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Cellular signaling protective against noise-induced hearing loss-A role for novel intrinsic cochlear signaling involving corticotropin-releasing factor?

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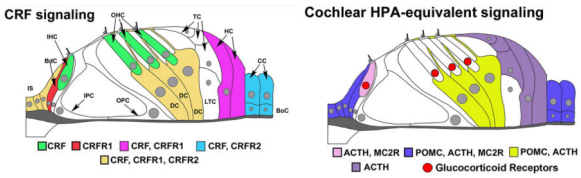
Abstract

Hearing loss afflicts approximately 15% of the world's population, and crosses all socioeconomic boundaries. While great strides have been made in understanding the genetic components of syndromic and non-syndromic hearing loss, understanding of the mechanisms underlying noise-induced hearing loss (NIHL) have come much more slowly. NIHL is not simply a significant mechanism by which individuals loose their hearing. Significantly, the incidence of noise-induced hearing is increasing, and is now involving ever younger populations. This may predict future significant medical problems. Current research has shown that even short-term exposures to loud sounds generating what was previously considered temporary hearing loss, actually produces an almost immediate and permanent loss of specific populations of auditory nerve fibers. Additionally, recurrent exposures to intense sound may hasten age-related hearing loss. While NIHL is a significant medical concern, to date, few compounds have delivered significant protection, arguing that new targets need to be identified. In this commentary, we will explore cellular signaling processes taking place in the cochlea believed to be involved in protection against hearing loss, and highlight new data suggestive of novel signaling not previously recognized as occurring in the cochlea, that is perhaps protective of hearing. This includes a recently described local hypothalamic-pituitary-adrenal axis (HPA)-like signaling system fully contained in the cochlea. This system may represent a local cellular stress-response system based on stress hormone release similar to the systemic HPA axis. Its discovery may hold hope for new drug therapies that can be delivered directly to the cochlea, circumventing systemic side effects.

Graphical Abstract



Keywords

Noise-induced hearing loss; Cochlea; Corticotropin-releasing hormone; Hair cell; Noise damage

1. Introduction

This commentary will cover aspects of the cochlea's response to loud sound, and ensuing noise-induced hearing loss, with the purpose of describing various cellular elements involved in acoustic traumas that could represent potential future drug targets. It should be recognized that significant differences exist among species typically used as experimental models in hearing loss research. Such differences concern the morphological and functional consequences of noise exposure, not least of which is the capacity for full regeneration of sensory cells found in birds, reptiles, and amphibians that is not present in mammals. Due to standard limitations of space, this commentary will concern itself only with the mammalian reaction to noise exposure.

The goal is to present to the non-specialist a directed overview of key components of the inner ear that are typically impacted by noise exposure, and to describe some of the biological consequences to these elements that occur following noise exposure. This commentary will examine theories of protection related to cochlear hair cells. Some of these ideas have been around for decades, and others are relatively recent. As usual, each model of protection has its pro's and con's, which will be explored. Importantly, to date, there has been no suggestion of whether the major models of protection may actually co-exist and explain protection under a varying auditory environment, rather than simply one model vanquishing the others. The problems of noise-induced hearing loss will be introduced first, followed by a discussion of what is currently known about the impact of noise in the inner ear. Along the way, components that could represent drug targets useful for recovery from noise-induced hearing loss will be highlighted. Finally, a newly described signaling system present in the cochlea will be discussed. A case will be made for how this signaling system may indicate that the previously described theories of cochlear hair cell protection can co-exist in a larger framework that includes cross-talk between these protective systems, and that could illustrate different requirements for protection in the naturally varying acoustic environment. Understanding endogenous protective systems in general is useful as more than an academic pursuit. As will be described below, significant societal burdens follow from hearing loss, and understanding endogenous protective signaling systems holds the promise of revealing novel, and potentially therapeutic future drug targets useful for mitigating consequences that typically follow from noise-induced damage.

1.1. The problem

Noise-induced damage and subsequent death of sensory cells of the cochlea, the hair cells, can result in permanent hearing loss and/or associated tinnitus, a persistent ringing in the ears. Hearing loss afflicts 15% of the US population (a similar incident rate occurs world-wide), much of it due to noise-induced damage. Of special concern is that noise-induced hearing loss is rising significantly in the adolescent population (20% of individuals aged 12-19 exhibit hallmarks of hearing loss). Significant research over many decades has

pursued the idea that the mammalian cochlea is able to protect itself from injury. Because the mammalian hair cells are not regenerated, protection, by way of intra- and intercellular signaling, should be considered a key attribute of the system. Without some mechanism to ensure the survival of sensory cells, any individual (and perhaps any species as a whole during early stages of mammalian evolution) is/was at risk of death due to an inability to recognize the approach of a predator, and the ability to communicate over distance following noise exposures. Whether any potential causal/correlation association exists between loss of regenerative abilities and the evolutionary movement toward encoding higher frequency sound has not been investigated.

1.2. The socio-economic burden of hearing loss

Other than congenital deafness, hearing loss is commonly associated with two main issues: aging and the work environment (we will not consider head trauma, tumors, genetic disorders, etc., in the context of this review). Thus, hearing loss is a common and significant medical complaint of the aged and workers operating in high ambient intensity sound environments or in environments in which unexpected high intensity sound exposure can occur. It is more difficult to estimate the economic impacts of hearing loss associated with the aging population because such loss is more often diagnosed near the end of a person's employment lifespan. Yet, upward of two-thirds of adults aged 70 or more have documented hearing and associated communication difficulties [1]. Those aged 65 or greater currently make up approximately 15% of the total US population, and is projected to rise to 21% (73 million people) by 2030, an increase of roughly 50%. Western European countries are projected to witness 24% (UK) to 40% (Germany) increase in the over-65 population during the same time frame (US Census Bureau International Database). A number of reports have indicated that the health-related quality of life (both physical and mental health components) is scored lower in respondents with degraded hearing abilities [1]. Although still controversial, there are also studies demonstrating that this lowered quality of life satisfaction score is also found among spouses of hearing impaired individuals (but this may be most robust when the male is impaired and the female spouse is surveyed concerning her health satisfaction) [1, 2]. Since occurrence of bilateral hearing loss considered at least of mild severity doubles for every 10 years beyond the age of 50, the aging population is poised to exert significant impacts on the general population with respect to communication-associated difficulties.

More easily assessed is the economic impact of hearing loss on those who are still of age to be active in the workforce. Data is especially abundant for military veterans and defense contractors. Significant economic impacts ensue as a consequence of hearing loss in these cohorts, including USD56 million paid by the US Dept. of Defense to civilian workers for noise-induced hearing loss in 2003 [3] and USD1.1 billion spent in 2005 by the US Veterans Administration for veterans with service-acquired hearing loss [4]. It is estimated that between 30-40% of US Naval and Air Force personnel, and 50% of US Army personnel exhibit hearing loss by the time they are 50 years old. Only mining and heavy construction are more damaging to hearing (survey published by www.audicus.com). In general, the ability to be, or remain, employed is significantly lessened with hearing loss. US unemployment rates for those with *any* hearing impairment, based on data from 2001, is

36.6% [5]. In 2000, Mohr estimated a lifetime loss of up to US$440000 in earnings [6], equivalent to over US$600000 in 2015 dollars, based on an annual inflation rate of 2.25% since 2000. Thus, the impacts of hearing loss, both personally, and to the general society, are significant.

2. Elements of the cochlea other than hair cells at risk for noise-induced damage

Qualities of damaging noise include high intensity, or long duration exposures. Damage can take the form of metabolic insult (excessive potassium influx, generation of intracellular peroxides and free radicals, etc.), or physical damage (shearing forces can ultimately rupture the hair cell body itself, or other delicate structures of the cochlea). The inner ear is composed of numerous cell types (Fig. 1), and it is very possible that each population has its own susceptibility to, and propensity for avoiding cellular damage when impacted by loud sound. While amphibians, reptiles, fish, and birds all possess the ability to regenerate severely damaged/dead hair cells, the evolution of the mammalian inner ear has produced a system that is, in general, attuned to higher frequencies and also unable to replace damaged hair cells. A key attribute of the auditory pathway is the frequency-place map that is maintained from the peripheral end organ along the central nuclei making up the pathway. A loss of significant numbers of hair cells in any place within the cochlea therefore results in a permanent hearing loss involving the frequency normally encoded at the region of damage. While significant efforts are underway to effectively induce a regeneration of mammalian hair cells, numerous other structures are also at risk of damage by noise, any one of which when damaged can lead to significant hearing impairment.

