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***In Vivo* Analysis of the Role of Metabotropic Glutamate Receptors in the Afferent Regulation of Chick Cochlear Nucleus Neurons**

Kathryn L. Carzoli and Richard L. Hyson *

Program in Neuroscience, Department of Psychology, The Florida State University, Tallahassee FL 32306-4301, USA

Abstract

Cochlea removal results in the death of approximately 20-30% of neurons in the chick nucleus magnocellularis (NM). One early event in NM neuronal degradation is the disruption of their ribosomes. This can be visualized in the first few hours following cochlea removal using Y10B, an antibody that recognizes ribosomal RNA. Previous studies using a brain slice preparation suggest that maintenance of ribosomal integrity in NM neurons requires metabotropic glutamate receptor (mGluR) activation. Isolating the brain slice *in vitro*, however, may eliminate other potential sources of trophic support and only allows for evaluation of the early changes that occur in NM neurons following deafferentation. Consequently, it is not known if mGluR activation is truly required for the maintenance of NM neurons in the intact system. The current experiments evaluated the importance of mGluRs *in vivo*. The effects of short-term receptor blockade were assessed through Y10B labeling and the effects of long-term blockade were assessed through stereological counting of NM neurons in Nissl-stained tissue. mGluR antagonists or vehicle were administered intracerebroventricularly following unilateral cochlea removal. Vehicle-treated subjects replicated the previously reported effects of cochlea removal, showing lighter Y10B-labeling and fewer Nissl-stained NM neurons on the deafened side of the brain. Blockade of mGluRs prevented the rapid activity-dependent difference in Y10B labeling, and in some cases, had the reverse effect, yielding lighter labeling of NM neurons on the intact side of the brain. Similarly, mGluR blockade over longer survival periods resulted in a reduction in number of cells on both intact and deafferented sides of the brain, and in some cases, yielded a reverse effect of fewer neurons on the intact side versus deafened side. These data are consistent with *in vitro* findings and suggest that mGluR activation plays a vital role in the afferent maintenance of NM neurons.

Keywords

Deafness; Nucleus Magnocellularis; mGluRs; Deafferentation; Cell death; Y10B

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*Corresponding Author: Richard Hyson, Telephone: 850-644-0349, Fax: 850-644-7730, hyson@psy.fsu.edu .

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1. Introduction

It is generally accepted that atypical sensory input during development can have dramatic and potentially damaging effects on the central nervous system that can persist into maturity. Compromised afferent input has been shown to result in neuronal atrophy, atypical innervations, and even neuronal death (Van der Loos and Woolsey, 1973; Born and Rubel, 1985; Frazier and Brunjes, 1988; Nucci et al., 2003). Although such effects of sensory deprivation have been widely documented, the transneuronal signals responsible for regulating sensory neurons still remain unclear.

The brain stem auditory system of the chick is a useful model for examining deafferentation-induced changes. Neurons in the chick cochlear nucleus, nucleus magnocellularis (NM), receive their sole excitatory input from the ipsilateral auditory nerve (Rubel and Parks, 1975; Parks and Rubel, 1978; Born et al., 1991). Consequently, unilateral cochlea ablation eliminates afferent input to only the ipsilateral NM and allows within-subject comparisons between the intact versus deafferented sides of the same brain.

Several events occur in NM neurons following cochlea ablation, including some rapid changes that can be visualized within the first few hours of deafferentation, such as the rise in intracellular calcium ($[Ca^{2+}]_i$) levels (Zirpel et al., 1995) and overall reduction in protein synthesis (Steward and Rubel, 1985). Later events conclude with the loss of Nissl staining of approximately 20-30% of NM neurons by 24 hrs and the ultimate death of this subpopulation of neurons within 2 days (Born and Rubel, 1985).

Within 6-12 hrs after cochlea removal, NM neurons appear to segregate into two populations: one population suffers a complete cessation of protein synthesis and eventually dies, while the other population continues to synthesize proteins, albeit at a reduced level, and survives (Steward and Rubel, 1985). The cessation of protein synthesis appears to be due to the dissociation of polyribosomes in NM neurons following cochlea removal (Rubel et al., 1991). One way to visualize the rapid activity-dependent changes in ribosomes following deafferentation is by using Y10B, a monoclonal antibody that recognizes a ribosomal epitope (Garden et al., 1994, 1995; Hyson and Rubel, 1995; Hyson, 1997, 1998).

Studies directed at identifying the signals necessary for preventing the early changes that occur in NM neurons following deafferentation have made use of an *in vitro* slice preparation of the chick auditory brain stem. In this condition, both auditory nerves are severed distally and an *in vivo* situation of unilateral cochlea ablation can be mimicked by unilaterally stimulating the auditory nerve fibers. Within 1 hr, NM neurons on the stimulated side of the slice show greater protein synthesis (Hyson and Rubel, 1989) and Y10B labeling (Hyson and Rubel, 1995; Hyson, 1997, 1998; Nicholas and Hyson, 2004) than the neurons on the opposite side of the same section.

