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

35 # INTRODUCTION

36

37 Hair cells, the sensory cells we use for hearing and balance, are extremely
38 sensitive to insult, and as such disorders of these sensory systems are common.
39 Recently environmental toxins such as heavy metals have gained attention as a
40 potential cause of hearing loss (Choi and Kim, 2014; Schaal et al., 2017). One of these
41 metals is cadmium. Cadmium can be found in some industrial run-off and individuals
42 can be exposed to it in certain occupations, via smoking or via contaminated food
43 (Faroon et al., 2012; IARC, 2012). Multiple studies have found a link between blood or
44 urinary cadmium levels and hearing or balance impairment (Choi et al., 2012; Choi and
45 Park, 2017; Dalton et al., 2020; Huh et al., 2018; Jung, 2019; Kang et al., 2018; Min et
46 al., 2012; Shargorodsky et al., 2011; Wang et al., 2020). Studies have also found
47 hearing loss and/or hair cell death in mammals and fish exposed to cadmium (Faucher
48 et al., 2006; Kim et al., 2008; Liu et al., 2014; Low and Higgs, 2015; Montalbano et al.,
49 2018; Ozcaglar et al., 2001; Schmid et al., 2020; Sonnack et al., 2015). There is
50 evidence that this toxicity may stem from reactive oxygen species generation. Increased
51 ROS production is seen following treatment with cadmium in the hair cell-like cell line
52 HEI-OC1, and antioxidant treatment protects against cadmium-induced cell death in
53 both HEI-OC1 cells and cochlear explants. Antioxidant treatment also protects against
54 cadmium-induced increases in auditory-brain stem response thresholds in mice (Kim et
55 al., 2008). However, the role of other potential cell-signaling pathways involved in
56 cadmium-induced cell death has not been extensively studied in hair cells to date.

57

58 In addition to its association with hearing loss, cadmium has also been linked to a
59 number of other health conditions including cancer, osteoporosis, and dysfunction of the
60 cardiovascular system, kidneys, liver, nervous system and reproductive system.
61 (Bhardwaj et al., 2021; Ferraro et al., 2010; Gallagher et al., 2008; García-Esquinas et
62 al., 2014; Hyder et al., 2013; Mezynska and Brzóska, 2018; Ruczaj and Brzóska, 2022;
63 Tellez-Plaza et al., 2012). While mechanisms of cadmium toxicity in hair cells are still
64 relatively unknown, they have been more extensively studied in other cell types. Multiple
65 cell types show an increase in calcium in response to cadmium (Shih et al., 2005; Smith
66 et al., 1989; Xu et al., 2011; Yamagami et al., 1998; Yang et al., 2007; Yeh et al., 2009).
67 In some cases this increase appears due to the release of calcium from endoplasmic
68 reticulum (ER) stores, as it can be blocked by an inositol 1,4,5-triphosphate (IP3)
69 receptor antagonist but not by the removal of extracellular calcium (Liu et al., 2016;
70 Smith et al., 1989; Yamagami et al., 1998). Cadmium has also been shown to increase
71 IP3 levels in cells (Benters et al., 1997; Hague et al., 2000; Smith et al., 1989;
72 Yamagami et al., 1998). However, in other cases removing extracellular calcium or
73 inhibiting calcium channels can block the cadmium-induced calcium increase
74 suggesting extracellular calcium is contributing to this increase (Liu et al., 2018; Xu et
75 al., 2011; Yeh et al., 2009).
76 This increase in calcium appears to be important for cadmium-induced cell death
77 as calcium chelation and IP3 receptor inhibition have both been shown to protect
78 against cadmium-induced toxicity (Liu et al., 2016; L. Wang et al., 2009; Wang et al.,
79 2008; Yang et al., 2008, 2007; Yeh et al., 2009). Calcium may ultimately be inducing
80 cell death through disruptions in the mitochondria and the production of reactive oxygen

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

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

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

149 ## MATERIALS AND METHODS
150

151 **Animals**

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

158

159 **Drug Treatments**

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

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

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

experiment, the average hair cells/neuromast number for each 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.

Confocal Imaging and Analysis of pAkt and pERK 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 planes separated by 1 μm were 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 were selected and planes separated by 1μm were imaged throughout the neuromast regardless of number with images ranging from 11-29 planes. Then 10 planes with minimal neuronal labeling were selected for subsequent analysis. One neuromast of the anterior lateral line was imaged per fish with the exact neuromast to be imaged selected based on which neuromasts were best positioned to image. Image analysis was conducted in Fiji. Maximum projection images were made for each 10-plane stack. The neuromast was outlined using the otoferlin label and the average fluorescent intensity of the pAkt or pERK signal was measured.

