

Real-time quantitative RT-PCR for low-abundance transcripts in the inner ear: analysis of neurotrophic factor expression

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Abstract

Real-time quantitative reverse transcription-PCR is a highly sensitive technology that allows high throughput quantification of gene expression. Application of this technique to the inner ear is potentially very important, but is not straightforward because tissue harvesting can be challenging, RNA yield from individual inner ears is low, and cDNA synthesis from scant RNA can be inefficient. To overcome these challenges, we tested many parameters and reagents, and developed an approach to reliably quantitate small changes in low-abundance transcripts. Using this technique we demonstrate the presence and quantify amounts of the neurotrophic factors neurotrophin 3 (NT-3), brain-derived neurotrophic factor (BDNF) and glial cell-line-derived neurotrophic factor (GDNF), in the cochlea and vestibular end organs of postnatal murine inner ear (P26). We show that out of the factors tested, BDNF is the only one differentially expressed between the cochlea and vestibular end organs, being 23.4 ± 0.3 times more abundant in the vestibular end organs. Within the cochlea, GDNF gene expression is 4.9 ± 0.2 times greater than NT-3 expression. Within the combined vestibular end organs, BDNF expression is 43.0 ± 1.5 times greater than NT-3 expression. Our results suggest that neurotrophic factors continue to play a role in the postnatal inner ear, in addition to their previously shown essential role during development.

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1. Introduction

Real-time quantitative reverse transcription-polymerase chain reaction (rt-RT-qPCR) is a highly sensitive technique that allows high throughput quantification of gene expression levels (Higuchi et al., 1993). This technique should be, in principle, of great aid to study gene expression in the inner ear, where the small tissue size prevents the use of techniques such as Northern blot. However, the inner ear has some specific characteristics that make reliable rt-RT-qPCR challenging. First, the ear is a small organ encapsulated in bone, which complicates reproducible surgical extraction of

anatomically distinct parts. Second, RNA yield from individual inner ear specimens is very low. Therefore, analysis of RNA quantity and quality by conventional methods (gel electrophoresis and spectrophotometry) would require a large fraction of each sample, leaving little sample for analysis of gene expression. Third, cDNA synthesis from the low amounts of RNA obtained from two mouse cochleae can be inefficient, resulting in dilute cDNA samples, making detection of low-abundance transcripts and small changes in these transcripts difficult. Thus, rt-RT-qPCR in inner ear samples is not straightforward.

In an effort to optimize methods to measure small changes in low-abundance transcripts in the murine inner ear, we tested many reaction parameters and current reagents. The techniques described in this paper were developed for murine specimens, but they could be easily adopted for other species. The appeal of de-

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veloping the techniques in mice is that mouse models have emerged as standards for characterizing mammalian gene function in vivo, through either transgenic or knockout animals. This paper focuses on one type of detection method, SYBR green, a fluorescent dye that binds specifically to the minor groove of double-stranded DNA. As the amount of double-stranded DNA increases during the amplification process of the PCR, so does the intensity of fluorescent signal. The SYBR green method is a general detection method that is accurate, reliable, fast, and less expensive than using fluorescent-labeled probes.

In this article we provide a detailed description of the technique, tools for data analysis and quality control, and we demonstrate the power of this technique by measuring the levels of expression of three neurotrophic factors in the cochlea and vestibular end organs of postnatal mice. The neurotrophic factors we focused on are neurotrophin 3 (NT-3), brain-derived neurotrophic factor (BDNF) and glial cell-line-derived neurotrophic factor (GDNF). These molecules have been shown to be critical for maintenance of inner ear innervation during development (reviewed by [Fritzsche et al., 1999](#)), and to be oto- and neuroprotective when exogenously added to inner ear (reviewed by [Altschuler et al., 1999](#)), but the levels of expression and roles of these molecules in the intact mature postnatal inner ear remain unclear. We studied the expression of neurotrophic factors in the mature postnatal (P26) ear and found that NT-3, BDNF and GDNF are expressed in cochlea and vestibular end organs, with GDNF mRNA being the most abundant in the cochlea, and BDNF mRNA being the most abundant in the vestibular end organs. These results suggest that GDNF provides trophic support in the postnatal cochlea. In the vestibular end organs, our results suggest that BDNF continues to provide a major trophic support postnatally, in addition to its previously shown essential role during development.

2. Materials and methods

2.1. Tissue dissection

The 26-day-old (P26) mice (FVBN strain) were euthanized in a CO₂ chamber, their coat wetted with 70% ethanol to prevent contamination of a surgical specimen with RNase-rich hairs, and decapitated. The skin of the head was inverted, and the ventral surface of the head exposed. Musculature over bulla cavities was removed and each bulla was pierced and removed to expose the inner ear. Otic capsules were removed and placed in a 35-mm tissue culture dish containing RNAlater (Ambion, Austin, TX, USA). The remnants of soft

tissue were removed from otic capsules and the cerebellar paraflocculi were removed from the cavity between semicircular canals. The bony otic capsule was removed by gently cracking the bone with fine forceps, thus exposing the membranous parts of the cochlea and vestibular end organs (i.e., the utricle, saccule, ampulae and semicircular canals). Two dissected cochleae from an animal were transferred into a well of a 24-well plate containing 400 µl of RNAlater. Similarly, vestibular end organs from an animal were placed in a different well. Dissected specimens from a single animal were stored at 4°C for up to 10 days before RNA extraction. Within that time period, there was no significant change in the subsequent yield or quality of total RNA, as evaluated by criteria described below.

2.2. Extraction of total RNA

Total RNA was purified using RNeasy spin columns (Qiagen, Valencia, CA, USA), whose advantages over phenol–chloroform-based procedures include simplicity of use, operator safety, speed and purity of the final product without the interference of autofluorescing chemicals such as phenol. Tissue stored in RNAlater was transferred using forceps into a sterile 2-ml tube. After addition of 600 µl of lysis buffer, the tissue was homogenized with a Polytron homogenizer (Kinematica AG, Cincinnati, OH, USA, probe PT-DA 1205/2EC) to maximize the yield and quality of total RNA. We found the polytron homogenizer to be more efficient than glass or plastic pestle-based homogenizers. The lysate was then applied to the spin column and RNA purified following the manufacturer's recommendations. Total RNA was eluted in 50 µl in two steps (30 µl followed by 20 µl).