Slice preparation studies have suggested that afferent regulation of ribosomal integrity requires the activity-dependent activation of metabotropic glutamate receptors (mGluRs) on NM neurons (Hyson, 1998; Nicholas and Hyson, 2004). Stimulated NM neurons do not show greater Y10B immunolabeling than unstimulated neurons if the slice is maintained in a buffer containing mGluR antagonists (Hyson, 1998; Nicholas and Hyson, 2004). These results are obtained even though antagonists have no obvious effects on excitatory postsynaptic potentials (EPSPs). Blockade of ionotropic glutamate receptors (iGluRs) with the non-NMDA antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and NMDA antagonist, (2R)-amino-5-phosphonopentanoate (APV), fails to block the activity-dependent difference in Y10B immunolabeling, even though CNQX completely blocks all EPSPs in NM neurons (Hyson, 1997). These studies suggest that glutamate, the neurotransmitter released from the auditory nerve (Nemeth et al., 1983), provides trophic support to NM

neurons through activation of mGluRs and that glutamate release controls electrical activity of NM neurons through activation of iGluRs.

One limitation of the previous studies is that the *in vitro* slice preparation isolates an individual brain slice from the rest of the body. This allows for the possibility that alternative sources of trophic support such as hormone release, neurotrophins, or factors being released from descending superior olivary nucleus projections have also been eliminated in the slice preparation. Consequently, it is not known if mGluR activation is truly required for maintaining neuronal integrity in the intact system or if mGluR activation is only required when other forms of trophic support are no longer present. A second limitation to previous *in vitro* experiments is that only rapid changes that occur following deafferentation can be examined in the acute slice preparation. Consequently, the role of mGluR activation for the long-term consequences of deafferentation cannot be examined *in vitro*. The current study tests the hypothesis that activation of mGluRs *in vivo* is necessary to maintain NM neurons. Rapid effects of cochlea removal were examined through the evaluation of ribosomal integrity, as measured by Y10B immunoreactivity, in the presence of mGluR antagonists administered into the IVth ventricle for 3 or 6 hrs following cochlea removal. The effect of mGluR blockade on neuronal survival was also assessed following continuous administration of mGluR antagonists into the IVth ventricle for periods of 1 or 5 days. If the loss of mGluR activation following deafferentation is what leads to cell death in NM neurons, then blockade of mGluRs should produce the same effects as cochlea removal. This would be expected to eliminate the difference between sides that has been previously observed in both Y10B immunolabeling and Nissl staining following unilateral cochlea removal.

2. Materials and Methods

2.1. Subjects

All subjects were 12-16 day post-hatch Ross X Ross chickens of either sex, hatched from eggs obtained from a local supplier (Pilgrim's Pride, Live Oak, FL, USA) and reared at Florida State University. The procedures used in these experiments were approved by the Animal Care and Use Committee at Florida State University and conform to the guidelines set forth by the National Institutes of Health. All efforts were made to minimize the number of animals used and their suffering.

2.1.2. 3 hr Infusion—To reduce the time between cochlea removal and application of antagonist, subjects first received a small craniotomy for insertion of the intraventricular injection pipette. They were then removed from the stereotaxic apparatus for cochlea removal surgery. Subjects were then placed back in the stereotaxic apparatus and the injection pipette was lowered into the IVth ventricle. Drug was administered during the 3 hr survival period prior to perfusion.

Chicks were anesthetized with a combination of 100 mg/kg ketamine and 10 mg/kg xylazine intramuscularly and mounted in a stereotaxic apparatus. Coordinates for intraventricular infusion of drugs were based on a stereotaxic atlas of the chick brain (Puelles, 2007). The injection probe entered the brain at approximately a 20° angle off vertical. Optimal coordinates for IVth ventricle placement of the pipette entering at this angle were 4.88 mm posterior and 2.7 mm lateral, where zero on each axis is the center point of centered ear bars, and approximately 7.2 mm deep from the surface of the brain. The skull was exposed and an opening was drilled at these coordinates. The bird was then removed from the apparatus and a unilateral cochlea ablation was performed immediately after obtaining the appropriate exposure in the skull. Subjects were maintained under anesthesia and, if necessary, booster injections of the anesthetic were administered. The tympanic membrane was punctured with

forceps and the columella was removed. The basilar papilla was then removed through the oval window with forceps and the chick was then placed back into position in the stereotax. The extracted basilar papilla was visually examined to confirm completed removal.

