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SCIENCE DIRECT
Hearing Research 216–217 (2006) 127–137
Hearing Research
www.elsevier.com/locate/heares

Research paper

Afferent regulation of neuron number in the cochlear nucleus: Cellular and molecular analyses of a critical period

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Received 14 January 2006; received in revised form 15 March 2006; accepted 16 March 2006

We dedicate this review to the memory of Dr. Larry Katz, Professor of Neurobiology at Duke University. Larry was a brilliant and remarkably creative neurobiologist who loved the study of plasticity and stability in the developing brain. Tragically, Larry died of melanoma on November 26th, 2005.

Abstract

The neurons of the cochlear nucleus are dependent on input from the auditory nerve for survival during a critical period of development in a variety of vertebrate species. The molecules that underlie this age-dependent vulnerability to deafferentation are for the most part unknown, although recent studies have begun to yield interesting candidate genes. Here, we review the studies that originally described the presence of afferent dependent neuron survival in the cochlear nucleus and the age-dependency of this effect, as well as more recent work that seeks to understand the mechanisms underlying the neuron loss that occurs and the basis of this critical period. While much of the past work on cochlear nucleus neuronal susceptibility has been conducted looking at one or two genes at a time, recent advances in genomics make it possible to screen tens of thousands of genes while looking for candidate genes that are determinants of the critical period response to afferent deprivation.

Keywords: Cochlear nucleus; Critical period; Microarray; Deafferentation; Neuron death

1. Introduction

Over 50 years ago, Levi-Montalcini (1949) and Hamburger and Levi-Montalcini (1949) first described the importance of both afferent and target-derived mechanisms in the regulation of neuron number in the developing nervous system. Since that time, the molecular and cellular signal transduction pathways that underlie target-derived regulation of neuron death and survival have been a large focus of attention and are well described in a variety of systems, most notably in developing dorsal root ganglion and spinal motor neurons deprived of their peripheral target cells. Intracellular events that follow binding of neurotrophins to cell surface receptors, including retrograde transport of signaling molecules to the cell body that lead to changes in gene transcription in the nucleus important for survival have been studied extensively (reviewed in Campenot and MacInnis, 2004). Afferent-dependent neuron survival has been less well appreciated in developmental neuroscience, and therefore the mechanisms that support this survival are not yet as well understood as target-derived factors. However, the role of afferent input in promoting neuron survival has been established and studied for many years in the auditory system as well as other sensory systems.

Removing afferent input by ablating peripheral sensory receptors in embryonic or young animals results in a variety of changes in target neurons, the most dramatic effect being neuron death (auditory, Born and Rubel, 1985; Nordeen et al., 1983; Parks, 1979; Trune, 1982; olfactory,

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doi:10.1016/j.heares.2006.03.016

Frazier and Brunjes, 1988; visual, Galli-Resta et al., 1993; somatosensory, Baldi et al., 2000). Importantly, the same sensory alterations experienced by older animals often have little or no effect on neuron survival (Born and Rubel, 1985; Brunjes and Borror, 1983; Hashisaki and Rubel, 1989; Moore, 1990; Mostafapour et al., 2000; Stern et al., 2001). This general pattern has been demonstrated and replicated in the auditory system of a variety of species. Specifically, ablation of the cochlea results in neuron death in the brainstem cochlear nucleus (CN) at young ages but not older ages. Interestingly in mammals, but not chickens, the end of CN neurons' dependency on afferent input parallels the time when hearing begins. This window of time when changes in sensory experience can affect the brain in a permanent manner is known as a "critical period". This term is really a shorthand way of stating that the presence of some tissue interaction, or the removal of a tissue interaction, has a larger consequence for the organism at one time of development than it does at another. Some authors make a distinction between "critical" and "sensitive" periods. We have abandoned the distinction because it is often a matter of semantic differences of opinion.

The term critical period is exemplified by the pioneering work of Lorenz (1958), who demonstrated the presence of a finite window of time during which appropriate signals are required for the development of an important, complex behavior in young birds called imprinting. There are many examples of critical periods that occur during postnatal development of complex behaviors, but the presence of critical periods have also been classically described during the formation of neural circuits and establishment of neuron number. The term "critical period" is not widely used to describe the time during which developing neurons are dependent on some form of trophic support, but the definition of critical period obviously encompasses this time window as well. Some populations of neurons have been found to be dependent on trophic support for survival during a finite period of time, while after that age the mature neurons will not die following trophic deprivation (Goedert et al., 1978; Snider et al., 1992).

The *effects* of sensory manipulations are well studied for many examples of critical periods during development of the nervous system. The most well known of these is derived from the classical work of Wiesel and Hubel (1963), Hubel and Wiesel (1964, 1970), but other examples abound in the visual, auditory, somatosensory, and chemosensory systems. However, the molecular natures of these critical periods are still essentially unknown in any system. Recent progress has been made with the advent of new technologies in the identification of single genes that can alter the timing of a critical period (e.g., Hensch, 2004; Mostafapour et al., 2002; Mostafapour et al., 2000; Walsh et al., 2004), but we are still far from understanding the full molecular nature that is permissive for this kind of plasticity in developing nervous systems.

In this review we address the following issues as they pertain to our understanding of the control of neuron survival in the cochlear nucleus by afferent input and the age-dependence of this phenomenon: (i) the development of eighth nerve synapses in the mammalian cochlear nucleus; (ii) mechanisms of cell death following afferent deprivation; (iii) the definition of this critical period in chickens and mammals; and (iv) what we currently know about the molecular basis of this critical period in mammals. Rubel et al. (2004) have recently reviewed some of these issues extensively. Here, we briefly address some of the same issues with a focus on understanding the molecular nature of the age-dependent vulnerability to afferent deprivation found in the mammalian CN.