2.1. Lateral wall and stria vascularis

Numerous structures/cell populations within the inner ear are at risk for damage following noise exposures. These include the sensory hair cells, which will be considered in depth below, and “supporting cells”. Supporting cells include cells within the lateral wall and stria vascularis of the cochlea, as well as cells surrounding the hair cells in what is termed the organ of Corti. The cells of the lateral wall and the stria vascularis together are responsible for setting and maintaining the endocochlear potential of the cochlea, which is the driving force for sensory transduction. Because these cells are presumed to be involved in ion recycling (Fig. 1), it is probable that all acoustic injury carries with it some degree of metabolic insult. Damage to this system can result in hair cell death [7] and profound hearing loss, but further discussion is beyond the scope of this commentary. In this commentary, we will focus on signaling systems involving the sensory hair cells and support cells immediately surrounding the hair cells as potential targets for drug therapies to combat noise-induced hearing loss. It should be recognized, however, that some of these signaling systems are also expressed by cells of the lateral wall and/or stria vascularis, and further research must be carried out to also help define roles of such signaling in these regions.

2.2. Stereocilia and Tip-links

The inner ear is a fluid-filled membranous labyrinth surrounded by bone. Within the membranous labyrinth are a series of either spot-like (maculae) or expanded collections of

sensory cells decorated with apical specializations, termed stereocilia. Due to the presence of these apical stereocilia, the sensory cells have historically been termed hair cells. Yet the stereocilia are neither hairs, nor stereocilia, but rather para-crystalline arrays of actin filaments surrounded by an extension of the apical plasma membrane [8, 9]. The stereocilia are not only morphologically impressive in their structure, but also critical for generating the receptor potentials induced by incoming vibrations conveyed through the fluid-filled spaces of the inner ear. Such vibrations are induced by either sound, encoded by the cochlea, or by movements of the head axially or changing orientation with respect to gravity, encoded by the semicircular canals or otolith organs (saccular and utricular maculae) of the vestibular portion of the inner ear respectively. Stereocilia are arranged in an increasing height, staircase-like organization, with each stereocilia connected to the next taller stereocilia via protein linkages termed tip links. Tip links are composed of cadherin 23 and protocadherin 15, and are most likely attached to mechanosensitive channels [10-13]. Receptor potentials are generated by the coordinated deflection of stereocilia (made possible by the tip links), which open still unidentified channels, although transmembrane-like channels are currently among the more favored candidates for the hair cell transduction channel [14, 15]. These channels are presumed to be associated with the tip links, and that tip links are critical for normal hearing [12, 16]. However, some evidence exists for hair cell mechanotransduction channel opening in the absence of tip links [17]. While there is good evidence that tip links damaged by drugs or noise can be regenerated in the mammalian inner ear [18], the stereocilia of the mammalian inner ear retain regenerative abilities only in the vestibular end organs and not the cochlea, although such regeneration can be induced [19]. The stereocilia undergo fast renewal via coordination between numerous proteins [20, 21], and mutations affecting stereocilia dynamics and integrity have been shown to cause deafness [22, 23]. Future drug targets should therefore include proteins involved in regenerating the stereocilia of cochlear hair cells. However, to date, this has not proven attractive as a target for restoration of hearing loss. Yet, one may argue that the most common noise-induced damage likely involves a loss of stereocilia following exposures to less traumatic sound intensities, and not primarily the loss of hair cells as a direct, primary consequence of noise exposure. Because of the ubiquitous nature of many proteins involved, directed application of drugs or gene therapy components will most likely have to be presented directly to the cochlea.

3. Vestibular involvement in noise-induced damage

As described above, the inner ear contains sensory end organs involved in vestibular function, as well as the process of hearing. Noise-induced damage is therefore associated not only with cochlear hair cell loss and subsequent hearing dysfunction. The membranous labyrinth is contiguous between the cochlear duct, housing the cochlear hair cells, and the vestibular portion of the inner ear, in which five hair cell structures (three cristae of the semi-circular canals, and one macula each of the utricle and saccule) encoding vestibular stimuli are located. Although vestibular dysfunction as a result of noise impact has been received much less attention than cochlear dysfunction following noise exposures, it has been long recognized that sound generates vibrations that invade the vestibule, especially the inferior portion containing the maculae of the saccule and utricle. Although understudied, convincing evidence exists demonstrating vestibular end organ damage as a result of sound

exposures [24-26]. The functional consequence of such damage is less clear, perhaps owing to active central compensatory mechanisms in patients, or simply because clinicians and researchers are not employing sufficiently sensitive diagnostic tests. While this topic will not be considered further, it should be recognized that the neural mechanisms of afferent nerve activation and modulation appear to be very similar (although perhaps not identical, e.g. [27]) to the processes used in the cochlea at the level of neurotransmitters and receptors, and most genes associated with cochlear hair cells are also expressed by vestibular hair cells. Yet, a description of various protective mechanisms associated with the cochlea discussed below have never been assessed as protective for the vestibular end organs. This represents a significant gap in our general knowledge of hair cell organs in general, and their survival under challenge.

4. Cochlear responses to sound exposures

Sound induces a variety of changes (molecular, structural, and physiological) in the cochlea. These changes serve as examples of the ear's functional capacity for adaptation in the face of different acoustic environments and sound qualities. From the point of view of hair cell function, sound can induce either permanent or temporary shifts in threshold, the historic indicator of the extent of damage. Some potentially protective responses induced by sound exposure are generated wholly by cells within the cochlea (intrinsic systems), while some are initiated by extra-cochlear sources (neural or endocrine) that feedback to the cochlea and change the state of various cell populations (extrinsic systems).

4.1. Permanent threshold shift (PTS)

PTS ensues following damage to the cochlea that is so severe that significant numbers of sensory and support cells die. Typically, PTS is defined as a permanent loss of hearing (identified by a non-recoverable shift threshold) that occurs in close temporal proximity to the acoustic challenge. Because the mammalian sensory cells do not regenerate, their loss is translated to permanent loss of sound-related neurotransmission that includes frequencies normally encoded by the cochlear region affected. PTS is often generated by a rupture of cell membranes and/or loss of integrity of cell adhesion molecules involved in separating the ionic compartments of the inner ear. PTS can be generated directly via exposures to extremely intense sounds (primary PTS), or secondarily by continued persistent oxidative stress (see below) that can occur even after the cessation of the stimulus, as one example. As will be discussed further below, permanent loss can also occur many years after an initial insult. Typical protective measures useful in guarding against occurrence of primary PTS include physical barriers, oftentimes including electronic noise cancellation systems, between the ear and the environment. Thus, protection against primary PTS includes engineering solutions more than biology-based solutions, and will not be considered further.

4.2. Temporary threshold shift (TTS)

TTS has historically been viewed as a relatively short-lived and fully reversible loss of hearing sensitivity that is not accompanied by structural damage. However, new data indicate that exposures producing TTS do generate permanent cochlear injury that results in supra-threshold response (functional) deficits, loss of pre- and postsynaptic (structural)

elements involved in afferent neurotransmission, and accelerated age-related hearing loss [28-32]. The long unrecognized deficits in supra-threshold responses following TTS are clinically important because the supra-threshold response range is critical for speech recognition and for filtering signal from noisy backgrounds, for example.

Inner hair cells are the sensory cell population of the cochlea responsible for sending approximately 95% of auditory information encoded in the cochlea to the brain via their connections with the spiral ganglion (auditory nerve associated) neurons. This is assessed by the number of the Type I ganglion cell fibers, which connect with the inner hair cell population, versus the Type II ganglion cell fiber count, which connects with the outer hair cells. The Type I fibers are further divided into two main populations of fibers: a low threshold, high spontaneous rate fiber, and a high threshold, low spontaneous rate fiber. Together, these fibers allow the enormous dynamic range (typically a range that includes a response to an intensity difference of 10^{12}) characteristic of a healthy cochlear response to sound. High spontaneous rate, low threshold fibers are especially important for setting hearing sensitivity, while low spontaneous rate, high threshold fibers are activated only at supra-threshold intensity levels. With TTS, it is the low spontaneous rate, high threshold fibers that are preferentially lost, explaining the permanent hearing deficits related to supra-threshold stimuli evident following TTS episodes while thresholds across frequencies are recovered or maintained. While there are also indications that the high spontaneous rate, low threshold fibers can also undergo synaptic pathology, this may take the form of glutamate receptor internalization, effectively disconnecting, in a functional sense, from the hair cell. Data suggest that some recovery of glutamate receptor surface expression occurs [32-34], but more work will need to be done to determine whether these represent functional synapses. The return to baseline thresholds, however, strongly suggests that this is the case. That low thresholds return following TTS conditions indicates such internalization may actually function as a protective mechanism, decreasing excitotoxic damage to the ganglion cell processes. However, it remains unclear precisely which signaling mechanisms limit TTS from evolving into permanent threshold loss, and that promote threshold recovery following TTS. As important is the question of why the high threshold, low spontaneous rate fibers die under the same conditions under which the low threshold, high spontaneous rate fibers recover. As described above, antioxidant pathways may have a role in this respect, given the greater damage and functional loss observed when antioxidant pathways are compromised. Additionally, the two main afferent fiber populations are known to have significantly different numbers of mitochondria, with the low threshold, high spontaneous rate fibers possessing more mitochondria than the high threshold, low spontaneous rate fibers [35]. Whether this ultimately equates to buffering capacity for oxidative stress or ionic challenges has not been determined.