2.3. DNase treatment of RNA

Total RNA from inner ear samples was treated both on-column and off-column. For on-column treatment, RNase-free DNase (Qiagen) was used for 30 min, otherwise following manufacturer's instructions. Upon RNA elution from the column, RNase-free DNase I kit (Invitrogen, Carlsbad, CA, USA) was used, closely following the manufacturer's protocols and assuming 1 µg of total RNA.

2.4. RNA quantification and assessment of quality

Since the quantity of total RNA from two cochleae was below the detection limit of regular spectrophotometers, a RiboProbe RNA quantitation reagent (Molecular Probes), which is a highly sensitive fluorescent nucleic acid stain, was initially used. An alternative approach, which we preferred, was based on Agilent

2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), which simultaneously determined RNA integrity and purity.

To determine if the RNA samples were significantly contaminated with genomic DNA, the samples were subjected to conventional PCR with primers for the housekeeping enzyme glyceraldehydes-3-phosphate dehydrogenase (GAPDH; forward: CACAGCTCATGC-CACTACTGCC, reverse: GCTCACCACCCTGTTG-CTGTAG). Since these primers were designed to recognize genomic sequence, the presence of a detectable PCR product on an ethidium-bromide-stained agarose gel indicated that the RNA sample was contaminated with genomic DNA, and thus was discarded. The 25 μ l PCR reaction contained: 1 μ l of RNA sample, 2.5 μ l of 10 \times PCR buffer with 1.5 mM MgCl₂ (GeneChoice, Frederick, MD, USA), 200 μ M each dNTP, 0.4 μ M each of forward and reverse primers, 1.25 U Taq polymerase (GeneChoice) and 18.75 μ l of H₂O. The cycling parameters were: 95°C for 1.5 min; 45 cycles of 93°C for 1 min, 60°C for 1 min and 72°C for 1 min; 72°C for 5 min.

2.5. cDNA synthesis

RT was performed with Omniscript (Qiagen), a reagent based on a modified reverse transcriptase that works efficiently when total RNA > 50 ng. For a 20 μ l reaction, the following reagents were used: 2 μ l of 10 \times buffer RT, 0.5 mM each dNTP, 1.5 μ M random hexamers, 10 U RNase inhibitor (Invitrogen), 4 U Omniscript reverse transcriptase, 1 μ l of RNase-free water and 10 μ l of sample RNA. RT was carried out at 37°C for 1 h, followed by 93°C for 5 min, then rapid cooling on ice. Samples without reverse transcriptase were processed in parallel and served as negative controls.

2.6. rt-qPCR

Measurements reported in this paper were made with an iCycler machine (BioRad, Hercules, CA, USA) and QuantiTect SYBR green PCR kit (Qiagen). The only idiosyncrasy of the iCycler system when used with the Qiagen's SYBR green kit is that samples need to be spiked with 10 nM fluorescein to ensure accurate detection of background fluorescence. A major advantage of the QuantiTect SYBR green PCR kit (Qiagen) is that all PCR reagents, except primers and template cDNA, are premixed and optimized for manufacturer-specified primer concentrations.

The following forward (F) and reverse (R) primers were used. For 18S rRNA (gene accession number X00686), F:CGGCTACCACATCCAAGGAA, R:GCTGGAATTACCGCGGCT (Hellstrom et al., 2001),

which generate a 187-bp amplicon. For BDNF (gene accession number NM_007540), F:GAT GCC GCA AAC ATG TCT ATG A, R:TAA TAC TGT CAC ACA CGC TCA GCT C, which generate a 82-bp amplicon. For NT-3 (gene accession number X53257), F:GCC CCC TCC CTT ATA CCT AAT G, R:CAT AGC GTT TCC TCC GTG GT, which generate a 83-bp amplicon. For GDNF (gene accession number U36499), F:GCC ACC ATT AAA AGA CTG AAA AGG, R:GCC TGC CGA TTC CTC TCT CT, which generate a 77-bp amplicon.

The measurements were carried out in a 96-well rt-RT-qPCR plate (BioRad). For each well, the 25 μ l reaction contained: 12.5 μ l of 2 \times QuantiTect SYBR green PCR master mix, 0.5 μ M each forward and reverse primer, 7.5 μ l of RNase-free H₂O and 2.5 μ l cDNA template. The cycling conditions were: 95°C for 15 min followed by 50 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 60 s. Upon PCR completion, PCR products were melted by gradually increasing the temperature from 54°C to 94°C in 0.5°C steps. Pipetting errors were minimized by using low-residue tips (Axygen Scientific, Union City, CA, USA). Each sample, loaded in quadruplicates, had a matched 'no-RT' control so that samples from up to 12 different animals were tested simultaneously. Having replicates of each sample allowed for determination of intra-assay reproducibility. In final analysis, the replicate that was farthest away from the mean was eliminated.

All animal procedures were approved by Children's Hospital Committee for the Use and Care of Animals. Children's Hospital is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

3. Results

3.1. Tissue dissection and extraction of total RNA

Accurate microdissection of different anatomical subsections of the inner ear membranous labyrinth is a key first step in ensuring reproducible amounts of starting material among different animals. When harvesting tissues from several animals, dissection can take a significant amount of time, during which RNA can degrade. We found that use of RNAlater improves the quality and quantity of RNA obtained from inner ear samples. RNAlater quickly permeabilizes tissue and protects RNA from hydrolysis by RNases, giving ample time for careful and thorough dissection. Since RNAlater can affect RNA purification (see below), reducing RNA yield, it is important to effectively remove this reagent.

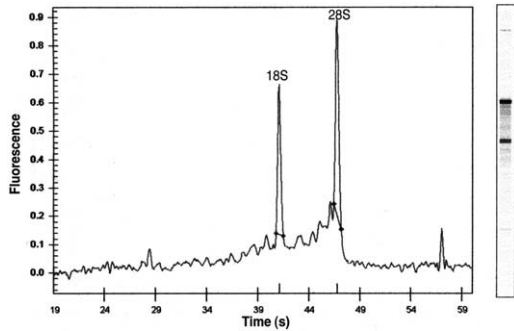


Fig. 1. Typical electropherogram of total RNA from two cochleae. Note two sharp peaks corresponding to 18S and 28S ribosomal RNA. The concentration of total RNA is 17.4 ng/ μ l.

