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1 **Ototoxin-induced cellular damage in neuromasts disrupts lateral line function in**
2 **larval zebrafish**
3
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14 **HIGHLIGHTS**
15
16 The functional effects of known ototoxins were studied in the zebrafish lateral line.
17
18 Ototoxins cause cellular damage to hair cells present in lateral line neuromasts.
19
20 Consequences of the damage are attenuated startle, rheotactic and avoidance reflexes.
21
22 The zebrafish ear may contribute to initiating the noise-evoked startle reflex.
23
24 When combined, zebrafish assays are sensitive pre-clinical detectors of ototoxicity.

25 **ABSTRACT**

26 The ototoxicity of a number of marketed drugs is well documented, and there is an
27 absence of convenient techniques to identify and eliminate this unwanted effect at a pre-
28 clinical stage. We have assessed the validity of the larval zebrafish, or more specifically
29 its lateral line neuromast hair cells, as a microplate-scale in vivo surrogate model of
30 mammalian inner ear hair cell responses to ototoxin exposure. Here we describe an
31 investigation of the pathological and functional consequences of hair cell loss in lateral
32 line neuromasts of larval zebrafish after exposure to a range of well known human and
33 non-human mammalian ototoxins. Using a previously described histological assay, we
34 show that hair cell damage occurs in a concentration-dependent fashion following
35 exposure to representatives from a range of drug classes, including the aminoglycoside
36 antibiotics, salicylates and platinum-based chemotherapeutics, as well as a heavy metal.
37 Furthermore, we detail the optimisation of a semi-automated method to analyse the
38 stereotypical startle response in larval zebrafish, and use this to assess the impact of
39 hair cell damage on hearing function in these animals. Functional assessment revealed
40 robust and significant attenuation of the innate startle, rheotactic and avoidance
41 responses of 5 day old zebrafish larvae after treatment with a number of compounds
42 previously shown to induce hair cell damage and loss. Interestingly, a startle reflex
43 (albeit reduced) was still present even after the apparent complete loss of lateral line hair
44 cell fluorescence, suggesting some involvement of the inner ear as well as the lateral
45 line neuromast hair cells in this reflex response. Collectively, these data provide
46 evidence to support the use of the zebrafish as a pre-clinical indicator of drug-induced
47 histological and functional ototoxicity.

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54 **Key words:** zebrafish, ototoxicity, hair cell, startle reflex, hearing function, neuromast,
55 rheotaxis, avoidance behaviour

56

56 **1. INTRODUCTION***

57 Ototoxins are compounds known to cause damage to the hearing and balance systems
58 of animals. The existence of ototoxins has been documented since the 1800s, most
59 notably with the emergence of new medicines such as the antimalarials in 1843,
60 aminoglycoside antibiotics in the 1940s and the cytotoxic chemotherapeutics in the
61 1970s (Schacht and Hawkins, 2006). In more recent times, it has been reported that
62 over 130 common medicines are ototoxic (Seligmann et al., 1996). It is currently
63 estimated that 9 million people in the UK suffer from hearing impairment, with many
64 more suffering from vestibular damage and tinnitus (AHL, 2011). In a proportion of these
65 individuals, hearing and vestibular damage will have been induced by common ototoxic
66 medications such as aminoglycosides, antimalarials, salicylates, loop diuretics and
67 antineoplastic agents (Rybak, 1986). Despite this, there is currently no standard pre-
68 clinical assay for drug-induced ototoxicity.
69
70 In recent years, studies have begun to explore the value of the zebrafish as a model for
71 assessing ototoxicity. Zebrafish are so-called “hearing specialists”, using a combination
72 of their acoustico-lateralis system, swim bladder and Weberian ossicles to detect sound
73 (Fay and Popper, 1974). The acoustico-lateralis system consists of an inner ear
74 (containing semicircular canals and the utricular, saccular and lagenar maculae) and a
75 lateral line system composed of mechanosensory neuromast organs (Bever and Fekete,
76 2002; Ghysen and Dambly-Chaudière, 2004; Metcalfe et al., 1985; Metcalfe, 1989;
77 Raible and Kruse, 2000; Whitfield et al., 2002). The function of the lateral line (LL) is to
78 detect local water flow and low frequency vibration, mediating behaviours such as
79 schooling and predator avoidance (Dijkgraaf, 1963; Ghysen and Dambly-Chaudière,
80 2004; Kaus, 1987). From 5 days post fertilisation (dpf) until adulthood, there are no
81 significant alterations in the sensitivity of the lateral line to vibratory stimuli, making 5dpf
82 an ideal age for both histological and functional studies (Zeddies and Fay, 2005).
83

* *Abbreviations*: AER, auditory evoked response; ALD, average large distance; dpf, days post fertilisation; DWC, dilution water control; GFP, green fluorescent protein; ISI, inter-stimulus interval; LL, lateral line; LLC, long-latency C-start; MTC, maximum tolerated concentration; pLL, posterior lateral line; SC, solvent control; SLC, short-latency C-start; SPL, sound pressure level; SR, seeker response; Vpp, voltage peak to peak.

84 The emphasis of previous studies of ototoxicity in larval zebrafish has been on the
85 histological damage caused to the neuromast hair cells (Chiu et al., 2008; Coffin et al.,
86 2009; Harris et al., 2003; Ou et al., 2007; Owens et al., 2007). More recent work has
87 focused on identifying genetic and chemical modulators of ototoxic damage and the
88 regeneration of LL hair cells (Brignull et al., 2009; Coffin et al., 2010; Ou et al., 2009; Ou
89 et al., 2010; Owens et al., 2008; Owens et al., 2009; Ton and Parng, 2005). In addition
90 to ototoxicity studies, the zebrafish has also been shown to be a useful model of human
91 congenital hearing loss (Whitfield, 2002), as well as demonstrating similar vestibular
92 functional circuits to humans (Mo et al., 2010).
93
94 In terms of quantifiable functionality, the acoustically-evoked startle reflex, or C-start, is
95 in place by 5 dpf, at around the same time the hair cells of the lateral line are proposed
96 to be mature and mechanotransductively active (Kimmel et al., 1974; Murakami et al.,
97 2003; Santos et al., 2006). This escape reflex, proposed to be mediated by the lateral
98 line, is instrumental in predator evasion and can also be evoked by touch and visual
99 cues (McHenry et al., 2009). High speed auditory-evoked responses (AERs) are
100 characterised by an initial whole body flexion (with an estimated angle of 90-220º
101 occurring in the first 6-14 milliseconds after stimulation), followed by a strong counter-
102 bend and subsequent smaller flexions. The response lasts approximately 40ms and is
103 initiated by Mauthner, MiD2cm and MiD3cm reticulospinal neurons (Kimmel et al., 1980;
104 Liu and Fetcho, 1999). The C-start can be described as short or long latency (SLC and
105 LLC respectively), with the SLC being the stronger response (Burgess and Granato,
106 2007). Similarly, the ability of larvae to orientate to, and swim against, currents is termed
107 rheotaxis (Arnold, 1974), and rheotactic behaviours can clearly be observed in free-
108 swimming larvae at 5 dpf. Again, rheotaxis has been shown to be mediated in part by
109 the lateral line (Johnson et al., 2007; Montgomery et al., 1997).
110
111 This study aimed to investigate the pathological and, perhaps more importantly, the
112 functional consequences of exposure to a range of known human and non-human
113 mammalian ototoxins in the zebrafish lateral line. This was achieved by quantifying the
114 amount of hair cell damage using a previously described vital dye-based fluorescence
115 assay, and by measuring the ability of animals to respond to auditory and other
116 mechanosensory cues via stereotypical AER, rheotactic and avoidance behaviours. To
117 our knowledge, this is the first study to use integrated auditory-evoked startle, rheotaxis

118 and seeker response assays to demonstrate the functional consequences of hair cell
119 damage in the larval zebrafish following exposure to a range of ototoxins. Our data
120 support the use of the zebrafish larva as a potentially valuable surrogate model for
121 assessing drug-induced damage to mammalian inner ear hair cells.
122

123 2. METHODS

124 *2.1 Animal husbandry*

125 Wild-type zebrafish (AB strain, Sheffield brood stock, UK) adults were used both at
126 Sheffield and Brixham Environmental Laboratory. *Tg(pou4f3::mGFP)* adult zebrafish
127 (Xiao et al., 2005) were used at Sheffield only. Adult fish were maintained on a 14 hour
128 light/10 hour dark cycle according to standard protocols (Nüsslein-Volhard and Dahm,
129 2002) and induced to breed using group spawning tanks. Egg collection and staging was
130 performed according to standard procedures (Kimmel et al., 1995; Nüsslein-Volhard and
131 Dahm, 2002). Larvae were raised at a density of 50 (or 128 in the case of the raising
132 density experiments) per 90 mm Petri dish (Sterilin, Newport, UK), at 28°C (± 0.5°C) in
133 standard E3 culture medium (5mM NaCl, 0.17mM KCl, 0.33 mM $CaCl_2$, 0.33 mM
134 $MgSO_4$, 0.0001% methylene blue). For vital dye staining, fish were transferred into E3
135 culture medium without methylene blue at 2 dpf to prevent methylene blue uptake into
136 cells.
137

138 All compound exposures, hair cell assessments, and functional testing were carried out
139 on larvae aged 5 dpf.
140

141 *2.2 Compound selection and treatments*

142 All positive compounds were selected based on reported ototoxic effects in humans and
143 non-human mammals (Brunton et al., 2006; Chiu et al., 2008; Joint Formulary
144 Committee, 2011; Linbo et al., 2006; Prestayko et al., 1979; Rybak, 1986), with the
145 exception of copper sulphate, which was chosen for its known ototoxicity in fish
146 (Hernández et al., 2006; Olivari et al., 2008).
147

148 Negative control compounds were selected that exhibited no reported ototoxicity in any
149 species, and had no additional reported behavioural, locomotor or visual effects (Brunton
150 et al., 2006; Colucci et al., 2002; Crino et al., 1999b; Crino et al., 1999a; Joint Formulary

151 Committee, 2011; Palumbo et al., 2010; Pinguet et al., 2000; Reller et al., 1973; Ribner
152 et al., 1983; Salvo et al., 2007; Sandler and Ettinger, 1999; Von der Maase et al., 1999).
153
154 All compounds and reagents were obtained from Sigma-Aldrich (UK), unless otherwise
155 stated (for test compound details, see Suppl. Table S1). Acute compound exposures
156 were performed in 24-well plates (Corning Costar, NY, USA) by immersion of 5 dpf
157 larvae in E3 alone (also referred to as dilution water control (DWC)), or E3 containing 0-
158 2% DMSO or methanol as a solvent control (SC). The Maximum Tolerated
159 Concentration (MTC) of each compound was determined by initial exposures (minimum
160 of 6 larvae per concentration for each trial) and was classed as the concentration of
161 compound that induced ≥20% mortality after a timed exposure (Suppl. Table S1).
162
163 Using the MTC data, appropriate concentration ranges and exposure times were defined
164 for the definitive compound exposures and subsequent hair cell damage and functional
165 assessments. Treatment times and concentrations were specific to each compound
166 tested (Suppl. Table S1). After compound exposure, larvae were rinsed 3 times with E3
167 and left to recover for a further 60 minutes prior to further processing. For each
168 exposure, an appropriate DWC/SC was used alongside a positive control, which was
169 neomycin (100 or 300 µM dependent upon assay type).
170
171 *2.3 Neuromast hair cell staining and scoring*
172 The fluorescent vital dyes DASPEI (2-[4-(dimethylamino)styryl]-N-ethylpyridinium iodide
173 (CAS No. 3785-01-1)) and FM1-43FX (Invitrogen; F-35355) were used to mark hair cells
174 within the lateral line neuromasts. After compound exposure and recovery, larvae were
175 incubated in E3 containing either DASPEI (0.05 mg/mL) for 20 minutes, or FM1-43FX (3
176 µM) for 45 seconds. All labelling was carried out under dark conditions. Following
177 DASPEI and FM1-43FX exposure, larvae were rinsed 3 times with E3. For both scoring
178 and imaging, larvae were anaesthetised in MS222 (0.5 mM 3-aminobenzoic acid ethyl
179 ester). Larvae were scored for fluorescence based on DASPEI labelling alone. For
180 scoring, larvae were observed under an epifluorescence dissecting microscope (Leica
181 MZ-12 FLIII) equipped with a GFP1 filter set (excitation 425/60 nm; barrier filter 480 nm).
182 Nine individual neuromasts of the posterior lateral line (pLL) that were present at 5 dpf
183 were scored for fluorescence (Fig. 2Ji). The scoring scheme used was based on
184 previous work (Harris et al., 2003) and was as follows: strong staining indicating the

185 presence of hair cells, score = 2; weak reduced staining indicating the presence of fewer
186 or damaged hair cells, score = 1; an absence of staining indicating the absence or
187 severe damage of hair cells, score = 0. See Fig. 2Jii, Jiii for examples of neuromasts
188 exhibiting strong and weak staining. Each fish was scored on both sides, giving a
189 maximum total score of 36 per larva. A minimum of 8 larvae per treatment group per trial
190 was scored and an average taken from 3 to 4 trials (except in the case of gemcitabine,
191 where 5 larvae per treatment group were assessed over a single trial). The concentration
192 relating to approximately a 50% reduction in score (IC_{50}) was ascertained for each
193 compound using either log(concentration of test compound) versus response (three
194 parameters) or log(concentration of test compound) versus response-variable slope (four
195 parameters) curve-fitting equations (Prism 5.0 (GraphPad Software Inc., USA)). The best
196 fit values (Fig. 3J) were used later as the IC_{50} for the startle assay. Six positive and three
197 negative compounds were selected for use in further behavioural assays to study the
198 functional consequences of ototoxin exposure.
199
200 *2.4 Startle assay*
201 *2.4.1 Experimental apparatus*
202 A schematic of the experimental setup used to assess lateral line functionality is shown
203 in Fig. 1. Vibratory stimuli (sinusoidal/square tone bursts) were generated using a 2.0
204 MHz PCGU1000 PC function generator and controlled using PClab2000SE generator
205 interface software (Velleman Inc., USA). Stimuli were amplified (SP103 amplifier;
206 Acoustic Solutions, UK) and directed through a vertically oriented 15” (381 mm) speaker
207 cone, housed in a wooden casing. Acoustic stimuli were delivered to the larvae through
208 a 6.4 mm thick translucent plastic base plate, bolted to the speaker case, to which the
209 microplate containing the larvae was fixed. Larvae under investigation were placed into
210 24-well plates with a single larva per well. In all cases, the external surfaces of the plates
211 were frosted using acetone. This served to provide a uniform opaque background
212 against which to observe animal movement, and also to prevent larvae from receiving
213 visual startle cues from animals in neighbouring wells. The 24-well plate was securely
214 bolted to the base plate. Wave output parameters were measured using a calibrated 35
215 MHz 4-channel digital storage oscilloscope (PM3305 (U), Phillips, Netherlands). Larval
216 startle responses (auditory-evoked high-speed movements) were captured using a high-
217 speed 0.3 megapixel digital video camera (GRAS-03K2M, Point Grey Research Inc.,
218 Canada) set at a 60 frames/second capture rate. Recordings were visualised and