Prior to drug loading, a pipette tip was filled with fast green dye. Once placed back in the apparatus, the pipette was filled with either vehicle control ($n = 3$), a general metabotropic glutamate receptor antagonist, (RS)- α -Methyl-4-carboxyphenylglycine, (MCPG, 3 mM, $n = 4$), a group I mGluR antagonist, (RS)-1-Aminoindan-1,5-dicarboxylic acid, (AIDA, 1 mM, $n = 4$), or a group II mGluR antagonist LY341495 (10 nM, $n = 3$). The vehicle was dextrose-free artificial cerebrospinal fluid (ACSF) consisting of (in mM) 130 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 26 NaHCO₃, and 1.25 NaH₂PO₄. Drug was administered intracerebroventricularly (icv) using periodic (approx. 2/sec) pressure pulses (10 psi, 5–7 msec duration) through a picospritzer (General Valve Corporation, Fairfield, NJ, USA) for the duration of 3 hrs or until 9.8 μ l of drug was injected. Correct placement of the pipette into the ventricle was determined by amplified multiunit extracellular recordings as well as by visual confirmation of the fast green dye during sectioning. Physiological recordings were made through the injection pipette using an AC amplifier (Grass SD 5, Grass Instruments, Quincy, MA, USA). Entrance into the ventricle was apparent by the dramatic reduction in unit activity. Further penetration resulted in a robust increase in activity as the pipette entered the brain stem and the pipette was retracted to the area of the ventricle.

2.1.3. 6 hr, 24 hr, 5 day Infusion—The preparation for longer duration infusion was similar to the 3 hr procedure except that drug was administered through a cannula into the IVth ventricle and continuous administration was achieved using a mini osmotic pump. Cannulae were inserted stereotactically and cemented in place with dental cement. Pumps were implanted subcutaneously through an incision in the upper back/lower neck. The incision was sealed with surgical glue. Following recovery from anesthesia, the birds were returned to their brooders. Drugs were administered into the IVth ventricle for 6 hrs, 24 hrs, or 5 days prior to perfusion ($n = 3$ per group).

One day prior to implantation surgery, mini osmotic pumps (Alzet, Durect Corp., Cupertino, CA, USA) were filled with vehicle control, MCPG, AIDA, or LY341495. Drug concentrations were calculated based on individual pump duration and release rate to achieve a constant rate of 1.8 to 2.0 μ g/hr (MCPG), 619 to 721 ng/hr (AIDA) or 9.4 to 10.6 pg/hr (LY341495) for the duration of the experiment. Filled pumps were attached to polyethylene tubing attached to the stainless steel arm of the guide cannula. Pumps were then placed in a 14 ml conical tube filled with 0.9% sterile saline and incubated at 37°C overnight, or for 12 hrs.

2.2. Anatomical Procedures

2.2.1. Tissue Preparation—Three hrs, 6 hrs, 24 hrs, or 5 days following cochlea removal, subjects were deeply anesthetized with 50 mg/kg pentobarbital and perfused with 0.9% saline followed by 4% paraformaldehyde. The brain stem was separated from the telencephalon and post-fixed for 1 hr in 4% paraformaldehyde followed by overnight cryoprotection in 30% sucrose/4% paraformaldehyde. The tissue was rapidly frozen in 2-methylbutane on dry ice.

2.2.2. Immunohistochemistry—Twenty μ m sections were collected from the brains of subjects surviving 3 hrs and 6 hrs following cochlea removal using a Leica CM 1850 cryostat (Leica Microsystems Inc., Bannockburn, IL, USA). Puncture marks were made in the ventral portion of the brain stem to identify the side of the cochlea removal. Sections were free-floated in a vial containing ice-cold phosphate-buffered saline (PBS). Sections

were then quenched for 20 min in 0.03% H₂O₂ in methanol. Following three 10 min washes in PBS, sections were placed in a 4% normal horse serum blocking solution for 10 min and then incubated on a rotator overnight in 1:500 Y10B at room temperature. The following day, sections were washed 3 × 10 min in PBS then incubated for 1 hr in 1:200 biotinylated horse anti-mouse in blocking solution. Sections were washed 3 × 10 min in PBS and then incubated in avidin-biotin-peroxidase complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 1 hr and then rinsed 2 × 10 min in PBS followed by a 10 min wash in 0.1 M phosphate buffer (PB). Sections were then reacted with diaminobenzidine tetrahydrochloride and 0.03% H₂O₂ for 15 min. Following 2 10 -min washes in 0.1M PB, sections were mounted onto slides and allowed to dry overnight. The following day, slides were cleared though graded ethanols and xylenes and coverslipped in DPX mounting medium (Sigma-Aldrich, St. Louis, MO, USA).

2.2.3. Densitometry—To objectively analyze the level of immunolabeling, densitometric measurements of NM neurons were obtained using a digital image analysis program (NIH ImageJ). The staining densities of NM neurons on the intact versus the deafferented side of the same tissue section were compared. For these analyses, the light levels and camera settings remained constant. Cells were visualized using a 40X objective and mean gray-scale densities over approximately 40 NM neurons/side in a given tissue section were measured beginning with cells at the medial edge of NM and proceeding laterally. At least 3 sections from each subject were measured. The investigator analyzing the tissue remained blind to the identities of the intact and deafferented sides of each section as well as to treatment group until after measurements were obtained. Differences in the density of labeling between subjects receiving ACSF or drug were compared using analysis of variance (ANOVA). To compare labeling differences across different subjects, the labeling density of each cell was transformed to a z-score based on the mean and standard deviation of the gray-scale density of cells on the intact side of the individual section.

