The role of calcium, Akt and ERK signaling in cadmium-induced hair cell death

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ABSTRACT

Exposure to heavy metals, an environmental toxin, has been shown to cause damage to a variety of different tissues and cell types including hair cells, the sensory cells of our inner ears responsible for hearing and balance. Elevated levels of one such metal, cadmium, has been associated with hearing loss and shown to cause hair cell death in multiple experimental models. While the mechanisms of cadmium-induced cell death have been extensively studied in other cell types they remain relatively unknown in hair cells. We have found that calcium signaling, known to be an important regulator of cadmium toxicity in other cell types, does not appear to play a significant role in cadmium-induced hair cell death. While calmodulin inhibition can partially protect hair cells this may be due to impacts on mechanotransduction activity, and inhibiting CaMKII, the IP3 receptor and the mitochondrial calcium uniporter all failed to protect against cadmium-induced hair cell death. We also found cadmium treatment increased pAkt levels in hair cells and pERK levels in supporting cells. This activation appears to

be protective in nature as inhibiting these pathways enhanced cadmium-induced hair cell death rather than protecting cells. Thus cadmium-induced hair cell death appears distinct from cadmium-induced cell death in other cell types where calcium, Akt and ERK signaling pathways all promote cell death.

INTRODUCTION

Hair cells, the sensory cells we use for hearing and balance, are extremely sensitive to insult and as such disorders of these sensory systems are common. Recently environmental toxins such as heavy metals have gained attention as a potential cause of hearing loss (Choi and Kim, 2014; Schaal et al., 2017). One of these metals is cadmium. Cadmium can be found in some industrial run-off and individuals can be exposed to it in certain occupations, via smoking or via contaminated food (Faroon et al., 2012; IARC, 2012). Multiple studies have found a link between blood or urinary cadmium levels and hearing or balance impairment (Choi and Park, 2017; Choi et al., 2012; Dalton et al., 2020; Huh et al., 2018; Jung, 2019; Kang et al., 2018; Min et al., 2012; Shargorodsky et al., 2011; Wang et al., 2020). Studies have also found hearing loss and/or hair cell death in mammals and fish exposed to cadmium (Faucher et al., 2006; Kim et al., 2008; Liu et al., 2014; Low and Higgs, 2015; Montalbano et al., 2018; Ozcaglar et al., 2001; Schmid et al., 2020; Sonnack et al., 2015). However, the mechanisms by which cadmium kills hair cells remain largely unknown beyond reactive oxygen species generation playing a role (Kim et al., 2008).

In addition to its association with hearing loss cadmium has been linked to a number of other health conditions including cancer, osteoporosis and dysfunction of the cardiovascular system, kidneys, liver, nervous system and reproductive system. (Bhardwaj et al., 2021; Ferraro et al., 2010; Gallagher et al., 2008; García-Esquinas et al., 2014; Hyder et al., 2013; Mezynska and Brzóska, 2018; Ruczaj and Brzóska, 2022; Tellez-Plaza et al., 2012). While mechanisms of cadmium toxicity in hair cells are still relatively unknown the mechanisms of cadmium toxicity in some of these other cell types have been more extensively studied. Multiple cell types show an increase in calcium in response to cadmium (Shih et al., 2005; Smith et al., 1989; Xu et al., 2011; Yamagami et al., 1998; Yang et al., 2007; Yeh et al., 2009) and calcium chelation as well as inhibition of calcium/calmodulin-dependent protein kinase II (CaMKII) or calmodulin have been shown to protect against cadmium-induced toxicity (Chen et al., 2011; Liu and Templeton, 2007; Liu et al., 2018; Wang et al., 2008; Yang et al., 2007; Yang et al., 2008; Yeh et al., 2009). Cadmium has also been shown to increase phosphorylated levels of Akt (pAkt), and Extracellular Receptor Kinase (pERK) in a calcium signaling dependent manner (Chen et al., 2008; Chen et al., 2011; Jiang et al., 2015; Xiao et al., 2009; Xu et al., 2011). Inhibitors of the Akt and ERK signaling pathways have been shown to protect against cadmium-induced cell toxicity (Chen et al., 2008; Iryo et al., 2000; Jiang et al., 2014; Jiang et al., 2015; Kim et al., 2005a; Wang et al., 2014; Zhang et al., 2017; Zhao et al., 2015).

Calcium, Akt and ERK signaling pathways have all been previously implicated in hair cell damage from either aminoglycoside antibiotic toxicity or noise (Battaglia et al., 2003; Brand et al., 2015; Chung et al., 2006; Esterberg et al., 2013; Fridberger et al.,

1998; Kurioka et al., 2015; Uemaetomari et al., 2009; Wang et al., 2019). Therefore, we felt they were strong candidates to investigate as potential signaling pathways involved in cadmium-induced hair cell death. Activation of ERK has also previously been seen in the cochlea in response to Cd and inhibition of ERK can protect HE-OC1 cells, a hair cell like cell line, from cadmium-induced cell death (Kim et al., 2008). We investigated the role of these signaling pathways in hair cell death, using the zebrafish lateral line. The lateral line is a sensory system in fish used to detect water motion that consists of clusters of hair cells known as neuromasts on the surface of the animal. Hair cells of the zebrafish lateral line have been shown to be functionally, molecularly and morphologically similar to mammalian hair cells (Nicolson, 2005; Whitfield, 2002). We have previously shown that cadmium causes reliable hair cell death in the zebrafish lateral line in a dose dependent manner (Schmid et al., 2020). In this study we found that co-treatment but not posttreatment of a calmodulin inhibitor with cadmium could significantly protect against cadmium-induced hair cell death. However, other calcium signaling pathway inhibitors including a CaMKII inhibitor, inositol 1,4,5-tripphosphate (IP3) receptor inhibitor and mitochondria calcium uniporter inhibitor failed to protect against cadmium-induced hair cell death. We also saw an increase in levels of pAkt in hair cells and pERK in supporting cells in response to cadmium, however, inhibition of these pathways enhanced hair rather than protected against cadmium-induced hair cell death. Therefore, we conclude that the signaling pathways responsible for cadmium-induced hair cell death appear distinct from those commonly seen in cadmium-induced toxicity in other cell types and partially distinct from those seen in other forms of hair cell toxicity.

METHODS

Animals

All experiments were carried out in 5 days post-fertilization (dpf) *AB strain wild-type zebrafish larvae. Fish larvae were housed in an incubator maintained at 28.5°C with a 14/10 hour light/dark cycle and raised in embryo media (EM) consisting of 1 mM $MgSO_4$, 150 μM KH_2PO_4, 42 μM Na_2HPO_4, 1 mM $CaCl_2$, 500 μM KCl, 15 mM NaCl, and 714 μM $NaHCO_3$. The Lafayette College Institution Animal Care and Use Committee approved all experiments.

Drug Treatments

The following drugs were used: cadmium chloride hemipentahydrate (Fisher Scientific), water soluble KN-93 (Abcam), MK-2206 (Millipore Sigma), PD184352 (Millipore Sigma), RU360 (Millipore Sigma), W-13 (Enzo) and Xestospongin (Abcam). Stock solutions of all drugs were made at 1000x their working dilution with the exception of cadmium chloride where a 5mM stock was made and PD184352 where a 10mM stock was made. Stock solutions for cadmium chloride, KN-93, and Ru360 were made in water. Stock solutions of all other drugs were made in dimethyl sulfoxide (DMSO, Millipore Sigma). All drugs were then diluted to their treatment concentrations in EM. For drugs diluted in DMSO the control groups contained DMSO at the same volume as the drug. For the water diluted drugs plain EM was used for the control group.

For the drug treatments fish were initially put into netwell inserts (Corning) in 6 well plates containing EM. The netwell inserts with the larvae were then moved to new plates containing the solutions the fish were to be treated in. At the end of treatment fish

were washed three times in plain EM, again by moving the netwell inserts to new plates, before fixation.

Immunostaining

Fish were anesthetized with MS-222 and then fixed for 2 hours at room temperature in 4% paraformaldehyde. Antibody labeling was carried out as previously described (Stawicki et al., 2014). The following primary antibodies were used: rabbit anti-parvalbumin (ThermoFisher, PA1-933) diluted at 1:1,000, mouse anti-otoferlin (Developmental Studies Hybridoma Bank, HCS-1) diluted at 1:100, rabbit anti-phopsho-Akt (Ser473) (Cell Signaling, 9271) diluted at 1:100, and rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling, 4370) diluted at 1:200.

