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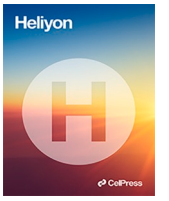

Research article

A Neurofilament-L reporter cell line for the quantification of early neuronal differentiation: A Bioassay for neurotrophic activities

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ABSTRACT

Background: Neurotrophic activity constitutes a crucial factor in the recovery from neurological injuries and is impaired in neurodegenerative disorders. Preclinical studies of neurotrophic factors to improve outcome of neurodegenerative diseases have yielded promising results. However, due to the complexity of these therapies, the clinical translation of this approach was so far not successful and more feasible treatments with neurotrophic activity may be promising alternatives. Therefore, highly sensitive and robust assays for compound screening are required.
New method: Nerve growth factor is known to induce Neurofilament-L (NF-L) expression in a rat pheochromocytoma cell line (PC12 cells) during early neuronal differentiation. We generated and characterized an enhanced green fluorescent protein (EGFP)-NF-L reporter PC12 cell line for the development of a cell-based assay (designated Neurofilament-L Bioassay) that allows straightforward quantification of early neuronal differentiation based on NF-L expression.
Results: Using Cerebrolysin® as a role model for a pharmacological compound that stimulates neurotrophic activity in the central nervous system, the Neurofilament-L Bioassay was proved to be a robust, specific, and reproducible method.
Comparison with existing method(s): It was already shown that NF-L expression correlates with neurite outgrowth in PC12 cells. Currently, quantification of neurite outgrowth is the most commonly used method to evaluate neuronal differentiation in PC12 cells, an approach that is time-consuming and of high variability.
Conclusions: This work describes the development of an EGFP-NF-L reporter PC12 cell-based assay as a robust and reproducible tool for "high throughput" compound screening for neurotrophic activity.

1. Introduction

Neurotrophic factors comprise a family of growth factors that are crucial for neuronal development as well as for the function and survival of adult neurons [1]. Upon acute neurological damage or neurodegenerative disease these factors are released to protect and regenerate the affected neurons. One important member of these neurotrophins is the nerve growth factor (NGF) [2,3].

The administration of NGF as a treatment of acute or degenerative neurological disorders is examined in various clinical studies, however, the use of NGF as therapy has its limitations since it is not able to cross the blood brain barrier and even showed adverse

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events in clinical trials [4]. To circumvent these challenges, there is an unmet need for the discovery of candidate molecules with neurotrophic activity for potential future drugs to treat neurodegenerative diseases. Possible alternatives that are explored are small molecule drugs [5] or low molecular weight peptide mixtures such as Cerebrolysin® [6].

Cerebrolysin® is a neuropeptide preparation consisting of biologically active, low molecular weight peptides, stabilized by free amino acids. It mimics the action of endogenous neurotrophic factors on brain protection and recovery and is currently used in multiple countries worldwide for treatment of ischemic and hemorrhagic stroke, traumatic brain injuries, different forms of dementia (vascular dementia, Alzheimer's disease), cognitive disorders and for the prevention of cognitive decline after brain injuries [6].

Neurotrophic factors are known to act pleiotropic; they promote neurogenesis, neuronal survival, neuronal differentiation, and maintenance of neuronal function [1]. One aspect of neurotrophic action, the neuronal differentiation, can be examined *in vitro* using a well-established cell model: the single cell clonal line PC12, derived from a rat pheochromocytoma by Greene and Tischler [7]. PC12 cells respond to NGF treatment by the formation of neurites, a major characteristic of neuronal differentiation. Currently, quantification of neurite outgrowth by counting the number of (i) cells with neurites, (ii) neurites per cell, (iii) branches per neurite or (iv) by measuring the length of the neurites represents the most common readout for the assessment of neuronal differentiation. However, this approach has its limitations as counting single neurites or measuring their lengths is a time-consuming and technically difficult task and consequently, the results often are highly variable.