2. Development and formation of the eighth nerve end-bulb synapses onto bushy neurons

A review of afferent influences on CN neuron survival would not be complete without first discussing the afferent terminals and their development in the cochlear nucleus. In the avian nucleus magnocellularis (NM) and mammalian anteroventral CN (AVCN), the predominant type of synapse is an elaborate network of axosomatic terminals known as the endbulb of Held (Lorente de No, 1981; Ryugo and Parks, 2003). There are few auditory nerve synaptic contacts onto the dendrites of mature bushy cells. Neurons in other subdivisions of the mammalian CN receive synapses with very different morphologies, usually simple bouton-like axon terminals (reviewed in Ryugo and Parks, 2003). The different synaptic morphologies contribute to the unique physiological response properties of different neuron types in the CN. For example, bushy neurons with endbulb synapses show a primary-like response type that is highly similar to activity in the auditory nerve, and thus preserves temporal information particularly important for binaural auditory processing.

Development of endbulb synapses onto avian NM neurons and bushy cells of the mammalian AVCN has been studied in some detail (reviewed in Rubel et al., 2004). In the mouse AVCN, Limb and Ryugo (2000) used small injections of neurobiotin into the cochlea at various postnatal ages to demonstrate that bouton-like synapses are present at birth on bushy neurons. There is not much further elaboration of these small bud-like terminal swellings until postnatal day (P)12–P14, when they increase in size to become 10–15-fold larger than those of the P1 mouse, although most still appear as bouton-like and not yet as endbulbs. The first terminations identifiable as endbulbs were observed in the P28 mouse. The endbulbs continue to elaborate until they reach a "mature" state around P50. Interestingly, maturation of the endbulb elaborations occurs after the onset of hearing in the mouse, at approximately P10–P12 (Ehret, 1976), suggesting a role for sensory experience in the development of synaptic morphology. However, the lack of sensory experience does not seem to simply halt development at this point as endbulb synapses in a deaf mutant mouse, shaker-2, are abnormal but do not appear to be immature (Limb and Ryugo, 2000).

The presence and morphological changes of synapses that occur over the first two weeks after birth alone may not account for any differences in age-dependent survival signaling from the afferent terminals. Thus, another important factor to consider when thinking about afferent regulation of neuron survival and the mechanisms that underlie the age-dependency of this phenomenon is what is the pattern and type of activity in the developing auditory nerve before and after the onset of hearing in mammals. Experiments in both the chick and gerbil after the onset of hearing indicate that metabolic and cell size changes observed in the CN after cochlear ablation can be attributed to the loss of sodium activated electrical activity in the eighth nerve (Born and Rubel, 1988; Hyson and Rubel, 1989; Pasic and Rubel, 1989; Sie and Rubel, 1992). It is not known what type or aspect of activity previous to hearing onset in the mammal is important for promoting neuron survival. Spontaneous activity has been reported in vivo in the cat and chick before the onset of hearing, and is suggested to be a general feature of developing sensory systems (Galli and Maffei, 1988; Lippe, 1994; Walsh and McGee, 1987). Although in vivo work in the mouse is still lacking, spiral ganglion cells in vitro from P0–P5 mice have been shown to have significant spontaneous activity (Lin and Chen, 2000). Currently, very little is known about spontaneous release of transmitters and other potential neurotrophic molecules independent of spike generation. Further observations and experiments on the patterns of afferent activity and spontaneous release of substances impinging on the CN neurons and how these events change over postnatal development, particularly around the onset of hearing, may help to identify specific signals underlying the critical periods we describe below.

3. Removing afferent input in young animals results in neuron death

Removing afferent input by ablation of the cochlea results in profound, but not complete, neuron death in the young cochlear nucleus. Levi-Montalcini (1949) first demonstrated that the survival of developing nucleus magnocellularis (NM) and nucleus angularis (NA) neurons depends on afferent input by removing the otocyst in early, embryonic day (E)2, chicken embryos. Interestingly, the initial development and number of NM neurons was unaffected by the lack of a normal otocyst. After E11, however, there was a 30% loss of neurons. This basic result was replicated and extended by Parks (1979). Many years after the Levi-Montalcini paper it was discovered that E11 corresponds to the time when the first auditory responses can be seen by electrophysiological methods (Saunders et al., 1973) and the first synaptically elicited action potentials are evoked in NM by stimulating the auditory nerve (Jackson et al., 1982). Born and Rubel (1985) then expanded on the earlier studies in chick to show that unilateral cochlear removal later in development, up to six weeks post-hatching, resulted in a similar 25–30% loss of NM neurons. The cell loss was remarkably rapid, most of it occurring within 2 days after afferent deprivation. Atrophy of the surviving neurons also occurred over this period.

During the same period, the effects of cochlear removal on CN neuron number in young mammals were also being analyzed. Trune (1982) showed that cochlear removal in a P6 mouse, allowed to survive for 39 days, resulted in an overall 66% loss of neurons in all three subdivisions of the CN. Later, Mostafapour et al. (2000) expanded on this study and showed that cochlear removals in young mice from P5 to P11 resulted in 25–61% AVCN neuron loss 14 days after the cochlear removal. In another mammal, the gerbil, Hashisaki and Rubel (1989) showed that unilateral cochlear ablation at P7 resulted in 35% neuron loss by 2 days after surgery, with a maximal loss of 58% by 14 days, again looking in AVCN only. These results were confirmed and extended to the entire gerbil CN by Tierney et al. (1997) who demonstrated an overall CN neuron loss of 52–88% when the cochlea was destroyed at ages P3–P7. The removal of auditory nerve input at an early postnatal age, P5, has also been shown to result in 50% CN neuron loss in the ferret (Moore, 1990).