219 processed using VideoTrack for Zebrafish™ software (Viewpoint Inc., France). The
220 Viewpoint software automated the allocation of each component of the movement of the
221 larvae into bins based on thresholds that were previously set following visual observation
222 of the speed of startle in 5 dpf larvae. The tracking software threshold was set at 21.1
223 mm s^{-1}, which was the level required to discriminate between stereotypical startle and
224 normal movement. This threshold was determined by comparing multiple video
225 recordings on a score by eye versus automation basis (data not shown).
226
227 [Figure 1 to go near here]
228
229 *2.4.2 Stimulus form*
230 Stimuli consisted of sinusoidal or square waves of either 440 or 540 ms duration. The
231 stimulus frequencies used were 40, 50, 100, 150, 200, 300, 400, and 500 Hz. For each
232 frequency, conditions were optimised for voltage peak to peak (Vpp) and amplifier
233 volume (-dBTP) to ensure purity (non-distortion) of the resultant sound wave (Suppl.
234 Table S2).
235
236 *2.4.3 Sound pressure level measurement and calibration*
237 The 17 mm diameter wells of a 24-well plate were too small for direct measurement of
238 the sound pressure level (SPL) using a standard hydrophone. Therefore, to measure the
239 SPL inside the wells of the microplate indirectly, a small microphone (TCM110 omni-
240 directional electret-condenser, RS Components Ltd., UK), waterproofed with a latex
241 sheath, was calibrated against a pre-calibrated SPL meter (Castle GA213; Castle Group
242 Ltd., UK) in air, and subsequently in wells filled with 2 mL of E3. Measurements were
243 taken over all test frequencies at previously optimised voltage settings (1.6–4.5 Vpp) for
244 a small range of amplifier volumes (-40 to -10 dBTP). The output of the microphone was
245 routed through the calibrated oscilloscope providing a direct measure between the SPL
246 meter and the microphone, and consequently the attenuation in air versus that in fluid.
247 The SPL meter was clamped at 35 mm above one of the four central wells (well 4C). In
248 addition, the microphone wire was adhered directly to the SPL meter, to ensure that it
249 was vertically positioned and adjacent to the meter. The microphone recordings in either
250 air or water were then taken inside the centre of the well adjacent to well 4C, 5 mm
251 above the bottom of the well.
252

253 *2.4.4 Determination of threshold responses of larvae*
254 Threshold responses of larvae were determined for each frequency to ensure that
255 maximum larval responses occurred at the lowest possible volume. A sub-threshold
256 stimulus was given, which represented the lowest audible volume, and this was followed
257 by 5 equally spaced sequential stimuli, up to a maximum volume threshold (the
258 maximum volume attainable before distortion of waveform). Stimulus volume was
259 chosen based on the best response at the lowest volume for each frequency. The
260 responses of 24 larvae were tested over 3 repeated stimuli for each individual frequency.
261
262 *2.4.5 Plate type*
263 To test whether the capacity of the assay could be increased by using more wells per
264 experiment, larval AERs were tested in either 24- or 48-well plates. For each individual
265 test, larvae were equilibrated on the startle platform for a minimum of 30 minutes.
266 Animals were then presented with eight selected frequencies (40, 50, 100, 150, 200,
267 300, 400 and 500 Hz) at previously optimised voltages and volumes, with a duration of
268 440 ms or 540 ms. The ISI was 60-80 seconds. For both conditions (24- and 48-well
269 plates), tests were carried out in triplicate with a total of either 24 or 48 larvae per trial.
270 All subsequent experiments described used the 24-well plate format.
271
272 *2.4.6 Wave type*
273 Twenty-four larvae (5 dpf) were individually loaded into each well of a microplate and
274 allowed to equilibrate for 30 minutes. Three stimuli (200Hz, 440 ms duration) of either a
275 square or sinusoidal waveform were presented with a minimum inter-stimulus interval
276 (ISI) of 60 seconds. The averaged AERs of the larvae for each wave type were then
277 compared.
278
279 *2.4.7 Density of raising*
280 To assess the effect of the density of culture on animal startle responses, larvae were
281 raised to 5 dpf at a density of 50 or 128 per Petri dish. Larvae were individually loaded
282 into microplates, and left to equilibrate on the apparatus, as previously described. Larvae
283 were presented with 10 identical sinusoidal stimuli of 200 Hz (440 ms duration, 60-80
284 second ISI). This trial was repeated 3 times for each set of animals (24 larvae per test
285 condition, per trial). To compare the response of animals raised at 50 or 128 per Petri
286 dish, an average large distance (ALD) measurement (distance travelled at >21.1 mm s^{-1})

287 was taken for each animal over the 10 stimuli, and the ALD for all animals across 3 trials
288 for each condition were calculated. To assess the development of fish housed at the two
289 different densities, larvae were raised as described (section 2.1) and imaged (for detail
290 see section 2.6).
291
292 *2.4.8 Choice of an appropriate interval for stimulus presentation*
293 To determine possible habituation to acoustic stimulation, the response of larvae to
294 varying ISIs was investigated. For each experimental trial, five separate ISIs (15, 30, 45,
295 60 and 120 seconds) were tested in turn on the same twenty-four test animals. Based on
296 previous studies, a minimum gap of 15 minutes was placed in between each interval
297 tested to allow animals to recover from any habituation (Best et al., 2008). For each ISI
298 tested, multiple sinusoidal stimuli of 200 Hz were presented to larvae. The habituation
299 experiment was carried out 3 times using different clutches of larvae. A test was also
300 performed with a 1 second ISI, to exemplify true habituation.
301
302 *2.4.9 Assessment of the larval startle response after ototoxin exposure*
303 Larvae were individually loaded into each well in a total of 2 mL E3 medium, and left
304 overnight. Three microplates were prepared per assessment, which provided data from
305 12 animals at each of the treatments. Before treatment, rheotaxis and seeker response
306 testing was performed (section 2.4.11). Larvae were then immersed in compound (see
307 section 2.2; Suppl. Table S1), and at the end of treatment, animals were rinsed 3 times
308 in E3 and rheotaxis and seeker response scoring was repeated. Larvae were then
309 transferred to frosted microplates and placed onto the startle platform to equilibrate. A
310 baseline recording of 60 seconds was taken at the beginning of equilibration to monitor
311 pre-stimulation movement. Next, 8 sinusoidal tone bursts (section 2.4.2) were presented
312 with a randomised ISI of 60-80 seconds. During each stimulus, a 10 second video
313 recording was taken to capture AERs. Each compound trial was carried out over 3
314 plates. Treatments were allocated by column using an in-house random number
315 generator to remove positional bias. For each plate, 3 control columns were assigned to
316 ensure that a startle response could be identified against a baseline recording. A single
317 pre-determined control column per plate was used for statistical analysis to make animal
318 numbers equal for each treatment group. To ensure identical exposure conditions for all
319 plates, treatments were staggered. Each compound was tested over 3 separate trials
320 using different clutches of larvae, at a temperature of 28±0.5°C.

321
322 *2.4.10 Baseline recording*
323 To examine possible sedation effects of test substances, we analysed activity profiles
324 from baseline recordings. Video footage of 60 seconds duration was taken for each plate
325 at the start of the equilibration time and the Viewpoint software was used to determine
326 the average speed of each animal. For comparison with animals sedated with
327 anaesthetic, larvae were immersed in 0.1 μg/mL or 0.0125 μg/mL MS222 for 30 minutes.
328 For the full startle assay and anaesthetic testing, a total of 72 larvae per trial were
329 examined. Each compound was tested over 3 trials using different clutches of larvae.
330
331 *2.4.11 Rheotaxis and seeker response (SR) testing*
332 To examine functional damage after neuromast disruption further, and/or drug-induced
333 behavioural effects, the same animals as in section 2.4.9 were subjected to tests for
334 rheotaxis and seeker approach responsiveness. For rheotaxis testing, 0.5 mL E3 was
335 dispensed at high speed through a pipette to the side of individual wells in turn, resulting
336 in a circular flow of fluid around the well. This was repeated, so that each larva was
337 tested twice. Rheotaxis behaviour was scored as follows: 2 = immediate rheotaxis
338 (orientation of the larva towards the direction of flow); 1 = rheotaxis observed on second
339 attempt; 0 = no rheotaxis. For seeker response (SR) evaluation, a previously described
340 method was used (Winter et al., 2008). Briefly, larvae were tested by gently approaching
341 them from behind with a 20 μL pipette tip (the "seeker"). The response was scored as
342 follows: 3 = uncompromised escape (immediate movement as soon as the tip touched
343 the water surface, at a distance from the larva); 2 = reduced escape, which required the
344 seeker to be moved towards the larva; 1 = escape following touch; and 0 = absence of
345 an escape with touch (see diagram in Graphical Abstract).
346
347 *2.5 Data collection and analysis*
348 For the DASPEI assay, data were collected manually by scoring (section 2.3) and all raw
349 data were analysed using Prism 5.0 (GraphPad Software Inc., USA). For the startle
350 assay, data were collected as previously described (section 2.4.1). Raw data from the
351 Viewpoint software were then processed using macros written in Microsoft Excel, and
352 analysed using Prism 5.0.
353

354 In all cases, datasets were assessed for deviations from a normal distribution using the
355 D'Agostino-Pearson normality test, and for equality of variances using Bartlett's statistic.
356 If tests indicated that a dataset deviated from a Gaussian distribution or had unequal
357 variances, non-parametric methods were applied (e.g. Kruskal-Wallis and Dunn's tests).
358 For all other datasets, parametric methods were appropriate (e.g. ANOVA).
359
360 In all analyses, we adopted a threshold value of 0.05α as statistically significant. In all
361 cases, error bars are plotted as SEM.
362
363 *2.6 Imaging*
364 Anaesthetised fish were mounted in 1% low melting point agarose (CAS No. 9012-36-6)
365 and imaged using a BX51 compound microscope, Camedia (C-3030ZOOM) camera and
366 Cell B software (Olympus, UK). Images were assembled using Adobe Photoshop
367 software (Adobe Inc., USA).
368
369 **3. RESULTS**
370 *3.1 Neuromast hair cell staining and scoring*
371 To assess the effect of compounds on hair cell integrity, we treated larvae (5 dpf) with
372 known mammalian ototoxins or control compounds, and then stained treated fish with
373 two vital dyes, DASPEI and FM1-43FX, that are preferentially taken up by hair cells. All
374 positive (ototoxic) compounds tested (neomycin, streptomycin, gentamicin, cisplatin,
375 aspirin, copper sulphate) resulted in reduced DASPEI staining and demonstrated a
376 statistically significant concentration-dependent reduction in DASPEI score, indicative of
377 hair cell damage or loss (Figs. 2 and 3). Staining of the nasal epithelium was not
378 affected by exposure to the various ototoxins tested; this served as an internal control for
379 the reliability of the DASPEI staining method. The observed reduction in DASPEI
380 staining also correlated with a decrease in FM1-43FX labelling in all cases, except with
381 aspirin treatment (Fig. 2Fiv). In the case of aspirin, FM1-43FX labelling was not strongly
382 reduced, indicating that some mechanotransductive activity remained after treatment.
383 Three of the four compounds selected as negative controls (amoxicillin, cefazolin,
384 melphalan) had no significant effect on hair cell labelling with either dye (Figs. 2 and 3).
385 However, gemcitabine gave an unexpected positive result in the initial DASPEI test
386 (Suppl. Fig. S1).
387

388 [Figure 2 to go near here]
389 [Figure 3 to go near here]
390
391 *3.2 Sound pressure level measurement and calibration*
392 Summary data from SPL calibrations are shown for each frequency in Suppl. Fig. S2.
393 Overall, there was a correlation of measurement between both media at individual fixed
394 frequencies. SPL meter readings were positively correlated with the readings taken in
395 both air and water (as the SPL meter reading increased, so did the SPL calculated from
396 the microphone recordings). As predicted, there was a difference in microphone SPL
397 measurements in air versus water for each frequency tested, varying from 14 to 24 dB
398 SPL. Interestingly, there was an increase in the SPL recorded with the microphone as
399 the frequency setting decreased, when compared with the SPL meter. This was
400 unexpected and is explained by the resonance of the apparatus, which could not be
401 detected by the SPL meter in air but was detectable in water using the microphone.
402
403 *3.3 Determination of threshold response of larvae to stimuli over a range of frequencies*
404 For all frequencies, the minimum startle responsiveness chosen was 40% (>40% of fish
405 responding to stimulus). A summary of the manual scoring data is shown in Suppl. Table
406 S3. 500 Hz was the highest frequency that elicited a larval response that could be
407 tested using the speaker. Above this frequency, no reproducible larval response could be
408 elicited, as the greater current required to drive stimulation at higher frequencies evoked
409 a double startle as the speaker audibly clicked off. In addition, fewer than 40% of larvae
410 per plate responded to even the loudest stimulus before distortion of the waveform at
411 frequencies of 800 Hz and above.
412
413 *3.4 Plate type*
414 As summarised in Figure 4A, we found a significant effect of plate type (24- versus 48-
415 well) on the startle response, with larvae in the 24-well plate displaying a stronger startle
416 response overall (two-way ANOVA; F (1, 32) = 8.72, P = 0.0058). The frequency of the
417 stimulus had no effect on response strength (two-way ANOVA; F (7, 32) = 0.72, P =
418 0.6529). It is noteworthy that plate type did not have the same effect at all frequency
419 values (two-way ANOVA; F (7, 32) = 0.66, P = 0.7028). Taken together, these data
420 supported the use of 24-well plates in the assay to ensure the most reproducible
421 reactions.

422
423 *3.5 Wave type*
424 We sought to determine whether an alternative stimulus wave shape (square) could elicit
425 an improved larval response compared with a sinusoid (Fig. 4B, 4C). We found that both
426 sinusoidal and square waveforms produced equally strong AERs (Paired *t*-test; t =
427 0.6267, 22 *d.f.*, P = 0.5373), with no difference in the proportion of responding larvae
428 (Paired *t*-test; t = 0.6146, 23 *d.f.*, P = 0.5449). We therefore chose to use the more
429 conventional sine wave in the final procedure.
430
431 *3.6 Density of raising*
432 We found that larvae raised at a density of 50 per Petri dish produced a significantly
433 stronger startle movement than those raised at 128 per dish (Mann Whitney U test; Fig.
434 4D; 2.09 ± 0.13 (50/dish) compared with 1.62 ± 0.16 mm s^{-1} (128/dish); *Mann Whitney U*
435 *Statistic* = 1743, 1 *d.f.*, P = 0.0033). Larvae raised at the lower density displayed
436 increased responsiveness to the stimulus when compared with those reared at the
437 higher density (Unpaired *t*-test; Fig. 4E; 0.85 ± 0.044 compared with 0.64 ± 0.039; t =
438 3.707, 142 *d.f.*, P = 0.0003). The developmental progression and health of the larvae,
439 however, did not appear to be affected by the altered rearing conditions. Qualitative
440 assessment revealed that larvae raised at both densities inflated their swimbladders,
441 and developed normal-sized jaw, ear and eye structures and a functional digestive tract
442 (Suppl. Fig. S3).
443
444 *3.7 Choice of an appropriate interval for stimulus presentation*
445 To determine whether there was any habituation at different inter stimulus intervals
446 (ISIs), we measured the startle response of individual larvae over 10 stimuli, and used
447 linear regression to analyse the data. Inducing a deliberate habituation using 1 second
448 ISIs showed that control fish were capable of learned habituation (Fig. 4F; Linear
449 regression: r^2 = 0.7882, F (1, 7) = 26.05, P = 0.0014). The responsiveness, but not
450 strength, of the AER was significantly decreased with an ISI of 30 seconds over 10
451 stimuli, which was indicative of habituation. Intervals of 45 seconds or longer did not
452 cause significant habituation (either in strength or responsiveness of AER; P >0.05). An
453 ISI of 60 seconds (with a random variation up to 80 seconds) was selected for further
454 work, as this did not cause habituation (Fig. 4F; Linear Regression: r^2 = 0.1179, F (1, 7)
455 = 0.9359, P = 0.3656). The initial stimulus induced a consistently stronger AER than