Thus, to overcome these limitations, researchers evaluate genes or proteins that are known to be expressed during NGF-induced differentiation of PC12 cells as biomarkers of neuronal differentiation [8,9]. A promising candidate is Neurofilament-L (NF-L), a neuron-specific intermediate filament expressed early in neuronal development that serves as cytoskeletal basis of neuronal cell extensions like axons together with Neurofilament-M and Neurofilament-H [10,11]. Ohuchi et al. [12] and Schimmelpfeng et al. [13] have shown that NF-L mRNA expression as well as protein amounts constitute valid markers to evaluate PC12 cell differentiation, additionally giving the opportunity of quantification.

Likewise using NF-L as a readout, our study describes the development and validation of a cell-based assay suitable for the screening of compounds that promote neuronal differentiation. Using established neurogenesis inducers such as NGF and Cerebrolysin® we demonstrate that these agents are able to induce early neuronal differentiation based on NF-L expression levels as a readout. To facilitate the analysis, an enhanced green fluorescent protein (EGFP)-NF-L reporter PC12 cell clone, designated PC12-B, was established, which allows higher throughput and a more robust quantification of NF-L expression.

2. Material & methods

2.1. Material

PC12 cells were obtained from the American Type Culture Collection (ATCC). PC12-B (EGFP-NF-L reporter PC12 cell clone) cells were derived through a monoallelic knock-in of EGFP at the NF-L locus at chromosome 15 of PC12 cells using CRISPR-Cas9 (Horizon Discovery Ltd).

Cerebrolysin® and an amino acid solution reflecting the free amino acid component of Cerebrolysin® were provided by EVER Neuro Pharma GmbH.

RPMI 1640 w/o Glutamine, FBS, Horse Serum, L-Glutamine, Penicillin/Streptomycin, WFI, Trypsin-EDTA solution and Dulbecco's PBS were obtained from Gibco™ (Thermo Fisher Scientific). Poly-L-Ornithine Hydrobromide, Prionex and Methanol were obtained from Sigma (Merck). Tween20 was obtained from Carl Roth, Normal Goat Serum and DAPI Staining Solution from abcam, human beta-NGF from alomone, antibodies for intracellular staining from Cell Signaling Technology and 7-AAD dye from BD Biosciences.

2.2. Cell culture

PC12 and PC12-B cells were maintained in RPMI 1640 medium containing 8.5 % Horse Serum, 4.3 % FBS, 1.7 mM L-Glutamine and 85.5 U/85.5 µg/ml Penicillin/Streptomycin on Poly-L-Ornithine Hydrobromide coated flasks at 37 °C and 5 % CO_2 and passaged twice a week. Cells were used with a biological age between 13 and 50 passages.

2.3. Intracellular staining

PC12 cells were seeded in Poly-L-Ornithine Hydrobromide coated 6-well plates one day before the first treatment with the indicated concentrations of Cerebrolysin® or human beta-NGF. The day after, half of the volume in the wells was replaced with fresh treatment solution. 5 days after seeding cells were harvested, fixed with ice-cold methanol, permeabilized (0.1 % Tween20), blocked (10 % Normal Goat Serum, 0.3 M L-Glycine) and stained with a monoclonal antibody against NF-L and an appropriate secondary antibody. Data acquisition using BD FACSCalibur, data analysis using BD FACSuite v1.4.

2.4. Fluorescence microscopy

PC12-B cells were seeded in Nunc Lab-Tek II CC2 Chamber Slide Systems (Thermo Fisher Scientific) directly before the first treatment with 100 µl/ml Cerebrolysin®. One and four days after, half of the volume in the well was replaced with fresh treatment solution respectively. 6 days after seeding cells were fixed with ice-cold methanol, stained with DAPI Staining Solution and observed using Zeiss Axio Vert.A1 fluorescence microscope.

2.5. Neurofilament-L Bioassay

PC12-B cells were seeded in Poly-L-Ornithine Hydrobromide coated 6-well plates directly before the first treatment with the indicated concentrations of human beta-NGF, Cerebrolysin®, amino acid solution, or Prionex. The day after, half of the volume in the wells was replaced with fresh treatment solution. 4 days after seeding cells were harvested using Trypsin-EDTA solution and EGFP-NF-L reporter expression was quantified using flow cytometry.