In summary, the percentage of CN neuron loss after afferent deprivation in young animals of a variety of species ranged from 25% to 88%. In mammals, evidence suggests that there is an equal amount of cell loss in each of the CN subdivisions. It is a fascinating question as to what might underlie this partial loss of neurons. Why do some neurons survive afferent deprivation while in many cases the majority of neurons die? Several hypotheses have been proposed to answer this question, and are reviewed in detail by Rubel et al. (2004). Briefly, one hypothesis suggests that there could be distinct populations of neurons with some intrinsic differences in susceptibility to afferent deprivation. However, there is no strong evidence that supports this idea. An alternate hypothesis is that there is not a bimodal population of cells to begin with, but that afferent deprivation results in the activation of cell death and survival pathways that compete within each neuron. Stochastic events and the particular state of each neuron at the time of deafferentation will then determine whether the death or survival signaling "wins".

4. Apoptotic mechanisms of cochlear nucleus neuron death

Cochlear nucleus cell death due to afferent deprivation happens relatively quickly in all species studied so far. In young chicks, maximal cell loss is reached by 48 h after cochlear removal (Born and Rubel, 1985). In gerbils, about 60% of maximal neuron loss is reached by 48 h, and in mice, maximal cell death occurs by 96 h following cochlear removal (Hashisaki and Rubel, 1989; Mostafapour et al., 2000). The relatively quick time course of neuron loss, in addition to morphological descriptions of how these neurons appear to degenerate, suggest they are likely to be dying by a set of processes that we refer to as apoptotic-like pathways. Apoptosis is a genetically regulated form of cell suicide that involves specific classes of genes like the Bcl-2 and caspase families. It can be characterized as having certain morphological features, such as membrane blebbing,

chromatin condensation, and DNA laddering (Hengartner, 2000; Rich et al., 1999). There are two main pathways described for apoptosis that are not mutually exclusive. In the first, the intrinsic pathway, Bcl-2 family members control the opening of a mitochondrial permeability transition pore through which cytochrome *c* can be released from the inner mitochondrial membrane after a stressful stimulus. In the cytoplasm, cytochrome *c* forms a complex called the apoptosome with caspase 9, Apaf-1, and ATP. The formation of this complex activates caspase 9, which goes on to cleave and activate other caspases, including the downstream executioner caspase 3. In the second pathway, called the extrinsic pathway, death receptors on the cell surface are activated by their ligands, which leads to the cleavage of caspase 8 inside the cell. Activated caspase 8 can then cleave and activate downstream executioner caspases without involving the mitochondria, although there can be crosstalk between these two canonical pathways.

One relatively common feature observed after an apoptotic stimulus is the dysregulation of intracellular calcium. Calcium overload can be toxic to neurons and has been demonstrated to underlie neuron death induced by pathological activation of ion channel receptors, for example NMDA channels (Choi, 1992). It is likely not a coincidence that two organelles intimately involved in many examples of apoptosis, the mitochondria and ER, are important for normal buffering of intracellular calcium levels. Calcium homeostasis and buffering may be particularly important in brainstem auditory neurons because of their consistently high levels of electrical activation (Rubel et al., 2004). Perhaps to deal with this, auditory neurons are rich in calcium binding proteins and other potential mechanisms of calcium control. Although high levels of calcium can be toxic to a cell, a variety of signal transduction pathways use calcium, several of which involve the activation of gene transcription in the nucleus. Physiological changes in intracellular calcium can activate the transcription of pro-survival genes, such as Bcl-2.

In the chick NM, a 3-fold increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in NM neurons is one of the earliest observable events following afferent deprivation (Zirpel et al., 1995). In vitro experiments demonstrated that this increase in $[Ca^{2+}]_i$ can be prevented by orthodromic stimulation but not antidromic stimulation (seen in Fig. 1) and that it is independent of AMPA receptor activation. Rather, maintenance of normal $[Ca^{2+}]_i$ homeostasis appears to depend on activation of one or more metabotropic glutamate receptors (Zirpel and Rubel, 1996). This conclusion is supported by recent studies using whole cell voltage clamp methods to study Ca^{2+} channel currents (Lu and Rubel, 2005). Zirpel et al. (1998) further demonstrated a strong correlation between $[Ca^{2+}]_i$ and amount of neuron death. Manipulations that increased the calcium concentration above physiological levels resulted in more NM cell death, while manipulations decreasing calcium levels, in the absence of afferent input, resulted in a decreased number of dying NM cells. However, the story is likely more complicated than simply increased calcium after afferent deprivation results in CN neuron apoptosis. Recent work has shown that the transcription factor CREB is activated in a subset of chick and mouse CN neurons by 1 h following cochlear ablation and, importantly, this activation is dependent on the increased calcium due to deafferentation (Zirpel et al., 2000). The transcription of anti-apoptotic genes, such as Bcl-2, can be regulated through a calcium-dependent signaling pathway involving CREB acting on the CRE element on the Bcl-2 gene (Riccio et al., 1999), suggesting that the elevated calcium levels could result in activation of both survival and death mechanisms. However, the precise role of CREB and its target genes in promoting CN neuronal survival after cochlear ablation is still unknown.

