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

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ABSTRACT

Exposure to heavy metals 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, have 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, which is known to play a role in cadmium-induced cell death in other cell types through calmodulin and CaMKII activation as well as IP3 receptor and mitochondrial calcium uniporter mediated calcium flow, 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. Removal of extracellular calcium, 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 may be protective as inhibiting these pathways enhances 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 all promote cell death.

Keywords: Akt, ERK, cadmium, calcium signaling, hair cells, ototoxicity

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 et al., 2012; Choi and Park, 2017; 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). There is evidence that this toxicity may stem from reactive oxygen species generation (ROS). Increased ROS production is seen following treatment with cadmium in the hair cell-like cell line HEI-OC1, and antioxidant treatment protects against cadmium-induced cell death in both HEI-OC1 cells and cochlear explants. Antioxidant treatment also protects against cadmium-induced increases in auditory-brain stem response thresholds in mice (Kim et al., 2008). However, the role of other potential cell-signaling pathways involved in cadmium-induced cell death has not been extensively studied in hair cells to date.

In addition to its association with hearing loss, cadmium has also 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, they have been more extensively studied in other cell types. 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). In some cases this increase appears due to the release of calcium from endoplasmic reticulum (ER) stores, as it can be blocked by an inositol 1,4,5-triphosphate (IP3) receptor antagonist but not by the removal of extracellular calcium (Liu et al., 2016; Smith et al., 1989; Yamagami et al., 1998). Cadmium has also been shown to increase IP3 levels in cells (Benters et al., 1997; Hague et al., 2000; Smith et al., 1989; Yamagami et al., 1998). However, in other cases removing extracellular calcium or inhibiting calcium channels can block the cadmium-induced calcium increase suggesting extracellular calcium is contributing to this increase (Liu et al., 2018; Xu et al., 2011; Yeh et al., 2009).

This increase in calcium appears to be important for cadmium-induced cell death as calcium chelation and IP3 receptor inhibition have both been shown to protect against cadmium-induced toxicity (Liu et al., 2016; L. Wang et al., 2009; Wang et al., 2008; Yang et al., 2008, 2007; Yeh et al., 2009). Calcium may ultimately be inducing cell death through disruptions in the mitochondria and the production of ROS as calcium

81 chelation can block cadmium-induced mitochondrial depolarization and increases in
82 ROS production (Xu et al., 2011; Yang et al., 2008, 2007; Yuan et al., 2013). Cadmium
83 is unable to increase ROS production when directly applied to isolated mitochondria
84 further supporting the role of calcium as a potential intermediary signal in this process
85 (Shih et al., 2005). Mitochondrial calcium also appears important in cadmium-induced
86 cell death as inhibition of the mitochondria calcium uniporter blocks cadmium-induced
87 cytochrome c release, mitochondrial depolarization and cell toxicity (Lee et al., 2005; Li
88 et al., 2003; Shih et al., 2005).

89 Downstream of calcium both calmodulin and calcium/calmodulin-dependent
90 protein kinase II (CaMKII) appear to be playing a role in cadmium-induced cell death.
91 Cadmium is able to directly activate calmodulin as well as increase calcium's activation
92 of calmodulin, and it is believed this may result in a toxic overactivation of this molecule
93 (Chao et al., 1984; Sutoo et al., 1990). Inhibition of both calmodulin and CaMKII can
94 protect cells against cadmium-induced cell death (Liu et al., 2018; Liu and Templeton,
95 2007).

96 Cadmium has also been shown to increase phosphorylated levels of Akt (pAkt),
97 and Extracellular Receptor Kinase (pERK) in vitro in a calcium-dependent manner
98 (Jiang et al., 2015; Xu et al., 2011). Increased pAkt levels have also been seen in the
99 brain in vivo in response to cadmium (Chen et al., 2014). The calcium-dependent
100 activation of these pathways appears to be through the activation of calmodulin and
101 CaMKII as inhabiting either of these molecules can block the cadmium-dependent
102 increase in pAkt and pERK (Chen et al., 2011; Fujiki et al., 2013; Liu et al., 2018; Xiao
103 et al., 2009; Xu et al., 2011). Akt and ERK activation appears to be playing a role in the

104 cell death process as inhibitors of both signaling pathways have been shown to protect
105 against cadmium-induced cell toxicity (Chen et al., 2008; Iryo et al., 2000; Jiang et al.,
106 2014, 2015; Y. K. Kim et al., 2005; Wang et al., 2014; Zhang et al., 2017; Zhao et al.,
107 2015).

108 Calcium, Akt and ERK signaling have also all been previously implicated in hair
109 cell damage from either aminoglycoside antibiotic toxicity or noise. Inhibiting calcium
110 movement from the ER or mitochondria has been shown to protect against hair cell
111 damage (Esterberg et al., 2014; Wang et al., 2019). There is evidence that a transfer of
112 calcium from the ER to the mitochondria in response to aminoglycoside treatment is
113 triggering ROS generation leading to hair cell death (Esterberg et al., 2016). The Akt
114 and ERK signaling pathways on the other hand are believed to play a protective role in
115 hair cell toxicity as inhibition of these pathways is most commonly shown to increase
116 hair cell damage (Battaglia et al., 2003; Brand et al., 2015; Chung et al., 2006; Haake et
117 al., 2009; Hayashi et al., 2013; Kurioka et al., 2015). Therefore, we felt these three
118 signaling molecules were strong candidates to investigate as potential signaling
119 pathways involved in cadmium-induced hair cell death. Activation of ERK has also
120 previously been seen in the cochlea in response to cadmium and inhibition of ERK can
121 protect HE-OC1 cells, a hair cell like cell line, from cadmium-induced cell death (Kim et
122 al., 2008).

123 We investigated the role of these signaling molecules in hair cell death using the
124 zebrafish lateral line. The lateral line is a sensory system in fish used to detect water
125 motion that consists of clusters of hair cells and support cells, collectively known as
126 neuromasts, on the surface of the animal. Support cells are glia-like cells surrounding

127 hair cells that have been shown to play key roles in the development, maintenance and
128 regeneration of hair cells (reviewed in Monzack and Cunningham, 2013). Hair cells of
129 the zebrafish lateral line have been shown to be functionally, molecularly and
130 morphologically similar to mammalian hair cells (reviewed in Nicolson, 2005; Whitfield,
131 2002).