According to their appearance in the forward/side scatter plot cells were gated, and viability was verified with a 7-Amino-Actinomycin D (7-AAD) dye staining (96.8 % ± 1 % of gated events; mean ± standard deviation, n = 24). The indicated relative expression represents the mean fluorescence intensity (MFI) of the samples divided by the MFI of untreated cells (control). The indicated relative potency represents the percentage of MFI of the samples relative to the MFI of the reference batch. Data acquisition using BD FACSLyric, data analysis using BD FACSuite v1.4.

2.6. Statistical analysis

Data are shown as box plot, scatter plot or mean ± standard deviation as indicated. Statistical analysis was performed using GraphPad Prism 7 (version 7.04).

3. Results

3.1. Cerebrolysin® induces Neurofilament-L expression in PC12 cells

PC12 cells have been previously used to quantify NGF-induced differentiation by means of NF-L mRNA expression or protein levels [12,13]. We could show that PC12 cells express NF-L not only after 3 days of treatment with NGF, but also with the neurotrophic drug Cerebrolysin® (Fig. 1A). Flow cytometry analysis revealed that around 17 to 55 % of the cells expressed NF-L upon treatment. NF-L was expressed reproducibly upon treatment with NGF or Cerebrolysin® significantly above control (untreated PC12 cells) (Fig. 1B); in short, at that point the flow cytometry data seemed very promising for the development of a cell-based assay, but was not feasible yet for high throughput analysis due to the elaborate method of intracellular staining.

3.2. Characterization of an EGFP-NF-L reporter PC12 cell clone for use in a cell-based assay

To facilitate NF-L expression analysis, a monoallelic knock-in of EGFP at the NF-L locus on chromosome 15 of PC12 cells was performed (Fig. 2A), resulting in EGFP-NF-L reporter PC12 cells, from which a single clone was picked and designated PC12-B. Correct targeting was verified by PCR sequencing and the presence of a full EGFP-NF-L protein was confirmed by Western blot analysis (data not shown). This cell clone expresses EGFP-NF-L reproducibly upon induction by either NGF or Cerebrolysin® treatment significantly above control (untreated PC12-B cells) (Fig. 2BCD).

The effect of various concentrations of Cerebrolysin® on the PC12-B cells was examined using 25 different Cerebrolysin® batches. Dose response curve analysis revealed that EGFP-NF-L expression levels are linear in a concentration range between 40 and 120 µl/ml

