Deafness-related plasticity in the inferior colliculus: gene expression profiling following removal of peripheral activity

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Abstract

The inferior colliculus (IC) is a major center of integration in the ascending as well as descending auditory pathways, where both excitatory and inhibitory amino acid neurotransmitters play a key role. When normal input to the auditory system is decreased, the balance between excitation and inhibition in the IC is disturbed. We examined global changes in gene expression in the rat IC 3 and 21 days following bilateral deafening, using Affymetrix GeneChip arrays and focused our analysis on changes in expression of neurotransmission-related genes. Over 1400 probe sets in the Affymetrix Rat Genome U34A Array were identified as genes that were differentially expressed. These genes encoded proteins previously reported to change as a consequence of deafness, such as calbindin, as well as proteins not previously reported to be modulated by deafness, such as clathrin. A subset of 19 differentially expressed genes was further examined using quantitative RT–PCR at 3, 21 and 90 days following deafness. These included several GABA, glycine, glutamate receptor and neuropeptide-related genes.

Expression of genes for GABA-A receptor subunits β2, β3, and γ2, plus ionotropic glutamate receptor subunits AMPA 2, AMPA 3, and kainate 2, increased at all three times. Expression of glycine receptor α1 initially declined and then later increased, while α2 increased sharply at 21 days. Glycine receptor α3 increased between 3 and 21 days, but decreased at 90 days. Of the neuropeptide-related genes tested with qRT–PCR, tyrosine hydroxylase decreased approximately 50% at all times tested. Serotonin receptor 2C increased at 3, 21, and 90 days. The 5B serotonin receptor decreased at 3 and 21 days and returned to normal by 90 days. Of the genes tested with qRT–PCR, only glycine receptor α2 and serotonin receptor 5B returned to normal levels of expression at 90 days. Changes in GABA receptor β3, GABA receptor γ2, glutamate receptor 2/3, enkephalin, and tyrosine hydroxylase were further confirmed using immunocytochemistry.

Keywords: auditory brainstem, immunocytochemistry, microarray, neurotransmitter, RT–PCR.

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Plasticity in the mature mammalian central nervous system is often induced by changes in neuronal activity. This response, called 'activity-dependent plasticity', can involve changes in amino acid neurotransmitters and receptors which, in turn, can reshape neuronal responses and information processing.

The auditory system provides an excellent model for examining activity-dependent plasticity, because the level of neuronal activity can be easily manipulated. Deafness (full or partial) and noise over-stimulation have been shown to lead to plastic changes in the mature central auditory pathways (Syka 2002; Moller 2005). Changes that have been reported following deafness include changes in the tonotopic map (Robertson *et al.* 1989; Rajan *et al.* 1993), in cell size (Sie and Rubel 1992; Dodson *et al.* 1994; Lustig *et al.* 1994; Lesperance *et al.* 1995; Willott and Bross 1996; Moore *et al.* 1997; Niparko and Finger 1997; Araki *et al.* 1998; Edmonds *et al.* 1999; Niparko 1999; Nishiyama *et al.* 2000), changes in synaptic contacts and synapse morphology (Kazee *et al.* 1995; Huchton *et al.* 1997; Ryugo *et al.* 1997; Niparko 1999; Russell and Moore 2002; Lee *et al.* 2003), in neurotransmitters and receptors as well as their release, uptake and binding (Caspary *et al.* 1995; Milbrandt *et al.* 2000; Potashner *et al.* 2000; Vale and Sanes 2002), in ion channels (Storey *et al.* 2002; Mhatre *et al.* 2004), in transcription factors (Illing *et al.* 1999; Illing and Michler 2001; von Hehn *et al.* 2004), in oxygen reactive species (Ohlemiller and Dugan 1999; Ohlemiller *et al.* 1999; Shi *et al.* 2002), and in stress response molecules (Myers *et al.* 1992; Billings *et al.* 1995; Verstreken *et al.* 1996; Oh *et al.* 2000; Van Campen *et al.* 2002; Wang *et al.* 2002; Yeom *et al.* 2003). These changes can lead to problems such as poor speech perception and tinnitus when there is partial or incomplete deafness and can influence the efficacy of cochlear prostheses following profound deafness.

An auditory brainstem region in which activity-dependent plastic changes have been well characterized is the inferior colliculus (IC), a major center of integration in the ascending as well as descending auditory pathways. Following deafness, there is a marked change in the balance of excitation and inhibition, resulting in changes in synaptic strength, inhibitory strength and evoked responses (Bledsoe *et al.* 1995; Wang *et al.* 1996; Mossop *et al.* 2000; Vale and Sanes 2002) These are associated with changes in inhibitory and excitatory amino acids and their receptors, particularly with changes in the production (Raza *et al.* 1994; Milbrandt *et al.* 2000; Mossop *et al.* 2000) and release (Bledsoe *et al.* 1995; Caspary *et al.* 1995) of GABA, as well as changes in GABA receptors (Caspary *et al.* 1995; Milbrandt *et al.* 2000; Vale and Sanes 2000).

Given the large and varied number of activity-dependent changes reported in the auditory brainstem following deafness, changes in expression for a large number of genes might be expected in the IC following deafness. Genes associated with amino acid neurotransmission and its regulation would be specific candidates for differential expression. Gene microarrays provide a method of screening thousands of genes simultaneously. Such an analysis can test multiple amino acid neurotransmission genes, identifying new genes that show changes, while confirming those already shown by other methods. By using this method, we can also identify genes associated with other known deafness-related changes, as well as identify changes in expression of genes associated with novel processes.

We therefore used Affymetrix rat U34A GeneChip arrays to examine changes in gene expression in the rat IC at two times following bilateral deafening. As expected, many genes encoding proteins involved in neurotransmission showed changes in expression. We verified a subset of these genes using quantitative RT–PCR (qRT–PCR) and immunocytochemistry. We were also able to identify genes associated with other known changes with deafness as well as genes that have not previously been shown to be activity dependent.

Materials and methods

Experimental design

Rats were checked for normal hearing by auditory brainstem response (ABR) prior to the study and were randomly assigned to either a group that received bilateral deafening or to an untreated, normal-hearing group. Treated rats and the age-matched controls were processed for gene microarrays at 3 and 21 days following deafness and for qRT–PCR and immunocytochemistry at 3, 21 and 90 days following deafening. These time points were chosen based on previous studies showing either transient or permanent changes in neurotransmitter and receptors at these times (Bledsoe *et al.* 1995; Sato *et al.* 2000). For gene microarray experiments, rats were randomly divided into three groups containing 18 animals per group: Group 1, normal hearing; group 2, bilaterally deafened by cochlear ablation and assessed at 3 days following deafening; and group 3, bilaterally deafened by cochlear ablation and assessed at 21 days following deafening. Each of these three groups was further divided into six subgroups containing three animals each. Therefore, six experimental replicates were performed per group, with the pooled inferior colliculi from three animals comprising one replicate. Thus, there were six biological replicates per experimental group, three groups and 18 total chips assessed. For qRT–PCR one of the six replicates from each group was randomly selected and run in triplicate on three separate occasions for each gene product examined (21 in all). For immunocytochemistry, 12 additional animals were analyzed, four in the normal group, three in the 3-days deafened group, two in the 21-days deafened group, and three in the 90-days deafened group.

Cochlear ablation

Male Sprague–Dawley rats obtained from Charles River Laboratories (Wilmington, MA, USA), were maintained on a 12-h reversed light–dark cycle and had free access to food and water. All studies were approved by the University Committee on the Use and Care of Animals. Rats weighing 185–275 g were deeply anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (8 mg/kg). ABR thresholds were measured at frequencies of 2, 10 and 20 kHz from 0 to 100 dB before surgery to confirm that all animals had normal hearing. Animals were bilaterally deafened by mechanical ablation of the cochlea. Hearing thresholds were again determined on anesthetized animals by ABR 3, 21 or 90 days following ablation,

to assess the degree of hearing loss. Only animals that sustained a threshold shift of 80 dB or greater were included in the study. This criterion for deafness was to ensure a homogenous group of deafened animals.

RNA isolation

Rats were anesthetized with a lethal dose (0.8–1 mL) of Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI, USA) and decapitated. Brains were rapidly removed and the right and left IC dissected away from the remaining midbrain with fine-tipped forceps and placed in RNAlater (Ambion, Austin, TX, USA). Tissues were homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) for 10 s with a Tissue-Tearor™ homogenizer (Biospec Products Inc., Bartlesville, OK, USA). RNA was isolated using a modification of the standard Trizol protocol. Briefly, chloroform was added to the homogenate, vortexed briefly, and centrifuged at 14 000 g. The aqueous phase was transferred to a Phase Lock Gel (heavy) tube (Eppendorf/Brinkmann, Westbury, NY, USA) and extracted with acid phenol : chloroform (1 : 1). The aqueous phase was decanted and RNA was precipitated with isopropanol. Contaminants were removed with RNeasy mini-spin columns (Qiagen, Chatsworth, CA, USA). RNA was eluted in 100 μL of diethyl pyrocarbonate (DEPC) treated water. RNA concentration was determined by UV spectrophotometry. RNA quality was assessed on an RNA 6000 Nano LabChip (Agilent, Palo Alto, CA, USA) using an Agilent 2100 Bioanalyzer, to assess the integrity of the 18S and 28S rRNA bands.

