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Expression of AMPA, Kainate, and NMDA Receptor Subunits in Cochlear and Vestibular Ganglia

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Glutamate is believed to be the principal afferent neurotransmitter in the peripheral auditory and vestibular systems. In this report, we present a comprehensive molecular analysis of ionotropic glutamate receptor gene expression in the cochlear and vestibular ganglia of the rat. Fourteen glutamate receptor subunits were studied: GluR1–4 (including flip and flop variants), GluR5–7, KA1&2, NR1, and NR2A-D. Reverse transcription of RNA followed by DNA amplification with the polymerase chain reaction was used for the initial analysis. Immunocytochemistry and *in situ* hybridization with subunit-specific oligonucleotides were subsequently used for cellular localization of receptor expression. AMPA (GluR2–4), kainate (GluR5&6 and KA1&2), and NMDA receptor (NR1 and NR2A-D) subunit expression was detected. Based on the relative amounts of mRNA detected by *in situ* hybridization, the predominant receptors expressed by cochlear and vestibular ganglion cells appear to be GluR2, GluR3, GluR4, GluR5, and NR1. At a moderate level were GluR6, NR2B, and NR2D. KA1, KA2, NR2A, and NR2C mRNAs were also expressed in ganglion cells, but at lower levels. Only the AMPA receptor subunit GluR1 and the kainate receptor subunit GluR7 were not found to be expressed in vestibulocochlear neurons. These studies suggest that functional AMPA, kainate, and NMDA receptors are present at the hair cell/vestibulocochlear nerve synapse.

[Key words: glutamate receptor,* in situ *hybridization, immunocytochemistry, auditory periphery, hair cell, rat]

Glutamate is the major excitatory neurotransmitter in the CNS and has been proposed as the principal neurotransmitter at the sensory hair cell/afferent nerve synapse in the cochlea and vestibule. Glutamate neurotransmission can be mediated by various ligand-gated ion channels, of which there are three subtypes. These subtypes, which are classified on the basis of sequence homologies and agonist affinities, are the AMPA receptors (GluR1–4), the kainate receptors (GluR5–7 and KA1&2), and the NMDA receptors (NR1 and NR2A–D). The AMPA and kainate receptors gate fast ionic currents, whereas the NMDA receptors mediate slower currents subject to voltage-dependent Mg^{2+} block (reviewed by Nakanishi, 1992; Sommer and Seeburg, 1992). The "orphan receptors" δ-1 and δ-2 (Yamazaki et al., 1992; Lomeli et al., 1993) comprise an additional subtype of ionotropic glutamate receptor that exhibits low expression levels in the CNS and serves an as yet unknown physiologic function. The ionotropic glutamate receptors exhibit additional complexity in subtype sequences via alternative mRNA splicing, resulting in the flip and flop variants for GluR1–4 (Sommer et al., 1990), the GluR4c variant (Gallo et al., 1992), and variants of GluR5, GluR6 (Gregor et al., 1993) and NR1 (Sugihara et al., 1992).

There is considerable evidence suggesting that glutamate or a structurally related molecule is the afferent neurotransmitter at the hair cell/vestibulocochlear nerve synapse. The criteria of neurotransmitter localization, replenishment, K^+-evoked release, pharmacologic intervention, and postsynaptic reception have been examined, and the majority of the evidence supports glutamatergic neurotransmission in the cochlea (reviewed by Eybalin, 1993). However, there remain several questions. These include whether a molecule other than glutamate activates auditory neurons (Sewell and Mroz, 1987), whether glutamate exhibits stimulus-evoked release, and whether glutamate activates postsynaptic receptors of the AMPA, kainate, or NMDA subtype(s) expressed by the eighth nerve. The receptor subtype has been the subject of several recent reports describing *in situ* hybridization of glutamate receptor mRNA expression in the cochlea; Ryan et al. (1991b) investigated GluR1–5 mRNA expression, whereas Safieddine and Eybalin (1992) and Kuriyama et al. (1993) examined NR1 receptor expression. As of yet, no report has been made of KA1&2 or NR2A–D gene expression. The presence (or absence) of NR2 receptors is particularly important since these subunits, in combination with NR1, are required for the formation of physiologically active NMDA receptors (Monyer et al., 1992).

In this report we describe a comprehensive molecular analysis of ionotropic glutamate receptor gene expression in the vestibulocochlear nerve. The expression of GluR1–4 (including the flip and flop variants), GluR5–7, KA1&2, NR1, and NR2A–D glutamate receptor subunits was examined. Reverse transcription of RNA followed by DNA amplification with the polymerase chain reaction (RT-PCR) was used for the initial analysis of glutamate receptor expression. Once the glutamate receptor complement of the cochlear and vestibular ganglia had been determined by RT-PCR, immunocytochemistry and *in situ* hybridization with subunit-specific oligonucleotides were used for the cellular localization of receptor expression. The application of

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Table 1. PCR primer sequences for glutamate receptor subunits

Subunit	Upstream primer (5′–3′)	Downstream primer (5′–3′)
GluR1–4	CCTTTGGCCTATGAGATCTGGATGTG	TCGTACCACCATTTGTTTTTCA
GluR5	GGTTTTTCACCCTTATCATCAT	GCACTTCAGGGACATTCTCAG
GluR6	TATGTTCTGCTGGCTTGCTTG	GCACTTCAGGGACATTCTCAG
GluR7	GGTTTTTCACCCTTATCATCAT	TGCTCCCGTTCCGCTGTCTTGC
KA1	GGTGTAATCTCCTGGTCAAC	GATGCTTCTGAGTGTCTGAG
KA2	TCGCCCGTGTCCTCAACTCA	CACCGACACCTCCTCAGACT
NR1	ACGGAATGATGGGCGAGC	GGCATCCTTGTGTCGCTTGTAG
NR2A	GGGGTTCTGCATCGACATCC	GACAGCAAAGAAGGCCCACAC
NR2B	GGGGTTCTGCATCGACATCC	GACAGCAAAGAAGGCCCACAC
NR2C	GGGGTTCTGCATCGACATCC	GACAGCAAAGAAGGCCCACAC
NR2D	CGATGGCGTCTGGAATGG	CTGGCAAGAAAGATGACCGC

Underlined bases denote mismatches.

the molecular techniques of RT-PCR, immunocytochemistry, and *in situ* hybridization permitted the extensive comparative analysis of glutamate receptor expression in cochlear and vestibular ganglion cells.

Materials and Methods

RT-PCR analysis. Cochlear and vestibular ganglion tissue was microdissected from adult male Sprague–Dawley rats (125–175 gm; 6–12 animals), the tissue was lysed in 4 M guanidinium thiocyanate, and then total RNA was isolated by phenol/chloroform extraction based on the rapid single step method of Chomczynski and Sacchi (1987). Alternatively, polyA$^+$ RNA was isolated directly from the tissue lysate with oligo(dT)-coated magnetic beads (Dynal, Lake Success, NY). The amount of RNA obtained was too small to reliably quantify without considerable loss of sample, and since RT-PCR was being used only as a survey method, RNA quantification was not necessary. First-strand cDNA was synthesized from oligo(dT)-primed RNA with Superscript reverse transcriptase (GIBCO–Bethesda Research Labs, Gaithersburg, MD). For the analysis of NR2A–D expression, first-strand cDNA synthesis utilized Superscript reverse transcriptase and random hexamer primers (Research Genetics, Huntsville, AL). The cDNA was amplified by the polymerase chain reaction (PCR) using degenerate primers for GluR1–4 (Lambolez et al., 1992), specific primers for GluR5, GluR6, GluR7, KA1, KA2, NR1, and NR2D, and degenerate primers for NR2A-C (Table 1). The sequences for the NR2 primers were provided by Audinat et al. (in press). PCR reaction conditions were as directed for AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA), typically using 40 cycles of amplification with annealing temperatures of 49°C (GluR1–4), 54°C (GluR5–7 and KA1&2), 55°C (NR1), and 52°C (NR2A–D). The presence of PCR fragments of the desired size was ascertained by agarose gel electrophoresis. In all cases, the utility of the primers was first examined using cDNA clones. Rat brain cDNA prepared in a manner similar to that from the ganglia was used as a positive control. Restriction enzyme digest of the GluR1–4 and NR2A-C PCR fragments (obtained with degenerate primers) was used for the identification of individual subunits. Flip and flop expression was detected by PCR amplification of the degenerate GluR1–4 fragment using variant-specific primers (Lambolez et al., 1992). Restriction enzyme digest was also used to verify the identities of the PCR products obtained for the kainate receptors and NR1 and NR2D. The receptor subunits and the enzymes used for the digests are as follows: GluR1, Bgl I; GluR2, Bsp1286 I; GluR3, Eco47 III; GluR4, EcoR I; GluR5, BspH I; GluR6, Hinc II; GluR7, Ava I; KA1, EcoR I; KA2, Eco52 I; NR1, Eco52 I; NR2A, Ban II; NR2B, BspH I; NR2C, Sca I; NR2D, Xho I (all enzymes supplied by New England Biolabs, Beverly, MA).

In situ *hybridization.* The modiolar portion of the cochlea containing the cochlear nerve ganglion tissue (with the vestibular ganglion attached) was dissected from adult male rats, frozen in isopentane/dry ice, cryostat sectioned at 18 μm, and mounted on silanated slides (Digene Diagnostics, Beltsville, MD). The sections were decalcified in fixative solution (5% EDTA in 4% paraformaldehyde) for 30 min at 4°C, acetylated, and washed immediately prior to *in situ* hybridization. Antisense oligonucleotides specific for each receptor subtype were synthesized on an Applied Biosystems PCRMate DNA synthesizer. The following oligonucleotide probes were identical to those utilized previously for *in situ* hybridization experiments: GluR1–4 (Keinänen et al., 1990), GluR1–4 flip and flop (Sommer et al., 1990), KA1 and KA2 (Herb et al., 1992), NR1 (Safieddine and Eybalin, 1992), and the probes for NR2A-C (Monyer et al., 1992). The antisense oligonucleotides for GluR5, GluR6, and NR2D were designed using the computer program Oligo (National Biosciences, Plymouth, MN). The oligonucleotide sequences are 5′-GTCTCTGTTGCCATCCGTCATGTTCAGCCCACTG-TTGGAGTTCCA-3′ (corresponding to amino acids 391–405 of GluR5; Bettler et al., 1990), 5′-TTCCTTTCTGACTTTCTGTCATATTCAGG-CCACTGGCTGGATCCC-3′ (amino acids 374–387 of GluR6; Egebjerg et al., 1991), and 5′-CCGCCCCGGGGTCCAGTGTCAGCGCTC-CTCGATGCTCCCAGCCCA-3′ (amino acids 210–225 of NR2D; Ishii et al., 1993). The oligonucleotides were 3′-end labeled by incubation with terminal deoxynucleotidyl transferase (Stratagene, La Jolla, CA) and ^{35}S-dATP (DuPont–New England Nuclear, Boston) for 5 min at 37°C. Labeled oligonucleotide [in hybridization buffer containing 50% formamide and 4× SSC (standard saline citrate, 0.15 M NaCl, 0.015 M sodium citrate)] was then applied to the sections at a final concentration of 8.3 pg/μl and slides were incubated at 40°C overnight. Controls for specificity of hybridization consisted of applying a 100-fold excess of unlabeled oligonucleotide in the presence of labeled probe. In addition, as a positive control, brain sections were subject to *in situ* hybridization in parallel with the inner ear sections. The sections were washed in 50% formamide/2× SSC at 40°C or 0.5× SSC at 45°C, dehydrated, dried, and then coated with a 1:1 dilution of NTB2 photographic emulsion (Kodak, Rochester, NY) in water. Slides were exposed for 28 to 32 d at 4°C, the emulsion was then developed, sections were stained with thionin, and the silver grains observed using light microscopy. All photographs and figures of the cochlear ganglion are displayed so that the scala vestibuli side of each ganglion is located towards the top of the figure.