3.2. DNase treatment of RNA

For rt-RT-qPCR, it is of paramount importance to minimize traces of genomic DNA, especially when studying mRNA transcripts from low-abundance genes and transcripts of intron-less genes. To this end, RNA samples need to be treated with RNase-free DNase. We found that both on-column and off-column DNase treatments are required to achieve sufficiently pure total RNA from inner ear samples, even though the off-column DNase treatment further dilutes RNA. The DNase treatment is necessary because the RNeasy spin column has a binding capacity of 100 μ g of RNA, which far exceeds the total RNA in our samples, thus leaving binding sites available for genomic DNA.

3.3. RNA quantification and assessment of quality

Typically, the amount of total RNA extracted from two cochleae is less than 1 μ g. Most spectrophotometers give accurate readings of absorbance at 260 nm (A_{260}) between 0.10 and 1.00 (corresponding to RNA concentrations of 4–40 μ g/ml), and the minimal volume required for analysis is usually 100 μ l. Thus, in most cases, more than 40% of a typical inner ear sample would have to be used to accurately measure RNA concentration by spectrophotometry. Similarly, RNA gel electrophoresis also requires a significant portion of the total RNA obtained from two cochleae. This problem can be overcome by using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The bioanalyzer separates nucleic acid by capillary electrophoresis in a chip with microfabricated channels and measures the amount of RNA by fluorescence, resulting in electropherograms, i.e., plots of fluorescence versus migration time in a microcapillary. Total RNA is deemed to be of ‘good quality’ when the electropherogram displays two sharp peaks corresponding to 18S and 28S ribosomal RNA (Fig. 1). The main advantage of the bioanalyzer is that it provides accurate

results with as little as 5 ng of RNA (less than 1% of the sample). Based on bioanalyzer measurements, our RNA yield from two mouse cochleae is 0.5–1 μ g (with RNA concentrations of 10–20 ng/ μ l). Although the bioanalyzer proved to be a powerful tool for quantifying and determining purity of dilute and scant RNA samples, we empirically found that the knowledge of the exact initial RNA concentration is not required for subsequent steps, so that proceeding to cDNA synthesis without prior RNA quantification is acceptable. As an alternative to assessment of RNA quality, we developed rt-RT-qPCR criteria for assessment of cDNA quality (see below), and the outcome is in general agreement with the outcome of the bioanalyzer analysis.

An important test for the quality of the RNA sample is to determine if it contains significant amounts of genomic DNA contamination. For this, RNA samples are subjected to conventional PCR before cDNA synthesis, using a set of primers that amplify genomic sequences. We used GAPDH primers, but any primer set that amplifies genomic sequence of any gene is an appropriate control for this step. If the PCR reaction is negative, the RNA sample is used for cDNA synthesis.

3.4. Primer and amplicon design

Special attention has to be given to primer and amplicon design for rt-RT-qPCR, which can be facilitated by commercial programs such as ‘Primer Express’ (Ap-

	s	r	E
◇ GDNF	-3.37	0.999	1.98
□ BDNF	-3.42	0.998	1.96
△ NT-3	-3.29	0.997	2.01
○ 18S	-3.40	0.999	1.97

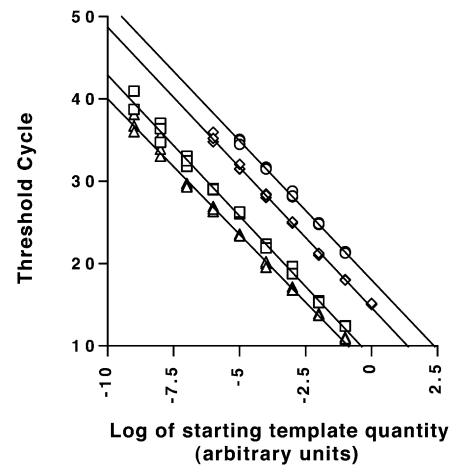


Fig. 2. Standard curves for 18S, NT-3, GDNF and BDNF primers. In the table, s=slope, and r=correlation coefficient of the standard curve; E=amplification efficiency, calculated from Eq. 1.