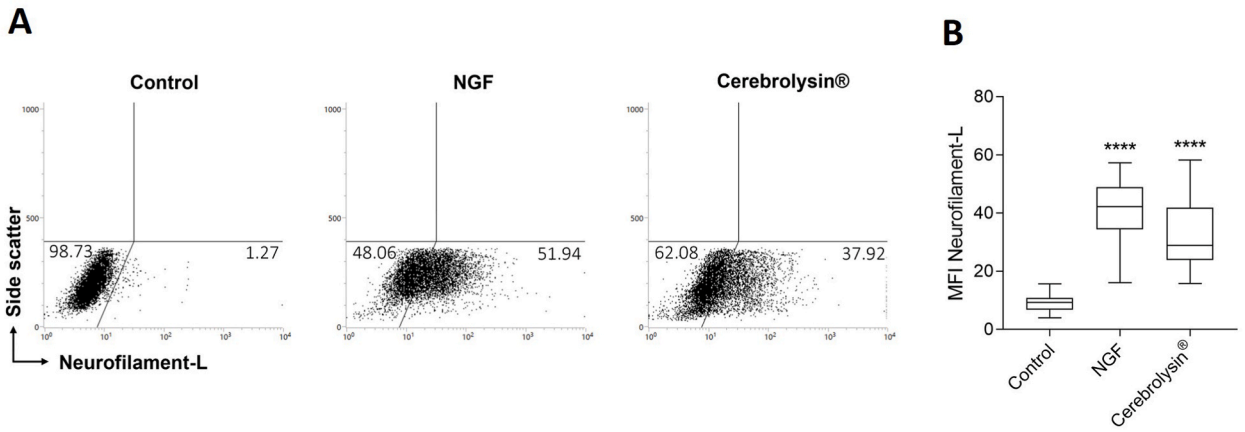

Fig. 1. Cerebrolysin® induces Neurofilament-L expression in PC12 cells. (A) Flow cytometry analysis representing Neurofilament-L expression of PC12 cells after treatment with 50 ng/ml NGF or 100 µl/ml Cerebrolysin® for 4 days. Untreated cells serve as a control. One representative out of 15 independent experiments. **(B)** Statistical analysis of MFI of NF-L from PC12 cells after treatment with 50 ng/ml NGF or 100 µl/ml Cerebrolysin® for 4 days. Boxes represent 25th to 75th percentile (including median), whiskers represent min to max. Control n = 19, NGF n = 15, Cerebrolysin® n = 31. ****$p < 0.0001$. Kruskal-Wallis test followed by multiple comparison of each condition against control, correction for multiple comparison using Dunn's test.