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

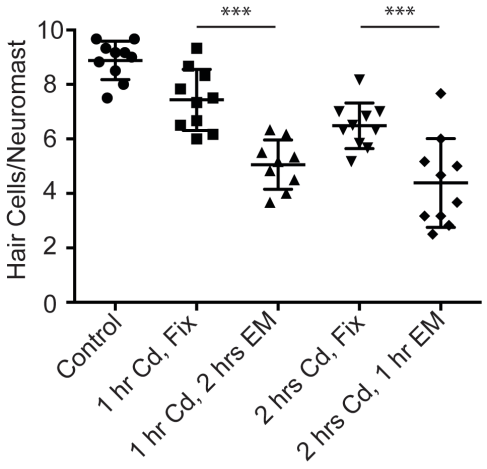
experiments where two or more different groups were tested against a range of calcium or cadmium doses, a two-way ANOVA was used with the multiple comparisons test recommended by Prism. Graphs of this data were represented by the average of each data set with error bars showing the standard deviation. For all other experiments, a one-way ANOVA was used again with the multiple comparison comparisons test recommended by Prism. 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-induced hair cell toxicity is mechanotransduction-activity dependent (Schmid et al., 2020). Extracellular calcium levels are known to impact hair cell mechanotransduction activity (reviewed in Fettiplace, 2017; Ó Maoiléidigh and Ricci, 2019) with calcium chelation completely blocking it (Assad et al., 1991). It has previously been shown that

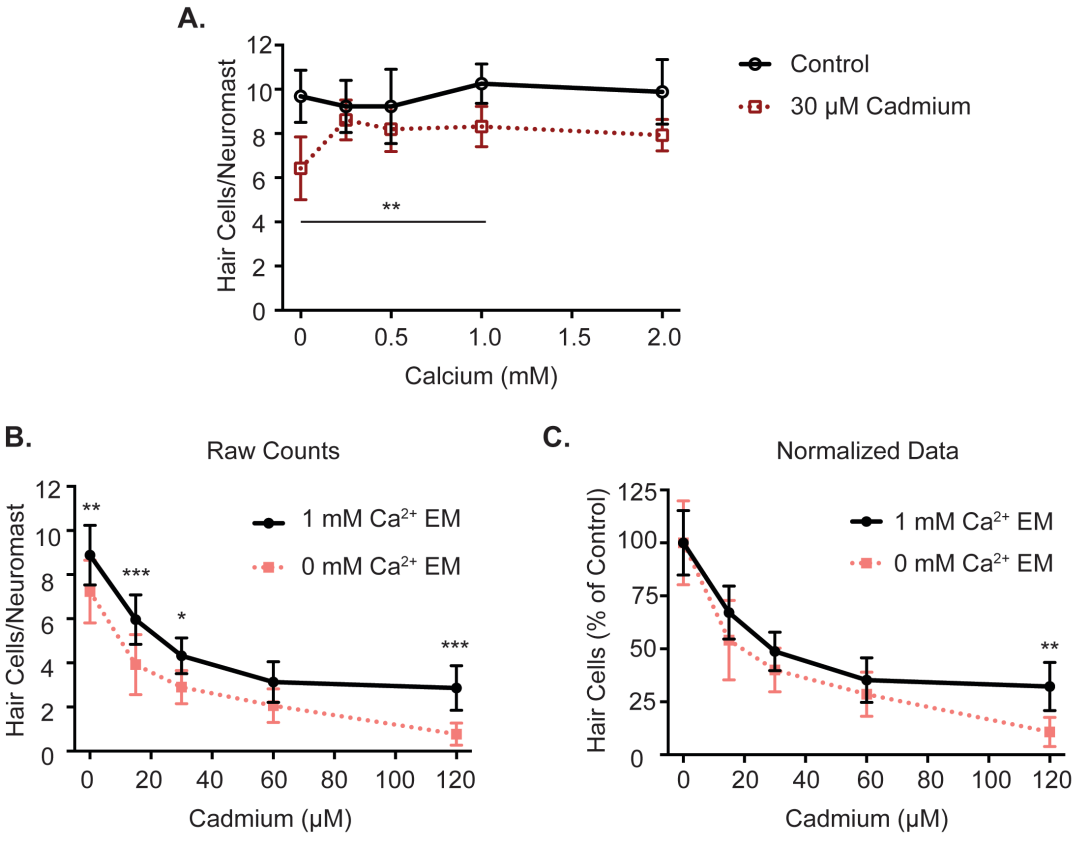
244 the entry and toxicity of other mechanotransduction-dependent ototoxins can be altered
245 by alterations in extracellular calcium levels for this reason (Coffin et al., 2009).
246 Therefore, to investigate whether calcium signaling was impacting cadmium-induced
247 hair cell death we first wanted to determine if there was continued hair cell death
248 following cadmium removal. If there was, we could potentially separate cadmium entry,
249 which is the phase of cadmium-induced hair cell death most likely to be
250 mechanotransduction dependent, from the subsequent cell death process. To do this
251 we treated fish with 30 μM of cadmium chloride for either 1 or 2 hours, fish were then
252 washed out of cadmium, and either fixed immediately or fixed three hours after the start
253 of the experiment. We found that following both the 1 and 2 hour cadmium chloride
254 treatment continued hair cell death was seen after cadmium removal (Figure 1).
255



256
257
258 **Figure 1: Cadmium causes continued cell death after removal**. Hair cells remaining after various
259 treatment paradigms with 30μM of cadmium. Fish were either treated with cadmium chloride for 1 hour
260 and immediately fixed (squares), treated with cadmium for 1 hour then left in EM for two hours before
261 fixation (triangles), treated with cadmium for 2 hours and then immediately fixed (upside down triangles),