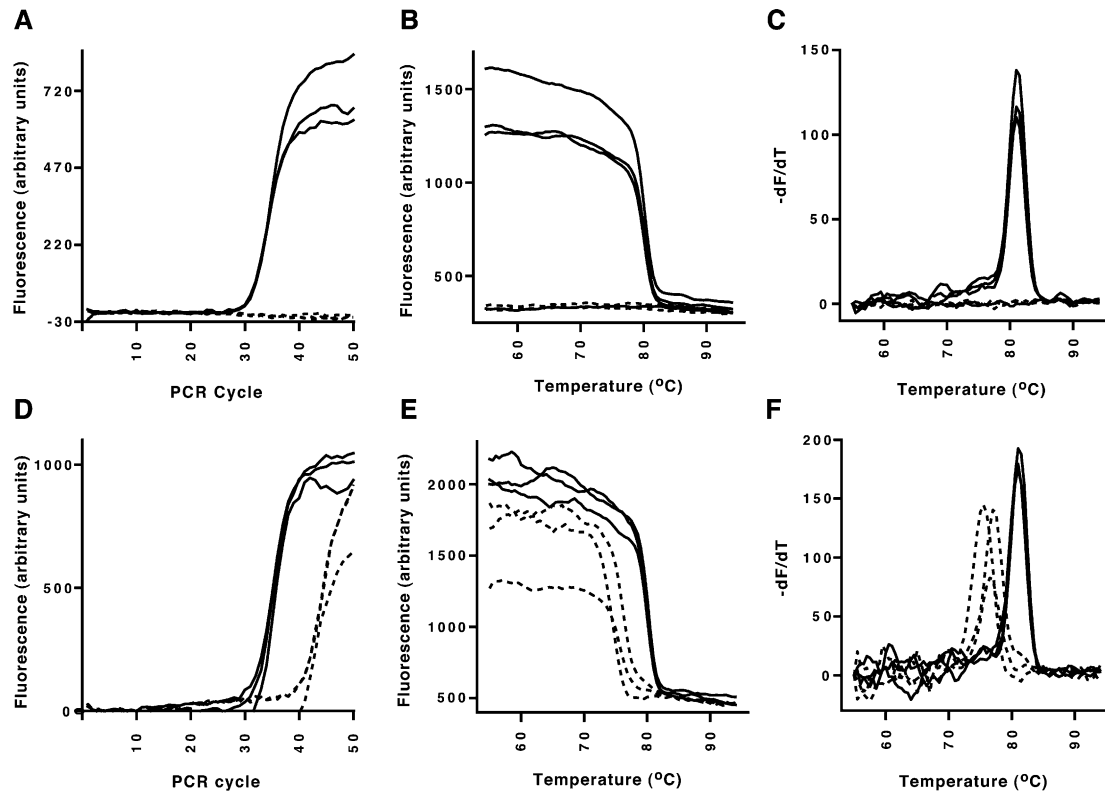


Fig. 3. Melting curve analysis for amplicons from two cochlear sample using GDNF primers. Triplicates of a single sample are overlaid; experimental samples ('RT sample') are shown with solid lines and no-RT controls with dotted lines. (A–C) Sample 511; (D–F) sample 460. The difference in the number of cycles between the experimental sample and no-RT control using 18S primers was 20.1 cycles (sample 511) or 22.5 cycles (sample 460).

plied Biosystems, Foster City, CA, USA). Specific primer requirements are discussed by Bustin (2000). When possible, primers should be targeted to separate exons to reduce the possibility of amplifying contaminating genomic DNA. Of course, this is not possible for genes without introns, such as some targets we have studied (18S rRNA, NT-3, BDNF). Even for genes with introns, such as GDNF, having intron-spanning primers is not absolutely required if genomic DNA is effectively removed. Overall, selecting for primers with constant and high amplification efficiency is more important than having intron-spanning primers. To verify that primers amplify the target amplicon, the PCR product is subjected to agarose gel electrophoresis, cloned and sequenced. Testing for amplification efficiency of primers, a key variable in the quantitative process, is described later.

3.5. The PCR reaction: fluorescence-versus-cycle curve and threshold cycle

The rt-RT-qPCR data are displayed as fluorescence versus PCR cycle number (e.g., Fig. 3A), where fluorescence is proportional to the amount of double-stranded DNA labeled by SYBR green, thus to the amount of

PCR product. We always found replicates to tightly overlap within the exponential phase of PCR, indicating high intra-assay reproducibility, even for very low-abundance genes (Fig. 3A). The replicates often differ in the abundance of the final PCR product, reflecting slight differences in reaction components, thermal cycling conditions or early mispriming events between adjacent wells of the same experimental plate (Bustin, 2000); these differences are inconsequential because all samples are analyzed in the exponential phase of PCR.

A key variable used to compare samples is the threshold cycle, C_T , defined as a PCR cycle at which fluorescence rises appreciably above background fluorescence (e.g., Fig. 4). When determining C_T for transcripts of the same gene arising from different samples on the same rt-qPCR plate, the same threshold fluorescence must be used for all samples. Because of this, the most prudent experimental design for relative mRNA quantification is to test for transcripts of a single gene (using a single set of primers) from as many different samples as possible in the same plate.

3.6. Standard curve

For meaningful quantitative comparison of levels of

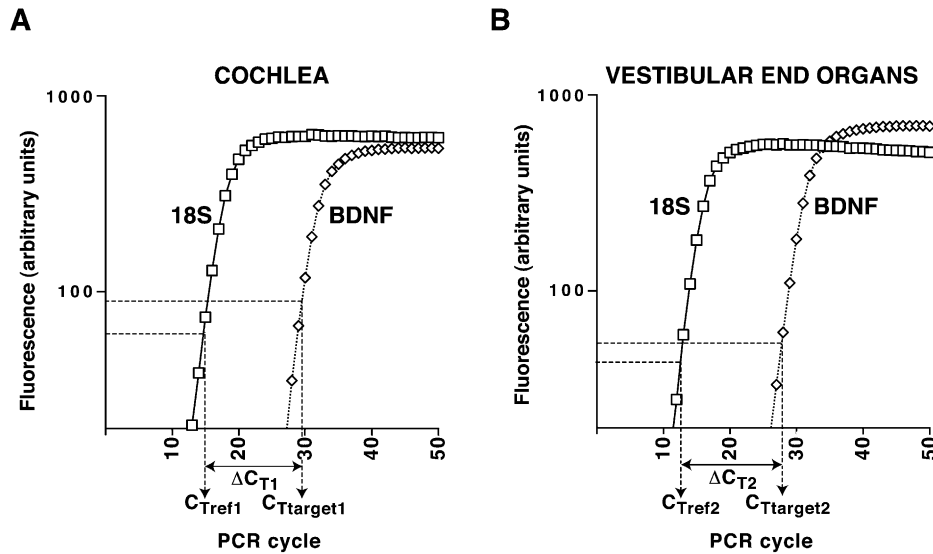


Fig. 4. An example of relative quantification of BDNF gene expression in vestibular end organs (B) relative to cochlea (A). For each sample, expression of the target gene BDNF (rhombs and dotted line) is normalized with 18S expression (squares and solid line) to arrive at $\Delta C_{T1} = \Delta C_{Ttarget1} - \Delta C_{Tref1}$ (A) and $\Delta C_{T2} = \Delta C_{Ttarget2} - \Delta C_{Tref2}$ (B). The expression of BDNF in the vestibular end organs relative to cochlea is calculated as $\Delta \Delta C_T = \Delta C_{T2} - \Delta C_{T1}$. Bars indicate the standard deviation.

expression of a specific gene among samples, amplification efficiency of the PCR must be constant over a wide range of target concentrations. Moreover, knowing amplification efficiencies of the different primer sets is critically important to choose the appropriate method of data analysis (as discussed below). Amplification efficiency and its constancy can be determined from a standard curve (Fig. 2), a plot of threshold cycle versus logarithm of the template amount, as follows:

$$\text{efficiency} = 10^{(-1/\text{slope})}. \quad (1)$$

Fig. 2 illustrates standard curves for all primers used in the study. All had a correlation coefficient of a linear fit close to 1, indicating constant amplification efficiency. Moreover, the selected primers displayed efficiencies close to the ideal amplification efficiency of two, i.e., doubling of amplicon during each PCR cycle, resulting in standard-curve slopes close to $-1/\log_2 = -3.32$. The standard curves in Fig. 2 were constructed using plasmids containing the relevant cDNAs since plasmids have optimal reproducibility over a wide range of concentrations, but a pure cDNA of known concentration would have been an acceptable alternative.