4.3. Intrinsic signaling responses of the cochlea protective against noise-induced hearing loss

4.3.1. Metabolic damage and oxidative stress in the cochlea—The cochlea is constantly stimulated by sound, and even under silence, remains basally active. This represents a potential for metabolic damage, via oxidative stress, at the cellular level of the tissue/organ. Oxidative stress in the cochlea results in excess generation of both reactive

oxygen species (ROS) and reactive nitrogen species (RNS). Both have been shown to accumulate in the cochlea following exposures to loud sound [36], and to induce a caspase-mediated apoptotic cell death program [37-39]. Importantly, ROS is observed within seconds of acoustic overexposure, and can also persist for up to 10 days following exposure. This has been interpreted as a spreading involvement of cells along the cochlear duct in apoptosis. Typical of oxidative damage, inflammation and production of pro-inflammatory cytokines (interleukin 6 and tumor necrosis factor alpha) are involved in the cochlea's response to intense sound [40, 41]. Intense sound can also induce generation of vasoactive lipid peroxidation products such as isoprostanes [42] that then restrict blood flow, inducing an ischemia/reperfusion injury, further exacerbating the direct oxidative stress injury. Despite the spiraling cascade of deleterious effects generated by oxidative stress, the fact that injury to the cochlea seems to occur over many days also suggests that there is an opportunity to intervene in this process.

Experimental treatments designed to deplete glutathione in animals have been shown to potentiate the damage caused by noise [43], and mice carrying targeted gene deletions involving copper/zinc superoxide dismutase (Sod1) or glutathione peroxidase (Gpx1) demonstrate a higher susceptibility to noise-induced damage and hearing loss [44, 45]. Numerous therapeutic compounds have been analyzed for their ability to combat oxidative stress and either restore normal cochlear function, or prevent further damage after the initial insult. These include compounds that enhance antioxidant activity and decrease the formation or level of reactive oxygen metabolites in the cochlea. Compounds tested include enzyme modulators designed to increase the levels of anti-oxidant enzymes, iron chelators, vitamins, and sulfhydryl (thiol) compounds. One of the more promising thiol compounds, N-acetyl-l-cysteine (L-NAC) is known to cross the blood/labyrinth barrier when administered systemically, and may possess oto-protective properties stemming from its ability to replenish glutathione levels, although conflicting data as to its oto-protective capabilities have been reported [46, 47]. Other compounds having similar actions either directly on glutathione levels or on other elements of the systemic antioxidant pathways (such as catalase, superoxide dismutase, glutathione reductase, etc.) are under active investigation as potential oto-protective drugs (see [48]).

Beyond invoking endogenous antioxidant systems to protect against noise overexposure, the cochlea is also able to simply decrease its sensitivity via modulation of its mechanical properties, thereby decreasing metabolic stress. This would decrease the physiological activity of the cells and presumably limit accumulation of reactive oxygen species formation. Yet, this strategy carries with it the potentially life-threatening inability to detect subtle environmental cues. Thus, to combat this cellular stress, the cochlea must balance its sensitivity with protection against metabolic and structural insults that occur with activity, and especially that occurs under conditions of sudden over-stimulation. The signaling systems involved in such balance have been recently hinted at, and is described in greater detail below. A more complete understanding of how the cochlea manages to balance the need to maintain the best sensitivity possible with protection against cellular stress may provide clues for future drug targets for prophylactic or post-exposure therapies useful in combating NIHL.

4.3.2. Purinergic signaling via P2X2—It has previously been shown that the cochlea maintains a basal level of releasable ATP associated with a biochemically isolatable vesicular fraction. This seems to largely reside in the stria vascularis, is increased to more than double the basal concentration by loud sound, and is released into the endolymphatic space where it can stimulate various cells of the cochlea that express purinergic receptors, including hair cells [49]. Furthermore, noise has been shown to up-regulate the $P2X_2$ purinergic receptor at both the transcription and translation levels [50]. Application of ATP into the endolymphatic compartment has been shown to decrease the overall resistance of the cochlear partition, which is also correlated with a decrease in the endocochlear potential (the driving force for transduction) [51]. This occurs by shunting K^+ currents through the ATP-gated channels. The result of this is that ATP-gated channels in the cochlea, especially the $P2X_2$ receptor, provide a shunt pathway that modulates the electrical potential across the apical surface of the hair cells, effectively modulating hearing sensitivity. It has been suggested that ATP activation of the $P2X_2$ receptors mediate gap junction-based K^+ recycling in the cochlea, which is important for homeostasis, and possibly also for reducing hearing sensitivity as described above [52, 53]. Recently, it has been shown that genetic null ablation of the $P2X_2$ receptor gene produces a mouse with greater vulnerability to permanent hearing loss following acoustic challenges, and that the $P2X_2$ receptor is necessary for the development of a TTS response to intense sound [54]. Interestingly, the $P2X_2$ null mouse line exhibits an apparent preservation of hearing sensitivity when exposed to mildly intense sound (85dB), a stimulus that would normally produce some degree of TTS. This then argues that TTS may be a form of adaptation responsible for maintenance of threshold integrity, albeit at the expense of supra-threshold physiology as described above.

Mutation of the P2RX2 gene (encoding the $P2X_2$ receptor subunit) in humans underlies a dominantly inherited and progressive sensorineural hearing loss, DFNA41 [55]. Affected families possess a heterozygous allele of the P2RX2 gene in which a single nucleotide change (G to T) alters the amino acid code from a valine to a leucine. This produces a progressive hearing loss that begins between 12 and 20 years of age, and also leaves the individual with an increased vulnerability to noise-induced hearing loss. When introduced in animals, the human mutation abolishes ATP-stimulated permeability of the $P2X_2$ receptors. Targeting the purinergic signaling system of the cochlea for drug therapies against noise-induced hearing loss has not been explored, however.

4.4. Extrinsic cochlear mechanisms involved in protection against noise-induced hearing loss

4.4.1. Cochlear conditioning—Following a sub-traumatic stimulus, the cochlea possesses an ability to adapt, in which it achieves a degree of tolerance against future, normally traumatizing sound. This is termed conditioning, and was first demonstrated using sound as a conditioning stimulus [56, 57]. Animals were exposed to sub-traumatic noise intensities (81dB) for 24 days, and then exposed to normally traumatizing intensities (105dB) of sound. In non-conditioned animals, nearly all of the outer hair cells, the hair cells most prone to noise-induced damage, were missing. While hair cell death also occurred in the conditioned animals, the number of missing hair cells was approximately 50% that of the unconditioned group. Functionally, preconditioning produced a complete recovery of

hearing thresholds, while the non-conditioned group exhibited a 14-35dB upward shift in threshold, signifying permanent hearing loss. Sound pre-conditioning has been demonstrated numerous times since this first report, and has been shown to be induced by as little as a 15 minute conditioning exposure [57]. Experiments have further demonstrated that the protection does not involve middle ear muscles [58]. Interestingly, the conditioning stimulus can be effective when delivered before (preconditioning) or following traumatizing sound exposures [59, 60]. Cochlear conditioning can also be produced by stimuli other than sound. Inducing changes to body temperature has proven effective in protecting against noise-induced hearing loss [61, 62], suggesting a role for systemic stress response in the etiology of cochlear conditioning. The idea of imparting protection to tissue against normally traumatic events is not new; ischemic damage to the brain [63] and heart [64, 65] can be lessened with conditioning, for example. While mechanisms, especially for cochlear protection, are still under active investigation, evidence suggests that conditioning up-regulates antioxidant enzyme activity [66, 67] in the cochlea. As will be further discussed below, a body-wide response to noise that includes activation of the hypothalamic-pituitary-adrenal gland (HPA) axis has also been implicated in cochlear conditioning [68]. However, as described below, there are also experimental data suggestive of an intrinsic signaling system potentially capable of inducing cochlear conditioning. However, more work needs to be done to move such theory from simply being speculative, to data-driven tests of hypotheses.