262 or treated with cadmium for 2 hours and then left in EM for 1 hour before fixation (diamonds). Control fish
263 were kept in EM for three hours (circles). There was a significant amount of cell death after cadmium
264 removal for both the 1 hour and 2 hours cadmium treatment paradigms (*** = $p<0.001$ by ANOVA and
265 Tukey post-hoc test).
266
267 As we saw significant continued cadmium-induced hair cell death in the paradigm
268 where fish were washed out of cadmium after one hour and then fixed two hours later
269 we used this treatment paradigm to test the role of alterations in extracellular calcium
270 levels following cadmium removal. We first tested a range of calcium concentrations by
271 treating fish with either EM or 30 μM cadmium chloride for 1 hour and then washing
272 them out of that solution and moving them into EM with calcium concentrations ranging
273 from 0-2 mM for 2 hours prior to fixation. We found the only calcium concentration
274 where a significant difference was seen in the amount of cadmium-induced hair cell
275 death as compared to the standard EM calcium concentration of 1mM was 0mM (Figure
276 2A). However, rather than protecting hair cells the removal of extracellular calcium
277 appeared to increase cadmium-induced hair cell death. We then treated fish with a
278 range of cadmium doses from 0-120 μM for 1 hour before washing them out of cadmium
279 and moving them to either standard EM with 1 mM calcium or EM with 0 mM calcium for
280 2 hours prior to fixation. Again, a significant increase in the amount of cadmium-induced
281 hair cell death was seen in the fish that were moved to 0 mM calcium following
282 cadmium treatment (Figure 2B). However, there was also a significant reduction in the
283 number of control hair cells in this condition similar to what has previously been seen
284 when zebrafish are put in calcium-free EM (Coffin et al., 2009). Therefore, to determine
285 whether the incubation in 0 mM calcium EM was sensitizing fish to cadmium-induced

286 hair cell death or simply killing hair cells on its own we normalized the data for both the
287 1 mM and 0 mM calcium EM groups to the 0 cadmium control for each group. We found
288 in this case while there is still a trend towards a decrease in hair cell number at all
289 cadmium concentrations tested, the only concentration that showed a significant
290 decrease was 120 μM cadmium (Figure 2C). Taken together this data suggests
291 alterations in calcium concentration following cadmium removal do not have dramatic
292 impacts on cadmium-induced hair cell death. However, due to the complications with
293 calcium's effect on mechanotransduction activity, we cannot rule out an impact from
294 extracellular calcium at earlier time points in the cell death process.
295



296
297

298 **Figure 2: Removal of extracellular calcium following cadmium treatment kills hair cells on its own**
299 **but does not dramatically impact cadmium-induced hair cell death. (**A) Hair cells remaining after a 1
300 hour treatment with either EM, control (solid line), or 30 μM cadmium chloride (dotted line) followed by a 2
301 hour incubation in EM containing varying concentrations of calcium. The only calcium concentration
302 where there was a significant difference in the number of hair cells following cadmium treatment
303 compared to the standard EM 1 mM calcium group was the 0 mM calcium group (** = $p<0.01$ by Two-
304 Way ANOVA and Tukey's post-hoc test). (B) Hair cells remaining after a 1 hour treatment with varying
305 doses of cadmium chloride followed by a 2 hour incubation in either EM containing 1mM calcium (solid
306 line) or EM containing 0 mM calcium (dotted line). A significant decrease in hair cells in the 0 mM calcium
307 group was seen for all concentrations of cadmium tested except 60 μM (* = $p<0.05$, ** = $p<0.01$, *** =
308 $p<0.001$, by Two-Way ANOVA and Šídák's post-hoc test). (C) The same data as in B, but normalized to
309 the zero cadmium control for each group. Following normalization, a significant decrease in hair cells in
310 the 0 mM calcium group was only seen at 120 μM cadmium. (** = $p<0.01$, by Two-Way ANOVA and
311 Šídák's post-hoc test). N = 10 fish for each group. Error bars = Standard deviation.
312

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

315 Calmodulin is a major intracellular receptor for calcium and calmodulin inhibition
316 has been shown to protect against cadmium-induced cell death (Niewenhuis and
317 Prozialeck, 1987; Xu et al., 2011; Yang and Yang, 1997). Calmodulin inhibition has also
318 previously been shown to protect against aminoglycoside-induced hair cell death
319 (Esterberg et al., 2013). To see if calmodulin inhibition could protect against cadmium-
320 induced hair cell death we first cotreated fish with 30 μM of the calmodulin inhibitor W-
321 13 hydrochloride and varying doses of calmodulin chloride ranging from 0-120 μM for
322 three hours. We found that calmodulin inhibition significantly reduced cadmium-induced