2.2.4. Nissl Staining—Fifty µm cryostat sections were collected from the brains of subjects surviving 24 hrs and 5 days following cochlea removal. Puncture marks were made in the ventral portion of the brain stem to identify the side of cochlea removal. Sections were mounted directly onto slides and every section containing NM was collected. Mounted sections were then dried and stained for Nissl. To assure adequate staining throughout the relatively thick section, slides were rehydrated in dH₂O for approximately 20 to 30 min. The slides were then transferred to Thionin for 10 min, rinsed twice with distilled water for 2 min each, followed by 2 changes of 70% ethanol for 5 min each. The slides were placed into 95% ethanol for a time period that varied depending on the darkness of the stain (approximately 20 min) and then they were transferred through two 3 min changes of 100% ethanol. Finally, sections were cleared with xylenes. Slides were coverslipped in DPX mounting medium and allowed to dry overnight.

2.2.5. Stereology—Nissl-stained sections containing NM neurons were analyzed using a stereological program (Stereo Investigator, Microbrightfield, Inc., Williston, VT, USA) to compare the number of neurons on the deafferented side versus the intact side. The optical fractionator, an unbiased and efficient stereological method, was used. Cell counts using this method are unaffected by tissue shrinkage and the objects are counted in an objective, systematic way. Each cell has an equal chance of being counted and the optical fractionator reduces the chance of double counting cells. The end result is an estimate of cell number in the nucleus. The parameters of the program were set such that a grid of 40×40 µm boxes separated by 100 µm were randomly placed over user-generated outlines of NM and the investigator counted the number of cells in each box. A cell was counted if its right-most portion was located inside the box or was touching the right side or top line of the box. Only

neurons that had a stained cytoplasm with a clearly distinguishable nucleolus were included in the analysis. Typical section thickness after processing was approximately 18 to 21 μm . To avoid double counting, only cells located between 3 μm and 18 μm from the top of the section were counted. A 1:2 series of the middle 65% of NM along its anterior-posterior axis was included in the analysis. The investigator performing the counting was blind to treatment condition.

3. Results

3.1. Immunohistochemistry

When ACSF was applied icv over 3 or 6 hrs, NM neurons on the intact side of the brain demonstrated darker Y10B immunolabeling when compared to NM neurons on the deafferented side of the same section. An example of this effect at 3 hrs can be seen in Fig. 1A-B. Application of the general mGluR antagonist, MCPG, effectively eliminated the activity-dependent difference in Y10B immunolabeling between sides of the same tissue sections (Fig. 1C-D). In fact, labeling often showed a reversed effect with NM neurons on the intact side showing less labeling than those on the deafferented side. This replicates the effects that were seen in previous *in vitro* experiments (Hyson, R.L. 1997). Relatively low concentrations of group I mGluR antagonist, AIDA, and group II mGluR antagonist, LY341495, also effectively eliminated the activity-dependent difference in Y10B labeling. Like MCPG, low concentrations of AIDA not only eliminated the difference in labeling between sides, but there appeared to be a trend towards lighter labeling on the intact side of the brain versus the deafferented side.

Visual impressions were confirmed through objective analyses of staining density. The staining densities of individual NM neurons on the intact versus the deafferented side of the same tissue section were compared. Densities for neurons on both sides of the brain at both survival periods showed a normal distribution, suggesting that deafferented neurons had not yet split into two populations based on this assay. A two-way mixed ANOVA was performed on the gray scale measurements using side of the section as the within-subject variable and drug as the between-subjects variable. This analysis on the 3 hr survival group revealed a reliable effect of drug treatment ($F(3, 10) = 5.87, P < 0.05$), no overall effect of side ($F(1, 10) < 1.0$), but importantly, a reliable drug treatment X side interaction ($F(3, 10) = 10.16, P < 0.01$). Post hoc (Newman-Keuls) pair-wise comparisons revealed that both the control and the MCPG-treated groups had reliable differences between sides, albeit in opposite directions ($P < 0.05$). Similarly, for the 6 hr survival group, the two-way ANOVA revealed no reliable effect of drug treatment ($F(3, 8) < 1.0$), no overall effect of side ($F(1, 8) < 1.0$), but importantly, a reliable drug treatment X side interaction ($F(3, 8) = 5.58, P < 0.05$). Post hoc pair-wise comparisons revealed that control animals were the only group to show a reliable difference between sides ($P < 0.05$).