The method of decalcification of cochlear and vestibular tissue sections prior to *in situ* hybridization (above) yielded optimal results. This method is a composite of two separate techniques: decalcification of cochleae prior to *in situ* hybridization, and *in situ* hybridization with cryosections. In our experience, the decalcification of whole cochlea prior to *in situ* hybridization, similar to the method of Ryan et al. (1991a), results in low hybridization signal with oligonucleotide probes. While *in situ* hybridization with cryosections normally would produce adequate results, cochlear cryosections that had not been decalcified exhibited high background due to the binding of labeled probe to bony tissue (data not shown). Thus, the methodology utilized for the *in situ* hybridization experiments consisted of brief decalcification of cryosections prior to hybridization with the oligonucleotide probe.

Quantitative analysis of receptor expression was achieved by both manual and computer-assisted counting of silver grains present over ganglion cells. Computer-assisted counting was based on a method described by McNamara (1993) and was achieved by using the particle analysis function of Image 1.47 (NIH, Bethesda, MD) on a captured video image that had been processed by unsharp masking (ADOBE PHOTOSHOP, Adobe Systems, Mountain View, CA). The computer-

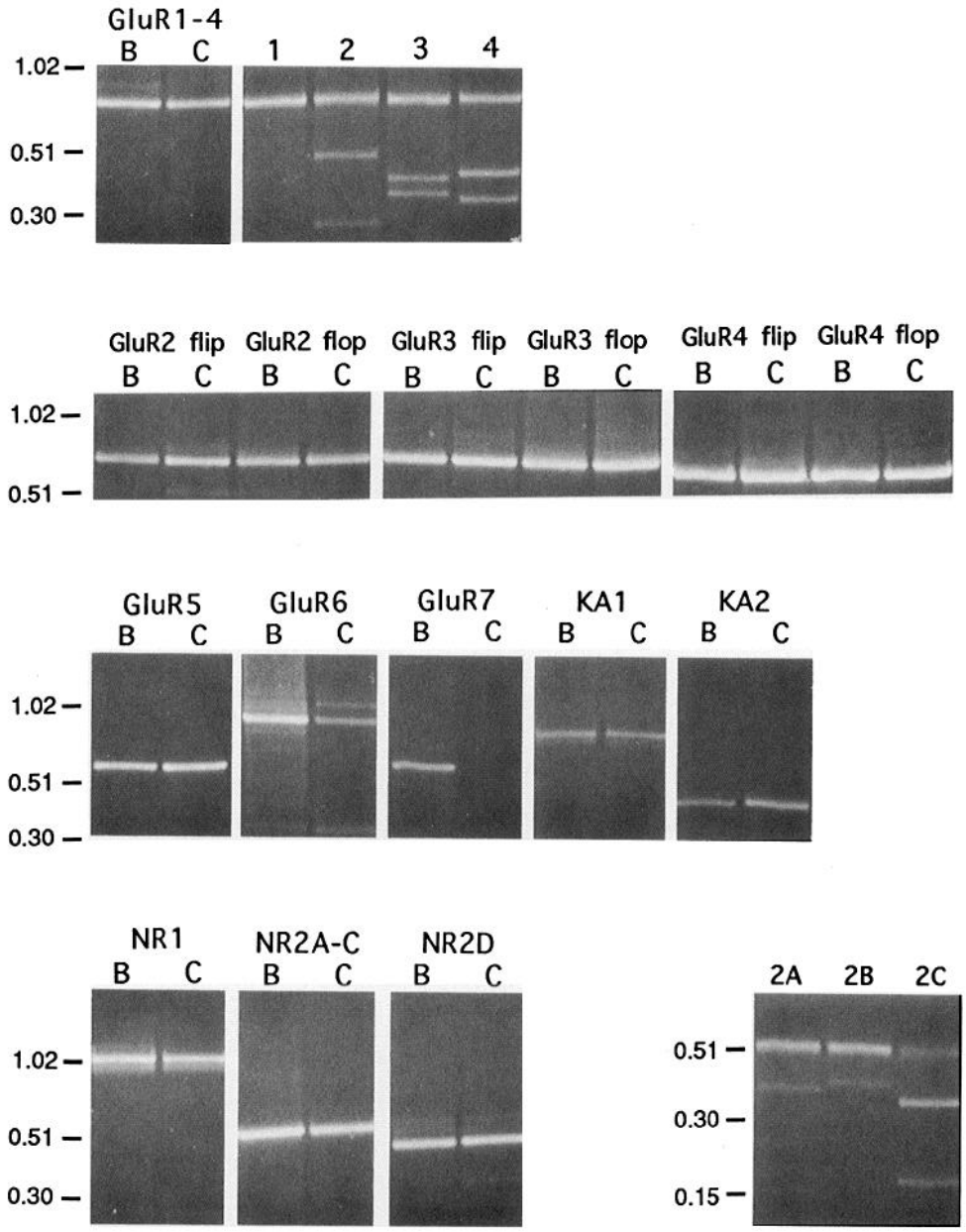

Figure 1. RT-PCR analysis of glutamate receptor subunit expression in the cochlear and vestibular ganglia. Each EtBr-stained gel illustrates the analysis for a specific receptor or receptors, with *lane B* = brain (positive control) and *lane C* = cochlear and vestibular ganglion. In addition, *lanes 1–4* = GluR1–4, respectively, detected by specific restriction enzyme digest of the cochlear and vestibular ganglion PCR fragment for combined GluR1–4 (note absence of specific fragments for GluR1); *lanes 2A–C* = NR2A–C, respectively, detected by restriction digest of cochlear and vestibular ganglion PCR fragment for combined NR2A–C (for enzymes used, see Materials and Methods). Molecular weight markers are in kilobase pairs.

assisted counts of silver grains were confirmed by manual grain counting over randomly selected cells. Ganglion cell area was measured using Image 1.47 and cell-specific grain counts were expressed per 100 μm². Background counts of silver grains were obtained from defined nontissue areas adjacent to the ganglia and were subtracted from ganglion cell-specific grain counts. Background levels for those oligonucleotides quantified extensively (see Results) ranged between 10% and 20% of the average grain density found overlying ganglion cells. In addition, background levels were compared to cell-specific hybridization for those probes characterized as having "low" expression. In all such cases, cell-specific grain density was at least twice background levels.

Immunocytochemistry. Polyclonal antibodies raised against C-terminal amino acid sequences of GluR1, GluR2/3, GluR4 (Wenthold et al., 1992), NR1 (Petralia et al., 1994a), and NR2A/B (Petralia et al., 1994b) were used to detect expression of receptor proteins in the cochlear ganglion. The vestibular ganglion was not examined in these experiments, since it had been studied previously (Petralia et al., 1994a,b; Petralia and Wenthold, unpublished observations). Cochleae were dissected from adult male rats after perfusion with 4% paraformaldehyde and then decalcified in fixative solution (5% EDTA in 4% paraformaldehyde) for 2 weeks at 4°C. After cryoprotection in 10% sucrose, the decalcified cochleae were frozen in isopentane/dry ice and stored at −70°C. Cochleae were cyrostat sectioned at 18 μm and sections mounted on silanated slides. Following an overnight incubation at 4°C with primary antibody (final concentration 2 μg/ml) in 10% goat serum in phosphate-buffered saline (PBS), sections were washed and then immunolabeled by the ABC avidin–biotin complex method (Vectastain kit, Vector Laboratories, Burlingame, CA). Immunostaining was visualized with diaminobenzidine tetrahydrochloride (DAB) and examined on a Zeiss Axiophot microscope. Control sections were incubated without primary antibody or with primary antibody preabsorbed with specific peptide (5 μg peptide/ml of antibody).

Results

RT-PCR analysis

The initial analysis of glutamate receptor subunit expression in the cochlear and vestibular ganglion utilized reverse transcription of RNA followed by DNA amplification (RT-PCR) with subunit-specific primers. Two different priming methods for reverse transcription (RT) were utilized in these experiments, with oligo-dT as the primer except when NR2 receptor expression

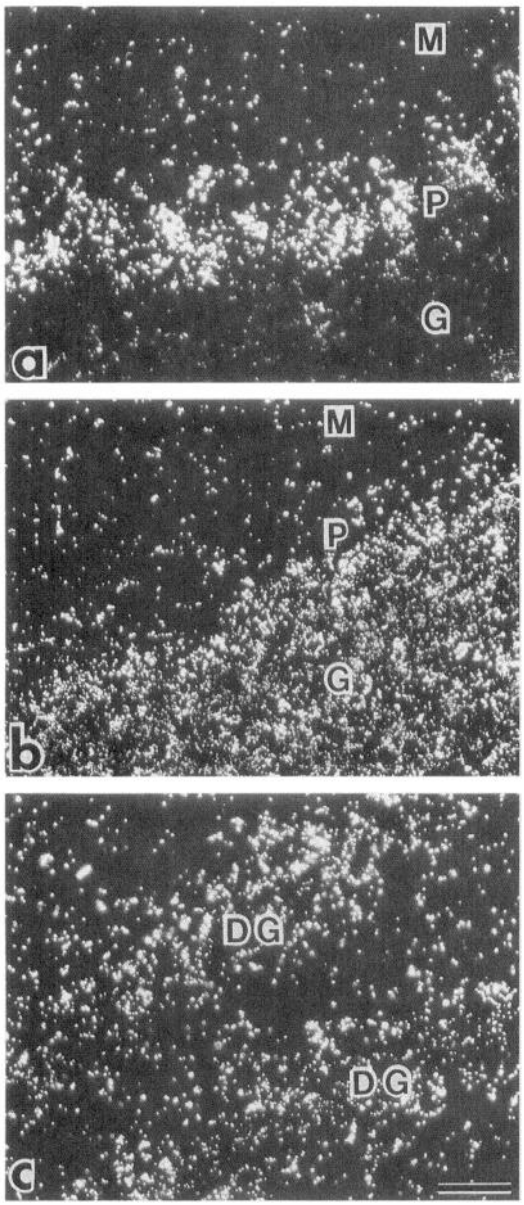

Figure 2. Dark-field photomicrographs of *in situ* hybridization for GluR5, GluR6, and NR2D. *a,* Expression of GluR5 in the Purkinje cell layer (*P*) of the cerebellum. *M* denotes the molecular layer, *G* denotes the granule cell layer. *b,* Expression of GluR6 in the granule cell layer (*G*) of the cerebellum. *c,* Expression of NR2D in dentate granule cells (*DG*) of the hippocampus. Scale bar, 40 μm.

