Delayed neurotrophin treatment supports auditory neuron survival in deaf guinea pigs

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As key factors in the development and maintenance of the auditory system, neurotrophins can prevent auditory neuron degeneration when applied within three to five days of deafening. We tested each of the neurotrophins BDNF, NT-3, NT-4/5 and NGF for their ability to support auditory neuron survival following a two-week

period of deafness in guinea pigs, when ~15% auditory neuron degeneration has already occurred. Although delayed, the treatment with each neurotrophin prevented further degeneration with similar efficacy. *NeuroReport* 15:1121–1125 © 2004 Lippincott Williams & Wilkins.

Key words: Delayed treatment; Neurotrophin; SGN survival

INTRODUCTION

Neurotrophins are key factors in the development and maintenance of the auditory system, and in the rescue of auditory neurons (spiral ganglion neurons; SGNs), from degeneration. Several studies report that neurotrophic factors from various families can prevent SGN degeneration in animal models of deafness [1–3] and can enhance the functional responsiveness of the auditory system [4]. Such studies have typically administered these agents within 3–5 days of deafening. Much less is known, however, about the effects of delayed neurotrophic factor treatment, which may be a clinically more realistic model, especially in cases of hearing loss such as presbycusis, which occur over long periods of time.

The duration of deafness prior to cochlear implantation is one of the key variables that affects post-operative performance, particularly in post-linguistically deafened adult patients [5]. In patients afflicted with a long period of deafness before implantation, perceptual benefits are consistently less than those in subjects with a short period of deafness before implantation [6]. Both post-operative speech recognition and the rate of improvement in performance over time appear to be lower and slower in patients who are deaf for a longer period prior to implantation [7,8]. This correlation between post-operative performance and duration of deafness may relate to the ongoing SGN degeneration that occurs following loss of hair cells [9], since a decreased SGN population may lead to decreased cochlear implant efficacy. Compounding the problem of ongoing SGN degeneration is the deafferentation that occurs following the onset of deafness, which has been reported to lead to decreased SGN functionality and increased excitation thresholds [10]. Consequently, increased periods of deafness

may inhibit the fidelity of the auditory signal by further decreasing the responsiveness of SGNs to electrical stimulation, leading to greater increases in excitation thresholds and further decreases in cochlear implant efficacy. If neurotrophic factors are to be considered for use in the clinical scenario, to maintain maximum SGN numbers and functionality, the efficacy of delayed neurotrophin treatment needs to be assessed. Specifically, it needs to be determined if neurotrophic factors can be effective in supporting SGN survival in cases of mid- to long-term deafness, when the degenerative processes have already commenced, or if they are only of benefit if applied shortly within the period of onset of hearing loss. Therefore, the current study investigated the effects of each of the members of the neurotrophin family, BDNF, NT-3, NT-4/5 and NGF, on SGN survival following a 2-week period of deafness in guinea pigs.

MATERIALS AND METHODS

Pigmented guinea pigs of both sexes (500–600 g) were used as the experimental subjects for this study under the approval and guidelines of the Animal Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital, Melbourne Australia. A total of 35 animals comprised the study, divided into seven groups. There were four treatment groups, in which all animals were deaf for 2 weeks, and then received intracochlear infusion of one of BDNF, NT-3, NT-4/5 or NGF for four weeks. The right cochleae in these animals acted as deafened, untreated internal controls. In addition there were three control groups: (a) normal hearing; (b) deafened, untreated (2 weeks); (c) deafened, untreated (6 weeks).

Experimental techniques were as previously described [11]. Briefly, animals were anaesthetised with ketamine (40 mg/kg; im) and xylazil (4 mg/kg; im), and normal auditory function was confirmed by measuring auditory brain stem responses (ABRs) to broadband click stimuli. Each animal was deafened using the loop diuretic frusemide (100 mg/kg; iv) and the ototoxic aminoglycoside kanamycin sulphate (400 mg/kg; sc). Two weeks following deafening the animals in the treatment groups were implanted with Alzet mini-osmotic pumps (model 2004), which delivered 10 µg neurotrophin directly into the left cochlea via a cannula inserted into the scala tympani.