456 subsequent stimuli, as shown by the one sample *t*-test (Fig. 4F; t (8) = 13.68, $P <$
457 0.0001; t (8) = 26.46, $P < 0.0001$ for 1 and 60 second ISIs respectively), and was
458 therefore removed from the datasets before performing regression tests. The maximum
459 number of stimuli chosen in the final startle protocol was 8.
460
461 [Figure 4 to go near here]
462
463 *3.8 Assessment of the larval startle response after ototoxin exposure*
464 As summarised in Figure 4, larvae treated with neomycin, streptomycin, gentamicin and
465 aspirin demonstrated a significantly reduced high-speed distance moved in response to
466 auditory stimulus. A reduction in startle response was (with the exception of gentamicin)
467 only detectable at the highest concentrations that damaged nearly all the hair cells,
468 based on data from the DASPEI assay. There was no statistically significant decrease in
469 AER at the IC_{50} level for any other compound, despite observing some reduction in high
470 speed larval movement. Compounds for which DASPEI staining was unaffected
471 (negative control compounds) had no significant effect on the response of animals to
472 auditory stimulation.
473
474 Interestingly, immersion in cisplatin and copper sulphate did not result in a significantly
475 decreased AER, even at the concentrations required to elicit complete loss of DASPEI
476 staining (Fig. 5D, E). As an independent assessment of hair cell integrity, we examined
477 GFP expression in the transgenic *Tg(pou4f3::mGFP)* line. In transgenic larvae treated
478 with copper sulphate, GFP expression was unaltered in pLL hair cells up to one hour
479 post-treatment, even though live hair cell labelling with DASPEI and FM1-43FX was
480 reduced. Conversely, neomycin treatment reduced GFP expression in the pLL (Fig. 6).
481 The presence of GFP-positive hair cells in the pLL of copper-treated larvae could
482 indicate that some hair cell function remains, accounting for the unaltered AER. This also
483 suggests that GFP expression in the *Tg(pou4f3::mGFP)* line is a more reliable indicator
484 of hair cell function than either DASPEI or FM1-43FX staining.
485
486 [Figure 5 to go near here]
487
488 At the top dose level of treatment with all ototoxins, the startle response was not
489 completely abolished, suggesting some maintenance of auditory function (Fig. 5). After

490 acute treatment with ototoxins, the inner ear hair cells of the transgenic
491 *Tg(pou4f3::mGFP)* line maintained a high level of GFP expression, even when the GFP-
492 positive cells of the pLL were absent (Fig. 6). This is likely to be because the ototoxins
493 were unable to access the inner ear, and so inner ear hair cells were protected from
494 damage. These data indicate a role for the inner ear in mediating the larval startle
495 response.
496
497 [Figure 6 to go near here]
498
499 *3.9 Baseline recording*
500 Analysis of baseline recordings revealed no significant effect on the average swimming
501 speed of treated larvae when compared with control groups for all but one of the test
502 substances (Table 1). Treatment with 100 μM neomycin resulted in a significant
503 decrease in average speed compared with untreated larvae (2.119 ± 0.23 compared with
504 3.467 ± 0.20 mm s^{-1} respectively; Kruskal-Wallis test, H = 15.83, 2 *d.f.*, P = 0.0004),
505 suggesting that neomycin had a sedative effect (Table 1). To assess this possibility,
506 neomycin was retested in the final startle protocol alongside two concentrations of
507 MS222 anaesthetic (0.1 μg/mL or 0.0125 μg/mL). The higher concentration of
508 anaesthetic induced a state of full sedation and non-responsiveness after 2 minutes. The
509 lower concentration was insufficient to cause full sedation after a 30 minute exposure
510 and caused only a mild decline in responsiveness. The average speed of neomycin-
511 treated larvae was significantly faster than those treated with the higher concentration of
512 MS222 (Suppl. Fig. S4A; 2.409 ± 0.27 versus 1.229 ± 0.21 mm s^{-1}; Dunn's test, P
513 <0.05), but not different from the lower concentration (2.409 ± 0.27 versus 2.572 ± 0.19
514 mm s^{-1}; Dunn's test, $P >0.05$). Thus, the effects of 100 μM neomycin treatment are
515 comparable to slight, not heavy, sedation.
516
517 [Table 1 to go near here]
518
519 *3.10 Rheotaxis and seeker response (SR) testing*
520 Significant disruption of rheotactic behaviour was observed after treatment with the
521 aminoglycosides (neomycin, streptomycin, gentamicin), cisplatin and aspirin (Table 2).
522 Surprisingly, copper sulphate treatment did not alter rheotactic behaviour significantly,
523 even at a concentration sufficient to damage the majority of hair cells based on the

524 DASPEI assay. Neither the solvent nor the negative controls showed any difference in
525 rheotaxis scoring before or after treatment.
526
527 In the seeker response (SR) test, all of the ototoxic compounds reduced the overall SR
528 score by at least 46% at the top-concentration level, indicative of a decreased
529 responsiveness to stimulation through the water (summarised in Table 2). Exposure to
530 ototoxins did not affect the ability of larvae to respond to direct touch in the SR assay,
531 suggesting that neuromuscular function remained intact. The negative controls,
532 amoxicillin and cefazolin, had no significant effect on SR score. Interestingly, however,
533 melphalan treatment caused a 14% reduction in responsiveness at the top concentration
534 of 400 μM. In all cases, treatment with solvent/dilution water control had no significant
535 effect on the SR score (Table 2).
536
537 [Table 2 to go near here]
538
539 To identify any subtle differences between MS222-induced light sedation and 100 μM
540 neomycin treatment, the rheotaxis and SR scores were analysed (Suppl. Fig. S4). Both
541 light sedation and neomycin treatment caused a reduction in rheotaxis score, but not a
542 complete loss of the response (1.981 ± 0.019 to 1.583 ± 0.173 (P <0.01) and 2.000 ± 0.0
543 to 1.194 ± 0.1 (P <0.0001) respectively; Bonferroni test). However, the effects of
544 neomycin on this behaviour were more pronounced (a reduction of 40.3% compared
545 with 20.2% for light sedation). In the SR test, scores were also significantly reduced for
546 both light sedation and neomycin treatment (2.426 ± 0.067 to 2.028 ± 0.169 (P <0.05)
547 and 2.463 ± 0.098 to 1.222 ± 0.073 (P <0.0001) respectively; Bonferroni test). Again, a
548 greater decline in responsiveness to the seeker was seen with neomycin treatment (a
549 reduction of 50.4% compared with 16.5% for light sedation). Thus, 100 μM neomycin
550 treatment is sufficient to induce a decline in movement comparable with slight sedation,
551 but displays stronger effects on rheotaxis and SR. This suggests that neomycin
552 treatment (at the level required to damage the majority of hair cells in the DASPEI assay)
553 is not simply inducing a sedative effect and that sedation alone does not account for the
554 observed loss of AER.
555

556 4. DISCUSSION

557 *4.1 Overview*

558 We have described the development and optimisation of a semi-automated startle assay
559 to assess the AER of larval zebrafish following exposure to a range of ototoxins,
560 together with assays for rheotaxis and avoidance behaviour. We have demonstrated that
561 ototoxin-induced cellular damage specific to the lateral line is sufficient to attenuate the
562 auditory-evoked high speed escape response, rheotactic behaviour and sensitivity of
563 motion detection in the larval zebrafish. We have determined that auditory-evoked startle
564 alone cannot act as an indicator of sub-pathological effects of ototoxins (Froehlicher et
565 al., 2009), but could be used in conjunction with additional histopathological (e.g.
566 DASPEI) and functional assays (e.g. rheotaxis and SR tests), as a reliable indicator of
567 the ototoxicity of new compounds.
568
569 *4.2 The DASPEI assay is a robust and sensitive indicator of ototoxicity*
570 A number of compounds is known to be toxic to human and mammalian hair cells
571 (Guthrie, 2008; Rybak and Ramkumar, 2007; Rybak et al., 2007). To examine if
572 zebrafish hair cells are similarly sensitive to a wide range of compounds, we exposed
573 zebrafish larvae to a series of compounds with known ototoxic effect in mammals, or to
574 negative controls with no known mammalian or human effect. To assay for hair cell
575 damage, we used the styryl dye DASPEI, which is readily taken up by sensory hair cells
576 present in the neuromasts of the lateral line and by cells of the nasal epithelium. A
577 scoring system was used to quantify ototoxin-induced hair cell damage in neuromasts of
578 the posterior lateral line, as the level of DASPEI staining has been shown to be
579 proportional to the number of hair cells present in the neuromast, using methods such as
580 phalloidin and acetylated tubulin labelling (Harris et al., 2003). The aminoglycoside
581 antibiotics neomycin, streptomycin and gentamicin, the platinum-based
582 chemotherapeutic cisplatin and the heavy metal salt copper sulphate were all sufficient
583 to induce concentration-dependent loss of DASPEI staining and to reduce FM1-43FX
584 staining in hair cells, indicating ototoxicity. This is in agreement with previously published
585 data in zebrafish (Chiu et al., 2008; Coffin et al., 2010; Harris et al., 2003; Hernández et
586 al., 2006; Ou et al., 2007; Ou et al., 2010; Owens et al., 2007; Owens et al., 2009; Ton
587 and Parng, 2005; Van Trump et al., 2010). In addition, we tested the salicylate aspirin, a
588 reported ototoxin in humans, and found that similar concentration-dependent hair cell
589 damage occurred with DASPEI but not FM1-43FX labelling.
590

591 A small subset of negative control compounds with similar therapeutic effect in humans
592 to the ototoxins, but no documented ototoxic side effects, were examined. These
593 included two antibiotics (the synthetic penicillin amoxicillin and the first generation
594 cephalosporin cefazolin), and two chemotherapeutics (the alkylating agent melphalan
595 and the anti-metabolite gemcitabine). The latter were selected as agents expected to
596 affect cell survival and differentiation, by disrupting DNA synthesis and cell division
597 (Brunton et al., 2006). Cefazolin and amoxicillin have not previously been investigated
598 for their ototoxic effects in the zebrafish. The chemotherapeutics melphalan and
599 gemcitabine have been shown to have no hair cell toxicity in a recent screen (Hirose et
600 al., 2011). Amoxicillin, cefazolin and melphalan had no significant effect on hair cell
601 staining, and therefore no ototoxic effect. In contrast to the findings of the published
602 screen (Hirose et al., 2011), gemcitabine exposure resulted in a significant reduction in
603 hair cell labelling, thus scoring as positive in our assay. A plausible reason for this
604 discrepancy is that DASPEI labels mitochondria, whereas Hirose and colleagues used
605 the nuclear dye YOPRO-1, which could be less sensitive to subtle cellular alterations
606 following treatment. One explanation for the lack of reported ototoxicity of gemcitabine in
607 humans is that patients treated with gemcitabine are typically treated with gemcitabine-
608 cisplatin adjuvant therapy (Joint Formulary Committee, 2011; Lee et al., 2004). It is
609 therefore possible that the known ototoxin, cisplatin, may mask any additional ototoxic
610 effect of the gemcitabine in patients. Additionally, the concentration of gemcitabine used
611 in our experiments is likely to be higher than the effective concentration used in humans,
612 due to the direct exposure of the neuromasts to the compound. The human ear is less
613 likely to encounter such high levels.
614
615 *4.3 Control compliance of larvae in the startle assay is dependent on many factors,*
616 *including raising density and inter stimulus interval*
617
618 The startle response (Bang et al., 2002; Kimmel et al., 1974; Zeddies and Fay, 2005)
619 formed the basis of a functional assay to measure physiological endpoints following
620 ototoxic insult and subsequent hair cell damage. One major obstacle in examining startle
621 responses is that the high speed AER of the larvae (at least in the case of SLCs) is
622 probabilistic (Burgess and Granato, 2007). In our investigation, the startle response
623 initially occurred in only approximately 40% of untreated larvae. A number of parameters
624 were refined in order to increase the percentage of control animals responding to stimuli,

625 thereby optimising the assay. In particular, raising density, ISI duration and plate type
626 were found to be key determinants of control compliance.
627
628 It has previously been reported that larval raising conditions can have a direct effect on
629 sensitivity to auditory stimuli, with larvae raised at lower densities showing significantly
630 greater responsiveness (Burgess and Granato, 2008). In agreement with Burgess and
631 Granato, our data show that AER is significantly increased in the lower raising density
632 condition. This effect is unlikely to be explained by a developmental delay, as larval
633 development appeared to be unaffected in the large sample of animals tested. More
634 likely, it is not unreasonable to assume that larvae raised at a higher density will come
635 into physical contact with other larvae more frequently, and experience a higher
636 background level of underwater motion. This added stimulation at higher raising
637 densities may desensitise the larval zebrafish to subsequent artificial vibratory
638 stimulation (Domenici, 2010).
639
640 It is well documented that repeated exposure to a given stimulus results in a progressive
641 reduction in response (Rankin et al., 2009; Thompson and Spencer, 1966). This
642 phenomenon, termed habituation, is present in the larval zebrafish, occurring as a
643 consequence of repeated exposure to tone bursts. To determine the appropriate
644 duration between stimuli for this investigation, the response to varying ISIs was
645 investigated. In agreement with previous work (Best et al., 2008), our data confirmed
646 that movement elicited by the first auditory stimulus is significantly greater than that
647 brought about by successive stimuli. This justifies the removal of the first stimulus from
648 further testing, as this immediate difference is not a habituation effect. In addition, we
649 were able to induce habituation deliberately using an ISI of 1 second, thus further
650 supporting that larvae are indeed capable of this behaviour. In our investigation,
651 habituation did not occur over repeated stimuli with an ISI of 45 seconds or more.
652 Previous studies have used an ISI of 15-20 seconds, which was shown to be insufficient
653 to induce habituation (Best et al., 2008; Burgess et al., 2009). One study (Zeddies and
654 Fay, 2005) used a much longer ISI of 105 ± 30 seconds, and in agreement with this, we
655 found that an ISI longer than 45 seconds prevented habituation. We propose that the
656 minor discrepancies in larval habituation are due to inter-strain variation and different
657 experimental methodology between studies. Based on these results, an ISI of 60
658 seconds with a random variation of up to 20 seconds was selected in order to prevent

659 habituation, and to streamline the experimental protocol to allow immediate video
660 processing.
661
662 In early pre-clinical screens, it is important to maximise the quality and throughput of
663 assays by optimising for sample size (i.e. *n* number). To test whether the capacity of our
664 assay could be increased by using more wells per experiment, without affecting the
665 acoustic properties or startle response of the animals, larval AERs were compared
666 between 24- and 48-well plates. These data indicated that the best response could be
667 observed using 24-well plates. Ultimately, this result limited the throughput of the assay
668 and introduced a necessity for staggered treatments to maintain an appropriate number
669 of animals per treatment group, but provided what appeared to be more robust data with
670 lower variability.
671
672 *4.4 The startle assay provides a readout of the functional consequences of ototoxic*
673 *damage*
674
675 Following optimisation of the startle assay, it was possible to assess the effects of the
676 positive and negative compounds on the AER. It was hypothesised that test substances
677 that caused a significant reduction in DASPEI fluorescence would also induce a
678 functional effect, in the form of a reduced response to sinusoidal tone bursts over a
679 range of frequencies. Results from the assay confirmed that certain ototoxins, including
680 all of the aminoglycoside antibiotics and aspirin, caused a decrease in AER at the
681 highest concentrations tested. These observations concur with data showing that lateral
682 line toxins attenuate the C-start in zebrafish and other fish species, and with
683 electrophysiological studies demonstrating that auditory evoked potentials can be
684 recorded from the brainstem and are reduced after gentamicin exposure in goldfish
685 (Brack and Ramcharitar, 2011; Faucher et al., 2006; Faucher et al., 2008; McHenry et
686 al., 2009; Ramcharitar and Selckmann, 2010; Ramcharitar and Brack, 2010; Weber,
687 2006). The neurotoxin lead is also reported to cause alterations in larval zebrafish startle
688 responses, although it is uncertain how closely this damage is linked to hair cell toxicity
689 (Rice et al., 2011).
690
691 *4.5 The startle assay is less sensitive than the DASPEI assay in detecting ototoxicity*
692