was analyzed. Since the long C-termini of the NR2 receptors (approximately 2 kb of coding sequence subsequent to TM4) caused difficulties in the conversion of oligo-dT primed mRNA into cDNA fragments containing the required sequences for PCR amplification, random hexamers were, instead, used to prime cDNA synthesis. For the PCR reaction, the use of degenerate or nondegenerate primers depended primarily on sequence similarities between subunits in addition to the practical limitations of PCR primer design (i.e., target sequences, duplex formation, cross-hybridization and mismatched bases). Due to these considerations, only the AMPA receptors (GluR1–4) (Lambolez et al., 1992) and the NMDA receptors NR2A-C were analyzed using both 5′ and 3′ degenerate PCR primers, whereas analysis

Table 2. Comparison of selected glutamate receptor subunit mRNA expression in cochlear and vestibular ganglion cells by quantitative analysis of *in situ* hybridization grain density [grains per 100 μm², mean ± SD (*n*)]

Subunit	Cochlea	Vestibule	
GluR2	6.9 ± 2.6 (apical) (20)	9.5 ± 4.5 (20)	*
	7.2 ± 3.1 (medial) (54)		*
	6.3 ± 2.7 (basal) (20)		**
GluR2 flip	12.3 ± 4.2 (20)	9.4 ± 3.0 (20)	*
GluR2 flop	8.3 ± 3.6 (20)	9.8 ± 3.2 (20)	
GluR3 flip	9.1 ± 2.9 (20)	6.4 ± 2.5 (20)	**
GluR3 flop	13.8 ± 4.9 (20)	9.4 ± 2.9 (20)	**
GluR5	11.2 ± 3.0 (68)	12.0 ± 3.2 (33)	
NR1	12.2 ± 2.8 (100)	11.7 ± 2.4 (20)	

Significant difference between cochlear and vestibular expression levels: $*p < 0.05$, $**p < 0.005$ (*t* test).

of GluR5 and GluR7 employed a common 5′ primer and a subunit-specific 3′ primer.

A relatively pure preparation of vestibulocochlear nerve ganglia can be obtained by microdissection and, thus, RT-PCR is a logical first step in the analysis of gene expression in this tissue. Preliminary RT-PCR analysis detected the expression of AMPA receptors in both the cochlear ganglion and the vestibular ganglion (as a degenerate fragment for GluR1–4). Since AMPA receptor expression was similar between both the cochlear and vestibular ganglia and since *in situ* hybridization would be utilized for cellular localization of detected transcripts, cochlear and vestibular RNA samples were combined for subsequent experiments in order to minimize the number of animals required while increasing the amount of mRNA available. RT-PCR analysis of combined cochlear/vestibular ganglion samples demonstrated the expression of the AMPA receptors. Restriction enzyme digest of the degenerate AMPA receptor PCR fragment detected GluR2, GluR3, and GluR4, but not GluR1 (Fig. 1). Flip and flop variants of the three AMPA receptors were also detected by PCR. The expression of the kainate receptors GluR5, GluR6, KA1, KA2, and the NMDA receptors (NR1 and NR2A–D) was also detected in the cochlear/vestibular ganglion. Digestion with restriction endonucleases specific for each amplified fragment verified the presence of the proper sequences, including NR2A, NR2B, and NR2C (which were originally obtained using degenerate primers). Of the 14 ionotropic receptor subunits examined by RT-PCR analysis, only GluR1 and GluR7 were not detected in the cochlear and vestibular ganglia (in contrast to brain samples, which did express GluR1 and GluR7 mRNA). In addition, no cochlear or vestibular ganglion-specific receptor splice variants were evident in the sequences amplified by PCR.

In situ *hybridization*

The results of the RT-PCR analysis of the cochlear and vestibular ganglion were used to determine which receptor subunits would be subject to cellular localization by *in situ* hybridization. Therefore, we investigated the cellular expression of all of the receptor subunits except GluR7. Although GluR1 had not been detected by RT-PCR analysis, its expression was examined by *in situ* hybridization in order to validate the use of RT-PCR as a survey method. The oligonucleotides utilized for *in situ* hybridization have all been previously published [GluR1–4 (Kein-

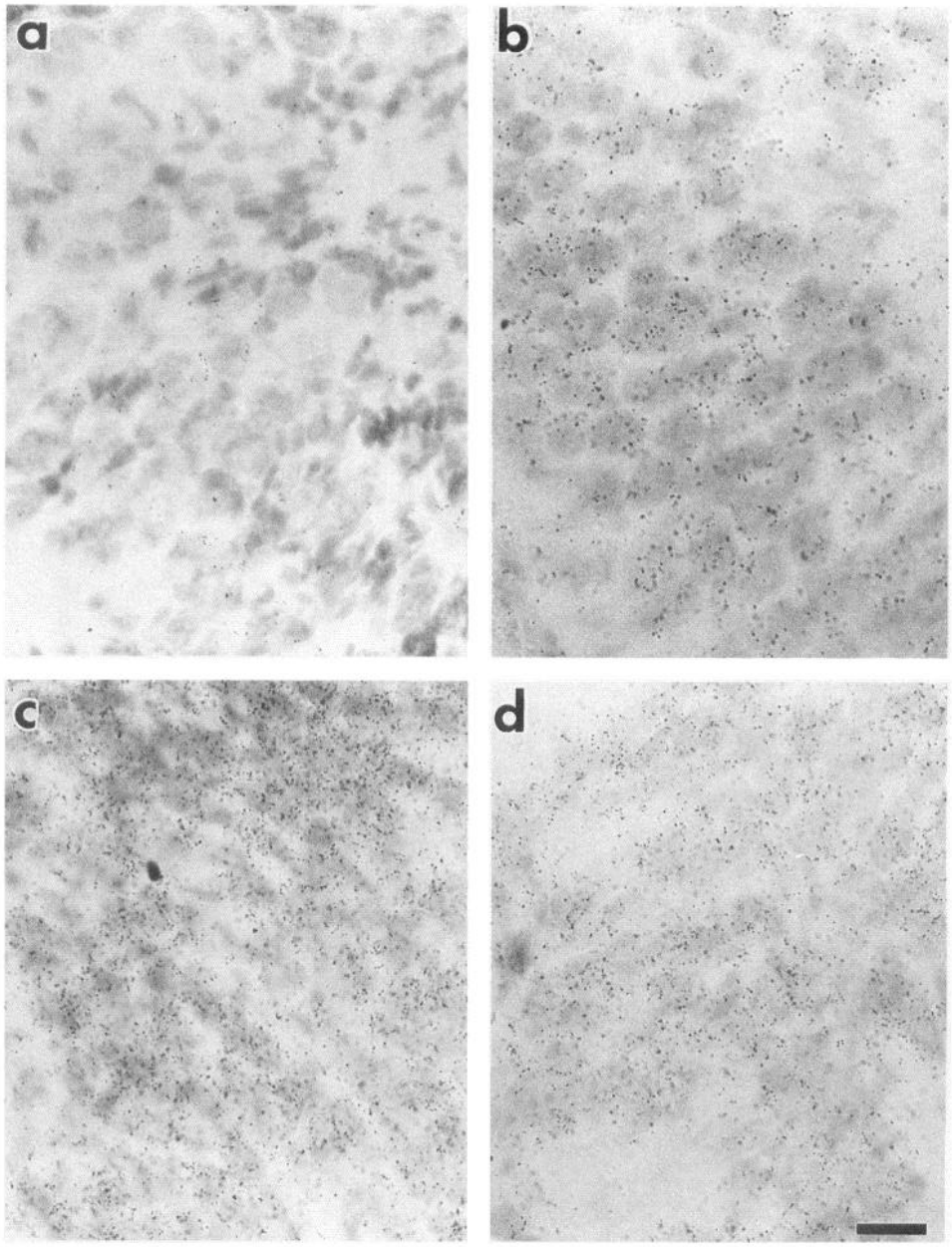

Figure 3. GluR1–4 mRNA expression in the cochlear ganglion. *a–d*, GluR1–4, respectively. Scale bar, 20 μm.

änen et al., 1990), GluR1–4 flip and flop (Sommer et al., 1990), KA1 and KA2 (Herb et al., 1992), NR1 (Safieddine and Eybalin, 1992), NR2A-C (Monyer et al., 1992)] except for those used for the detection of GluR5, GluR6, and NR2D. Each of these oligonucleotides exhibited hybridization patterns that were in agreement with previous reports (Bettler et al., 1990; Egebjerg et al., 1991; Ishii et al., 1993). Specifically, GluR5 hybridization was found at high levels in the Purkinje cell layer of the cerebellum, GluR6 hybridization was high in cerebellar granule cells, and NR2D hybridization was moderate in the dentate granule cells of the hippocampus (Fig. 2). All previously-published oligonucleotides except that for NR2B exhibited hybridization patterns in brain that were similar to earlier reports (data not shown). The NR2B-specific oligonucleotide did exhibit a moderate level of hybridization in cerebellar granule cells, which we interpret as cross-hybridization with NR2C. Since NR2B expression was found to be higher than NR2C, particularly in the cochlear ganglion, most NR2B hybridization signal in cochlear and vestibular tissues is specific for NR2B mRNA. As a control for nonspecific hybridization, a 100-fold excess of unlabeled oligonucleotide diminished hybridization for all probes in both brain and vestibulocochlear nerve sections to near background levels.