3.7. Criteria for evaluation of data

A final test for the quality of the rt-RT-qPCR reaction involves testing for potential confounding signals such as significant production of PCR products based on genomic DNA contamination and primer dimer formation. We tested for the former using control samples with reverse transcriptase omitted from the cDNA re-

action mix, and for the latter using melting curve analysis.

3.8. Controls without reverse transcriptase

Since rt-RT-qPCR is a more sensitive technique than ethidium bromide gel electrophoresis, RNA samples that appear not to have genomic DNA contamination by conventional PCR and ethidium bromide agarose gel electrophoresis ('no-RT' controls) can show detectable product when using rt-qPCR. Therefore, it is important to perform a no-RT control for each sample with rt-qPCR. If DNase treatment is performed, the C_T of no-RT controls is usually high ($C_T > 30$), thus not having significant impact on the quantification of the highly abundant mRNA transcripts whose C_T is many cycles smaller. However, for low- or very low-abundance transcripts whose $C_T > 30$, contamination with genomic DNA can preclude quantification.

We found that the difference in the number of cycles between C_T of the cDNA sample and no-RT control (i.e., 'no RT-RT criterion') for the reference gene 18S ribosomal RNA is a good predictor of whether quantification of low-abundance mRNA transcripts within the same sample will be possible. Using 32 cochlear samples, we found that the average difference between the sample cDNA and no-RT control for 18S gene was 20.6 cycles with a standard deviation of 2.4. Thus, we established a criterion that for 18S primers that we used, the no RT-RT difference had to be at least 18.2 cycles ($20.6 - 2.4 = 18.2$). This meant, assuming the ideal amplification efficiency of 2 (see Fig. 2), that genomic DNA was 3×10^5 times less abundant than cDNA. In this

particular data set, 93.8% (30/32) of samples met the ‘18S criterion’.

To quantify the levels of expression of neurotrophic factors, we generated 27 cochlear and vestibular samples, 26 of which met the ‘18S criterion’. Of these 26 samples, 18 had no detectable product in no-RT controls with NT-3, BDNF and GDNF primers. However, 5 of no-RT controls for BDNF and 9 of no-RT controls for GDNF primers (but never no-RT controls for NT-3 primers) gave rise to detectable amplicons. The C_T s of these amplicons from no-RT controls were substantially lower than C_T s of the samples. In those cases we applied the criterion that the difference in C_T of the sample and no-RT control for low-abundance transcripts had to be at least 10 cycles for data to be acceptable for further analysis. This criterion assured that non-specific amplicons were more than 1000-fold less abundant than target amplicons. Overall, the two criteria - one for 18S primers, the other for primers for low-abundance transcripts - selected for 74% (20/27) of samples (12 cochlear and 8 vestibular).

3.9. Melting curve

Since SYBR green binds to any double stranded DNA, the possible contribution of artifacts such as primer-dimers to the total fluorescence needs to be determined. A way to do that is to generate a melting curve (Ririe et al., 1997) that displays fluorescence (F) as a function of increasing temperature (T). When DNA melts, there is an abrupt decrease in fluorescence (e.g., Fig. 3B), which is most easily appreciated by plotting the negative derivative of the melting curve, $-dF/dT$ versus T (e.g., Fig. 3C).

Melting curve analysis is illustrated in Fig. 3 for GDNF primers applied to two different cDNA samples. In Fig. 3A,C, a single PCR product was present in experimental samples ($C_T = 31.30 \pm 0.05$), resulting in a melting curve with a single and sharp transition; no product was detected in no-RT controls (dashed lines). In Fig. 3D–F, although a single PCR product was present in experimental samples ($C_T = 32.04 \pm 0.09$), no-RT control gave rise to non-specific amplicons ($C_T = 40.9 \pm 0.4$) with lower and varied melting temperatures. Although this sample had met the ‘RT’–‘no-RT’ criterion for 18S, it was not used in further analysis because threshold cycles of the experimental sample and no-RT control differed by less than 10 cycles. Overall, we detected primer–dimer artifacts occasionally, and only in controls that had reverse transcriptase omitted from the cDNA reaction mixture, i.e., ‘no-RT’ controls. The occurrence of non-specific amplicons was minimized – and for the most part completely eliminated, even for very low-abundance templates – with careful primer design.

3.10. Data analysis

The ‘ $\Delta\Delta C_T$ ’ method (Livak and Schmittgen, 2001) is the most commonly used approach to analyze rt-RT-qPCR data to compare the levels of expression of a particular target gene under different experimental conditions, or the levels of expression of two genes in the same sample (one referred to as calibrator, the other as target). In this method, the amount of target gene normalized to an endogenous reference (e.g., a housekeeping gene), and relative to the calibrator is $2^{-\Delta\Delta C_T}$. The details of $\Delta\Delta C_T$ derivation are provided by Livak and Schmittgen, 2001. Briefly, $\Delta\Delta C_T = \Delta C_{T1} - \Delta C_{T2}$, where ΔC_{T1} is the difference in threshold cycles for target and endogenous reference genes, and ΔC_{T2} is the difference in threshold cycles for the calibrator and the same endogenous reference.