Hair Cell Counts

To perform hair cell counts fish were observed on an Accu-Scope EXC-350 microscope using the 40X objective. The number of hair cells in the following neuromasts OP1, M2, IO4, O2, MI2 and MI1 (Raible and Kruse, 2000) were counted for each larvae using either the parvalbumin or otoferlin stain. The total number of hair cells counted for that fish were then divided by 6 to get an average number of hair cells per neuromast. To normalize data for the cadmium and ERK inhibitor experiment the average hair cells/neuromast number for each DMSO or PD184352 treatment group was divided by the average hair cells/neuromast number for their respective control groups with no cadmium and then multiplied by 100 to be converted to a percentage. All statistics were calculated in GraphPad Prism 6.

Signaling Molecule Levels

To calculate pAkt and pERK levels fish stained with either the pAkt or pERK antibodies and the otoferlin antibody were imaged on a Zeiss LSM800 confocal microscope using the Zen Blue software. For pAkt the approximate center of the neuromast was selected and 10 stacks separated by 1 μm was imaged for each neuromast. For pERK there was significant neuronal labeling in the control condition that we did not want to include in our analysis. Therefore, the top and bottom of the neuromast was selected and stacks separated by 1μm were imaged throughout the neuromast regardless of number. Then 10 stacks with minimal neuronal labeling were selected for subsequent analysis. One neuromast of the anterior lateral line was imaged per fish. Image analysis was conducted in Fiji. Maximum projection images were made for each stack. The neuromast was outlined using the otoferlin label and the average fluorescent intensity of the pAkt or pERK signal was measured. All statistics were calculated in GraphPad Prism 6.

RESULTS

Cotreatment but not pretreatment with a calmodulin inhibitor protects against cadmium-induced hair cell death

Previous research has shown that calcium plays an important role in cadmium-induced cell death in many cell types. Calmodulin is a major intracellular receptor for calcium and calmodulin inhibition has been shown to protect against cadmium-induced cell death (Niewenhuis and Prozialeck, 1987; Xu et al., 2011; Yang and Yang, 1997).

Calmodulin inhibition has also previously been shown to protect against aminoglycoside-induced hair cell death (Esterberg et al., 2013). To see if calmodulin inhibition could protect against cadmium-induced hair cell death we first cotreated fish with 30 μM of the calmodulin inhibitor W-13 hydrochloride and varying doses of calmodulin chloride ranging from 0-120 μM for three hours. We found that calmodulin inhibition significantly reduced cadmium-induced hair cell death at lower doses of cadmium chloride, 15 and 30 μM, but not higher doses, 60 and 120 μM (Figure 1A).

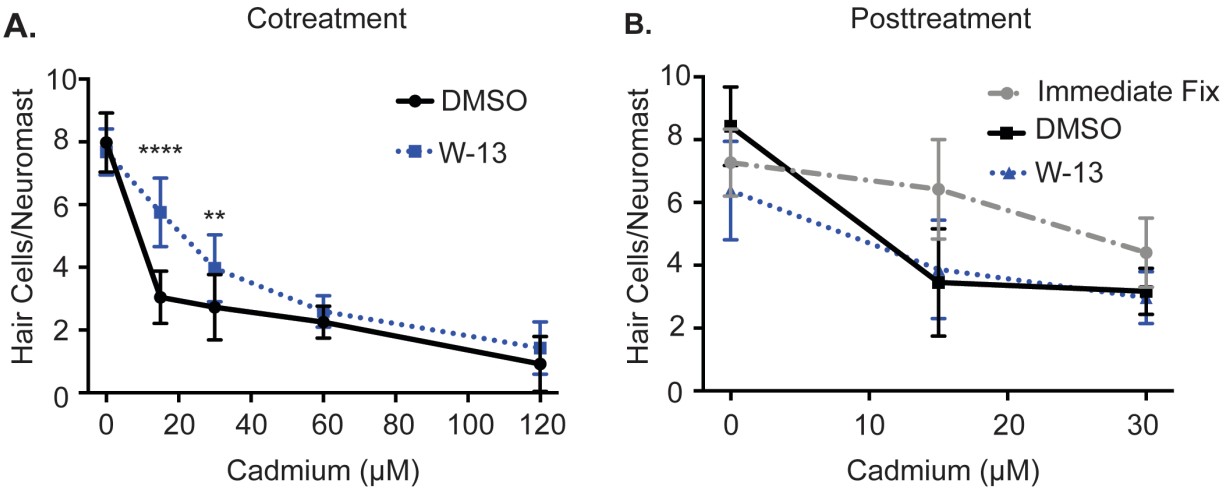

Figure 1: Calmodulin inhibitor cotreatment but not pretreatment protects against cadmium-induced hair cell death. (A) Hair cells remaining after a 3 hour cotreatment of either DMSO, a vehicle control, (solid line), or 30 μM of the calmodulin inhibitor W-13 (dotted line) and varying doses of cadmium chloride. W-13 cotreatment caused a significant reduction in hair cell death seen at lower cadmium doses (** = $p<0.01$, **** = $p<0.0001$ by Two-Way ANOVA and Šídák's post-hoc test). (B) Hair cells remaining after a 1 hour treatment with varying doses of cadmium chloride and either immediate fixation (dashed

line), or a two hour treatment with DMSO (solid line) or W-13 (dotted line). No significant protection was seen with W-13 treatment. n = 10 fish for each group. Error bars = Standard deviation.

Calmodulin inhibition has also previously been shown to inhibit rapid apical endocytosis in hair cells, a process dependent on mechanotransduction activity (Seiler and Nicolson, 1999). Inhibiting mechanotransduction can protect against cadmium-induced hair cell death potentially by impairing cadmium influx into hair cells (Schmid et al., 2020). We therefore wanted to distinguish between whether calmodulin inhibition was affecting mechanotransduction and cadmium uptake or the actual cell death process once cadmium was in the cell. To do this we first confirmed that there was continued hair cell death after the removal of calmodulin by treating fish with 30 μM of cadmium chloride for either 1 or 2 hours and then fixing either immediately or three hours after the start of the experiment. We found that following both the 1 and 2 hour cadmium chloride treatment continued hair cell death was seen after cadmium removal (Figure 2). We then tested whether inhibiting calmodulin only after cadmium removal would still significantly protect against cadmium-induced hair cell death. To do this fish were treated with varying ranges of cadmium chloride for one hour and then immediately fixed or washed out of cadmium-chloride and placed into DMSO or 30 μM W-13 for two hours before fixation. We again saw continued hair cell death after cadmium-chloride removal, however, we no longer saw any protection from calmodulin inhibition (Figure 1B). This suggests the protection seen with cotreatment with the calmodulin inhibitor may be due to impacts on mechanotransduction-dependent processes, though we can also not rule out a distinct early role for calmodulin in the cell death process.

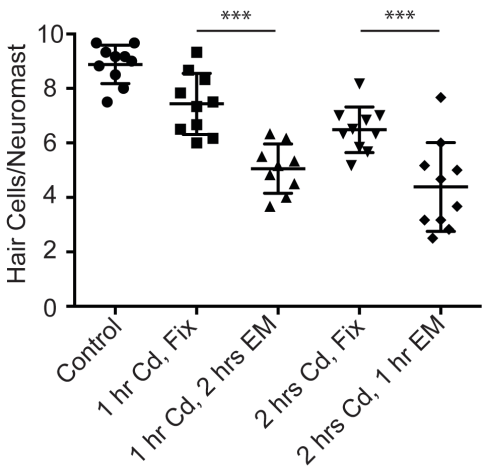

Figure 2: Cadmium causes continued cell death after removal. Hair cells remaining after various cadmium treatment paradigms. Fish were either treated with cadmium chloride for 1 hour and immediately fixed (squares), treated with cadmium for 1 hour then left in EM for two hours before fixation (triangles), treated with cadmium for 2 hours and then immediately fixed (upside down triangles), or treated with cadmium for 2 hours and then left in EM for 1 hour before fixation (diamonds). Control fish were kept in EM for three hours (circles). There was a significant amount of cell death after cadmium removal for both the 1 hour and 2 hours cadmium treatment paradigms (*** = $p<0.001$ by ANOVA and Tukey post-hoc test).