To further examine the between-group differences in the effects of cochlea removal, density measurements were converted to z-scores based on the mean and standard deviation of labeling density measured on the intact side of each section. This transformation reduces the contribution of differences in labeling density between sections that might be attributed to variation in processing variables. The density of labeling following this transform is the number of standard deviations away from the mean labeling observed on the intact side of the section, regardless of the overall darkness of labeling. The mean z-scores for each group are displayed in Fig. 2. A one-way ANOVA revealed a reliable effect of drug treatment for both the 3 hr (Fig. 2A) and 6 hr (Fig. 2B) survival groups ($F(3, 10) = 7.33, F(3, 8) = 6.31$, respectively, $P < 0.01$). For both survival periods, post hoc (Newman-Keuls) comparisons showed that the mean z-score for cells on the deafferented side of the brain in the control

group differed from all 3 of the drug treatment groups ($P < 0.05$), which did not reliably differ from each other.

Labeling in a non-auditory region (abducens) was also measured on both sides of the brain. This was done to confirm that the drugs specifically influenced the effect of cochlea removal on NM neurons by ruling out overall drug effects on neuronal labeling or side-specific drug effects that may have resulted from cannula placement. There were no reliable effects of drug ($F < 1.0$), side ($F < 1.0$), or their interaction ($F(3, 8) = 2.3$, $P > 0.14$) on labeling of abducens neurons.

3.2. Nissl

The previously reported effects of cochlea removal were replicated in subjects that had vehicle administered into the IVth ventricle. The appearance of “ghost” cells, which lack Nissl staining but have an intact cell membrane, was observed 24 hrs following cochlea removal and fewer Nissl-stained cells were found on the deafferented side of the brain at both 24 hr and 5 day survival periods. Administration of mGluR antagonists eliminated the difference in cell number between sides and, in some cases, a trend towards a reversed effect, with fewer cells on the intact side of the brain, was observed. An example of these effects can be seen in Fig. 3.

NM neurons were counted by stereological methods. Fig. 4 displays the raw cell count data (A, B) and the average percent difference in the number of cells on each side of the brain (C, D). As can be seen, there were approximately 25% fewer Nissl-stained cells on the deafened side of the brain in comparison to the intact side of the brain in vehicle treated subjects at both 24 hrs and 5 days. This is in line with previous reports of 20–30% cell death in NM neurons following cochlea removal (Born and Rubel, 1985). The difference between sides was eliminated in all drug-treated groups and if anything, mGluR blockade resulted in a trend towards a reversed effect such that there were fewer cells on the intact side of the brain in comparison to the deafferented side. Unexpectedly, administration of mGluR antagonists for 24 hrs post-cochlea removal (4A, C) resulted in deafened sides of the brain having more neurons than the deafened side of control brains ($P < 0.05$ in each group). Administration of mGluR antagonists 5 days post-cochlea removal (4B, D) resulted in deaf and intact sides of drug-treated brains looking similar to deaf sides of control group. There appeared to be cell loss on both sides of the brain in the drug-treated groups, eliminating the differences between sides. In some cases, there was a trend towards a reversed effect with fewer cells on the intact side of the brain in comparison to the deafferented side, but these differences did not reach statistical significance. MCPG-treated groups yielded a 13.8% difference between sides with fewer cells counted on the intact versus the deafferented side of the tissue. Similarly AIDA-treated subjects showed a 10.4% difference towards the same reversal trend while LY341495-treated subjects demonstrated a smaller difference between sides 6%, albeit in the same reversed pattern.

A separate two-way mixed ANOVA was performed for each survival period on the stereological estimates of cell number, using side of the brain as the within-subject variable and drug as the between-subjects variable. Similar results were observed at both time points. Both analyses revealed no reliable effect of drug treatment ($F(3, 8) < 1.0$, $F(3, 8) = 2.6$, for 24 hrs and 5 days, respectively, $P > 0.10$). There was an overall effect of side ($F(1, 8) = 7.48$, $P < 0.05$) for the 24 hr survival period, but not for the 5 day survival period ($F(1, 8) < 1.0$). Importantly, both analyses revealed a reliable drug treatment X side interaction ($F(3, 8) = 49.52$, $P < 0.01$, $F(3, 8) = 16.58$, $P < 0.001$). Post hoc (Newman-Keuls) pair-wise comparisons revealed that there were reliably fewer cells on the deafferented side of the brain in control groups at both the 24 hr and 5 day survival periods, ($P < 0.05$). At the 24 hr survival period, both MCPG and AIDA showed reliable differences in cell number between

the two sides, albeit in the opposite direction of the control group. At this time, the number of cells on the intact side of the control group was reliably greater than the number of cells on either side of the brain in the other groups, with the exception of the deafferented side of the AIDA group. At 5 days, only the control group showed a reliable difference between deafferented and intact sides of the brain. The intact side of the control group had a reliably greater number of NM neurons than either side of any of the other groups at this survival period.

Statistical analyses were also carried out on the percent difference scores using a one-way ANOVA. There was a reliable effect of treatment at both survival periods ($F(3, 8) = 51$ and 11.5 for 24 hr and 5 day survival periods, respectively, $P < 0.01$). Post hoc (Newman-Keuls) comparisons showed that percent difference scores for the control group at 24 hrs post-cochlea removal differed from all 3 of the drug treatment groups ($P < 0.05$), and that LY341495 differed reliably from the MCPG-treated group. For the 5 day survival period, post hoc (Newman-Keuls) comparisons showed that the mean percent difference for the control group differed from all 3 of the drug treatment groups ($P < 0.05$), which did not reliably differ from each other.