693 Positive compounds that induced cellular damage, namely cisplatin and copper
694 sulphate, were not shown to have any significant effect on response in the startle assay.
695 The fact that the startle assay could not detect AER decreases for these compounds is
696 best explained by the high variability of response between test animals and not by drug-
697 induced activity changes leading to desensitisation to stimulation (as there was no
698 change in baseline activity of larvae). As the hair cells of the lateral line were damaged
699 after treatment with both compounds, the undamaged hair cells of the inner ear may
700 have allowed for an uncompromised response. Additionally, some lateral line hair cell
701 function may have remained even in the absence of DASPEI and FM1-43FX staining,
702 thus explaining the differences in AER effects between aminoglycosides, cisplatin and
703 copper sulphate. However, the startle assay is advantageous in that it does not suffer
704 from one of the main drawbacks of the use of larval zebrafish for early toxicology
705 screens, whereby uptake of compound is chemistry-dependent. Poor uptake of
706 compounds in other assays can result in low potency for some compounds when based
707 on applied concentration (Redfern et al., 2008; Richards et al., 2008; Winter et al.,
708 2008). In all of our assays, cells are directly exposed to the applied concentration of
709 compound in the aqueous medium, avoiding this problem.
710
711 The positive control, neomycin, gave consistent results, with a decrease in AER
712 observed in 89% of cases. Overall, the startle assay was only sensitive at high
713 concentrations that were sufficient to bring about complete hair cell damage (based on
714 DASPEI staining). The reason for this low comparative sensitivity could at least in part
715 be due to the stringent method of defining the startle movement. In this assay,
716 movements below a high-speed threshold were not recorded as startle responses.
717 These movements tended to include a weak C-bend but not the full range of motion
718 expected from a stereotypical startle. The inclusion of these less marked responses in
719 future may be sufficient to increase the initial control compliance levels, and therefore
720 any compound-induced decreases in AER would prove more significant.
721
722 *4.6 The ear may contribute to larval startle responses*
723
724 Acute exposure of larvae to ototoxins is thought to cause damage specific to the lateral
725 line that does not affect the ear (Blaxter and Fuiman, 1989; Matsuura et al., 1971). This
726 is likely to be because ototoxins applied to the medium in which the larvae are swimming

727 are unable to access the ear: inner ear hair cells are susceptible to ototoxic damage if
728 compounds are injected directly into the otic lumen (L. Buck, data not shown). In our
729 assays, although startle responses were significantly attenuated for the aminoglycosides
730 and aspirin at concentrations that abolished pLL DASPEI staining completely, the
731 reaction was not entirely diminished. Additionally, we found that acute treatment with
732 ototoxins did not affect the levels of GFP expression or the morphology of inner ear hair
733 cells. These findings are of real value as they imply that the ear contributes to initiating
734 the noise-evoked startle. This would support the previous implication that the sacculus
735 partly mediates the startle response (Zeddies and Fay, 2005). Further investigation will
736 be necessary to separate out the roles of the ear versus the lateral line for this
737 behaviour.
738
739 *4.7 Rheotaxis and seeker response assays offer sensitive readouts of ototoxin-induced*
740 *functional damage*
741
742 Rheotactic behaviour in response to artificial circular currents was examined in larvae
743 treated with ototoxins. The mechanosensory lateral line is a key mediator of rheotactic
744 behaviour in zebrafish (Johnson et al., 2007) and other fish species (Montgomery et al.,
745 1997). We hypothesised that lateral line-specific hair cell damage induced by ototoxins
746 would lead to a decrease in rheotaxis score but that this decline in behaviour would not
747 be observed in solvent- and negative control-treated larvae. Our data supported this
748 hypothesis in the main. This is in line with data from other fish species, such as whitebait
749 and blind cave fish, in which ototoxins and heavy metals are sufficient to raise the
750 threshold for rheotactic responses (Baker and Montgomery, 2001; Montgomery et al.,
751 1997). It was surprising that copper sulphate did not affect rheotaxis in our experiments,
752 as this effect has previously been observed in larval zebrafish in response to copper
753 exposure (Johnson et al., 2007). The effects of copper may have gone undetected in our
754 investigation due to the presence of pLL hair cells (based on GFP expression in the
755 *Tg(pou4f3::mGFP)* line) even in the absence of live cell labelling with DASPEI. These
756 results cannot be explained by alterations in baseline activity of larvae (e.g. seizure
757 induction). Nevertheless, testing for rheotactic disturbance proved more sensitive than
758 the startle assay by direct comparison (Suppl. Table S4).
759

760 The seeker response (SR) test served to answer two questions; are larvae responsive to
761 water flow disturbances, and do they retain neuromuscular function after compound
762 exposure? It was predicted from previous data based on neomycin alone that ototoxin
763 treatment would cause a decreased responsiveness to water flow (McHenry et al.,
764 2009). It was also hypothesised that negative compounds would have no discernable
765 effect. This was true for all compounds, except melphalan, which gave an unexpected
766 minor decrease in SR score. As melphalan treatment did not alter the activity profile of
767 the larvae, the result could not be attributed to a sedative effect. Reduction in SR score
768 is a third functional consequence of lateral line hair cell damage.
769
770 *4.8 Sedation and seizure induction do not account for alterations in functional responses*
771 *to exogenous stimuli*
772
773 To exclude the possibility that the reduction in startle response after exposure to
774 ototoxins was due to sedative effects, we analysed the activity profiles of the larvae from
775 baseline recordings. With the exception of neomycin, all test substances had no
776 significant effect on the overall movement of larvae. When compared with the
777 anaesthetic MS222, it appeared that the decreased activity caused by neomycin
778 exposure might have been due to slight sedative effects. Interestingly, the decrease in
779 baseline activity of neomycin-treated larvae could not solely be attributed to sedative
780 effects, as neomycin treatment displayed more pronounced effects on rheotaxis and
781 seeker response than light sedation. These findings lead us to believe that a more
782 subtle, compound-specific effect is occurring with neomycin treatment.
783
784 *4.9 CONCLUDING REMARKS*
785 These data are the first to demonstrate that larval zebrafish can experience deficits in
786 detecting auditory and vibratory stimuli, and in orientation to current flow, after cellular
787 damage induced by a range of ototoxins. Our findings collectively recapitulate the
788 functional hearing and vestibular effects manifested in humans and non-human
789 mammals after exposure to these ototoxins, thereby further supporting the use of
790 zebrafish as a pre-clinical indicator of drug-induced ototoxicity.
791
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801

802 **AUTHOR CONTRIBUTIONS**

803 Project and experimental design: LMJB, MJW, WSR, TTW

804 Experimental work: LMJB

805 Preparation of the article: LMJB, MJW, WSR, TTW

806

807 **APPENDIX A**

808 *Supplementary data*

809 [Supplementary Figures S1-4 and Supplementary Tables S1-4 to go here]

810

810 **REFERENCES**

811 AHL. Action on Hearing Loss. 2011. From http://www.actiononhearingloss.org.uk/your-
812 hearing/about-deafness-and-hearing-loss/statistics/adults-who-are-deaf-or-have-
813 a-hearing-loss-in-the-uk.aspx.
814 Arnold, G. P., 1974. Rheotropism in fishes. Biol. Rev. Camb. Philos. Soc. 49, 515-576.
815 Baker, C. F., Montgomery, J. C., 2001. Sensory deficits induced by cadmium in banded
816 kokopu, Galaxias fasciatus, juveniles. Envir. Biol. Fish. 62, 455-464.
817 Bang, P. I., Yelick, P. C., Malicki, J. J., Sewell, W. F., 2002. High-throughput behavioral
818 screening method for detecting auditory response defects in zebrafish. J.
819 Neurosci. Methods 118, 177-187.
820 Best, J. D., Berghmans, S., Hunt, J. J., Clarke, S. C., Fleming, A., Goldsmith, P., Roach,
821 A. G., 2008. Non-associative learning in larval zebrafish.
822 Neuropsychopharmacology 33, 1206-1215.
823 Bever, M. M., Fekete, D. M., 2002. Atlas of the developing inner ear in zebrafish. Dev.
824 Dyn. 223, 536-543.
825 Blaxter, J. H. S., Fuiman, L. A. 1989. Function of the free neuromasts of marine teleost
826 larvae. The Mechanosensory Lateral Line, Neurobiology and Evolution. Coombs,
827 S., Gorner, P., Munz, H. (eds.). New York, Springer-Verlag: 481-499.
828 Brack, C. L., Ramcharitar, J. 2011. Assessment of lateral line function: A potential
829 technique for studies in ototoxicity. J. Clin. Neurosci. (doi:
830 10.1016/j.jocn.2011.06.008.)
831 Brignull, H. R., Raible, D. W., Stone, J. S., 2009. Feathers and fins: non-mammalian
832 models for hair cell regeneration. Brain. Res. 1277, 12-23.
833 Brunton, L., Lazo, J., Parker, K. 2006. Goodman & Gilman's The Pharmacological Basis
834 of Therapeutics, 11th Edition. New York; London, McGraw-Hill.
835 Burgess, H. A., Granato, M., 2007. Sensorimotor gating in larval zebrafish. J. Neurosci.
836 27, 4984-4994.
837 Burgess, H. A., Granato, M., 2008. The neurogenetic frontier: Lessons from misbehaving
838 zebrafish. Brief. Funct. Genomic. Proteomic. 7, 474-482.
839 Burgess, H. A., Johnson, S. L., Granato, M., 2009. Unidirectional startle responses and
840 disrupted left-right co-ordination of motor behaviors in *robo3* mutant zebrafish.
841 Genes Brain Behav. 8, 500-511.
842 Chiu, L. L., Cunningham, L. L., Raible, D. W., Rubel, E. W., Ou, H. C., 2008. Using the
843 zebrafish lateral line to screen for ototoxicity. J. Assoc. Res. Otolaryngol. 9, 178-
844 190.
845 Coffin, A. B., Reinhart, K. E., Owens, K. N., Raible, D. W., Rubel, E. W., 2009.
846 Extracellular divalent cations modulate aminoglycoside-induced hair cell death in
847 the zebrafish lateral line. Hear. Res. 253, 42-51.
848 Coffin, A. B., Ou, H., Owens, K. N., Santos, F., Simon, J. A., Rubel, E. W., Raible, D. W.,
849 2010. Chemical screening for hair cell loss and protection in the zebrafish lateral
850 line. Zebrafish 7, 3-11.
851 Colucci, G., Giuliani, F., Gebbia, V., Biglietto, M., Rabitti, P., Uomo, G., Cigolari, S.,
852 Testa, A., Maiello, E., Lopez, M., 2002. Gemcitabine alone or with cisplatin for
853 the treatment of patients with locally advanced and/or metastatic pancreatic
854 carcinoma: a prospective, randomized phase III study of the Gruppo Oncologia
855 dell'Italia Meridionale. Cancer 94, 902-910.
856 Crino, L., Scagliotti, G. V., Ricci, S., De Marinis, F., Rinaldi, M., Gridelli, C., Ceribelli, A.,
857 Bianco, R., Marangolo, M., Di Costanzo, F., Sassi, M., Barni, S., Ravaioli, A.,
858 Adamo, V., Portalone, L., Cruciani, G., Masotti, A., Ferrara, G., Gozzelino, F.,
859 Tonato, M., 1999a. Gemcitabine and cisplatin versus mitomycin, ifosfamide, and

860 cisplatin in advanced non-small-cell lung cancer: A randomized phase III study of
861 the Italian Lung Cancer Project. J. Clin. Oncol. 17, 3522-3530.
862 Crino, L., Mosconi, A. M., Scagliotti, G., Selvaggi, G., Novello, S., Rinaldi, M., Della
863 Giulia, M., Gridelli, C., Rossi, A., Calandri, C., De Marinis, F., Noseda, M.,
864 Tonato, M., 1999b. Gemcitabine as second-line treatment for advanced non-
865 small-cell lung cancer: A phase II trial. J. Clin. Oncol. 17, 2081-2085.
866 Dijkgraaf, S., 1963. The functioning and significance of the lateral-line organs. Biol. Rev.
867 Camb. Philos. Soc. 38, 51-105.
868 Domenici, P., 2010. Context-dependent variability in the components of fish escape
869 response: integrating locomotor performance and behavior. J. Exp. Zool. A Ecol.
870 Genet. Physiol. 313, 59-79.
871 Faucher, K., Fichet, D., Miramand, P., Lagardère, J. P., 2006. Impact of acute cadmium
872 exposure on the trunk lateral line neuromasts and consequences on the "C-start"
873 response behaviour of the sea bass (*Dicentrarchus labrax* L.; Teleostei,
874 Moronidae). Aquat. Toxicol. 76, 278-294.
875 Faucher, K., Fichet, D., Miramand, P., Lagardère, J. P., 2008. Impact of chronic
876 cadmium exposure at environmental dose on escape behaviour in sea bass
877 (*Dicentrarchus labrax* L.; Teleostei, Moronidae). Environ. Pollut. 151, 148-157.
878 Fay, R. R., Popper, A. N., 1974. Acoustic stimulation of the ear of the goldfish (*Carassius*
879 *auratus*). J. Exp. Biol. 61, 243-260.
880 Froehlicher, M., Liedtke, A., Groh, K. J., Neuhauss, S. C., Segner, H., Eggen, R. I.,
881 2009. Zebrafish (*Danio rerio*) neuromast: promising biological endpoint linking
882 developmental and toxicological studies. Aquat. Toxicol. 95, 307-319.
883 Ghysen, A., Dambly-Chaudière, C., 2004. Development of the zebrafish lateral line.
884 Curr. Opin. Neurobiol. 14, 67-73.
885 Guthrie, O. W., 2008. Aminoglycoside induced ototoxicity. Toxicology 249, 91-96.
886 Harris, J. A., Cheng, A. G., Cunningham, L. L., MacDonald, G., Raible, D. W., Rubel, E.
887 W., 2003. Neomycin-induced hair cell death and rapid regeneration in the lateral
888 line of zebrafish (*Danio rerio*). J. Assoc. Res. Otolaryngol. 4, 219-234.
889 Hernández, P. P., Moreno, V., Olivari, F. A., Allende, M. L., 2006. Sub-lethal
890 concentrations of waterborne copper are toxic to lateral line neuromasts in
891 zebrafish (*Danio rerio*). Hear. Res. 213, 1-10.
892 Hirose, Y., Simon, J. A., Ou, H. C., 2011. Hair Cell Toxicity in Anti-cancer Drugs:
893 Evaluating an Anti-cancer Drug Library for Independent and Synergistic Toxic
894 Effects on Hair Cells Using the Zebrafish Lateral Line. J Assoc Res Otolaryngol
895 6.
896 Johnson, A., Carew, E., Sloman, K. A., 2007. The effects of copper on the morphological
897 and functional development of zebrafish embryos. Aquat. Toxicol. 84, 431-438.
898 Joint Formulary Committee 2011. British National Formulary. Joint Formulary
899 Committee. London, British Medical Association and Royal Pharmaceutical
900 Society.
901 Kaus, S., 1987. The Effect of Aminoglycoside Antibiotics on the Lateral Line Organ of
902 Aplocheilus lineatus (Cyprinodontidae). Acta. Otolaryngol. 103, 291-298.
903 Kimmel, C. B., Patterson, J., Kimmel, R. O., 1974. The development and behavioral
904 characteristics of the startle response in the zebra fish. Dev. Psychobiol. 7, 47-
905 60.
906 Kimmel, C. B., Eaton, K. B., Powell, S. L., 1980. Decreased fast-start performance of
907 zebrafish lacking Mauthner neurons. J. Comp. Physiol. A Neuroethol. Sens.
908 Neural. Behav. Physiol. 140, 343-350.
909 Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., Schilling, T. F., 1995. Stages
910 of embryonic development of the zebrafish. Dev. Dyn. 203, 253-310.