Following the neurotrophin delivery (28 days), the animals were euthanised with pentobarbitone sodium (160 mg/kg) and perfused with 10% neutral buffered formalin (NBF), and the cochleae were harvested and processed for histology. Paraffin sections were cut at 5 µm and stained with dilute acidified thionin. The auditory neuron survival rate across each cochlea was determined by calculating neuronal density (the number of surviving neurons/mm²). A one-way ANOVA was carried out to determine the statistical significance of neurotrophin treatment on damaged SGNs, with a difference considered significant at $p < 0.05$. The presence of the neurotrophin trk receptors in these cochleae was assessed using standard immunohistochemistry (IHC). Each trk receptor antibody (Santa Cruz) was used at 1:100; and a standard neurofilament 200 kDa was used at 1:800. Sections were incubated in primary antibody overnight at 4°C. Following primary antibody incubation, the ABC method of a rabbit standard Vectastain kit (Vector, Burlingame, CA, USA) was used, as all primary antibodies were raised in rabbit. The biotinylated secondary antibody was diluted 1:200 and applied for 2 h at room temperature. The sections were then incubated in ABC reagents, diluted 1:100, for 1 h at room temperature, and labelling was subsequently visualized by reaction with the chromogen substrate diaminobenzidine (DAB; Vector, Burlingame, CA, USA). The immunostaining results were assessed in a qualitative manner, simply referring to either the presence or absence of a positive staining signal.

RESULTS

Delayed treatment with each of the neurotrophins supports auditory neuron survival in deafened guinea pigs: In normal hearing, control guinea pigs the mean ($\pm$ s.e.m.) neuronal density across both left and right ears was 652 ± 18 neurons/mm². These neurons displayed morphological and histological characteristics typical of healthy cells, with large cell bodies, round nuclei and identifiable nucleoli. In addition, the peripheral dendrites could be clearly seen in the osseous spiral lamina (OSL), reaching as far as the base of the hair cells. The healthy, intact hair cells within the organ of Corti were also easily identifiable in these sections.

Ototoxin exposure led to a complete loss of inner and outer hair cells, which was apparent in all deafened animals. Progressive auditory neuron degeneration was consequently observed, with 544 ± 13 neurons/mm² remaining after 2 weeks of deafness, equivalent to 83% survival compared to normal hearing controls. After 6 weeks of deafness, neuronal density rates were reduced to an average of 342 ± 16 neurons/mm², a survival rate of ~52%. The majority of neurons in these (deafened, untreated) cochlear

Fig. 1. The effects of delayed neurotrophin treatment on SGN survival following deafening. Guinea pigs were deaf for two weeks, then received intracochlear infusion of one of BDNF, NT-3, NT-4/5 or NGF for 4 weeks; time-matched, untreated controls were deaf for 6 weeks. Each neurotrophin prevented the SGN degeneration that occurred in time-matched, untreated controls. The results are expressed as neuronal density (neurons/mm²) with mean $\pm$ SEM, and as a percentage of normal hearing controls. $n = 5$ for each treatment group; * $p < 0.001$ vs time-matched untreated controls (deaf 6 weeks).

Table 1. Delayed neurotrophin treatment prevents SGN degeneration. Treatment with each of the members of the neurotrophin family prevented the ongoing degenerative effects of hearing loss. The number of surviving neurons is expressed as mean $\pm$ SEM.