The mechanisms by which conditioning is protective for the inner ear seem to be attractive targets for therapeutic intervention, especially given its retrograde protective abilities. The major concern is that if the classic HPA axis is indeed the major initiator of conditioning, significant systemic involvement may be required to produce the conditioning effect. This could prove an impossible barrier to using a drug-based initiation of conditioning for hearing protection, although behavioral training remains an interesting possibility. However, as will be discussed further below, there is new data on stress-related signaling in the cochlea that suggest a site-directed (middle ear) application of drugs designed to initiate an HPA-like response could prove useful in protection of hearing.

4.4.2. The Olivocochlear System—The olivocochlear (OC) system is a system of neurons that reside in the superior olivary complex of the brainstem that send axons to innervate the cells of the cochlea. Two main pools of OC neurons exist in most mammals: those termed the lateral OC system cells that innervate the inner hair cell region, synapsing largely with ganglion cell processes below the inner hair cell somas, and those termed the medial OC system cells that directly innervate the outer hair cells. The prevalent theory concerning mechanisms of cochlear protection is that olivocochlear (OC) system activation is protective against NIHL [69]. The exact mechanism by which protection is generated is complex, being a combination of both neural and mechanical consequences, although it is assumed that the modulation of basilar membrane mechanical properties, via OC driven modulation of outer hair cell electromotility, is presumed to be the major effector. This model has not changed substantially over the past several decades. While evidence exists that OC stimulation can provide protection against NIHL under specific conditions, other studies employing electrical or acoustic stimulation of the OC bundle often fail to reveal

protection against acoustic injury [70]. Additionally, not all potentially damaging sounds efficiently activate the OC system [71-73]. Discrepancies over the intensity levels normally encountered in the environment versus those required for OC-based protection in the lab (typically greater than 100dB) suggest that the OC system may not protect against most noise exposures outside of the laboratory setting [74]. Slow effects from the medial OC (MOC) system normally assumed to underlie protection [75] are maximal only at high frequencies [71], and require non-physiologically intense long duration OC stimulation for its generation [71]. Unilateral surgically de-efferented ears show no difference in threshold shifts compared to non-lesioned ears following *moderately* intense noise exposure, suggesting that the outcome is no different in the absence or presence of the "protective" MOC [75, 76]. Human data seem to also raise concerns over the protective role of the OC system. A recent clinical report found no significant correlation between measures of efferent suppression of distortion product otoacoustic emissions (DPOAEs, a readout of outer hair cell activity and indicative of OC system activation and effects on the outer hair cells), and protection against TTS [77]. Yet, other recent experiments designed to illustrate the protective nature of the OC system have reported protective effects against moderate intensity noise [78]. Significantly, de-efferentation resulted in a greater loss of hearing and afferent synapses associated with the inner hair cells. The experimental design, however, used a relatively non-physiologic stimulus paradigm (1 week continuous noise exposures), limiting interpretation of the results. Additionally, since the efferent innervation was completely lost via surgical transection of the OC bundle, it is unclear whether the results are a product of losing classical OC-mediated effects (via the nicotinic receptors) or whether the results are produced secondary to loss of some other component of efferent- based action beyond the typical mechanical modulation associated with classic medial OC function. These and other reports [74] spanning almost three decades of research underscore the debates and lingering concerns regarding the relative efficacy, and especially the primacy, of the protective nature of the OC system.

4.4.3. The Hypothalamic-Pituitary-Adrenal (HPA) Axis—A second model for cochlear protection against acoustic injury has more recently been advanced, and is based on evidence suggesting that stress activated HPA axis signaling [68, 79-82] initiates the cochlear conditioning described above. The key steroid hormone released by HPA activity is the glucocorticoid corticosterone in rodents (cortisol in humans), with a lesser amount of the mineralocorticoid aldosterone also being released. Glucocorticoid receptors are expressed by numerous cells of the cochlea, including hair cells, ganglion neurons, and cells within the lateral wall/stria vascularis [83, 84]. Exposure to moderately intense sounds generating a TTS (100dB, 45 minutes) has been shown to raise plasma corticosterone levels [85]. Conditioning to a moderate intensity noise (85B) for 15 minutes results in significant ACTH release detectable as elevated plasma ACTH levels, and protects against glucocorticoid receptor down-regulation typically induced by noise trauma. The effects of conditioning were blocked with adrenalectomy or pharmacological treatment blocking glucocorticoid receptor-mediated activity [68]. Given the wide-ranging effects of glucocorticoid-mediated effects in the body, the mechanisms involved are sure to be complex. But one possible mechanism is the regulation of the immune response in the inner ear following noise trauma. Inflammatory cells and associated pro-inflammatory cytokines have been demonstrated in

the cochlea after noise exposure [86, 87]. Glucocorticoids are intimately involved in suppressing the synthesis, release, and cellular effects of inflammatory cytokines.

Similar to the argument for an olivocochlear role in protection, a number of caveats of the systemic HPA axis as a *primary* protective system for the cochlea have arisen. These include: 1) activation time for systemic response (10-15 minutes); 2) sound intensities that do not rise to the threshold of systemic HPA activation, but can nonetheless be deleterious to the cochlea (e.g. recurring moderate intensity sounds); and 3) inability of the systemic response to protect *both* ears following unilateral conditioning, and to protect cochlear regions outside those immediately surrounding the stimulated basilar membrane regions. First, the HPA axis response to systemic stress is not optimally conducive to fully protect the cochlea. Specifically, exposure to moderate noise levels that still can produce TTS may not be sufficiently stressful to trigger activation of the HPA axis [88]. Additionally, following typical HPA axis activation, plasma ACTH levels reflective of HPA activation do not peak for ~2-30 minutes [89], and levels of plasma corticosterone, the effector molecule of HPA axis activity, peak 10-30 minutes after ACTH [90]. Thus, HPA axis mediated protective signals may be relevant only well after termination of potentially traumatizing sound stimuli, arguing against its *primary* role as an early response protective system. Second, published results indicate that HPA axis activation may provide only partial, and perhaps even minimal, protection against NIHL, at least following moderate-intensity sound exposures. While pre-conditioning is presumed to exert protective effects by activating the systemic HPA axis, in animals with prior unilateral reversible cochlear occlusion, protection against NIHL occurs only in the exposed ear [91]. A systemic response should provide wide spread protection. Additionally, pre-conditioning protection is lost when the pre-conditioning and subsequent traumatizing stimuli are separated by more than 3-octaves [92-94]. Collectively, these results seemingly argue against HPA axis activation as a primary source of protection against noise-induced hearing loss resulting from any but the most intense (systemic stress-inducing) sound exposures. However, these results begin to hint at local (cochlear) signaling that shares some of the elements of the systemic HPA axis as being important for protection against acoustic injury.

5. A Novel Theory Concerning Protective Mechanisms Against NIHL

5.1. The cochlear HPA-equivalent signaling system

A potentially new and widespread signaling system in the cochlea was hinted at in our work demonstrating the expression of urocortin, a member of the corticotropin releasing factor (CRF) family of peptides, in the lateral OC system, and both known CRF receptors (CRFR1 and CRFR2) in the majority of cells of the cochlea [95]. In our ongoing efforts to identify underlying cellular signaling involved in protection against functional and structural damage typically resulting in NIHL, we have recently described the expression of a number of proteins in the mouse cochlea that collectively represent a putative CRF-based, HPA-like signaling system. This system (Fig. 1) includes local expression of all the major stress-response signaling molecules (POMC, ACTH, MC2R (the ACTH receptor), CRFR1, and CRFR2) commonly associated with HPA axis-mediated systemic signaling [96, 97]. Most cells within the organ of Corti, the region directly around the hair cells, express CRF, the

primary endogenous ligand for the CRF receptors. Interestingly, most cells in this region also express one or both CRF receptors, but the hair cells do not express either CRF receptor, and ganglion cells do not express CRFR1, but do express CRFR2 [97, 98]. In the HPA axis, neurons of the hypothalamus that release CRF express both CRF and glucocorticoid receptors, and CRF and corticosterone feedback onto these cells represents an important inhibitory feedback that helps to terminate the stress response. While it is presumed that activation of hair cells releases CRF, lack of CRFR expression ensures that such release cannot directly participate in cessation of release. However, the hair cells express glucocorticoid receptors. It is therefore probable that cessation of CRF release from hair cells occurs by decreased auditory stimulus transduction, and inhibition of future release may occur via transcriptional regulation involving the hair cell glucocorticoid receptors.