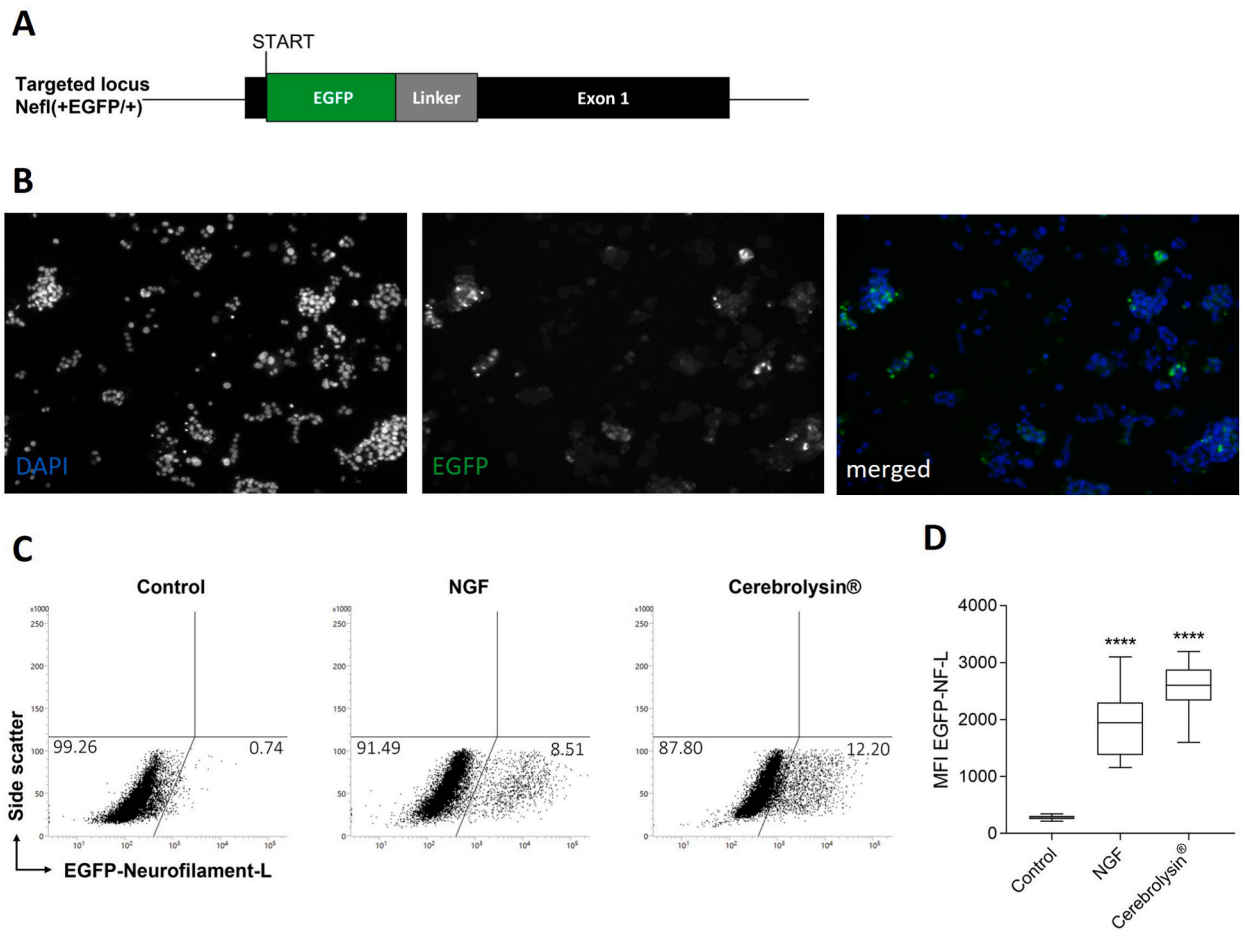

Fig. 2. Characterization of the EGFP-NF-L reporter PC12 cell line: PC12-B. (A) Scheme of the monoallelic knock-in of EGFP at the Neurofilament-L locus. Cell line generation was performed by targeting the locus of interest via CRISPR/Cas9. **(B)** Fluorescence microscopy of PC12-B cells after 6 days of 100 μl/ml Cerebrolysin® treatment. **(C)** Flow cytometry analysis representing EGFP-Neurofilament-L expression of PC12-B cells after treatment with 50 ng/ml NGF or 100 μl/ml Cerebrolysin® for 4 days. Untreated cells serve as a control. One representative out of 25 independent experiments. **(D)** Statistical analysis of MFI of EGFP from PC12-B cells after treatment with 50 ng/ml NGF or 100 μl/ml Cerebrolysin® for 4 days. Untreated cells serve as a control. Boxes represent 25th to 75th percentile (including median), whiskers represent min to max. Control n = 31, NGF n = 28, Cerebrolysin® n = 25. ****p < 0.0001. Kruskal-Wallis test followed by multiple comparison of each condition against control, correction for multiple comparison using Dunn's test.

Cerebrolysin® (Fig. 3A).

In order to reduce assay complexity and to be able to measure Cerebrolysin®'s biological activity with higher throughput, we decided to use a single treatment concentration within the linear range, namely the 100 μl/ml Cerebrolysin® concentration. Repeated measurements of different batches showed the high technical precision of the cell-based assay as well as the excellent batch to batch consistency (Fig. 3B).

3.3. Development of a PC12-B cell-based assay

For biological assays, it is common to use a defined reference standard that each measurement is calibrated against [14]. Since there is no (inter)national reference standard available for Cerebrolysin®, we defined one batch as an in-house reference that all the measurements were compared to, resulting in the final unit "relative potency [%]". The selection of the reference batch is carried out based on its overall quality attributes such as representativeness of peptide composition and other physicochemical parameters. The reference batch can be kept constant for several years providing a highly stable baseline for the assay normalization. Furthermore, we designated this cell-based assay "Neurofilament-L Bioassay".

We could already show that the Neurofilament-L Bioassay is able to evaluate Cerebrolysin®'s NF-L inducing capacity, however, to be approved as a specific method, it must be able to differentiate compounds that are not able to induce NF-L expression. Specificity of the Neurofilament-L Bioassay was verified by measuring the free amino acids component (no peptides) of Cerebrolysin® as well as an unspecific peptide mixture (Prionex); both preparations were not able to induce EGFP-NF-L reporter expression in the PC12-B cells