Inhibition of other calcium signaling molecules fails to protect against cadmium-induced hair cell death

We next wanted to test whether inhibiting other calcium signaling molecules would impact cadmium-induced hair cell death. Calmodulin is known to activate CaMKII and inhibiting CaMKII has been shown to protect against cadmium-induced cell death in multiple cell types (Chen et al., 2011; Liu and Templeton, 2007; Liu et al., 2018). However, this is not always the case as other researchers have found CaMKII inhibition had either no impact on cadmium-induced toxicity (Ha et al., 2016) or caused an

increase in cadmium-induced cell death (Xiao et al., 2009). To test if CaMKII inhibition would impact cadmium-induced hair cell death we cotreated fish with 10 μM of the CaMKII inhibitor KN-93 and varying doses of cadmium chloride for three hours. We found CaMKII inhibition did not significantly impact cadmium-induced hair cell death (Figure 3A).

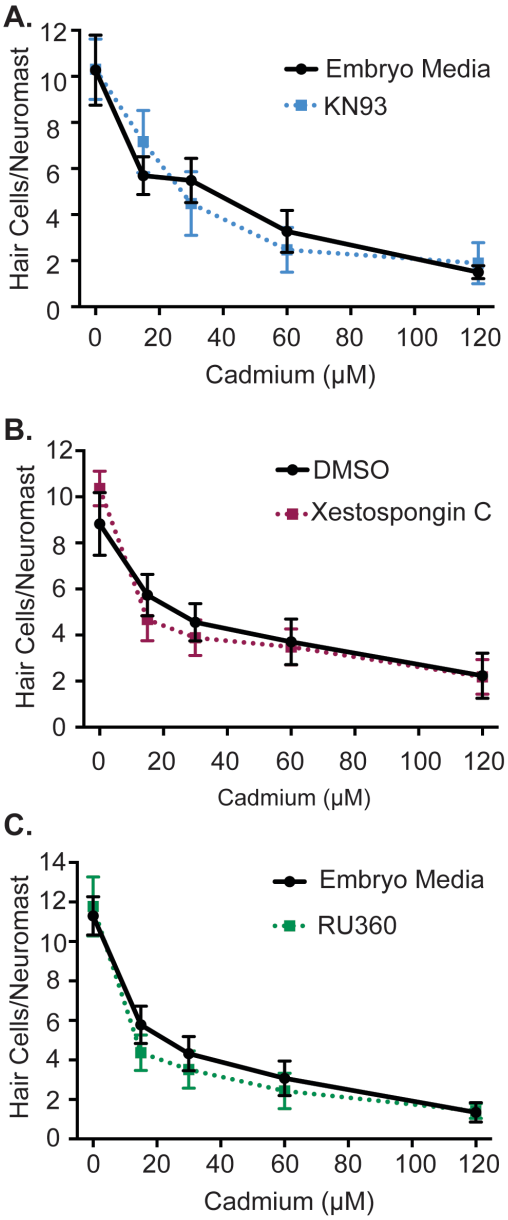

Figure 3: Inhibiting calcium signaling molecules fails to protect against cadmium-induced hair cell death. Hair cells remaining after a three hour cotreatment of either plain EM or DMSO (solid lines), or (A) 10 μM of the CaMKII inhibitor KN-93, (B) 1 μM of the IP3 receptor inhibitor Xestospongin C, or (C) 500 nM of the mitochondrial uniporter inhibitor RU360 (dotted lines) and varying doses of cadmium chloride. None of the inhibitors significantly protected against cadmium-induced hair cell death. n = 10 fish/group. Error bars = standard deviation.

In other cell types there has also been evidence that calcium from the endoplasmic reticulum (ER) and mitochondria play a role in cadmium-induced toxicity and that blocking either the IP3 receptor or the mitochondria calcium uniporter can protect cells against cadmium-induced toxicity (Lee et al., 2005; Liu et al., 2016; Liu et al., 2018; Shih et al., 2005; Wang et al., 2008; Wang et al., 2009; Wang et al., 2022). However, as with CaMKII this is not always seen with one group failing to see an impact to cadmium toxicity when blocking the mitochondrial uniporter in neurons (Tang et al., 2021). ER and mitochondrial calcium have also been implicated in other forms of hair cell toxicity with IP3 receptor antagonists protecting against aminoglycoside-induced hair cell death and mitochondria uniporter antagonists protecting against aminoglycoside and noise-induced hair cell death (Esterberg et al., 2014; Wang et al., 2019). To see if ER or mitochondrial calcium were playing a role in cadmium-induced hair cell death we cotreated fish for three hours with either 1 μM of the IP3 receptor inhibitor Xestospongin C or 500 nM of the mitochondrial uniporter inhibitor RU360 and varying doses of cadmium chloride for three hours. In both cases the inhibitor failed to significantly protect against cadmium-induced hair cell death (Figure 3B & C).

Cadmium increases Akt and ERK signaling in neuromasts

In many cell types cadmium increases the level of phosphorylated Akt and ERK (Chen et al., 2008; Chen et al., 2011; Iryo et al., 2000; Kim et al., 2005b; Liu and Templeton, 2007; Misra et al., 2003; Xiao et al., 2009). This includes in the cochlea and HE-OC1 cells where researchers have previously seen an increase in pERK levels in response to cadmium (Kim et al., 2008). Activation of the Akt and ERK signaling pathways by cadmium appears to be a downstream consequence of calcium signaling as chelating calcium, or inhibiting calmodulin or CaMKII has been shown to block the activation of these pathways (Chen et al., 2008; Chen et al., 2011; Fujiki et al., 2014; Xiao et al., 2009; Xu et al., 2011). Activation of these pathways has also been seen in hair cells or supporting cells in response to other forms of hair cell damage. Aminoglycoside treatment has been shown to increases pAkt levels in organ of Corti explants (Chung et al., 2006), and increases of pERK in supporting cells of the organ of Corti has been seen in response to aminoglycoside, cisplatin and noise damage (Herranen et al., 2018; Ingersoll et al., 2020; Lahne and Gale, 2008; Maeda et al., 2013).

We wished to determine if these pathways would be activated in either hair cells or supporting cells of the zebrafish lateral line in response to cadmium. To do this we treated fish with 30 μM of cadmium chloride for one hour and then fixed and stained with an antibody for either pAkt or pERK. We found that treatment with cadmium significantly increased the levels of pAkt seen in hair cells (Figure 4A & B). This increase was lost when fish were cotreated with cadmium and 5 μM of the Akt inhibitor MK-2206. We also saw a significant increase in pERK levels following cadmium treatment that was lost when fish were cotreated with cadmium and 3 μM of the MEK

inhibitor PD184352 which suppresses ERK phosphorylation (Figure 4C & D). However, unlike pAkt labeling which appeared localized to hair cells the pERK labeling appears localized to supporting cells (Figure 4). This is in agreement with past research in mammals showing increased pERK in support cells but not hair cells in response to other forms of hair cell damage (Herranen et al., 2018; Ingersoll et al., 2020; Lahne and Gale, 2008; Maeda et al., 2013).