323 hair cell death at lower doses of cadmium chloride, 15 and 30 μM, but not at higher
324 doses, 60 and 120 μM (Figure 3A).
325 However, calmodulin inhibition has also previously been shown to inhibit rapid
326 apical endocytosis in hair cells as measured by rapid FM1-43 uptake, a process
327 dependent on mechanotransduction activity (Seiler and Nicolson, 1999). As mentioned
328 above inhibiting mechanotransduction can protect against cadmium-induced hair cell
329 death (Schmid et al., 2020), therefore, we tested whether inhibiting calmodulin only after
330 cadmium removal would still significantly protect against cadmium-induced hair cell
331 death. To do this fish were treated with varying doses of cadmium chloride from 0-30
332 μM for one hour, washed out of cadmium, and then immediately fixed or placed into
333 DMSO or 30 μM W-13 for two hours before fixation. Using this treatment paradigm we
334 no longer saw protection from calmodulin inhibition (Figure 3B). This suggests the
335 protection seen with cotreatment with the calmodulin inhibitor may be due to impacts on
336 mechanotransduction-dependent processes, though we can also not rule out a distinct
337 early role for calmodulin in the cell death process.
338 Calmodulin is known to activate CaMKII and inhibiting CaMKII has also been
339 shown to protect against cadmium-induced cell death in multiple cell types (Chen et al.,
340 2011; Liu et al., 2018; Liu and Templeton, 2007). Therefore, as our calmodulin results
341 did now show a clear protective impact we, subsequently tested if CaMKII inhibition
342 would impact cadmium-induced hair cell death. To do this we cotreated fish with 10 μM
343 of the CaMKII inhibitor KN-93, a dose previously shown to be effective in zebrafish
344 (Rothschild et al., 2013), and varying doses of cadmium chloride for three hours. We
345 found CaMKII inhibition did not significantly impact cadmium-induced hair cell death

346 (Figure 3C). This further supports the idea that calmodulin and CaMKII are not playing a

347 role in cadmium-induced hair cell death outside of calmodulin's impact on

348 mechanotransduction activity.

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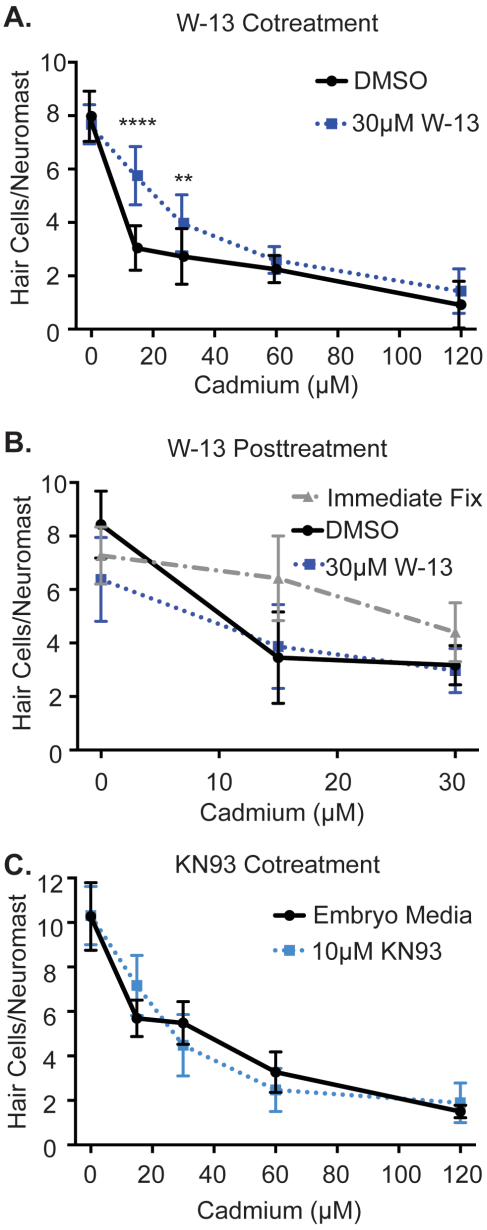

351

352 **Figure 3: Calmodulin inhibitor cotreatment protects against cadmium-induced hair cell death**
353 **whereas calmodulin inhibitor posttreatment and CaMKII inhibitor cotreatment fail to protect.** (A)
354 Hair cells remaining after a 3 hour cotreatment of either DMSO, a vehicle control, (solid line), or 30 μM of
355 the calmodulin inhibitor W-13 (dotted line) and varying doses of cadmium chloride. W-13 cotreatment
356 caused a significant reduction in hair cell death seen at lower cadmium doses (** = $p<0.01$, **** =
357 $p<0.0001$ by Two-Way ANOVA and Šídák's post-hoc test). (B) Hair cells remaining after a 1 hour
358 treatment with varying doses of cadmium chloride and either immediate fixation (dashed line), or a two
359 hour post-treatment with DMSO (solid line) or W-13 (dotted line). No significant protection was seen
360 comparing the DMSO and W-13 groups by Two-Way ANOVA and Tukey's post-hoc test. (C) Hair cells
361 remaining after a 3 hour cotreatment of either plain embryo media or 10 μM of the CaMKII inhibitor KN-93
362 and varying doses of cadmium chloride. No significant difference was seen between the two groups by
363 Two-Way ANOVA. n = 10 fish for each group. Error bars = Standard deviation.
364