The AMPA receptor subunits GluR2, GluR3, and GluR4 were each detected in cochlear and vestibular ganglion cells by *in situ* hybridization (Figs. 3, 4 Table 2), with nearly all cells exhibiting some level of probe hybridization. In concert with the RT-PCR analysis, hybridization to GluR1 mRNA was not detected. Although absolute quantitative comparisons of expression levels for the different receptor subunits are not possible, levels of expression can be inferred from the intensity of a hybridization signal. In this context, AMPA receptor mRNA expression can be viewed as being moderate to high for GluR2–4 in the cochlear and vestibular ganglia. Similarly, all of the flip and flop variants of GluR2–4 were expressed at moderate levels except for GluR4 flip, which was expressed at low levels in both the

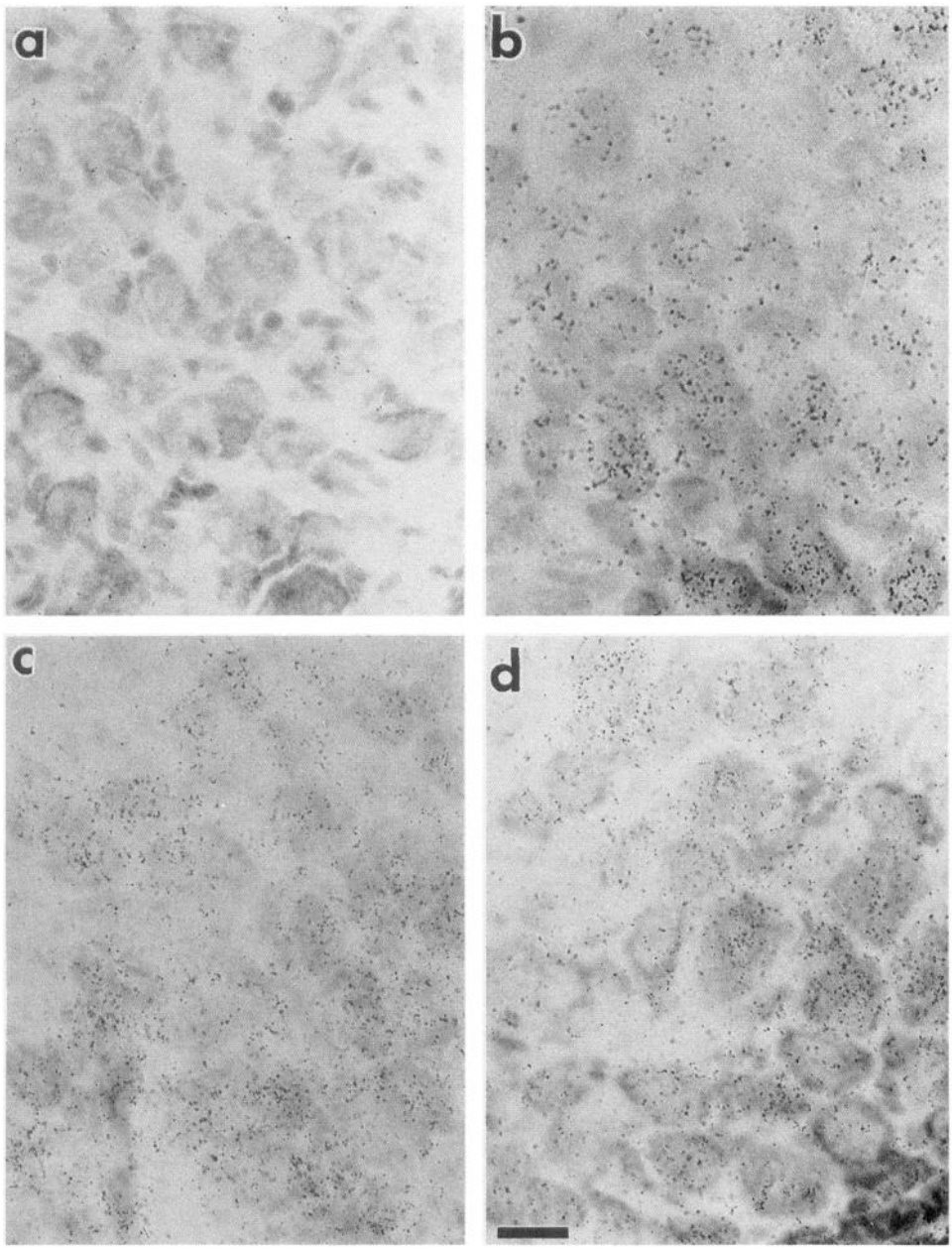

Figure 4. GluR1–4 mRNA expression in the vestibular ganglion. *a–d,* GluR1-4, respectively. Scale bar, 20 μm.

cochlear and vestibular ganglia (Figs. 5; Table 2). For the kainate receptors, GluR5 mRNA exhibited high levels of expression, GluR6 mRNA expression was moderate, and KA1 and KA2 mRNAs were expressed at low to moderate levels (Figs. 7, 8; Table 2). The NMDA receptors NR1, NR2B, and NR2D were expressed at moderate to high levels, whereas NR2A and NR2C mRNA expression was low (Figs. 9, 10; Table 2). For those glutamate receptors exhibiting moderate to high mRNA levels, including GluR2-5 and NR1, all of the ganglion cells in a section exhibited some level of receptor expression. There was no evidence of heterogeneous expression of these receptor subunits (i.e., expression in certain cells with no expression in others). This may also be the case for those receptors that exhibit low levels of expression such as KA1 and NR2A. However, the low expression levels detected by *in situ* hybridization preclude direct confirmation that KA1 and NR2A are expressed in all ganglion cells.

Quantitative analysis of hybridization patterns

On a qualitative level, the *in situ* hybridization results suggest that cochlear and vestibular ganglion cells express a similar glutamate receptor complement. Since cochlear and vestibular ganglia sections were placed jointly on a slide and, thus, were subject to identical hybridization conditions, quantitative analysis of grain density for individual *in situ* hybridization probes can be used to examine whether tissue-specific differences in expression exist. Moreover, receptor expression can be compared along the tonotopic axis of the cochlear ganglion (i.e., from base to apex) to examine the contribution, if any, of glutamate receptors to auditory neuron tuning. Finally, receptor expression can be compared between cells of an individual cochlear ganglion to determine whether cell position or size is related to expression levels.

GluR2 (including flip and flop), GluR5, and NR1 were chosen

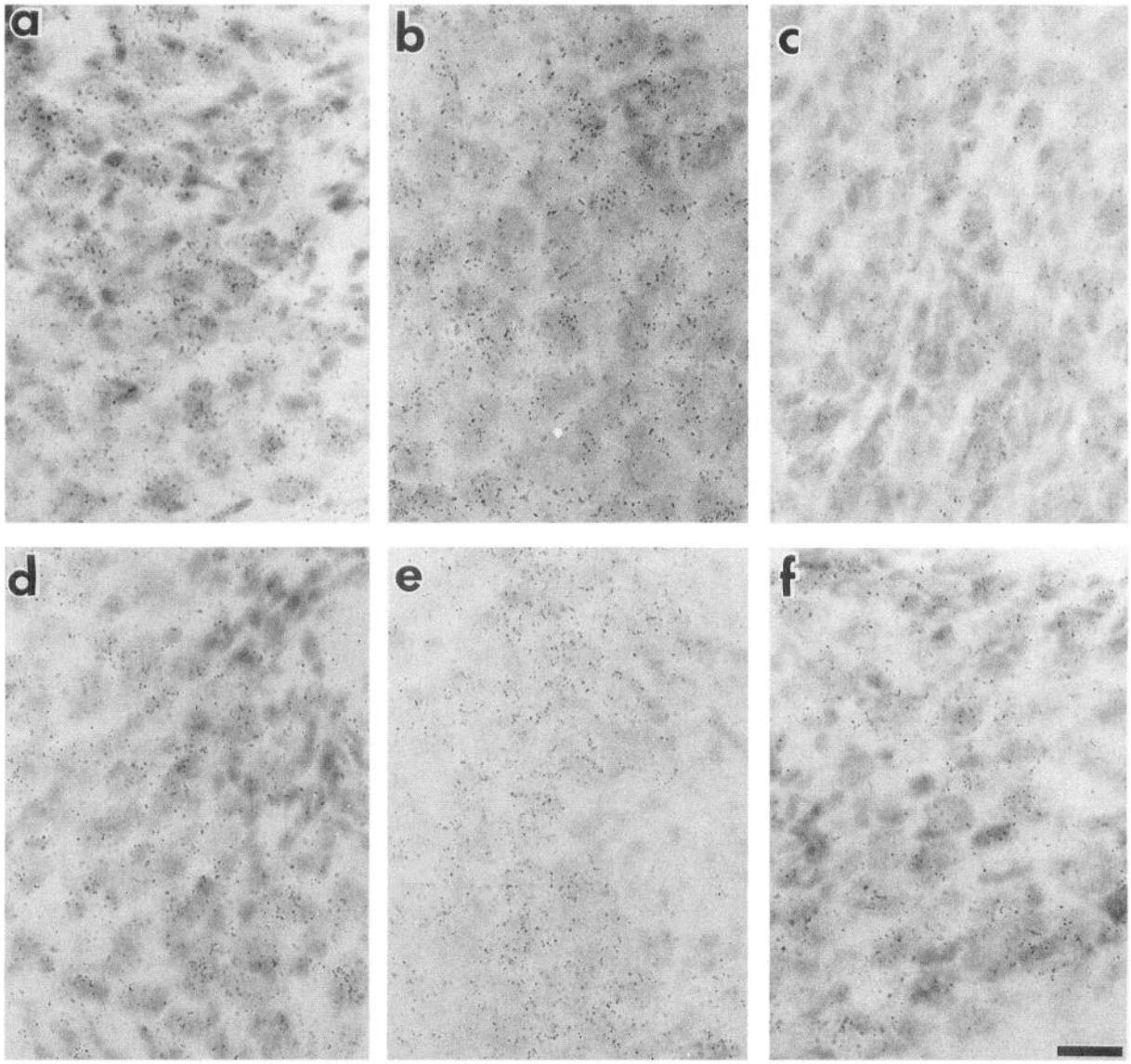

Figure 5. GluR2–4 flip and flop mRNA expression in the cochlear ganglion. *a* and *d* = GluR2 flip, flop. *b* and *e* = GluR3 flip, flop. *c* and *f* = GluR4 flip, flop. Scale bar, 20 μm.

for quantitative analysis since they are each members of a different receptor subtype and all three exhibit high levels of expression in the vestibulocochlear nerve. Furthermore, GluR2 has been shown to be a critical subunit for the AMPA receptor since its presence results in a channel impermeable to calcium ions. GluR3 flip and flop were also quantified since visual comparison of Figures 5 and 6 suggested differences in expression between the cochlear and vestibular ganglia. While various neurochemical and anatomical differences exist between cochlear base and apex (reviewed in Eybalin, 1993), expression levels of GluR2 did not differ along this tonotopic axis of the cochlea (Table 2). Similar results were obtained for GluR5 and NR1 (data not shown). Total GluR2 expression is slightly higher in the vestibular ganglion, while GluR2 flip, GluR3 flip, and GluR3 flop expression is slightly higher in the cochlear ganglion. No significant difference between cochlear and vestibular ganglion cells was observed for GluR5 and NR1 expression.