5.1.1. Functional differences for steroid hormone signaling originating from the cochlea versus the HPA axis—Given this CRF-based, HPA-equivalent signaling system of the cochlea, which includes all of the signaling molecules normally associated with HPA axis-induced release of glucocorticoids and mineralocorticoids, there seems to be a strong possibility that the cochlea may be an extra-adrenal steroidogenic tissue capable of releasing its own glucocorticoids. Preliminary data from our lab (Vetter and Yee, 2014 ARO abstracts) suggests that acute cochlear explants cultured in defined, serum-free media release both corticosterone and aldosterone at a basal, constitutive rate detectable by ELISA, and in response to both CRF and ACTH bath application. Notably, CRFR1 nulls are not responsive to CRF application, and do not release glucocorticoids. Given that the cochlea is exposed to steroid hormones present in the circulation following activation of the HPA axis, two questions arise: to what extent is this local HPA-like system functionally redundant to the systemic stress response induced by HPA axis activity? To what extent is the underlying origin of steroid hormones relevant for cochlear protection? We hypothesize that the *source of* steroid hormone release *is key for cochlear protection.* This hypothesis is based on: 1) a considerable time lag (see above) between exposure to traumatizing sounds and activation and delivery of the systemic response signals that may prevent cochlear damage; 2) laterality of sound induced insults may obligate one ear to receive greater protection than the other; 3) differential protection may be required along the frequency place map of the cochlear duct; 4) a local signaling system permitting an almost immediate response to rapidly changing stimuli that may be “averaged out” if central processing is required. The current challenge is that each of these points must still be vigorously investigated. But such data will help establish to what degree a cochlear HPA-like system represents an independent cell stress response signaling system.

5.2. Loss of function data indicates roles for cochlear CRF initiated signaling as a protective system against noise-induced hearing loss

In an attempt to reveal the functional role(s) of the cochlear CRF-based signaling, we examined mice carrying null mutations for each of the CRF receptors, CRFR1 and CRFR2. Global ablation of CRFR1 results in significant elevation of auditory thresholds (Fig. 2), even when supplemental corticosterone is provided as replacement therapy for lost CRF-induced adrenal secretions. Supra-threshold responses of the CRFR1 null mice were also significantly depressed compared to wild type mice (Fig. 2), similar to the situation

following TTS as described above. Especially interesting are the findings that loss of CRFR1 activity impacts glutamate receptor expression and innervation to the inner hair cells. Lack of CRFR1 activity results in increased GluA4 expression levels, the consequences and interpretations of which are discussed more in relation with CRFR2 null mice, below. Loss of CRFR1 activity results in a significant loss of afferent fibers innervating the pillar side of the inner hair cells. This population of afferent fibers are the low threshold, high spontaneous rate fibers that are preferentially spared following sound exposures giving rise to TTS, as described above. Loss of these fibers probably explains the higher thresholds that were measured in the CRFR1 null mice. The documented fiber loss following CRFR1 gene ablation suggests that CRF-mediated signaling may be critical for their maintenance following TTS-inducing stimuli. Because the ganglion cells do not express CRFR1, such signaling must come either via CRFR2, or indirectly via CRF-mediated signaling involving neighboring support cells. Concerning the latter possibility, we have shown that CRFR1 is expressed in a supporting cell population termed border cells, which are intimately juxtaposed to the inner hair cell. The border cells express GLAST (glutamate transporter) and glutamine synthetase, the enzyme that converts glutamate to glutamine in the glutamate-glutamine cycle. Ablation of CRFR1 did not alter the expression level of GLAST, at least suggesting the continued ability for normal removal of glutamate from the synaptic pole of the inner hair cell. However, the expression level of glutamine synthetase was approximately half that of the wild type level. Because the role of the glutamate-glutamine cycle is to manage a recycling of amino acids involved in producing glutamate for afferent neurotransmission back into the inner hair cell while bypassing the potential for excitotoxicity, this data suggests a role for CRFR1 signaling in maintaining afferent synaptic tone. Without proper glutamate-glutamine recycling, the hair cell will become deficient in glutamate neurotransmitter. For a cell specialized in rapid release, which is critical for encoding auditory stimuli, this can quickly degrade auditory nerve responses and central processing. This data also indicates a strong possibility for cross-talk between the classic olivocochlear system, here specifically the lateral olivocochlear fibers, which express urocortin (the endogenous ligand for CRFR2), and the cochlear CRF signaling system by way of the CRFR2 expressed by Type 1 spiral ganglion cells and CRFR1, expressed by the border cells as described above (Fig. 3). Finally, constitutive loss of CRFR1 produced inner hair cells that were significantly smaller than those in wild type mice. This suggests a role for CRF signaling in the development and/or maintaining proper structure of the inner hair cell population, and could have consequences for hair cell survival. An understanding of noise-induced hearing loss has thus far typically focused on the survival of the outer hair cell population since this is the population more prone to noise-induced damage/death. Few indications have been forthcoming of molecules involved in maintaining the structural integrity of the inner hair cell population.

The results from a CRFR2 null mouse line indicate a diametrically opposed function from that observed following loss of CRFR1 expression. Ablation of CRFR2 results not only in significantly greater hearing sensitivity (indicated by extremely low ABR thresholds), but also a greater degree of hearing loss following exposure to loud (100dB), or even low/moderate intensity background (50dB) noise not usually injurious to wild type mice [97, 98] (Fig. 4). These data suggest that CRFR2 activity serves to limit susceptibility to noise-

induced hearing loss, and to balance hearing sensitivity with such susceptibility. Our data indicate that the altered functional output of the CRFR2 null cochlea occurs via complex, multifactorial mechanisms. These include: 1) abnormally high GluR expression in cochleae of CRFR2 null mice under basal, quiet conditions; 2) altered ion recycling; and 3) loss of normal Akt/PKB signaling.

GluA2/3 expression levels are down-regulated by noise in wild type mice, and are similarly down-regulated in the CRFR2 null mice. However, the initial expression level of GluA2/3 in the cochleae of CRFR2 null mice is approximately half that of wild type mice. This is accompanied by a non-significant upward trend of GluA4 expression in the null animals under quiet conditions. Expression levels of GluA4 increase slightly under noise conditions in the wild type mice, but increase approximately 40% above the wild type levels following noise exposure (almost doubling the quiet condition levels). These expression level states following loss of CRFR2 suggest that hyperacute hearing is generated by a decrease in GluA2, which is known to be Ca^{++} impermeable. Thus, it is possible that CRF signaling in the cochlea controls the threshold of hearing by modulating glutamate receptor subunit expression, and perhaps membrane trafficking/insertion. To explain how more sensitive hearing occurs despite a loss of GluA2/3, we envision a greater number of GluRs that are Ca^{++} permeable following loss of CRFR2 activity. The significant rise in GluA4 expression under noise conditions in the CRFR2 null mice may lead to a greater excitotoxic response compared to wild type mice, giving rise to a more significant hearing loss.

Data from the CRFR2 null mice indicate that ion recycling is also impacted by the loss of CRFR2 activity. The $P2X_2$ receptor, described above as a driver of the K^+ shunt pathway controlling hearing sensitivity and involved in susceptibility to noise-induced hearing loss, trends toward up-regulation in the CRFR2 null cochlea, suggesting that perhaps the null cochlea should be more resistant to hearing loss. However, connexin 26 and 30, which are critical for allowing ion recycling through the support cell population, are significantly down-regulated under resting conditions. While noise exposure does increase expression of both genes, the expression levels remain lower than the wild type state. This suggests that CRF signaling in the cochlea controls the gap junctions by which ions are passed between cells. It is through these junctions that hair cells are protected from an ionic imbalance by shuttling ions out of the local environment, and by which K^+ is ultimately returned to the endolymph.