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

367 We next wanted to test whether calcium flow from the ER or mitochondria would
368 impact cadmium-induced hair cell death as blocking the IP3 receptor and the
369 mitochondria calcium uniporter has been shown to protect other cell types against
370 cadmium-induced toxicity (Lee et al., 2005; Liu et al., 2016, 2018; Shih et al., 2005; S.
371 H. Wang et al., 2009; Wang et al., 2008, 2022). These inhibitors have also been shown
372 to protect hair cells from other forms of toxicity. (Esterberg et al., 2014; Wang et al.,
373 2019). To test this in hair cells we cotreated fish for three hours with either 1 μM of the
374 IP3 receptor inhibitor Xestospongin C or 500 nM of the mitochondrial uniporter inhibitor
375 RU360, doses that have previously been shown to protect against aminoglycoside-
376 induced hair cell death in the zebrafish lateral line (Esterberg et al., 2014), and varying

377 doses of cadmium chloride for three hours. In both cases, the inhibitor failed to
378 significantly protect against cadmium-induced hair cell death with RU360 causing a
379 slight enhancement of cadmium-induced hair cell death at the lowest cadmium dose
380 (Figure 4). Thus, our results indicate calcium flow from the ER and mitochondria is not
381 playing a significant role in cadmium-induced hair cell death.
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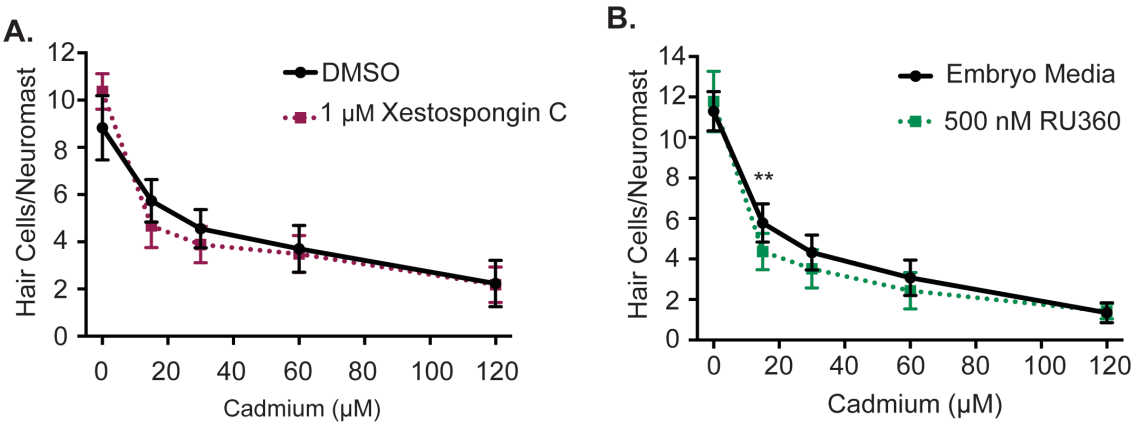


383

384 **Figure 4: Inhibiting ER and mitochondria calcium transfer does not protect against cadmium-**
385 **induced hair cell death.** Hair cells remaining after a three hour cotreatment of either EM or DMSO (solid
386 lines), or (A) 1 μM of the IP3 receptor inhibitor Xestospongin C, or (B) 500 nM of the mitochondrial
387 uniporter inhibitor RU360 (dotted lines) and varying doses of cadmium chloride. Neither of the inhibitors
388 significantly protected against cadmium-induced hair cell death with RU360 showing a significant
389 increase in cadmium-induced hair cell death at the lowest dose (** = $p<0.01$= by Two-Way ANOVA and
390 Šídák's post-hoc test). N = 10 fish/group. Error bars = standard deviation.
391

392 **Cadmium increases Akt and ERK signaling in neuromasts**

393 In many cell types cadmium increases the level of phosphorylated Akt and ERK
394 (Chen et al., 2008, 2011; Fujiki et al., 2014; Iryo et al., 2000; J. Kim et al., 2005; Kim et
395 al., 2008; Liu and Templeton, 2007; Misra et al., 2003; Xiao et al., 2009; Xu et al.,

396 2011). Activation of these pathways has also been seen in hair cells or supporting cells
397 in response to other forms of hair cell damage (Chung et al., 2006; Herranen et al.,
398 2018; Ingersoll et al., 2020; Lahne and Gale, 2008; Maeda et al., 2013). Based on this
399 evidence we tested whether the Akt or ERK pathways would be activated in either hair
400 cells or supporting cells of the zebrafish lateral line in response to cadmium. To do this
401 we treated fish with 30 μM of cadmium chloride for one hour and then fixed and stained
402 with an antibody for either pAkt or pERK. We found that treatment with cadmium
403 significantly increased the levels of pAkt seen in hair cells (Figure 5A & B). This
404 increase was lost when fish were cotreated with cadmium and 5 μM of the Akt inhibitor
405 MK-2206. We also saw a significant increase in pERK levels following cadmium
406 treatment that was lost when fish were cotreated with cadmium and 3 μM of the MEK
407 inhibitor PD184352 which suppresses ERK phosphorylation (Figure 5C & D). However,
408 unlike pAkt labeling which appeared localized to hair cells, pERK labeling appears
409 localized to supporting cells (Figure 5B & D). This is in agreement with past research in
410 mammals showing increased pERK in support cells but not hair cells in response to
411 other forms of hair cell damage (Herranen et al., 2018; Ingersoll et al., 2020; Lahne and
412 Gale, 2008; Maeda et al., 2013).

