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Subchronic alteration of vestibular hair cells in mice: implications for multisensory gaze stabilization

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1 **Abstract**

2 The functional complementarity of the vestibulo-ocular reflex (VOR) and optokinetic reflex
3 (OKR) allows for optimal combined gaze stabilization responses (CGR) in light. While sensory
4 substitution has been reported following complete vestibular loss, the capacity of the central
5 vestibular system to compensate for partial peripheral vestibular loss remains to be
6 determined. Here, we first demonstrate the efficacy of a 6-week subchronic ototoxic protocol
7 in inducing transient and partial vestibular loss which equally affects the canal- and otolith-
8 dependent VORs. Immunostaining of hair cells in the vestibular sensory epithelia revealed
9 that organ-specific alteration of type I, but not type II, hair cells correlates with functional
10 impairments. The decrease in VOR performance is paralleled with an increase in the gain of
11 the OKR occurring in a specific range of frequencies where VOR normally dominates gaze
12 stabilization, compatible with a sensory substitution process. Comparison of unimodal OKR
13 or VOR versus bimodal CGR revealed that visuo-vestibular interactions remain reduced
14 despite a significant recovery in the VOR. Modeling and sweep-based analysis revealed that
15 the differential capacity to optimally combine OKR and VOR correlates with the
16 reproducibility of the VOR responses. Overall, these results shed light on the multisensory
17 reweighting occurring in pathologies with fluctuating peripheral vestibular malfunction.

18 **Introduction**

19 The vestibular system is well-preserved amongst vertebrates, participating in essential
20 functions such as balance, postural control and, together with the optokinetic system, gaze
21 stabilization (Straka et al., 2016; Wibble et al., 2022). Beyond these recognized roles,
22 vestibular signals also contribute to cognitive processes e.g spatial orientation and navigation
23 (Cullen, 2019), or body representation (Lopez et al., 2012; Facchini et al., 2021). Because of
24 its involvement in many basic functions important in our daily life, vestibular pathologies
25 affecting the inner ear are associated with a significant deterioration of the well-being of
26 patients (Möhwald et al., 2020) and represent an important public health concern (Agrawal
27 et al., 2009, 2013).

28 Research on post-lesion plasticity following permanent vestibular loss has shed light on the
29 neural plastic mechanisms that follow a chronic unilateral or bilateral vestibular lesion, a
30 process referred as “vestibular compensation” (Brandt et al., 1997; Cullen et al., 2010;

31 Beraneck and Idoux, 2012). The compensation taking place after the lesion is known to involve
32 dynamical multisensory reweighting of proprioceptive and visual inputs and of internal
33 efferent copies (Cullen et al., 2010; Sadeghi et al., 2012; Sadeghi and Beraneck, 2020). While
34 total and permanent lesions offer the experimental opportunity to characterize drastic
35 cellular and molecular changes triggered by the total silencing of the vestibular endorgans,
36 they imperfectly mimic clinical situations where peripheral vestibular function loss is only
37 partial and/or transient (Bisdorff et al., 2009; Lopez-Escamez et al., 2015; Brandt and
38 Dieterich, 2017). To better model fluctuating inner ear function, protocols based on
39 subchronic exposure to an ototoxic substance, 3,3'-iminodipropionitrile (IDPN) were first
40 introduced in the rat (Seoane et al., 2001; Sedó-Cabezón et al., 2014, 2015; Martins-Lopes et
41 al., 2019) and more recently in the mouse (Greguske et al., 2019). Subchronic exposure to
42 IDPN in drinking water at low doses allowed for progressive ototoxicity, leading to a partial
43 and largely reversible loss of function. Although the subchronic IDPN protocol was shown to
44 cause postural and locomotor deficits (Martins-Lopes et al., 2019), its effects on the gaze
45 stabilizing reflexes, namely the vestibulo-ocular and optokinetic reflexes, have not yet been
46 described.

47 The primary objective of the present study is first to assess how subchronic exposure to IDPN
48 may affect the function of the different vestibular endorgans. To that end, we took advantage
49 of our recently described methodology (Simon et al., 2020, 2021) using canal-specific and
50 otolith-specific tests. Quantification of the vestibulo-ocular reflexes is a sensitive and specific
51 method to assess the functionality of the sensory-motor vestibular pathway; it is the most
52 used test in clinics, and highly correlates to quality-of-life reports in patients suffering from
53 acute peripheral diseases (Möhwald et al., 2020).

54 The secondary objective is to determine whether visual substitution occurs following
55 transient and partial vestibular loss. Optogenetic stimulation of the vestibular pathway
56 demonstrated the recruitment of circuits involved in visual processing at the midbrain,
57 thalamic and cortical regions (Leong et al., 2019). Recent imaging studies in rodents have
58 shown that acute vestibular loss triggers brain-wide adaptive plasticity in circuits known to
59 be involved in visual processing (Zwergal et al., 2016; Grosch et al., 2021). In addition, it was
60 previously shown that OKR plasticity is triggered during vestibular compensation following a
61 permanent vestibular lesion (Faulstich et al., 2006; Nelson et al., 2017).