Cell size and position have physiological implications in the cochlear ganglion. Cell size is an indication of cell type, such that smaller cells [less than 125 μm^2 in the rat (Berglund and Ryugo, 1991)] are putative type II ganglion cells that project exclusively onto outer hair cells. The type I cells, each of which projects exclusively to a single inner hair cell, are usually larger and considerably more abundant (approximately 90 to 95% of cochlear ganglion cells). GluR2 expression is highest in smaller cochlear ganglion cells, whereas GluR5 and NR1 expression is relatively constant between cells of different sizes (Fig. 11). In the cat, and presumably in other species, the position of a cell within a cochlear ganglion is related to its spontaneous discharge rate. Ganglion cells located near the scala vestibuli are more likely to exhibit low to medium spontaneous discharge rates, while cells having high spontaneous rates are more evenly distributed within the ganglion (Kawase and Liberman, 1992). No specific expression pattern for GluR2, GluR5, or NR1 was observed between cells of a representative cochlear ganglion (Fig. 12), particularly in cells likely having low spontaneous rates (which are located at the upper portion of the figure). However, since spontaneous rate of cochlear ganglion cells is not totally

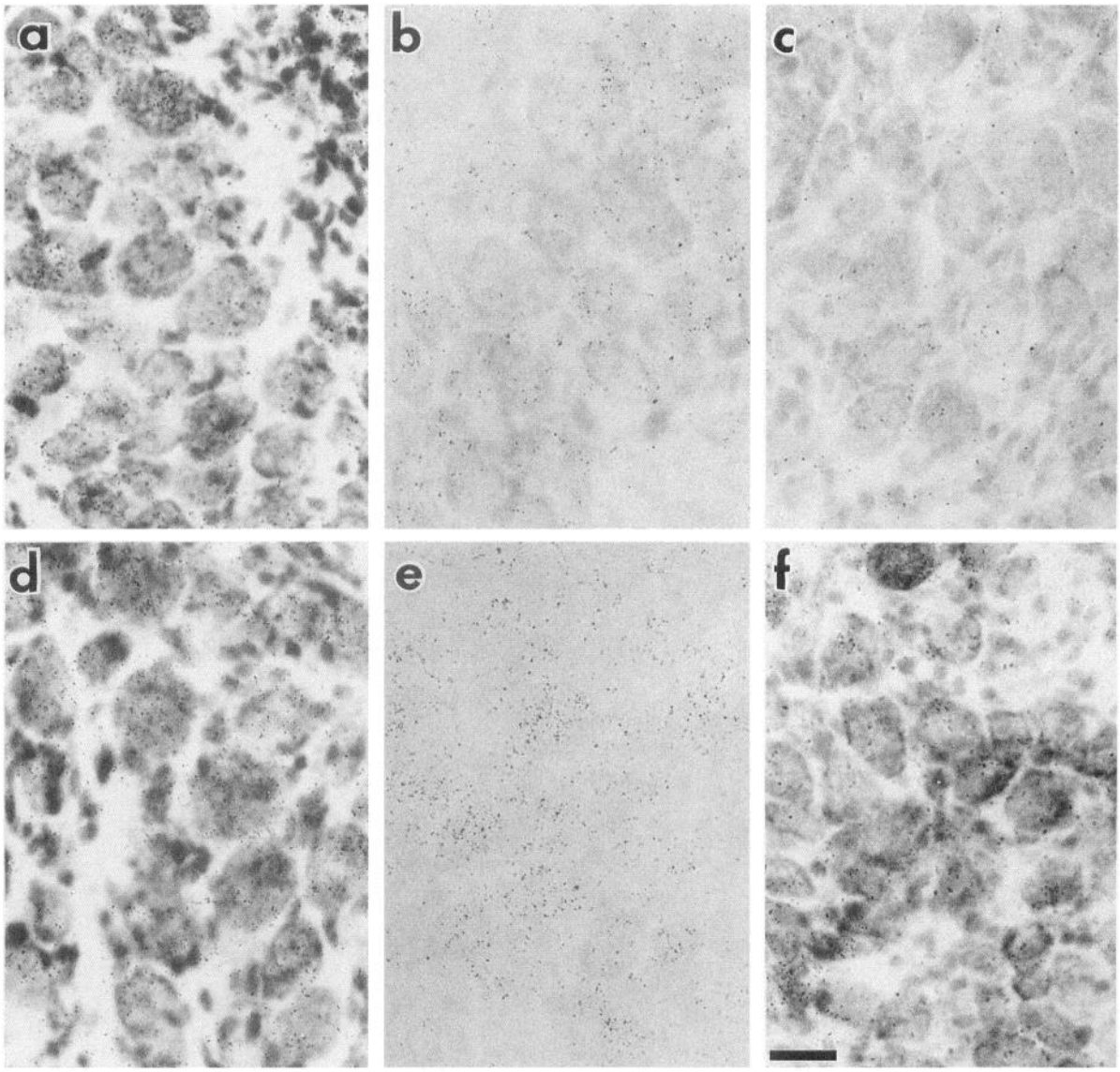

Figure 6. GluR2–4 flip and flop mRNA expression in the vestibular ganglion. *a* and *d* = GluR2 flip, flop. *b* and *e* = GluR3 flip, flop. *c* and *f* = GluR4 flip, flop. Scale bar, 20 μm.

dependent on cell position, the presence or absence of a correlation between receptor expression and spontaneous rate cannot be absolutely determined. The figure does illustrate that ganglion cells exhibit varying levels of grain density, but these levels followed a normal distribution around the average grain density and were randomly distributed in the ganglion.

Immunocytochemistry

Immunocytochemistry with subunit-specific anti-peptide antibodies to GluR2/3, GluR4, NR1, and NR2A/B was utilized to confirm the expression of these receptor proteins in the cochlear ganglion. Immunochemistry was applied in parallel with *in situ* hybridization since it has been determined that NMDAR1 mRNA transcripts are not translated into receptor proteins in PC12 cells (Sucher et al., 1993). Although a number of other receptor transcripts had been detected with *in situ* hybridization, the presence of their respective receptor proteins was not examined by immunocytochemistry because either no antibodies were available or the *in situ* hybridization signal was too low to warrant it. Anti-GluR2/3 antibody and anti-NR1 showed the highest levels of immunolabeling in cochlear ganglion cells, while anti-GluR4 and anti-NR2A/B immunolabeling was detected at lower levels (Fig. 13). Immunolabeling of presumed glial cells in the cochlear ganglion was observed with the GluR4 and NR1 antibodies. Anti-GluR1 antibody did not immunolabel ganglion cells, and control sections were similarly unlabeled (data not shown). GluR2/3, GluR4, NR1, and NR2A/B immunostaining was exhibited by all neuronal cells of a particular cochlear ganglion, which is consistent with the results of the *in situ* hybridization experiments. In addition, these results are consistent with previous experiments that detected GluR2/3, GluR4, NR1, and NR2A/B immunostaining in the vestibular ganglion (Petralia et al., 1994a,b; Petralia and Wenthold, unpublished observations).

Discussion

Using RT-PCR, *in situ* hybridization and immunocytochemistry for the comprehensive analysis of ionotropic glutamate receptors

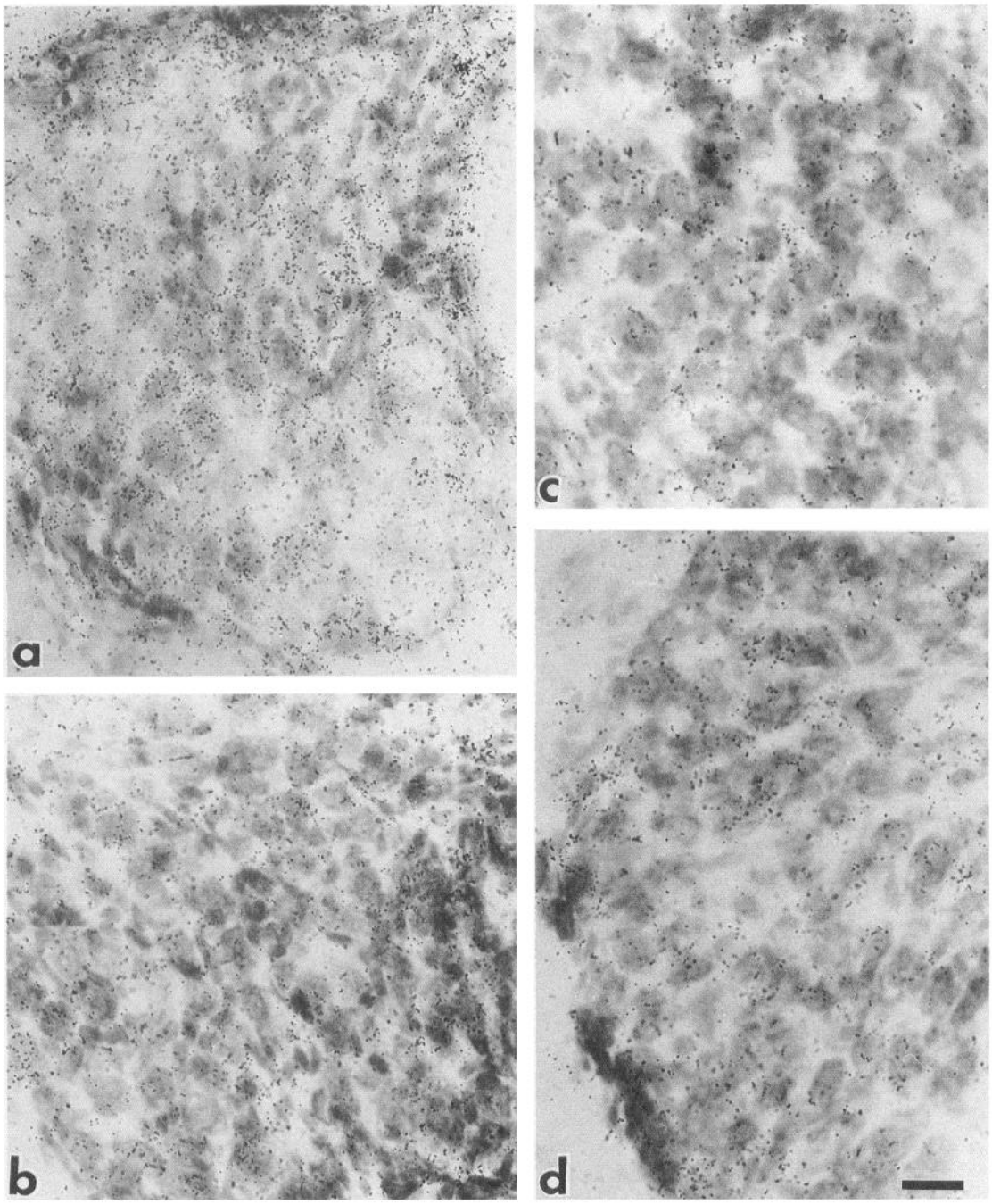

Figure 7. Kainate receptor subunit mRNA expression in the cochlear ganglion. *a* and *b* = GluR5, GluR6. *c* and *d* = KA1, KA2. Scale bar, 20 μm.

in the cochlear and vestibular ganglion, we have demonstrated the expression of AMPA (GluR2–4), kainate (GluR5&6 and KA1&2), and NMDA (NR1 and NR2A–D) receptor subunits. The predominant receptors expressed by cochlear and vestibular ganglion cells appear to be GluR2, GluR3, GluR4, GluR5, and NR1. GluR6, NR2B, and NR2D mRNAs were expressed at moderate levels, and KA1, KA2, NR2A, and NR2C mRNAs were expressed at low levels in ganglion cells. In addition, immunocytochemistry detected GluR2/3, GluR4, NR1, and NR2A/B protein expression at moderate to high levels in cochlear ganglion cells, with NR1 and GluR4 immunolabeling also being detected in putative glial cells. GluR4 immunoreactivity within the glia of the cochlear ganglion resembles staining of satellite cells in dorsal root ganglia (Tachibana et al., 1994); however, the physiological role of glial GluR4 may be difficult to determine because of the extensive expression of glutamate receptors in ganglion cells. Of the 14 glutamate receptor subunits examined in this study, only the AMPA receptor subunit GluR1 and the kainate receptor subunit GluR7 were not detected in vestibulocochlear neurons.