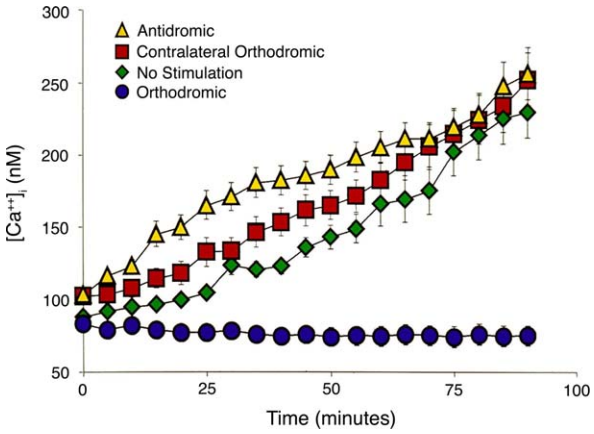

Fig. 1. Fluorescent imaging was used to evaluate changes in $[Ca^{2+}]_i$ in NM neurons in a slice preparation while stimulating either the eighth nerve (orthodromic), the NM axons as they cross the midline (antidromic), or the opposite eighth nerve (contralateral) as compared to no stimulation. From Zirpel and Rubel (1996).

Bcl-2 mRNA levels were shown to increase in chick NM neurons 6 and 12 h after a unilateral cochlear removal in a subset of neurons. Surprisingly, the number of neurons expressing Bcl-2 at these times is correlated with the number of neurons that eventually go on to die (30%, Wilkinson et al., 2002). It is unclear if this is a causal event in the death of these neurons, or if it is a compensatory response that contributes to the overall survival of the remaining 70% of neurons. The relative levels of two other pro-apoptotic molecules have also been assessed after unilateral cochlear removal in chick NM. Caspase 9 was activated by 3 h after deafferentation, peaked at 12 h, and was back to control levels after 4 days (2 days after maximal cell loss; Wilkinson et al., 2003). Cytochrome *c* protein levels also were found to increase by 6 h following deafferentation. However, it was not released into the cytoplasm; all the detectable protein was found still in the mitochondrial membranes. These results suggest that a caspase-dependent apoptotic process is involved in NM neuron death, but perhaps not through the mitochondrial cytochrome *c* pathway.

There are also several indications of apoptosis in young mouse AVCN neurons after deafferentation. The number of cells that labeled with TUNEL, a marker of DNA break-

down, increased at 12 h following cochlear removal, and peaked at 48 h, preceding the time of maximal neuron loss (Mostafapour et al., 2000). The downstream executioner caspase 3 is also activated in AVCN neurons at about 12–24 h following deafferentation, returning to control levels by 48 h (Mostafapour et al., 2002). The role of calcium in mammalian CN cell death is currently unknown. More work will need to be done to address whether the calcium increases observed in the chick also occur in a mammal after cochlear removal, but together these data strongly point to roles for calcium homeostasis and apoptotic genes in underlying the CN neuronal death response to deafferentation in young animals.

5. Critical period: age-dependent vulnerability

One of the most interesting observations made during studies of afferent deprivation in the auditory and other sensory systems is the dramatic age dependency of vulnerability to cell death. The first demonstration of this effect in the auditory system came from the work of Born and Rubel (1985), who found that somewhere between 6 and 66 weeks of age post-hatching in a broiler chicken, CN neurons are no longer dependent on auditory nerve input for survival. Recently, this phenomenon in the chick has been studied in more detail due to indications that breed affects CN neuron susceptibility to cochlear removal at older ages (Edmonds et al., 1999). Two different breeds of chickens commonly used for auditory research are egg layers and broiler chickens. Interestingly, CN neurons in egg layer chickens continue to show vulnerability to cochlear removal at old ages, while broiler chickens do not (Edmonds et al., 1999). One difference between these breeds is that most aging broiler chickens have cochlear degeneration, while the egg layers do not. Further studies demonstrated that in adult broiler chickens with *normal* healthy cochleas, CN neurons continue to be vulnerable to loss of afferent input (Smittkamp et al., 2005). This extended period of time in chickens when cochlear ablation results in neuron death is different in a couple of potentially important ways from the critical period described in several mammalian species.

First, the length of the time period when CN neurons are reliant on afferent input for survival is much shorter in ferrets, gerbils, and mice compared to chickens, as seen in Fig. 2. Moore (1990) found that CN neurons were susceptible to cochlear ablation at age P5 in the ferret, but the critical period was closed by the next age examined, P24. Hashisaki and Rubel (1989) observed that this period of susceptibility to afferent deprivation closed between 1 week and 20 weeks postnatal in the gerbil. The sharpness of the critical period closure was not fully appreciated until 1997, when Tierney and Moore carefully looked at the effect of unilateral cochlea removal on CN neuron survival when the surgery was done every two days between P3 and P18. Neurons in the entire CN were highly susceptible to cochlear removal at ages up to and including P7 (52% neuron loss), but only two days later at P9, the next age examined, there was no longer any significant cell loss. In the mouse, the critical period closes more gradually than found in the gerbil, but is still relatively tight. AVCN neurons were found to be susceptible to cochlear removal at ages up to and including P11, where neuron loss ranged from 25% to 61%, but by P14, there was no longer any significant loss of neurons (Mostafapour et al., 2000). Fig. 3 shows examples of AVCN sections in mice that were operated on at P5 and P21. Cochlear ablation has no observable effect on P21 cell survival (Mostafapour et al., 2000).

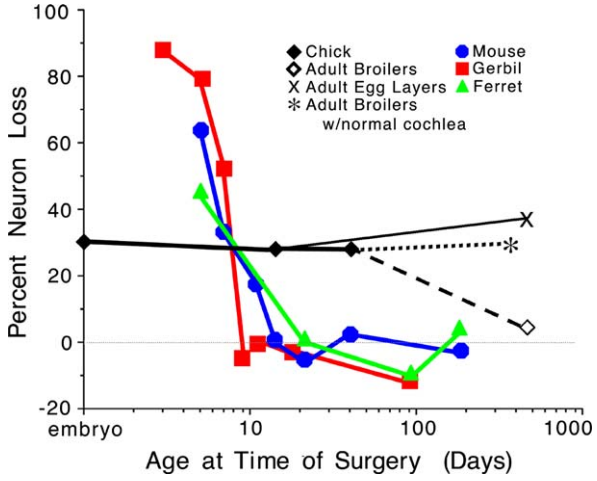

Fig. 2. Percentages of maximal neuron loss in the CN ipsilateral to a cochlear removal compared to the contralateral side in three mammalian species and two breeds of chicken are plotted against age at the time of surgery (postnatal or posthatch days plotted on a log scale). Note the extended critical period present in the chicken compared to the shorter periods of susceptibility seen in the mouse, gerbil, and ferret. Data presented in this figure are means re-plotted from previous publications (Born and Rubel, 1985; Edmonds et al., 1999; Moore, 1990; Mostafapour et al., 2000; Parks, 1979; Smittkamp et al., 2005; Tierney et al., 1997).