Hybridization of Affymetrix GeneChips

This study utilized high-density oligonucleotide microarrays (Gene-Chips, Affymetrix, Santa Clara, CA, USA) that detect the expression levels of 8799 known genes and expressed sequence tags (ESTs; Rat Genome U34A Array). Preparation of cRNA, hybridization and scanning of the arrays were performed according to manufacturer's protocols. Briefly, 5 μg of total RNA was used to generate double-stranded cDNA by reverse transcription using a cDNA synthesis kit (Superscript Choice System, Gibco-BRL, Rockville, MD, USA) that employs an oligo(dT)$_{24}$ primer containing a T7 RNA polymerase promoter 5′ to the poly(dT) (Genset, La Jolla, CA, USA), followed by second-strand synthesis. Biotin-labeled cRNA was prepared from the double-stranded cDNA by *in vitro* transcription with T7 RNA polymerase in the presence of biotin-11-CTP and biotin-16-UTP (Enzo, Farmingdale, NY, USA), then purified over RNeasy columns (Qiagen). Fifteen micrograms of cRNA was fragmented at 94°C for 35 min in 40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate, then added to 300 μL of hybridization cocktail (100 mM MES, 1 M NaCl, 20 mM EDTA, 0.01% Tween 20) containing 0.1 mg/mL herring sperm DNA (Promega, Madison, WI, USA), and 500 μL/mL acetylated bovine serum albumin (BSA; Gibco-BRL). Prior to hybridization, the cocktails were heated to 94°C for 5 min, equilibrated at 45°C for 5 min, then centrifuged (16 000 g) at room temperature (27°C) for 5 min. Aliquots of this hybridization cocktail containing 10 μg of fragmented cRNA were hybridized to HuGeneFL arrays at 45°C for 16 h in a rotisserie oven at 60 r.p.m. The arrays were washed under non-stringent conditions (6 × Saline Sodium Phosphate EDTA (SSPE) at 25°C), followed by a stringent wash [100 mM 2-(N-morpholino) ethane sulphonic Acid, MES) (pH 6.7), 0.1 M NaCl, 0.01% Tween 20 at 50°C], then stained with streptavidin-phyco-

erythrin (Molecular Probes, Eugene, OR, USA), washed with 6 × SSPE, incubated with biotinylated anti-streptavidin IgG, stained again with streptavidin–phycoerythrin, and washed again with 6 × SSPE. Arrays were scanned with the GeneArray scanner (Affymetrix) and image analysis was performed with GeneChip software (Affymetrix).

Microarray data analysis

Affymetrix U34A GeneChips contain a set a set 16 pairs of short (25-mer) oligonucleotide probes for each gene (a probe set). The paired probes consist of a 'perfect match' (PM) probe that is identical to a particular sequence of the gene and a 'mismatch' (MM) probe that has the central base switched with its complement. PM probes are used to estimate transcript abundance, whereas MM probes are intended to provide a measure of background binding for each PM probe. Overall transcript abundance for each gene is estimated by calculating a summary statistic for each probeset (an 'expression value'). There are several methods for computing expression values [MAS5, dChip, robust multiarray average (RMA), etc.]; we chose to use an RMA as implemented in the 'affy' package of Bioconductor (http://www.bioconductor.org). RMA performs three operations: probe-specific background correction to compensate for non-specific binding using PM distribution rather than PM-MM value; probe-level multichip quantile normalization to unify PM distributions across all chips; and robust probe-set summary of the log-normalized probe-level data by median polishing. This method is based on recent studies that used dilution and spike-in experiments, adding known amount of specific transcripts to the preparation, to evaluate different methods of calculating intensity values and normalizing GeneChip data. RMA was directly compared with other GeneChip analysis software such as dChip or MAS5 (Irizarry *et al.* 2003a,b). These studies found that RMA gave the most accurate representation of 'known' datasets.

Statistical analysis

Statistical Analysis of Microarrays (SAM; http://www-stat.stanford.edu/~tibs/SAM) was used to perform two-sample t-tests on the log transformed (log base 2) intensity values obtained from 18 GeneChips (six biological replicates from each of three experimental groups). By permuting the data to estimate the percentage of genes identified at random, a user of SAM can calculate a false discovery rate (FDR), the probability that the change in expression of a given gene is erroneously called significant, and thereby assign statistical significance to differences in intensity values across experimental groups (Tusher *et al.* 2001). Genes with an FDR of 5% or less were considered significant in the current study.

Quantitative RT–PCR

A subset of significantly expressed genes was selected for further study, based on the importance of amino acid neurotransmitters and neuropeptides in the ascending auditory pathways. Real-time PCR was performed on a randomly selected pool of RNA (from pools used in GeneChip experiments) 3 and 21 days following cochlear ablation. The 90-day group consisted of three pools of mRNA, each derived from three animals. cDNA was generated from 1 μg of total RNA using Superscript II (Invitrogen). The cDNA was diluted to 200 μL and 2.5 μL was used for each real-time PCR reaction. Real-time PCR reactions were performed using Assays-by-Design

TaqMan MGB assays (Applied Biosystems, Foster City, CA, USA), which use 5′ nuclease chemistry with two unlabeled PCR primers (Table S1; 900 μM each) and an FAM dye-labeled TaqMan minor groove binder (MGB) probe (250 μM), and TaqMan Universal PCR Master Mix. Reactions were performed on an ABI Prism 7900, and results were analyzed using ABI Prism 7900 Sequence Detection System software (Applied Biosystems).

We tested 19 neurotransmission-related genes at the 3-, 21- and 90-day time points in triplicate. These genes were GABA-A receptors β2, β3, γ1, γ2; glutamate decarboxylase 2; glycine α1, α2, α3; glutamate receptors AMPA 2 (GluR 2), AMPA 3 (GluR 3); glutamine synthetase 1 (glns); glutaminase (gls); glutamate receptor kainate 2 (GluR K2); 5-hydroxytryptamine (serotonin) receptors 5B, 2C; pre-proenkephalin; pre-pronociceptin (neuropeptide nociceptin); activity and neurotransmitter-induced early gene protein 4 (ania-4); and tyrosine hydroxylase (TH).

We used the $2^{-\Delta_1\Delta_2 Ct}$ method to calculate the fold change in gene expression (Livak and Schmittgen 2001). For each gene at each time point, we determined the cycle threshold (Ct), the cycle number at which PCR products are increasing exponentially. To normalize the data, the threshold value for ribosomal subunit S16 was subtracted from threshold values for each gene at each time point (Δ_1). Then the threshold value of the control group for each gene was subtracted from the experimental threshold value (Δ_2). The final value was then expressed as logarithm to base 2. Using this formula $[2^{-(\Delta_1\Delta_2 Ct)}]$, we calculated the relative differences between experimental and control groups as fold change. The average value of triplicate PCR reactions was normalized to the average value for the S16 ribosomal protein for each sample and then compared with the expression value of the control group.

Immunocytochemistry

From the group of genes confirmed by qRT-PCR, we selected five genes to determine the cellular localization and distribution of their products in the IC, using immunocytochemistry. Two of these genes, TH and pre-proenkephalin, showed decreases and three genes, GluR 2/3, GABA-A receptor subunit β3 and subunit γ2, increased in expression. The distribution of labeled neurons and processes were examined in a qualitative manner in sections from the brachium to the mid rostro-caudal region of the IC. Normal animals ($n = 4$) were compared with eight deafened animals (three 3-day deaf, two 21-day deaf, three 90-day deaf). Animals were anesthetized with Fatal Plus (0.8–1 mL) and then received vascular perfusion with phosphate buffer followed by a fixative containing 4% paraformaldehyde in phosphate buffer. Brains were removed and post-fixed for 2 h in the same fixative. Brains were then rinsed in phosphate-buffered saline (PBS) and placed in 10% sucrose in PBS for 12–16 h at 4°C. The auditory brainstem was then rapidly frozen onto a cryostat chuck using OCT compound (Fisher Scientific, Pitsburgh, PA, USA) and 12 μm frozen coronal sections were cut through the IC. Sections were mounted on Fisher Plus slides and antibodies were used to localize the protein products of these five differentially expressed genes within the IC.

Cryostat sections were incubated with a primary antibody, TH (Novus Biologicals, Littleton, CO, USA, 1 : 400), pre-proenkephalin (PPENK; Fitzgerald Industries, Concord, MA, USA; 1 : 100), GluR 2/3 (Chemicon, Temecula, CA, USA; 1 : 50), GABA β3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 50), or

GABA γ2 (Alomone Laboratories, Jerusalem, Israel; 1 : 4000) and normal serum from the species in which the secondary antibody was raised (donkey or goat; 1 : 200; Jackson Laboratories Bar Harbor, ME, USA) in PBS with 0.3% triton X-100 for 24 h at 4°C in a humid chamber. All subsequent incubations and rinses (three times for 5 min in PBS) were carried out at room temperature. The sections were then rinsed and incubated for 1 h with Alexa fluor 594 donkey anti-goat (1 : 100) or Alexa fluor 568 goat anti-rabbit (1 : 100) secondary antibody (Molecular Probes) in 0.3% triton X-100. After rinsing, slides were coverslipped using gel/mount (Biomedia Corp., Foster City, CA, USA).

Histological analysis

Images of immunoreactive cells, fibers, and puncta in the IC were obtained from sections using a Zeiss LSM confocal microscope. The characteristics: somatic, dendritic, punctate, as well as the amount and location of the immunoreactive label within the IC, were noted for each experimental group, and a blind qualitative assessment was made by two investigators. For each antibody, a *z*-series was collected for each experimental group studied. The *z*-series was then montaged and one maximum projection image compiled.

Results

Identification of differentially expressed genes following deafening

From the 8799 probe sets interrogated on the Affymetrix GeneChips, we found over 1400 probe sets that showed differential expression at either 3 or 21 days following deafness, with a false discovery rate (FDR) of 5% or less. Of these, 411 showed increased expression after 3 days of deafness, and 38 had decreased expression (Table S2). By 21 days following deafness, 700 probe sets had increased expression and 331 decreased (Table S3). With the additional restriction of a twofold or greater increase in expression, 38 probe sets met this criterion at 3 days (Table 1) and 57 probe sets at 21 days (Table 2). With the additional restriction of a decrease of approximately 20% or more, 34 probe sets showed decreased expression 3 days following deafness (Table 3) and 104 decreased at 21 days following deafness (Table 4).