911 Lee, N. S., Byun, J. H., Bae, S. B., Kim, C. K., Lee, K. T., Park, S. K., Won, J. H., Hong,
912 D. S., Park, H. S., 2004. Combination of gemcitabine and cisplatin as first-line
913 therapy in advanced non-small-cell lung cancer. Cancer Res. Treat. 36, 173-177.
914 Linbo, T. L., Stehr, C. M., Incardona, J. P., Scholz, N. L., 2006. Dissolved copper triggers
915 cell death in the peripheral mechanosensory system of larval fish. Environ.
916 Toxicol. Chem. 25, 597-603.
917 Liu, K. S., Fetcho, J. R., 1999. Laser ablations reveal functional relationships of
918 segmental hindbrain neurons in zebrafish. Neuron 23, 325-335.
919 Matsuura, S., Ikeda, K., Furukawa, T., 1971. Effects of streptomycin, kanamycin,
920 quinine, and other drugs on the microphonic potentials of goldfish sacculus. Jpn.
921 J. Physiol. 21, 579-590.
922 McHenry, M. J., Feitl, K. E., Strother, J. A., Van Trump, W. J., 2009. Larval zebrafish
923 rapidly sense the water flow of a predator's strike. Biol. Lett. 5, 477-479.
924 Metcalfe, W. K., Kimmel, C. B., Schabtach, E., 1985. Anatomy of the posterior lateral line
925 system in young larvae of the zebrafish. J. Comp. Neurol. 233, 377-389.
926 Metcalfe, W. K. 1989. Organisation and development of the zebrafish posterior lateral
927 line. The mechanosensory lateral line: neurobiology and evolution. Coombs, S,
928 Gorner, P,Munz, H. New York, Springer-Verlag**:** 147-159.
929 Mo, W., Chen, F., Nechiporuk, A., Nicolson, T., 2010. Quantification of vestibular-
930 induced eye movements in zebrafish larvae. BMC Neurosci. 11, 110.
931 Montgomery, J. C., Baker, C. F., Carton, A. G., 1997. The lateral line can mediate
932 rheotaxis in fish. Nature 389, 960-963.
933 Murakami, S. L., Cunningham, L. L., Werner, L. A., Bauer, E., Pujol, R., Raible, D. W.,
934 Rubel, E. W., 2003. Developmental differences in susceptibility to neomycin-
935 induced hair cell death in the lateral line neuromasts of zebrafish (Danio rerio).
936 Hear. Res. 186, 47-56.
937 Nüsslein-Volhard, C., Dahm, R. 2002. Zebrafish: A Practical Approach. Hames, BD.
938 Oxford, Oxford University Press.
939 Olivari, F. A., Hernández, P. P., Allende, M. L., 2008. Acute copper exposure induces
940 oxidative stress and cell death in lateral line hair cells of zebrafish larvae. Brain.
941 Res. 1244, 1-12.
942 Ou, H. C., Raible, D. W., Rubel, E. W., 2007. Cisplatin-induced hair cell loss in zebrafish
943 (*Danio rerio*) lateral line. Hear. Res. 233, 46-53.
944 Ou, H. C., Cunningham, L. L., Francis, S. P., Brandon, C. S., Simon, J. A., Raible, D. W.,
945 Rubel, E. W., 2009. Identification of FDA-approved drugs and bioactives that
946 protect hair cells in the zebrafish (*Danio rerio*) lateral line and mouse (*Mus*
947 *musculus*) utricle. J. Assoc. Res. Otolaryngol. 10, 191-203.
948 Ou, H. C., Santos, F., Raible, D. W., Simon, J. A., Rubel, E. W., 2010. Drug screening
949 for hearing loss: using the zebrafish lateral line to screen for drugs that prevent
950 and cause hearing loss. Drug Discov. Today 15, 265-271.
951 Owens, K. N., Cunningham, D. E., MacDonald, G., Rubel, E. W., Raible, D. W., Pujol,
952 R., 2007. Ultrastructural analysis of aminoglycoside-induced hair cell death in the
953 zebrafish lateral line reveals an early mitochondrial response. J. Comp. Neurol.
954 502, 522-543.
955 Owens, K. N., Santos, F., Roberts, B., Linbo, T., Coffin, A. B., Knisely, A. J., Simon, J.
956 A., Rubel, E. W., Raible, D. W., 2008. Identification of genetic and chemical
957 modulators of zebrafish mechanosensory hair cell death. PLoS Genet. 4,
958 e1000020.
959 Owens, K. N., Coffin, A. B., Hong, L. S., Bennett, K. O., Rubel, E. W., Raible, D. W.,
960 2009. Response of mechanosensory hair cells of the zebrafish lateral line to
961 aminoglycosides reveals distinct cell death pathways. Hear. Res. 253, 32-41.

962 Palumbo, A., Bringhen, S., Bruno, B., Falcone, A. P., Liberati, A. M., Grasso, M., Ria, R.,
963 Pisani, F., Cangialosi, C., Caravita, T., Levi, A., Meloni, G., Nozza, A., Pregno,
964 P., Gabbas, A., Callea, V., Rizzo, M., Annino, L., De Stefano, V., Musto, P.,
965 Baldi, I., Cavallo, F., Petrucci, M. T., Massaia, M., Boccadoro, M., 2010.
966 Melphalan 200 mg/m^2 versus melphalan 100 mg/m^2 in newly diagnosed myeloma
967 patients: a prospective, multicenter phase 3 study. Blood 115, 1873-1879.
968 Pinguet, F., Culine, S., Bressolle, F., Astre, C., Serre, M. P., Chevillard, C., Fabbro, M.,
969 2000. A phase I and pharmacokinetic study of melphalan using a 24-hour
970 continuous infusion in patients with advanced malignancies. Clin. Cancer Res. 6,
971 57-63.
972 Prestayko, A. W., D'Aoust, J. C., Issell, B. F., Crooke, S. T., 1979. Cisplatin (cis-
973 diamminedichloroplatinum II). Cancer Treat. Rev. 6, 17-39.
974 Raible, D. W., Kruse, G. J., 2000. Organization of the lateral line system in embryonic
975 zebrafish. J. Comp. Neurol. 421, 189-198.
976 Ramcharitar, J., Selckmann, G. M., 2010. Differential ablation of sensory receptors
977 underlies ototoxin-induced shifts in auditory thresholds of the goldfish (*Carassius*
978 *auratus*). J. Appl. Toxicol. 30, 536-541.
979 Ramcharitar, J. U., Brack, C. L., 2010. Physiological dimensions of ototoxic responses in
980 a model fish species. J. Clin. Neurosci. 17, 103-106.
981 Rankin, C. H., Abrams, T., Barry, R. J., Bhatnagar, S., Clayton, D. F., Colombo, J.,
982 Coppola, G., Geyer, M. A., Glanzman, D. L., Marsland, S., McSweeney, F. K.,
983 Wilson, D. A., Wu, C. F., Thompson, R. F., 2009. Habituation revisited: an
984 updated and revised description of the behavioral characteristics of habituation.
985 Neurobiol. Learn. Mem. 92, 135-138.
986 Redfern, W. S., Waldron, G., Winter, M. J., Butler, P., Holbrook, M., Wallis, R., Valentin,
987 J. P., 2008. Zebrafish assays as early safety pharmacology screens: paradigm
988 shift or red herring? J. Pharmacol. Toxicol. Methods 58, 110-117.
989 Reller, L. B., Karney, W. W., Beaty, H. N., Holmes, K. K., Turck, M., 1973. Evaluation of
990 cefazolin, a new cephalosporin antibiotic. Antimicrob. Agents Chemother. 3, 488-
991 497.
992 Ribner, B. S., Raeder, R., Hollstein, M., Freimer, E. H., 1983. A randomized study
993 comparing clinical efficacy and safety of thienamycin formamidine
994 (MK0787)/renal dipeptidase inhibitor (MK0791) and cefazolin. J. Antimicrob.
995 Chemother. 12, 387-391.
996 Rice, C., Ghorai, J. K., Zalewski, K., Weber, D. N., 2011. Developmental lead exposure
997 causes startle response deficits in zebrafish. Aquat Toxicol 105, 600-608.
998 Richards, F. M., Alderton, W. K., Kimber, G. M., Liu, Z., Strang, I., Redfern, W. S.,
999 Valentin, J. P., Winter, M. J., Hutchinson, T. H., 2008. Validation of the use of
1000 zebrafish larvae in visual safety assessment. J Pharmacol Toxicol Methods 58,
1001 50-58.
1002 Rybak, L. P., 1986. Drug ototoxicity. Annu. Rev. Pharmacol. Toxicol. 26, 79-99.
1003 Rybak, L. P., Whitworth, C. A., Mukherjea, D., Ramkumar, V., 2007. Mechanisms of
1004 cisplatin-induced ototoxicity and prevention. Hear. Res. 226, 157-167.
1005 Rybak, L. P., Ramkumar, V., 2007. Ototoxicity. Kidney Int. 72, 931-935.
1006 Salvo, F., Polimeni, G., Moretti, U., Conforti, A., Leone, R., Leoni, O., Motola, D., Dusi,
1007 G., Caputi, A. P., 2007. Adverse drug reactions related to amoxicillin alone and in
1008 association with clavulanic acid: data from spontaneous reporting in Italy. J.
1009 Antimicrob. Chemother. 60, 121-126.
1010 Sandler, A., Ettinger, D. S., 1999. Gemcitabine: single-agent and combination therapy in
1011 non-small cell lung cancer. Oncologist 4, 241-251.

1012 Santos, F., MacDonald, G., Rubel, E. W., Raible, D. W., 2006. Lateral line hair cell
1013 maturation is a determinant of aminoglycoside susceptibility in zebrafish (*Danio*
1014 *rerio*). Hear. Res. 213, 25-33.
1015 Schacht, J., Hawkins, J. E., 2006. Sketches of otohistory. Part 11: Ototoxicity: drug-
1016 induced hearing loss. Audiol. Neurootol. 11, 1-6.
1017 Seligmann, H., Podoshin, L., Ben-David, J., Fradis, M., Goldsher, M., 1996. Drug-
1018 induced tinnitus and other hearing disorders. Drug Saf. 14, 198-212.
1019 Thompson, R. F., Spencer, W. A., 1966. Habituation: a model phenomenon for the study
1020 of neuronal substrates of behavior. Psychol. Rev. 73, 16-43.
1021 Ton, C., Parng, C., 2005. The use of zebrafish for assessing ototoxic and otoprotective
1022 agents. Hear. Res. 208, 79-88.
1023 Van Trump, W. J., Coombs, S., Duncan, K., McHenry, M. J., 2010. Gentamicin is
1024 ototoxic to all hair cells in the fish lateral line system. Hear. Res. 261, 42-50.
1025 Von der Maase, H., Andersen, L., Crino, L., Weinknecht, S., Dogliotti, L., 1999. Weekly
1026 gemcitabine and cisplatin combination therapy in patients with transitional cell
1027 carcinoma of the urothelium: a phase II clinical trial. Ann. Oncol. 10, 1461-1465.
1028 Weber, D. N., 2006. Dose-dependent effects of developmental mercury exposure on C-
1029 start escape responses of larval zebrafish *Danio rerio*. J. Fish Biol. 69, 75-94.
1030 Whitfield, T. T., Riley, B. B., Chiang, M. Y., Phillips, B., 2002. Development of the
1031 zebrafish inner ear. Dev. Dyn. 223, 427-458.
1032 Whitfield, T. T., 2002. Zebrafish as a model for hearing and deafness. J. Neurobiol. 53,
1033 157-171.
1034 Winter, M. J., Redfern, W. S., Hayfield, A. J., Owen, S. F., Valentin, J. P., Hutchinson, T.
1035 H., 2008. Validation of a larval zebrafish locomotor assay for assessing the
1036 seizure liability of early-stage development drugs. J. Pharmacol. Toxicol.
1037 Methods 57, 176-187.
1038 Xiao, T., Roeser, T., Staub, W., Baier, H., 2005. A GFP-based genetic screen reveals
1039 mutations that disrupt the architecture of the zebrafish retinotectal projection.
1040 Development 132, 2955-2967.
1041 Zeddies, D. G., Fay, R. R., 2005. Development of the acoustically evoked behavioral
1042 response in zebrafish to pure tones. J. Exp. Biol. 208, 1363-1372.
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1045 **FIGURE AND TABLE LEGENDS**

1046 **Figure 1. Experimental setup used to evoke startle responses in larval zebrafish.**
1047 (A) Stimuli were generated with a PC function generator, amplified, and directed through
1048 a speaker cone. Acoustic stimuli were delivered to the larvae through a plastic base
1049 plate, bolted to the speaker case. AERs were captured using a high speed digital video
1050 camera. Recordings were visualised and processed using VideoTrack software. The
1051 threshold used to distinguish between normal and stereotypical startle-type locomotor
1052 activity (shown in red) was 21.1 mm s^{-1}. (B) Treatment and testing paradigm used in the
1053 startle, SR and rheotaxis assays.
1054

1055 **Figure 2. The effects of compounds on DASPEI and FM1-43FX staining in the pLL.**
1056 Ototoxin treatment reduced DASPEI and FM1-43FX staining in the hair cells of the pLL
1057 compared to control larvae. Other staining, including the nasal epithelium and ear, was
1058 not affected in treated larvae (Rows A-F). Treatment with negative control compounds
1059 did not reduce the brightness of DASPEI or FM1-43FX labelling in the pLL compared to
1060 control larvae (Rows G-I). The images shown are representative of all fish tested (12
1061 larvae per treatment group). Scale bar = 800 μm. (Ji) Schematic showing typical pattern
1062 of neuromasts in a 5 dpf zebrafish larva. Smaller neuromasts from the second wave of
1063 deposition are indicated (II-1, II-2), but were not scored for the purpose of the DASPEI
1064 assay. The remaining 9 neuromasts of the pLL (P1-9) were scored on both sides. (Jii-iii)
1065 Exemplar images of neuromast staining with DASPEI. Image (Jii) is representative of a
1066 healthy neuromast given a score of 2 in the fluorescence assay. Image (Jiii) is
1067 representative of a damaged neuromast, given a score of 1. Scale bar = 20 μm.
1068

1069 **Figure 3. Concentration-response relationships of known ototoxins and negative**
1070 **control substances in larval zebrafish**. (A-F) Exposure to the ototoxins neomycin,
1071 streptomycin, gentamicin, cisplatin, aspirin and copper sulphate caused a significant
1072 concentration-dependent decrease in DASPEI staining, indicative of hair cell damage
1073 (Kruskal-Wallis test, followed by Dunn's multiple comparison test). Exposure time and
1074 concentration range were as described in Suppl. Table S1. (G-I) Exposure to the
1075 negative controls amoxicillin (G), cefazolin (H) and melphalan (I) had no significant effect
1076 on DASPEI staining (Kruskal-Wallis test, followed by Dunn's multiple comparison test).
1077 Minimum of 29 fish per group (pooled from 3 or more experimental trials). Statistical
1078 significance compared with the control group is indicated by asterisks. * $P < 0.05$, ** $P <$