132 We have previously shown that cadmium causes reliable hair cell death in the
133 zebrafish lateral line within three hours in a dose-dependent manner, and therefore
134 used that treatment paradigm in this study (Schmid et al., 2020). We first found that
135 removal of extracellular calcium following cadmium treatment caused an increase in hair
136 cell death both in control conditions and following cadmium treatment. We also found
137 that co-treatment of a calmodulin inhibitor with cadmium could significantly protect
138 against cadmium-induced hair cell death. However, when the calmodulin inhibitor was
139 applied after fish were washed out of cadmium, a condition we refer to as
140 posttreatment, protection was no longer seen. Additionally, other calcium signaling
141 pathway inhibitors including a CaMKII inhibitor, inositol IP3 receptor inhibitor, and
142 mitochondria calcium uniporter inhibitor failed to protect against cadmium-induced hair
143 cell death. We also saw an increase in levels of pAkt in hair cells and pERK in
144 supporting cells in response to cadmium, however, inhibition of these pathways
145 enhanced hair cell death rather than protecting against it. Therefore, we conclude that
146 the signaling pathways responsible for cadmium-induced hair cell death appear distinct
147 from those commonly seen in cadmium-induced toxicity in other cell types and partially
148 distinct from those seen in other forms of hair cell toxicity.
149

150 # MATERIALS AND METHODS

151

152 **Animals**

153 All experiments were carried out in 5 days post-fertilization (dpf) *AB strain wild-
154 type zebrafish larvae. Fish larvae were housed in an incubator maintained at 28.5°C
155 with a 14/10 hour light/dark cycle and raised in embryo media (EM) consisting of 1 mM
156 $MgSO_4$, 150 μM KH_2PO_4, 42 μM Na_2HPO_4, 1 mM $CaCl_2$, 500 μM KCl, 15 mM NaCl, and
157 714 μM $NaHCO_3$. The Lafayette College Institution Animal Care and Use Committee
158 approved all experiments.

159

160 **Drug Treatments**

161 The following drugs were used: cadmium chloride hemipentahydrate (Fisher
162 Scientific), water-soluble KN-93 (Abcam), MK-2206 (Millipore Sigma), PD184352
163 (Millipore Sigma), RU360 (Millipore Sigma), W-13 (Enzo) and Xestospongin (Abcam).
164 Stock solutions for cadmium chloride, KN-93, and Ru360 were made in water. Stock
165 solutions of all other drugs were made in dimethyl sulfoxide (DMSO, Millipore Sigma).
166 All drugs were then diluted to their treatment concentrations in EM. For drugs diluted in
167 DMSO the control groups contained DMSO at the same volume as the drug. For the
168 water diluted drugs EM was used for the control group.

169 For the drug treatments, fish were initially put into netwell inserts (Corning) in 6
170 well plates containing EM. The netwell inserts with the larvae were then moved to new
171 plates containing the solutions the fish were to be treated in. At the end of treatment,
172 fish were washed three times in EM, again by moving the netwell inserts to new plates,
173 between treatments or before fixation. For experiments where fish were moved to EM

174 with reduced calcium levels after cadmium treatment, they were washed three times in
175 zero calcium EM instead of standard EM before being moved to the reduced or zero
176 calcium EM and then returned to standard EM immediately prior to fixation.
177
178 **Immunostaining**
179 Fish were anesthetized with MS-222 and then fixed for 2 hours at room
180 temperature in 4% paraformaldehyde. Antibody labeling was carried out as previously
181 described (Stawicki et al., 2014). The following primary antibodies were used: rabbit
182 anti-parvalbumin (ThermoFisher, PA1-933) diluted at 1:1,000, mouse anti-otoferlin
183 (Developmental Studies Hybridoma Bank, HCS-1) diluted at 1:100, rabbit anti-phospho-
184 Akt (Ser473) (Cell Signaling, 9271) diluted at 1:100, and rabbit anti-phospho-p44/42
185 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling, 4370) diluted at 1:200.
186
187 **Hair Cell Counts**
188 For hair cell counts fish were either stained with just parvalbumin or costained
189 with otoferlin and parvalbumin. In the majority of experiments the parvalbumin stain was
190 used for counts, however, in fish that were costained with otoferlin the otoferlin stain
191 was used if the parvalbumin stain was too dim to reliably count hair cells. Within a single
192 experiment the same antibody was always used for all counts. To perform hair cell
193 counts fish were observed on an Accu-Scope EXC-350 microscope using the 40X
194 objective. The number of hair cells in the following neuromasts OP1, M2, IO4, O2, MI2,
195 and MI1 (Raible and Kruse, 2000) were counted for each larvae. The total number of
196 hair cells counted for that fish was then divided by 6 to get an average number of hair

197 cells per neuromast. To normalize data for the zero calcium and ERK inhibitor
198 experiment, the average hair cells/neuromast number for each treatment group was
199 divided by the average hair cells/neuromast number for their respective control groups
200 with no cadmium and then multiplied by 100 to be converted to a percentage.
201
202 **Confocal Imaging and Analysis of pAkt and pERK levels**
203 To calculate pAkt and pERK levels fish stained with either the pAkt or pERK
204 antibodies and the otoferlin antibody were imaged on a Zeiss LSM800 confocal
205 microscope using the Zen Blue software. For pAkt, the approximate center of the
206 neuromast was selected and 10 planes separated by 1 μm were imaged for each
207 neuromast. For pERK, there was significant neuronal labeling in the control condition
208 that we did not want to include in our analysis. Therefore, the top and bottom of the
209 neuromast were selected and planes separated by 1μm were imaged throughout the
210 neuromast regardless of number with images ranging from 11-29 planes. Then 10
211 planes with minimal neuronal labeling were selected for subsequent analysis. One
212 neuromast of the anterior lateral line was imaged per fish with the exact neuromast to
213 be imaged selected based on which neuromasts were best positioned to image. Image
214 analysis was conducted in Fiji. Maximum projection images were made for each 10-
215 plane stack. The neuromast was outlined using the otoferlin label and the average
216 fluorescent intensity of the pAkt or pERK signal was measured.
217
218 **Statistics and Graphical Representation of Data**

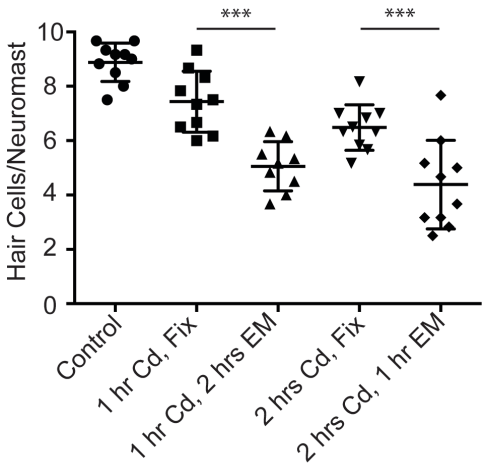
All experiments were carried out with n=10 animals per group. Statistics were calculated in GraphPad Prism 6 which was also used to generate graphs. For experiments where two or more different groups were tested against a range of calcium or cadmium doses, a two-way ANOVA was used with the either a Tukey's or Šídák's multiple comparisons test depending on which was recommended by Prism. The multiple comparisons test used for each experiment is mentioned in the figure legend. Graphs of this data were represented by the average of each data set with error bars showing the standard deviation. For all other experiments, a one-way ANOVA was used again with a Tukey's multiple comparison test as all groups were compared. Graphs of this data show the individual data points as well as the average and standard deviations. For experiments where the ANOVA showed a significant difference between relevant groups the significance found by the multiple comparisons tests are shown on the graphs as asterisks.