The second major difference observed between the critical period of mammals and the extended period of susceptibility in chickens is the timing relative to the onset of function in the auditory system. In mammals, this critical period closes around the onset of hearing, but this is not the case for chickens, where the window remains open long after the onset of hearing (Born and Rubel, 1985; Moore, 1990; Mostafapour et al., 2000; Tierney et al., 1997). If there is a causal relationship between the onset of hearing function and the ending of this mammalian critical period it is still unknown, but the correlation between these events is highly suggestive of a role for changing activity patterns and/or levels in helping to define the length of this window.

6. Molecular mechanisms underlying this critical period

The short window of this critical period in mammals suggests that there may be only a few changes in gene or protein expression underlying the switch from afferent-dependent to afferent-independent neuronal survival. However, the molecular basis underlying this differential susceptibility to cochlear ablation in the CN is not known.

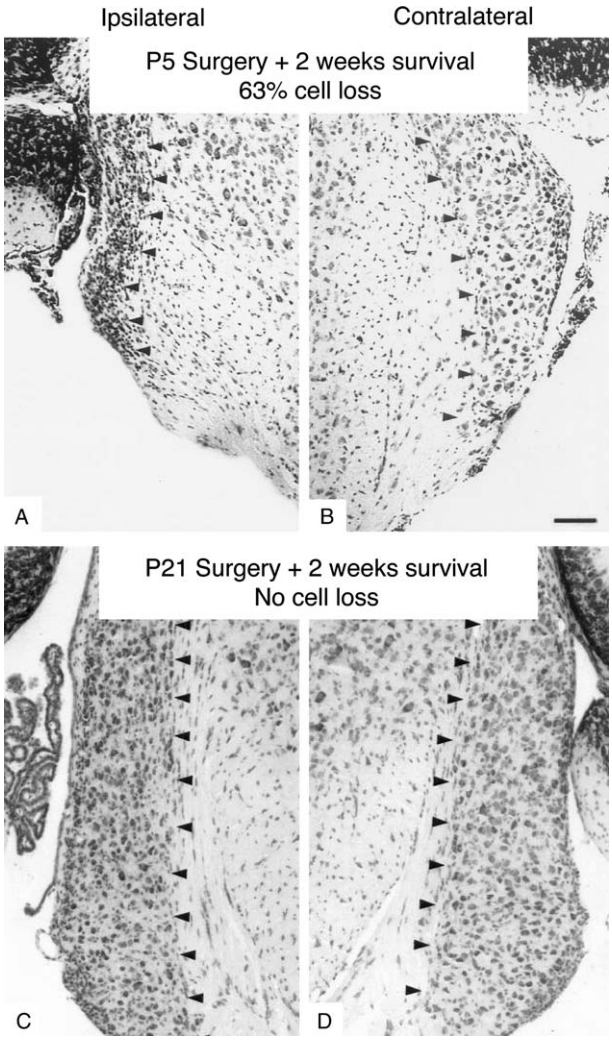

Fig. 3. Comparison of AVCN sections on sides ipsilateral to cochlear ablation with the contralateral sides two weeks after surgery was done at P5 (A, B) and P21 (C, D). There was a gross reduction in size of the nucleus and neuron number at P5, but not P21. Scale bar = 100 μm. Figure modified from Mostafapour et al. (2000).

What happens during maturation to render CN neurons less susceptible to loss of afferent input? What are the molecules that underlie the critical period window, and why does it close? The mechanisms that define a critical period could include changes in expression of mRNA or protein, changes in post-translational modifications or even intracellular trafficking of proteins. For all examples, it is implicit that there is a developmental switch in either expression or function of one or many genes that defines the window of the critical period.

These questions have been difficult to address in the past, but are becoming within reach because of advances in genome and proteome technologies, particularly in the mouse. Two criteria for identifying genes responsible for the critical period response and closure are: (i) a critical period gene must show a change in expression or function in parallel with the end of this critical period; (ii) manipulation of the expression or function of a gene, or set of genes, can alter the timing of the critical period response.

The first approach taken to identify candidate genes responsible for the critical period in the CN has been to address the second criterion above. Based on the findings that AVCN neurons die an apoptotic-like death, Mostafapour et al. (2000, 2002) tested the hypothesis that a known regulator of apoptosis could alter the timing of CN neurons' sensitivity to death by cochlear removal. The role of the anti-apoptotic Bcl-2 gene in preventing cell death and closing the critical period in mature neurons after cochlear removal was tested in mice with the Bcl-2 gene knocked out (Bcl-2 null mutant). In addition, the potential role of Bcl-2 in protecting immature CN neurons was assessed in mice that overexpressed the Bcl-2 gene (Bcl-2 overexpressor). These results are shown in Fig. 4. In the Bcl-2 null mutant mouse, cochlear removal at ages older than P24 resulted in significantly more neuron loss in the AVCN compared with wild type mice of the same background strain (38% vs. −7%). This 38% neuron loss in the Bcl-2 null mutants at ages after the critical period lies within the range of neuron loss observed during the wild type critical period, suggesting that the critical period has been extended in these mutant mice. However, whether or not the critical period ever closes at later ages was not analyzed in these mice, nor were ages during the wild type critical period analyzed for comparison. On the other hand, Bcl-2 overexpression in a young transgenic mouse protected CN neurons from afferent deprivation to a level seen at ages after the critical period normally. These results together suggested that increased levels of Bcl-2 protein could close the critical period window. This has to be interpreted cautiously, however, as it is not clear from these studies whether Bcl-2 itself is the sole molecule responsible for the switch from afferent-dependent to -independent survival, or if overexpression of this strongly anti-apoptotic molecule has tipped the natural balance of death and survival effectors toward survival.

