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AFFERENT REGULATION OF OXIDATIVE STRESS IN THE CHICK COCHLEAR NUCLEUS

A. H. NICHOLAS AND R. L. HYSON*

Department of Psychology, Program in Neuroscience, Florida State University, Tallahassee, FL 32306-1270, USA

Abstract—The chick auditory brain stem has been a useful model system for examining the afferent-dependent signals that regulate postsynaptic neurons. Like other sensory systems, compromised afferent input results in rapid death and atrophy of postsynaptic neurons. The present paper explores the possible contributions of an oxidative stress pathway in determining neuronal fate following deafferentation. Levels of reactive oxygen species, lipid damage measured by 4-hydroxynonenal formation, and a compensatory reactive oxygen species-induced response regulated by glutathione s transferase M1 and the reactive oxygen species-sensitive transcriptional factor, nuclear respiratory factor 1 were examined. Unilateral cochlea removal surgery was performed on young posthatch chicks. Labeling in the cochlear nucleus, nucleus magnocellularis, on opposite sides of the same tissue sections were compared by densitometry. The results showed a dramatic increase in reactive oxygen species in the deafferented nucleus magnocellularis by 6 h following cochlea removal. This increase in reactive oxygen species was accompanied by lipid damage and a compensatory upregulation of both glutathione s transferase M1 and nuclear respiratory factor 1. Double-labeling revealed that glutathione s transferase M1 expression was highest in neurons that were likely to survive deafferentation, as assessed immunocytochemically with Y10b, a marker for ribosomal integrity. Together, these data suggest reactive oxygen species are generated and a compensatory detoxifying pathway is upregulated in the first few hours following deafferentation. This is consistent with the hypothesis that oxidative stress plays a role in determining whether a given neuron survives following deafferentation.

Key words: reactive oxygen species, auditory system, nucleus magnocellularis, deafferentation, cell death, antioxidant.

Oxidative stress is the production of reactive oxygen species (ROS) beyond the cell's antioxidant capacity. While potentially toxic, ROS are necessary for normal cellular activities and are kept in balance by an antioxidant system. If the equilibrium between antioxidants and ROS is perturbed in favor of the latter, critical proteins, lipids and nucleic acids, essential to cellular homeostasis are oxidized (Ohinata et al., 2000; Cheng et al., 2001; Kelada et al., 2003). Often, these altered products initiate cellular cascades that culminate in apoptosis or necrosis (Tjalkens et al., 1998; Cheng et al., 2001). Although the relationship between oxidative stress and cell death is well documented in neurodegenerative diseases, such as Alzheimer's, Huntington's, and muscular dystrophies (Andersen, 2004; Cui et al., 2004), its role in the cascades of events that determine survival following deafferentation remains a mystery.

The chick auditory brain stem has been a useful model for examining the factors that regulate the life and death of postsynaptic CNS neurons following deafferentation. Its relatively simple and bilaterally symmetrical organization allows for within-subject comparative analysis. In this system, the ipsilateral auditory nerve provides the sole source of excitatory input to the cochlear nucleus, nucleus magnocellularis (NM). Thus, unilateral cochlea removal results in complete loss of excitatory input to the ipsilateral NM but leaves the contralateral side unaffected, thereby allowing comparison of NM neurons on the intact side versus the deafferented side of the same brain.

The elimination of sensory experience by cochlea ablation produces several dramatic changes in NM neurons. A few days (2–3) following cochlea removal, approximately 20–30% of NM neurons die, while the remaining show a reduction in soma size (Born and Rubel, 1985). Approximately 6 h following deafferentation, protein synthesis assays show two diverging and distinct pathways in NM. One population of NM neurons shows a reduce level of protein synthesis while the other, approximately 20–30% of the neurons, shows complete cessation of protein synthesis (Steward and Rubel, 1985). Similar effects of deafferentation can also be observed using Y10b, an antibody that recognizes ribosomal RNA (Garden et al., 1994). Prior to dissociating into two populations, decreased protein synthesis and Y10b labeling across all NM neurons can be visualized as early as one hour following deafferentation (Hyson and Rubel, 1989; Garden et al., 1994; Hyson, 1995). These early changes occur across the entire population of deafferented cells, therefore the factors that determine whether a given NM neuron will follow the survival pathway or the death pathway are still unknown. It is possible that the balance between pro-survival and pro-death mechanisms approaches a threshold that leaves some cells incapable of recovery. The precise nature of these pro-survival and pro-death influences, however, is also uncertain.

*Corresponding author. Tel: +1-850-644-5824; fax: +1-850-644-7739.
E-mail address: hyson@psy.fsu.edu (R. L. Hyson).

Abbreviations: ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; ASK1, apoptosis signal-regulating kinase; BSA, bovine serum albumin; GSH, glutathione; H_2DCFDA, carboxyl-2,7-dichlorodihydrofluorescin diacetate; JNK, jun N-terminal kinase; GSTM1, glutathione-S-transferase M1; GSTs, glutathione-S-transferases; mGluRs, metabotropic glutamate receptors; NM, nucleus magnocellularis; PBS, phosphate-buffered saline; PKC, protein kinase c; ROS, reactive oxygen species; 4-HNE, 4-hydroxynonenal.