62 We report that 6 weeks of IDPN subchronic treatment affects both the canal- and otolith-
63 dependent vestibulo-ocular reflexes and that organ-specific loss of type I hair cells (HC)
64 correlates with individual mice's impairments. We show that optokinetic adaptive
65 compensation is frequency-specific and delayed with respect to the VOR changes. OKR
66 changes occur at the frequencies where physiologically the vestibular inputs dominate visuo-
67 vestibular gaze stabilization. We demonstrate that despite the significant recovery of their
68 vestibulo-ocular reflexes, the visuo-vestibular integration remains notably impaired in some
69 IDPN-treated mice. We suggest that the "noisiness" of the recovered vestibular signal affects
70 their capacity to optimally combine visual and vestibular responses. Overall, these results
71 shed light on the dynamic of multisensory reweighting in patients suffering from fluctuating
72 peripheral vestibular malfunction.

73 Results

74 Effects of the subchronic treatment of IDPN on the canal- and otolith-dependent VOR.

75 To investigate the effects of the IDPN on vestibulo-ocular reflexes (VOR), animals were
76 exposed to the ototoxic compound in the drinking water for six weeks (Treatment period),
77 followed by 6 weeks of standard drinking water without IDPN (Washout period). The VOR
78 were quantified every two weeks using canal-specific and otolith-specific tests. Horizontal
79 sinusoidal rotations in the dark were performed and oculomotor responses were recorded
80 using video-oculography (Figure 1A) to study the impact of IDPN treatment on the canal-
81 dependent angular VOR (aVOR). Typical raw aVOR traces are shown in Figure 1B. At week 6
82 (W6) the amplitude of the eye movements was distinctly reduced compared to W0, while at
83 W12 the amplitude of the response appeared partially restored. The dynamics of decrease
84 and recovery of the mean aVOR gain over the course of the protocol are reported in Figure
85 1C for both IDPN (n=21) and SHAM (n=22) groups. The evolution over time of the aVOR gain
86 is significantly different for the two groups of mice (ANOVA Weeks x Group interaction,
87 $F(6,246)=29,949, p<10^{-4}$). Before treatment, both groups responded similarly to the sinusoidal
88 stimulations and their aVOR gain remained unchanged through 2 weeks of treatment (W2).
89 However, starting W4 the aVOR gain of IDPN group significantly decreased with respect to
90 W0 (Newman-Keuls post hoc test: IDPN W0 vs W4 $p<10^{-4}$, see Table 1) and was significantly
91 lower compared with the SHAM group (W4, IDPN vs SHAM $p<10^{-4}$). The aVOR gain of the IDPN
92 group remained lower compared to SHAM through the rest of the protocol (W6 IDPN vs SHAM
93 $p<10^{-4}$, W8 IDPN vs SHAM $p<10^{-4}$, W10 IDPN vs SHAM $p<10^{-4}$, W12 IDPN vs SHAM $p<10^{-4}$)
94 with a minimum reached at W6 corresponding to $\sim 2/3$ of aVOR loss (IDPN W4 vs W6 $p<10^{-4}$,
95 IDPN W6 vs W8 $p=0,043762$). At the end of the six weeks of washout, the mean aVOR
96 significantly improved (IDPN W6 vs W12 $p<10^{-4}$) to levels observed at W4 (IDPN W4 vs W12
97 $p=0.561969$). Notably, the amplitude and dynamic of gain changes were similar for all
98 frequencies $>0.2\text{Hz}$ (see Figure 1 supplement 1A). However, at the lowest frequency tested
99 (0.2Hz), aVOR gain decrease reached significance only at W6.

100 aVOR responses were further modified by significant phase leads that affected all frequencies
101 starting W6 (Figure 1 supplement 1B, ANOVA repeated measures Weeks x Group Interactions
102 $F(6, 246)=14.528, p<10^{-4}$). Overall, canal responses remained unaffected until week 2, but

103 the amplitude and/or timing of the aVOR was abnormal from week 4 until the end of the
104 protocol, despite a significant recovery of angular VOR responses observed during the
105 washout period.

106 To determine whether otolith-dependent VOR was also affected by IDPN treatment, ocular-
107 counter roll responses (OCR) were tested during static lateral inclination in the range $\pm 40^\circ$
108 (OCR; Figure 1D). Examples of raw traces and quantification of the response are shown in
109 Figure 1E, and mean gain of the OCR of each group is plotted in Figure 1F (n=13 IDPN, n=14
110 SHAM). The modulations of responses amplitude were significantly different between IDPN
111 and SHAM groups (ANOVA repeated measures Weeks x Group Interaction $F(6, 150) = 7.7411$
112 $p < 10^{-4}$). We note that there was a significant difference between SHAM and IDPN during the
113 initial measurements at W0, before any treatment. However, at this timepoint mice were not
114 yet separated into different groups. This incidental difference completely disappeared on the
115 measurement performed at W2 (W2 IDPN vs SHAM $p = 0.8135$). While the gain of the SHAM
116 group stays in a 0.5-0.6 range over the whole duration of the protocol, the responses of the
117 IDPN group significantly decreased at W4 compared to the SHAM group (W4 IDPN vs SHAM
118 $p < 10^{-4}$). This decrease was larger at W6 (IDPN W4 vs W6 $p = 0.023$) and stayed significantly
119 different from the SHAM group until W8. At W10 and W12, the OCR of IDPN group recovered
120 to a level comparable to the SHAM group (W10 IDPN vs SHAM $p = 0.1262$, W12 IDPN vs SHAM
121 $p = 0.3385$).