RESULTS

Alterations in extracellular calcium levels following cadmium treatment do not cause dramatic changes in cadmium-induced hair cell death

Previous research has shown that calcium plays an important role in cadmium-induced cell death in many cell types. In some cell types, it appears that extracellular calcium is important as chelating it or treating cells with calcium channel blockers can protect against cadmium-induced toxicity (Liu et al., 2018; Yang et al., 2008; Yeh et al., 2009). Unfortunately, determining the role of extracellular calcium in cadmium-induced hair cell toxicity is complicated by the fact that we have previously shown that cadmium-

243 induced hair cell toxicity is mechanotransduction-activity dependent (Schmid et al.,
244 2020). Extracellular calcium levels are known to impact hair cell mechanotransduction
245 activity (reviewed in Fettiplace, 2017; Ó Maoiléidigh and Ricci, 2019) with calcium
246 chelation completely blocking it (Assad et al., 1991). It has previously been shown that
247 the entry and toxicity of other mechanotransduction-dependent ototoxins can be altered
248 by alterations in extracellular calcium levels for this reason (Coffin et al., 2009).
249 Therefore, to investigate whether calcium signaling was impacting cadmium-induced
250 hair cell death we first wanted to determine if there was continued hair cell death
251 following cadmium removal. If there was, we could potentially separate cadmium entry,
252 which is the phase of cadmium-induced hair cell death most likely to be
253 mechanotransduction dependent, from the subsequent cell death process. To do this
254 we treated fish with 30 μM of cadmium chloride for either 1 or 2 hours, fish were then
255 washed out of cadmium, and either fixed immediately or fixed three hours after the start
256 of the experiment. We found that following both the 1 and 2 hour cadmium chloride
257 treatment continued hair cell death was seen after cadmium removal (Figure 1).
258



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260

261 **Figure 1: Cadmium causes continued cell death after removal**. Hair cells remaining after various
262 treatment paradigms with 30μM of cadmium. Fish were either treated with cadmium chloride for 1 hour
263 and immediately fixed (squares), treated with cadmium for 1 hour then left in EM for two hours before
264 fixation (triangles), treated with cadmium for 2 hours and then immediately fixed (upside down triangles),
265 or treated with cadmium for 2 hours and then left in EM for 1 hour before fixation (diamonds). Control fish
266 were kept in EM for three hours (circles). There was a significant amount of cell death after cadmium
267 removal for both the 1 hour and 2 hours cadmium treatment paradigms (*** = $p<0.001$ by ANOVA and
268 Tukey post-hoc test).
269

270 As we saw significant continued cadmium-induced hair cell death after cadmium
271 removal, we next wanted to test if this continued cell death could be altered by changing
272 extracellular calcium levels. To do this we used the treatment paradigm where fish were
273 treated with cadmium chloride for one hour, washed out of cadmium, and then fixed two
274 hours later. We first tested a range of calcium concentrations by treating fish with either
275 EM or 30 μM cadmium chloride for 1 hour and then washing them out of that solution
276 and moving them into EM with calcium concentrations ranging from 0-2 mM for 2 hours
277 prior to fixation. We found the only calcium concentration where a significant difference
278 was seen in the amount of cadmium-induced hair cell death as compared to the
279 standard EM calcium concentration of 1mM was 0mM (Figure 2A). However, rather than
280 protecting hair cells the removal of extracellular calcium appeared to increase cadmium-
281 induced hair cell death. We then treated fish with a range of cadmium doses from 0-120
282 μM for 1 hour before washing them out of cadmium and moving them to either standard
283 EM with 1 mM calcium or EM with 0 mM calcium for 2 hours prior to fixation. Again, a
284 significant increase in the amount of cadmium-induced hair cell death was seen in the
285 fish that were moved to 0 mM calcium following cadmium treatment (Figure 2B).

286 However, there was also a significant reduction in the number of control hair cells in this
287 condition similar to what has previously been seen when zebrafish are put in calcium-
288 free EM (Coffin et al., 2009). Therefore, to determine whether the incubation in 0 mM
289 calcium EM was sensitizing fish to cadmium-induced hair cell death or simply killing hair
290 cells on its own we normalized these data for both the 1 mM and 0 mM calcium EM
291 groups to the 0 cadmium control for each group. We found in this case while there is still
292 a trend toward a decrease in hair cell number at all cadmium concentrations tested, the
293 only concentration that showed a significant decrease was 120 μM cadmium (Figure
294 2C). Taken together this data suggests alterations in calcium concentration following
295 cadmium removal do not have dramatic impacts on cadmium-induced hair cell death.
296 However, due to the complications with calcium's effect on mechanotransduction
297 activity, we cannot rule out an impact from extracellular calcium at earlier time points in
298 the cell death process.
299