Finally, CRFR2 signaling seems to be critically involved in controlling Akt1 (also known as protein kinase B, PKB). Akt is known to be involved in cell metabolism and survival, and activation of Akt inhibits the apoptosis pathway. Additionally, Akt is known to function as a protective signaling cascade against glutamate excitotoxicity in the hippocampus [99]. Akt expression levels are significantly lower under both quiet and noise conditions in the CRFR2 null mice. More importantly, the phosphorylation of two critical amino acids, T308 and S473, does not occur normally in the CRFR2 null mice. Phosphorylation of these residues is critical for activation of downstream Akt processes that include activation of p70S6K (which itself blocks an inhibitor, PDCD4, of protein synthesis while activating an enhancer, S6 ribosomal protein, of protein synthesis) and inhibition of apopotosis. Further, quantitative mass spectrometry experiments carried out on a cell line derived from inner ear tissue

demonstrated that activation of CRFR2 protects against a rise in reactive oxygen species generation induced by aminoglycoside treatment, and blocks the inhibition of superoxide dismutase that is typically observed following aminoglycoside treatment. Not surprisingly, then, caspase 3 activity, critically important in apoptosis, is also blocked by activation of CRFR2. A hierarchical clustering of the mass spectrometry data indicated that signal transduction, enriched for growth factors and adhesion proteins, and elements involved in protein degradation were up-regulated following CRFR2 activation. These data suggest that mechanisms of cellular support/survival following CRFR2 activity include inter-cellular signaling (growth factors) and an increase in a cell's ability to deal with unwanted, and perhaps malformed proteins that could otherwise exert metabolic load on a cell [100].

5.3. Synthesis of cochlear CRF-based signaling

Together, data concerning local CRF signaling within the cochlea, and its role in cochlear function strongly suggest a significant protective role against hearing loss is produced by local CRF-based signaling. Despite data highlighting shortcomings of previous cochlear protection models as described above, one should recognize that extant data also suggest that each of the current major models described above could explain cochlear protection under certain circumstances. Thus, such data may indicate an over-arching protective system with numerous signaling systems at its core. Each model may actually represent a protective mechanism that “comes online” under varying acoustic environments. We hypothesize that a continuum exists in the cochlea's response to sound that includes aspects of the major models previously proposed, plus local, intrinsic cochlear stress axis signaling. As signals increase in intensity, or more likely, in duration under natural conditions, new protective modes may become activated.

One may envision a spreading involvement of cells releasing CRF with increasing intensity of sound and consequent organ of Corti displacement following basilar membrane movements. Thus, the summed magnitude of CRF release offers functional feature selectivity in the model, and may encode the magnitude of local cellular stress correlated with sound exposures. Two possible mechanisms of homeostatic maintenance in the face of sound over-exposure follow from the expression pattern of the receptors. First, activation of the hair cells by shearing forces that occur during exposures to moderate sound intensities may activate CRF signaling to either induce a pre-conditioning protective effect, or when faced with moderately intense sounds, control the set-point for TTS, below which there is protection against significant structural and excitotoxicity damage. This could explain how the active cochlea is normally maintained against metabolic loads present during everyday activity. Above this, TTS may then be generated, partially as a mechanism protective of thresholds. Here again, CRF signaling may be involved in helping to maintain those afferent fibers that are involved in setting threshold, and this may also represent a mechanism by which TTS is limited from an obligatory evolution into PTS, which is defined as a loss of threshold sensitivity. Second, with increasing sound exposures, CRF signaling in lateral support cells (as one example) may modulate ion recycling/homeostasis, thereby maintaining proper ionic balance and protecting hair cells against damage or death. Since this “cochlear stress axis” is completely contained within the cochlea, no delays would be incurred between acoustic over-exposure and activation of protective mechanisms, in

contrast to the current models of cochlear protection based on extra-cochlear feedback-based mechanisms. With truly intense sounds, and/or sounds that have an especially long duration, classic olivocochlear activity, which is based largely on altering mechanical responses of the cochlea to incoming stimuli, and/or HPA axis signaling may then take over as the predominant protective system.

6. Future Directions

6.1. Clinical trials and approaches to pharmacological treatment of NIHL

Given the number of people potentially affected by NIHL, and the current lack of therapies for either prevention or mitigation of damage following noise exposures, there has been increasing interest in formulating new drug-based approaches to this problem. Currently, two clinical trials are examining the use of antioxidants for treatment of NIHL, and as such, hold the promise of not just being restorative, but perhaps even being useful as a prophylactic protective drug. Trial NCT01345474 (Campbell, SIU) is examining the use of D-methionine for alleviating NIHL. This trial is in Phase III, and is sponsored in part by the US Dept. of Defense. Another trial, performed by Sound Pharmaceuticals, has examined the use of ebselen, a molecule that induces the activity of glutathione peroxidase. Early evidence suggests some decreases in the incidence, severity, and duration of TTS. Another clinical trial, NTC02049073 (Lieu, Wash Univ. Sch. Med.), takes a different approach and is examining the combined use of Zonisamide (an anti-epileptic drug approved for partial seizures) with methylprednisolone (a glucocorticoid medication). This trial has not officially started patient recruitment as of the date of writing, but points to the strategy of using FDA-approved drugs toward off-target processes not initially proposed during original FDA approval. All of these drugs are potentially useful not only as a treatment following exposure, but may also find use as prophylactic treatments useful for at-risk populations.

As described above, there are numerous mechanisms that are involved in normal hearing, such as ion recycling by both the support cells on the basilar membrane and the cells of the spiral ligament and stria vascularis, buffering of oxidative stress/ROS, maintenance of healthy hair cells and the afferent and efferent synapses they are involved with (see [101] for review). It is possible that pharmacologic intervention targeting molecules involved in any of these processes may hold promise for alleviation of trauma resulting from exposures to loud sound. The main bottleneck in examining compounds for use in treating NIHL is that the molecules targeted are expressed in other compartments throughout the body, including the heart and the brain. Thus, oral medications for treating NIHL may always run into significant issues related to impacts on extra-cochlear tissue. This may limit prophylactic oral interventions to compounds that bolster anti-oxidant abilities unless ear drops can be devised to carry significant doses of compounds across the tympanic membrane, into the middle ear, and finally across the round window membrane, and still maintain concentration/potency significant enough to affect the targeted tissue/cells. It should be noted that for any pharmacologic intervention to succeed, the patient must present with limited pathological changes to the inner ear, and this means that treatment should start as soon as possible following insult.

6.2. Co-morbidities with hearing loss (potential increased susceptibility to NIHL) of interest to work on the cochlear CRF signaling system

There are numerous reports of co-morbidities associated with hearing loss. The difficulty for assessing these states is in unraveling causality. In the context of this report on novel cochlear CRF signaling, it is of interest to recognize that patients with psoriasis may be at risk for sudden sensorineural hearing loss (SSNHL) [102]. These patients are also at risk for cardiovascular disease, diabetes, and hypertension. CRF signaling occurs in skin, and is involved in local inflammatory responses [103], and has been associated with the intensity of psoriasis [104]. Given the systemic and local inflammatory state of psoriasis patients and the role of inflammation in these other diseases associated with psoriasis, it may be that in a similar manner, inflammation and associated local CRF/steroid hormone signaling within the inner ear could be contributors to SSNHL. Indeed, expression levels of Toll-like receptor genes (TLRs) are significantly elevated in patients with SSNHL [105]. Common treatment for SSNHL is intratympanic steroid injections. In light of our data demonstrating a full CRF-related signaling system in the inner ear, one may begin to ask whether other drugs targeting steroid hormone synthesis/release might also be good candidates to treatment.

Perhaps of unusually special interest are studies beginning to identify associations between hearing loss and dementia. Such associations are made after controlling for the variety of other aging phenomena. The risk for all-cause dementia begins to rapidly rise once hearing loss exceeds 40dB [106]. Additionally, accelerated cognitive decline, and decreases in both regional and whole brain volume is associated with hearing loss [107-109]. An unexplored, but common element between the novel aspects of hearing described in this review and the dementia/neuropathology of Alzheimer's is that CRF is known to accelerate both the anatomical and cognitive decline in Alzheimer's Disease [110]. Whether abnormalities in CRF, its release, or in the receptors could explain degenerative processes that take place in the inner ear are open questions that we are actively pursuing.