Genes showing the largest increase in expression included several that encode proteins involved in neurotransmission, such as synaptosomal-associated protein (SNAP 25), GABA-A receptor epsilon, and microtubule-associated protein 2 (MAP2; Tables 1 and 2). Genes with the greatest decrease in expression included pre-proenkephlin (PENK), early growth response 1 (ERG1), and activity-regulated cytoskeletal-associated protein (ARC; Tables 3 and 4). Among those probe sets showing large increases or decreases in expression, several were 'unknowns', or genes that have not yet been identified. Of the 1480 genes showing significant differential expression, 83 were classified as

Table 1 Genes with greater than 2 fold increase in expression 3 days following deafness

Gene Title	UniGene ID	Fold Change	q-value (%)	Probe Set ID
Unknown	Unknown	3.97	0.40	M13101cds_f_at
aquaporin 4	Rn.90091	3.97	0.40	U14007_at
Unknown	Unknown	3.77	0.40	U83119_f_at
LINE retrotransposable element 3	Unknown	3.75	0.40	M13100cds#6_f_at
Unknown	Unknown	3.68	0.40	X53581cds#5_f_at
Unknown	Unknown	3.65	0.40	X05472cds#1_s_at
Unknown	Unknown	3.36	0.40	X61295cds_s_at
LINE retrotransposable element 3	Unknown	3.35	0.40	M13100cds#2_s_at
visinin-like 1	Rn.105934	3.22	0.40	D10666_at
Unknown	Unknown	3.17	0.40	X07686cds_s_at
synaptosomal-associated protein	Rn.107689	3.09	0.40	AB003991_g_at
LINE retrotransposable element 3	Unknown	2.92	0.40	M13100cds#4_f_at
synaptosomal-associated protein	Rn.107689	2.84	0.40	AB003992_s_at
synaptosomal-associated protein	Rn.107689	2.59	0.40	AB003991_at
Similar to pinin; DNA segment, Chr 12, ERATO Doi 512, expressed (LOC368070), mRNA	Rn.38987	2.59	0.40	rc_AI639151_at
LINE retrotransposable element 3	Unknown	2.54	0.40	M13100cds#3_f_at
LINE retrotransposable element 3	Unknown	2.53	0.40	M13100cds#5_s_at
Transcribed seq. with moderate similarity to protein pdb:1LBG (E. coli) B Chain B, Lactose Operon Repressor Bound To 21-Base Pair Symmetric Operator Dna, Alpha Carbons Only	Rn.24387	2.36	0.40	rc_AA891476_at
cadherin 22	Rn.108785	2.35	0.40	D83349_at
Similar to RNA-binding region (RNP1, RRM) containing 2; splicing factor (CC1.3); coactivator of activating protein-1 and estrogen receptors (LOC362251), mRNA	Rn.8555	2.33	0.40	rc_AA800126_at
Unknown	Unknown	2.31	0.40	X53581cds#3_f_at
Similar to heterogeneous nuclear ribonucleoprotein A2/B1 (LOC362361), mRNA	Rn.4057	2.25	0.40	rc_AA799511_g_at
Ac1054 mRNA, complete cds	Rn.3412	2.25	0.40	rc_AA893212_at
gamma-aminobutyric acid A receptor, alpha 1	Unknown	2.18	0.40	L08490cds_at
Transcribed sequences	Rn.25117	2.18	0.40	rc_AA799804_at
microtubule-associated protein 2	Rn.10484	2.17	0.40	X17682_s_at
Transcribed sequences	Rn.98479	2.15	0.40	rc_AA874873_at
Similar to N-terminal aceyltransferase 1 (LOC310399), mRNA	Rn.12587	2.14	2.89	rc_AI177404_at
microtubule-associated protein 2	Rn.10484	2.13	0.40	X53455cds_s_at
catalase	Rn.3001	2.07	0.40	rc_AA926149_g_at
Similar to heterogeneous nuclear ribonucleoprotein A2/B1 (LOC362361), mRNA	Rn.4057	2.06	0.40	rc_AA799511_at
ectonucleotide pyrophosphatase/phosphodiesterase 2	Rn.20403	2.06	0.40	D28560_at
Nclone10 mRNA	Rn.32307	2.05	0.40	rc_AI071866_s_at
diacylglycerol kinase, beta	Rn.11413	2.04	0.71	rc_AA818983_at
LINE retrotransposable element 3	Unknown	2.04	0.40	M13100cds#1_g_at
chimerin (chimaerin) 1	Rn.11166	2.01	1.39	X67250_at
solute carrier family 3, member 1	Rn.11196	2.01	0.40	M80804_s_at
Transcribed sequences	Rn.22663	2.00	0.40	rc_H31859_at

unknown, 191 were expressed sequence tags (ESTs) and 119 were transcripts that represent a different region of one of the known genes. This left 1087 known genes (Tables S2 and S3).

As a first step in understanding functions of differentially expressed genes, we used the gene ontology (GO) classification of genes (http://www.godatabase.org/cgi-bin/

Table 2 Genes with greater than 2 fold increase in expression 21 days following deafness

Gene Title	UniGene ID	Fold Change	q-value (%)	Probe Set ID
aquaporin 4	Rn.90091	4.32	0.31	U14007_at
Unknown	Unknown	4.26	0.54	M13101cds_f_at
LINE retrotransposable element 3	Unknown	4.01	0.54	M13100cds#6_f_at
Unknown	Unknown	4.00	0.54	U83119_f_at
Unknown	Unknown	3.99	0.63	X05472cds#1_s_at
Unknown	Unknown	3.91	0.31	X53581cds#5_f_at
Unknown	Unknown	3.85	0.63	X61295cds_s_at
LINE retrotransposable element 3	Unknown	3.61	0.63	M13100cds#2_s_at
LINE retrotransposable element 3	Unknown	3.54	1.17	M13100cds#4_f_at
Unknown	Unknown	3.51	0.63	X07686cds_s_at
Similar to pinin; DNA segment, Chr 12, ERATO Doi 512, expressed (LOC368070), mRNA	Rn.38987	3.20	0.31	rc_AI639151_at
visinin-like 1	Rn.105934	3.03	0.31	D10666_at
synaptosomal-associated protein	Rn.107689	2.94	0.31	AB003991_g_at
LINE retrotransposable element 3	Unknown	2.92	0.31	M13100cds#3_f_at
synaptosomal-associated protein	Rn.107689	2.86	0.31	AB003992_s_at
LINE retrotransposable element 3	Unknown	2.82	1.38	M13100cds#5_s_at
Nclone10 mRNA	Rn.32307	2.75	0.31	rc_AI071866_s_at
Unknown	Unknown	2.71	0.54	X53581cds#3_f_at
Similar to trinucleotide repeat containing 6; EDIE; GW182 autoantigen (LOC308971), mRNA	Rn.6839	2.67	0.31	rc_AA875500_at
Similar to N-terminal aceyltransferase 1 (LOC310399), mRNA	Rn.12587	2.58	0.31	rc_AI177404_at
synaptosomal-associated protein	Rn.107689	2.52	0.31	AB003991_at
Transcribed seq. with moderate similarity to protein pdb:1LBG (E. coli) B Chain B, Lactose Operon Repressor Bound To 21-Base Pair Symmetric Operator Dna, Alpha Carbons Only	Rn.24387	2.49	0.31	rc_AA891476_at
microtubule-associated protein 2	Rn.10484	2.37	1.38	X17682_s_at
LINE retrotransposable element 3	Unknown	2.32	0.63	M13100cds#1_g_at
catalase	Rn.3001	2.27	0.31	rc_AA926149_g_at
myristoylated alanine rich protein kinase C substrate	Rn.9560	2.24	0.31	rc_AA955167_s_at
Unknown	Unknown	2.24	2.10	X05472cds#3_f_at
Transcribed sequences	Rn.98479	2.22	0.31	rc_AA874873_at
Rattus norvegicus focal adhesion kinase (FAK) mRNA, alternative 5'UTR	Rn.806	2.22	0.31	rc_AA874926_at
Similar to heterogeneous nuclear ribonucleoprotein A2/B1 (LOC362361), mRNA	Rn.4057	2.20	0.31	rc_AA799511_g_at
microtubule-associated protein 2	Rn.10484	2.20	2.08	rc_AI228850_s_at
Transcribed sequences	Rn.15385	2.19	0.31	rc_AI639410_s_at
microtubule-associated protein 2	Rn.10484	2.19	1.64	X53455cds_s_at
gamma-aminobutyric acid A receptor, alpha 1	Unknown	2.18	0.54	L08490cds_at
Transcribed sequences	Rn.16596	2.17	0.31	rc_AI638960_g_at
Similar to RNA helicase A (LOC304859), mRNA	Rn.107359	2.17	0.31	rc_AI639188_at
dynamin 1-like	Rn.10830	2.12	0.31	AF020212_s_at
LIC-2 dynein light intermediate chain 53/55	Rn.31981	2.11	0.31	rc_AA891132_s_at
chimerin (chimaerin) 1	Rn.11166	2.11	0.31	X67250_at
Ac1054 mRNA, complete cds	Rn.3412	2.11	2.08	rc_AA893212_at
solute carrier family 24 (sodium/potassium/calcium exchanger), member 2	Rn.74242	2.11	0.31	AF021923_at
sodium channel, voltage-gated, type 2, alpha 1 polypeptide	Rn.89192	2.11	0.31	M22254_at
Similar to cAMP-dependent protein kinase, beta-2-catalytic subunit (PKA C-beta-2) (LOC310986), mRNA	Rn.3502	2.10	5.03	D10770_s_at
casein kinase II beta subunit	Rn.11095	2.09	0.31	L15619_at
phospholipase C, beta 1	Rn.45523	2.09	0.31	L14323_at

