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Functional Pattern of Increasing Concentrations of Brain-Derived Neurotrophic Factor in Spiral Ganglion: Implications for Research on Cochlear Implants

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Abstract

BACKGROUND: As previously various studies have suggested application of brain-derived neurotrophic factor (BDNF) may be considered as a promising future therapy for hearing deficits, in particular for the improvement of cochlear neurone loss during cochlear implantation.

AIM: The present study's aim was to establish the upper threshold of the concentration of BDNF in Naval Medical Research Institute (NMRI) mice spiral ganglion outgrowth.

METHODS: Spiral ganglion explants were prepared from post-natal day 4 (p4) (NMRI) mice of both sexes under the approval and guidelines of the regional council of Hearing Research Institute Tubingen.

RESULTS: Spiral ganglion explants were cultured at postnatal days 4 in the presence of different concentrations of BDNF as described under methods. We chose an age of postnatal day (P4) and concentrations of BDNF 0; 6; 12.5; 25 and 50 ŋg/ml. Averaged neurite outgrowth is measured in 4 different cultures that were treated with different concentrations. Results show that with increasing concentrations of BDNF, the neurite density increases.

CONCLUSION: The present finding show evidence that BDNF has a clear incremental effect on the number of neurites of spiral ganglia in the prehearing organ, but less on the neurite length. The upper threshold of exogenous BNDF concentration on spiral ganglion explant is 25 ŋg/ml. This means that concentration beyond this level has no further incremental impact. Therefore our suggestion for hydrogel concentration in NMRA mice in future research should be 25 ŋg/ml.

Introduction

Over the past 20 years, cochlear implants (CIs) have been developed to improve hearing in patients with hearing impairment [1]. The CI is among the most important medical and biotechnological achievements because it allows individuals who had never heard or had lost their hearing to perceive sound [1, 2]. The levels to which neurotrophic factors can rescue the auditory nerve from differentiation-induced degeneration and promote regrowth are of both basic scientific and clinical interest [2]. Such regrowth may enhance the therapeutic efficacy of cochlear prostheses [3, 4]. In 2014, Pinyon and colleagues tested gene therapy on deaf guinea pigs [1]. Briefly, guinea pigs were made deaf with a drug that destroyed cochlear hair cells, and then cDNA encoding brain-derived neurotrophic factor (BDNF) was delivered to the cochlea by electroporation. The successful entry was detected using green fluorescent protein as a reporter. The electroporated cells began to release BDNF, which stimulated the outgrowth of neurones in the direction of the electrodes. The results were much better with added BDNF. However, the new nerves began to die because the cells no longer produced BDNF [5, 6]. Modification of the electroporated cDNA may facilitate the longer-term production of BDNF in the cells.

The present study was performed to calculate

the upper threshold of exogenous BDNF concentration in spiral ganglion outgrowth.

Materials and Methods

Our institutional ethics committee provided approval for this research on 10 June 2009. This study was performed by German laws regulating the use of animals in laboratory and experiments for research purposes (TSchG, 4§ (3)). All international and EU ethical norms were rigorously respected. All data were collected and processed using Microsoft Excel (Microsoft, Redmond, WA, USA) and SPSS 17.0 (SPSS, Chicago, IL, USA). The requirement for informed consent was waived due to the nature of the study.

Spiral ganglion explant culture

Spiral ganglion explant cultures were prepared from postnatal day 4 (p4) Naval Medical Research Institute (NMRI) mice of both sexes by the guidelines of the regional council. The animals were rapidly decapitated in accordance with German laws governing the care and use of animals (TSchG, §4 (3)). All subsequent procedures were performed under sterile conditions with confocal microscopy (Zeiss, Oberkochen, Germany). The skin was removed, and the skull was opened mid-sagittally. For this experiment, 20 mice were sacrificed, one mouse per ear. After removal of the brain, the temporal bone was harvested and transferred to a sterile petri dish filled with ice-cold HEPES-buffered Hanks' balanced salt solution (HHBSS). The bony capsule of the cochlea was removed to expose the membranous parts of the cochlea. The stria vascular, the organ of Corti, and the bone around the modiolus were removed, separating the entire spiral ganglion. The apical part of the spiral ganglion was transferred into a sterile culture dish coated with laminin, poly-D-lysine, collagen, and fibronectin (BD Biosciences, Franklin Lakes, NJ, USA). Spiral ganglion cultures were cultivated in serum-free medium. Neurobasal media was enriched by adding 2% B27 supplement, 0.2% penicillin, two mM HEPES, and 2.5% glutamine. BDNF (R&D Systems, Wiesbaden, Germany) was added to the medium. Cultivation was performed in an incubator (Heraeus, Hanau, Germany) at 37°C with 95% humidity and 5% CO_2 for 96 hours.