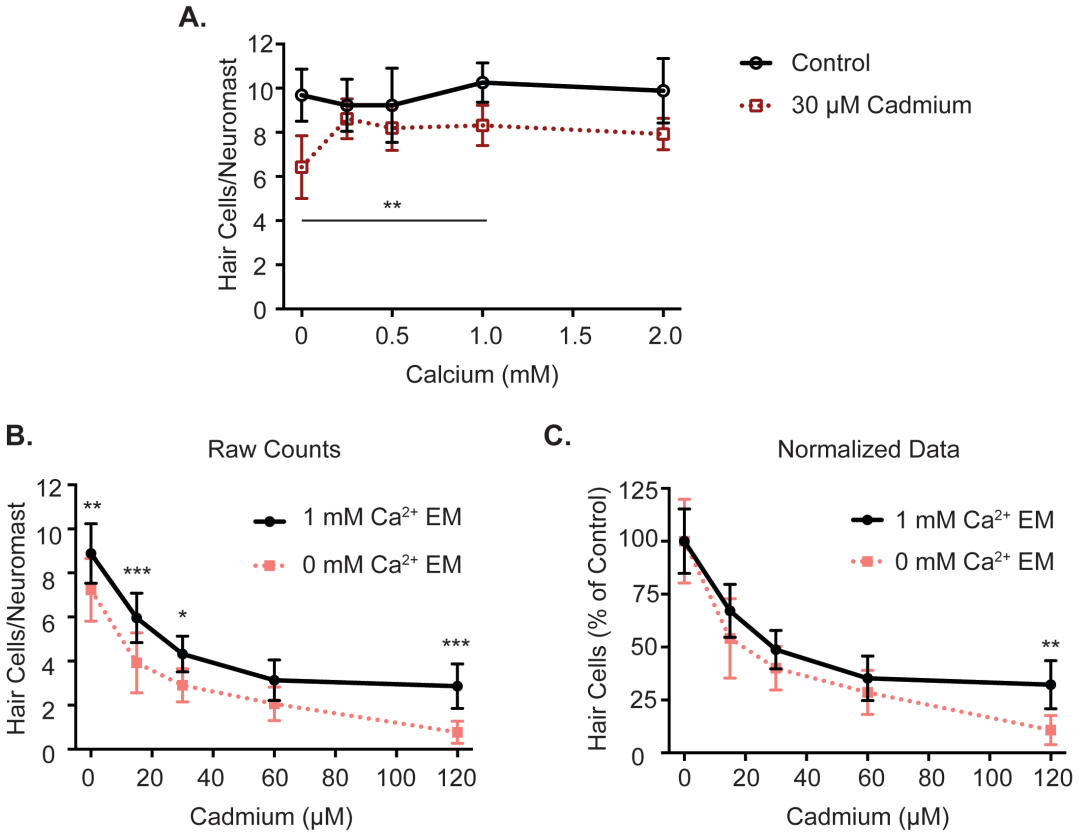

Figure 2: Removal of extracellular calcium following cadmium treatment kills hair cells on its own but does not dramatically impact cadmium-induced hair cell death. (A) Hair cells remaining after a 1 hour treatment with either EM, control (solid line), or 30 μM cadmium chloride (dotted line) followed by a 2 hour incubation in EM containing varying concentrations of calcium. The only calcium concentration where there was a significant difference in the number of hair cells following cadmium treatment compared to the standard EM 1 mM calcium group was the 0 mM calcium group (** = $p<0.01$ by Two-Way ANOVA and Tukey's post-hoc test). (B) Hair cells remaining after a 1 hour treatment with varying doses of cadmium chloride followed by a 2 hour incubation in either EM containing 1mM calcium (solid line) or EM containing 0 mM calcium (dotted line). A significant decrease in hair cells in the 0 mM calcium group was seen for all concentrations of cadmium tested except 60 μM (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, by Two-Way ANOVA and Šídák's post-hoc test). (C) The same data as in B, but normalized to the zero cadmium control for each group. Following normalization, a significant decrease in hair cells in

314 the 0 mM calcium group was only seen at 120 μM cadmium. (** = p<0.01, by Two-Way ANOVA and
315 Šídák's post-hoc test). N = 10 fish for each group. Error bars = Standard deviation.
316

317 **Calmodulin and CaMKII inhibitors do not consistently protect against cadmium-**
318 **induced hair cell death**

319 Calmodulin is a major intracellular receptor for calcium and calmodulin inhibition
320 has been shown to protect against cadmium-induced cell death (Niewenhuis and
321 Prozialeck, 1987; Xu et al., 2011; Yang and Yang, 1997). Calmodulin inhibition has also
322 previously been shown to protect against aminoglycoside-induced hair cell death
323 (Esterberg et al., 2013). To see if calmodulin inhibition could protect against cadmium-
324 induced hair cell death we first cotreated fish with 30 μM of the calmodulin inhibitor W-
325 13 hydrochloride and varying doses of calmodulin chloride ranging from 0-120 μM for
326 three hours. We found that calmodulin inhibition significantly reduced cadmium-induced
327 hair cell death at lower doses of cadmium chloride, 15 and 30 μM, but not at higher
328 doses, 60 and 120 μM (Figure 3A).

329 However, calmodulin inhibition has also previously been shown to inhibit rapid
330 apical endocytosis in hair cells as measured by rapid FM1-43 uptake, a process
331 dependent on mechanotransduction activity (Seiler and Nicolson, 1999). As mentioned
332 above inhibiting mechanotransduction can protect against cadmium-induced hair cell
333 death (Schmid et al., 2020), therefore, we tested whether inhibiting calmodulin only after
334 cadmium removal would still significantly protect against cadmium-induced hair cell
335 death. To do this fish were treated with varying doses of cadmium chloride from 0-30
336 μM for one hour, washed out of cadmium, and then immediately fixed or placed into

337 DMSO or 30 μM W-13 for two hours before fixation. Using this treatment paradigm we
338 no longer saw protection from calmodulin inhibition (Figure 3B). This suggests the
339 protection seen with cotreatment with the calmodulin inhibitor may be due to impacts on
340 mechanotransduction-dependent processes, though we can also not rule out a distinct
341 early role for calmodulin in the cell death process.

342 Calmodulin is known to activate CaMKII and inhibiting CaMKII has also been
343 shown to protect against cadmium-induced cell death in multiple cell types (Chen et al.,
344 2011; Liu et al., 2018; Liu and Templeton, 2007). Therefore, as our calmodulin results
345 did now show a clear protective impact we, subsequently tested if CaMKII inhibition
346 would impact cadmium-induced hair cell death. To do this we cotreated fish with 10 μM
347 of the CaMKII inhibitor KN-93, a dose previously shown to be effective in zebrafish
348 (Rothschild et al., 2013), and varying doses of cadmium chloride for three hours. We
349 found CaMKII inhibition did not significantly impact cadmium-induced hair cell death
350 (Figure 3C). This further supports the idea that calmodulin and CaMKII are not playing a
351 role in cadmium-induced hair cell death outside of calmodulin's impact on
352 mechanotransduction activity.
353
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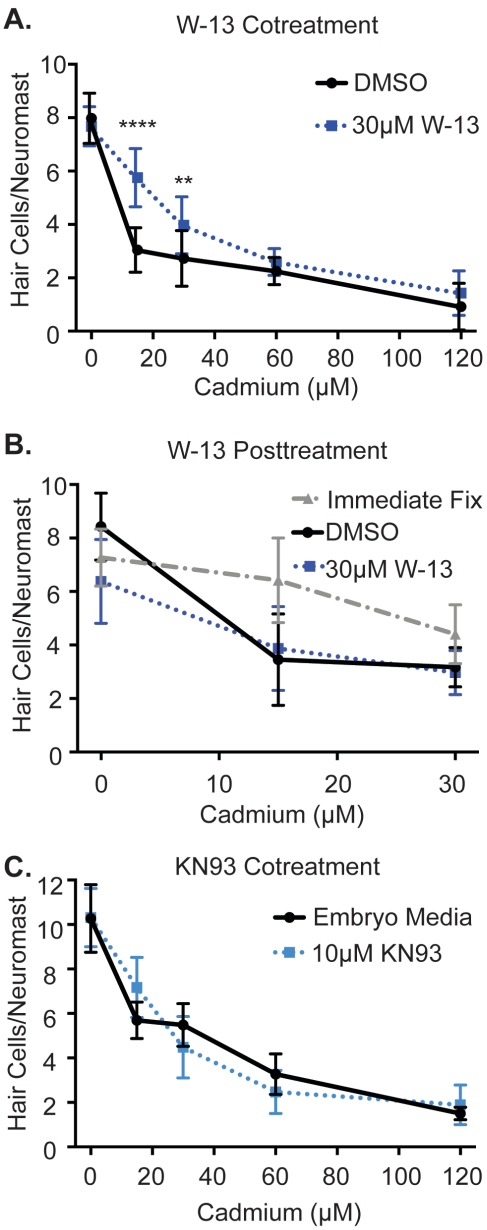