4. Discussion

Cochlea removal results in rapid cellular and metabolic changes in the avian cochlear nucleus, NM, and concludes with the ultimate death of 20-30% of these neurons. One of the earliest changes following deafness in NM neurons is a change in ribosomal activity (Steward and Rubel, 1985). This change in function corresponds with a change in antigenicity for Y10B, a monoclonal antibody that recognizes a ribosomal epitope (Garden et al., 1994, 1995; Hyson and Rubel, 1995; Hyson, 1997, 1998). Previous studies aimed at identifying the important transneuronal signals involved in the regulation of NM neurons have utilized a brain slice preparation. These studies have demonstrated that auditory nerve activity is necessary to maintain ribosomal Y10B antigenicity and protein synthesis (Hyson and Rubel, 1989, 1995). Further investigation *in vitro* has shown that mGluR activation is required to prevent changes in NM ribosomes (Hyson, 1998; Nicholas and Hyson, 2004) as well as to prevent a rise in $[Ca^{2+}]_i$ that takes place within the first few hours following deafferentation (Zirpel et al., 1995; Zirpel and Rubel, 1996).

Previous studies utilizing the *in vitro* slice preparation allow for the possibility of eliminating alternative sources of trophic support. Consequently, it is not known if mGluR activation is truly required for maintaining ribosomal integrity in the intact system. Additionally, while it is possible to conclude that mGluR activation is necessary to prevent changes in ribosomal function short-term *in vitro* preparations, it still remains unclear whether the loss of mGluR activation is enough to induce the ultimate demise in NM neurons. The current study demonstrated that mGluRs are important for maintaining NM neurons in the intact system. At early time points in the cell death cascade, data from control (vehicle-treated) subjects replicated previous work showing a decrease in Y10B antigenicity following cochlea removal. When treated with mGluR antagonists, however, this difference between sides was not observed. These findings are consistent with previous *in vitro* studies (Hyson, 1998; Nicholas and Hyson, 2004) and confirm that the loss of mGluR activation results in the early metabolic changes that are observed in NM neurons following cochlea removal. Long-term mGluR blockade resulted in the death of neurons, even on the intact side of the brain. The commonly reported deafferentation-induced cell loss was replicated in vehicle-treated subjects, but subjects treated with mGluR antagonists showed fewer cells on both sides of the brain and this treatment eliminated the effect of cochlea removal. The most parsimonious explanation of these findings is that administration of antagonists

prevented the trophic effects of mGluR activation on both sides of the brain, thereby eliminating differences normally observed following unilateral deafferentation.

One unexpected finding was that, in some cases, administration of mGluR antagonists for 24 hrs post-cochlea removal resulted in both deafened and intact sides of the brain appearing to have more labeled cells than the deafened side of control brains (see Fig. 4A). One would expect to see deafened and intact sides of drug-treated brains to look similar to the deafened side of control brains if mGluR activation was the key to maintaining NM neurons. This suggests that mGluR blockade could have a transient neuroprotective influence during the first 24 hrs following cochlea removal, but longer blockade results in cell death. An alternative explanation, however, relates to a technical limitation of the study and the measurement of cell number at the 24 hr survival time. It is possible that variability in Nissl staining interfered with the determination of cell number in the 24 hr survival group. The presumably dying “ghost” (Nissl-negative) cells were not included in the analysis of cell number in these tissue sections. In the relatively thick sections that are required for accurate stereology, “ghost” cells are difficult to discern since cells are overlapping in these sections. Additionally, variation in the overall level of Nissl staining may have led to inaccurate measurements of cell number by counting cells that may have been considered to be “ghost” cells in more lightly stained tissue. It is possible that in order to obtain sections stained darkly enough to perform the stereology, drug-treated brains were relatively over-stained and more “live” cells were counted. This possible technical limitation does not apply to the 5 day survival period since “ghost” cells are no longer present and the dying cells have completely degenerated by this time.