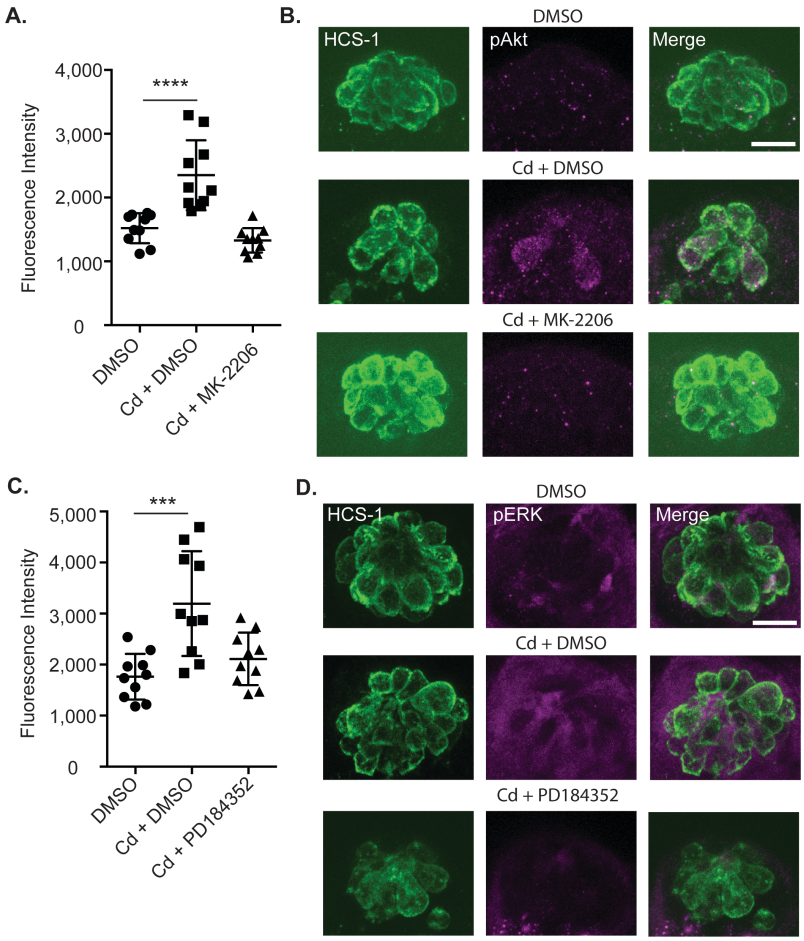


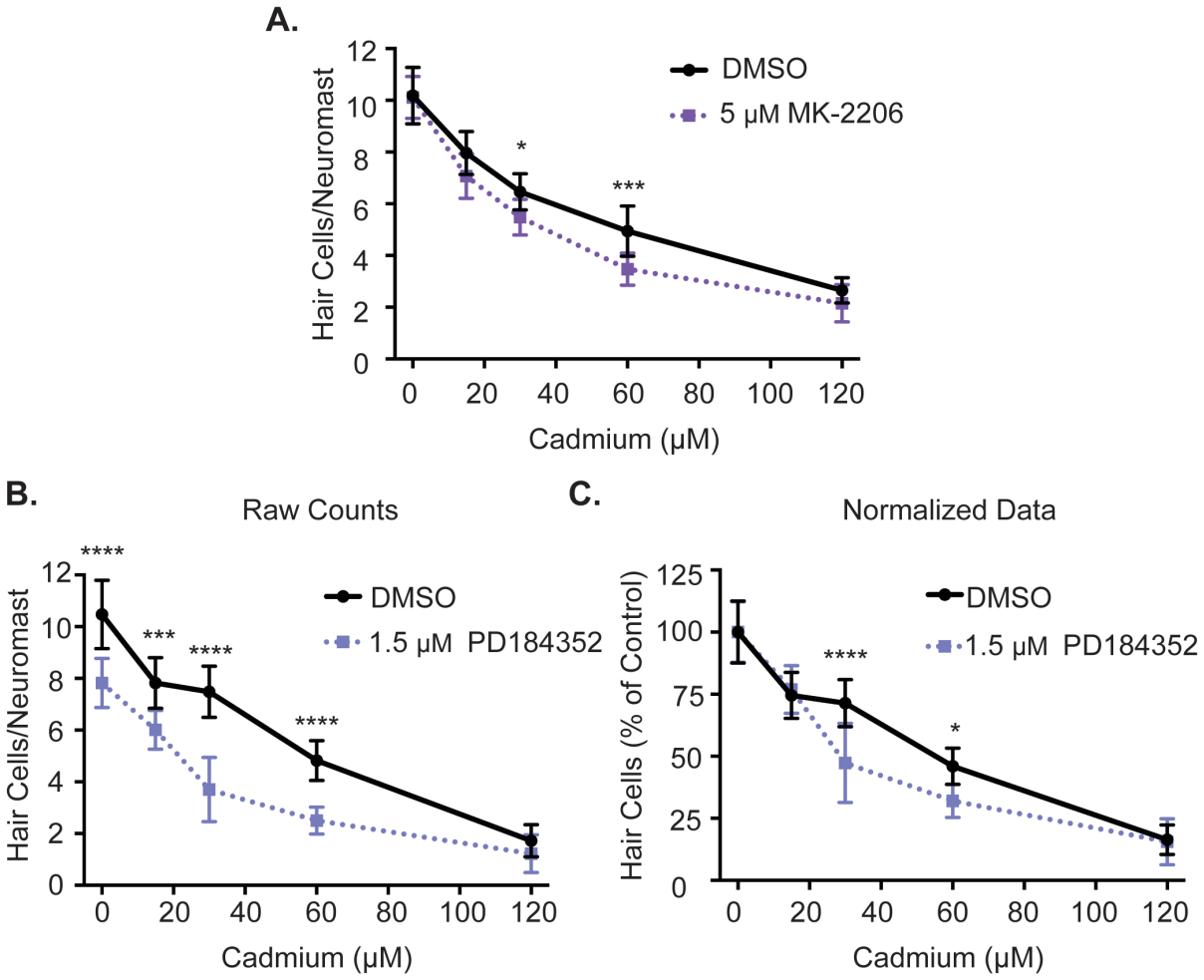
Figure 5: Cadmium treatment increases pAkt levels in hair cells and pERK levels in supporting cells. (A) Quantification of the fluorescence intensity of pAkt in fish treated for 1 hour with either DMSO alone, 30μM cadmium chloride (Cd) + DMSO, or 30μM Cd and 5 μM of MK-2206. (**** = p<0.0001 by ANOVA 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.