122 Sub-chronic treatment of IDPN was also investigated through the dynamic Off Vertical Axis
123 Rotation (OVAR) test (Figure 1 supplement 1C), which primarily reflects maculo-ocular
124 (dominantly otolithic) responses integrated by central vestibular pathways. The maculo-
125 ocular reflex (MOR) bias decreased significantly compared to the SHAM group (ANOVA Weeks
126 x Group, $F(6, 132) = 10.076$, $p < 10^{-4}$) starting W2 of treatment (W2 IDPN vs SHAM $p = 0.0018$),
127 demonstrating that ototoxicity already affected the vestibular system at this early time point.
128 The maximal decrease was reached at W6 (W6 IDPN vs SHAM $p = 0.00012$) and recovery led
129 to normal responses at W12 (W12 IDPN vs SHAM $p = 0.4709$).

130

131 **Comparison of otolith- and canal-dependent plasticity in individuals.**

132 To compare the dynamic of canal- and otolith-dependent VOR alterations, the paired VOR
133 and OCR gains measured in individuals from the IDPN group (n=13) are plotted together
134 (Figure 2). Alteration in canal- and otolith-dependent responses followed a very similar time

135 course (OCR vs aVOR at 1Hz on figure 2A; similar patterns were obtained with the other tested
136 frequencies (not shown)).

137 To investigate further the organ-specific responses, the individual values of VOR gain at 1Hz
138 (left panel) and OCR slope (right panel) are plotted in Figure 2B. These plots show the
139 variability of the responses observed in different individuals. 2/3 of the mice had aVOR
140 responses lowered by more than 50%, while 1/3 had milder aVOR impairments. In most cases,
141 however, the aVOR gain decreased notably between W4 and W6 and started to recover from
142 W8 (compare individual slopes in Figure 2B). Similarly, OCR gains were variable between
143 individuals. However, the dynamic of the variations in otolith-dependent responses followed
144 a pattern comparable to that of the canal-dependent changes.

145 To determine whether the changes in canal- and otolith-dependent responses were
146 proportional, individual variations in gain since pre-treatment of the aVOR and OCR were
147 compared at W6 (Figure 2C) and W12 (Figure 2D). At W6, there was a significant correlation
148 between the amplitude of the changes observed in aVOR and OCR, such that canalar and
149 otolithic loss of function was proportional (slope of regression line: 0.5586). Despite the
150 general recovery, this significant correlation was preserved at W12: the recovery in canal-
151 dependent responses was proportional to the recovery in otolith-dependent responses (slope
152 of regression line: 0.5566). Overall, these results suggest that the subchronic IDPN treatment
153 similarly affects the vestibulo-ocular reflexes that depend on semi-circular canals and on the
154 otolithic organs, respectively.

155

156 **Effects of the IDPN exposure on the number of hair cells in the vestibular epithelia.**

157 To correlate the vestibular loss of function to the structural changes induced by the ototoxic
158 compound, vestibular epithelia were dissected and labelled to assess the number of hair cells
159 (HC) in the organs. One horizontal semi-circular canal and one utricle were harvested at W6
160 for n=7 IDPN and n=4 SHAM, and at W12 for n=8 IDPN and n=4 SHAM, and each organ
161 epithelium was divided into the central and peripheral region to differentiate the possible
162 participation of zone- and organ-specific HC to the vestibular function. Vestibular HC were
163 labelled with type-specific immunomarkers in both endorgans (Figure 3). Confocal
164 immunostaining data are presented in Figure 3A1 for the horizontal semi-circular canals and
165 Figure 3C1 for the utricle. 6-week long treatment of IDPN exposure led to a significant
166 reduction in the labelling of type I HC in the canal crista (quantified in Figure 3A2, Non

167 parametric Kruskal-Wallis test, SHAM vs IDPN Spp1, $p=0.0322$; SHAM vs IDPN CASPR1,
168 $p=0.0268$; Figure 3 supplement 3A1 and B1 for the peripheral zones) and in the otolith macula
169 (Figure 3C2, Non parametric Kruskal Wallis test SHAM vs IDPN Spp1, $p=0.0279$; SHAM vs IDPN
170 CASPR1, $p=0.0343$). However, the treatment did not alter type II HC-specific labelling as the
171 number of Calre+ cells in the IDPN group was similar to the SHAM in all regions of either
172 vestibular endorgans (Figure 3A2, canal crista, Calre: Non parametric Kruskal-Wallis test IDPN
173 vs SHAM, $p>0.9999$; Figure 3C2, otolith macula, Calre: SHAM vs IDPN, $p>0.9999$). Importantly,
174 the total number of HC marked with Myo7a (labelling both type I and type II HC) was not
175 significantly different between the IDPN-treated mice and the SHAM, suggesting that the
176 alteration of type I HC is not associated with cell loss. Moreover, at W12 the number of type
177 I HC labelled in the IDPN-treated mice was no longer significantly different from the SHAM
178 group in either endorgans (Figure 3A3 for crista; Figure 3C3 for macula). The count of type II-
179 specific markers and of non-specific HC markers was not statistically different between SHAM
180 and IDPN either, so that overall no difference persisted at W12, indicating a structural
181 recovery at the end of the washout period.