Figure 3: Calmodulin inhibitor cotreatment protects against cadmium-induced hair cell death whereas calmodulin inhibitor posttreatment and CaMKII inhibitor cotreatment fail to protect. (A) Hair cells remaining after a 3 hour cotreatment of either DMSO, a vehicle control, (solid line), or 30 μM of the 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

363 hour post-treatment with DMSO (solid line) or W-13 (dotted line). No significant protection was seen
364 comparing the DMSO and W-13 groups by Two-Way ANOVA and Tukey's post-hoc test. (C) Hair cells
365 remaining after a 3 hour cotreatment of either plain embryo media or 10 μM of the CaMKII inhibitor KN-93
366 and varying doses of cadmium chloride. No significant difference was seen between the two groups by
367 Two-Way ANOVA. n = 10 fish for each group. Error bars = Standard deviation.
368

369 **Inhibition of the IP3 Receptor and Mitochondrial Uniporter fail to protect against**
370 **cadmium-induced hair cell death**

371 We next wanted to test whether calcium flow from the ER or mitochondria would
372 impact cadmium-induced hair cell death as blocking the IP3 receptor and the
373 mitochondria calcium uniporter has been shown to protect other cell types against
374 cadmium-induced toxicity (Lee et al., 2005; Liu et al., 2016, 2018; Shih et al., 2005; S.
375 H. Wang et al., 2009; Wang et al., 2008, 2022). These inhibitors have also been shown
376 to protect hair cells from other forms of toxicity. (Esterberg et al., 2014; Wang et al.,
377 2019). To test this in hair cells we cotreated fish for three hours with either 1 μM of the
378 IP3 receptor inhibitor Xestospongin C or 500 nM of the mitochondrial uniporter inhibitor
379 RU360, doses that have previously been shown to protect against aminoglycoside-
380 induced hair cell death in the zebrafish lateral line (Esterberg et al., 2014), and varying
381 doses of cadmium chloride for three hours. In both cases, the inhibitor failed to
382 significantly protect against cadmium-induced hair cell death with RU360 causing a
383 slight enhancement of cadmium-induced hair cell death at the lowest cadmium dose
384 (Figure 4). Thus, our results indicate calcium flow from the ER and mitochondria is not
385 playing a significant role in cadmium-induced hair cell death.
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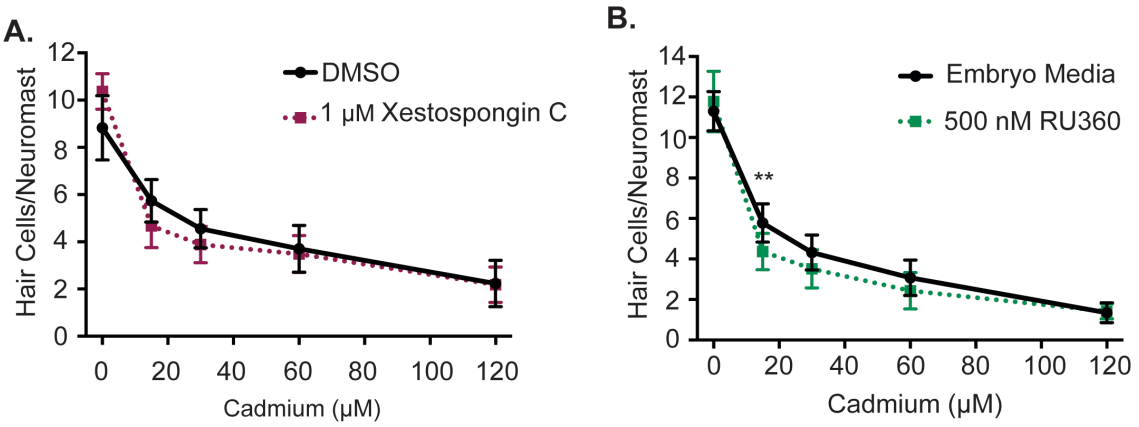


Figure 4: Inhibiting ER and mitochondria calcium transfer does not protect against cadmium-induced hair cell death. Hair cells remaining after a three hour cotreatment of either EM or DMSO (solid lines), or (A) 1 μM of the IP3 receptor inhibitor Xestospongin C, or (B) 500 nM of the mitochondrial uniporter inhibitor RU360 (dotted lines) and varying doses of cadmium chloride. Neither of the inhibitors significantly protected against cadmium-induced hair cell death with RU360 showing a significant increase in cadmium-induced hair cell death at the lowest dose (** = $p<0.01$= by Two-Way ANOVA and Šídák's post-hoc test). N = 10 fish/group. Error bars = standard deviation.

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, 2011; Fujiki et al., 2013; Iryo et al., 2000; J. Kim et al., 2005; Kim et al., 2008; Liu and Templeton, 2007; Misra et al., 2003; 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 (Chung et al., 2006; Herranen et al., 2018; Ingersoll et al., 2020; Lahne and Gale, 2008; Maeda et al., 2013). Based on this evidence we tested whether the Akt or ERK 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

406 with an antibody for either pAkt or pERK. We found that treatment with cadmium
407 significantly increased the levels of pAkt seen in hair cells (Figure 5A & B). This
408 increase was lost when fish were cotreated with cadmium and 5 μM of the Akt inhibitor
409 MK-2206. We also saw a significant increase in pERK levels following cadmium
410 treatment that was lost when fish were cotreated with cadmium and 3 μM of the MEK
411 inhibitor PD184352 which suppresses ERK phosphorylation (Figure 5C & D). However,
412 unlike pAkt labeling which appeared localized to hair cells, pERK labeling appears
413 localized to supporting cells (Figure 5B & D). This is in agreement with past research in
414 mammals showing increased pERK in support cells but not hair cells in response to
415 other forms of hair cell damage (Herranen et al., 2018; Ingersoll et al., 2020; Lahne and
416 Gale, 2008; Maeda et al., 2013).