Another single molecule that has been shown to alter the timing of this critical period, although not eliminate it com-

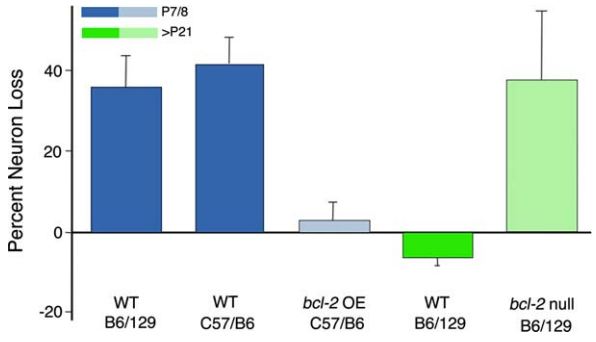

Fig. 4. Bcl-2 regulation alters the critical period for deafferentation-induced cell death in AVCN. This figure is redrawn from data presented in Mostafapour et al. (2000) and Mostafapour et al. (2002), showing AVCN cell loss in wild type mice (strain control, either C57/B6 or B6/129) and mutant mice with alterations in expression of the anti-apoptotic gene, Bcl-2. WT = wild type, OE = overexpressor. Means ± SEMs are shown.

pletely, is the protein tyrosine phosphatase, SHP-1 (Zhao and Lurie, 2004). SHP-1 is upregulated in glial cells following injury, and may normally play a role in regulating signal transduction pathways such as the Jak/Stat and MAPK cascades. In a naturally occurring mutant, the motheaten mouse (me/me), SHP-1 levels are reduced or eliminated. Zhao and Lurie (2004) sought to determine the possible effect of this gene on the critical period response. Me/me mice at ages P5, P14, and P21 were subjected to a cochlear removal and allowed to survive for various times up to 4 days, when maximal neuron loss during the critical period has already occurred. Mutation of this gene did not affect neuron loss at either P5 or P21, but at P14, an age right at the end of the critical period window, there was an increased amount of cell death compared to wild type mice. The lack of functional SHP-1 therefore seems to *extend* the critical period, but does not keep it open indefinitely as there is no increased neuron death after cochlear removal in P21 me/me mice. Interestingly, a positive correlation was found in the motheaten P14 mice between markers for activated microglia and neuronal death. However, there was also an increase in activated microglia labeling, albeit much smaller, in wild type P21 mice after cochlear ablation when no significant neuron death occurred. It is unclear if the large increase in microglia activation at P14 caused the increased neuron death but, whatever the mechanism may be, changes in glial gene expression, in addition to changes in neuronal gene expression, could underlie the switch during development from afferent-dependent to -independent survival.

Although these genes, Bcl-2 and SHP-1, both seem to fit with the second criterion stated above in some way, the first criterion was not answered as to whether Bcl-2 or SHP-1 protein or mRNA levels change in parallel with the end of the critical period. In fact, it appears that Bcl-2 protein and mRNA levels do not change dramatically between P7 and P21 (Harris, personal observation, Harris et al., 2005b). Given that these two disparate genes both can have an effect on the critical period response, it seems likely that many genes, perhaps in several cell types, play a role in defining this critical period.

To identify candidate genes that actually change in expression level in parallel with the end of the critical period, and to identify genes that are differentially regulated by afferent deprivation as a function of age of the organism, our lab decided to take an unbiased screening approach using microarray technology. This approach is based on two hypotheses that are neither mutually exclusive nor inclusive of the list of cellular events that may control this critical period. The first hypothesis is that developmental differences in baseline mRNA levels of particular genes could predispose CN neurons to death during the critical period or survival after the critical period. The second hypothesis is that deprivation produced by cochlea removal in neonatal animals will induce rapid upregulation or downregulation in the expression of genes that are different from those induced by the same manipulation after the critical period.

The first hypothesis was recently examined (Harris et al., 2005b). Two different microarray platforms were used to survey >22,690 mouse genes for changes in relative constitutive levels of mRNA between 3 ages; during the critical period at P7, at the closing of the critical period at P14, and one week after the critical period ends at P21. Interestingly, there were only 31 genes whose expression levels changed significantly between P14 and P21, despite the fact that hearing development changes radically over this period. Over 1000 genes were identified as changing in expression in a statistically meaningful way during compared to after the critical period. Approximately half were higher during the critical period (P7) and half were higher after the critical period (P14 and P21). These 1000 genes of various functions can all be considered candidates for underlying the critical period, but based on the work of Mostafapour et al. (2000, 2002), it seems likely that more genes involved in apoptosis, like Bcl-2, could be involved. An analysis of known anti- and pro-apoptotic genes that were screened with these particular microarrays, shown in Table 1, revealed that 9 of 11 genes with known survival functions showing a change in expression over the critical period were expressed at a significantly higher level *after* the critical period ends. These anti-apoptotic genes included several heat shock proteins, alphaB crystallin, hsp12, and hsp105, as well as two members of the anti-apoptotic Bcl-2 family, Bcl-w and Bcl-x. Interestingly, the mRNA levels of Bcl-2 itself were unchanged from P7 to P21. Strikingly, 6 of 7 genes with known death functions showing a relative change in expression over these ages were expressed at a higher level *during* the critical period. These pro-death genes included apoptotic Bcl-2 family members, Bid and Bok, as well as Caspases 3 and 7. These very exciting findings suggest that CN neurons during the critical period may be in a baseline gene expression situation that favors death, while more mature neurons are in a baseline situation that favors survival. Postnatal development may involve an overall net increase in survival mechanisms in mature neurons, by upregulating survival promoting genes and downregulating death promoting genes.

