerebrosylin (5%), in contrast to NGF, did not induce process outgrowth in naïve PC12 cells, but protected from CoCl2-induced loss of viability and cell shrinkage (13.1 ± 0.6 μm) indicating the presence of a protective activity.

3.2. Cerebrosylin and to a lesser extent NGF protect naïve PC12 cells from CoCl2-induced metabolic collapse

The MTS assay was used as readout for cell viability/proliferation and metabolic activity. In naïve unstimressed PC12 cells, neither Cerebrosylin, nor NGF or Prionex had any effect on metabolic activity.
Fig. 1. Cerebrolysin and NGF protect PC12 cells from CoCl₂-induced cell death. The cells were stimulated for 24 h with medium, 5% Cerebrolysin (CB), 5% Prionex (UP), 100 ng/mL NGF, 150 μM CoCl₂, 150 μM CoCl₂ + 5% CB, 150 μM CoCl₂ + 5% UP, 150 μM CoCl₂ + 100 ng/mL NGF. (A) Shows the number of PC12 cells after treatment with the different substances. Note the significant decrease in cell number after 150 μM CoCl₂, 150 μM CoCl₂ + 5% Prionex and 150 μM CoCl₂ + 100 ng/mL NGF treatment. The cell death induced by these treatments is also seen in the microscopic pictures of (C). (B) Shows the mean diameter of PC12 cells, which is significantly decreased after 150 μM CoCl₂, 150 μM CoCl₂ + 5% Prionex and increased after NGF treatment compared with medium control conditions. The NGF induced neurite growth is seen in (C). Asterix marks a p-value of p < 0.05 with ANOVA, Student–Newman–Keuls Method (* compared to medium control). The values are presented as mean ± SD (N = 3).

Fig. 2. Cerebrolysin and to a lesser extent NGF protect naïve PC12 cells from CoCl₂ induced-metabolic collapse. (A) The metabolic activity of Cerebrolysin (CB), NGF and Prionex (UP) did not differ significantly from medium control conditions. (B) Treatment with 150 μM CoCl₂ compromised the metabolic activity. This toxic effect of CoCl₂ was significantly counteracted by Cerebrolysin and NGF (*). The protective effect of 2%, 3% and 5% Cerebrolysin (#) was significantly higher compared with NGF. Prionex had no protective effect. Asterix marks p-value of p < 0.05 (* compared to control). The values are presented as mean ± SD (N = 3). (A) Kruskal–Wallis One Way Analysis of Variance on Ranks, Dunn’s Method. (B) ANOVA, Tukey Test.
of the cells (Fig. 2A). The CoCl₂ treatment (150 μM) significantly reduced the cell viability (OD values: medium control: 0.65 ± 0.07 vs. 150 μM CoCl₂: 0.23 ± 0.03) (Fig. 2A and B). While the metabolic breakdown of PC12 cells was only partly prevented by NGF and not at all by the unrelated peptidemix Prionex, Cerebrolysin had a dose-dependent protective effect, indicating a Cerebrolysin-specific activity (Fig. 2B). This was further supported by the fact that the protective effect of 2%, 3% and 5% Cerebrolysin was significantly higher compared with NGF and reached unstressed control conditions of proliferative activity (OD values: medium control: 0.65 ± 0.07 vs. 5% Cerebrolysin: 0.70 ± 0.03).

3.3. Cerebrolysin protects differentiated PC12 cells from CoCl₂-induced metabolic collapse

In the next experiments we tested if Cerebrolysin has protective effects on differentiated PC12 cells. NGF treatment for 6 days induced the differentiation of PC12 cells as indicated by neurite outgrowth (Fig. 3A). In differentiated PC12 cells no significant cell stress at 150 μM CoCl₂ was detected (OD values: medium control: 0.84 ± 0.12 vs. 150 μM CoCl₂: 0.79 ± 0.03) whereas at elevated CoCl₂ concentrations (300 μM) metabolic activity was clearly abolished (OD value: 0.09 ± 0.01). Cerebrolysin reversed the negative effect of 300 μM CoCl₂ in a dose dependent manner (Fig. 3B). Neither NGF nor Prionex exerted a significant effect on cell survival, which clearly supports a Cerebrolysin-specific effect also in differentiated PC12 cells.