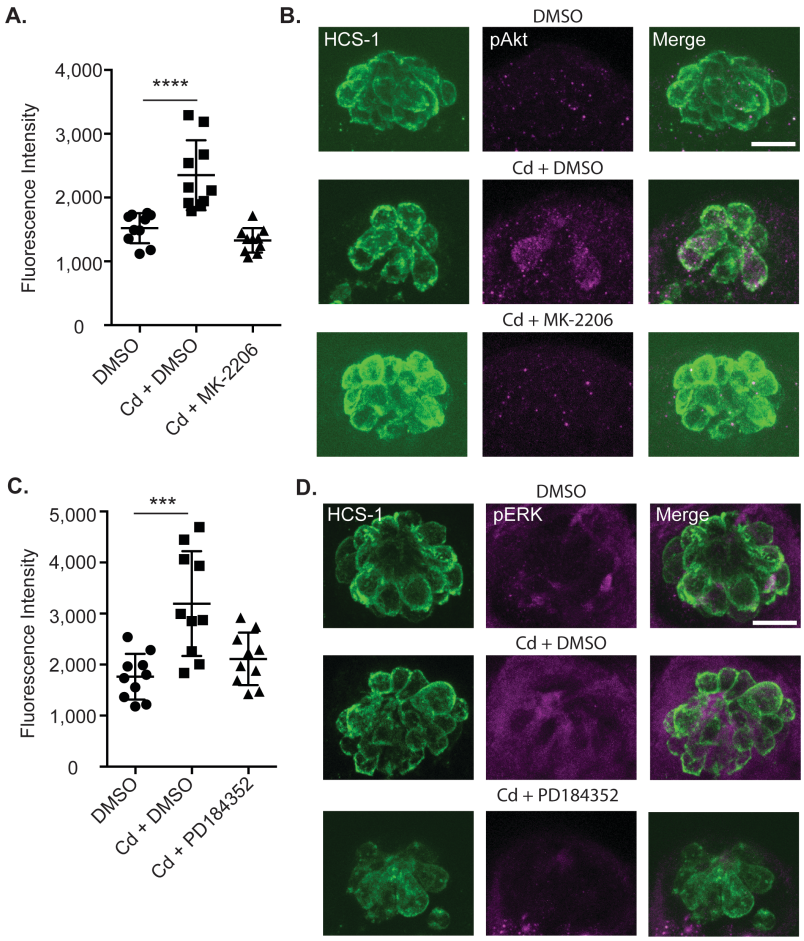


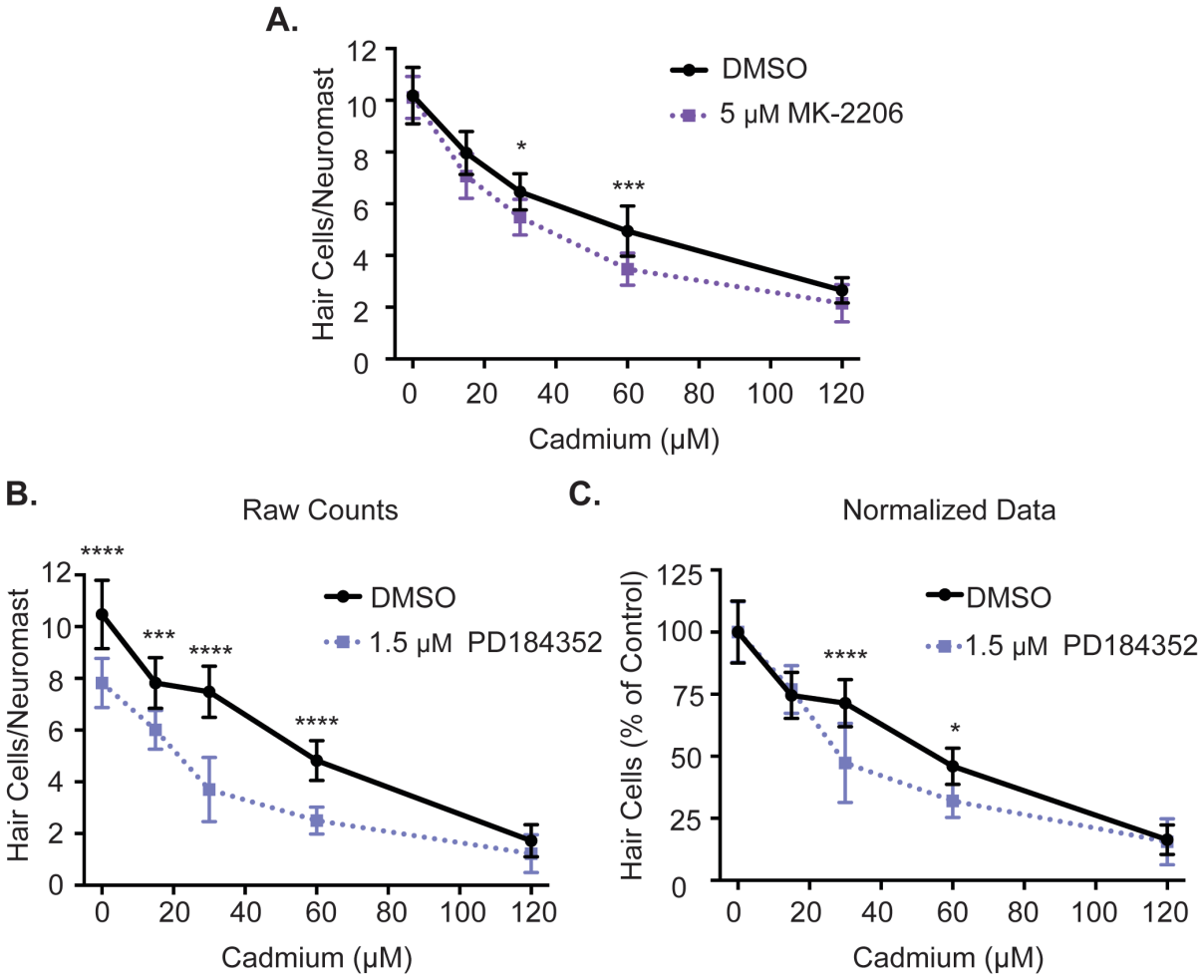
Figure 5: Cadmium treatment increases pAkt levels in hair cells and pERK levels in supporting cells. (A) Quantification of the fluorescence intensity of pAkt in fish treated for 1 hour with either DMSO alone, 30μM cadmium chloride (Cd) + DMSO, or 30μM Cd and 5 μM of MK-2206. (**** = p<0.0001 by ANOVA and Tukey post-hoc test). (B) Images of a neuromast from the DMSO alone group (top), the Cd + DMSO group (middle), and Cd + MK-2206 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, 30μM Cd + DMSO, or 30μM 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 (middle), and Cd + MK-2206 group (bottom). Hair cells are labeled with the HCS-1 antibody in green (left) and pERK in magenta (center). Scale bar = 10 μm.

430 **Inhibiting Akt and ERK signaling pathways increases cadmium-induced hair cell**
431 **death**

432 Having seen an increase in pAkt and pERK levels following cadmium treatment,
433 we wanted to determine whether the Akt or ERK signaling pathways impacted
434 cadmium-induced hair cell death. Akt and/or ERK inhibitors have been shown to protect
435 against cadmium-induced toxicity in multiple cell types (Chen et al., 2008; Iryo et al.,
436 2000; Jiang et al., 2015; J. Kim et al., 2005; Kim et al., 2008; Wang et al., 2014; Zhang
437 et al., 2017), suggesting these pathways normally function to promote cadmium-induced
438 cell death. In hair cells blocking Akt or ERK signaling commonly increases hair cell
439 death, suggesting these pathways may be activated to protect hair cells (Battaglia et al.,
440 2003; Brand et al., 2015; Chung et al., 2006; Haake et al., 2009; Hayashi et al., 2013;
441 Kurioka et al., 2015).