The main assumption of the $\Delta\Delta C_T$ method is that amplification efficiencies of all genes are identical and optimal. However, Livak and Schmittgen (2001) did not discuss the more generalized expression for calculation of relative gene expression levels when assumptions of the $\Delta\Delta C_T$ method are not met. Such a generalized expression follows from their approach and we discuss it here. Specifically, the normalized gene expression, NGE, during exponential phase of PCR can be defined as:

$$\text{NGE} = \frac{(E_{\text{target}})^{-\overline{C_{T\text{target}}}}}{(E_{\text{ref}})^{-\overline{C_{T\text{ref}}}}} \quad (2)$$

where E_{target} and E_{ref} are respectively amplification efficiencies of the target and endogenous reference genes, which are determined from standard curves using Eq. 1, and $\overline{C_{T\text{target}}}$ and $\overline{C_{T\text{ref}}}$ are respectively mean threshold cycles for the target and reference genes, which are determined from replicates. The NGE is equivalent to X_N/K (Livak and Schmittgen, 2001), where X_N is the ratio of the initial number of target and reference molecules and K is the ratio of the threshold fluorescence for the target and reference. The NGE is also the reciprocal of the quantity NE for normalized gene expression as defined by Muller et al., 2002. The variance of NGE, σ_{NGE}^2 , is calculated from the variances of $\overline{C_{T\text{target}}}$ and $\overline{C_{T\text{ref}}}$, denoted as $\sigma_{\overline{C_{T\text{target}}}}^2$ and $\sigma_{\overline{C_{T\text{ref}}}}^2$ respectively, by keeping linear terms in Taylor expansion of the expression for variance of a quotient:

$$\sigma_{\text{NGE}}^2 = (\text{NGE})^2 [\ln(E_{\text{target}})^2 \sigma_{\overline{C_{T\text{target}}}}^2 + \ln(E_{\text{ref}})^2 \sigma_{\overline{C_{T\text{ref}}}}^2]. \quad (3)$$

By dividing NGE of the target— NGE_1 by NGE of the calibrator— NGE_2 , we arrive at a generalized expression for calculating the amount of target gene, normalized to an endogenous reference and relative to a calibrator: $\text{NGE}_1/\text{NGE}_2$. The associated variance, denoted as $\sigma_{\text{NGE}_1/\text{NGE}_2}^2$, is:

$$\sigma_{NGE_1/NGE_2}^2 = \frac{1}{NGE_2^2} \sigma_{NGE_1}^2 + \frac{NGE_1^2}{NGE_2^4} \sigma_{NGE_2}^2. \quad (4)$$

To determine whether the difference in experimentally determined normalized gene expression of two targets is statistically significant, we use the bootstrap method (Efron and Tibshirani, 1993; Stankovic and Guinan, 1999). A major advantage of the bootstrap method is that it makes no assumptions about underlying statistical distributions (such as the assumption of Gaussian distribution). In the bootstrap method, the actual data are used to provide an estimate of the underlying distribution space of possible data by repeatedly randomly sorting the actual data and calculating statistic on each trial. The goal is to determine whether a certain value of a statistic might have arisen from chance. For example, for NGE_1 determined from p samples, and NGE_2 determined from q samples, we calculate the statistic, S , as $S = NGE_1 - NGE_2$. To determine if this value might have arisen by chance, we randomly sort (using Matlab 5.3, The MathWorks Inc., Natick, MA, USA) all NGE data into the ‘pseudo- NGE_1 ’ group containing p samples and the ‘pseudo- NGE_2 ’ group containing q samples, and calculate the same statistic (now called S_{pseudo}). The random sorting is repeated many times (we use 5000 to reach convergence) and a histogram of S_{pseudo} is plotted. This histogram provides an estimate of the real distribution of S , if NGE_1 is not different from NGE_2 in characteristics that determine S . The S from the original data is considered to be significant at the 0.05 level if it falls further from the mean than 95% of the S_{pseudo} histogram values.

3.11. Comparison of NT-3, BDNF and GDNF gene expression in the inner ear

The type of analysis outlined above allowed us to compare levels of NT-3, BDNF and GDNF mRNA in the cochlea and the combined vestibular end organs (i.e., the saccule, utricle, ampullae and semicircular canals) in healthy, wild-type mice. We compared 12 cochlear samples and eight vestibular samples from 26-day-old mice (P26); four vestibular samples consisted of peripheral vestibular organs from two animals since pooling was used in initial experiments. The pooled and non-pooled data followed the same trend. For all analyses, relative quantification of gene expression was used. Data are reported as mean \pm standard error of the mean.

For each of the neurotrophic factors under study, we compared the levels of gene expression in the cochlea and vestibular end organs. For this comparison, it was appropriate to use the $\Delta\Delta C_T$ method since slopes of the standard curves for each of the neurotrophic factors were less than 0.1 different from the slope of the stan-

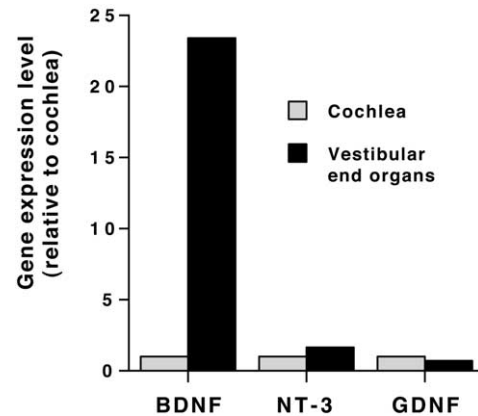


Fig. 5. Relative quantification of NT-3, BDNF and GDNF gene expression in vestibular end organs ($n=8$) relative to cochlea ($n=12$). For each of the neurotrophic factors, the amount of cochlear expression was set to 1. All error bars (depicting standard error of the mean) are smaller than the figure's resolution.

dard curve for 18S RNA, which was used as a reference (Fig. 2). We found BDNF to be the only neurotrophic factor tested that is differentially expressed between cochlear and vestibular samples, with BDNF gene expression in the vestibular end organs being 23.4 ± 0.3 -fold greater than in the cochlea ($\Delta\Delta C_T = -4.55 \pm 0.02$; $P < 0.00001$). There were no significant differences in the expression of NT-3 ($\Delta\Delta C_T = -0.73 \pm 0.04$; $P = 0.22$) and GDNF ($\Delta\Delta C_T = 0.52 \pm 0.04$; $P = 0.25$) between the cochlear and vestibular samples (Fig. 5).