3.4. Cerebrolysin protects PC12 cells from CoCl₂-induced apoptosis

Since the observed cell shrinkage, the reduction in cell viability and the reduced metabolic activity were indicative for an apoptotic cell death, the activation of Caspases 3 and 7 in response to CoCl₂ was analyzed. Cerebrolysin induced a small increase in Caspases 3/7 in unstressed naïve PC12 cells compared to NGF treatment (Fig. 4A). However, these small changes did not correspond to reduced cell viability as demonstrated by the MTS readout (Fig. 2A). The treatment with 150 μM CoCl₂ induced a significant increase (approximately 7.4 fold) of Caspase 3/7 activity compared to control conditions (Fig. 4A and B). Cerebrolysin exerted a strong protective effect by inducing a significant, dose-dependent decrease of Caspase 3/7-activity already at 0.5% final concentration (Fig. 4B) and completely abolished Caspase 3/7 activation at higher concentrations. NGF also induced a significant protective effect, which however showed no dose-dependence in the range of 20–100 ng/mL and was significantly smaller than the effect of 1% Cerebrolysin. Since the unspecific peptidemix Prionex even increased the amount of Caspases 3/7 in CoCl₂-treated PC12 cells, this anti-apoptotic effect of Cerebrolysin was found to be specific.

PC12 cell differentiation caused a time-dependent massive increase of Caspase 3/7 activity in PC12 cells. After 6 days of differentiation neither 150 μM nor 300 μM CoCl₂ were able to increase the Caspase 3/7 activity. Therefore the apoptotic potential of CoCl₂ could not be studied with the Caspase-Glo® 3/7 Assay Kit. This increase of Caspase 3 activity in differentiated cells has been previously described as well as the fact that the neuronal Caspase 3 has not only apoptotic effects but mediates also non-apoptotic, physiological functions, such as synaptic plasticity (Fernando et al., 2005).

3.5. Decreased superoxide levels in CoCl₂-stressed PC12 cells co-treated with Cerebrolysin

One of the central hallmarks of neuronal, in particular of hypoxia-induced neuronal cell death is the production of ROS. To analyze the generation of ROS upon treatment with CoCl₂, we used a FACS based assay kit that allows to separately address the formation of superoxide anions and other reactive oxygen species. Treatment with CoCl₂ (150 μM) induced a clear and specific production of superoxides, which was completely reversed by Cerebrolysin (5%), whereas NGF (100 ng/mL) only partly reversed superoxide production and Prionex (5%) did not display any protective effect at all (Fig. 5). CoCl₂ induced superoxide production in 70% of the cells. This effect was in accordance with the previous observations, where the majority of PC12 cells underwent apoptotic cell death upon treatment with CoCl₂.

3.6. The neuroprotective effect of Cerebrolysin and NGF correlates with phosphorylation of GSK3β

Finally, we analyzed in CoCl₂-treated PC12 cells one of the most relevant signaling cascade for cell survival, namely the Akt/GSK3β signaling. Treatment of CoCl₂-challenged PC12 cells with Cerebrolysin (5%) led to a strong increase in the levels of phosphorylated GSK3β Serine 9 (S9) (Fig. 6), but had neither effect on...
phosphorylation of Ser473 of Akt nor on Thr308 of Akt (data not shown). NGF (100 ng/mL) also induced GSK3β phosphorylation independent of Akt-signaling pathway. Prionex (5%) was not able to target this pathway in CoCl2-stressed cells and showed therefore comparable low phospho-GSK3β levels as 150 μM CoCl2 only (Fig. 6).

4. Discussion

The treatment of PC12 cells constitutes a well-established in vitro model of hypoxia that involves generation of ROS, suppression of mTor signaling (Zhong et al., 2014) and activation of the p38-MAPK-iNOS pathway (Lan et al., 2013). Here we demonstrate that Cerebrolysin protects PC12 cells from CoCl2-induced cell death. Our findings expand the illustrated neuroprotective and neurorestorative properties of Cerebrolysin in different neuronal in vitro systems (Hutter-Paier et al., 1996; Hartbauer et al., 2001; Gutmann et al., 2002; Zhang et al., 2013a,b). The protective effect of Cerebrolysin is supported by the complete and specific suppression of CoCl2-induced production of superoxides, prevention of CoCl2-triggered activation of Caspases 3/7 and maintenance of the metabolic activity of stressed naïve PC12 cells.

Furthermore we compared the effects observed with Cerebrolysin to the neurotrophin NGF. It is noteworthy that NGF and Cerebrolysin had different effects on CoCl2-challenged PC12 cells suggesting diverse underlying mechanisms. NGF promoted neurite outgrowth in naïve PC12 cells and displayed only partial protection from CoCl2-induced cell death. In differentiated PC12 cells, NGF showed no protective activity at all; suggesting, that the positive effect of NGF is related to its cell differentiation activity.

**Fig. 4.** Cerebrolysin protects naïve PC12 cells from CoCl2-induced apoptosis. (A) Cerebrolysin (CB 1–5%) induced slightly significant (*) higher RIU values compared to NGF. (B) Cerebrolysin (CB 0.5–5%) reversed significantly (**) the CoCl2 treatment-induced Caspase 3/7 activation. The protective effect of Cerebrolysin (CB 1–5%) was significantly (#) higher when compared to NGF. Prionex (UP) even elevated the effect of CoCl2 on caspase activation. Asterix marks p-value of p < 0.5 (**compared to control). The values are presented as mean ± SD (N = 3). (A) Kruskal–Wallis One Way Analysis of Variance on Ranks, Tukey Test. (B) ANOVA, Tukey Test.