442 To determine if the Akt or ERK pathways were impacting cadmium-induced hair
443 cell death, we cotreated fish with either 5 μM of the Akt inhibitor MK-2206, or 1.5 μM of
444 the MEK inhibitor PD184352, which inhibits ERK activation, and varying doses of
445 cadmium chloride for three hours. A smaller dose of PD184352 was used in this
446 experiment than the pERK levels experiment due to increased hair cell toxicity from
447 PD184352 alone with the longer treatment time. We found both drugs caused a
448 decrease in hair cell number following cadmium treatment particularly, at the 30 and 60
449 μM cadmium chloride doses (Figure 6A & B). However, as noted above PD184352
450 caused a loss of hair cells independent of cadmium treatment, a similar loss of hair cells
451 from simple ERK inhibition has also previously been seen in cochlear explants
452 (Battaglia et al., 2003). Therefore, to determine if PD184352 was enhancing cadmium-

453 induced hair cell death or simply killing hair cells on its own, we normalized the data for
454 both the DMSO and PD184352 groups to the zero cadmium control (Figure 6C). In
455 doing so we still saw a significant increase in cadmium-induced hair cell death at the 30
456 and 60 μM cadmium chloride doses. Thus, we conclude that unlike in other cell types
457 Akt and ERK signaling do not appear to be facilitating cadmium-induced hair cell death
458 and may be playing a protective role similar to what is seen in other forms of hair cell
459 toxicity.
460



461

462 **Figure 6: Inhibiting Akt and ERK signaling increases cadmium-induced cell death.** (A) Hair cells
463 remaining after a 3 hour cotreatment of either DMSO (solid line) or 5 μM of the Akt inhibitor MK-2206

464 (dotted line) and varying doses of cadmium chloride. MK-2206 cotreatment caused a significant increase
465 in cadmium-induced hair cell death. (B) Hair cells remaining after a 3 hour cotreatment of either DMSO, a
466 vehicle control, (solid line), or 1.5 μM of the ERK pathway inhibitor PD184352 (dotted line) and varying
467 doses of cadmium chloride. PD184352 caused a significant decrease in the number of hair cells seen. (C)
468 The same data as in B, but normalized to the zero cadmium control for each group. PD184352 caused a
469 significant increase in the amount of cadmium-induced hair cell death seen. (* = $p<0.05$, *** = $p<0.001$,
470 **** = $p<0.0001$, by Two-Way ANOVA and Šídák's post-hoc test). N = 10 fish for each group. Error bars =
471 Standard deviation.
472

473 ## DISCUSSION
474

475 Overall we found that cadmium-induced hair cell death showed both some
476 distinctions and similarities with cadmium-induced cell death in other cell types and
477 other forms of hair cell toxicity. One of the distinctions was that we failed to find a
478 significant role for calcium signaling. This is in contrast to both cadmium-induced cell
479 death in other cell types and other forms of hair cell death where calcium appears to be
480 a key mediator in the cell death process (Esterberg et al., 2014, 2013; Liu et al., 2016;
481 L. Wang et al., 2009; Wang et al., 2008, 2019; Xu et al., 2011; Yang et al., 2008, 2007;
482 Yeh et al., 2009). We did however, see activation of both the Akt and ERK signaling
483 pathways. These two pathways are known to be activated both in response to cadmium
484 in other cell types (Chen et al., 2008, 2011; Fujiki et al., 2013; Iryo et al., 2000; J. Kim et
485 al., 2005; Kim et al., 2008; Liu and Templeton, 2007; Misra et al., 2003; Xiao et al.,
486 2009; Xu et al., 2011) and in other forms of hair cell toxicity (Chung et al., 2006;
487 Herranen et al., 2018; Ingersoll et al., 2020; Lahne and Gale, 2008; Maeda et al., 2013).
488 Though unlike in other cell types, inhibiting these signaling pathways enhanced

489 cadmium-induced hair cell death rather than protecting against it (Chen et al., 2008; Iryo
490 et al., 2000; Jiang et al., 2014, 2015; Y. K. Kim et al., 2005; Wang et al., 2014; Zhang et
491 al., 2017; Zhao et al., 2015) similar to what has been shown in other forms of hair cell
492 toxicity (Battaglia et al., 2003; Brand et al., 2015; Chung et al., 2006; Haake et al., 2009;
493 Hayashi et al., 2013; Kurioka et al., 2015).

494 While most of our manipulations of calcium signaling failed to protect against
495 cadmium-induced hair cell death, one manipulation that did cause significant protection
496 was the cotreatment of cadmium and a calmodulin inhibitor. It has been shown that
497 cadmium can activate calmodulin in lieu of calcium and it has been hypothesized this
498 may be one potential mechanism of cadmium-induced toxicity (Chao et al., 1984; Sutoo
499 et al., 1990). Treatment with inhibitors blocking calmodulin-dependent signaling
500 pathways has been shown to protect against cadmium in multiple cell types and tissues
501 including the kidneys, osteoblasts, and testes (Ha et al., 2016; Niewenhuis and
502 Prozialeck, 1987; Yang and Yang, 1997). However, these experiments are complicated
503 in hair cells by the fact that calmodulin inhibition also inhibits rapid apical endocytosis in
504 hair cells, a process dependent on mechanotransduction activity (Seiler and Nicolson,
505 1999). As inhibiting mechanotransduction activity is known to inhibit cadmium-induced
506 hair cell death (Schmid et al., 2020) we cannot definitively say that the protection we
507 see from calmodulin inhibition is due to a role for calmodulin in the cell death process.
508 The fact that protection is no longer seen when the calmodulin inhibitor is added after
509 cadmium treatment, rather than as a cotreatment, and the fact that CaMKII inhibition
510 was unable to protect hair cells from cadmium-induced hair cell death further supports

511 the idea that calmodulin inhibition is mainly impacting hair cell mechanotransduction
512 activity and cadmium entry rather than the cell death process.
513 We also failed to see protection against cadmium-induced hair cell death using
514 other calcium signaling manipulations including removing extracellular calcium and
515 inhibiting the IP3 receptor and mitochondrial calcium uniporter. These manipulations
516 have been shown to protect against cadmium-induced cell death in other cell types (Lee
517 et al., 2005; Liu et al., 2016, 2018; Shih et al., 2005; Wang et al., 2008; Xu et al., 2011).
518 However, these previous studies were carried out in mitotic cultured cells, including
519 mesangial cells, PC-12 cells, proximal tubule cells, osteoblasts, and osteosarcoma
520 cells, whereas our study was carried out *in vivo* in postmitotic cells, so this may be the
521 reason for the discrepancy. Indeed another study looking at neurons, another
522 postmitotic cell, failed to see protection from cadmium-induced cell death when
523 inhibiting the mitochondrial calcium uniporter (Tang et al., 2021). Calcium signaling in
524 general and specifically calmodulin, calcium release via the IP3 receptor, and activation
525 of the mitochondrial uniporter have all been shown to be important for the progression
526 of mitosis (Lagos-Cabré et al., 2020; Nugues et al., 2022; Rasmussen and Means,
527 1989; Takuwa et al., 1995; Zhao et al., 2019). Therefore, mitotic cells may be more
528 sensitive to cadmium-induced alterations in these calcium signaling pathways. Also,
529 even in mitotic cells, there have been discrepancies over the role of calcium signaling in
530 cadmium-induced cell death. For example, when looking at CaMKII inhibition a study in
531 an osteoblast cell line showed no effect (Ha et al., 2016), and a study in mesangial cells
532 showed an increase in cell death (Xiao et al., 2009).