1079 0.01, *** $P < 0.001$, ns $P > 0.05$ (applies to all subsequent figures and tables). The first
1080 observed statistical effect is denoted by the hash symbol (#). (J) IC_{50} values were
1081 approximated using either log(concentration of test compound) versus response (three
1082 parameters) or log(concentration of test compound) versus response - Variable slope
1083 (four parameters) curve-fitting equations.
1084
1085 **Figure 4. Factors contributing to the control compliance of larval AERs.** (A) Overall,
1086 untreated larvae displayed stronger AERs when arrayed into microplates containing 24
1087 wells compared with 48 wells (two-way ANOVA; F (1, 32) = 8.72, P = 0.0058). (B, C)
1088 Paired *t*-tests indicated that both sine and square waveforms elicited equally strong
1089 startle responses (based on the strength of response and the percentage of animals
1090 responding). (D, E) A density of 50 larvae per Petri dish gave both the strongest startle
1091 response (Mann Whitney test) and the highest percentage of untreated larvae startling
1092 (unpaired *t*-test), when compared with a higher density of 128 larvae per Petri dish. (F)
1093 The habituation profile of larvae over 10 repeated stimuli, with an ISI of 1 second or 60
1094 seconds. For graphs (C) and (E), statistical testing was performed on values that had
1095 been normalised using Arcsine transformation. See Methods, sections 2.4.5 to 2.4.8, for
1096 *n* numbers.
1097
1098 **Figure 5. Specific ototoxins caused a concentration-dependent decrease in larval**
1099 **AER following histological damage.** The positive control was neomycin 100μM.
1100 Minimum of 36 larvae pooled from 3 experimental repeats per condition. All data were
1101 analysed using the Kruskal-Wallis test, followed by Dunn's multiple comparison test.
1102 Statistical significance compared with the control group is indicated by asterisks. For
1103 details of compound exposures see Suppl. Table S1.
1104
1105 **Figure 6. The effects of copper sulphate and neomycin exposure on the ear and**
1106 **pLL of *Tg(pou4f3::mGFP)* larvae.** Treatment with neomycin and copper sulphate (E-H)
1107 did not affect GFP expression in the hair cells of the inner ear when compared to control
1108 treatment (A-D). These images were taken at both the anterior and posterior focal planes
1109 in live animals. A, anterior macula; P, posterior macula; arrowheads indicate the cristae
1110 of the semicircular canals. Scale bar = 200 μm. Treatment with neomycin (K) but not
1111 copper sulphate (L) caused a strong decrease in GFP expression in hair cells of the pLL,

1112 when compared to control treatment (I and J). Scale bar = 500 µm. Images are
1113 representative of an *n* of 12 per treatment group.
1114
1115 **Table 1. Movement profiles of drug-treated larvae helped to identify potentially**
1116 **sedative effects of compounds.** Neomycin treatment reduced the average speed of
1117 unstimulated larvae (Kruskal-Wallis test; H = 15.83, 2 *d.f.*, P = 0.0004). All other
1118 histologically positive and negative compounds had no significant reduction in average
1119 speed of the larvae over a 60 second recording. Minimum of 36 larvae pooled from 3
1120 experimental repeats per condition. Statistical tests performed were one-way ANOVA
1121 (followed by Dunnett's multiple comparison tests) or Kruskal-Wallis test (followed by
1122 Dunn's multiple comparison test). For details of compound exposures see Suppl. Table
1123 S1.
1124
1125 **Table 2. Rheotaxis and seeker response scores decreased in a concentration**
1126 **dependent manner with ototoxin exposure.** Data are presented as the percent
1127 reduction in score following treatment with either the water/solvent control, test
1128 compound or positive control for all test substances. Statistical data from post-tests are
1129 displayed in the adjacent column (for all post-test data, *d.f.* = 1). All data were analysed
1130 using two-way ANOVA, followed by Bonferroni multiple comparison tests. Minimum of 36
1131 larvae pooled from 3 experimental repeats per condition. For details of compound
1132 exposure, see Suppl. Table S1.

Test Substance	Significant Change in Overall Baseline Activity?	Statistical Data (ANOVA/ Kruskal-Wallis)	Concentration Required to Significantly Increase/Reduce Activity Level?
Neomycin	Yes	$H = 15.83$, 2 *d.f.*, $P = 0.0004$	100 µM ($P < 0.05$, Dunn's post-test)
Streptomycin	No	$F(2,93) = 0.6848$, $P = 0.5067$	-
Gentamicin	No	$H = 1.261$, 2 *d.f.*, $P = 0.5324$	-
Cisplatin	Yes	$F(2,100) = 4.231$, $P = 0.0172$	-
Aspirin	No	$F(2,94) = 1.655$, $P = 0.1966$	-
Copper Sulphate	No	$F(2,94) = 1.825$, $P = 0.1669$	-
Amoxicillin	No	$H = 4.977$, 2 *d.f.*, $P = 0.0831$	-
Cefazolin	No	$F(2,98) = 0.3355$, $P = 0.7158$	-
Melphalan	No	$F(2,98) = 0.6207$, $P = 0.5397$	-

Buck et al., Table 1

Test Substance	Seeker Response Score								Rheotaxis Score							
	Percentage Change in Score After Compound Exposure and Significance Level of Change								Percentage Change in Score After Compound Exposure and Significance Level of Change							
	DWC/SC		IC_{50}/Mid-dose		Top-dose		+ Control		DWC/SC		IC_{50}/Mid-dose		Top-dose		+ Control	
Neomycin	2.6	t=0.4261, ns	-27.8	t=4.497, **	-61.6	t=9.505, ***	-61.3	t=9.634, ***	-0.4	t=0.0673, ns	-22.2	t=4.310, **	-36.1	t= 7.004, ***	-36.8	t=7.071, ***
Streptomycin	6.3	t=1.449, ns	-35.6	t=8.132, ***	-48.9	t=11.11, ***	-61.2	t=14.09, ***	0	t=0.0, ns	-6.9	t=1.443, ns	-15.3	t=3.175, *	-34.7	t=7.217, ***
Gentamicin	4.4	t=0.9912, ns	-40.2	t=8.769, ***	-49.6	t=10.60, ***	-59.5	t=13.42, ***	-2.8	t=0.686, ns	-15.3	t=3.773, **	-22.2	t=5.488, ***	-26.4	t=6.517, ***
Cisplatin	8.7	t=1.621, ns	-17.8	t=3.511, *	-55.8	t=10.98, ***	-51.4	t=9.859, ***	0.5	t=0.1240, ns	-5.6	t=1.488, ns	-11.1	t=2.977, *	-16.5	t=4.341, **
Aspirin	0.8	t=0.1249, ns	-37.7	t=5.725, ***	-45.8	t=7.063, ***	-59.9	t=9.337, ***	-1.4	t=0.2073, ns	-15.3	t=2.280, ns	-21.3	t=3.178, *	-38.9	t=5.803, ***
Copper Sulphate	1.7	t=0.2966, ns	-35.4	t=5.753, ***	-46.9	t=8.007, ***	-63.5	t=11.45, ***	-5.6	t=0.8251, ns	-11.1	t= 1.650, ns	-18.1	t=2.682, ns	-30.6	t=4.538, **
Amoxicillin	9.0	t=1.687, ns	-0.7	t=0.1350, ns	2.2	t=0.405, ns	-60.6	t=11.54, ***	-2.8	t=0.9177, ns	-5.6	t=1.835, ns	-5.6	t=1.835, ns	-22.2	t=7.341, ***
Cefazolin	3.5	t=0.9288, ns	-8.4	t=2.303, ns	-5.9	t=1.613, ns	-62.8	t=17.05, ***	0	t=0.0, ns	-2.3	t=0.5692, ns	-1.6	t=0.3997, ns	-45.3	t=11.29, ***
Melphalan	-1.4	t=0.3107, ns	-6.9	t=1.553, ns	-13.7	t=3.107, *	-58.2	t= 13.28, ***	-1.4	t=0.2955, ns	-4.2	t=0.8866, ns	0	t=0.0, ns	-29.5	t= 6.287, ***