182 To determine how the loss of HC correlates with organ-specific functional tests, the numbers
183 of type I and II HC in the central region of the horizontal semi-circular canals are plotted as a
184 function of the mean aVOR gain for mice at W6 and W12 (Figure 3B1). Both markers of type
185 I HC (CASPR1 and Spp1, left panels) were significantly correlated with the aVOR gain (linear
186 regression, Spp1: $r^2=0.5128$, $p=0.0001$; CASPR1: $r^2=0.4948$, $p=0.0002$) whereas the rather
187 constant number of type II HC in the ampulla did not correlate with the variation observed in
188 aVOR function. Finally, to determine if the correlation was due to the recovery occurring
189 between W6 and W12, the number of CASPR+ cells of IDPN mice counted at W12 are plotted
190 as a function either of the individual aVOR gain at W12 (pink squares) or aVOR gain at W6
191 (black circles), paired by an arrow (Figure 3B2). The recovery of the vestibular function
192 between W6 and W12 induces a shift toward the W12 linear regression, reinforcing the
193 notion that the recovery of function was related to the increase in the number of hair cells
194 with normal expression of biochemical markers.

195 The numbers of type I HC and II HC found in the striolar region of the utricle are similarly
196 plotted in relation to the OCR gain (linear regression, Spp1: $r^2=0.6011$, $p<0.0001$; CASPR1:
197 $r^2=0.6342$, $p<0.0001$, Figure 3D1). Again, the otolithic function correlated with the number of
198 type I HC, and not type II HC in the central region. Furthermore, the correlation found

199 between the otolithic function and the number of type I HC related to the increase in the
200 number of hair cells after the recovery period (Figure 3C2). A similar correlation between
201 organ-specific function and the number of type I HC was also found in the peripheral regions
202 of these organs (see Figure 3 supplement 3C1, 3C2 for the SCC, and Figure 3 supplement 3D1,
203 3D2 for the utricle).

204 Taken together, comparison at W6 and W12 of non-specific HC and type I-specific HC markers
205 suggest that the ototoxic effect induces a transient biochemical alteration of type I HC rather
206 than a definitive hair cell degeneration. Overall, IDPN-induced alteration of canal and otolith
207 functions are correlated with the ototoxic effect on type I, and not type II, HC in the central
208 and peripheral zones of vestibular endorgans.

209

210 **Effects of the subchronic treatment of IDPN on the optokinetic reflex.**

211 To determine the effects of the subchronic treatment of IDPN on the optokinetic reflex (OKR),
212 mice were tested with sinusoidal rotations of a virtual drum (Figure 4A). An example of raw
213 traces of the evoked horizontal eye movements is shown in Figure 4B for different timepoints
214 (0.5Hz at 10deg.s⁻¹ optokinetic stimulation). The mean OKR gain for all tested frequencies is
215 plotted in Figure 4C for SHAM (n=12) and IDPN-treated mice (n=12). The mean OKR gain of
216 IDPN mice was significantly different from the mean OKR gain of SHAM mice at W8
217 (interaction between Weeks and Group $F(6,132)=2.9845$, $p=0.0091$; IDPN W0 vs W8,
218 $p=0.0216$, see Table 2) where it reached its peak (IDPN W0 vs W8, $p=0.00037$). The gain stayed
219 significantly larger compared to the SHAM through the end of the washout period (W12 IDPN
220 vs SHAM, $p=0.046$).

221 To determine whether this OKR modulation was frequency-specific, the gain of the 5 tested
222 frequencies was compared for W0, W6, W8 and W12 (Figure 4D). There was at W0 no
223 difference between SHAM and IDPN mice (Figure 4D, left panel, W0). At W6, there was a
224 significant difference between the gain of the IDPN and SHAM groups limited to the
225 frequencies 0.5 and 1Hz (W6 IDPN vs. SHAM, 0.5Hz, $p=0.013117$; W6 IDPN vs. SHAM, 1Hz,
226 $p<10^{-4}$). At W8, the gains measured at frequencies above 0.2Hz were significantly increased
227 compared to the SHAM group (W8 IDPN vs. SHAM, 0.33Hz, $p=0.027687$; 0.5Hz, $p=0.0025$; 1Hz,
228 $p=0.00015$). At W12, the gain remained high for both 0.5 and 1Hz whereas the gain at 0.33Hz
229 was no longer significantly larger than the SHAM group (W12 IDPN vs. SHAM, 0.33Hz, ns;
230 0.5Hz, $p=0.0069$; 1Hz, $p=0.00078$). Globally, responses at higher frequencies (0.33, 0.5 and 1

231 Hz) were significantly increased (ANOVA Weeks x Group x Frequencies interaction, $F(24,$
232 $528)=6.5870$, $p<10^{-4}$) whereas responses at the lowest frequencies (0.1 and 0.2Hz) were not
233 significantly modulated. Additionally, these changes in gain for frequencies $>0.2\text{Hz}$ were not
234 accompanied by changes in the timing (phase) of the OKR (Figure 4E) (ANOVA, Weeks x Group
235 x Frequencies interaction, $p=0.3802$).

236

237 **Relation between VOR decrease and optokinetic increase**

238 To correlate the changes in vestibular pathway with the frequency-specific changes observed
239 in the optokinetic pathway, we compared the responses in aVOR and OKR obtained at the
240 frequencies that were common between the 2 tests (i.e 0.2 Hz, 0.5 Hz and 1Hz) for $n=12$ SHAM
241 and $n=12$ IDPN treated mice by comparing the increase in the OKR and decrease in VOR
242 relative to W0 (Δ mean gain, see Figure 5). To determine whether the changes in VOR and OKR
243 were proportional, we compared the paired decrease and increase in the mean Δ aVOR and
244 Δ OKR gain after 8 weeks of treatment, when the difference between the 2 reflexes peaked
245 (Figure 5A). The vast majority ($n=11/12$) of IDPN-treated mice showed a decrease in aVOR
246 and an increase in OKR (top left quadrant corner, Figure 5B), which was not the case for SHAM
247 mice (blue squares). However, there was no correlation between the amplitude of the VOR
248 decrease and the amplitude of the OKR increase, i.e mice that had the greatest VOR loss did
249 not show the largest OKR increase (slope in the regression line: 0.27, $p=0.0834$).