533 The fact that blocking the IP3 receptor and mitochondrial calcium uniporter failed
534 to protect against cadmium-induced hair cell death also contrasted with what has
535 previously been shown in other forms of hair cell death (Esterberg et al., 2014; Wang et
536 al., 2019). However, while there are clearly some overlapping mechanisms between
537 different forms of hair cell death, drug screens frequently find drugs that can protect
538 against only a subset of ototoxic insults (Coffin et al., 2013; Owens et al., 2009; Vlasits
539 et al., 2012) showing there are distinct mechanisms by which ototoxic compounds
540 induce cell death in hair cells.

541 While we did not find a significant impact of calcium signaling in cadmium-
542 induced hair cell death we did see an increase in Akt and ERK signaling similar to what
543 has been seen in other cell types. This was somewhat unexpected as in other cell types
544 this increase is calcium signaling dependent (Chen et al., 2011; Fujiki et al., 2013; Jiang
545 et al., 2015; Liu et al., 2018; Xiao et al., 2009; Xu et al., 2011). However, it has
546 previously been shown that ERK signaling can be activated in supporting cells even
547 following calcium chelation (Lahne and Gale, 2008). One potential alternative
548 mechanism for the activation of these pathways includes Ras and Raf signaling which
549 has previously been implicated in activating ERK signaling following hair cell damage
550 (Battaglia et al., 2003; Lahne and Gale, 2008). Another alternative activation route is
551 growth factor signaling. Increases in growth factor receptors have been seen after hair
552 cell damage (Lee and Cotanche, 1996; Zine and de Ribaupierre, 1999), and inhibiting
553 the IGF1R receptor has been shown to also increase susceptibility to aminoglycoside-
554 induced hair cell damage (Alassaf and Halloran, 2021), suggesting a role for growth
555 factor signaling in protecting hair cells from damage.

556 While the activation of ERK and Akt signaling was similar to what was seen in
557 other forms of cadmium-induced cell death, the fact that inhibiting these pathways
558 increased rather than protected against cell death was unexpected. This, however, is
559 not unprecedented. While many studies have found blocking Akt or ERK activation
560 protects cells against cadmium-induced cell death (Chen et al., 2008; Iryo et al., 2000;
561 Jiang et al., 2015; J. Kim et al., 2005; Zhang et al., 2017) others have found that it has
562 no effect (Låg et al., 2005; Liu and Templeton, 2007; Rigon et al., 2008; Wang et al.,
563 2008), and still others that death is increased similar to what we observed (Chao and
564 Yang, 2001; Chuang et al., 2000; Xiao et al., 2009). Some of this disparity may come
565 from differences in treatment paradigms. For example, in astrocytes ERK inhibition
566 protects against chronic but not acute cadmium treatment (Jiang et al., 2015). The type
567 of cell death also appears important as in macrophages ERK inhibition can protect
568 against necrosis but not apoptosis induced by cadmium (J. Kim et al., 2005).
569 Unlike what we found in calcium signaling, the role of the Akt and ERK signaling
570 pathways in cadmium-induced hair cell death appears similar to what has previously
571 been shown for other forms of hair cell damage. These pathways are activated in
572 response to both noise and other ototoxic drugs (Chung et al., 2006; Herranen et al.,
573 2018; Ingersoll et al., 2020; Lahne and Gale, 2008; Maeda et al., 2013), and inhibiting
574 them has been shown to increase hair cell death (Battaglia et al., 2003; Brand et al.,
575 2015; Chung et al., 2006; Haake et al., 2009; Hayashi et al., 2013; Kurioka et al., 2015).
576 Particularly striking is the fact that ERK activation is seen not in the hair cells
577 themselves but rather in the support cells. This has also been seen in the mammalian

578 inner ear in response to aminoglycosides, cisplatin, and noise (Herranen et al., 2018;
579 Ingersoll et al., 2020; Lahne and Gale, 2008; Maeda et al., 2013).
580 Cadmium is known to damage support cells (Ding et al., 2020), and thus this
581 ERK activation could be coming from a direct effect of cadmium on the support cells.
582 Alternatively, the damaged hair cells could be sending a signal to the support cells to
583 induce ERK activation. It has been shown that the damage-induced release of ATP and
584 gap junction signaling are important for support cell ERK activation following mechanical
585 injury of hair cells (Lahne and Gale, 2008). Once ERK is activated in support cells it
586 may mediate its protective effect by modulating support cell released HSP70. ERK
587 activation is important for HSP70 expression and folding (Lim et al., 2019; Xu et al.,
588 2018; Zhai et al., 2019). Support cells are known to release HSP70 in exosomes
589 following heat shock, with that HSP70 subsequently protecting hair cells from
590 aminoglycoside-induced hair cell death (Breglio et al., 2020; May et al., 2013). The
591 mechanism by which Akt activation is working in hair cell death is likely more
592 straightforward as it is activated in the hair cells themselves. The activation of Akt
593 signaling is well known to promote cell survival and inhibit cell death and multiple
594 apoptotic regulators are Akt substrates (reviewed in Downward, 2004; Franke et al.,
595 2003), providing a potential mechanism by which Akt activation could protect hair cells
596 from cadmium-induced cell death.
597 The highly conserved activation and potential protective nature of Akt and ERK
598 pathways suggest enhancing their activation could be a potential target for protecting
599 hair cells from damage, however, as with other forms of cadmium-induced cell death,
600 there are other examples where the activation of ERK appears to promote hair cell

death (Lahne and Gale, 2008; So et al., 2007). The reasons behind these dual roles of ERK would need to be further investigated before ERK activation could be used as a potential therapeutic target.

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AUTHOR CONTRIBUTIONS

Jennifer Galdieri: investigation, writing – original draft. **Chloe Adams:** investigation, writing – review & editing. **María Padilla:** investigation, writing – review & editing. **Tamara Stawicki:** conceptualization, methodology, formal analysis, investigation, writing – original draft and revision, and review and editing.

COMPETING INTERESTS

The authors have no competing interests to declare

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