6.3. A place for cochlear CRF-based signaling in thinking about hearing loss

A model incorporating the various known cochlear protective systems predicts cross talk and synergistic activity between the systems. Exploring this hypothesis should be a major goal for future research into noise-induced hearing loss and protective systems. Major questions to resolve include a direct assessment of whether CRF is released from hair cells, and what effect olivocochlear activity, which typically inhibit outer hair cells via the hair cell nicotinic ACh receptor/SK2 channel, has on such release. One would hypothesize that olivocochlear activity would limit CRF release from outer hair cells. What would be the functional consequences of such an inhibition of release? If hair cell CRF release is the first step in release of glucocorticoids from other cells on the cochlea, does this indicate a need to limit steroid hormone release in the inner ear under certain circumstances, and if so, what does such limitation serve for cochlear function?

Probably the most important questions stemming from basic research on noise-induced hearing loss are concerned with pharmacological treatment of this disorder. While numerous targets have been suggested as potential drug targets for mitigating noise-induced hearing loss in the past, all of which have been indicated based on known signaling systems believed

to be important in cochlear protection (antioxidants, possible modulators of olivocochlear function), none have yet met the promise of delivering a robust therapy useful for combating hearing loss. As described above, a number of clinical trials are underway to address the paucity of available therapies. We believe that the recent discovery of a local CRF-based signaling system in the cochlea delivers a number of new therapeutic targets for exploration. While there have been reports of successful glucocorticoid therapies in restoring some hearing to patients with sudden sensorineural hearing loss (SSNL), success seems attributable to numerous different factors that vary between patients. Success rates are also less than optimal. Revelation of a potential glucocorticoid release mechanism local to the cochlea opens up numerous new drug targets that could not have been previously approached due to involvement of systemic consequences. Drugs that could enhance or block CRFR activation, POMC cleavage or ACTH release can now be considered because these drugs can be introduced locally via middle ear injections or even more directed round window applications. This route of administration should bypass all of the myriad concerns over systemic steroid therapies, which limit dosage and length of time under medication. Future therapies will likely take the form of treatment following unexpected, potentially traumatizing exposures, but perhaps more importantly should also be prophylactic in nature, such that high-risk groups can be protected. The prophylactic therapy strategy will demand an oral delivery mechanism, much as the current clinical trials examining anti-oxidants are now taking. High-risk groups will always have to be counseled in the proper use of physical protection systems as well, even if prophylactic drugs are used. This would protect against unexpected exposures to severe sound pressure levels, and can only help in combination with drug treatment. Difficulties in enforcing the use of physical protection devices, however, are well known, suggesting that education and societal norms/expectations must continue to evolve.

As described above, the rising occurrence of noise-induced hearing loss will require better therapies, and given the rather uninspiring success rate to date, expansion of potential targets for drugs seems the best way of moving forward in assessing future therapeutics with the best chance for successfully treating noise-induced hearing loss.

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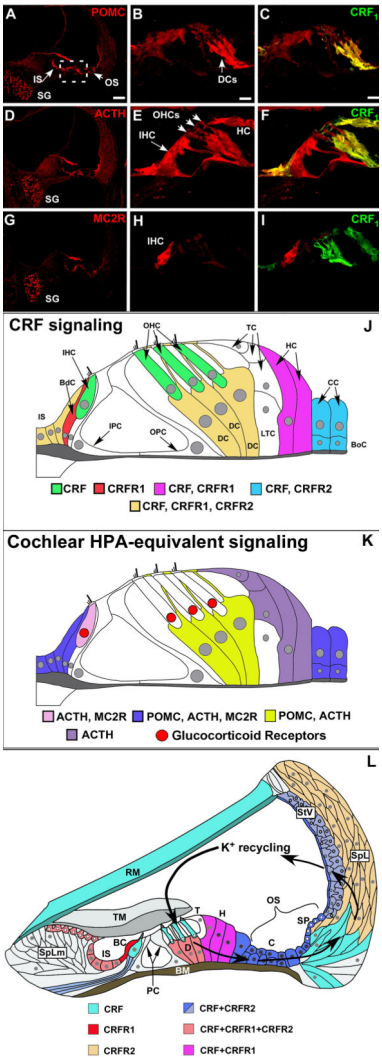

Figure 1. The cochlea expresses an HPA equivalent signaling system

Immunofluorescent labeling of POMC, ACTH, and MC2R reveals expression of classic HPA components in the cochlea. (A, D, G) POMC and ACTH are expressed in inner and outer sulcus cells lining the cochlear duct (IS and OS, respectively). MC2R is expressed in these regions to a lesser extent. All components are expressed in the spiral ganglion cells (SG). The organ of Corti region is boxed in A and is the region from which the higher magnification illustrations (J, K) were produced. (B) Higher magnification of the organ of Corti region reveals intense POMC labeling in the Deiter's Cells (DCs) and this expression overlaps with CRFR1-GFP label (C). (E, F) ACTH shows less immunolabeling in Deiter's cells compared to POMC, but an intense labeling of the inner hair cell (IHC). (H, I) Finally, in the organ of Corti region, MC2R shows an intense and specific labeling for the IHC and a lack of Deiter's cell labeling. (J, K) Expression of CRF, CRFR1, and HPA components in the cochlea is mapped for clarity. CRF signaling molecules are expressed in the cochlea as defined in the immunostaining panels, and are re-created here in schematic fashion. Cells of the inner sulcus (IS) express CRF, CRFR1, and CRFR2. CRF alone is expressed in the inner and outer hair cells (IHC, OHC respectively) of the cochlea, shown in green. These CRF-

positive sensory cells are juxtaposed by support cells such as the border cell (BdC, shown in red), which expresses CRFR1, and the Deiter's cells (DC, cells directly below the outer hair cells and shown in dark yellow) that express CRF, CRFR1, and CRFR2. In addition to the Deiter's cells, Tectal cells (TC) and Lateral Tunnel Cells (LTC) are directly apposed to the outer hair cells and Deiter's cells, but do not express any CRF signaling components. Support cells located more laterally include the Hensen's cells (HC), which flank the Tectal cells and Lateral Tunnel Cells laterally and express CRF, CRFR1, and the Claudius cells (CC) and Boettcher cells (BoC), which express CRF and CRFR2. Thus there is a potential for juxtacrine interaction between hair cells and support cells in their immediate vicinity. The inner sulcus cells medial to the border cell and support cells lateral to the organ of Corti express CRF, CRFR1, and CRFR2, suggesting autocrine and paracrine communications in these peripheral support cells that could also involve the hair cell populations. Finally, molecules of the classic HPA signaling system are expressed in the cochlea as defined in the immunostaining panels, and are schematically mapped for clarity. POMC, ACTH, and to a lesser extent, MC2R are expressed in inner sulcus cells, border cell near the IHC, and the lateral-most support cells which include Claudius cells and Boettcher cells (blue). Deiter's cells express POMC and ACTH (depicted in yellow), but with little to no expression of MC2R. ACTH and its receptor, MC2R, are expressed in the IHC (pink), suggesting a convergence of HPA signaling on the afferent auditory transducer. ACTH alone seems to be expressed in the Hensen's and Tectal cells (purple), with no discernable POMC expression found to date. Localization of previously described glucocorticoid receptors within the organ of Corti (based on Terakado et al., 2011) is indicated by red nuclei, and demonstrates spatial proximity between cells expressing ACTH and MC2R and cells expressing glucocorticoid receptors. (L) A radial cross section of the cochlear duct is shown with the major cells coded for CRF signaling molecule expression.

Scale bar in A represents 60μm and pertains to A, D, G. Scale bar in B represents 10μm and pertains to all other panels. HC = Hensen's cell. CRFR1 designates CRFR1-GFP immunolabeling. (permissions: photomicrographs reprinted from [97] with permission. Schematic figures J, K were re-drawn and annotated with permission from an original provided by Dr. M. Charles Liberman, Mass Eye and Ear Infirmary, Boston, MA, and schematic L adapted with permission from Jentsch, 2000).