Pharmacological and electrophysiological experiments have provided considerable evidence for the presence of AMPA/kainate receptors in the cochlea and vestibule. Experiments with isolated rat cochlear ganglion cells demonstrated the presence of

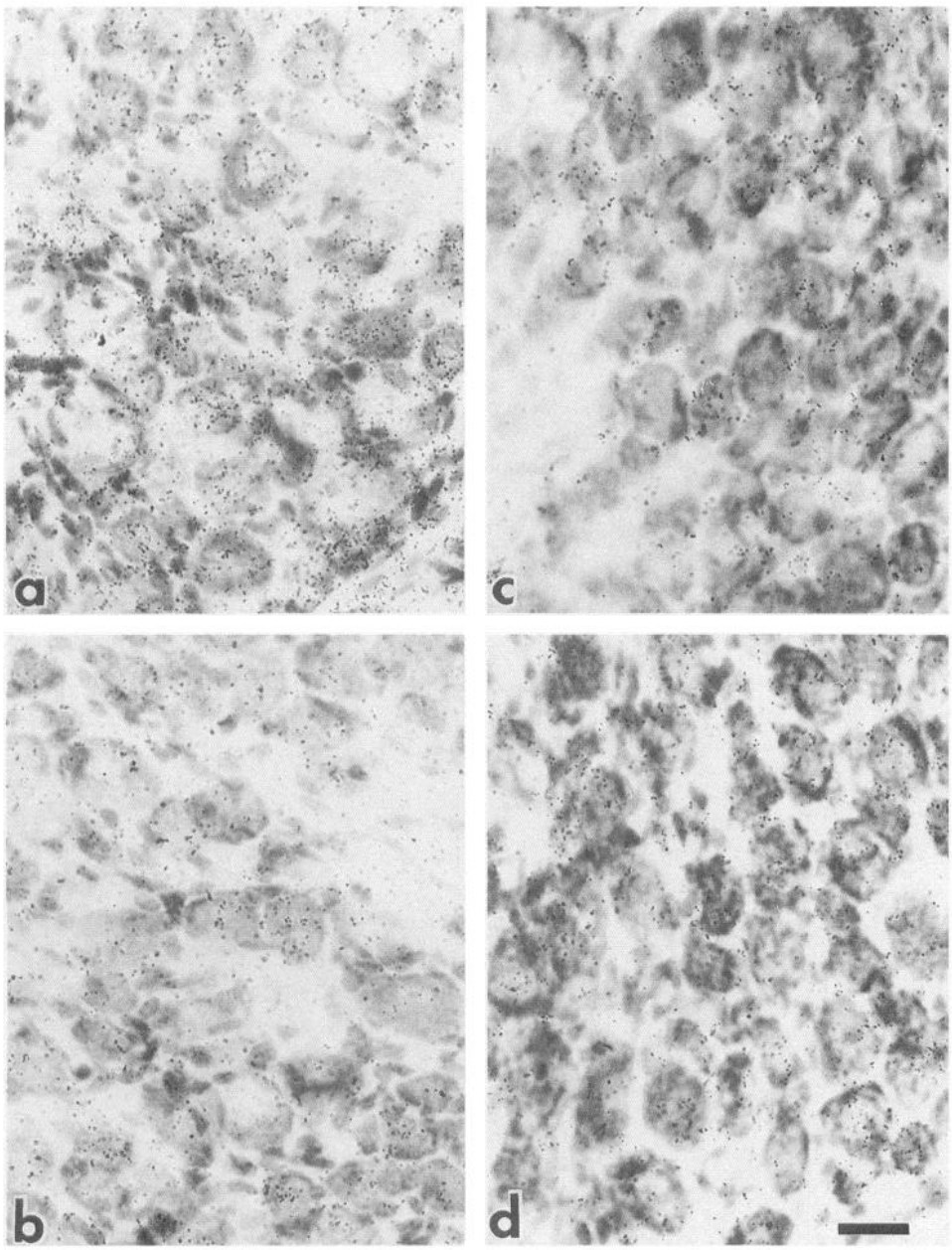

Figure 8. Kainate receptor subunit mRNA expression in the vestibular ganglion. *a* and *b* = GluR5, GluR6. *c* and *d* = KA1, KA2. Scale bar, 20 μm.

functional AMPA/kainate receptors (Nakagawa et al., 1991), and cochlear perfusion experiments in the guinea pig have determined that AMPA is slightly more effective than kainate in reducing the compound auditory nerve action potential (Jenison et al., 1986; Puel et al., 1991b). There is also electrophysiological evidence for AMPA/kainate receptors in the frog vestibular system (Prigioni et al., 1994). The high levels of expression of GluR2–4 in the vestibulocochlear nerve are consistent with AMPA receptors being the primary mediators of afferent neurotransmission, and it is likely that cochlear and vestibular AMPA receptors are composed of five-membered heteromeric complexes of GluR2, GluR3, and GluR4. The putative receptor subunit composition differs from previous reports that detected GluR2 and GluR3 but not GluR4 expression in the cochlear ganglion (Ryan et al., 1991b), and we attribute this to methodological differences regarding decalcification procedures. An additional level of complexity is provided by the expression of GluR2&3 flip and flop isoforms and GluR4 flop, in that the flip and flop variants of individual GluR subunits differ in their physiological properties and their developmental expression profile in the brain (Sommer et al., 1990; Monyer et al., 1991). Although each AMPA receptor subunit makes a distinct contribution to the functional properties of the receptor complex, the contribution of GluR2 is particularly important since AMPA receptors are not permeable to Ca^{2+} if the GluR2 subunit is present (Hollmann et al., 1991). Thus, most, if not all, of the AMPA receptors expressed at the afferent nerve synapse with the hair cell should exhibit low Ca^{2+} permeability.

Kainate receptor subunits (GluR5–7 and KA1&2) have been found to be widely expressed in the CNS by *in situ* hybridization experiments, consistent with the broad distribution of high-affinity kainate binding sites (reviewed by Hollmann and Heinemann, 1994). However, fast-desensitizing high-affinity kainate receptors have not been widely demonstrated in the CNS. Functional

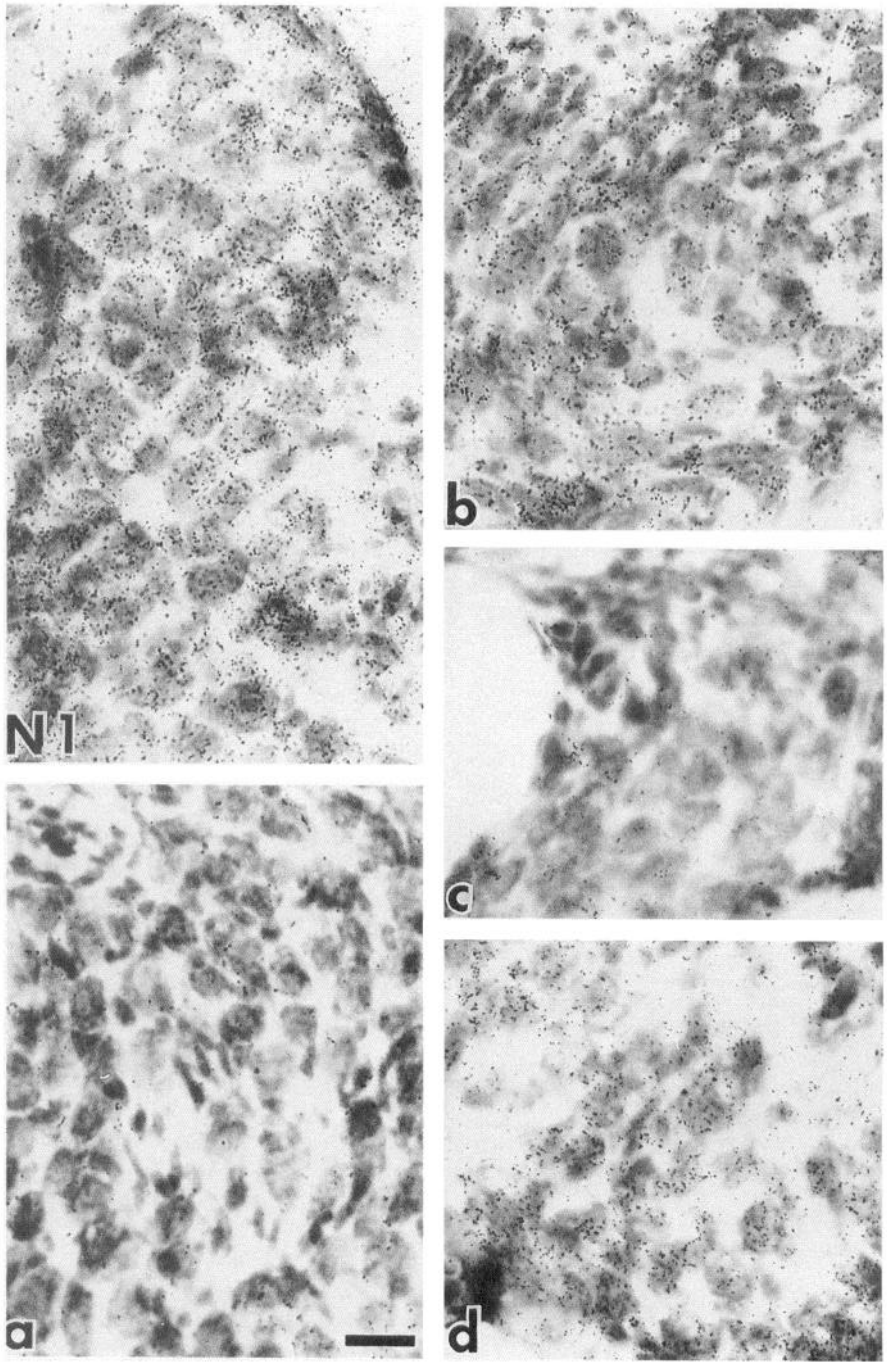

Figure 9. NMDA receptor subunit mRNA expression in the cochlear ganglion. *N1* represents NR1; *a–d* represents NR2A–D, respectively. Scale bar, 20 μm.

kainate-gated ion channels have been found in the dorsal root ganglion (Huettner, 1990) in sensory neurons that express high levels of GluR5 (Bettler et al., 1990). These functional channels have properties similar to those of recombinant homomeric GluR5 subunits (Sommer et al., 1992). When expressed as recombinant homomers, GluR5 and GluR6 form functional channels (Egebjerg et al., 1991; Sommer et al., 1992), whereas homomeric GluR7, KA1, or KA2 do not (Werner et al., 1991; Bettler et al., 1992; Herb et al., 1992; Sakimura et al., 1992). All the receptor subunits form kainate binding sites when expressed as homomers, and coexpression of KA2 with GluR5 or GluR6 generated functional kainate receptors with characteristics different from those of homomeric receptors (Herb et al., 1992; Sakimura et al., 1992). This is taken as evidence that kainate receptors *in vivo* are heteromers of GluR5, GluR6, and KA2, and likely incorporate GluR7 and KA1 as well. Immunoprecipitation analysis of detergent-solubilized brain has demonstrated that KA2 and GluR6&7 are associated in the same molecular complex (Wenthold et al., 1994).