Table 2 Continued

Gene Title	UniGene ID	Fold Change	q-value (%)	Probe Set ID
gap junction membrane channel protein alpha 1	Rn.10346	2.08	3.49	rc_AI029183_s_at
glutamate receptor, ionotropic, 2	Rn.91361	2.07	1.13	M38061_at
discs, large homolog 1 (Drosophila)	Rn.89331	2.07	0.31	U14950_at
matrin 3	Rn.29774	2.06	0.31	M63485_at
Similar to RNA-binding region (RNP1, RRM) containing 2; splicing factor (CC1.3); coactivator of activating protein-1 and estrogen receptors (LOC362251), mRNA	Rn.8555	2.06	0.31	rc_AA800126_at
Transcribed sequences	Rn.25117	2.06	0.31	rc_AA799804_at
Transcribed sequences	Rn.22663	2.05	0.63	rc_H31859_at
gamma-aminobutyric acid A receptor, gamma 2	Unknown	2.05	0.31	L08497cds_at
chimerin (chimaerin) 1	Rn.11166	2.04	0.31	rc_AA894317_s_at
dynamin 1-like	Rn.10830	2.04	0.31	AF019043_at
solute carrier family 3, member 1	Rn.11196	2.02	3.49	M80804_s_at
Transcribed sequences	Rn.15956	2.00	0.31	rc_AI639088_s_at

amigo/go.cgi), which assigns differentially expressed genes to groups based on molecular function and biological processes (Tables S2 and S3). Of these genes, over 300 in the 3-day deaf group and over 600 in the 21-day deaf group could not be classified, either because they were unknown genes or because they have not been formally assigned a molecular function or biological process based on GO terminology. Nonetheless, we were able to identify many neurotransmitter-related genes (Table 5 and Tables S2 and S3) that showed changes in expression and may contribute to auditory plasticity following deafening.

Glutamate-related changes in expression

GeneChip analysis identified many glutamate-related genes with significant and sustained increases in expression following deafening (Table 5). Of these excitatory neurotransmission related genes, five were further assessed with qRT-PCR and showed comparable changes (Fig. 1a). GluR 2 showed significant increases of 2.7-fold at 3 days, 2.9-fold at 21 days and 1.7-fold at 90 days. GluR 3 expression increased 3.3-fold and 3.0-fold at 3 and 21 days, respectively, and was still 2.7-fold higher than the control at 90 days. The expression of GluR K2 increased 2.5-fold at 3 days, 2.6-fold at 21 days, and was still 1.7-fold higher at 90 days. Similarly, glutaminase expression increased more than a 3-fold at both 3 and 21 days, and was still 2.6-fold higher at 90 days. Glutamine synthetase (glns) expression did not increase following deafening, with no significant change in glns expression at 3 days, and a modest decrease at 21 days. Similarly, qRT-PCR detected no change in expression (< 15%) at 3 and 21 days, with a somewhat greater (24%) decrease at 90 days. None of the glutamate-related transcripts returned to normal levels of expression by 90 days (Table 5).

GABA-A receptor subunits

Increased gene expression was seen in the three major types (α, β and γ) of GABA-A receptor subunits using GeneChip analysis (Table 5). The $\beta2$, $\beta3$, $\gamma1$ and $\gamma2$ subunits of this inhibitory receptor were further investigated with qRT–PCR (Fig. 1b). GABA-A receptor $\beta2$ expression increased more than 4-fold at 3 and 21 days, and was still 3-fold higher at 90 days following deafening. GABA-A $\beta3$ and $\gamma2$ mNRA levels were > 2-fold higher at 3 and 21 days, with a return towards normal levels at 90 days. GABA-A receptor $\gamma1$ subunit mRNA levels increased in the 3-day deaf group by GeneChip analysis, while the qRT-PCR data did not show an increase until 21 days and showed a return to near normal levels at 90 days. For GAD 65 there were small increases of 1.28-, 1.51- and 1.33-fold at 3, 21 and 90 days, respectively.

Glycine receptor subunits

GeneChip results for the $\alpha2$ subunit of the glycine receptor showed a 1.6-fold increase in expression at 3 and 21 days (Table 5), while qRT–PCR detected little change (below 15%) at 3 days, a 2.9-fold increase at 21 days, and a return to normal levels of expression by 90 days following deafening (Fig. 1c). GeneChip analysis detected no significant change in the expression of glycine receptors $\alpha1$ or $\alpha3$ (Table 5), and qRT–PCR detected little change (below 15%) in glycine receptor subunit $\alpha1$ at 3 days. By qRT–PCR, there was a slight increase of 30% in glycine $\alpha1$ at 21 days and an almost 1.5-fold increase by 90 days (Fig. 1c and Table 5). Glycine receptor $\alpha3$ had increased levels of expression (2.4-, 3.5- and 2.0-fold, respectively) at each of these three time points.

Other genes assessed by qRT–PCR

While GeneChip analysis did not detect a decrease in TH until 21 days (Table 5), qRT–PCR showed an approxi-

Table 3 Genes with greater than 1.25 fold (25%) decrease in expression 3 days following deafness

Gene Title	UniGene ID	Fold Change	Deaf / Normal	q-value (%)	Probe Set ID
early growth response 1	Rn.9096	-3.91	0.26	0.40	AF023087_s_at
Unknown	Unknown	-3.30	0.30	0.40	U75397UTR#1_s_at
early growth response 1	Rn.9096	-3.23	0.31	0.40	M18416_at
VGF nerve growth factor inducible	Rn.9704	-2.83	0.35	0.40	M74223_at
galectin-related inter-fiber protein	Rn.26894	-2.22	0.45	0.40	AF082160_at
Similar to testican-2 protein (LOC361840), mRNA	Rn.66831	-2.08	0.48	4.02	rc_AI639294_at
activity regulated cytoskeletal-associated protein	Rn.10086	-1.82	0.55	0.40	U19866_at
preproenkephalin, related sequence	Rn.10015	-1.80	0.55	0.40	S49491_s_at
Unknown	Unknown	-1.72	0.58	1.98	rc_AI014135_g_at
hydroxysteroid 11-beta dehydrogenase 1	Rn.888	-1.61	0.62	0.40	rc_AI105448_at
Similar to Leucine-rich repeat-containing 8 (LOC311846), mRNA	Rn.33512	-1.56	0.64	2.89	rc_AA875002_at
Similar to carboxylesterase (LOC291863), mRNA	Rn.108790	-1.51	0.66	5.46	rc_AA800851_s_at
Transcribed sequences	Rn.3130	-1.48	0.68	1.98	rc_AA874803_g_at
Rgc32 protein	Rn.3504	-1.46	0.68	0.93	AF036548_g_at
Rat mRNA for beta-tubulin T beta15	Rn.37849	-1.42	0.70	0.40	X03369_s_at
Unknown	Unknown	-1.41	0.71	1.98	X03347cds_g_at
protein tyrosine phosphatase, receptor type, N	Rn.11097	-1.41	0.71	4.02	D38222_s_at
Max	Rn.4210	-1.40	0.71	2.89	D14448_s_at
calcium/calmodulin-dependent protein kinase II alpha subunit	Rn.107499	-1.40	0.71	4.02	J02942_at
phosphoglycerate mutase 1	Rn.123549	-1.40	0.72	5.46	rc_AI169417_s_at
chemokine (C-X3-C motif) ligand 1	Rn.107266	-1.38	0.73	0.40	AF030358_g_at
synaptic vesicle glycoprotein 2 a	Rn.11264	-1.37	0.73	5.46	L05435_at
Unknown	Unknown	-1.37	0.73	1.39	U17837UTR#1_at
protein kinase, cAMP-dependent, catalytic, alpha	Rn.103828	-1.37	0.73	1.98	X57986mRNA_s_at
farensyl diphosphate synthase	Rn.2848	-1.36	0.73	2.89	rc_AI180442_at
phosphodiesterase 4A	Rn.91357	-1.36	0.74	2.89	M25348_s_at
ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	Rn.3454	-1.36	0.74	2.89	rc_AI171844_g_at
Similar to 2700038E08Rik protein (LOC293725), mRNA	Rn.43300	-1.36	0.74	4.02	rc_AA860024_at
pleckstrin homology, Sec7 and coiled/coil domains 3	Rn.10673	-1.33	0.75	2.89	U83897_at
Similar to mitochondrial ribosomal protein S6 (LOC288253), mRNA	Rn.41455	-1.33	0.75	5.46	rc_AI639387_at
laminin receptor 1 (67kD, ribosomal protein SA)	Rn.999	-1.29	0.77	5.46	D25224_at
proteasome (prosome, macropain) subunit, beta type 4	Rn.6169	-1.28	0.78	4.02	L17127_g_at
Unknown	Unknown	-1.28	0.78	4.02	D13623_g_at
Transcribed sequences	Rn.6620	-1.27	0.79	1.98	rc_AA800693_g_at

mately 50% decrease in TH expression at 3, 21 and 90 days following deafness (Fig. 1d). Prepronociceptin (PNOC) and pre-proenkephalin (PENK) expression levels also decreased at these three times. Serotonin receptor 5B (5-HT 5B) decreased approximately 20% at 3 and 21 days following deafness, but returned to normal by 90 days. The activity- and neurotransmitter-induced gene, ANIA4, had comparable slight increases in expression at 3 days with both GeneChip analysis and qRT–PCR. There was, however, a difference at 21 days, where an increase was seen in the GeneChip analysis, while there was a large decrease in expression (80–90%) at 21 and 90 days detected by qRT–PCR.