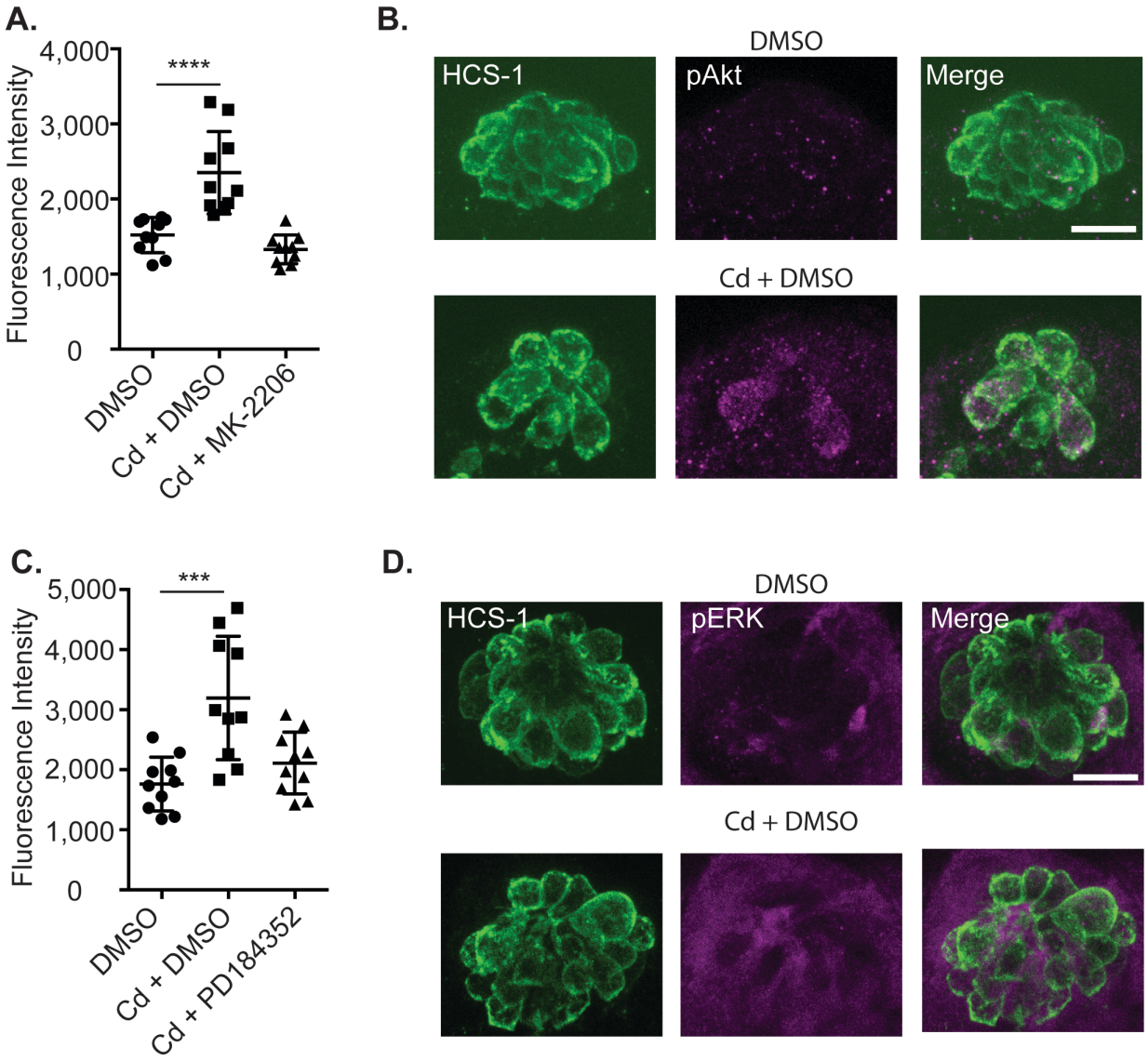

Figure 4: Cadmium treatment increases pAkt levels in hair cells and pERK levels in supporting cells. (A) Quantification of the fluorescence intensity of pAkt in fish treated for 1 hour with either DMSO alone, cadmium chloride (Cd) + DMSO, or Cd and 5 μM of MK-2206. (**** = $p<0.0001$ by ANOVA and Tukey post-hoc test). (B) Images of a neuromast from the DMSO alone group (top) and the Cd + DMSO group (bottom). Hair cells are labeled with the otoferlin (HCS-1) antibody in green (left) and pAkt in magenta (center). (C) Quantification of the fluorescence intensity of pERK in fish treated for 1 hour with either DMSO alone, Cd + DMSO, or Cd and 3 μM of PD184352. (*** = $p<0.001$ by ANOVA and Tukey post-hoc test). (D) Images of a neuromast from the DMSO alone group (top) and the Cd + DMSO group (bottom). Hair cells are labeled with the HCS-1 antibody in green (left) and pERK in magenta (center). Scale bar = 10 μm.

Akt and ERK signaling pathways protect hair cells against cadmium-induced hair cell death

Having seen an increase in pAkt and pERK levels following cadmium treatment we next wanted to determine whether the Akt or ERK signaling pathways impacted cadmium-induced hair cell death. Akt and/or ERK inhibitors have been shown to protect against cadmium-induced toxicity in multiple cell types (Chen et al., 2008; Iryo et al., 2000; Jiang et al., 2015; Kim et al., 2005b; Wang et al., 2014; Zhang et al., 2017) including the hair cell like HE-OC1 cell (Kim et al., 2008) suggesting these pathways normally function to promote cadmium-induced cell death. However, in other cell types inhibiting these pathways has been shown to have no effect on cadmium-induced toxicity (Låg et al., 2005; Liu and Templeton, 2007; Rigon et al., 2008; Wang et al., 2008) or to increase toxicity (Chao and Yang, 2001; Chuang et al., 2000; Xiao et al., 2009). As mentioned above increases in pAkt and pERK are also seen in response to other forms of hair cell injury (Chung et al., 2006; Herranen et al., 2018; Ingersoll et al.,

2020; Lahne and Gale, 2008; Maeda et al., 2013). In hair cells blocking Akt or ERK signaling most commonly increases hair cell death suggesting these pathways are activated to protect hair cells (Battaglia et al., 2003; Brand et al., 2015; Chung et al., 2006; Haake et al., 2009; Hayashi et al., 2013; Kurioka et al., 2015). Though there are some examples where blocking ERK can protect against damage to hair cells or HE-OC-1 cells (Lahne and Gale, 2008; So et al., 2007).

To determine if the Akt or ERK pathways were impacting cadmium-induced hair cell death, we cotreated fish with either 5 μM of the Akt inhibitor MK-2206, or 1.5 μM of the MEK inhibitor PD184352, which inhibits ERK activation, and varying doses of cadmium chloride for three hours. A smaller dose of PD184352 was used in this experiment than the pERK levels experiment due to increased hair cell toxicity from PD184352 alone with the longer treatment time. We found both drugs caused a decrease in hair cell number following cadmium treatment particularly at the 30 and 60 μM cadmium chloride doses (Figure 5A & B). However, as noted above PD184352 caused a loss of hair cells independent of cadmium treatment. To determine if PD184352 was enhancing cadmium-induced hair cell death or simply killing hair cells on its own, we normalized the data for both the DMSO and PD184352 groups to the zero cadmium control (Figure 5C). In doing so we still saw a significant increase in cadmium-induced hair cell death at the 30 and 60 μM cadmium chloride doses. Thus, we conclude that similar to what is seen in other forms of hair cell damage the Akt and ERK pathways appear to be functioning to protect hair cells.