250 To determine whether the parallel changes in OKR and VOR are frequency-specific, Figure 5C
251 compares the gains measured at W0 and W8 for each frequency. At 0.2Hz, there was a
252 significant decrease in aVOR (IDPN W0 vs. W8 0.2Hz $p<10^{-4}$), however the OKR was not
253 modified. On the other hand, at 0.5 and 1 Hz significant VOR decreases (IDPN W0 vs. W8,
254 0.5Hz, $p<10^{-4}$; 1Hz, $p<10^{-4}$) were accompanied by a significant OKR increase (IDPN W0 vs. W8,
255 0.5Hz, $p<10^{-4}$; 1Hz, $p<10^{-4}$). To examine this frequency-selective increase, the percentage of
256 change in the OKR gain is plotted as a function of the *vestibular weight*, determined as the
257 ratio between the aVOR and aVOR+OKR values at week 0. The *vestibular weight* therefore
258 represents the frequency-dependent relative influence of the vestibular signal on gaze
259 stabilization. Figure 5D shows that at week 8, the change in the OKR occurred at the
260 frequencies for which the *vestibular weight* is dominant ($>50\%$), and that the increase in OKR
261 was positively correlated with the *vestibular weight* among frequencies (slope of the
262 regression line: 1.235, $r^2=0.6299$, $p<0.0001$). Overall, these results suggest that the increase

263 in OKR gain observed at high frequencies could correspond to a “visual substitution” occurring
264 primarily in the range where the vestibular inputs are normally dominating gaze stabilization.
265

266 **Visuo-vestibular interactions following alteration of vestibular inputs**

267 To investigate whether OKR increase did constitute a “visual substitution” that maintained
268 optimal gaze stabilization at light despite vestibular loss, we investigated how IDPN-treated
269 mice integrated vestibular and visual inputs. To this end, a session of combined visual and
270 vestibular stimulation (aVOR in light, here referred as *Combined Gaze Response* or CGR
271 condition) was performed at W6 and at W12 (n=19 IDPN; n=12 SHAM). A model (see methods)
272 was implemented to predict the theoretical CGR (visuo-vestibular) gain and phase from aVOR
273 and OKR (unimodal) measured values. The model was first optimized to correctly predict the
274 average SHAM CGR gains and phases (Figure 6A) from their individual aVOR and OKR
275 responses, and then applied to the individual IDPN data. The predicted and observed CGR
276 gain (Model factor) were compared for each treatment group (ANOVA Group x Model,
277 $F(1,29)= 12.236$, $p=0.0013$): as expected, model predictions for SHAM well-matched
278 experimental data (Post-hoc Newman-keuls, SHAM MODEL vs SHAM CGR, $p=0.41$). When
279 applied to IDPN mice the model predicted that their CGR gain should be lower compared to
280 SHAM (shaded blue and red areas on Figures 6A and 6B, Post-hoc Newman-keuls, SHAM
281 MODEL vs IDPN MODEL, $p=0.00176$), suggesting that even an optimal combination of
282 unimodal responses could not restore completely normal CGR in IDPN-treated mice. The CGR
283 gains observed for IDPN-treated mice actually revealed how their visuo-vestibular responses
284 were not only reduced compared to SHAM (compare solid blue and red lines in Figure 6A-B,
285 Post-hoc Newman-keuls, SHAM CGR vs IDPN CGR, $p=0.00055$), but were even inferior to the
286 prediction of the model (compare solid red line and shaded red area in Figure 6B, Post-hoc
287 Newman-keuls, IDPN MODEL vs IDPN CGR, $p=0.00041$). This suggests that the IDPN-treated
288 mice sub-optimally combined their residual/compensated vestibular and visual reflexes to
289 stabilize gaze. Figure 6C illustrates the relationship between the measured CGR gain and the
290 prediction of the model for all tested frequencies. For SHAM animals, CGR gain values were
291 positioned along the unity line that represents a close match between predicted and
292 measured CGR (mean \pm SEM: SHAM MODEL 0.8255 \pm 0.053; SHAM CGR 0.8375 \pm 0.042). For
293 IDPN-treated mice, the responses formed 2 subgroups that did not correspond to different
294 frequencies (all frequencies found in either subgroup). The first subgroup was intermingled