Immunocytochemical distribution of differentially expressed genes in the IC

Immunocytochemistry was used to examine the distribution of five proteins in the IC; GABA β3, GABA γ2, glutamate receptor 2/3 (Fig. 2), TH, and PPENK (Fig. 3). These immunolabeling studies identified the neurons and cellular elements containing products ultimately produced by the gene. We also examined whether changes in expression following deafness could be translated into changes in production and/or localization and whether the changes were sufficient to be appreciated in a qualitative appraisal. Comparing the 3- and 21-day deafened group with the normal group, there were not sufficient differences in the

Table 4 Genes with greater than 1.25 fold (25%) decrease in expression 21 days following deafness

Gene Title	UniGene ID	Fold Change	Deaf / Normal	q-value (%)	Probe Set ID
VGF nerve growth factor inducible	Rn.9704	-2.72	0.37	0.31	M74223_at
early growth response 1	Rn.9096	-2.18	0.46	3.49	AF023087_s_at
Unknown	Unknown	-2.02	0.49	5.03	U75397UTR#1_s_at
serum/glucocorticoid regulated kinase	Rn.4636	-1.98	0.51	5.03	L01624_at
protein tyrosine phosphatase, non-receptor type 16	Rn.98260	-1.83	0.55	2.08	S74351_s_at
protein tyrosine phosphatase, non-receptor type 16	Rn.98260	-1.82	0.55	3.49	S81478_s_at
preproenkephalin, related sequence	Rn.10015	-1.82	0.55	0.31	S49491_s_at
activity regulated cytoskeletal-associated protein	Rn.10086	-1.79	0.56	0.31	U19866_at
Similar to carboxylesterase (LOC291863), mRNA	Rn.108790	-1.77	0.56	0.54	rc_AA800851_s_at
ATP-binding cassette, subfamily C (CFTR/MRP), member 8	Rn.11187	-1.74	0.58	3.49	rc_AA799786_s_at
nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	Rn.12550	-1.65	0.61	1.64	X63594cds_g_at
neuronal d4 domain family member	Rn.42906	-1.61	0.62	4.54	X66022mRNA#1_s_at
immediate early gene transcription factor NGFI-B	Rn.10000	-1.61	0.62	3.49	U17254_g_at
synaptic vesicle glycoprotein 2 a	Rn.11264	-1.60	0.63	2.10	L05435_at
Similar to talin (LOC313494), mRNA	Rn.7715	-1.57	0.64	5.03	rc_AA800962_at
protein tyrosine phosphatase, non-receptor type 16	Rn.98260	-1.55	0.64	1.13	U02553cds_s_at
Similar to RIKEN cDNA 1110020C13 (LOC363004), mRNA	Rn.2990	-1.53	0.65	3.49	rc_AA800199_at
Similar to transformation/transcription domain-associated protein (LOC288471), mRNA	Rn.92036	-1.52	0.66	2.34	rc_AA800513_at
nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	Rn.12550	-1.52	0.66	1.64	X63594cds_at
Similar to T-cell differentiation antigen (LOC311635), mRNA	Rn.25180	-1.51	0.66	2.69	rc_AA799761_at
RT1 class II, locus Bb	Rn.20089	-1.49	0.67	4.02	M36151cds_I_at
discs, large homolog 4 (Drosophila)	Rn.9765	-1.49	0.67	0.31	M96853_at
Unknown	Unknown	-1.45	0.69	0.31	X03347cds_g_at
myosin heavy chain 11	Rn.94969	-1.45	0.69	5.03	X16261_i_at
peroxiredoxin 6	Rn.42	-1.45	0.69	2.08	AF014009_at
macrophage galactose N-acetyl-galactosamine specific lectin	Rn.779	-1.44	0.69	2.89	J05495_at
protein tyrosine phosphatase, receptor type, N	Rn.11097	-1.44	0.70	3.49	D38222_s_at
Transcribed sequences	Rn.4292	-1.43	0.70	3.49	rc_AA799347_at
Transcribed sequences	Rn.6676	-1.43	0.70	5.03	rc_AA800971_at
Rat mRNA for beta-tubulin T beta15	Rn.37849	-1.42	0.70	1.38	X03369_s_at
Similar to RIKEN cDNA 4930415K17 (LOC291670), mRNA	Rn.105695	-1.41	0.71	2.08	rc_H33001_at
Similar to DNA segment, Chr 19, ERATO Doi 386, expressed (LOC361064), mRNA	Rn.30496	-1.41	0.71	4.54	rc_AA892753_s_at
Transcribed sequences	Rn.6620	-1.39	0.72	1.13	rc_AA800693_g_at
hydroxysteroid 11-beta dehydrogenase 1	Rn.888	-1.39	0.72	1.13	rc_AI105448_at
disks large-associated protein 4	Rn.11279	-1.38	0.72	2.89	U67140_g_at
calreticulin	Rn.974	-1.38	0.72	2.89	D78308_at
solute carrier family 2,member 1	Rn.3205	-1.38	0.72	1.17	S68135_s_at
calcium/calmodulin-dependent protein kinase II alpha subunit	Rn.107499	-1.38	0.72	2.08	J02942_at
Similar to 26S proteasome non-ATPase regulatory subunit 11 (26S proteasome regulatory subunit S9) (26S proteasome regulatory subunit p44.5) (LOC303353), mRNA	Rn.11861	−1.38	0.72	1.17	rc_AA892831_s_at
Rgc32 protein	Rn.3504	-1.38	0.73	2.69	AF036548_at
Jun-B oncogene	Rn.15806	-1.38	0.73	2.34	rc_AA891041_at
endothelin converting enzyme-like 1	Rn.45803	-1.37	0.73	1.17	Y16188_at
Transcribed sequences	Rn.3130	-1.37	0.73	1.17	rc_AA874803_g_at
c-fos oncogene	Rn.103750	-1.37	0.73	1.64	X06769cds_at
Similar to cDNA sequence BC019776 (LOC316842), mRNA	Rn.64557	-1.37	0.73	2.10	rc_AI639012_at
Similar to Tubulin alpha-4 chain (Alpha-tubulin 4) (LOC316531), mRNA	Rn.92961	-1.36	0.73	4.54	rc_AA800948_at
Unknown	Unknown	-1.36	0.73	1.17	S82649_s_at

Table 4 Continued

Gene Title	UniGene ID	Fold Change	Deaf / Normal	q-value (%)	Probe Set ID
Similar to guanylate kinase (LOC303179), mRNA	Rn.36522	-1.36	0.73	0.31	rc_AA800291_at
Unknown	Unknown	-1.36	0.73	0.31	U17837UTR#1_at
farensyl diphosphate synthase	Rn.2848	-1.36	0.73	1.38	rc_AI180442_at
nerve growth factor, gamma	Rn.11331	-1.36	0.74	5.03	M19647_i_at
Unknown	Unknown	-1.36	0.74	4.54	AFFX-BioB-5_at
ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	Rn.3879	-1.35	0.74	5.03	U00926_at
ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	Rn.3454	-1.35	0.74	1.64	rc_AI171844_g_at
mast cell protease 4	Rn.10698	-1.34	0.74	2.10	U67907_f_at
Similar to R31449_3 (LOC314641), mRNA	Rn.2928	-1.34	0.74	1.17	rc_AA799473_at
calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	Rn.10961	-1.34	0.75	4.02	U73503_at
Transcribed sequence with weak similarity to protein ref:NP_075568.1 (H.sapiens) hypothetical protein FLJ20989 [Homo sapiens]	Rn.2859	-1.34	0.75	2.89	rc_AA891551_at
Similar to cation-transporting atpase (LOC290673), mRNA	Rn.3697	-1.33	0.75	1.64	rc_AA893621_at
Similar to 17,000 dalton myosin light chain (LOC362816), mRNA	Rn.969	-1.33	0.75	3.49	rc_AA875523_i_at
apolipoprotein A-I	Rn.10308	-1.33	0.75	4.54	J02597cds_s_at
tumor-associated protein 1	Rn.32261	-1.32	0.76	2.89	AB015432_s_at
Similar to DGCR6 protein (DiGeorge syndrome critical region 6 homolog) (LOC303794), mRNA	Rn.22467	-1.32	0.76	1.38	rc_AA799732_at
Similar to 2700038E08Rik protein (LOC293725), mRNA	Rn.43300	-1.32	0.76	2.10	rc_AA860024_at
ribosomal protein L14	Rn.108039	-1.32	0.76	3.49	X94242_at
Transcribed sequence with strong similarity to protein ref:NP_062269.1 (M.musculus) cysteine and histidine rich 1; cysteine and histidine-rich cytoplasmic protein [Mus musculus]	Rn.8316	−1.32	0.76	5.03	rc_AA891802_at
prepronociceptin	Rn.87935	-1.32	0.76	0.63	S79730_s_at
Similar to KIAA1064 protein (LOC308385), mRNA	Rn.98823	-1.32	0.76	0.31	rc_AA893980_at
methylenetetrahydrofolate reductase	Rn.10494	-1.31	0.76	5.03	U57049_at
Rgc32 protein	Rn.3504	-1.31	0.76	3.49	AF036548_g_at
Unknown	Unknown	-1.31	0.76	5.03	L48618_at
CDNA clone MGC:72845 IMAGE:6917939, complete cds	Rn.119365	-1.31	0.76	4.54	rc_AI103236_at
Transcribed sequence with strong similarity to protein ref:NP_037465.1 (H.sapiens) EH domain-binding mitotic phosphoprotein [Homo sapiens]	Rn.14814	−1.30	0.77	1.17	rc_AA893612_at
von Hippel-Lindau syndrome homolog	Rn.11059	-1.30	0.77	0.63	U14746_at
ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	Rn.3454	-1.30	0.77	5.03	rc_AI171844_at
solute carrier family 12, member 2	Rn.11523	-1.30	0.77	5.03	AF086758_at
Similar to tumor-rejection antigen SART3 (LOC304582), mRNA	Rn.101480	-1.30	0.77	0.31	rc_AI639476_s_at
phosphoglycerate mutase 1	Rn.123549	-1.30	0.77	4.02	rc_AI169417_s_at
Transcribed sequence with moderate similarity to protein ref:NP_037529.1 (H.sapiens) over-expressed breast tumor protein [Homo sapiens]	Rn.1327	−1.30	0.77	5.03	rc_AA891796_at
Similar to hypothetical protein MGC6696 (LOC293719), mRNA	Rn.2847	-1.29	0.77	0.63	rc_AA800039_s_at
Max	Rn.4210	-1.29	0.77	2.10	D14448_s_at
Transcribed sequence	Rn.114062	-1.29	0.77	2.34	rc_AA892154_g_at
lactate dehydrogenase A	Rn.107896	-1.29	0.77	2.34	M54926_at
ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	Rn.3879	-1.29	0.77	5.03	U00926_g_at
ribosomal protein L18	Rn.484	-1.29	0.78	4.02	M20156_at
phosphodiesterase 4A	Rn.91357	-1.29	0.78	2.10	M26715_at