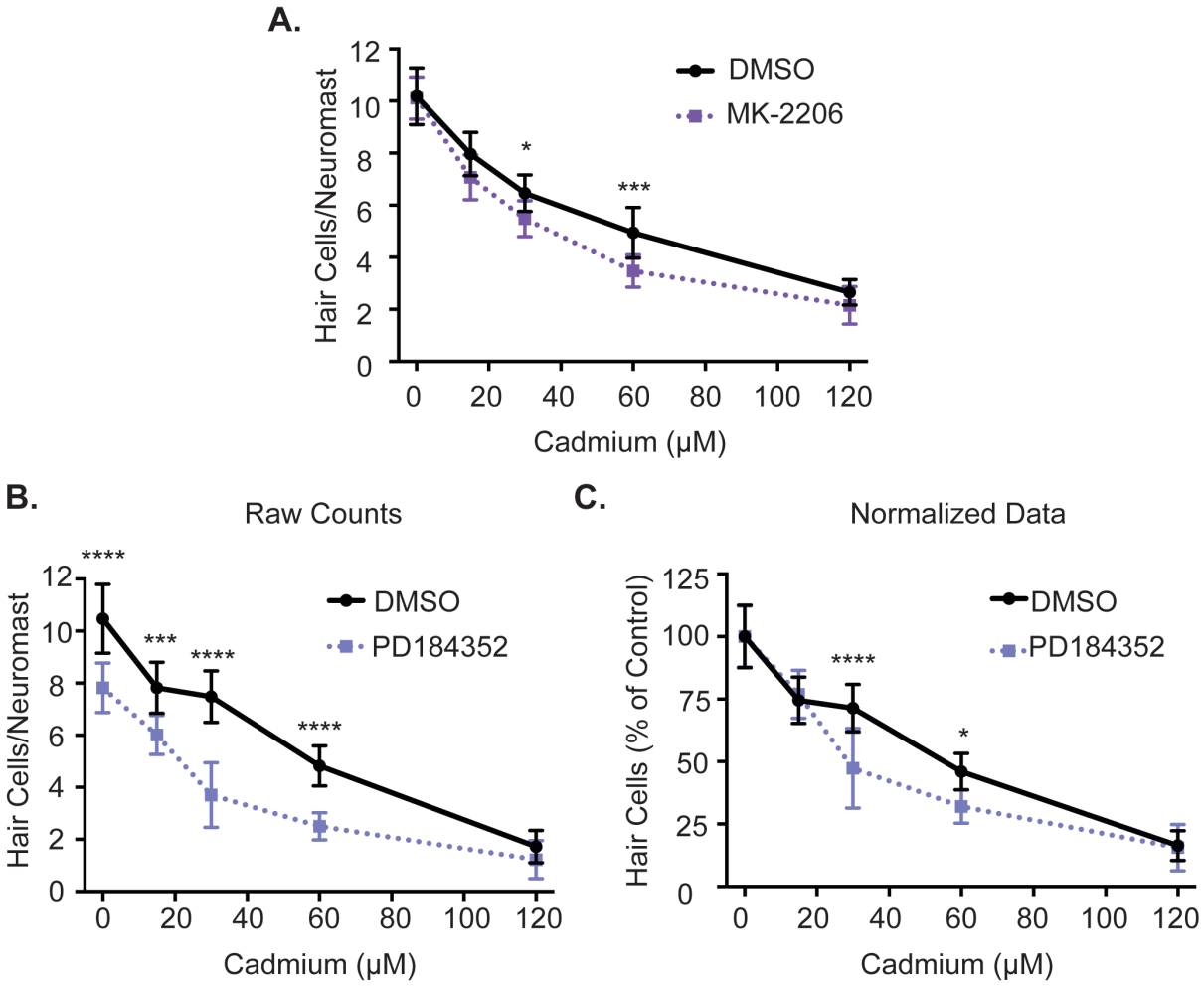

Figure 6: Akt and ERK signaling work to protect hair cells from cadmium-induced cell death. (A) Hair cells remaining after a 3 hour cotreatment of either DMSO (solid line), or 5 μM of the Akt inhibitor MK-2206 (dotted line) and varying doses of cadmium chloride. MK-2206 cotreatment caused a significant increase in cadmium-induced hair cell death. (B) Hair cells remaining after a 3 hour cotreatment of either DMSO, a vehicle control, (solid line), or 1.5 μM of the ERK pathway inhibitor PD184352 (dotted line) and varying doses of cadmium chloride. PD184352 caused a significant decrease in the number of hair cells seen. (C) The same data as in B, but normalized to the zero cadmium control for each group. . PD184352 caused a significant increase in the amount of cadmium-induced hair cell death seen. (* = $p<0.05$, *** = $p<0.001$, **** = $p<0.0001$, by Two-Way ANOVA and Šídák's post-hoc test). n = 10 fish for each group. Error bars = Standard deviation.

DISCUSSION

We have shown that cadmium-induced hair cell death largely does not depend on the activation of calcium, Akt and ERK signaling pathways the way other forms of cadmium-induced cell death does. The lack of dependence on calcium signaling in cadmium-induced hair cell death is also distinct from other forms of hair death. However, cadmium-induced hair cell toxicity does show some overlap with other forms of hair cell damage in that the Akt and ERK signaling pathways are activated not as part of the death mechanism, as seen in other forms of cadmium toxicity, but rather to help and protect cells against damage.

One area where we did see an overlap between cadmium-induced cell death mechanisms in hair cells and other cell types was with calmodulin. It has been shown that cadmium can activate calmodulin in lieu of calcium and it has been hypothesized this may be one potential mechanism of cadmium-induced toxicity (Chao et al., 1984; Sutoo et al., 1990). Treatment with inhibitors blocking cadmium-dependent signaling pathways have been shown to protect against cadmium in multiple cell types and tissues including the kidneys, osteoblasts, and testes (Ha et al., 2016; Niewenhuis and Prozialeck, 1987; Yang and Yang, 1997). However, these experiments are complicated in hair cells by the fact that calmodulin inhibition also inhibits rapid apical endocytosis in hair cells, a process dependent on mechanotransduction activity (Seiler and Nicolson, 1999). As inhibiting mechanotransduction activity is known to inhibit cadmium-induced hair cell death (Schmid et al., 2020) we cannot definitively say that calmodulin inhibition is working directly on the cell death process. Particularly as calmodulin inhibition can no

longer protect against cadmium-induced hair cell death when the inhibitor is added after cadmium treatment rather than as a cotreatment.

We failed to see cadmium-induced hair cell death protection from any other calcium signaling inhibitor we tested including inhibitors for CaMKII, the IP3 receptor, and the mitochondrial calcium uniporter. Inhibitors of all these molecules have been shown to protect against cadmium-induced cell death in other cell types (Chen et al., 2011; Lee et al., 2005; Liu and Templeton, 2007; Liu et al., 2016; Liu et al., 2018; Shih et al., 2005; Wang et al., 2008). However, these previous studies were carried out in mitotic cultured cells, including mesangial cells, PC-12 cells, proximal tubule cells, osteoblasts, and osteosarcoma cells, whereas our study was carried out *in vivo* in postmitotic cells, so this may be the reason for the discrepancy. Indeed another study looking at neurons, another postmitotic cell, failed to see protection from cadmium-induced cell death when inhibiting the mitochondrial calcium uniporter (Tang et al., 2021). The fact that blocking the IP3 receptor and mitochondrial calcium uniporter failed to protect against cadmium-induced hair cell death also differed from what has been shown in other forms of hair cell death (Esterberg et al., 2014; Wang et al., 2019). However, while there are clearly some overlapping mechanisms of different forms of hair cell deaths drug screens frequently find drugs that can protect against only a subset of ototoxic insults (Coffin et al., 2013; Owens et al., 2009; Vlasits et al., 2012) showing there are distinct mechanisms by which these compounds induce cell death in hair cells.

In terms of Akt and ERK signaling we found that while these pathways were activated in either hair cells or supporting cells in response to cadmium similar to what has been seen in other cell types, their activation appeared to be playing a role in

protecting hair cells rather than in the death process. This is not unprecedented. While many studies have found blocking Akt or ERK activation protects cells against cadmium-induced cell death (Chen et al., 2008; Iryo et al., 2000; Jiang et al., 2015; Kim et al., 2005b; Zhang et al., 2017) others have found that it has no effect (Låg et al., 2005; Liu and Templeton, 2007; Rigon et al., 2008; Wang et al., 2008), and still others that death is increased similar to what we see (Chao and Yang, 2001; Chuang et al., 2000; Xiao et al., 2009). Some of this disparity may come from differences in treatment paradigms. For example, in astrocytes ERK inhibition protects against chronic but not acute cadmium treatment (Jiang et al., 2015). The type of cell death also appears to matter as in macrophages ERK inhibition can protect against necrosis but not apoptosis induced by cadmium (Kim et al., 2005b).

Unlike what we found in calcium signaling the role of the Akt and ERK signaling pathways in cadmium-induced hair cell death appears similar to what has previously been shown for other forms of hair cell damage. These pathways have been shown to be activated in response to both noise and other ototoxic drugs (Chung et al., 2006; Herranen et al., 2018; Ingersoll et al., 2020; Lahne and Gale, 2008; Maeda et al., 2013) and blocking them has been shown to increase hair cell death (Battaglia et al., 2003; Brand et al., 2015; Chung et al., 2006; Haake et al., 2009; Hayashi et al., 2013; Kurioka et al., 2015). Particularly striking is the fact that ERK activation is seen not in the hair cells themselves but rather the support cells both in the zebrafish lateral line in response to cadmium, and in the mammalian cochlea in response to aminoglycosides, cisplatin and noise (Herranen et al., 2018; Ingersoll et al., 2020; Lahne and Gale, 2008; Maeda et al., 2013). The highly conserved activation and protective nature of these

pathways suggests enhancing their activation could be a potential target for protecting hair cells from damage, however, as with cadmium-induced cell death there are other examples where the activation of ERK appears to promote cell death (Lahne and Gale, 2008; So et al., 2007). The reasons behind these dual roles of ERK would need to be worked out before it could be used as a potential therapeutic.

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REFERENCES

Battaglia, A., Pak, K., Brors, D., Bodmer, D., Frangos, J. A. and Ryan, A. F. (2003). Involvement of ras activation in toxic hair cell damage of the mammalian cochlea. *Neuroscience* **122**, 1025–1035.

Bhardwaj, J. K., Panchal, H. and Saraf, P. (2021). Cadmium as a testicular toxicant: A Review. *J. Appl. Toxicol.* **41**, 105–117.