In some cases, mGluR blockade resulted in a reversed effect; neurons on the intact side were more lightly labeled than neurons on the deafferented side of the section, or there were more cells on the deafened side versus the intact side. This reversed effect was not entirely unexpected since similar results have been observed with previous *in vitro* experiments. While unilateral stimulation of the auditory nerve *in vitro* results in greater protein synthesis (Hyson and Rubel, 1989) and Y10B antigenicity on the stimulated side of the brain slice (Hyson and Rubel, 1995), unilateral antidromic stimulation of NM neurons produces lighter labeling on the stimulated side of the slice (Hyson and Rubel, 1995; Hyson, 1998). Additionally, unilateral stimulation of the auditory nerve *in vitro* in the presence of mGluR antagonists sometimes results in lighter labeling on the stimulated side of the slice (Hyson, 1998; Nicholas and Hyson, 2004). Similar results are also observed when measuring $[Ca^{2+}]_i$ levels. Stimulation of the auditory nerve *in vitro* prevents a slow rise in $[Ca^{2+}]_i$ in NM neurons, but if stimulated in the presence of mGluR antagonists the stimulated neurons show a more robust rise in $[Ca^{2+}]_i$ than that observed in unstimulated neurons (Zirpel and Rubel, 1996). One interpretation of these phenomena is that postsynaptic activity in NM neurons without trophic support from mGluR activation is more detrimental than just complete blockade of activity. Stimulation of the auditory nerve, or having an intact cochlea, will result in glutamate release from the auditory nerve. In addition to activating mGluRs, glutamate will activate iGluRs, which will lead to generation of action potentials in NM neurons. Blockade of mGluRs will still allow activation of iGluRs and the action potentials in NM neurons (Hyson, 1997). The *in vitro* studies using antidromic stimulation (Hyson and Rubel, 1989, 1995; Hyson, 1998) suggest that action potentials, per se, are detrimental to the neuron because the stimulated side of the slice shows lighter labeling. Consequently, mGluR blockade sets up a condition of having action potentials in the NM neuron without the trophic support to counteract their detrimental effects.

Although it is clear that mGluR activation is important for maintaining NM neurons, a couple of limitations to our current understanding will require further study. First, it is not known if mGluR activation is sufficient for maintaining NM neurons or if other activity-

dependent factors are also necessary. Glutamate and an mGluR agonist, $\pm$ trans 1-amino-1,3-cyclopentadecarboxylic acid (t-ACPD), attenuate the rise in $[Ca^{2+}]_i$ in NM neurons produced by KCl depolarization (Lachica et al., 1995). mGluR agonists have also been shown to modulate Ca^{2+} currents (Lu and Rubel, 2005). This suggests that mGluR activation may be sufficient to partially regulate Ca^{2+} in NM neurons, but it is not known if mGluR activation is sufficient for regulating the ribosomes or the ultimate viability of these cells. A second issue requiring future study is exactly how activation of mGluRs allows for neuroprotection. Several theories of mGluR-mediated neuroprotection have emerged in recent literature and it has been suggested that different mGluR subtypes may be involved. In cultured cortical cells, for example, group II mGluRs have been shown to exhibit neuroprotective effects against excitotoxic cell death (for review, see Monyer et al., 1992; Bruno et al., 1998) while in primary neuronal hippocampal cultures, mGluR groups I and III provide enhanced protection from nitric oxide-induced cell death (Maiese et al., 2000).

While there are various mechanisms by which the different mGluRs could provide for neuroprotection, these mechanisms can be generally categorized as having either direct or indirect effects on the neuron. One of the direct effects of mGluR activation on NM neurons is the maintenance of $[Ca^{2+}]_i$ (Zirpel and Rubel, 1996; Zirpel et al., 1998; Lu and Rubel, 2005). It is generally accepted that Ca^{2+} homeostasis is critically important for cellular survival, and a threefold increase in $[Ca^{2+}]_i$ is one of the earliest changes observed in NM neurons following cochlea removal (Zirpel et al., 1995). Activation of mGluRs on NM neurons can affect $[Ca^{2+}]_i$ through a number of different pathways including through the activation of protein kinase A (PKA), or protein kinase C (PKC) (Zirpel et al., 1998), through the modulation of Ca^{2+} release from intracellular stores (Kato et al., 1996), or through inhibition of L-type channels (Lachica et al., 1995) and Ca^{2+} permeable AMPA receptors (Zirpel et al., 2000). Typically, stimulation of group I mGluRs results in the activation of PKC within cells and groups II and III lead to activation of PKA (for review, see Conn and Pin, 1997). In the present studies, both group I and group II antagonists eliminated the difference between sides following cochlea removal, suggesting that activation of both groups are important for neuronal survival in this system. The combined effect of blocking both mGluR groups appeared to be more detrimental to NM neurons than the blockade of selective groups, as would be expected if both types of receptors contribute to cell survival. The differences in effect seen between drugs, however, could simply be due to differences in the effective potency of the concentrations used in these studies. The group-selective antagonists were used at relatively low concentrations in order to maintain their specificity.

A possible indirect mechanism by which mGluR activation could lead to NM neuron protection is by maintaining the balance between excitation and inhibition in these neurons. Activation of mGluRs has been shown to suppress GABA release to NM neurons, and blockade of mGluRs increases GABA-evoked depolarizations (Lu, 2007). The activation of GABA_A receptors on NM neurons produces a depolarization even though GABA's main effect is inhibitory. This increase in GABA-evoked depolarizations following mGluR blockade could contribute to the detrimental effect of NM neuron activity.