Testing of the second hypothesis is underway (Harris et al., 2005a). The results of this analysis are yielding provocative results as well. For example, it appears that afferent deprivation in animals past the critical period triggers changes in gene expression in the CN that are as great as that seen during the critical period, but different genes are affected. How these different patterns of gene expression result in a different pattern of neuronal death remains unknown.

In summary, the exact group of genes that actually underlies the switch from afferent-dependent to -independent survival is at present unknown, but the new data and hypotheses generated using microarray technology should lead us even closer to elucidating the molecular mechanisms that define this and other critical periods during neuronal maturation. At the very least, these data provide a rich source of testable hypotheses and candidate molecules that we would not have suspected to play a role in the past.

Table 1
Differences in expression of known apoptotic genes surveyed with arrays

	Critical period	Post-critical period		Critical period	Post-critical period
Pro-apoptotic			*Anti-apoptotic*		
AIF			AlphaB crystallin		★
AIP-1 (alix)		★	A1		
Apaf-1			API5		
Bad			Bcl-2		
Bak1			Bcl-w		★
Bax			Bcl-x		★
Bid	★		Boo/Diva		
Bik			CREB		
Bim			Gelsolin		★
Bok	★		Hsp12		★
CAD			Hsp25	★	
Caspase 12			Hsp70		
Caspase 3	★		Hsp105		★
Caspase 6			IAP1		
Caspase 7	★		ICAD		
Caspase 8			Mcl-1		
Caspase 9			Mef2d		★
Cytochrome *c*			Metallothionein I		★
DP5, Hrk			Metallothionein II		★
P53	★		Naip		
P75	★		NF κB		
Smac/Diablo			Survivin	★	
			Xiap		

Genes with established pro- and anti-apoptotic roles that are represented on either of two microarrays are listed. In a survey of these genes, 6 out of 7 with pro-apoptotic roles were constitutively expressed higher during the critical period at P7. Nine out of eleven differentially expressed genes with anti-apoptotic roles were constitutively expressed higher after the critical period at P14 and P21. Genes showing a statistically reliable change in mRNA expression levels during compared to after the critical period are indicated with a ★ (for details see Harris et al., 2005b). Unmarked genes did not show any reliable differences in expression between P7 and P14 or P21. Figure is modified from Harris et al. (2005b).

7. Conclusions, future directions, and speculations

Neurons of the cochlear nucleus, like neurons in a variety of other brain regions in all sensory systems, are dependent on afferent input for survival during a critical period of development. The mechanisms and signals that are responsible for this survival are a focus of research in several laboratories, and a picture is beginning to emerge about how the afferent supply supports neuron survival during this critical period and what occurs to lead to cell death after deafferentation (reviewed by Rubel et al., 2004). Less is known about the molecular basis that underlies this critical period of age-dependent vulnerability. In particular, what are the biochemical and molecular events underlying the transition of neurons from afferent-dependent survival to a state of relative stability, where survival is independent of afferent activity or the integrity of a major excitatory input. A few studies have attempted to define a role for single candidate genes based on previous findings with mixed results (Mostafapour et al., 2002; Mostafapour et al., 2000; Zhao and Lurie, 2004). A newer approach we have taken, acknowledging that important events such as regulating neuron number in the brain is not likely due to a single gene, has been to first identify genes showing a change in expression that parallels the timing of the critical period (Harris et al., 2005b). At the very least, we have been able to identify changes in gene expression levels for tens of thousands of genes in the postnatal mouse CN in *one* experiment versus the traditional "one gene at a time" approach. This important baseline data provides a wealth of information for CN researchers in all fields. The entire microarray datasets are freely available in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov, series number GSE2390). The next step, identifying genes that are differentially upregulated or downregulated at the different ages will also provide a wealth of data, but no definitive answers about one or two genes that control this critical period (Harris et al., 2005a). The more data we get, the more it appears that there will not be a single unitary answer. This is not to say that predictions are not possible, but the models will not be simple additive ones. Single gene studies like the ones cited above are crucial, however, to test for functional roles of candidates identified in large-scale microarray screens. Additionally, the development of technology that could enable the manipulation of expression of *groups* of genes in one experiment will likely be essential. It will be the combination of these screening and function experiments that will most successfully identify the genes that underlie this critical period during development of the cochlear nucleus.

The use of genomics and proteomics has created a new way of thinking about and conducting systems biology. Most bio-behavioral scientists are trained to be reductionist thinkers. The application of these types of tools to tradi-

tional problems, however, can force us to explore and think beyond a single gene, a single protein, and beyond a single cell. The mouse cochlear nucleus is undergoing many developmental changes over the first three postnatal weeks, most notably the onset of hearing, around the same time the critical period of afferent dependence is occurring. The timing of the critical period and the death or survival response of the neurons to afferent deprivation could conceivably be affected by any of these changes, as there is currently no strong evidence suggesting that only changes in the postsynaptic neuron cause this switch. A schematic model illustrating some of the developmental changes in the mouse CN during and after the critical period is shown in Fig. 5. There are five main points presented in this model based on observations made in the mouse CN. First, glial cell proliferation is still occurring through the first postnatal week (Martin and Rickets, 1981), so there are more glia (yellow, astrocytes; blue, microglia) present in the CN after the critical period. Second, spherical bushy neurons (purple) increase in size between P7 and P14 (Limb and Ryugo, 2000). Third, eighth nerve terminals increase in size 10–15-fold between ages during compared to just after the critical period (Limb and Ryugo, 2000). Importantly, it is still unknown if and how synapses change functionally with these changes in morphology. Fourth, there is increased myelination (green, oligodendrocytes; Harris and Westrum, personal observation), and fifth, more vascularization after the critical period at P14 compared to P7 (red, blood vessel; Harris, personal observation).