In addition to comparing neurotrophic factor expression between cochlear and vestibular samples, we were interested in the relative expression of NT-3, BDNF and GDNF genes within the cochlear and vestibular samples. This entailed two steps: (1) normalizing expression of each neurotrophic factor with 18S, and (2) calibrating normalized BDNF and GDNF expression with the normalized NT-3 expression. For the second step it was not appropriate to use the $\Delta\Delta C_T$ method since slopes of the standard curves for NT-3, BDNF and GDNF differed by more than 0.1 (Fig. 2). Therefore, we used Eqs. 2–4 for both steps. As expected from studies that demonstrated an essential role for BDNF during development of innervation of the vestibular end organs (reviewed by Fritzsche et al., 1999), we found BDNF to be expressed at 43.0 ± 1.5 -fold greater levels than NT-3 ($P < 0.00001$), and 17.8 ± 2.5 -fold greater levels than GDNF ($P < 0.00001$) (Fig. 6B) in this tissue. No statistically significant difference was detected between expression of GDNF and NT-3 ($P = 0.89$). Surprisingly, in the cochlear samples, expression of GDNF was 4.9 ± 0.2 -fold greater than NT-3 expression ($P < 0.00001$) and 2.0 ± 0.2 -fold greater than BDNF expression although the latter barely met the criterion for statistical significance ($P = 0.048$). We detected no sta-

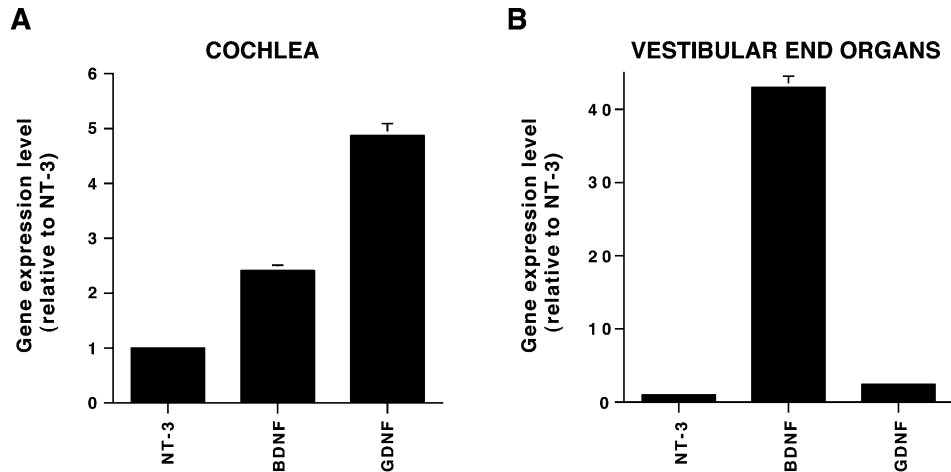


Fig. 6. Quantification of expression of BDNF and GDNF relative to NT-3 in cochleae (A) and vestibular end organs (B). For comparative purposes, NT-3 expression was set to 1. Note the change in the scale of the ordinate between (A) and (B).

tistically significant difference between BDNF and NT-3 expression ($P=0.30$) (Fig. 6A).

4. Discussion

4.1. Quantification of gene expression: relative or absolute

rt-qPCR can be used to quantify gene expression in absolute units (e.g., number of transcripts per cell, per unit mass of tissue or per concentration of total RNA) or relative to expression of a reference gene. For absolute quantification, on a single rt-RT-qPCR plate, an absolute standard curve has to be constructed for each individual amplicon to ensure accurate RT and PCR amplification (Bustin, 2000). Relative quantification determines the change in steady-state gene expression and is often adequate. When analyzing expression of multiple genes from a sample of total RNA, relative quantification is faster and less expensive than absolute quantification because (1) standard curves for each of the amplicons can be constructed on a separate rt-RT-qPCR plate, (2) standard curves are constructed only once, and (3) less rt-RT-qPCR reagents are used. An additional advantage of relative compared to absolute quantification for inner ear samples – where a small amount of starting RNA is a limiting factor – is that more target genes can be analyzed from the same sample of total RNA.

For relative quantification, testing expression of a single gene per rt-RT-qPCR plate from as many different samples as possible is a preferred experimental design because the same threshold fluorescence applies to all samples, and thus threshold cycles from all samples are directly comparable. In addition, if target mRNA

levels are not vastly different among different samples, quantification is straightforward since exponential phases of amplification overlap for different samples.

4.2. Amount of tissue necessary for rt-RT-qPCR analysis of inner ear samples

We found that RNA extracted from a single mouse (two cochleae or two sets of peripheral vestibular organs) is of sufficient quality and quantity to perform accurate measurements of at least five target genes. This is important for studies in which the number of experimental animals available is small and the target genes are expressed at low levels. We routinely measured with high inter- and intra-assay reproducibility (e.g., Fig. 3A) levels of expression of very low-abundance genes with C_T between 30 and 35 (Fig. 3A) in dilute inner ear samples from a single animal. Nevertheless, pooling tissues from several animals may result in cleaner RNA, and larger amounts of RNA should make it possible to study more target genes. Pooling of tissue is especially important when studying gene expression in fractions of cochlear or vestibular tissues, such as the sensory, neural or lateral wall components (e.g., Stover et al., 2000).

4.3. Data normalization

Correct interpretation of the collected data is arguably the most important part of a rt-RT-qPCR experiment. The choice of the appropriate reference gene is of paramount importance. Ideally, the reference gene should be unaffected by the experimental treatment, should be expressed at roughly the same level as the mRNA under study, and should be expressed at a constant level among different tissues of an organism at all

stages of development (Bustin, 2000). Since the ideal reference does not exist, it is important to be aware of the limitations of the most commonly used reference gene (reviewed in detail by Bustin, 2000). In the inner ear literature that deals with rt-qPCR and RT-PCR, the following reference genes have been used: GAPDH (Zheng et al., 1999; Uno et al., 2000; Uno et al., 2002), 18S rRNA (Yoshida et al., 1999; Liberman et al., 2002), tubulin (Staecker et al., 2001), and β -actin (Sawada et al., 2002). Recent evidence suggests that some of these reference genes, which have been traditionally used in quantitative Northern blots, are not appropriate for rt-RT-qPCR. For example, GAPDH level of expression varies significantly among human individuals, with experimental treatment, with developmental stage and cell cycle (reviewed by Bustin, 2000). We use 18S rRNAs as endogenous reference because its levels are less likely to vary under conditions that affect the expression of mRNAs since various rRNA transcripts are generated by distinct polymerases.