Brand, Y., Levano, S., Radojevic, V., Naldi, A. M., Setz, C., Ryan, A. F., Pak, K., Hemmings, B. A. and Bodmer, D. (2015). All Akt isoforms (Akt1, Akt2, Akt3) are involved in normal hearing, but only Akt2 and Akt3 are involved in auditory hair cell survival in the mammalian inner ear. *PLoS One* **10**, e0121599.

Chao, J. I. and Yang, J. (2001). Opposite roles of ERK and p38 mitogen-activated

protein kinases in cadmium-induced genotoxicity and mitotic arrest. *Chem. Res. Toxicol.* **14**, 1193–1202.

Chao, S., Suzuki, Y., Zysk, J. and Cheung, W. (1984). Activation of calmodulin by various metal cations as a function of ionic radius. *Mol. Pharmacol.* **26**, 75–82.

Chen, L., Liu, L., Luo, Y. and Huang, S. (2008). MAPK and mTOR pathways are involved in cadmium-induced neuronal apoptosis. *J. Neurochem.* **105**, 251–261.

Chen, S., Xu, Y., Xu, B., Guo, M., Zhang, Z., Liu, L., Ma, H., Chen, Z., Luo, Y., Huang, S., et al. (2011). CaMKII is involved in cadmium activation of MAPK and mTOR pathways leading to neuronal cell death. *J. Neurochem.* **119**, 1108–1118.

Choi, Y.-H. and Kim, K. (2014). Noise-Induced Hearing Loss in Korean Workers: Co-Exposure to Organic Solvents and Heavy Metals in Nationwide Industries. *PLoS One* **9**, e97538.

Choi, Y.-H. and Park, S. K. (2017). Environmental Exposures to Lead, Mercury, and Cadmium and Hearing Loss in Adults and Adolescents: KNHANES 2010-2012. *Environ. Health Perspect.* **125**, 067003.

Choi, Y.-H., Hu, H., Mukherjee, B., Miller, J. and Park, S. K. (2012). Environmental cadmium and lead exposures and hearing loss in U.S. adults: the National Health and Nutrition Examination Survey, 1999 to 2004. *Environ. Health Perspect.* **120**, 1544–50.

Chuang, S. M., Wang, I. C. and Yang, J. L. (2000). Roles of JNK, p38 and ERK mitogen-activated protein kinases in the growth inhibition and apoptosis induced by cadmium. *Carcinogenesis* **21**, 1423–1432.

Chung, W. H., Pak, K., Lin, B., Webster, N. and Ryan, A. F. (2006). A PI3K pathway

mediates hair cell survival and opposes gentamicin toxicity in neonatal rat organ of corti. *JARO - J. Assoc. Res. Otolaryngol.* **7**, 373–382.

Coffin, A. B., Williamson, K. L., Mamiya, A., Raible, D. W. and Rubel, E. W. (2013). Profiling drug-induced cell death pathways in the zebrafish lateral line. *Apoptosis* **18**, 393–408.

Dalton, D. S., Schubert, C. R., Pinto, A., Fischer, M. E., Huang, G.-H., Klein, B. E. K., Klein, R., Pankow, J. S., Paulsen, A. J., Tsai, M. Y., et al. (2020). Cadmium, obesity, and education, and the 10-year incidence of hearing impairment: The beaver dam offspring study. *Laryngoscope* **130**, 1396–1401.

Esterberg, R., Hailey, D. W., Coffin, A. B., Raible, D. W. and Rubel, E. W. (2013). Disruption of intracellular calcium regulation is integral to aminoglycoside-induced hair cell death. *J. Neurosci.* **33**, 7513–7525.

Esterberg, R., Hailey, D. W., Rubel, E. W. and Raible, D. W. (2014). ER-mitochondrial calcium flow underlies vulnerability of mechanosensory hair cells to damage. *J. Neurosci.* **34**, 9703–19.

Faroon, O., Ashizawa, A., Wright, S., Tucker, P., Jenkins, K., Ingerman, L. and Rudisill, C. (2012). *Toxicological Profile for Cadmium*. Agency for Toxic Substances and Disease Registry (US), Atlanta (GA).

Faucher, K., Fichet, D., Miramand, P. and Lagardère, J. P. (2006). Impact of acute cadmium exposure on the trunk lateral line neuromasts and consequences on the “C-start” response behaviour of the sea bass (Dicentrarchus labrax L.; Teleostei, Moronidae). *Aquat. Toxicol.* **76**, 278–294.

Ferraro, P. M., Costanzi, S., Naticchia, A., Sturniolo, A. and Gambaro, G. (2010).

Low level exposure to cadmium increases the risk of chronic kidney disease: analysis of the NHANES 1999-2006. *BMC Public Health* **10**, 304.

Fridberger, A., Flock, Å., Ulfendahl, M. and Flock, B. (1998). Acoustic overstimulation increases outer hair cell Ca2+ concentrations and causes dynamic contractions of the hearing organ. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 7127–7132.

Fujiki, K., Inamura, H. and Matsuoka, M. (2014). Detrimental effects of Notch1 signaling activated by cadmium in renal proximal tubular epithelial cells. *Cell Death Dis.* **5**, e1378.

Gallagher, C. M., Kovach, J. S. and Meliker, J. R. (2008). Urinary Cadmium and Osteoporosis in U.S. Women ≥50 Years of Age: NHANES 1988–1994 and 1999–2004. *Environ. Health Perspect.* **116**, 1338–1343.

García-Esquinas, E., Pollan, M., Tellez-Plaza, M., Francesconi, K. A., Goessler, W., Guallar, E., Umans, J. G., Yeh, J., Best, L. G. and Navas-Acien, A. (2014). Cadmium Exposure and Cancer Mortality in a Prospective Cohort: The Strong Heart Study. *Environ. Health Perspect.* **122**, 363–370.

Ha, T., Burwell, S., Goodwin, M., Noeker, J. and Heggland, S. (2016). Pleiotropic roles of Ca +2/calmodulin-dependent pathways in regulating cadmium-induced toxicity in human osteoblast-like cell lines. *Toxicol. Lett.* **260**, 18–27.

Haake, S. M., Dinh, C. T., Chen, S., Eshraghi, A. A. and Van De Water, T. R. (2009). Dexamethasone protects auditory hair cells against TNFα-initiated apoptosis via activation of PI3K/Akt and NFκB signaling. *Hear. Res.* **255**, 22–32.

Hayashi, Y., Yamamoto, N., Nakagawa, T. and Ito, J. (2013). Insulin-like growth factor 1 inhibits hair cell apoptosis and promotes the cell cycle of supporting cells by

activating different downstream cascades after pharmacological hair cell injury in neonatal mice. *Mol. Cell. Neurosci.* **56**, 29–38.

Herranen, A., Ikäheimo, K., Virkkala, J. and Pirvola, U. (2018). The Stress Response in the Non-sensory Cells of the Cochlea Under Pathological Conditions—Possible Role in Mediating Noise Vulnerability. *JARO - J. Assoc. Res. Otolaryngol.* **19**, 637–652.

Huh, D. A., Choi, Y. H., Ji, M. S., Moon, K. W., Yoon, S. J. and Sohn, J. R. (2018). Comparison of Pure-Tone Average Methods for Estimation of Hearing Loss Caused by Environmental Exposure to Lead and Cadmium: Does the Pure-Tone Average Method Which Uses Low-Frequency Ranges Underestimate the Actual Hearing Loss Caused by Environmental Le. *Audiol. Neurootol.* **23**, 259–269.

Hyder, O., Chung, M., Cosgrove, D., Herman, J. M., Li, Z., Firoozmand, A., Gurakar, A., Koteish, A. and Pawlik, T. M. (2013). Cadmium Exposure and Liver Disease among US Adults. *J. Gastrointest. Surg.* **17**, 1265–1273.

IARC (2012). Cadmium and cadmium compounds. In *Arsenic, Metals, Fibres, and Dusts IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, pp. 121–145. Lyon, France: International Agency for Research on Cancer.

Ingersoll, M. A., Malloy, E. A., Caster, L. E., Holland, E. M., Xu, Z., Zallocchi, M., Currier, D., Liu, H., He, D. Z. Z., Min, J., et al. (2020). BRAF inhibition protects against hearing loss in mice. *Sci. Adv.* **6**, eabd0561.