Buck et al., Table 2

A

Function Generator

CCD Camera

Viewpoint Tracking Software

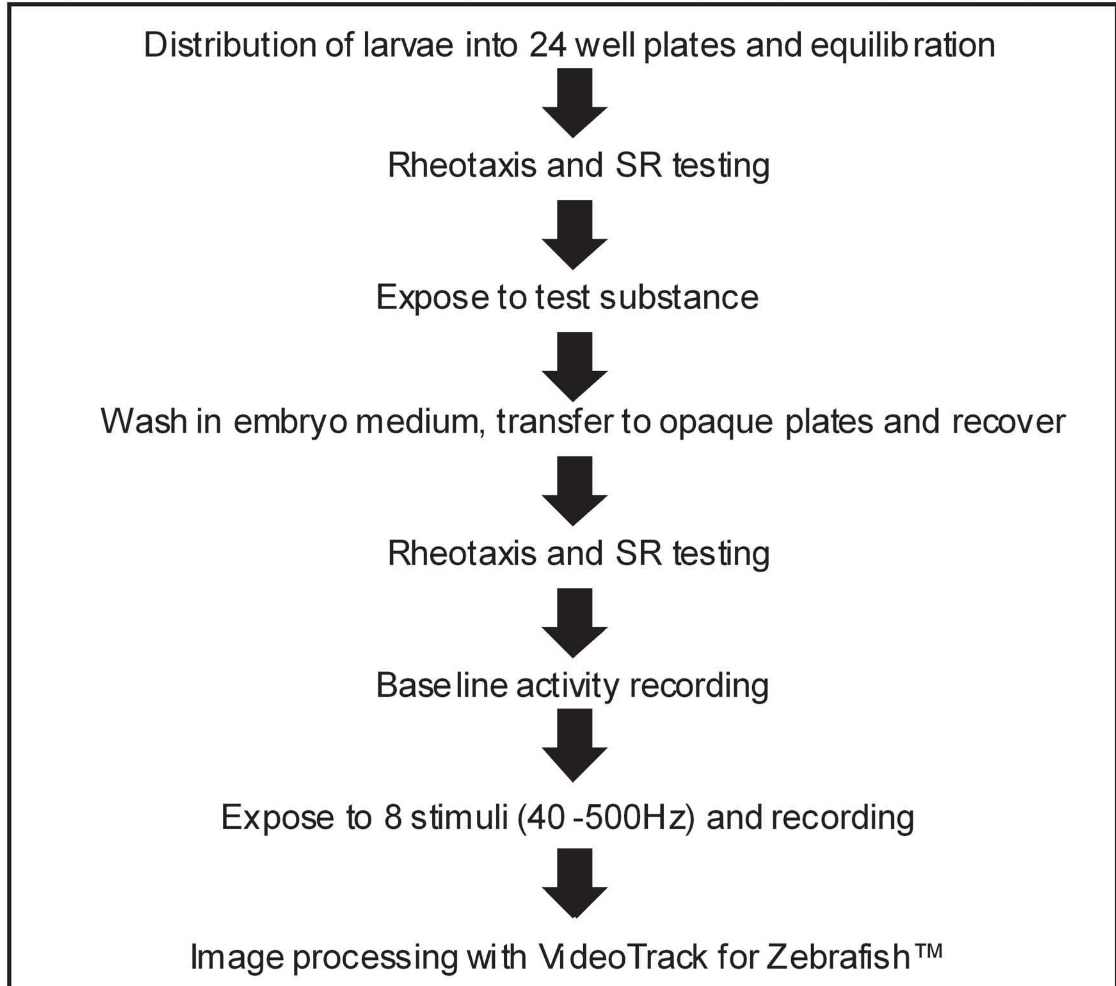
Speaker

Amplifier

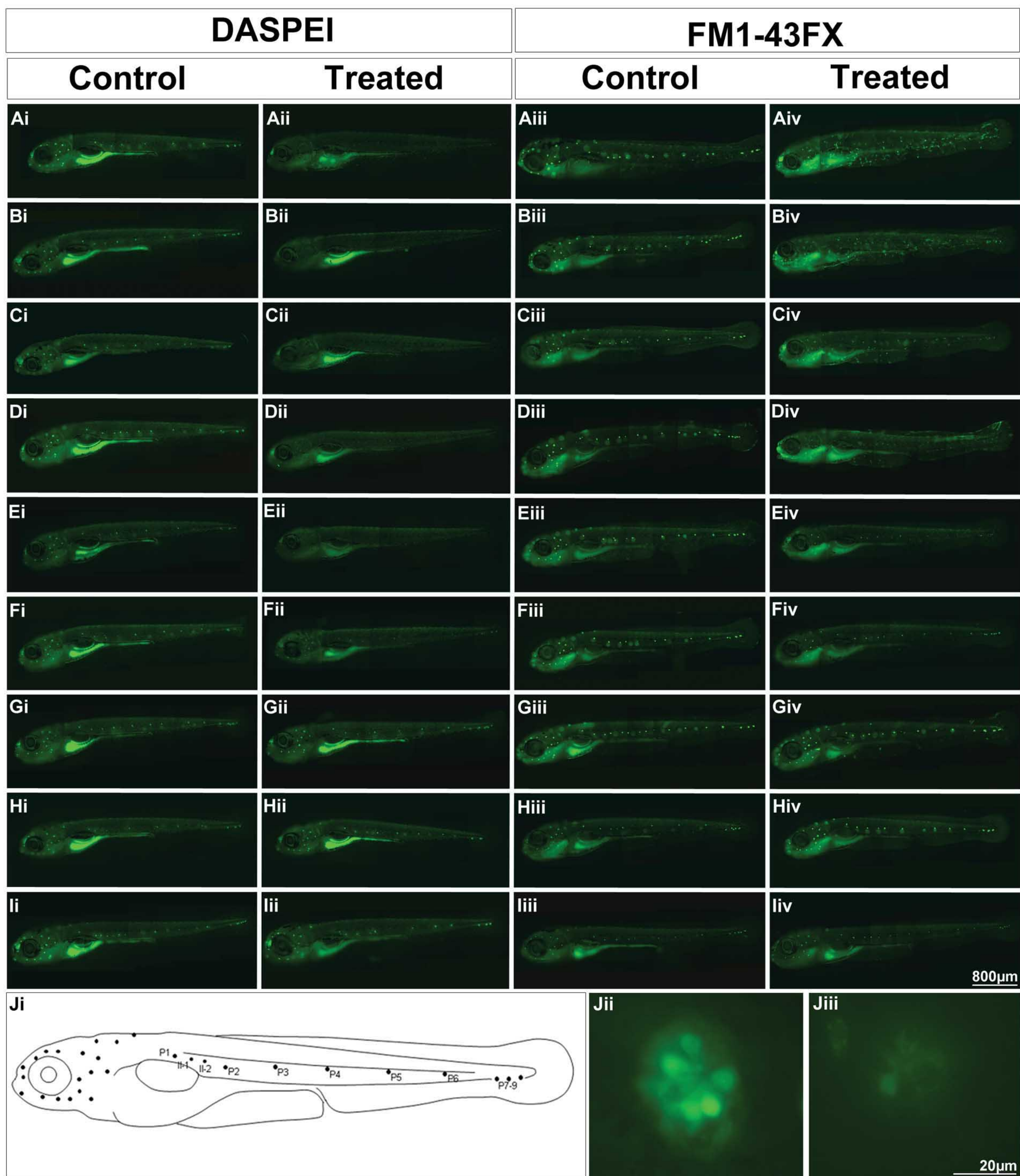
X 3

24 Well Plate containing fish

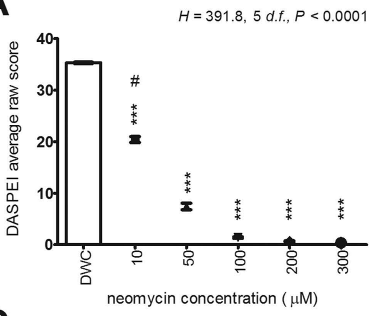
B

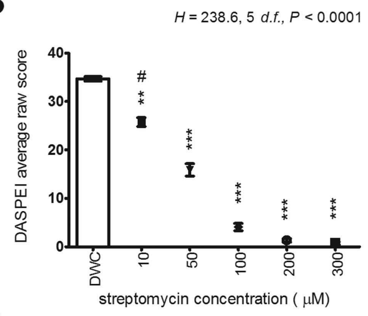


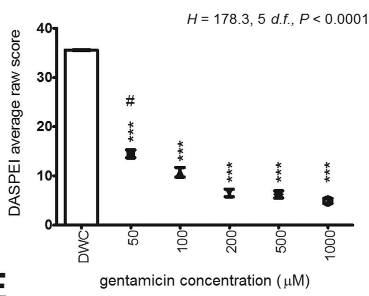
Buck et al., Figure 1

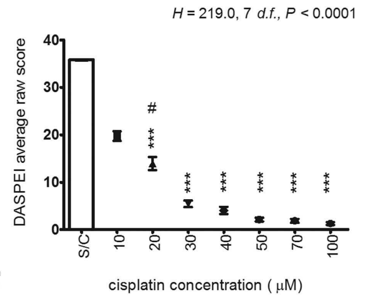


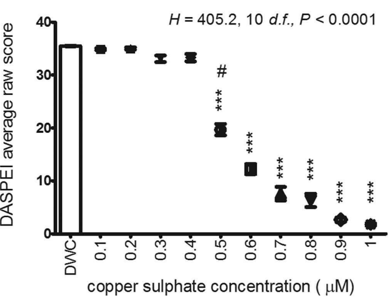
Buck et al., Figure 2

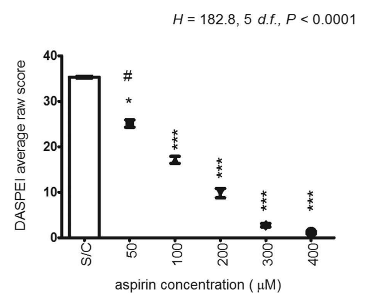


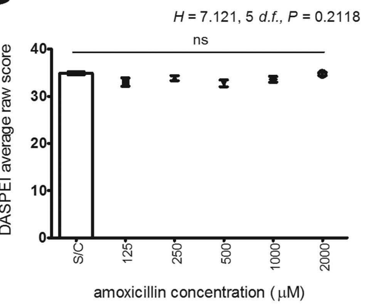


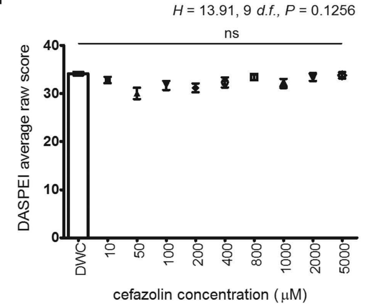


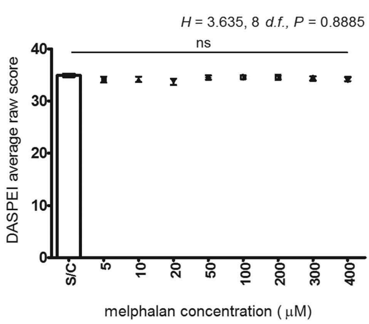








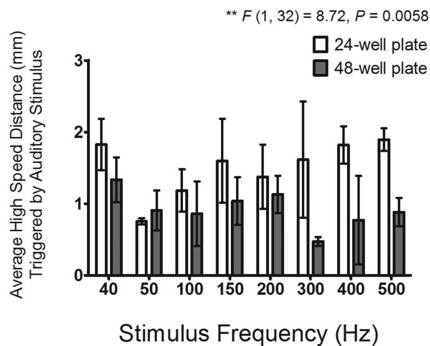


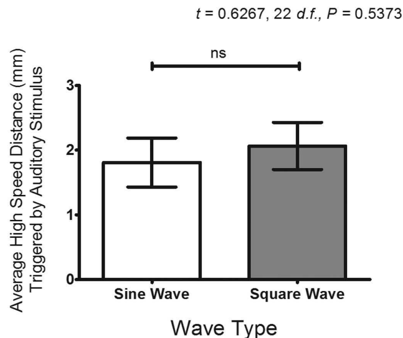


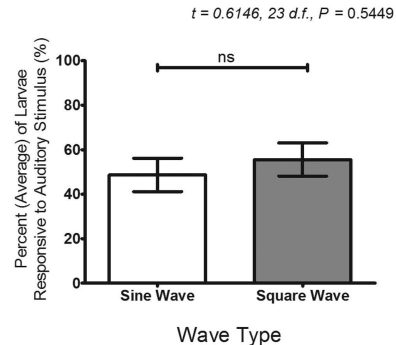
J

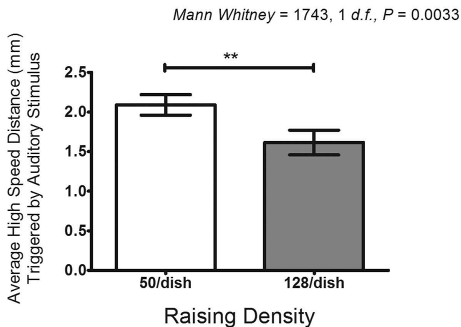
Test Substance	Estimated IC_{50} (μM)	IC_{50} 95% Confidence Level (μM)
Neomycin	13.90	9.99 to 19.32
Streptomycin	40.36	20.20 to 80.67
Gentamicin	24.89	11.98 to 51.75
Aspirin	193.10	86.90 to 429.10
Cisplatin	14.46	8.20 to 25.48
Copper (II) sulphate	0.53	0.49 to 0.58

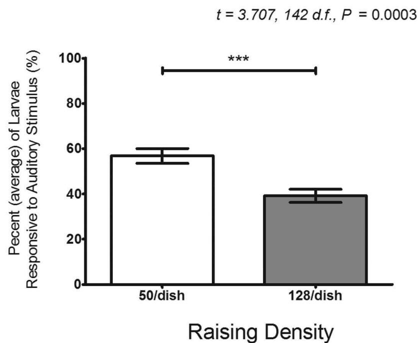
Buck et al., Figure 3

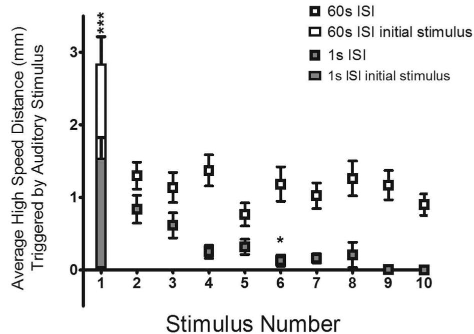




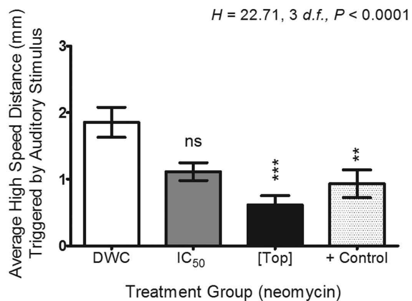


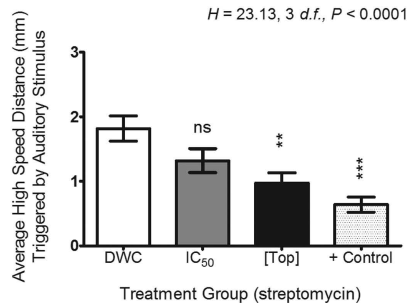


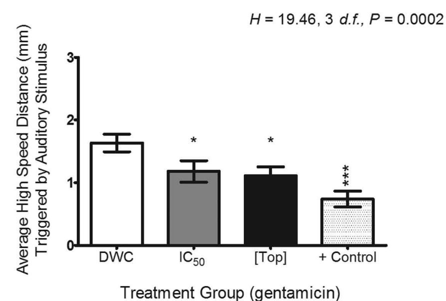


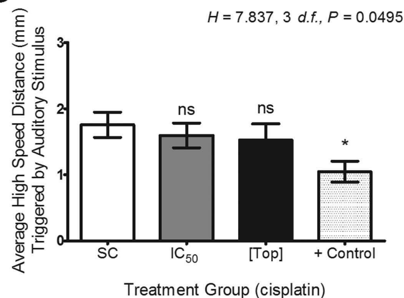


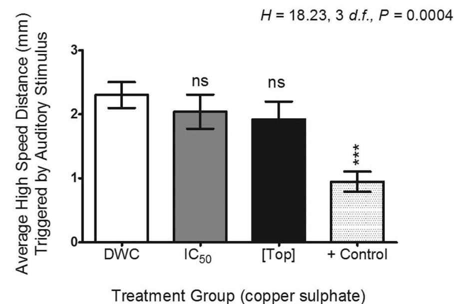
Buck et al., Figure 4

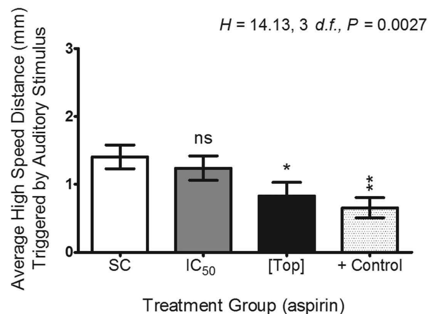


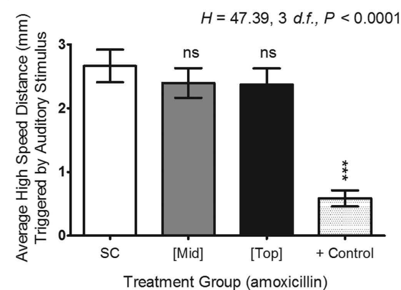


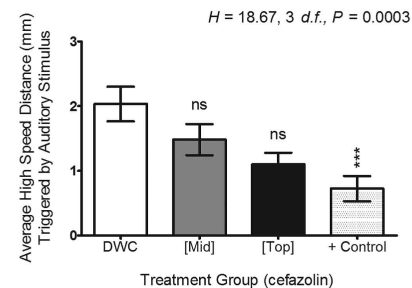


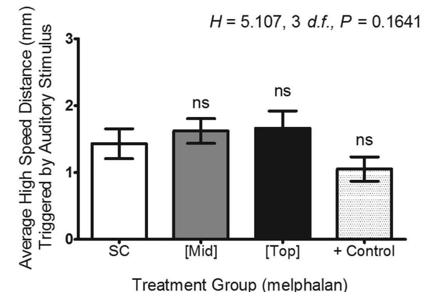




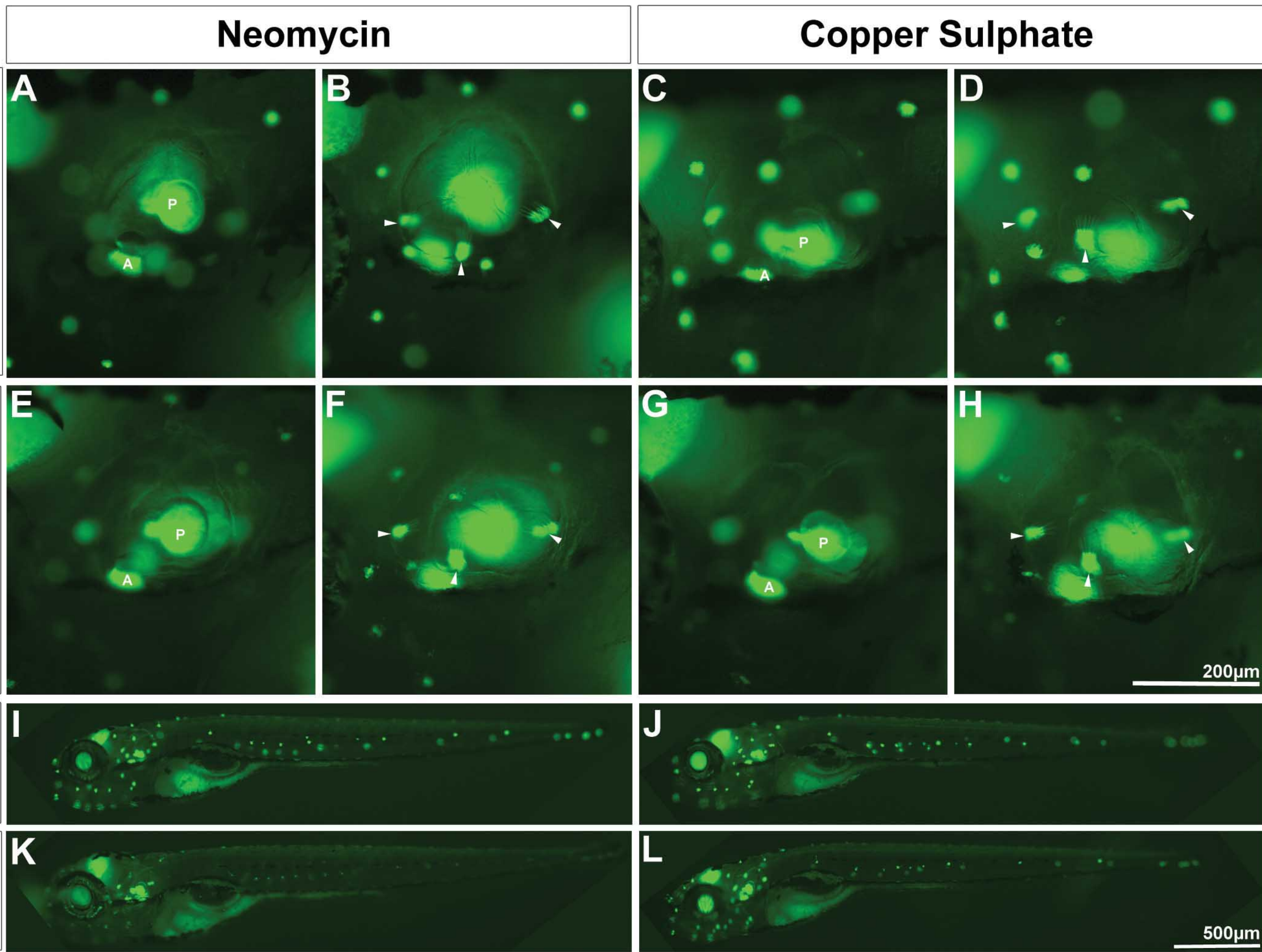








Buck et al., Figure 5



Buck et al., Figure 6

1 **SUPPLEMENTARY FIGURE AND TABLE LEGENDS**
2
3 **Supplementary Figure S1. Concentration-response data for gemcitabine**
4 **hydrochloride.** (A) Exposure to gemcitabine hydrochloride caused a significant
5 concentration-dependent decrease in DASPEI staining, indicative of hair cell damage
6 (One-way ANOVA followed by Dunnett's tests; F (4, 21) = 197.3, P <0.0001). Exposure
7 time and concentration range as described in Suppl. Table S1. 5 larvae per treatment
8 group were assessed over a single trial. (B) The estimated IC_{50} value for gemcitabine
9 (log(concentration of test compound) vs. response (three parameters) curve-fitting
10 equation).
11
12 **Supplementary Figure S2. The relationship between sound pressure level**
13 **measurements and microphone SPL in air versus water.**
14
15 **Supplementary Figure S3. Development was unaffected by altering the density at**
16 **which larvae are raised.** (A-D) Representative images of larvae reared at a density of
17 50 per Petri dish (taken from a sample of 150 animals). (E-H) Representative images of
18 larvae reared at a density of 128 per Petri dish (taken from a sample of 128 animals).
19 Note normal development of eye, body pigmentation, body length, and swimbladder
20 inflation for all animals. Scale bar = 500 μm.
21
22 **Supplementary Figure S4. The effects of neomycin versus MS222 on the**
23 **movement profile, rheotaxis and SR score of larvae.** (A-B) Examination of the
24 baseline movement profiles of larvae treated with top concentration neomycin or
25 anaesthetic (analysed using Kruskal-Wallis test and Dunn's post-tests). (C-D) Rheotaxis
26 and SR scores alter after immersion in neomycin or anaesthetic. Data from three
27 experimental trials were analysed using Two-way ANOVA, followed by Bonferroni
28 multiple comparison tests. Statistical significance level (α) is denoted by asterisks (*).
29 For *n* numbers see section 2.4.10.
30
31 **Supplementary Table S1 Compound exposure details.** Treatments were performed
32 as shown for each assay. MTC was defined as the concentration of compound to elicit
33 more than 20% mortality within a single treatment group. For *n* numbers see section 2.2.
34

35 **Supplementary Table S2 Individual stimulus settings.** The stimulus duration and
36 factors controlling the signal amplitude are shown for each individual frequency tested.
37
38 **Supp Table S3 Threshold testing in control larvae.** Amplifier volume settings that
39 elicited an average response of more than 40% of fish over three experimental trials are
40 shown. Fewer than 40% of fish responded at 800Hz, even at the top volume before
41 waveform distortion. For *n* numbers see section 2.4.4.
42
43 **Supplementary Table S4 The comparative sensitivities of the histological and**
44 **functional assays.** The various sensitivities are presented for each method. The most
45 sensitive assay is decided first by lowest detectable concentration effect and then by
46 significance level (α) at that concentration.