Condition	No. surviving SGNs	% Survival
Normal	652 ± 18	100%
Deaf (2 weeks)	544 ± 13	83%
Deaf (6 weeks)	342 ± 16	52%
Deaf (2 weeks) + NT-3 (4 weeks)	567 ± 33	87%
Deaf (2 weeks) + BDNF (4 weeks)	555 ± 23	85%
Deaf (2 weeks) + NT-4/5 (4 weeks)	554 ± 39	85%
Deaf (2 weeks) + NGF (4 weeks)	518 ± 12	79%

sections showed morphological changes indicative of degeneration, including shrunken somata and misshapen nuclei, and few peripheral processes remained within the OSL. Delayed treatment with each of the neurotrophins, after a 2 week period of deafness, prevented the auditory neuron degeneration that was seen in time-matched, untreated controls (Fig. 1). The neurotrophins protected the survival of SGNs with the following potency: NT-3 > BDNF = NT-4/5 > NGF. These data are summarised in Table 1. In each of these cases, the survival rates were statistically significant ($p < 0.001$) when compared with the survival of auditory neurons in the time-matched, ototoxin-exposed, untreated inner ears. Furthermore, these neurons were similar in appearance to those in the normal hearing specimens, with large and round cell bodies, obvious nuclei and nucleoli, and peripheral processes apparent within the OSL (Fig. 2).

The neurotrophin trk receptors are present in the normal and damaged adult guinea pig cochlea: All cohorts (normal; deaf, untreated; deaf, neurotrophin-treated) showed positive labelling of the neuronal cell bodies when immunostained against the neurotrophin trk receptors (trkA, trkB and trkC). TrkB staining was also seen on the central axons,

Fig. 2. Photomicrographs depicting the lower basal Rosenthal's canal from the left (neurotrophin-treated) and right (untreated) cochlea of an animal from each of the neurotrophin-treated experimental groups: (a) BDNF, (b) NT-3, (c) NT-4/5 and (d) NGF. Sections were stained with dilute acidified thionin. It is apparent that delayed treatment with each of the neurotrophins prevents SGN degeneration, with the left neurotrophin-treated sections showing an increased number of surviving neurons as compared to the contralateral untreated sections. Arrows point to auditory neurons; arrow heads to vacuous spaces from which neurons have degenerated.

and on some non-neuronal cells within the spiral ganglion. Similar staining patterns were observed across all cohorts, with positive labelling localised to the same regions in each cochlea. Representative images of the immunostaining results are shown in Fig. 3.

Immunohistology with NF200 showed labelling of cell bodies and central axons of the cochleae of animals from each experimental group. NF staining was also present in the peripheral processes of normal hearing animals, and in animals treated with each of the neurotrophins. Predictably, in the deafened (untreated) control animals, neurodegeneration of the peripheral processes had occurred and no NF staining was observed.

DISCUSSION

Delayed neurotrophin treatment supports SGN survival: Neurotrophin treatment following two weeks of deafness in guinea pigs prevented SGN degeneration in comparison to time-matched untreated controls. In part, these results support findings from a previous study, in which Shah *et al.* investigated the protective effects of NGF on SGNs following a 2 week period of deafness, and observed that NGF treatment led to a significant increase in the number of surviving SGNs compared to untreated controls [12]. These results also expand upon previous studies in which BDNF and NT-3 prevent SGN degeneration after shorter periods of deafness [1–3]. Importantly, the potential of neurotrophins

to be used in clinical situations has been enhanced by the current discovery that they can have positive survival effects on SGN when degenerative processes are well underway.

In addition, each of the neurotrophins supported SGN survival with similar efficacy, suggesting that following trauma, SGNs have the capacity to respond to any of the neurotrophins to obtain trophic support for maintained survival. Importantly, this may also mean that SGNs may be responsive to a combination of neurotrophins, or even neurotrophic factors from various families. The stimulation of numerous receptors and their subsequent second messenger signalling pathways may therefore result in enhancement of the survival signal in an additive or synergistic fashion. Indeed, *in vitro* studies have demonstrated that combined neurotrophic factor treatment results in enhanced SGN survival. For example, the co-administration of BDNF and NT-3, and therefore the simultaneous activation of the trkB and trkC receptors, synergistically promotes SGN survival in comparison to treatment with either factor alone [13,14]. This may have important implications for therapeutic applications, as optimal SGN survival following deafening may require combined neurotrophic factor therapy. The effect of delayed neurotrophic factor treatment has also been studied in relation to auditory function. In a study by Yamagata *et al.*, evoked ABR (EABR) thresholds of deafened guinea pigs increased during a 2 week period of deafness, however treatment with BDNF