Experiments in the cochlea have characterized the glutamate receptors as being either AMPA/kainate or NMDA-preferring because, until recently, the pharmacological differentiation of AMPA and kainate receptors had been difficult. This distinction can now be elucidated with the use of concanavalin A, which selectively potentiates the responses of kainate receptors (Werner et al., 1991), and cyclothiazide, which selectively enhances AMPA receptor responses (Partin et al., 1993; Wong and Mayer, 1993). At present, however, the only evidence for the expression

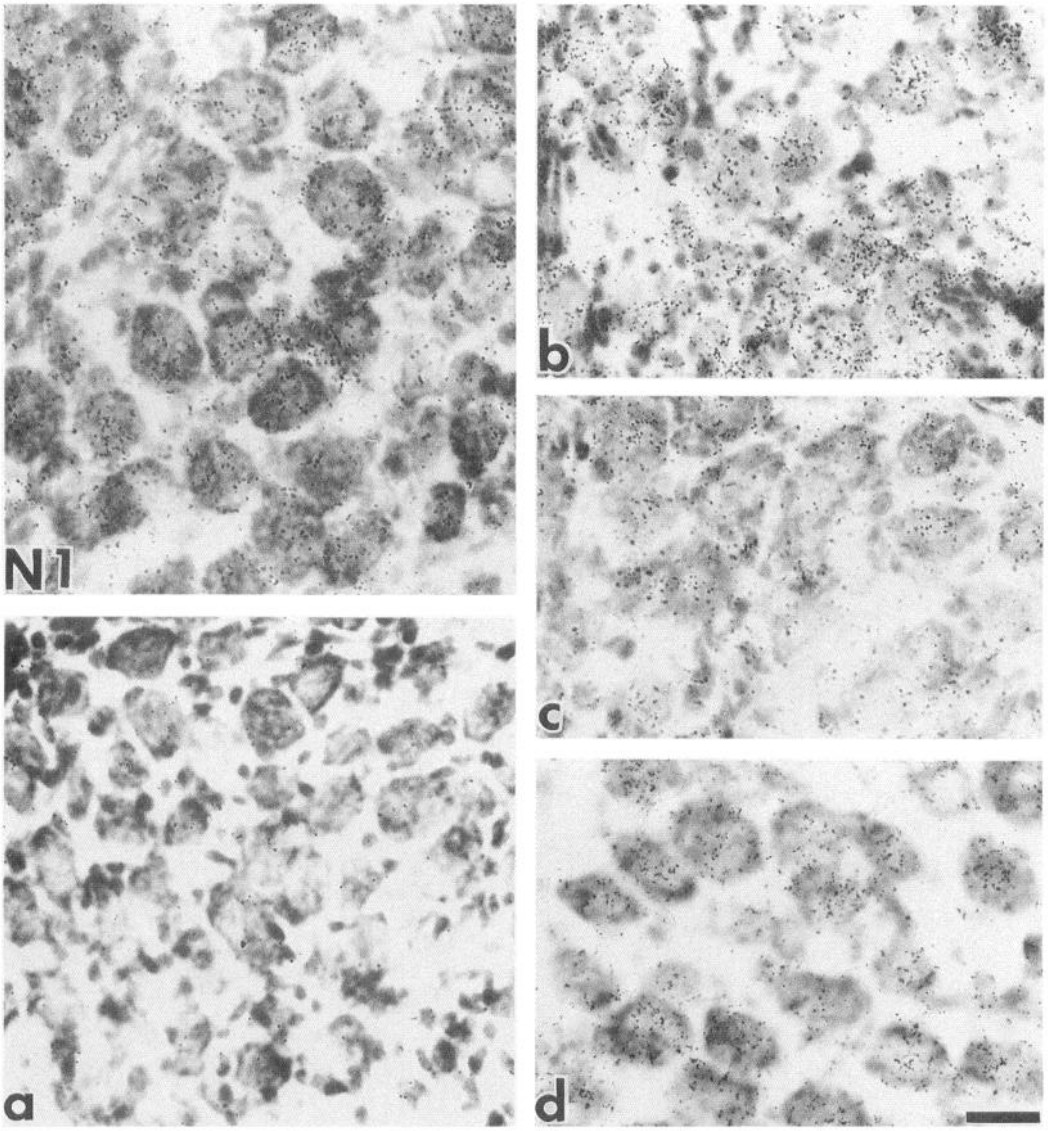

Figure 10. NMDA receptor subunit mRNA expression in the vestibular ganglion. *N1* represents NR1; *a–d* represents NR2A–D, respectively. Scale bar, 20 μm.

of kainate receptors in the cochlea and vestibule is the *in situ* hybridization experiments reported here. GluR5 is the predominant kainate receptor subunit detected in the vestibulocochlear nerve, and it is probable that homomeric GluR5 receptors, or receptors in which GluR5 is the predominant subunit, are present at the postsynaptic membrane.

NMDA receptors are distinguished from other glutamate receptors by their distinct functional properties, which include voltage-dependent Mg^{2+} blockade and Ca^{2+} permeability. Homomeric NR1 subunit expression in *Xenopus* oocytes does produce functional channels with these properties (Moriyoshi et al., 1991), whereas NR2 subunits do not form channels when expressed independently or in combination with other NR2 subunits (Monyer et al., 1992). Heteromeric NR1 and NR2 expression results in channels with distinct properties dependent on the NR2 subunit (Monyer et al., 1992) and the NMDA response of the NR1/NR2 receptor is several-fold greater than that of the NR1 homomer (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992). Thus, NMDA receptors *in vivo* are likely to be heteromers made up of both NR1 and NR2 subunits. In contrast to the AMPA receptors, NMDA receptors exhibit slow gating kinetics (Johnson and Ascher, 1987) and, therefore, as in the brain, it is expected that NMDA receptors are not the primary transducers of sensory information in the cochlea and vestibule. In fact, physiological and pharmacological evidence for functional NMDA receptors in the auditory and vestibular systems has been mixed. For example, NMDA did not induce any response in isolated rat cochlear ganglion cells, while chicken cochlear ganglion cells did respond to NMDA in the absence of Mg^{2+} (Nakagawa et al., 1991). Other evidence in the mammalian cochlea suggests that NMDA receptors may take part in sensory transduction at high levels of auditory stimulation (Puel et al., 1991a). NR1 mRNA has been localized to cochlear and vestibular ganglion cells previously (Saffiediene and Eybalin, 1992; Kuriyama et al., 1993; Fujita et al., 1994), while we demonstrate expression of both NR1 and NR2 mRNA and protein in vestibulocochlear neurons. Our results are consistent with the hypothesis that there are func-

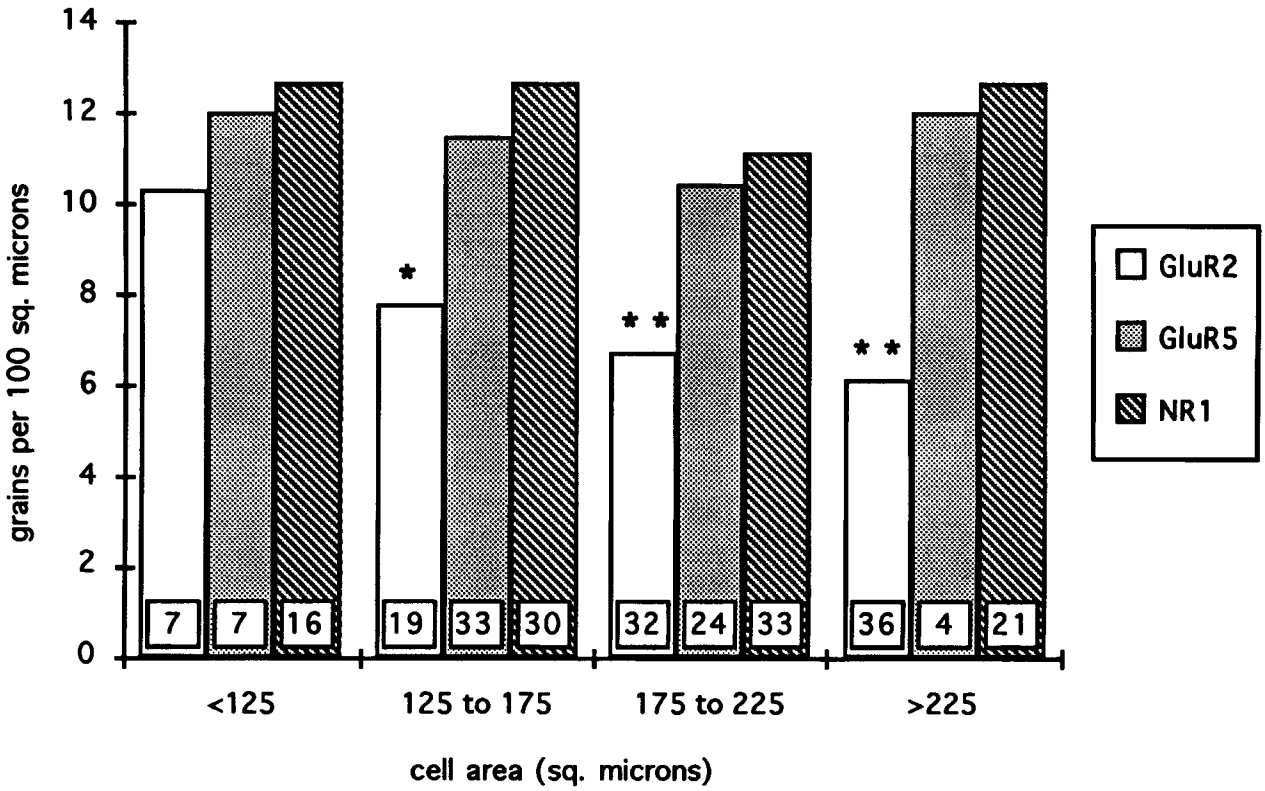

Figure 11. Quantitative analysis of GluR2, GluR5, and NR1 grain density in cochlear ganglion cells of various sizes. Significant difference from GluR2-expressing cells < 125 μm²: $*p < 0.05$, $**p < 0.005$ (*t*-test). Number of cells analyzed is shown at the bottom of each column.