426 **Inhibiting Akt and ERK signaling pathways increases cadmium-induced hair cell**
427 **death**

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

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

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



457

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

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

469 ## DISCUSSION
470

471 Overall we found that cadmium-induced hair cell death showed both some
472 distinctions and similarities with cadmium-induced cell death in other cell types and
473 other forms of hair cell toxicity. One of the distinctions was that we failed to find a
474 significant role for calcium signaling. This is in contrast to both cadmium-induced cell
475 death in other cell types and other forms of hair cell death where calcium appears to be
476 a key mediator in the cell death process. We did however, see activation of both the Akt
477 and ERK signaling pathways. These two pathways are known to be activated both in
478 response to cadmium in other cell types and in other forms of hair cell toxicity. Though
479 unlike in other cell types, inhibiting these signaling pathways enhanced cadmium-
480 induced hair cell death rather than protecting against it similar to what has been shown
481 in other forms of hair cell toxicity.

482 While most of our manipulations of calcium signaling failed to protect against
483 cadmium-induced hair cell death, one manipulation that did cause significant protection
484 was the cotreatment of cadmium and a calmodulin inhibitor. It has been shown that

485 cadmium can activate calmodulin in lieu of calcium and it has been hypothesized this
486 may be one potential mechanism of cadmium-induced toxicity (Chao et al., 1984; Sutoo
487 et al., 1990). Treatment with inhibitors blocking calmodulin-dependent signaling
488 pathways has been shown to protect against cadmium in multiple cell types and tissues
489 including the kidneys, osteoblasts, and testes (Ha et al., 2016; Niewenhuis and
490 Prozialeck, 1987; Yang and Yang, 1997). However, these experiments are complicated
491 in hair cells by the fact that calmodulin inhibition also inhibits rapid apical endocytosis in
492 hair cells, a process dependent on mechanotransduction activity (Seiler and Nicolson,
493 1999). As inhibiting mechanotransduction activity is known to inhibit cadmium-induced
494 hair cell death (Schmid et al., 2020) we cannot definitively say that the protection we
495 see from calmodulin inhibition is due to a role for calmodulin in the cell death process.
496 The fact that protection is no longer seen when the calmodulin inhibitor is added after
497 cadmium treatment, rather than as a cotreatment, and the fact that CaMKII inhibition
498 was unable to protect hair cells from cadmium-induced hair cell death further supports
499 the idea that calmodulin inhibition is mainly impacting hair cell mechanotransduction
500 activity and cadmium entry rather than the cell death process.
501 We also failed to see protection against cadmium-induced hair cell death using
502 other calcium signaling manipulations including removing extracellular calcium and
503 inhibiting the IP3 receptor and mitochondrial calcium uniporter. These manipulations
504 have been shown to protect against cadmium-induced cell death in other cell types (Lee
505 et al., 2005; Liu et al., 2016, 2018; Shih et al., 2005; Wang et al., 2008; Xu et al., 2011).
506 However, these previous studies were carried out in mitotic cultured cells, including
507 mesangial cells, PC-12 cells, proximal tubule cells, osteoblasts, and osteosarcoma

508 cells, whereas our study was carried out *in vivo* in postmitotic cells, so this may be the
509 reason for the discrepancy. Indeed another study looking at neurons, another
510 postmitotic cell, failed to see protection from cadmium-induced cell death when
511 inhibiting the mitochondrial calcium uniporter (Tang et al., 2021). Calcium signaling in
512 general and specifically calmodulin, calcium release via the IP3 receptor, and activation
513 of the mitochondrial uniporter have all been shown to be important for the progression
514 of mitosis (Lagos-Cabré et al., 2020; Nugues et al., 2022; Rasmussen and Means,
515 1989; Takuwa et al., 1995; Zhao et al., 2019). Therefore, mitotic cells may be more
516 sensitive to cadmium-induced alterations in these calcium signaling pathways. Also,
517 even in mitotic cells, there have been discrepancies over the role of calcium signaling in
518 cadmium-induced cell death. For example, when looking at CaMKII inhibition a study in
519 an osteoblast cell line showed no effect (Ha et al., 2016), and a study in mesangial cells
520 showed an increase in cell death (Xiao et al., 2009).