Fig. 3. Immunohistochemical staining of cochlear sections from adult deafened, neurotrophin-treated guinea pigs: (a) Neurofilament 200 kDa, (b) trkA, (c) trkB and (d) trkC. Staining against neurofilament clearly labels the neuronal cell bodies and central processes. Staining against each of the neurotrophin receptors shows the presence of these receptors on the neuronal cell bodies, and trkB was also localised to the central projections. These staining patterns were regardless of the neurotrophin treatment used, and in fact were also seen in normal hearing and deafened, untreated controls. Arrows point to auditory neurons (ANs), peripheral processes (pp) and central processes (cp).

plus CNTF led to decreased EABR thresholds [15]. Therefore, even in delayed circumstances neurotrophic factors can have a positive effect on electrophysiological sensitivity. It is of course important to realise that the rate of SGN degeneration in the guinea pig is much more rapid than that seen in the human deaf ear. Animal studies have reported a significant loss of neurons 2 weeks after ototoxin exposure [16], and ~50% degeneration as early as six weeks after deafening, as illustrated in the present study. In comparison, SGN degeneration in human patients extends over years [17,18]. However, regardless of the rate of SGN degeneration, the current results provide evidence that delayed neurotrophin treatment, commencing when the degenerative processes are well underway, can still protect the SGNs from further degeneration. More recently, longer periods of deafness prior to neurotrophic factor treatment have also been investigated. Treatment with BDNF and FGF-1 (fibroblast growth factor-1) has been shown to significantly enhance SGN survival in 3- and 6-week deaf guinea pigs, compared with untreated controls [19]. Importantly, this study also demonstrated that the degree of SGN survival enhancement decreased as the period of deafness increased [19], indicating that there is likely a critical period during which neurotrophic factors need to be administered in order to have beneficial effects. Indeed, it still remains to be determined precisely how long after the degenerative process commences that SGNs can be rescued. In our study the expression pattern of the trk receptors is

consistent with previous analysis of trk receptor expression within the developing cochlea [20–22]. These results therefore suggest that these expression patterns are maintained throughout life, even during periods of neuronal degeneration, and further support the theory that the neurotrophins are essential for maintained SGN integrity. By contrast, neurofilament labelling demonstrated the importance of neurotrophic factors for maintained SGN survival throughout adulthood, with peripheral processes clearly present in normal and neurotrophin-treated specimens, but in various stages of degeneration in the deafened, untreated cochleae. The presence of each of the trk receptors in both deafened and neurotrophin-treated cochleae suggests that following injury or trauma, the SGNs have the capacity to respond to any or all of the neurotrophins in order to maximise chances of survival. Indeed, this was shown to be true with the survival results observed in the current study. This is important in the context of designing pharmacological therapies for auditory deficits, as it indicates that any or all of these trophic molecules may be able to be used in the clinical scenario to enhance the size and integrity of the surviving SGN population.

CONCLUSION

Each of the neurotrophins can protect SGNs from ototoxin-induced degeneration when applied 2 weeks after deafening. In addition, each of the neurotrophin trk receptors are

expressed within the mature guinea pig cochlea, are similarly expressed within all turns of the cochlea, and these staining patterns are regardless of hearing status or the neurotrophin used. These results suggest that SGNs may have the capacity to respond to any or all of the neurotrophins following injury. As such, in cases of long-term deafness, combined neurotrophin treatment may be the ultimate means to protecting SGNs from degeneration.

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