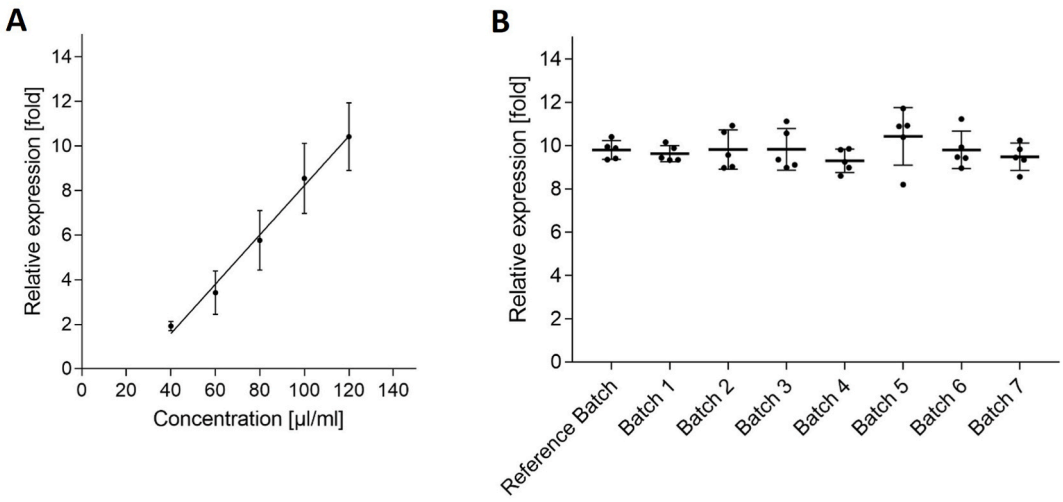

Fig. 3. EGFP-NF-L reporter expression is linear and precise. (A) Cerebrolysin® dose response of EGFP-NF-L expression (MFI of EGFP in Cerebrolysin®-treated relative to untreated cells). Linear regression of dose response data (shown as mean ± standard deviation) from n = 25 different Cerebrolysin® batches; $R^2 = 0.99$. **(B)** Relative EGFP-NF-L expression (MFI of EGFP in Cerebrolysin®-treated relative to untreated cells). n = 5 independent measurements per indicated batch, shown as scatter plot with mean ± standard deviation.

(Fig. 4). The repeated measurements of NGF in the Neurofilament-L Bioassay revealed significant NF-L inducing capacity. Fig. 4 additionally shows the robustness of the Neurofilament-L Bioassay, since the large number of measurements was carried out by different operators, on different days.

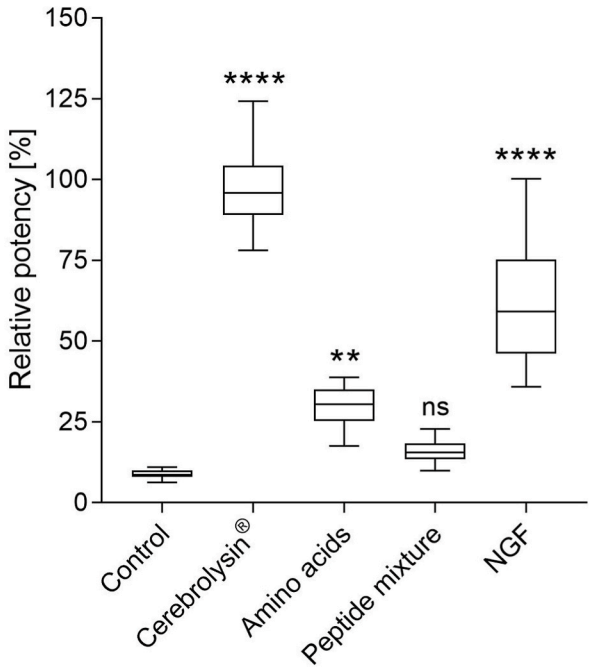

Fig. 4. EGFP-NF-L reporter expression upon Cerebrolysin® treatment is specific and robust. Neurofilament-L Bioassay data from PC12-B cells treated with 50 ng/ml NGF or 100 µl/ml of Cerebrolysin®, the amino acid component of Cerebrolysin® or an unspecific peptide mixture for 4 days. Untreated cells serve as a control. Boxes represent 25th to 75th percentile (including median), whiskers represent min to max. Control n = 25, Cerebrolysin® n = 50 different batches, amino acids n = 25, peptide mixture n = 25, NGF n = 50. ****$p < 0.0001$, **$p < 0.01$, ns = not significant. Kruskal-Wallis test followed by multiple comparison of each condition against control, correction for multiple comparison using Dunn's test.