Table 4 Continued

Gene Title	UniGene ID	Fold Change	Deaf / Normal	q-value (%)	Probe Set ID
solute carrier family 2,member 1	Rn.3205	-1.28	0.78	1.64	M13979_at
Transcribed sequences	Rn.98517	-1.28	0.78	2.34	rc_AA800719_at
ATPase, vacuolar, 14 kD	Rn.6167	-1.28	0.78	4.54	U43175_at
potassium voltage-gated channel, KQT-like subfamily, member 1	Rn.9779	-1.28	0.78	1.46	U92655_at
Similar to eukaryotic translation initiation factor 3 subunit 7; eukaryotic translation initiation factor 3, subunit 7 (zeta, 66/67kD); translation initiation factor eIF3 p66 subunit (LOC362952), mRNA	Rn.3463	−1.28	0.78	2.89	rc_AA891553_at
myosin heavy chain, polypeptide 7	Rn.48663	-1.27	0.78	2.10	X15939_f_at
insulin 1	Rn.962	-1.27	0.79	2.34	E00001cds_f_at
major intrinsic protein of eye lens fiber	Rn.23532	-1.27	0.79	4.54	X53052cds_g_at
peroxiredoxin 6	Rn.42	-1.27	0.79	5.03	rc_AA892041_at
nucleoside diphosphate kinase	Rn.927	-1.27	0.79	5.03	M91597_s_at
Transcribed sequences	Rn.13477	-1.27	0.79	2.69	rc_AA893124_at
c-fos oncogene	Rn.103750	-1.27	0.79	2.89	X06769cds_g_at
laminin receptor 1 (67kD, ribosomal protein SA)	Rn.999	-1.26	0.79	1.64	D25224_at
proline-rich protein 15	Unknown	-1.26	0.79	2.34	M83567_s_at
isovaleryl coenzyme A dehydrogenase	Rn.147	-1.26	0.79	2.69	rc_AI102838_s_at
Tonin	Rn.9882	-1.26	0.79	4.02	M26534_i_at
Similar to non-receptor protein tyrosine kinase Ack (LOC303882), mRNA	Rn.98335	-1.26	0.79	2.69	rc_H31144_g_at
methyl CpG binding protein 2	Rn.9680	-1.26	0.79	2.69	M94064_at

immunolabeling to be striking for any of the antibodies used, either in the number of labeled elements and/or the intensity of labeling. However, for many of these proteins there were large enough changes in immunolabeling in the 90-day deaf group compared with labeling in the normal hearing control group (Figs 2 and 3) to be obviously different in a qualitative appraisal.

GABA receptor subunit immunolabeling
Numerous neurons were immunolabeled for GABA β3 throughout the three regions of the IC of normal hearing animals with the labeling having a punctate appearance and many prominent neurons located throughout the nucleus with the fewest labeled neurons observed in the central nucleus (Fig. 2a). In deafened animals the neuropil labelling was comparable with modestly increased levels of label in 3-day and 21-day deaf groups, but the 90-day deaf group was strikingly increased compared with the 3-day and 21-day deaf groups and controls, with the label for the subunit outlining many neurons, including several primary dendrites (Fig. 2b).

Neurons immunostained for the γ2 subunit of the GABA-A receptor were also distributed throughout the IC, with a punctate label filling the somata of the cells. A large number of these neurons were found in the central nucleus (Fig. 2c). In animals deafened for 3 days, neither the distribution nor the number of neurons immunostained for the γ2 subunit appeared to change, nor did the intensity of labeling. However, in the 21-day and 90-day deafened groups the number of labeled cells increased, particularly in the central nucleus of the IC (Fig. 2d) with more primary dendrites being labeled.

Glutamate receptor subunit immunolabeling
An antibody that immunolabels glutamate receptors containing both the 2 and 3 subunits (GluR 2/3) was used. GluR 2/3 immunolabel filled cell bodies and the labeling was found primarily in small to medium-sized cells (≤ 20 μm) scattered throughout the central nucleus of the IC. No obvious differences in labeling were found in the 3- and 21-day deaf groups compared with that of the control group, with a modest increase in the 90-day deaf group in the number of labeled cells. Immunolabeled neurons formed clusters or patches within the central nucleus of the IC (Fig. 2e) that become more prominent in the 90-day deaf group (Fig. 2f).

Neuropeptide immunolabeling
In normal animals there were very few PENK labeled cell bodies observed in the IC. However, there were labeled fibers and puncta throughout the nucleus, with a higher concentration of label in the dorsal cortex (Fig. 3a). While there was no apparent diminution of label in the 3- and 21-day deaf groups when compared with the control group, in the 90-day deaf group there was a general decrease in labeling of fibers and puncta in deafened animals (Fig. 3b) with fairly intense labeling maintained in the dorsal cortex.

Table 5 Comparison of gene expression 3 and 21 days after deafening: GeneChip vs qRT-PCR data

Gene Name	unigene id	3 day vs. control		21 day vs. control		90 day vs. control[c]
		GeneChip[a]	qRT-PCR[b]	GeneChip[a]	qRT-PCR[b]	qRT-PCR[b]
GLUTAMATE RELATED GENES						
glutamate receptor, ionotropic, 2 [d]	Rn.91361	1.70	2.67	1.61	2.87	1.70
glutamate receptor, ionotropic, 2 [d]	Rn.91361	1.53	2.67	1.23	2.87	1.70
glutamate receptor, ionotropic, 3	Rn.74049	1.25	3.34	1.07	2.95	2.73
glutamate receptor, ionotropic, kainate 2	Rn.87696	1.32	2.47	1.23	2.61	1.67
glutaminase (gls)	Rn.5762	1.52	3.50	1.44	3.13	2.62
glutamine synthetase 1 (glns)	Rn.2204	no change	—	-1.14	—	-1.32
GABA RELATED GENES						
GABA A receptor b 2	Rn.48880	1.61	4.42	no change	4.26	3.35
GABA A receptor b 3	Rn.53967	1.48	3.07	no change	2.81	2.62
GABA A receptor g 1	Rn.10366	1.33	—	1.41	1.48	—
GABA A receptor g 3	Rn.103894	1.88	2.15	2.05	2.37	1.70
glutamate decarboxylase 2 (GAD 65)	Rn.29951	1.41	1.28	1.52	1.51	1.33
GLYCINE RELATED GENES						
glycine a 1	Rn.10109	no change	—	no change	1.30	1.39
glycine a 2	Rn.10379	1.56	—	1.57	2.86	—
glycine a 3	Rn.10038	no change	2.37	no change	3.54	2.50
OTHER GENES						
5-hydroxytryptamine (serotonin) receptor 5B	Rn.10572	no change	-1.22	-1.17	-1.19	—
5-hydroxytryptamine (serotonin) receptor 2C [d]	Rn.9935	1.39	2.19	1.65	2.18	2.30
5-hydroxytryptamine (serotonin) receptor 2C [d]	Rn.9935	no change	2.19	1.86	2.18	2.30
preproenkephalin, related sequence	Rn.10015	-1.82	—	-1.82	-1.22	-1.25
prepronociceptin (neuropeptide nociceptin) (N23K)	Rn.87935	no change	-1.69	-1.32	-2.27	-1.40
activity and neurotransmitter-induced early gene protein 4 (ania-4)	Rn.80575	1.28	1.18	1.33	-7.14	-7.89
tyrosine hydroxylase	Rn.11082	no change	-2.33	-1.18	-2.04	-2.28

[a.] Change in intensity, expressed as fold change

[b.] Expressed as fold change ($2^{|\Delta\Delta Ctl|}$), with the decreased expression indicated by a minus sign.

Note: The exponent in the fold change calculation is expressed as an absolute value.

[c.] No GeneChips run for this group

[d.] Same gene but different probe set on GeneChip

— Less that 15% change

Tyrosine hydroxylase immunolabeled neurons were found throughout the IC of normal animals. However, there were so many intensely immunolabeled fibers and puncta observed throughout the IC, immunolabeling of cells was obscured. Specifically, bundles of immunolabeled fibers were found coursing through the dorsal cortex (Fig. 3c). While there was only a slight decrease in labeling in the 3-day deaf group, there were dramatic reductions in labeled cells and fibers in the 21-day and 90-day deaf groups throughout the IC when comparing deafened (Fig. 3d) and normal hearing animals (Fig. 3c).

Discussion

GeneChip analysis using SAM identified large numbers of genes as showing significant differential expression across groups in the rat IC 3 and 21 days following bilateral cochlear ablation. The level of expression and the magnitude of change are also often considered when assigning functional relevance to an identified change in expression. We have chosen not to add these constraints to our assessment as, even small changes, when they appear consistently (as shown by a test of significance), can have functional implications. Information on magnitude of changes in gene expression, however, is reported in Table 5.