Iryo, Y., Matsuoka, M., Wispriyono, B., Sugiura, T. and Igisu, H. (2000). Involvement of the extracellular signal-regulated protein kinase (ERK) pathway in the induction of apoptosis by cadmium chloride in CCRF-CEM cells. *Biochem. Pharmacol.* **60**,

1875–1882.

Jiang, C., Yuan, Y., Hu, F., Wang, Q., Zhang, K., Wang, Y., Gu, J., Liu, X., Bian, J. and Liu, Z. (2014). Cadmium induces PC12 cells apoptosis via an extracellular signal-regulated kinase and c-Jun N-terminal kinase-mediated mitochondrial apoptotic pathway. *Biol. Trace Elem. Res.* **158**, 249–258.

Jiang, J. H., Ge, G., Gao, K., Pang, Y., Chai, R. C., Jia, X. H., Kong, J. G. and Yu, A. C. H. (2015). Calcium Signaling Involvement in Cadmium-Induced Astrocyte Cytotoxicity and Cell Death Through Activation of MAPK and PI3K/Akt Signaling Pathways. *Neurochem. Res.* **40**, 1929–1944.

Jung, D. J. (2019). Association between cadmium exposure and hearing impairment: a population-based study in Korean adults. *Yeungnam Univ. J. Med.* **36**, 141–147.

Kang, G. H., Uhm, J. Y., Choi, Y. G., Kang, E. K., Kim, S. Y., Choo, W. O. and Chang, S. S. (2018). Environmental exposure of heavy metal (lead and cadmium) and hearing loss: data from the Korea National Health and Nutrition Examination Survey (KNHANES 2010–2013). *Ann. Occup. Environ. Med.* **30**, 22.

Kim, Y. K., Kim, H. J., Kwon, C. H., Kim, J. H., Woo, J. S., Jung, J. S. and Kim, J. M. (2005a). Role of ERK activation in cisplatin-induced apoptosis in OK renal epithelial cells. *J. Appl. Toxicol.* **25**, 374–382.

Kim, J., Kim, S. H., Johnson, V. J. and Sharma, R. P. (2005b). Extracellular signal-regulated kinase-signaling-dependent G2/M arrest and cell death in murine macrophages by cadmium. *Environ. Toxicol. Chem.* **24**, 3069–3077.

Kim, S.-J., Jeong, H.-J., Myung, N.-Y., Kim, M., Lee, J.-H., So, H., Park, R.-K., Kim, H.-M., Um, J.-Y. and Hong, S.-H. (2008). The protective mechanism of

antioxidants in cadmium-induced ototoxicity in vitro and in vivo. *Environ. Health Perspect.* **116**, 854–62.

Kurioka, T., Matsunobu, T., Satoh, Y., Niwa, K., Endo, S., Fujioka, M. and Shiotani, A. (2015). ERK2 mediates inner hair cell survival and decreases susceptibility to noise-induced hearing loss. *Sci. Rep.* **5**, 16839.

Låg, M., Refsnes, M., Lilleaas, E. M., Holme, J. A., Becher, R. and Schwarze, P. E. (2005). Role of mitogen activated protein kinases and protein kinase C in cadmium-induced apoptosis of primary epithelial lung cells. *Toxicology* **211**, 253–264.

Lahne, M. and Gale, J. E. (2008). Damage-induced activation of ERK1/2 in cochlear supporting cells is a hair cell death-promoting signal that depends on extracellular ATP and calcium. *J. Neurosci.* **28**, 4918–4928.

Lee, W. K., Bork, U., Gholamrezaei, F. and Thévenod, F. (2005). Cd(2+)-induced cytochrome c release in apoptotic proximal tubule cells: role of mitochondrial permeability transition pore and Ca(2+) uniporter. *Am. J. Physiol. Renal Physiol.* **288**, F27–F39.

Liu, Y. and Templeton, D. M. (2007). Cadmium activates CaMK-II and initiates CaMK-II-dependent apoptosis in mesangial cells. *FEBS Lett.* **581**, 1481–1486.

Liu, H., Ding, D., Sun, H., Jiang, H., Wu, X., Roth, J. A. and Salvi, R. (2014). Cadmium-induced ototoxicity in rat cochlear organotypic cultures. *Neurotox. Res.* **26**, 179–89.

Liu, F., Li, Z.-F., Wang, Z.-Y. and Wang, L. (2016). Role of subcellular calcium redistribution in regulating apoptosis and autophagy in cadmium-exposed primary rat proximal tubular cells. *J. Inorg. Biochem.* **164**, 99–109.

Liu, W., Xu, C., Ran, D., Wang, Y., Zhao, H., Gu, J., Liu, X., Bian, J., Yuan, Y. and Liu, Z. (2018). CaMKⅡ mediates cadmium induced apoptosis in rat primary osteoblasts through MAPK activation and endoplasmic reticulum stress. *Toxicology* **406–407**, 70–80.

Low, J. and Higgs, D. M. (2015). Sublethal effects of cadmium on auditory structure and function in fathead minnows (Pimephales promelas). *Fish Physiol. Biochem.* **41**, 357–369.

Maeda, Y., Fukushima, K., Omichi, R., Kariya, S. and Nishizaki, K. (2013). Time Courses of Changes in Phospho- and Total- MAP Kinases in the Cochlea after Intense Noise Exposure. *PLoS One* **8**, e58775.

Mezynska, M. and Brzóska, M. M. (2018). Environmental exposure to cadmium-a risk for health of the general population in industrialized countries and preventive strategies. *Environ. Sci. Pollut. Res. Int.* **25**, 3211–3232.

Min, K.-B., Lee, K.-J., Park, J.-B. and Min, J.-Y. (2012). Lead and Cadmium Levels and Balance and Vestibular Dysfunction among Adult Participants in the National Health and Nutrition Examination Survey (NHANES) 1999–2004. *Environ. Health Perspect.* **120**, 413–417.

Misra, U. K., Gawdi, G. and Pizzo, S. V. (2003). Induction of mitogenic signalling in the 1LN prostate cell line on exposure to submicromolar concentrations of cadmium+. *Cell. Signal.* **15**, 1059–1070.

Montalbano, G., Capillo, G., Laurà, R., Abbate, F., Levanti, M., Guerrera, M. C., Ciriaco, E. and Germanà, A. (2018). Neuromast hair cells retain the capacity of regeneration during heavy metal exposure. *Ann. Anat. - Anat. Anzeiger* **218**, 183–

189.

Nicolson, T. (2005). The genetics of hearing and balance in zebrafish. *Annu. Rev. Genet.* **39**, 9–22.

Niewenhuis, R. and Prozialeck, W. (1987). Calmodulin inhibitors protect against cadmium-induced testicular damage in mice. *Biol. Reprod.* **37**, 127–133.

Owens, K. N., Coffin, A. B., Hong, L. S., Bennett, K. O., Rubel, E. W. and Raible, D. W. (2009). Response of mechanosensory hair cells of the zebrafish lateral line to aminoglycosides reveals distinct cell death pathways. *Hear. Res.* **253**, 32–41.

Ozcaglar, H. U., Agirdir, B., Dinc, O., Turhan, M., Kilinçarslan, S. and Oner, G. (2001). Effects of cadmium on the hearing system. *Acta Otolaryngol.* **121**, 393–7.

Raible, D. W. and Kruse, G. J. (2000). Organization of the lateral line system in embryonic zebrafish. *J. Comp. Neurol.* **421**, 189–198.

Rigon, A., Cordova, F., Oliveira, C., Posser, T., Costa, A., Silva, I., Santos, D., Rossi, F., Rocha, J. and Leal, R. (2008). Neurotoxicity of cadmium on immature hippocampus and a neuroprotective role for p38 MAPK. *Neurotoxicology* **29**, 727–734.

Ruczaj, A. and Brzóska, M. M. (2022). Environmental exposure of the general population to cadmium as a risk factor of the damage to the nervous system: A critical review of current data. *J. Appl. Toxicol.*

Schaal, N., Slagley, J., Zreiqat, M. and Paschold, H. (2017). Effects of combined exposure to metals, solvents, and noise on permanent threshold shifts. *Am. J. Ind. Med.* **60**, 227–238.

Schmid, C., Alampi, I., Briggs, J., Tarcza, K. and Stawicki, T. M. (2020).

Mechanotransduction Activity Facilitates Hair Cell Toxicity Caused by the Heavy Metal Cadmium. *Front. Cell. Neurosci.* **14**,.