**Fig. 5.** Cerebrolysin decreases superoxide levels in CoCl2-stressed PC12 cells. A representative example of FACS based analysis of ROS/superoxide generation is shown. Prionex (positive control) induced ROS and superoxide production. CoCl2 (150 μM) induced only superoxide production in 68% of the PC12 cells. An effect, which was completely reversed by 5% Cerebrolysin (<5% superoxide production).
Cerebrolysin, in contrast, did not induce neurite outgrowth, but strongly promoted cell survival of CoCl2-treated PC12 cells, independent of the final CoCl2 concentration (150 μM for naive and 300 μM for differentiated PC12 cells). The higher CoCl2 concentration, which was necessary to impair differentiated PC12 cells, indicates a higher stress resistance of these cells. An effect which has been previously described by Eksheyan and Aw (2005), who demonstrated a decreased vulnerability of differentiated PC12 cells due to enhanced peroxide reduction and increased redox cycle enzyme activities.

The CoCl2-induced neurotoxicity of PC12 cells involves a number of different pathways that might be targeted by Cerebrolysin. For example, CoCl2 exposure releases cytochrome c from mitochondria into the cytosol resulting in the activation of pro-apoptotic caspases and pro-apoptotic proteins such as Bax (Jung and Kim, 2004; Linford and Dorsa, 2002). In addition, it involves the death receptor pathway since CoCl2 upregulates expression of Fas and Fas-L in PC12 cells (Jung and Kim, 2004). Furthermore, CoCl2 induces the generation of ROS and the transcriptional change of hypoxia-related genes such as hypoxia inducible factor (HIF-1), p53 and p21, thereby mimicking hypoxia (Chandel et al., 1998; Wang et al., 2000). More recently it was also demonstrated that CoCl2 induced hypoxia is associated with suppression of mTor signaling (Zhong et al., 2014) and p38-MAPK activation (Lan et al., 2013). In the present study we found that also the phosphorylation of GSK3β was susceptible to CoCl2 induced hypoxia which is in agreement with previous findings in vascular smooth muscle cells using a physical model of hypoxia (Loberg et al., 2002).

In the present study, Cerebrolysin led to the phosphorylation of GSK3β at Serine 9 (Ser9) a modification associated with inhibition of the kinase, which among others consequently reduced the apoptosis rate (Cole, 2012). Phosphorylation of hypoxia inducible factor 1 (HIF-1) via GSK3β was demonstrated to prime the protein for proteasomal degradation (Mottet et al., 2003; Flugel et al., 2007). Since HIF-1 constitutes a key regulator that facilitates adaptation of cells from normoxia to hypoxia conditions, the protective effects of Cerebrolysin might at least partially be mediated via this pathway (Ke and Costa, 2006).

The observed GSK3βS9 phosphorylation appears to be not mediated via the Akt-signaling pathway in the CoCl2-stressed PC12 cells, as Cerebrolysin did not induce Akt phosphorylation at Ser473 or Thr308 in this experimental setup. This missing Akt activation suggests that the GSK3β phosphorylation at Ser9 might be mediated by different kinases, such as serum and glucocorticoid inducible kinase (SGK1) (Wyatt et al., 2006) or protein kinase A (Fang et al., 2000), which can phosphorylate GSK3β at Ser9. However the relevant pathways for GSK3βS9 phosphorylation upon treatment with Cerebrolysin are not yet fully characterized. Moreover, this finding might be clinically relevant for Alzheimer’s disease, since GSK3β activity has been involved in the production of amyloid beta (Rockenstein et al., 2006).

5. Conclusion
To summarize, Cerebrolysin is able to protect PC12 cells from CoCl2-induced apoptosis as shown by the reduction of Caspase 3/7-activity, inhibition of cell shrinkage and reduction of cell diameter. Furthermore, Cerebrolysin is able to restore the metabolic activity in CoCl2-stressed naive and differentiated PC12 cells. These protective effects might be associated with a decreased production of superoxides and the involvement of GSK3β pathway.

Conflict of interest
K.H, V.F, D.M, H.M, S.W are employees of EVER Neuro Pharma GmbH.

Acknowledgements
The present work was supported by a grant from EVER Neuro Pharma GmbH; by the European Union’s Seventh Framework Program (FP7/2007–2013) under grant agreements no. HEALTH-F2-2011-278850 (INMIND) and HEALTH-F2-2011-279288 (IDEA); and by the State of Salzburg (to L.A.).

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