3.4. Validation of the Neurofilament-L Bioassay

The excellent reproducibility of our PC12-B cell-based assay also allows to monitor biological activity of consecutive drug batches and implementation of the assay in quality control testing of pharmaceutical compounds. This concept was applied to the testing of consecutive batches of Cerebrolysin® by reducing assay complexity and verifying assay performance according to requirements set forth by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) [15].

In accordance with this guideline, we showed the linear relationship of EGFP-NF-L expression and Cerebrolysin® concentrations from 40 to 120 µl/ml and proved accuracy within this range (percentage recovery between 91 % and 112 %). Precision (8.85 % CV), robustness and specificity (Fig. 4) were evaluated for the 100 µl/ml Cerebrolysin® concentration.

Finally, the Neurofilament-L Bioassay was validated according to ICH guideline Q2 "Validation of analytical procedures" for its being specific, linear, accurate, precise, and robust for the measurement of Cerebrolysin®'s biological activity.

4. Discussion

Greene and Tischler [7] anticipated already in 1976 that the PC12 cells serve as "a useful model system for the study of […] development and differentiation of neural stem cells; initiation and regulation of neurite outgrowth" [7]. Indeed, evaluating neurite outgrowth of PC12 cells upon distinct treatments became a widespread tool in neurobiology to investigate neuronal differentiation. Schimmelpfeng et al. [13] refined the approach of quantifying neuronal differentiation revealing the correlation between neurite outgrowth and NF-L expression. With the Neurofilament-L Bioassay we seized on this idea but went one step further in terms of practicability and throughput: introducing a monoallelic EGFP reporter into the NF-L locus allows us to directly quantify neuronal differentiation. Furthermore, the dispense of staining procedures significantly improves reproducibility and robustness of the assay setup. We characterized the PC12-B cells for the measurement of two well established promoters of neurotrophic activity, the nerve growth factor and Cerebrolysin®, and furthermore validated the Neurofilament-L Bioassay for Cerebrolysin®. However, the assay setup may not be limited to these compounds, it could be used to screen for neurotrophic activity in any substance with relatively high throughput. Moreover, the accessible and straightforward approach of measuring the EGFP reporter makes the PC12-B cell clone a useful model system for studying NF-L signaling.

Neurodegenerative and acute neurological diseases (like stroke or traumatic brain injury) are both accompanied by the loss or damage of neurons. In order to cope with these disorders, substances that protect and help re-build neurons are urgently needed. Previous work already showed Cerebrolysin®'s beneficial effects on neurogenesis and differentiation by mimicking the action of neurotrophic factors [16]. Furthermore, a stabilizing and protective effect on axons was described for Cerebrolysin® based on increased phosphorylated Neurofilament-H levels [16,17]. Our data show that Cerebrolysin® enhances the expression of the developmental earliest neuronal intermediate filament, NF-L, which provides further insight into its neurotrophic activity.

4.1. Conclusion/Outlook

The Neurofilament-L Bioassay constitutes a powerful tool to screen for neurotrophic activity and to quantify the capability of any substance to induce early neuronal differentiation. Furthermore, the PC12-B cells could serve as a useful model system for studying Neurofilament-L signaling.

CRediT authorship contribution statement

Lisa-Franziska Seidl: Investigation, Methodology, Validation, Visualization, Writing – original draft. **Stefan Winter:** Supervision, Writing – review & editing. **Ludwig Aigner:** Supervision, Writing – review & editing. **Julia Schartner:** Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Julia Schartner reports financial support was provided by EVER Neuro Pharma GmbH; Julia Schartner reports a relationship with EVER Neuro Pharma GmbH that includes: employment; Lisa-Franziska Seidl reports a relationship with EVER Neuro Pharma GmbH that includes: employment; Stefan Winter reports a relationship with EVER Neuro Pharma GmbH that includes: employment.

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Abbreviations

EGFP: enhanced green fluorescent protein
ICH: International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
NF-L: Neurofilament-L
NGF: nerve growth factor
PC12: pheochromocytoma cell line
PC12-B: EGFP-NF-L reporter PC12 cell line