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

529 While we did not find a significant impact of calcium signaling in cadmium-
530 induced hair cell death we did see an increase in Akt and ERK signaling similar to what

531 has been seen in other cell types. This was somewhat unexpected as in other cell types
532 this increase is calcium signaling dependent (Chen et al., 2011; Fujiki et al., 2013; Jiang
533 et al., 2015; Liu et al., 2018; Xiao et al., 2009; Xu et al., 2011). However, it has
534 previously been shown that ERK signaling can be activated in supporting cells even
535 following calcium chelation (Lahne and Gale, 2008). One potential alternative
536 mechanism for the activation of these pathways includes Ras and Raf signaling which
537 has previously been implicated in activating ERK signaling following hair cell damage
538 (Battaglia et al., 2003; Lahne and Gale, 2008). Another alternative activation route is
539 growth factor signaling. Increases in growth factor receptors have been seen after hair
540 cell damage (Lee and Cotanche, 1996; Zine and de Ribaupierre, 1999), and inhibiting
541 the IGF1R receptor has been shown to also increase susceptibility to aminoglycoside-
542 induced hair cell damage (Alassaf and Halloran, 2021), suggesting a role for growth
543 factor signaling in protecting hair cells from damage.

544 While the activation of ERK and Akt signaling was similar to what was seen in
545 other forms of cadmium-induced cell death, the fact that inhibiting these pathways
546 increased rather than protected against cell death was unexpected. This, however, is
547 not unprecedented. While many studies have found blocking Akt or ERK activation
548 protects cells against cadmium-induced cell death (Chen et al., 2008; Iryo et al., 2000;
549 Jiang et al., 2015; J. Kim et al., 2005; Zhang et al., 2017) others have found that it has
550 no effect (Låg et al., 2005; Liu and Templeton, 2007; Rigon et al., 2008; Wang et al.,
551 2008), and still others that death is increased similar to what we observed (Chao and
552 Yang, 2001; Chuang et al., 2000; Xiao et al., 2009). Some of this disparity may come
553 from differences in treatment paradigms. For example, in astrocytes ERK inhibition

554 protects against chronic but not acute cadmium treatment (Jiang et al., 2015). The type
555 of cell death also appears important as in macrophages ERK inhibition can protect
556 against necrosis but not apoptosis induced by cadmium (J. Kim et al., 2005).
557 Unlike what we found in calcium signaling, the role of the Akt and ERK signaling
558 pathways in cadmium-induced hair cell death appears similar to what has previously
559 been shown for other forms of hair cell damage. These pathways are activated in
560 response to both noise and other ototoxic drugs (Chung et al., 2006; Herranen et al.,
561 2018; Ingersoll et al., 2020; Lahne and Gale, 2008; Maeda et al., 2013), and inhibiting
562 them has been shown to increase hair cell death (Battaglia et al., 2003; Brand et al.,
563 2015; Chung et al., 2006; Haake et al., 2009; Hayashi et al., 2013; Kurioka et al., 2015).
564 Particularly striking is the fact that ERK activation is seen not in the hair cells
565 themselves but rather in the support cells. This has also been seen in the mammalian
566 inner ear in response to aminoglycosides, cisplatin, and noise (Herranen et al., 2018;
567 Ingersoll et al., 2020; Lahne and Gale, 2008; Maeda et al., 2013).
568 Cadmium is known to damage support cells (Ding et al., 2020), and thus this
569 ERK activation could be coming from a direct effect of cadmium on the support cells.
570 Alternatively, the damaged hair cells could be sending a signal to the support cells to
571 induce ERK activation. It has been shown that the damage-induced release of ATP and
572 gap junction signaling are important for support cell ERK activation following mechanical
573 injury of hair cells (Lahne and Gale, 2008). Once ERK is activated in support cells it
574 may mediate its protective effect by modulating support cell released HSP70. ERK
575 activation is important for HSP70 expression and folding (Lim et al., 2019; Xu et al.,
576 2018; Zhai et al., 2019). Support cells are known to release HSP70 in exosomes

following heat shock, with that HSP70 subsequently protecting hair cells from aminoglycoside-induced hair cell death (Breglio et al., 2020; May et al., 2013). The mechanism by which Akt activation is working in hair cell death is likely more straightforward as it is activated in the hair cells themselves. The activation of Akt signaling is well known to promote cell survival and inhibit cell death and multiple apoptotic regulators are Akt substrates (reviewed in Downward, 2004; Franke et al., 2003), providing a potential mechanism by which Akt activation could protect hair cells from cadmium-induced cell death.

The highly conserved activation and potential protective nature of Akt and ERK pathways suggest enhancing their activation could be a potential target for protecting hair cells from damage, however, as with other forms of cadmium-induced cell death, 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.

ACKNOWLEDGEMENTS

This work was supported by a Lafayette College Academic Research Committee faculty research grant to TMS and the Lafayette College Neuroscience program. We thank Amy Badillo for assistance with zebrafish care.

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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