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One possibility is that the response to oxidative stress predicts the survivability of a given neuron. A few studies have reported changes that suggest a perturbation of ROS homeostasis in NM. First, intracellular calcium is elevated within an hour of deafferentation (Zirpel et al., 1995). Elevated intracellular calcium has been shown to cause mitochondrial dysfunction that increases levels of ROS (review, Brookes et al., 2004). Additionally, 6 h following deafferentation, the mitochondria dramatically proliferate (Hyde and Durham, 1994). This is accompanied by a transient increase in oxidative enzyme capacity (Durham and Rubel, 1985; Hyde and Durham, 1990). Given that the mitochondrion is the major source of ROS production, a robust increase could cause elevated levels of ROS. Increases in ROS, however, can be counterbalanced by compensatory increases in antioxidant mechanisms. Perhaps the balance between ROS production and these compensatory responses determine whether an NM neuron will survive or die following deafferentation.

Typically, ROS indiscriminately attack polyunsaturated fatty acids of membranes consequently disrupting cellular integrity. Lipid peroxidation can be determined by measuring 4-hydroxynonenal (4-HNE), an α,β-unsaturated aldehyde that is produced from the reaction of ROS with the n-6 polyunsaturated lipids in the cellular membrane. The elevation of intracellular 4-HNE has been shown to activate the jun N-terminal kinase (JNK) pathway (Forman et al., 2003) and to cause cell cycle arrest (Yang et al., 2003) and apoptosis (Awasthi et al., 2003). In contrast, other studies have shown that the normal formation of 4-HNE can initiate differentiation and proliferation (Cheng et al., 1999), and the rise in levels of 4-HNE leads to the synthesis of the antioxidant, glutathione (GSH) (Liu et al., 1998).

Glutathione-S-transferases (GSTs) are one group of antioxidant effectors that respond to a variety of stresses (review, Hayes et al., 2005). These enzymes are classified into five classes (alpha, mu, pi, theta and zeta) based upon protein sequence, substrate specificity, and immunological properties (Clark, 1989; Rouimi et al., 1996). GSTs protect cellular macromolecules from electrophilic attack through the conjugation of GSH to oxidized molecules such as 4-HNE. There is an emerging body of literature documenting the integral roles of GSTs in cell survival and death. For instance, numerous laboratories have shown that elevated levels of GST are associated with resistance to apoptosis (Kodym et al., 1999; Cumming et al., 2001). Moreover, forced expression of glutathione-S-transferase M1 (GSTM1) blocked apoptosis signal-regulating kinase (ASK1) -dependent cell death in cultured cells (Cho et al., 2001). It has been suggested that the induction of GST is an early adaptive response to oxidative stress; a response that is conserved across various species (review, Hayes et al., 2005).

Changes in GST expression have been shown to be regulated by Nrf1, the ROS-sensitive transcription factor. The increase in levels of ROS causes the translocation of Nrf1 into the nucleus resulting in the upregulation of detoxifying genes (Kang et al., 2004). The relevance of Nrf1 in this pathway is underscored by a study that showed Nrf1 deficiency results in early embryonic lethality and severe oxidative stress (Leung et al., 2003). The aim of the present study is to determine whether deafferentation inflicts oxidative damage but induces a compensatory change in GSTM1 expression in NM neurons. Toward this end, changes in ROS, 4-HNE, GSTM1, and Nrf1 were examined in NM following deafferentation. The results from this study are consistent with the hypothesis that an oxidative signaling pathway plays a role in the control of cell death following deafferentation.

EXPERIMENTAL PROCEDURES

Subjects

All subjects were Ross×Ross chickens that were hatched and reared at Florida State University. The procedures used in these experiments were approved by the Animal Care and Use Committee at The 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 potential suffering. Subjects were 7–10 days posthatch for the experiments using immunocytochemistry. This age was chosen because the auditory system is presumed to be relatively mature by this time and because the majority of previous studies on the time course of changes following cochlea removal have been performed at this age (Rubel et al., 1990). The studies examining change in ROS required that younger chicks be used in order to obtain adequate dye loading.

Cochlea removal

A unilateral cochlear ablation was performed under halothane anesthesia. The ear canal was enlarged with small scissors then the tympanic membrane was punctured with forceps. The columella was removed, and the basilar papilla was extracted through the oval window using forceps. Finally, the middle ear cavity was packed with gelfoam, and the incision was closed with surgical adhesive. After recovery from anesthesia, the animals were placed back in their housing unit and allowed to survive for varying periods of time. The purpose of this surgical procedure was to eliminate all afferent drive to the ipsilateral NM while leaving the innervations to the contralateral NM intact.

Changes in ROS

All subjects were one day old post-hatched Ross×Ross chickens. Following unilateral cochlea removal surgery, the animals were allowed to survive for 1 (n=4), 6–8 (n=7), or 12 h (n=3).

Brain slicing for ROS detection

Following the survival period, subjects were anesthetized with halothane and decapitated. The brainstem was rapidly dissected from the cranium and mounted onto a custom-built stage for slicing using a vibrating-blade microtome. Dissecting and slicing were done in an artificial cerebrospinal fluid (ACSF) bath. The ACSF contained (in mM) NaCl, 130; KCl, 3; $CaCl_2$, 2; $MgCl_2$, 2; $NaHCO_3$, 26; NaH_2PO_4, 1.25; D-glucose, 10 and was oxygenated using a 95% O_2, and 5% CO_2 gas mixture. Care was taken during the dissection to preserve the auditory nerve on both sides of the brainstem. The brainstem was affixed to the microtome stage using cyanoacrylate glue and a 30% gelatin compound was placed under the lateral edges of the brainstem to provide additional support. A 150-μm, bilaterally symmetrical, coronal slice was obtained containing NM and the auditory nerve on both sides.