4.4. Relative quantification of expression of neurotrophic factors within the inner ear

While it is clear that neurotrophic factors are expressed and play important roles in the developing inner ear, their expression and roles in the postnatal and adult inner ear are not well understood. Numerous studies have analyzed postnatal inner ear expression of BDNF, NT-3 and GDNF at the RNA level (using in situ hybridization or RT-PCR) or protein level [using immunostaining, ELISA (enzyme-linked immunosorbent assay) or Western blots], but many of the results remain contradictory. The high degree of specificity of rt-RT-qPCR has the potential to provide a reliable measure of neurotrophic factor mRNA expression that could be used to validate and interpret the results generated with other techniques. Furthermore, although quantification of mRNA levels does not necessarily directly translate into quantification of biologically active protein, these measurements can provide important insights into developmental processes and into the mechanism of inner ear pathogenesis. For example, rt-RT-qPCR allows for future quantitatively accurate and in depth analyses of the temporal pattern of expression of neurotrophic factors to the degree not achievable by other techniques. Moreover, finding alterations in the levels of mRNA for specific neurotrophic factors in the context of animal models of inner ear dysfunction provides insights into the mechanisms of these defects.

Our experiments not only demonstrated that NT-3, BDNF and GDNF are present within the cochlea and vestibular end organs of postnatal mice, but also quantify their relative levels of expression within the P26 inner ear. The relatively high levels of GDNF mRNA

in postnatal cochlea suggest an important postnatal trophic role for GDNF. Earlier studies found GDNF mRNA and protein in adult rat (Nosrat et al., 1996; Nam et al., 2000; Stover et al., 2000; Stover et al., 2001). A trophic role for GDNF in adult cochlea was suggested by experiments that demonstrated increase in cochlear GDNF levels following acoustic trauma (Nam et al., 2000; Altschuler et al., 2002; Kanzaki et al., 2002), suggesting that increased GDNF expression was an injury response likely aimed at protecting a damaged cochlea.

In the vestibular end organs, BDNF mRNA is very abundant (Fig. 6B), suggesting that BDNF signaling has important roles in the mature vestibular end organs, beyond those it plays role during development (reviewed by Fritzsche et al., 1999). Although not as abundant as BDNF, GDNF and NT-3 are also expressed at significant levels in the vestibular end organs, comparable to those in the cochlea (Fig. 5). The importance of NT-3 for vestibular function was demonstrated in NT-3 heterozygous mice that – after unilateral surgical labyrinthectomy – took twice the time to recover from balance deficits as their littermate controls (Gacek and Khetarpal, 1998). In contrast, BDNF heterozygous knockout mice had no delay in behavioral recovery. This suggested that NT-3 is the early trophic regulator of vestibular compensation. Authors of that study reasoned that NT-3 in central vestibular pathways had to be responsible, since NT-3 was thought to be absent from the adult vestibular end organs. In light of our findings, however, we suggest that the NT-3 involved in vestibular compensation most likely derives from the vestibular periphery. Beside the present study, we are not aware of any other study showing GDNF expression within vestibular end organs. Our findings suggest that it would be interesting to study the roles of GDNF in the adult vestibular system.

4.5. Alternative rt-qPCR methods

In this paper we focused on the simplest, fastest and least expensive version of the fluorescent rt-qPCR – the SYBR green method – where the fluorescent signal is generated by a dye intercalated into double-stranded DNA. Variants of this technique include hybridization probes (TaqMan or molecular beacons) that rely on fluorescence resonance energy transfer (Heid et al., 1996). Even for the variants of the technique, the overall principles of sample preparation and data analysis presented in this paper still apply.

4.6. rt-qPCR for inner ear samples

Although rt-RT-qPCR has already been applied to inner ear samples (Yoshida et al., 1999; Zheng et al.,

1999; Uno et al., 2000; Staecker et al., 2001; Uno et al., 2002; Liberman et al., 2002; Sawada et al., 2002), there is a wide range of the reagents used, how data were analyzed, and how much detail about specific steps of rt-RT-qPCR were provided. In order to critically evaluate quality of rt-RT-qPCR data, we propose that the following steps always be outlined when using this technique: (1) the nature of the sample, e.g., whether inner ear samples included the otic capsule, solution in which the sample was microdissected, and what method of tissue homogenization was used, (2) amplification efficiency of all primers and constancy of amplification efficiency for a wide range of template concentrations; this is gathered from the slope and correlation coefficient of standard curves, (3) primer sequences and lengths of expected amplicons since amplicon length most strongly affects amplification efficiency, (4) rt-RT-qPCR plate setup, specifying whether samples were run in replicates and whether standards were run on the same plate as experimental samples; this allows evaluation of the appropriateness of absolute vs. relative quantification, (5) whether no-RT controls were included in rt-RT-qPCR experiments; this allows quantification of the degree of genomic DNA contamination that may be undetectable by ethidium bromide gel electrophoresis, but may be significant enough to preclude detection of small changes in low-abundance transcripts, (6) whether melting curve analysis, gel electrophoresis, and sequencing of rt-RT-qPCR products were used to verify amplicons, (7) what reference gene was used and whether the reference changed with experimental conditions; sometimes, two or more appropriate housekeeping genes may be used as reference to add strength to data interpretation, especially if all reference genes give similar expression profiles for a given sample, and (8) specifics of how data were analyzed and whether all assumptions embedded in a specific data analysis method, such as the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), were verified. Addressing all of the above steps will allow correct data interpretation and verification of the experimental findings by the scientific community at large.

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