Activity-dependent changes in the expression of several genes shown to be differentially expressed, including, tyrosine kinase, JUN, EGR1, calbindin, MAP and protein kinase C (PKC), have previously been demonstrated in the auditory brainstem (Garcia *et al.* 2000; Illing 2001; Kotak and Sanes 2002; Michler and Illing 2003; Zhang *et al.* 2004). Increased neuronal activity is known to activate immediate early genes C-FOS, JUN and EGR1 (Akins *et al.* 1996), which are often used as markers of neuronal
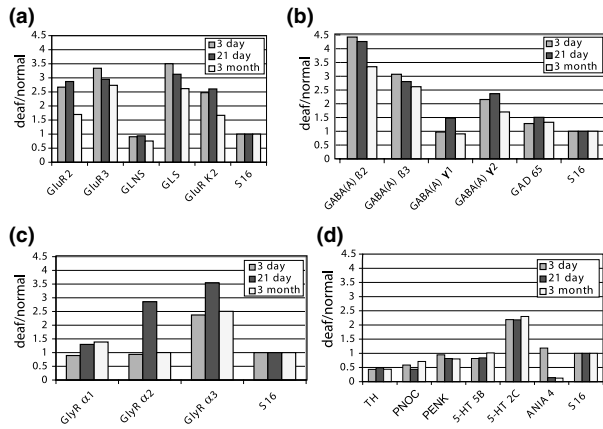
Fig. 1 Neurotransmitter-related genes are differentially expressed following deafferentation. Bar graphs of qRT–PCR showing differential gene expression of glutamate-related (a), GABA-related (b), glycine-related (c) and neuropeptide-related (d) genes following bilateral cochlear ablation. Data are expressed as a ratio, gene expression from deafened animals divided by gene expression from normal animals. Grey bars represent gene expression from 3-day deaf animals, black bars represent expression levels of 21-day deaf groups and white bars represent expression from 3-month deaf animals. Only two of the 19 genes tested (GlyR α2 and 5-HT 5B) had returned to normal levels of expression by 90 days.

activity. As expected, JUN and EGR1 showed large decreases in expression following deafening. The present study also demonstrated changes in the levels of mRNA for various neurotransmission-related genes that have been studied previously. Our results are consistent with and extend previous analyses.

Glutamate receptor modulation

There are several receptor subtypes responsible for the binding of excitatory neurotransmitter. In our study, the level of expression for two AMPA receptor subunits (GlurR 2 and GluR 3) and one kainate receptor subunit (GluR K2) were significantly higher at all times following deafening. The GluR 2–GluR 3 receptor combination is believed to be continuously delivered to the synapse in a non-activity-dependent manner, while the GluR1–GluR2 receptor combination, as well as those containing GluR 4, are delivered to the synapse in an activity-dependent manner (Malinow *et al.* 2000; Passafaro *et al.* 2001; Shi *et al.* 2001).

Genes for ras, CamK2, mitogen-activated protein kinase, and protein kinase A, each implicated in the insertion of AMPA receptors into the synapse, showed increased expression. Gene expression for PKC and rap, implicated in the removal of AMPA receptors from the synapse, also increased. This may indicate a high rate of turn-over/delivery of the constitutive receptor (GluR2–GluR3) and an increase in the insertion of the more plastic receptor combination

containing the GluR 1 and GluR 2 receptor subunits. We also found decreased expression of glutamine synthetase, a key enzyme in the recycling of glutamate and increased expression of glutaminase, an enzyme that catalyzes the reaction of glutamine to glutamate.

GABA-related genes

The current study demonstrated sustained increases in the expression in three GABA-A receptor subunit types, with high levels of expression for β2, β3 and γ2 even at 90 days following bilateral cochlear ablation. This suggests compensatory postsynaptic mechanisms that increase expression of these inhibitory receptors at the synapse when presynaptic transmitter function decreases.

The GABA-A γ2 receptor subunit plays a role in transport and placement of the receptor complexes (Kittler and Moss 2003; Schweizer *et al.* 2003). We found increased expression of the GABA-A γ2 subunit along with differential expression of other genes associated with placement of functional receptors into the synapse, such as clathrin (Lee *et al.* 2002; Blanpied *et al.* 2003), dynamin (Okamoto *et al.* 2001; Bobanovic *et al.* 2002; Carvelli *et al.* 2002), and adaptor protein-2 (Kittler *et al.* 2002). There was also increased expression of cAMP-dependent protein kinase, PKC, and tyrosine kinase which modulate GABA receptors through phosphorylation of β and γ subunits. Interestingly, we found increased expression of β3 subunits where phosphorylation leads to increased activity of the receptor, perhaps leading to more 'sensitive' GABA-A receptors following deafness.

Glycine receptor modulation

Studies (Kungel *et al.* 1997; Piechotta *et al.* 2001) have found low levels of expression of the 'immature' glycine receptor α2 subunit in post-natal animals. We found increased expression of the inhibitory receptor's α2 subunit following deafness. The α2 subunit may confer such functional properties as a mean increase in channel open time (Takahashi *et al.* 1992; Mangin *et al.* 2002), an increase of the desensitization constant, and an increase in the duration of the postsynaptic current. The function of glycine receptors has been shown to be enhanced by phosphorylation of α subunits by cAMP-dependent protein kinase, calcium-dependent calmodulin kinase II (CamK), PKC (Legendre 2001) and the glycine β subunit by tyrosine kinase (Caraiscos *et al.* 2002), all of which increased in expression in the current study with the exception of CamK2a and CamK2g which decreased in expression.

Other neurotransmission-related genes

There was decreased TH in the IC following deafness, which correlates well with recent findings by Niu *et al.* (2004) that acoustic trauma decreases TH in the lateral olivocochlear system. Nociceptin and enkephalin also showed decreased
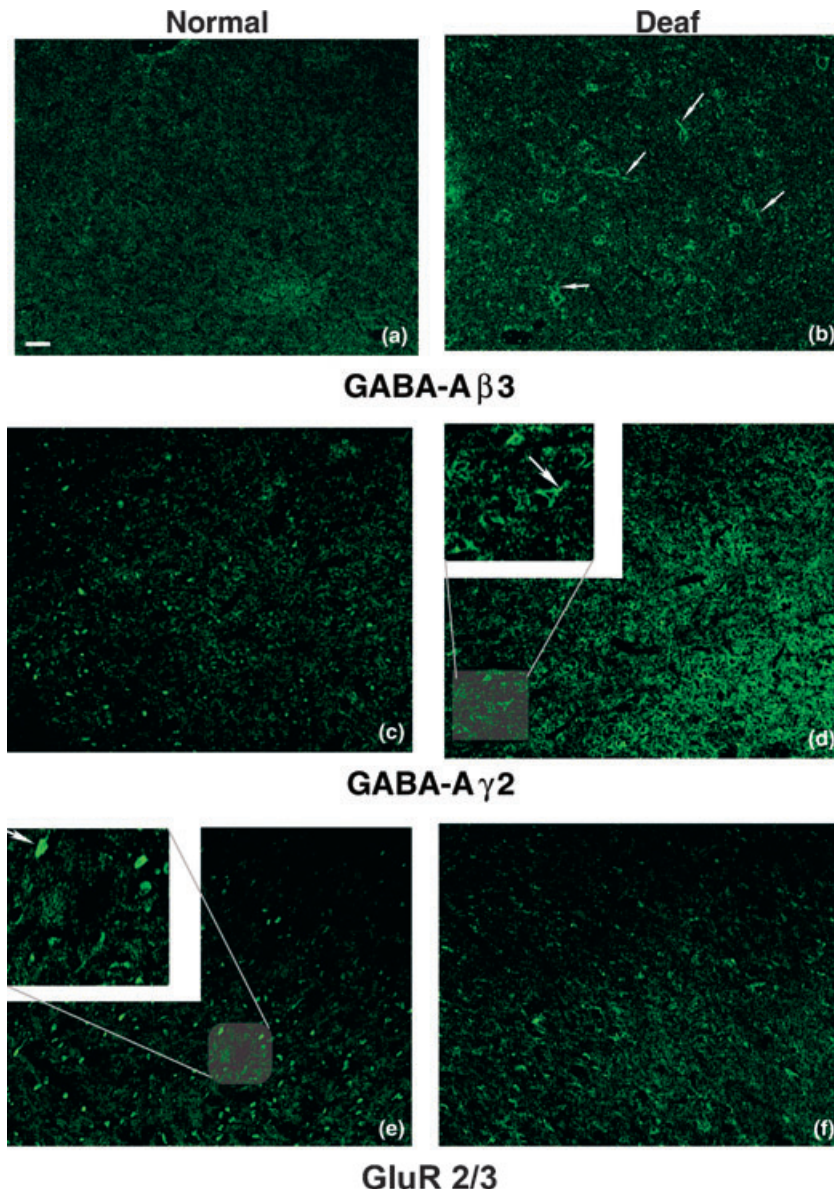
Fig. 2 Changes in immunoreactivity for GABA (a–d) and glutamate (e, f) receptor subunits in the central nucleus of the inferior colliculus. Photomicrographs of subunits for GABA-A β3 (a, b) GABA-A γ2 (c, d) and GluR 2/3 (e, f) receptor immunoreactive cells and/or fibers. Immunoreactivity from animals deafened for 90 days (b, d, f) is more robust when compared with labeling from normal animals (a, c, d). Following deafness, the labeling for GABA-A β3 and GABA-A γ2 is found in more primary dendrites (arrows in b and d) when compared with labeling in the normal hearing group (a, c). Labeling for GluR 2/3 is primarily concentrated in the soma of the neuron in the normal hearing group (arrow in e), however, following deafness the neuropil shows more label (f). Calibration bar = 100 μm.

expression in the current study. Nociceptin receptor knockout mice have a longer recovery period when compared with wild-type mice following noise exposure (Nishi *et al.* 1997), suggesting a protective role for nociceptin. Serotonin receptors are present in the IC (Li *et al.* 2004), and have a purported role in synaptogenesis (Niitsu *et al.* 1995) and neuronal plasticity (Julius 1991; Kojic *et al.* 2000). Many of the differentially expressed neuropeptide-related genes have been implicated in the modulation of GABA, glycine, and/or glutamate receptor function. For example, serotonin receptors (Cai *et al.* 2002) regulate GABA-A receptor activity. Dopamine can enhance kainate receptor-mediated activity (Knapp and Dowling 1987). In addition, opiates can exert an inhibitory affect on AMPA and kainate receptors (Kolaj and Randic 1996).