295 with the SHAM responses, with measured CGR values slightly lower than predicted values
296 (MODEL 0.7457 ± 0.028 ; CGR 0.6644 ± 0.052). The second subgroup represented mice for which
297 the measured CGR significantly underperformed the predicted CGR (MODEL 0.4685 ± 0.022 ;
298 CGR 0.2005 ± 0.01). To investigate the difference between the two subgroups, the VOR, OKR
299 and CGR values were plotted for the $n=19$ IDPN mice (Figure 6D). A hierarchical cluster
300 analysis (see methods) suggested the presence of two main clusters that differ from the
301 clusters observed considering only the aVOR gain (see dendrogram in Figure 6D). The first
302 cluster ($\Delta+$, $n=12$; in green in Figure 6D, E, and F) comprised individuals with CGR values higher
303 than OKR (positive values on $\Delta = \text{CGR} - \text{OKR}$), i.e with gaze stabilization at light better in the
304 presence of vestibular stimulation. The second cluster ($\Delta-$, $n=7$; depicted in orange) is
305 composed of individuals with low CGR (<0.4). IDPN mice from the $\Delta-$ cluster have degraded
306 responses during bimodal visuo-vestibular stimulation compared to unimodal OKR
307 stimulation (negative value of Δ indicates CGR responses lower than OKR responses). Notably,
308 this degraded CGR is not solely a consequence of a low VOR gain, as some $\Delta+$ mice identified
309 by the second differentiation of the clustering analyses (depicted with grey diamond symbols
310 in Figure 6D, and indicated as $\Delta_{+(-)}$) also showed low unimodal VOR gain responses.
311 We reasoned that the worsening of the gaze responses in the bimodal condition compared
312 to the OKR-only condition could be a consequence of a poorly reliable, noisy vestibular signal.
313 Based on a cycle-to-cycle sweep analysis, two additional features of the VOR responses were
314 quantified (see methods): the mean amplitude of the raw sweep during VOR stimulation and
315 the VOR response reproducibility between sweeps. The mean VOR raw sweep amplitude
316 could not statistically differentiate $\Delta-$ and $\Delta_{+(-)}$ mice (Figure 6E, orange triangle vs green
317 diamonds $p=0.0857$), suggesting that vestibular signal of comparable amplitude improved or
318 deteriorated the CGR response in the $\Delta_{+(-)}$ and $\Delta-$ mice, respectively. On the other hand, the
319 VOR reproducibility clearly discriminated $\Delta-$ and $\Delta_{+(-)}$ mice (Figure 6F, orange triangle vs green
320 diamonds, $p=0.0001$). There was a strong correlation between the inter-sweep reproducibility
321 and the capacity of the mice to stabilize gaze in presence of vestibular inputs at light. This
322 result suggests that the incapacity of the $\Delta-$ group to compute optokinetic signals in the
323 presence of vestibular stimulation could relate to the poor reliability of the vestibular signal.
324 Overall, these data suggest that in some situations the presence of a degraded vestibular
325 signal of significant amplitude (panel 6E) but poorly reliable (panel 6F) can be detrimental to
326 properly stabilize gaze in presence of visual and vestibular inputs.

327 **Discussion**

328 **IDPN as a model of partial and transitory loss of vestibular function**

329 IDPN has long been established as an ototoxic compound targeting vestibular HC in the
330 different vestibular endorgans of rats (Llorens et al., 1993, 1994; Llorens and Demêmes, 1994)
331 and later in guinea-pig, frogs and mice (Soler-Martin et al., 2007; Greguske et al., 2019). Its
332 ototoxic vestibular effects were used as a tool to study extraocular muscle development
333 (Brueckner et al., 1999) or more recently to induce permanent vestibular loss in mice (Yang
334 et al., 2019; Zeng et al., 2020) and study hair cell regeneration (Sayyid et al., 2019). The sub-
335 chronic, reversible protocol used in our study was validated previously in rats (Sedó-Cabezón
336 et al., 2015; Martins-Lopes et al., 2019; Maroto et al., 2021) and mice (Boadas-Vaello et al.,
337 2017; Greguske et al., 2019) with postural/locomotor quantification of vestibular loss. We for
338 the first time demonstrate that the subchronic protocol leads to a progressive and partly
339 reversible loss of vestibulo-ocular reflexes. These previous studies further demonstrated
340 some of the cellular mechanisms associated with the progressive loss of postural control:
341 ototoxic effects lead to the early dismantlement of calyceal junction, followed by synaptic
342 uncoupling, both of which were shown to be reversible, while continuation of the IDPN
343 treatment would lead to hair cell extrusion and long-term, permanent lesion. HC loss was also
344 demonstrated to occur in a central to peripheral order within vestibular epithelia, and in crista
345 to utricule to saccule order (see Sedó-Cabezón et al. 2015). Maroto et al (2021) convincingly
346 demonstrated that type I HC show greater sensitivity than type II HC to IDPN subchronic
347 exposure. Given these data, a primary goal of the present study was to try to correlate the
348 loss of VOR functions to organ-specific, zone-specific, and cell-type-specific effects.

349 Loss and substantial recovery of the vestibular function quantified by VOR measures were
350 found to be correlated with the number of type I HC in both canals and otoliths. A parallel
351 was previously established between HC integrity and VOR following ototoxic protocols in mice
352 (Cassel et al., 2019; Yang et al., 2019; Zeng et al., 2020), but these were established at the
353 population level. Here, we for the first time specifically correlated the loss of HC and loss of
354 VOR function both at the individual level and in an organ-specific way. aVOR and OCR tests
355 demonstrated a parallel decrease in canal- and otolith-dependent functions, respectively. The
356 6 week-long treatment was followed by a 6 week-washout period, allowing a significant but