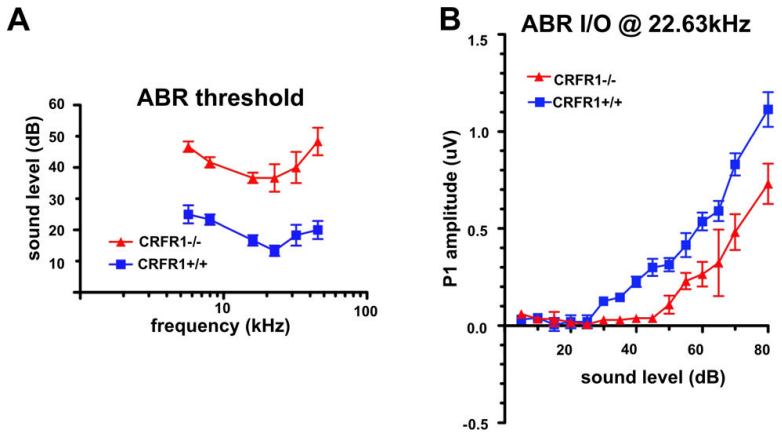

Figure 2. Elimination of CRFR1 causes auditory impairment

(A) Auditory brainstem response (ABR) thresholds were measured in wild type and CRFR1$^{-/-}$ mice. Symbols mark the average threshold observed at each frequency tested (5.66, 8, 16, 22.65, 32, 45.25 kHz) ± SEM. CRFR1$^{-/-}$ exhibited a 20-30 decibel (dB) increase in ABR thresholds. (B) The amplitude versus sound level relationship of the 22 kHz wave 1 obtained during ABR analysis was plotted. The loss of sensitivity of CRFR1$^{-/-}$ mice is reflected in the absent threshold up to 45dB stimulus intensity. Once the CRFR1$^{-/-}$ wave 1 response began to grow, however, the slope of the amplitude growth was identical, although the peak current was 35% less than that of the CRFR1$^{+/+}$ mice (2-way ANOVA, $F_{1,122} = 66.49$, $p<0.0001$).

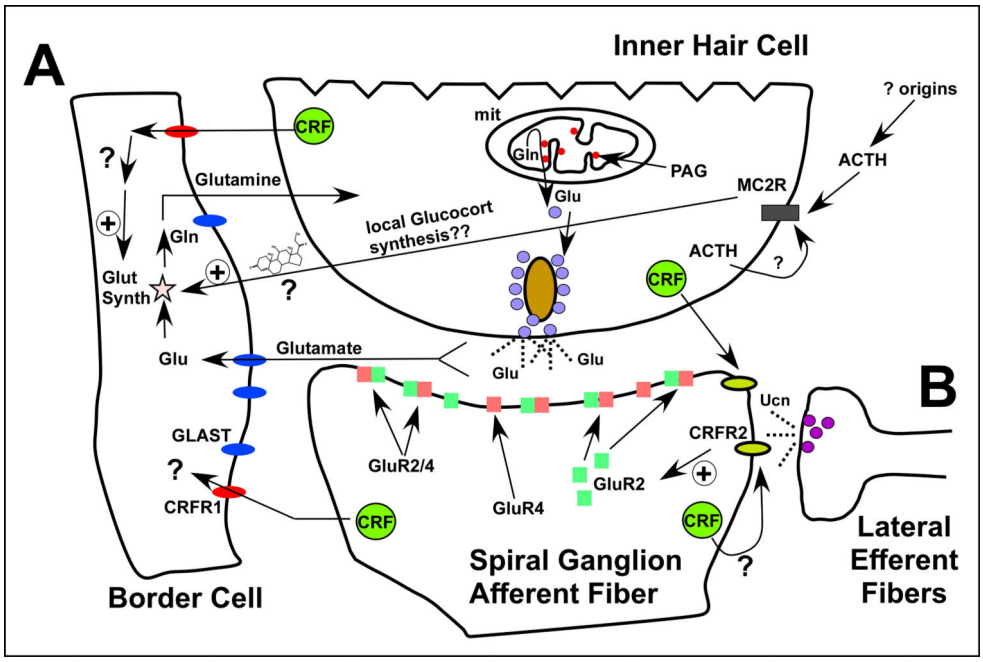

Figure 3. CRF signaling and HPA effects on the cochlear afferent synapse

A) CRFR1 expressed on the border cell adjacent to the inner hair cell regulates glutamate-glutamine cycling by promoting expression of Glutamine Synthetase (GS). In the absence of CRFR1, GS levels are significantly reduced, suggesting impaired glutamate-glutamine cycling and, by extension, impaired afferent function. The specific ACTH receptor melanocortin receptor 2 (MC2R) is expressed in the inner hair cell, suggesting an important convergence of HPA signaling here. Stimulation of MC2R at the IHC may lead to production and release of glucocorticoids, which could have an impact on glutamine synthetase expression and thereby impact the glutamate-glutamine cycle. B) Release of Ucn1 from the lateral efferent terminals and/or release of CRF from the inner hair cells could alter the molecular composition of the AMPA-class glutamate receptors as has been shown in various regions of the brain. CRF-related signal modification of the degree to which GluRs include GluR2 subunits may help determine the basal cochlear sensitivity, stimulus encoding, and susceptibility for excitotoxicity.

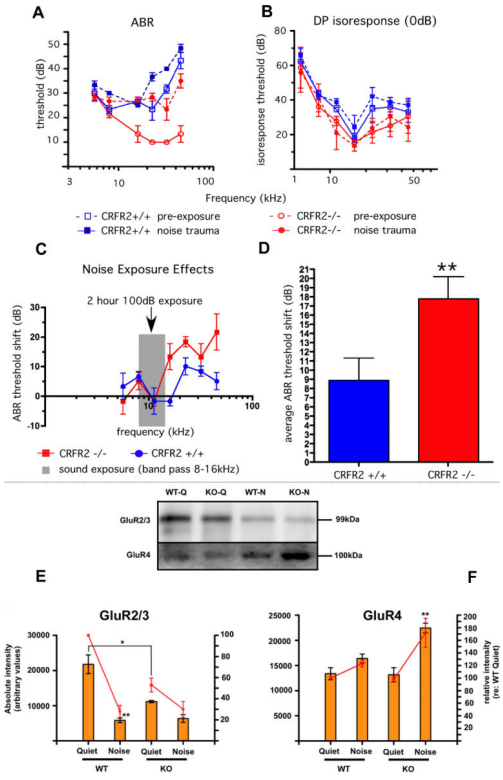

Figure 4. Analysis of auditory function of CRFR2$^{-/-}$ mice

CRFR2$^{-/-}$ and wild type mice were born and raised in an acoustic attenuation chamber in cages on standard wire rack shelving. (A, B) At approximately two months of age, baseline ABR and distortion product otoacoustic emissions (DPOAE) thresholds (a measure of outer hair cell functionality) were measured and plotted as a function of stimulus frequency (solid lines). Mice were then exposed within 24hrs to the 8-16kHz high intensity (100dB) sound for 2hrs. Two weeks post-exposure, mice were again tested for ABR and DPOAE thresholds, and post-trauma results (dashed lines) were plotted over baseline results obtained prior to exposure (solid lines). Note that ABR thresholds were altered following exposure, (A), while DPOAE thresholds were not (B). (C) ABR threshold shifts were calculated and plotted as a function of stimulus frequency. Gray region represents bandwidth of traumatizing sound. CRFR2$^{-/-}$ mice underwent significantly greater permanent ABR threshold shifts than wild type mice. (D) The average ABR threshold shift was computed, and a repeated measure ANOVA used to calculate statistical significance. On average, wild type mice underwent a 9dB threshold shift, while the CRFR2$^{-/-}$ mice underwent an 18dB threshold shift ($p<0.01$). (E) Glutamate receptor subunit expression levels are altered in CRFR2$^{-/-}$ mice. GluR2/3 and GluR4 western blotting was performed to quantify expression levels in wild type and knockout quiet reared (WT-Q, KO-Q respectively) and wild type and knockout noise exposure reared (WT-N, KO-N respectively) mice. Absolute intensity (left ordinate and bar graph) and relative intensity normalized to the wild type quiet expression level (right ordinate and line graph) were plotted for GluR2/3 expression. Under quiet conditions, CRFR2$^{-/-}$ mice expressed roughly half the level of wild type mice for GluR2/3 ($p=0.0168$). Following constant exposure to noise, wild type mice down-regulated GluR2/3 expression approximately 80% ($p=0.0044$). However, no statistical difference (2-way ANOVA with

Bonferroni's post-hoc test) was found in GluR2/3 expression between wild type mice under noise conditions and CRFR2$^{-/-}$ mice under quiet or noise conditions. (F) Expression levels of GluR4 were similarly probed and plotted. No significant difference was observed in wild type versus CRFR2$^{-/-}$ GluR4 levels under quiet conditions. GluR4 expression did not change significantly with sound exposure in wild type mice. However, in CRFR2$^{-/-}$ mice GluR4 levels increased significantly, reaching levels approximately 70% above quiet conditions ($p=0.0053$). Thus, GluR2/3 expression is down regulated in CRFR2$^{-/-}$ mice under both quiet and noise conditions, while GluR4 becomes over-expressed under noise.