Spiral ganglion explant immunocytochemistry

Spiral ganglion explants were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), (Invitrogen, Karlsruhe, Germany) for 20 minutes, washed, and permeabilised with PBS containing 0.2% Triton-X for 5 minutes. Non-specific binding was blocked by incubation with 1% bovine albumin serum in PBS for 1 hour. Explants were incubated with primary antibodies, anti-neurofilament 200 kDa (NF200), and anti-glial fibrillary acidic protein (anti-GFAP) (1:800 dilution) at 4°C overnight. After three washes in 0.2% Triton-X in PBS, secondary fluorescence-labelled antibodies (cyan (Cy3) and Alexa 488) were added at a dilution of 1:400 (Table 1). The spiral ganglion explants were incubated for 1 hour at room temperature. Cell bodies were marked with 4',6-diamidino-2-phenylindole (DAPI) fluorescent DNA stain. Explants were rinsed with PBS, mounted on the microscope slide with fluorescent dye [with Alexa Fluor (Thermo Fisher Scientific, Waltham, MA, USA) and observed under normal epifluorescence using a Zeiss microscope. We counted only the number of neurites and not their length, as this experiment focused only on the trophic effect.

Table 1: Dual staining for identification of receptors on neurones in explants of immature spiral ganglia treated with 6, 12.5, 25, or 50 ng/ml BDNF at P4

Primary and secondary antibodies	0 ng/ml BDNF[1]	6 ng/ml BDNF	12.5 ng/ml BDNF	25 ng/ml BDNF	50 ng/ml BDNF	Category-Number
Neurofilament 200 mouse	1:400	1:400	1:500	1:800	1:800	Sigma #0142
GFAP[2] rabbit	1:400	1:400	1:600	1:800	1:800	DakoCytomation #Z0334
Cy 3[3] goat anti-rabbi	1:400	1:400	1:400	1:400	1:1000	ImmunoResearch Jackson 800/367/5296

1, Brain-derived Neurotrophic Factor; 2, Glial Fibrillary Acid Protein; 3, Cyan 3 (colour).

As described in the Methods section, spiral ganglion explants were cultivated in the presence of different concentrations of BDNF at postnatal day 4 (P4). Specifically, at P4, samples were treated with different concentrations of exogenous BDNF: group 1 (control), 0 ng/ml; group 2; 6 ng/ml; group 3,;12.5 ng/ml; group 4; 25 ng/ml; and group 5,;50 ng/ml. Twenty spiral ganglia were used in this experiment (four ears per group).

Statistical analysis

All data were analysed and are expressed as means ± standard deviation. Statistical analyses were conducted using Microsoft Office Excel 2013. The median neurite number, standard deviation, and *p*-value are shown in Table 2. The Kruskal–Wallis H test was used to determine the statistical significance of differences involving five independent variables. The significance of differences between groups was examined with the Mann–Whitney test using SPSS. In this study, the mean is indicated by the x-bar symbol ($\bar{x}$), the standard deviation is indicated by SD, the median is indicated by Me, and the 95% confidence

interval is expressed as 95% CI. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Results

The Kruskal–Wallis H test indicated statistically significant differences in individual neurite numbers among the different BDNF concentrations (H = 15.542, DF = 4, $p = 0.002371$): BDNF = 0 ng/ml, mean rank, 2.5.

The K statistic was 16.542733, and there were four degrees of freedom.

The significance of differences between groups was tested with the Mann–Whitney test. There were no significance differences between groups 4/5 ($p = 0.88$). The correction was performed with Bonferroni test. There were significance differences among groups except groups 4/5.

Table 2: Neurite number in spiral ganglion explants treated with 6, 12.5, 25, or 50 ng/ml BDNF at P4

BDNF concentration (ng)	Individual neurite number	Statistics
0	4 16 11 15	$\bar{X}$ = 11.5 SD = 5.45 95% CI = 5.36 – 17.63 *n* = 4, Mean rank = 2.5
6	20 35 29 32	$\bar{X}$ = 29.0 SD = 6.48 95% CI = 22.87 – 35.13 *n* = 4, Mean rank = 8.0
12.5	21 34 30 41	$\bar{X}$ = 31.5 SD = 8.35 95% CI = 25.37 – 37.63 *n* = 4, Mean rank = 9.0
25	55 44 51 48	$\bar{X}$ = 50.0 SD = 4.65 95% CI = 43.37 – 55.63 *n* = 4, Mean rank = 16.37
50	52 50 48 50	$\bar{X}$ = 50.0 SD = 1.63 95% CI = 43.87 – 56.13 *n* = 4, Mean rank = 16.62
Kruskal–Wallis test H	H = 16.542733 (DF = 4, *n* = 20)	*p* = 0.002371