357 partial recovery of the aVOR, and complete recovery of the OCR. The individual correlations
358 between these functional tests and the number of HC counted in the ampullae and maculae
359 of the mice were found to be particularly significant, both during the trough (W6) of VOR
360 function and after the recovery period (W12) (Figure 3). We, however, did not find any
361 difference between the HC loss in the central vs. peripheral zones of the organs, and can
362 therefore not conclude on any putatively differential implication of these areas in the VORs.
363 Effects of the IDPN treatment on the aVOR was evidenced by a gain decrease starting W4,
364 later associated with a significant phase lead at W6 (Figure 1 supplement 1A,1B). The dynamic
365 of these changes might be related to the amount of HC progressively affected by the
366 treatment. The modifications affected all frequencies; however, responses at the lowest
367 tested frequency (0.2Hz) tended to be less affected and to recover first. These results could
368 suggest that type I HC are crucial element to encode both the amplitude and timing of the
369 VOR, particularly for the more dynamic stimulations. How IDPN progressively impairs the
370 encoding of information by the HC, and how population-coding influence both VOR features
371 in the natural range of head-movements should be the focus of future dedicated studies.
372 Overall, these organ-specific structure-function correlations confirm that the vestibulo-ocular
373 reflex can serve as a proxy indicating the status of the vestibular endorgans. It further
374 validates the use of the aVOR and OCR as relevant tests reflecting HC integrity at the level of
375 the ampullae and maculae, respectively.

376

377 **Differential alteration of type I versus type II hair cells**

378 The subchronic treatment affected type I HC more than type II HC, in accordance with
379 previous reports (Llorens et al. 1993; Maroto et al. 2021). Susceptibility to IDPN was also
380 reported to vary between different mouse strains (Boadas-Vaello et al., 2017; Wilkerson et
381 al., 2018). Interestingly, we used a different strain of mice than the one used in a previous
382 subchronic experiment (Greguske et al. 2019) and have not observed any type II HC loss
383 either, nor differences between males and females (data not shown).

384 Hence, IDPN treatment induces a loss of cell markers specific to type I HC, with no effects on
385 type II markers. Two types of markers were used to identify type I HC. *Spp1* targeted a protein
386 located on the neck of the hair cell (McInturff et al., 2018), while *CASPR1* is located at the
387 calyceal junction of the afferent terminal, and has been proven to be necessary for the
388 functionality of the synapse between the HC and its connected afferent (Sousa et al., 2009).

389 Though it is not possible to compare for one individual the number of cells before and after
390 treatment, the number of cells labelled for either of these proteins decreased significantly
391 compared to SHAM group, whose numbers are consistent between W6 and W12. The loss of
392 CASPR1 marker induced by IDPN sub-chronic treatment has been linked to HC detachment
393 from the calyx terminal and loss of vestibulo-spinal reflexes, and both CASPR1 expression and
394 vestibular function recover during washout (Sedó-Cabezón et al., 2015; Greguske et al., 2019;
395 Maroto et al., 2021). Only after a longer IDPN exposure does HC extrusion occur, associated
396 with persistent functional deficits. In theory, the loss of vestibular function observed in both
397 canal- and otolith-dependent VOR at W6 could be linked to loss of type I HC. In adult rodents,
398 however, the regeneration of hair cells leads to the formation of cells with type II HC features
399 (González-Garrido et al., 2021), so the recovery of type I cellular markers at W12 is likely not
400 a result of cell regeneration. Also, the apparent dramatic loss of CASPR1 and Spp1 HC (90%)
401 at W6 was in contrast with the much smaller and not significant loss in Myo7a cells (29%),
402 supporting the conclusion that the sensory cells persisted in the epithelium despite CASPR1
403 loss, and that the recovery in the number of type I HC observed at W12 was more likely the
404 result of molecular repair, not cell regeneration. In addition, we observed a similar loss of
405 Spp1 marker at W6, normally located in the neck of the hair cell. As such the loss of vestibular
406 function could rather be attributed to a global disorganization of the synapse and defective
407 hair cell function, associated with a transitory large decrease of the Spp1 and CASPR1 proteins
408 and a smaller decrease in Myo7a. In accordance with this hypothesis, the number of Spp1 and
409 CASPR1 positive cells after the washout period at W12 was not significantly different from the
410 SHAM group and correlated to the recovery of the vestibular function observed at W12 for
411 the IDPN mice (Figure 3C2). The altered expression of proteins in type I HC induced by the
412 sub-chronic treatment of IDPN seems to be reversible in most individuals. One possibility is
413 that definitive extrusion of type I HC occurred in the most susceptible individuals that did not
414 show recovery after the end of treatment. This in fact was the case in the 2 individuals tested
415 at W12 with immuno-histochemistry: CASPR1 mean value (\pm S.E.M) of 18.5 ± 5.5 compared to
416 34.5 ± 4.359 for IDPN with recovery and 41.75 ± 1.215 for SHAM). It was previously reported in
417 the pigeon that type I HC and calyx afferents take longer (12 weeks) to regenerate following
418 aminoglycoside toxicity, while type II and boutons endings regenerated in a week (Zakir and
419 Dickman, 2006). Similarly, recovered innervation for type I HC was delayed compared to type

420 II HC in mice (Kim et al., 2022). A longer recovery period could be investigated in the case of
421 the severely-affected mice to confirm the absence of recovery in the longer term.