Seiler, C. and Nicolson, T. (1999). Defective calmodulin-dependent rapid apical endocytosis in zebrafish sensory hair cell mutants. *J. Neurobiol.* **41**, 424–434.

Shargorodsky, J., Curhan, S. G., Henderson, E., Eavey, R. and Curhan, G. C. (2011). Heavy Metals Exposure and Hearing Loss in US Adolescents. *Arch. Otolaryngol. Neck Surg.* **137**, 1183–1189.

Shih, Y. L., Lin, C. J., Hsu, S. W., Wang, S. H., Chen, W. L., Lee, M. T., Wei, Y. H. and Shih, C. M. (2005). Cadmium toxicity toward caspase-independent apoptosis through the mitochondria-calcium pathway in mtDNA-depleted cells. In *Annals of the New York Academy of Sciences*, pp. 497–505. New York Academy of Sciences.

Smith, J. B., Dwyer, S. D. and Smith, L. (1989). Cadmium evokes inositol polyphosphate formation and calcium mobilization: Evidence for a cell surface receptor that cadmium stimulates and zinc antagonizes. *J. Biol. Chem.* **264**, 7115–7118.

So, H., Kim, H. J., Lee, J. H., Park, C., Kim, Y., Kim, E., Kim, J. K., Yun, K. J., Lee, K. M., Lee, H. Y., et al. (2007). Cisplatin cytotoxicity of auditory cells requires secretions of proinflammatory cytokines via activation of ERK and NF-κB. *JARO - J. Assoc. Res. Otolaryngol.* **8**, 338–355.

Sonnack, L., Kampe, S., Muth-Köhne, E., Erdinger, L., Henny, N., Hollert, H., Schäfers, C. and Fenske, M. (2015). Effects of metal exposure on motor neuron development, neuromasts and the escape response of zebrafish embryos.

Neurotoxicol. Teratol. **50**, 33–42.

Stawicki, T. M., Owens, K. N., Linbo, T., Reinhart, K. E., Rubel, E. W. and Raible, D. W. (2014). The zebrafish merovingian mutant reveals a role for pH regulation in hair cell toxicity and function. *Dis. Model. Mech.* **7**, 847–856.

Sutoo, D., Akiyama, K. and Imamiya, S. (1990). A mechanism of cadmium poisoning: the cross effect of calcium and cadmium in the calmodulin-dependent system. *Arch. Toxicol.* **64**, 161–164.

Tang, J., Duan, W., Deng, P., Li, H., Liu, C., Duan, Y., Feng, M. and Xu, S. (2021). Cadmium disrupts mitochondrial distribution and activates excessive mitochondrial fission by elevating cytosolic calcium independent of MCU-mediated mitochondrial calcium uptake in its neurotoxicity. *Toxicology* **453**, 152726.

Tellez-Plaza, M., Navas-Acien, A., Menke, A., Crainiceanu, C. M., Pastor-Barriuso, R. and Guallar, E. (2012). Cadmium Exposure and All-Cause and Cardiovascular Mortality in the U.S. General Population. *Environ. Health Perspect.* **120**, 1017–1022.

Uemaetomari, I., Tabuchi, K., Nakamagoe, M., Tanaka, S., Murashita, H. and Hara, A. (2009). L-type voltage-gated calcium channel is involved in the pathogenesis of acoustic injury in the cochlea. *Tohoku J. Exp. Med.* **218**, 41–47.

Vlasits, A. L., Simon, J. A., Raible, D. W., Rubel, E. W. and Owens, K. N. (2012). Screen of FDA-approved drug library reveals compounds that protect hair cells from aminoglycosides and cisplatin. *Hear. Res.* **294**, 153–165.

Wang, S. H., Shih, Y. L., Ko, W. C., Wei, Y. H. and Shih, C. M. (2008). Cadmium-induced autophagy and apoptosis are mediated by a calcium signaling pathway.

Cell. Mol. Life Sci. **65**, 3640–3652.

Wang, S. H., Shih, Y. L., Lee, C. C., Chen, W. L., Lin, C. J., Lin, Y. S., Wu, K. H. and Shih, C. M. (2009). The role of endoplasmic reticulum in cadmium-induced mesangial cell apoptosis. *Chem. Biol. Interact.* **181**, 45–51.

Wang, B., Xiao, J. L., Ling, Y. H., Meng, X. J., Wu, B., Yang, X. Y. and Zou, F. (2014). BNIP3 upregulation by ERK and JNK mediates cadmium-induced necrosis in neuronal cells. *Toxicol. Sci.* **140**, 393–402.

Wang, X., Zhu, Y., Long, H., Pan, S., Xiong, H., Fang, Q., Hill, K., Lai, R., Yuan, H. and Sha, S. H. (2019). Mitochondrial Calcium Transporters Mediate Sensitivity to Noise-Induced Losses of Hair Cells and Cochlear Synapses. *Front. Mol. Neurosci.* **11**, 469.

Wang, D.-H., Xu, H., Zheng, Y.-H., Gu, D.-S., Zhu, Y.-J., Ren, Y., Wang, S.-C., Yang, L. and Xu, L.-W. (2020). Environmental exposure to lead and cadmium and hearing loss in Chinese adults: A case-control study. *PLoS One* **15**, e0233165.

Wang, T., Zhu, Q., Cao, B., Cai, Y., Wen, S., Bian, J., Zou, H., Song, R., Gu, J., Liu, X., et al. (2022). Ca 2+ transfer via the ER-mitochondria tethering complex in neuronal cells contribute to cadmium-induced autophagy. *Cell Biol. Toxicol.* **38**,.

Whitfield, T. T. (2002). Zebrafish as a model for hearing and deafness. *J. Neurobiol.* **53**, 157–171.

Xiao, W., Liu, Y. and Templeton, D. M. (2009). Pleiotropic effects of cadmium in mesangial cells. *Toxicol. Appl. Pharmacol.* **238**, 315–326.

Xu, B., Chen, S., Luo, Y., Chen, Z., Liu, L., Zhou, H., Chen, W., Shen, T., Han, X., Chen, L., et al. (2011). Calcium Signaling Is Involved in Cadmium-Induced

Neuronal Apoptosis via Induction of Reactive Oxygen Species and Activation of MAPK/mTOR Network. *PLoS One* **6**, e19052.

Yamagami, K., Nishimura, S. and Sorimachi, M. (1998). Cd2+ and Co2+ at micromolar concentrations mobilize intracellular Ca2+ via the generation of inositol 1,4,5-triphosphate in bovine chromaffin cells. *Brain Res.* **798**, 316–319.

Yang, X. F. and Yang, Y. N. (1997). Protective effects of calcium antagonists on cadmium-induced toxicity in rats. *Biomed. Environ. Sci.* **10**, 402–407.

Yang, P. M., Chen, H. C., Tsai, J. S. and Lin, L. Y. (2007). Cadmium induces Ca2+-dependent necrotic cell death through calpain-triggered mitochondrial depolarization and reactive oxygen species-mediated inhibition of nuclear factor-κB activity. *Chem. Res. Toxicol.* **20**, 406–415.

Yang, C. S., Tzou, B. C., Liu, Y. P., Tsai, M. J., Shyue, S. K. and Tzeng, S. F. (2008). Inhibition of cadmium-induced oxidative injury in rat primary astrocytes by the addition of antioxidants and the reduction of intracellular calcium. *J. Cell. Biochem.* **103**, 825–834.

Yeh, J. H., Huang, C. C., Yeh, M. Y., Wang, J. S., Lee, J. K. and Jan, C. R. (2009). Cadmium-induced cytosolic Ca2+ elevation and subsequent apoptosis in renal tubular cells. *Basic Clin. Pharmacol. Toxicol.* **104**, 345–351.

Zhang, R., Zhu, Y., Dong, X., Liu, B., Zhang, N., Wang, X., Liu, L., Xu, C., Huang, S. and Chen, L. (2017). Celastrol Attenuates Cadmium-Induced Neuronal Apoptosis via Inhibiting Ca2+-CaMKII-Dependent Akt/mTOR Pathway. *J. Cell. Physiol.* **232**, 2145–2157.

Zhao, H., Liu, W., Wang, Y., Dai, N., Gu, J., Yuan, Y., Liu, X., Bian, J. and Liu, Z.

(2015). Cadmium induces apoptosis in primary rat osteoblasts through caspase and mitogen-activated protein kinase pathways. *J. Vet. Sci.* **16**, 297–306.