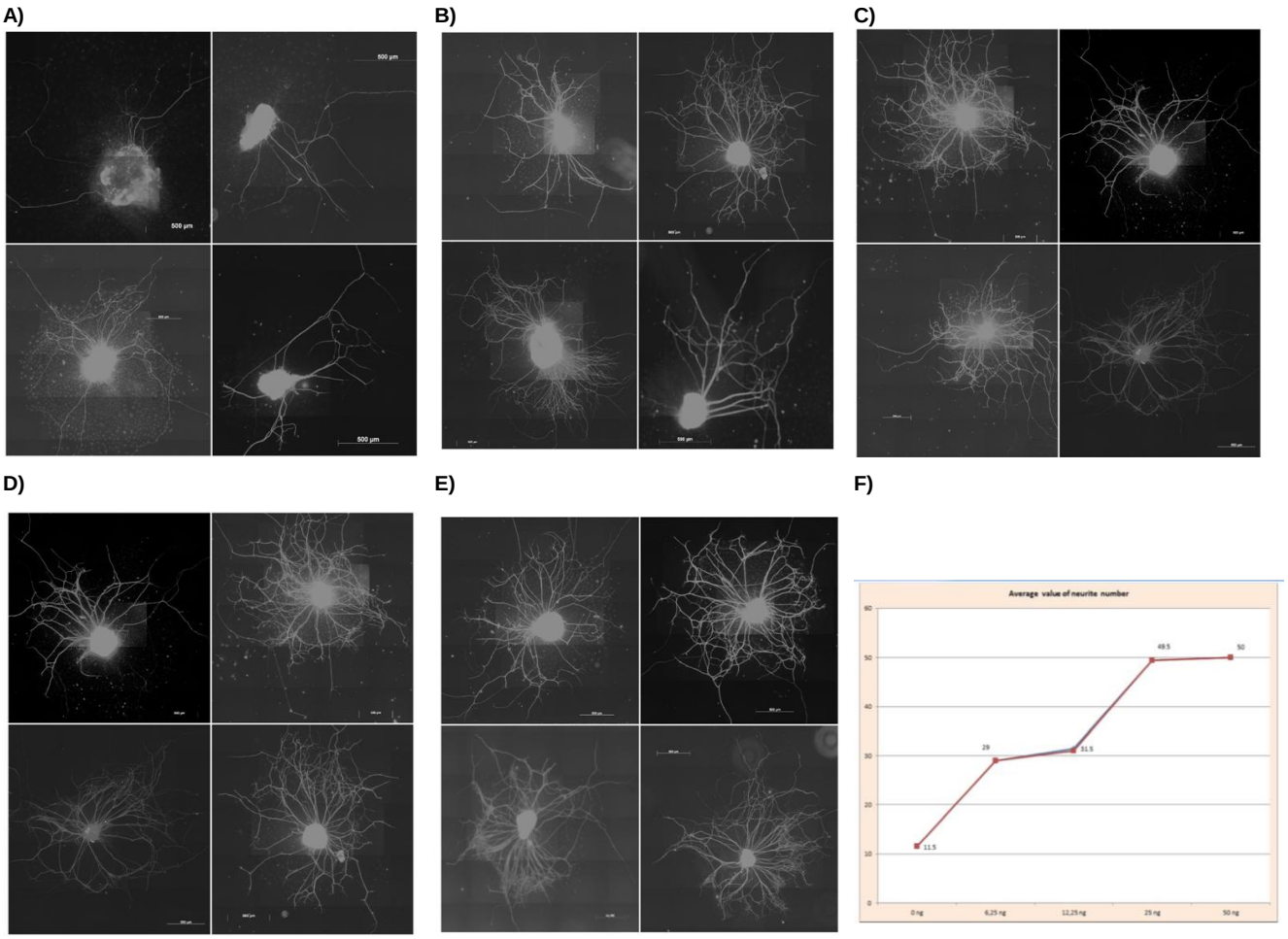

Figure 1: Spiral ganglion explants of NMRI mouse at P4. Staining performed with anti-NF200 and anti-GFAP antibodies. Scale bar represents 500 µm. 352 x 352 mm (72 x 72 DPI). A) BDNF = 0 ng/ml, mean rank, 2.5; B) BDNF = 6 ng/ml, mean rank, 8.0; C) BDNF = 12.5 ng/ml, mean rank, 9.0; D) BDNF = 25 ng/ml, mean rank, 16.37; E) BDNF = 50 ng/ml, mean rank, 16.62; F) Neurite number in spiral ganglion explants with 0, 6, 12.5, 25 ng, or 50 ng/ml BDNF at P4.