Finally, mGluR activation could have an indirect neuroprotective effect on NM neurons by working at neighboring glial cells. Previous research in the chick auditory brain stem has demonstrated that there is rapid growth of astrocytic processes in NM following cochlea removal (Canady and Rubel, 1992; Rubel and MacDonald, 1992; Canady et al., 1994). It has been demonstrated that mGluRs are present on glial cells in several systems (D'Antoni et al., 2008). For example, cortical glial cells exposed to mGluR agonists have been shown to be highly neuroprotective when transferred to mixed cultures that have been exposed to toxic levels of NMDA (Bruno et al., 1997). It has also been suggested that substances

released from glial cells in the hippocampus can influence local synapses (Liu et al., 2004). If this is the case in NM neurons, then it is possible that mGluR activation could alter the release of some substance from astrocytes, which, in turn, could act on NM neurons. If these substances were providing trophic support, then blockade of mGluRs would result in the elimination of this support.

5. Conclusions

The present experiments support the hypothesis that activation of mGluRs is necessary to maintain NM neurons and that the loss of mGluR activation following deafness is a critical factor leading to neurodegeneration. Activation of these receptors appears to be involved in both the regulation of ribosomes in NM neurons and their ultimate survival.

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Abbreviations

AC	adenyl cyclase
t-ACPD	± trans 1-amino-1,3-cyclopentadecarboxylic acid
ACSF	artificial cerebrospinal fluid
AIDA	(RS)-1-Aminoindan-1,5-dicarboxylic acid
ANOVA	analysis of variance
APV	(2R)-amino-5-phosphonopentanoate
[Ca²⁺]_i	intracellular calcium concentration
cAMP	cyclic adenosine monophosphate
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
EPSP	excitatory postsynaptic potential
icv	intracerebroventricular
iGluR	ionotropic glutamate receptor
MCPG	(RS)-α-Methyl-4-carboxyphenylglycine
mGluR	metabotropic glutamate receptor
NM	nucleus magnocellularis
PBS	phosphate buffered saline
PB	phosphate buffer
PKA	protein kinase A
PKC	protein kinase C
eCBs	endocannabinoids
CB1	cannabinoid receptor 1

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Figure 1.

Photomicrographs of intact and deafferented sides of vehicle-treated (top) and MCPG-treated subjects. Drugs were applied icv for 3 hrs following cochlea removal. Tissue sections were immunolabeled with Y10B, a monoclonal antibody that recognizes a ribosomal epitope. In ACSF (vehicle)-treated animals, there is darker Y10B immunolabeling on the intact side of the brain section (**A**) in comparison to the deafferented side (**B**). Application of MCPG blocked the difference in Y10B immunolabeling between intact (**C**) and deafferented (**D**) sides of the same tissue sections. Valid comparisons can only be made between opposite sides of the same tissue sections since differences in overall staining intensity between sections could be attributable to a variety of processing variables.

Figure 2.

Mean z-scores of labeling density of NM neurons on the deafferented side of the brain for subjects treated with vehicle, or mGluR antagonists at 3 hrs (**A**) and 6 hrs (**B**) post-cochlea removal. Negative numbers indicate that labeling was lighter on the deaf side of the section. There was a robust difference in labeling between intact and deafferented sides of the sections from ACSF (vehicle)-treated subjects. Treatment with mGluR antagonists MCPG (mixed Group I and II), AIDA (Group I), and LY341495 (Group II) all resulted in an elimination of the activity-dependent difference seen in the vehicle-treated subjects. For both MCPG and AIDA there was a trend towards a reversed effect, where immunolabeling was darker in cells on the deafferented side in comparison to those on intact side of the tissue section. This reversal was only statistically reliable ($P < 0.05$) in the MCPG-treated group at 3 hrs post-cochlea removal. The mean z-score value of the ACSF (vehicle)-treated group was statistically ($P < 0.05$) greater than the mean z-score of the other three groups for both 3 and 6 hr survival periods.

Figure 3.

Photomicrographs of intact and deafferented sides of vehicle-treated (top) and AIDA-treated subjects (bottom). Drugs were applied icv for 24 hours following cochlea removal. Tissue sections were stained for Nissl. In ACSF (vehicle)-treated animals, there is greater cell number on the intact side of the brain section (**A**) in comparison to the deafferented side (**B**). Application of AIDA blocked the difference in cell number between intact (**C**) and deafferented (**D**) sides of the same tissue sections.

Figure 4.

Cell counts of deafened versus intact side of brains stained for Nissl at 24 hrs (A) and 5 days (B) post-cochlea removal. These data are transformed to percentage difference in cell number between sides for the 24 hr (C) and 5 day (D) survival periods. Negative numbers indicate that there were fewer cells on the deaf side of the brain. At both 24 hrs and 5 days vehicle-treated subjects demonstrated approximately 25% fewer Nissl-stained cells on the deafened side of the brain in comparison to the intact side of the brain. Treatment with mGluR antagonists MCPG (mixed Group I and II), AIDA (Group I), or LY341495 (Group II) all prevented the deafness-induced difference in cell number between the two sides and in some cases resulted in a reliable difference between sides in the opposite direction.