Other functionally related groups

Previous studies of auditory brainstem plasticity found that deafness affects ion channels (Li *et al.* 2001; Sivaramakrishnan and Oliver 2001; Tzounopoulos and Stackman 2003; von Hehn *et al.* 2004). We found potassium inwardly rectifying channel, subfamily J, members 4 and 11 decreased at both 3 and 21 days, while the potassium voltage-gated channels, Shal-related family, members 2 and 3 increased at 21 days and the potassium voltage-gated channel, KQT-like subfamily, member 1 decreased in expression at 21 days.

Changes in expression of genes associated with deafness-related changes in cell size, dendrites, dendritic spines and synaptic characteristics might be expected. We found the cytoskeleton-related genes Map 2, laminin γ1, protein kinase

Normal   Deaf

Leu - Enkephalin
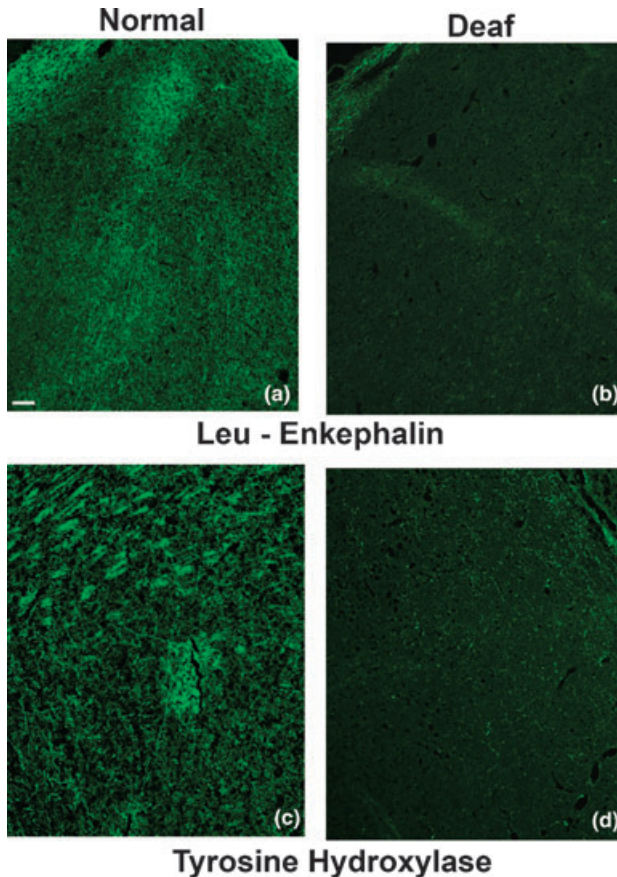

Tyrosine Hydroxylase

Fig. 3 Changes in immunoreactivity for enkephalin (a, b) and tyrosine hydroxylase (c, d) in the central nucleus of the inferior colliculus. Immunoreactivity is decreased in 90-day deaf animals (b, d) when compared with immunoreactivity from normal animals (a, c). Calibration bar = 100 μm.

2 and 6, and ankyrin all increased in expression, while Map Tau and ARC decreased. Genes involved in vesicular trafficking such as SNAP 25, Munc 13–3, and synaptic vesicle glycoprotein 2b increased, while synaptic vesicle glycoprotein 2a decreased in expression.

Several stress-related genes increased in expression and remained increased at 21 days following deafening. These included heat shock proteins 40, 60 and 86, as well as caspase 2, nucleostemin, super oxide dismutase 2, and cyclin (L and G1).

Second messenger-related genes can have direct effects on gene expression and protein production. In this study, the G-protein receptor-related genes endothelin receptor type B, neuropeptide Y receptor Y1, neuropeptide Y receptor Y5, and the cannabinoid receptor 1 gene increased at 21 days, and prostaglandin E receptor 4 (subtype EP4), adrenergic receptor, alpha 2c, galanin receptor 1, gonadotropin-releasing hormone receptor, cholecystokinin B receptor, neurotensin receptor 2, and angiotensin II receptor, type 1 (AT1A) decreased.

Given the many genes differentially expressed, there should also be changes in genes involved in regulation of transcription. We found decreased expression of methyl CpG binding protein 2 (MeCP2), which normally acts to repress gene transcription. This decreased expression could be coupled to increased transcription of some of its target genes although, in this study, one such target, brain derived neurotrophic factor (BDNF), showed decreased expression. Decreased expression of both BNDF and PENK in the current study is interesting in the context of recent studies which showed that application of an enkephalin agonist increases BDNF expression in frontal cortex and hippocampus (Torregrossa *et al.* 2004). Bone morphogenetic protein (BMP), an important signaling molecule, is modulated by a group of BMP antagonists. We found increased expression of BMP-4B, along with decreased expression of noggin, one of the major BMP antagonists. Up-regulation of BMP-4B expression and down-regulation of the BMP-antagonist noggin would lead to increased BMP4 signaling through associated SMADs. Other genes in the GO transcriptional regulation classification that increased in expression included cadherin 2, myosin Ib, myelin-associated oligodendrocytic basic protein, and vascular endothelial growth factor receptor. While Delta-like 1 (*Drosophila*) had decreased expression.

Technical considerations

We found excellent correspondence in the direction and amount of change with GeneChip analysis and qRT-PCR for most genes at most times following deafness. However, in the 3-day deaf group GABA γ1 and glycine α2 receptor subunits showed 33 and 56% increases, respectively, in the GeneChip analysis, while there was no significant change in either by qRT–PCR. qRT–PCR did show increases in both GABA γ1 and glycine α2 receptor subunits at 21 days. In the 21-day deaf group, neurotransmitter-induced early gene protein had only minor expression changes in GeneChip analysis and a 64% increase and 86% decrease, respectively, by qRT–PCR. Glycine receptor α1 and α3 subunits did not show significant changes in the GeneChip analysis but showed increases by qRT–PCR, at 21 and 90 days, a modest increase for α1 and large increases for α3. Neurotransmission-related genes often have low levels of constitutive expression and may therefore prove more challenging when trying to detect changes in such genes by GeneChip analysis when compared with qRT–PCR, perhaps a more sensitive measure. We performed qRT–PCR analysis on eight genes with low FDRs, but small changes in expression (ratio less than 1.5) at 3 days following deafness. Six of eight showed greater changes in expression levels by qRT–PCR.

The low constitutive expression of neurotransmission-related genes may contribute to the greater variability in intensity levels, as measured by GeneChip analysis. The present study addresses the issue of variability by using an

increased sample size, using RMA for normalization that more accurately reflects the expression level of genes, as well as using a statistical method sufficiently stringent in assigning significance. We used SAM to compare gene expression of deafened and normal animals, and to calculate the FDR. A surprisingly large number of genes showed FDRs of 5% or less, suggesting that increased numbers of replicates and RMA can enhance the sensitivity of microarray analysis. Decreasing variability allowed identification of numerous and diverse activity-dependent genes within the inferior colliculus.

We then followed these experiments and analyses with qRT–PCR and immunocytochemical studies for a representative population of our data that we hypothesized would be responsive to decreased activity. Although the specific cell types that undergo modulation as a result of deafness were not differentiated in this examination, we were able to detect changes in both the quantity and intensity of immunolabeling within the central nucleus of the inferior colliculus. The genes that were confirmed with these methods may play an important role in deafness-related plasticity and merit further study in other nuclei within this system. Additionally, many of the other genes identified as differentially expressed in Tables 1–5 and Tables S2 and S3, but not further examined in our study, may also be important and warrant further study.

The present study provides evidence that gene microarrays can provide a useful method for studying deafness-induced plasticity in the central auditory system. By surveying a large pool of neurotransmitter-related genes, we have been able to identify important patterns of expression among these genes. In response to 3 and 21 days of deafness, within the IC, the system seems to compensate for decreased inhibitory input by increasing the expression of highly efficacious inhibitory receptors as well as the molecular machinery necessary for more efficient delivery of these receptors to the synapse. Future studies will include examining different times after deafness as well as studies in additional auditory brainstem regions.

Our results lay the groundwork for future studies that can focus on specific genes, identified in the present study, in greater detail. Certainly, the genes that have not returned to normal levels of expression by the latest time assessed in this study will be of particular interest when considering return of hearing with cochlear prostheses, at different times following deafness. Indeed, there is a correlation between how well a post-lingually deafened patient responds to the cochlear prosthesis and the duration of deafness, with those implanted at earlier times following deafness having the best outcomes (Kileny *et al*. 1991; Blamey *et al*. 1992; Gantz *et al*. 1993). Results of the current study will also provide a normative database and metric to examine reversal at different times following deafness, relationships that have not previously been studied in much detail.

Supplementary material

The following material is available from:
   http://www.blackwellpublishing.com/products/journals/suppmat/JNC/JNC3090/JNC3090sm.htm
   Table S1. Primers used for qRT-PCR.
   Table S2. Genes differentially expressed 3 days after deafening.
   Table S3. Genes differentially expressed 21 days after deafening.

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