Discussion

Sensorineural hearing loss is caused by loss of neurotrophic factors, leading to cochlear hair cell damage, which is followed by degeneration of spiral ganglion cells [7–10].

Schwieger et al. reported that the functionality of CIs is dependent on the number and excitability of surviving spiral ganglion neurones (SGNs) [11]. Spatial separation between the SGNs located in the bony axis of the inner ear and the CI, which is inserted into the scala tympani, results in suboptimal performance in CI patients and may be decreased by attracting the SGN neurites toward the electrode contacts. Neurotrophic factors, including BDNF, can support neuronal survival and neurite outgrowth [12–14]. Postnatal Sprague–Dawley rats (P2–4) of both sexes were dissected to harvest tissue for cell culture experiments. The number of seeded neurones was determined after 4 hours of incubation.

Neuron-specific staining of the control indicated 805.6 ± 90 neurones/well. The survival rates under various conditions were calculated by determining the number of surviving neurones about the mean seeding density of the respective plate. After two days of incubation, the untreated control showed a survival rate of 3.57% ± 0.52%. Compared with the negative control, the neuronal survival rates in wells treated with 50 ng/ml BDNF (18.64% ± 0.79%) or 100 ng/ml ciliary neurotrophic factor (CNTF) (12.51% ± 2.27%) were significantly increased (BDNF: $p < 0.001$; CNTF: $p < 0.01$). BDNF showed a significantly higher neuroprotective effect than CNTF ($p < 0.05$). The highest neuronal survival rate was observed in SGNs treated with a combination of 50 ng/ml BDNF and 100 ng/ml CNTF (40.69% ± 3.52%; $p < 0.001$) compared with all other conditions. We concluded that the combination of BDNF and CNTF has great potential to increase both neuronal survival and the number of bipolar neurones *in vitro* and to regenerate retracted nerve fibres.

In agreement with our study, BDNF has been shown to increase the number of SGNs with increasing levels of exogenous BDNF [15–17]. This effect was demonstrated in this study in groups treated with different exogenous BDNF concentrations, ranging from 0 to 25 ng/ml. The results reflected significant differences in the numbers of individual neurites according to the different BDNF concentrations ($H = 15.542$, $DF = 4$, $p = 0.002371$): BDNF = 0 ng/ml, mean rank, 2.5; BDNF = 6.25 ng/ml, mean rank, 8.0; BDNF = 12.25 ng/ml, mean rank, 9.0; BDNF = 25, mean rank, 16.37; and BDNF = 50 ng/ml, mean rank, 16.62. To avoid the influence of other substances, such as CNTF, we used purely exogenous BDNF added to the cell culture medium.

Studies with other animals using similar methods yielded similar results [17]. Lake et al. reported that treating deafened cats with neurotrophins can improve SGN survival and functionality [17–24]. Neonatal deafened cats provided the first evidence of the neurotrophic effects of BDNF in the developing auditory system [24–25]. We used NMRI mice at P4 in the present study and concluded that BDNF has an increasing effect on the number of SGNs in this animal model.

Stöver et al. reported that intracochlear administration of BDNF (50 ng/ml) for 14 days starting on the seventh day after ototoxic treatment resulted in significantly improved survival of SGNs. They also demonstrated that BDNF, NT-3, NT-4/5, and NGF, each administered at a concentration of 62.5 µg/ml, can prevent the further degeneration of these cells after 14 days of deafness. Even where therapy with BDNF + FGF, NT-3 or CNTF was delayed until 1–6 weeks after deafening, this therapy still significantly protected the cells from degeneration [26].

Our results agreed with this previous study in that 50 ng/ml of exogenous BDNF resulted in significantly improved SGN survival. In the present study, however, there were no significant differences in neurite number or outgrowth between spiral ganglion explant groups 4 and 5 treated with 25 ng/ml and 50 ng/ml BDNF, respectively ($p = 0.88$). Thus, it would be possible to reduce the costs associated with the use of hydrogel containing BDNF by using a lower concentration while achieving the same clinical effect.

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