Test compound	CAS number	DASPEI assay		Startle, SR and Rheotaxis Assays		MTC (>20% Mortality) (mM)
		Concentration range (μM)	Exposure time (Hours)	Concentrations Tested (μM)	Exposure time (Hours)	
Neomycin trisulphate salt hydrate	1405-10-3	0-300	1	0, 14, 100	1	0.5
streptomycin sulphate salt	3810-74-0	0-300	1	0, 40, 200	1	4
gentamicin sulphate	1405-41-0	0-1000	1	0, 25, 1000	1	>10
cisplatin	15663-27-1	0-100	2	0, 14, 100	2	1
aspirin	50-78-2	0-400	1	0, 193, 300	1	1.5
copper (II) sulphate	7758-98-7	0-1	1	0, 0.5, 1	1	0.15
amoxicillin	26787-78-0	0-2000	2	0, 1000, 2000	2	12
cefazolin sodium salt	27164-46-1	0-5000	1	0, 2500, 5000	1	>20
melphalan	148-82-3	0-400	0.5	0, 200, 400	1	>0.5
gemcitabine hydrochloride	122111-03-9	0-5000	0.5	-	-	1.5

Buck et al., Suppl. Table S1

Frequency (Hz)	Stimulus Duration (ms)	Amplitude of Signal	
		Voltage (Vpp)	Volume (dB TP)
40	540	2.3	-15
50	440	1.6	-15
100	440	2.1	-25
150	440	2.7	-35
200	440	2.2	-30
300	440	3.8	-40
400	440	4.3	-41.25
500	440	4.5	-30

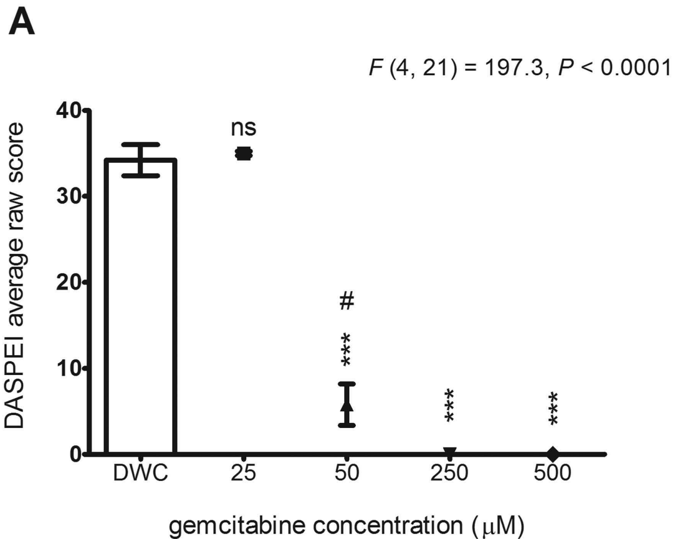
Buck et al., Suppl. Table S2

Frequency (Hz)	Amplifier volume (-dBTP)	Average Number of Larvae Responding Over 3 Repeats	Percentage of Larvae Responding (%)
40	15	12.67	52.78
50	15	9.67	40.28
100	25	12	50
150	40	10	41.67
200	30	14.67	61.1
300	40	9.67	40.28
400	43.75	11.67	48.61
500	30	11.3	47.2
800	16.25	8.67	36.1

Buck et al., Suppl. Table S3

Test Substance	DASPEI Assay		Startle Assay		SR Assay		Rheotaxis Assay		Most Sensitive Assay?
	Lowest Detectable Concentration Effect (µM)	Significance *P* value	Lowest Detectable Concentration Effect (µM)	Significance *P* value	Lowest Detectable Concentration Effect (µM)	Significance *P* value	Lowest Detectable Concentration Effect (µM)	Significance *P* value	
Neomycin	10	*	100	*	14	**	14	**	DASPEI
Streptomycin	10	*	200	*	40	***	200	*	DASPEI
Gentamicin	50	*	25	*	25	***	25	**	SR assay
Aspirin	50	*	300	*	193	***	300	*	DASPEI
Cisplatin	20	*	-	ns	14	*	100	*	SR assay
Copper (II) sulphate	0.5	*	-	ns	0.5	***	-	ns	SR assay

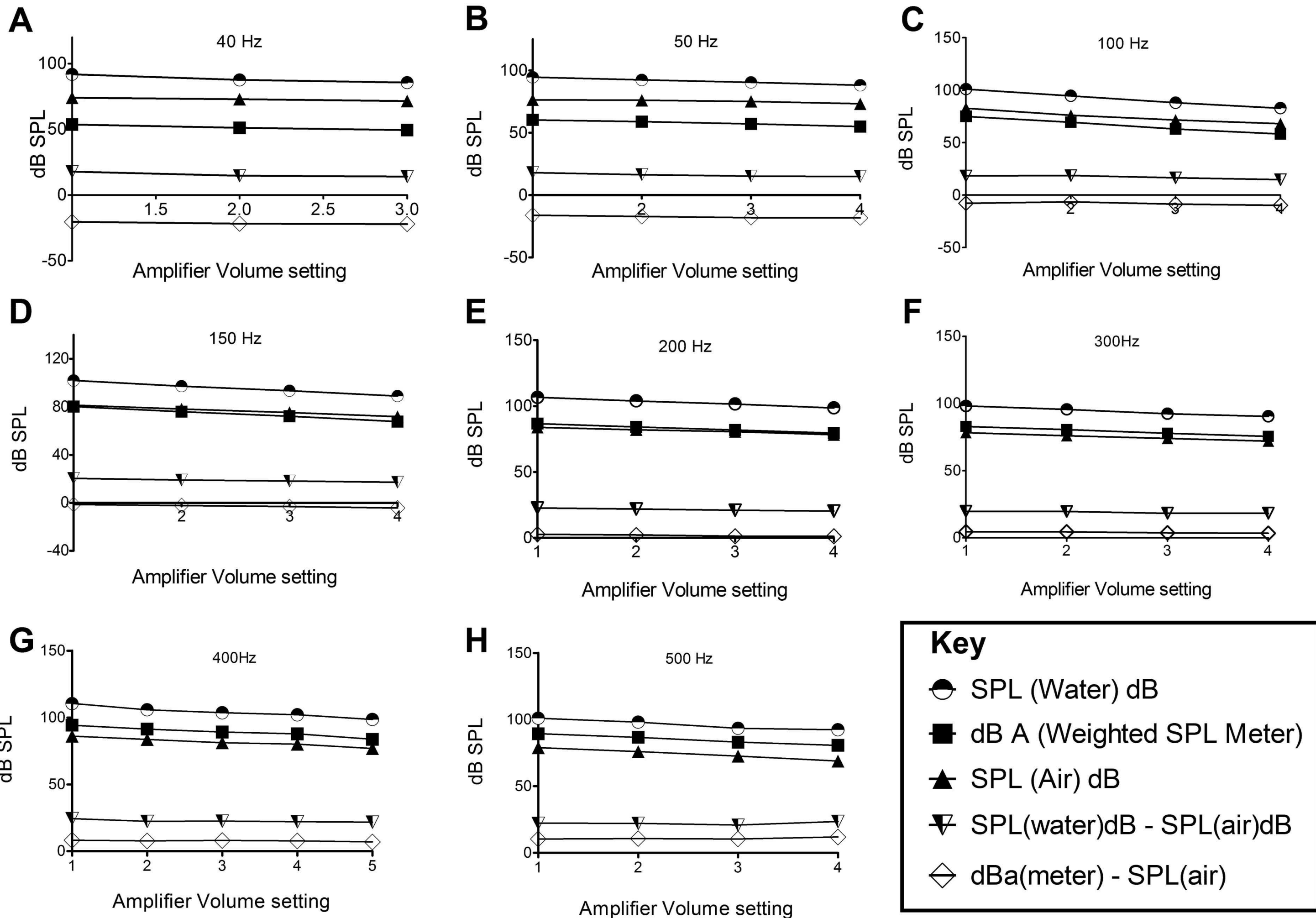
Buck et al., Suppl. Table S4



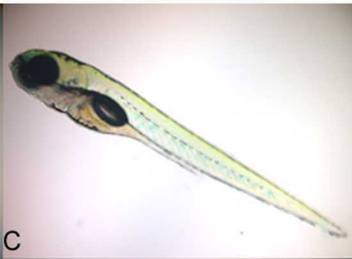
B

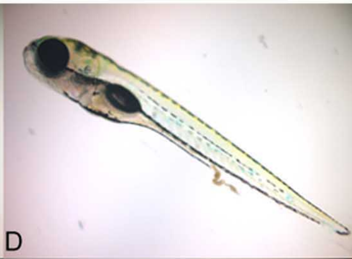
Predicted IC_{50} (μM)	95% Confidence Levels IC_{50} (μM)
59.25	24.03 - 146.1

Buck et al., Suppl. Fig. S1

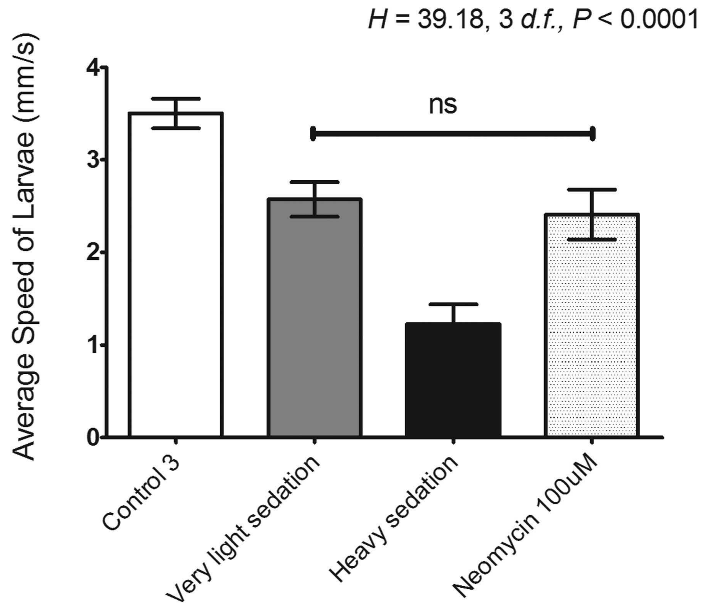


Buck et al., Supp. Fig. S2



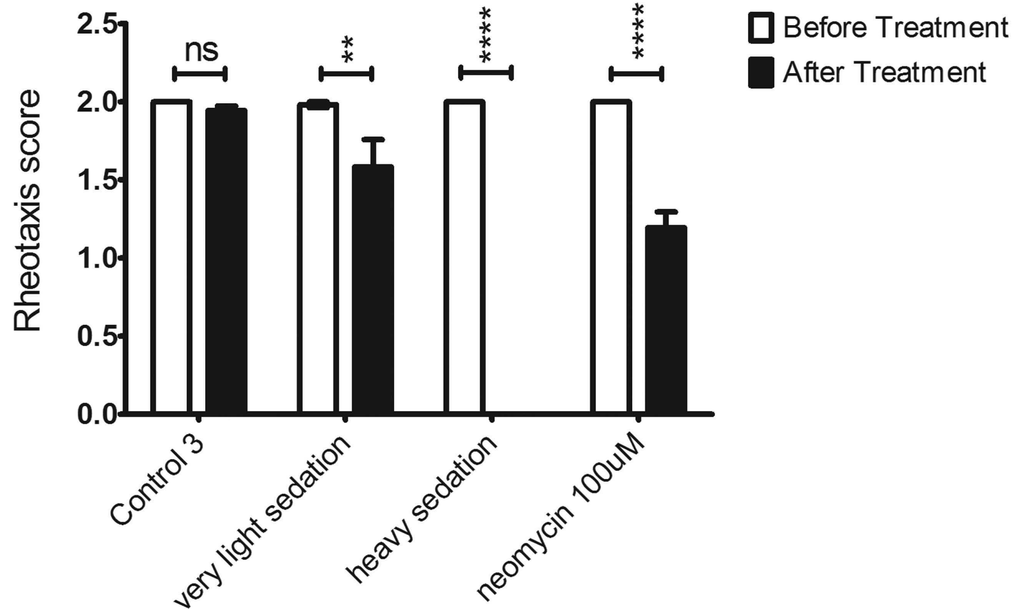


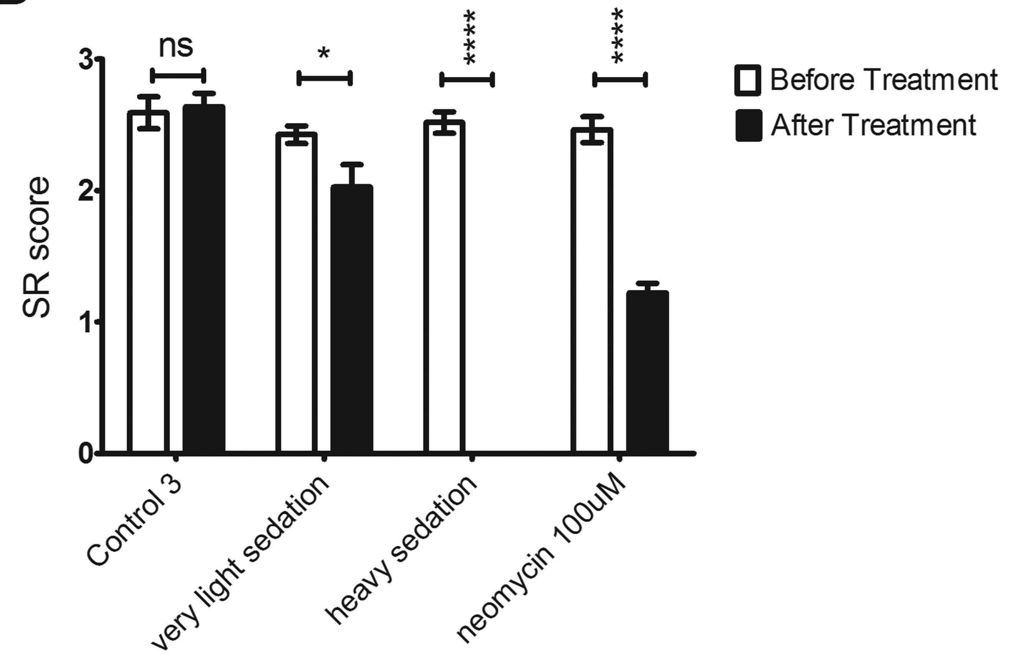
Buck et al., Suppl. Fig. S3



B

Baseline Movement Recordings: Summarised Results			
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
Control 3 vs. Very light sedation	24.87	Yes	*
Control 3 vs. Heavy sedation	60.64	Yes	***
Control 3 vs. Neomycin 100uM	33.8	Yes	***
Very light sedation vs. Neomycin 100uM	8.933	No	ns
Heavy sedation vs. Neomycin 100uM	-26.84	Yes	*





Buck et al., Suppl. Fig. S4