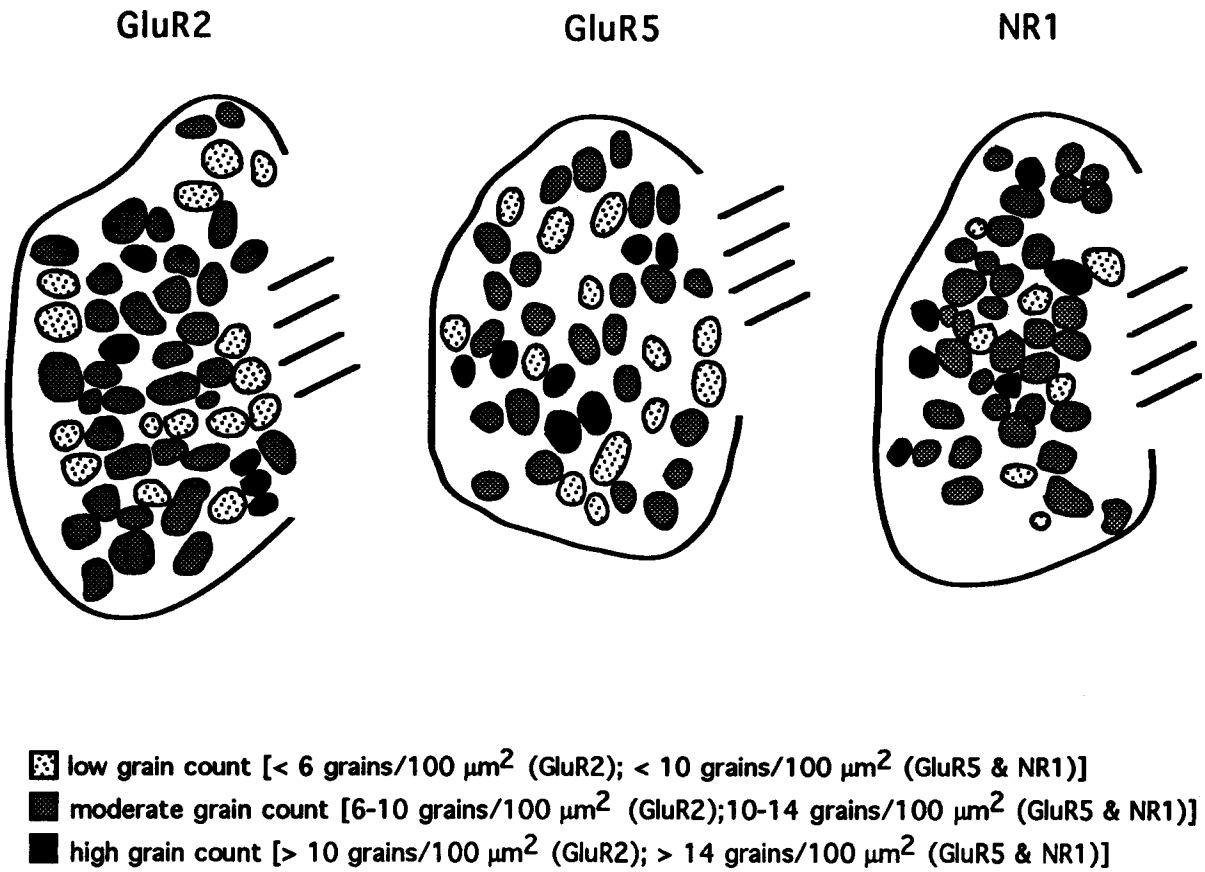

Figure 12. Distribution of GluR2, GluR5, and NR1 grain density in cells of representative cochlear ganglia. For each subunit, $n = 3$. Afferent dendrites are indicated by the *skewed lines.*

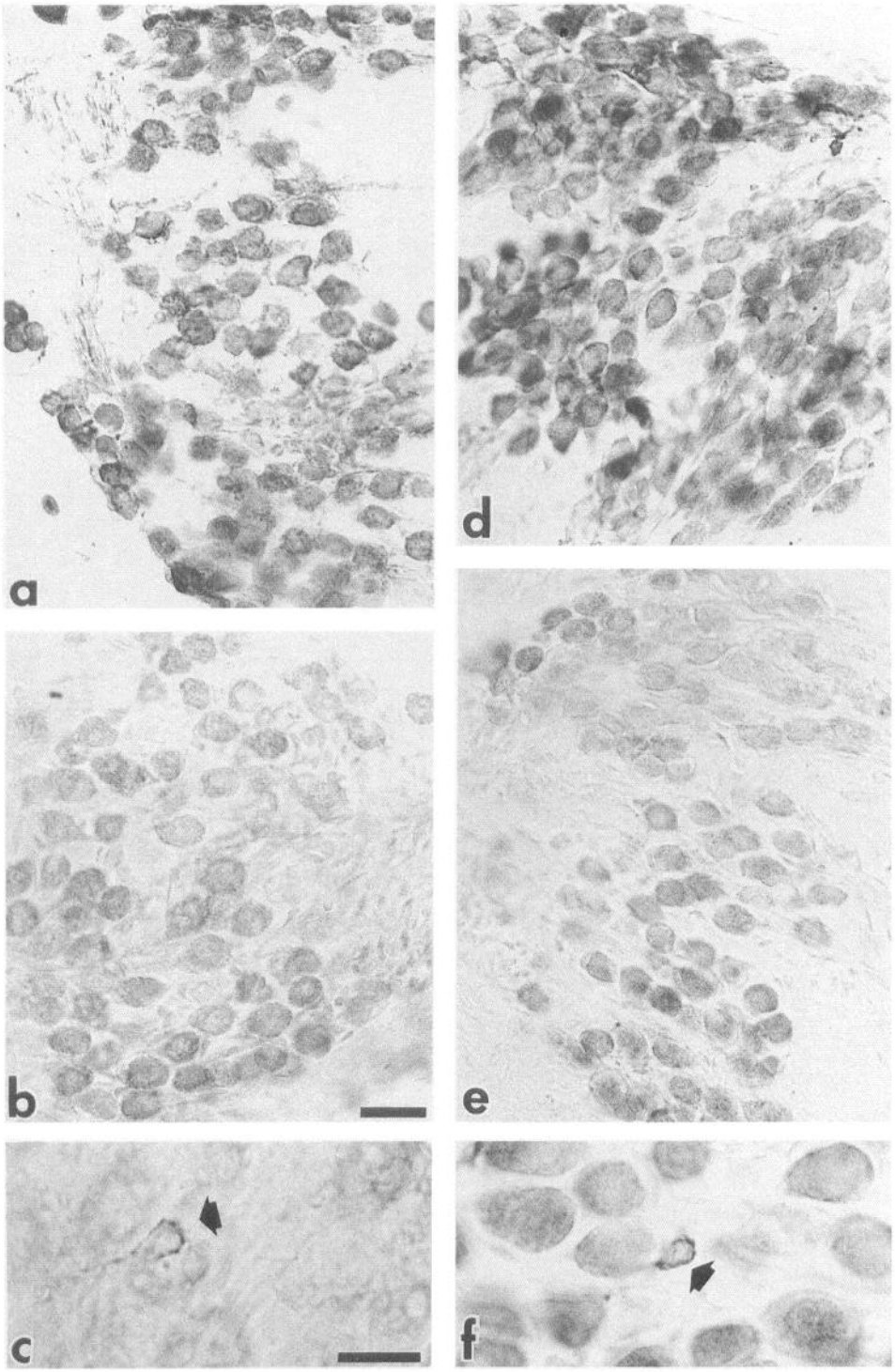

Figure 13. Immunocytochemical analysis of glutamate receptor expression in the cochlear ganglion. *a* = GluR2/3. *b* = GluR4. *c* = GluR4 immunolabeling of an apparent glial cell (*arrow*). *d* = NR1. *e* = NR2A/B. *f* = NR1 immunolabeling of an apparent glial cell (*arrow*). Scale bar (whole cochlear ganglia; *a, b, d,* and *e*), 20 μm. Scale bar (glial cells; *c* and *f*), 10 μm.

tional NMDA receptors present at the hair cell/vestibulocochlear nerve synapse.

Cochlear and vestibular ganglion neurons express 12 individual subunits that could form functional AMPA, kainate, and NMDA receptors. The subunits are presumably expressed at the hair cell/afferent nerve synapse since, in general, immunochemical data in the brain shows no evidence of presynaptic glutamate receptor expression (Petralia and Wenthold, 1992; Petralia et al., 1994a), and, more specifically, there is no evidence of presynaptic glutamate receptor expression by cochlear ganglion cells at axonal termini in the cochlear nucleus (Petralia and Wenthold, unpublished observations). The single afferent process of a Type I cochlear ganglion cell or a vestibular ganglion cell synapses directly with a single hair cell and, therefore, this suggests that AMPA, kainate, and NMDA receptors are coexpressed at the same synapse. Subunit expression levels were extraordinarily homogeneous in the cochlear and vestibular ganglia; no obvious differences were seen between cochlear turns, expression levels did not exhibit exceptional differences between cochlear and vestibular ganglia, and, except for GluR2, expres-

sion levels were independent of cochlear ganglion cell size. The increased expression of GluR2 in the smaller cells of the cochlear ganglion may be related to the specialized function of these putative type II ganglion cells, which innervate outer hair cells exclusively. The absence of GluR1 expression in both the cochlear and vestibular ganglion cells, as well as the neurons of the cochlear nucleus that receive primary auditory input (Hunter et al., 1993), is another demonstration of the similarity in receptor complement for this sensory system. The overall homogeneity of glutamate receptor subunit expression in the cochlear and vestibular ganglia suggests that these receptors perform a critical task during sensory transduction.

The role of glutamate neurotransmission in pathologic disorders of the cochlea and vestibule is complicated by the diversity of receptor subtypes. Excitotoxicity and ischemic damage in the cochlea may be a result of calcium influx through NMDA receptors, resulting in calcium-induced ganglion cell death if overstimulated (Choi, 1988). Both NMDA and kainate exert toxic effects on cultured adult rat cochlear neurons (Lefebvre et al., 1991). A recent report by Puel et al. (1994) implicates AMPA/kainate receptors as the primary mediator of acute excitotoxic injury to cochlear nerve dendrites. NMDA receptors may otherwise be involved in chronic, long-term pathologies of the cochlea and vestibule that result in ganglion cell death. Further investigation can now focus on trauma-induced changes in glutamate receptor expression in the mammalian vestibulocochlear nerve. In addition, the molecular analysis provided here will prove useful for future research that relies on molecular information, such as antisense oligonucleotide experiments, knockout strategies, and gene transfer protocols.

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