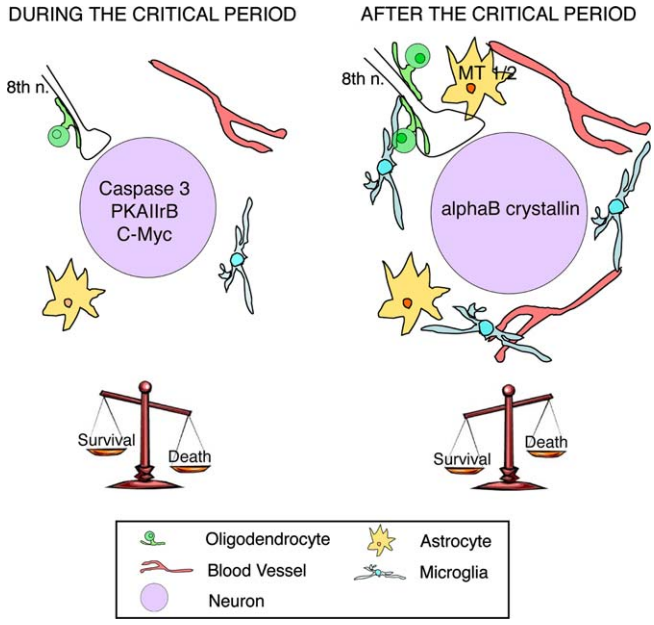

Fig. 5. A schematic model depicting changes that occur over postnatal development in parallel with the timing of the critical period for afferent-dependent neuron survival in the mouse CN. Note that there are more glial cells, increased somal size, increased size of eighth nerve terminals, increased myelination, and increased vascularization present after the critical period compared to during the critical period. These changes could impact the timing of the critical period, but how or if they do at present is unknown. There is higher mRNA expression of pro-death genes in the CN during the critical period relative to the CN after the critical period, and higher mRNA expression of pro-survival genes after the critical period ends. These changes in gene expression suggest that under baseline conditions, the CN during the critical period is predisposed to show a death response. While in the more mature CN, the natural balance under baseline conditions would favor a survival response.

The five examples of changes described above in the developing CN could perhaps influence the response of the CN neurons illustrated here to afferent deprivation. Already Zhao and Lurie (2004) have shown a change in glial gene expression can affect the time when the critical period ends. In addition, changes in the amount or pattern of activity impinging on neurons could define the suite of genes expressed in CN neurons, and perhaps astrocytes as well. Differences in eighth nerve activity, if they exist between these ages before and after hearing onset, could create the different baseline levels of gene expression observed using microarrays. Recently, dorsal root ganglion neurons in culture were stimulated with one of two trains of action potentials differing only in temporal parameters, but both having the same total number of action potentials. Gene expression was then measured using microarrays and it was discovered that the two different stimuli resulted in completely non-overlapping changes in gene expression (Fields et al., 2005). This is perhaps not too surprising because it is known that different routes of calcium entry into the cell and different patterns of phosphorylation of transcription factors, such as CREB, can result in the transcription of different target genes at a particular time (West et al., 2001).

All of the cell types shown in Fig. 5 (and more) were present in the mRNA samples derived for the microarray studies performed by Harris et al. (2005a,b). Therefore the localization of genes of interest is of particular importance in generating new hypotheses. During this critical period, three candidate genes that could promote cell death following afferent deprivation were localized to neurons. After the critical period, one of the pro-survival candidate genes, alphaB crystallin was found in neurons (although not bushy cells), while a second candidate, metallothionein 1/2, was localized to glial cells. Based on the most recent results using microarrays, we speculate that under normal baseline conditions during the critical period the CN is primed for a death response. With increasing age, gene expression changes to tip the balance in favor of a survival response. This seems likely to be a common phenomenon in the developing nervous system (Benn and Woolf, 2004), and an extremely important mechanism for promoting the survival of mature neurons for the lifetime of the organism.

This point may be worth some additional emphasis with respect to studies of the developmental neurobiology of sensory and motor systems in general. The study of structural and functional *plasticity* in the developing brain has captivated the attention of scores of neurobiologists over the past half century, and has generated literally thousands of papers in the scientific literature. On the other hand, our recent data stress the fact that a major change that occurs during the initial development of hearing is the upregulation of

expression of an entire group of genes that could promote *stability* of the cells in the cochlear nucleus in the face of metabolic and structural challenges. More globally, whereas behavioral plasticity is clearly important, every day we remember how to walk and talk, and most of our memories remain intact for decades. Without doubt, this remarkable feat is facilitated by the stability of neuronal populations and circuitry in the brain. Such considerations beg the question of how much attention we are paying toward understanding the neural mechanisms underlying the structural and functional stability of the brain. Fortunately, other groups have also recently stressed this point of view (Mizrahi and Katz, 2003; Mizrahi et al., 2004; Turrigiano and Nelson, 2004). We would suggest that for both conceptual and clinical reasons, this topic of stability deserves much greater emphasis in the future.

Acknowledgments

The authors' work discussed in this review was supported by NIDCD Grants DC03829, DC04661, DC05361, and DC07001.

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