422

423 **Evidence for the role of type I HC in the vestibulo-ocular pathways**

424 To our knowledge, the direct implication of type I and type II HC in the VOR was never directly
425 tested. Type I and type II HC differ by many features, including their anatomical location
426 within the epithelium, electrophysiological properties, morphology and innervation by
427 afferents (Eatock and Songer, 2011). Seemingly, irregular afferents have been described as
428 predominantly innervating the central area and striolar zones of the ampullae and maculae
429 with calyx and dimorphic synaptic contacts, while regular afferents make dimorphic and
430 buttons contacts predominantly within the peripheral zones of cristae and extrastriolar zones
431 of maculae (Goldberg, 2000; Eatock and Songer, 2011; Contini et al., 2022). Afferents with
432 bouton terminals that contact only type II HC have regular discharge, while afferents with
433 calyx terminals that contact only type I HC have irregular discharge. However, most regular
434 and irregular afferents are fed by both type I and type II HC (Goldberg et al., 1992).
435 Functionally, regular afferents recorded in the monkey show a lower detection threshold than
436 irregular afferents (Sadeghi et al., 2007), while irregular afferents showing higher gains and
437 phase leads (mouse: Lasker et al., 2008; Cullen, 2019) would be better optimized for encoding
438 high dynamic stimuli (Cullen, 2019). Central VOR neurons receive a mixture of regular and
439 irregular afferent inputs (Highstein et al., 1987; Boyle et al., 1992; Goldberg, 2000), with
440 irregular afferents constituting $\sim 1/3$ of their excitatory drive (Highstein et al., 1987; Boyle et
441 al., 1991).

442 While the role of type I and type II HC in the VOR remains to be determined, previous studies
443 have emphasized the importance of the regular afferents and regular central vestibular
444 neurons in the vestibulo-ocular pathway. Functional ablation targeting irregular afferents
445 suggested that VOR might function normally with only intermediate and regular afferent
446 inputs (Minor and Goldberg, 1991). Functionally identified VOR neurons (i.e. PVP) were
447 recently demonstrated to have heterogeneous discharge variability (high or low), with the
448 most regular units particularly well-suited to faithfully encode the compensatory eye
449 movements generated during natural stimulation (Mackrous et al., 2020).

450 Based on this literature, it would be tempting to infer that only the most regular and tonic
451 elements of the entire vestibular pathway are responsible for the VOR. However, the

452 correspondence between hair cell properties and afferents/central neurons properties is only
453 partial, such that the two phasic/irregular and tonic/regular channels for head motion signals
454 are constituted of both types I and type II HC (Baird et al., 1988; Goldberg et al., 1990 p.90)
455 and afferents (irregular and regular) (Goldberg et al. 2000; Eatock and Songer 2011; Beraneck
456 and Straka, 2011). Carey and colleagues (1996) previously reported a better correlation of
457 VOR recovery with type I than with type II HC following ototoxic lesions in the chick. Overall,
458 the result of ototoxic studies, including the present one, demonstrate a fundamental role of
459 type I HC in the encoding of vestibular signals that drive the vestibulo-ocular reflexes, even in
460 the relatively low range (Carriot et al., 2017) of head movements tested. It has also been
461 recently shown that both type I and type II HC actually contribute to otolithic vestibular
462 evoked potential responses (i.e. vestibulo-spinal pathway), previously described as mostly
463 type I-specific (Sayyid et al, 2019). Overall, our results are compatible with the hypothesis of
464 a convergence of heterogeneous peripheral neural elements at the level of central vestibular
465 nuclei, where intrinsic properties of central vestibular neurons (Straka et al., 2005; Beraneck
466 and Idoux, 2012) supplemented by network properties (Beraneck et al., 2007; Pfanzelt et al.,
467 2008; Beraneck and Straka, 2011) would differentially integrate vestibular signals further
468 processed in the different functional pathways (Sadeghi and Beraneck, 2020; Mackrous et al.
469 2020).

470

471 **Visuo-vestibular interactions after IDPN treatment**

472 OKR plasticity following vestibular loss concerned gain, and not phase, modifications for
473 frequencies $>0.33\text{Hz}$. Previous studies have reported an increase in OKR gain following a
474 permanent vestibular lesion for a non-specific range of frequencies (Shinder et al., 2005;
475 Faulstich et al., 2006; Nelson et al., 2017). One key to understand the frequency-specific OKR
476 plasticity could be the physiological dominance of vestibular inputs in the gaze stabilization
477 process at high frequencies (Faulstich et al., 2004). The visual inputs could be reweighted and
478 potentiated specifically in the range where the vestibular loss has the most impact on gaze
479 stabilization (Figure 5D).

480 The VOR and OKR work synergistically to stabilize gaze by compensating for head and visual
481 surround movements, respectively (França de Barros et al., 2020). If the reflexive eye
482 movements are not perfectly compensatory, an error signal (e.g retinal slip) is produced that
483 drives adaptation of the VOR (Boyden et al., 2004; Dean and Porrill, 2014; Shin et al., 2014)

484 and OKR (Glasauer, 2007; Kodama and du Lac, 2016). In an effort to understand the
485 integration of VOR and OKR, Holland and colleagues (2020) recently proposed that OKR would
486 account for the retinal slip not compensated by VOR. Within that framework, the changes in
487 the OKR could happen preferentially as a function of the vestibular weight, i.e in the range of
488 movements where the loss of vestibular inputs generates the largest retinal slip. While
489 present data are evidence for frequency-specific adaptation, how this relates to alteration in
490 vestibular inputs and/or visual feedback signals remains to be determined.

491 The increase in OKR gain could be seen as a substitution for the decrease in the VOR. However,
492 even after VOR recovery, the optimal integration of OKR and VOR (as predicted by our
493 MODEL) led to a degraded combined gaze response (CGR) with respect to SHAM mice. Even
494 worse, the measured CGR was inferior to the predicted CGR (Figure 6B). This observation
495 suggests that the integration of visual and vestibular signals during CGR is more than a mere
496 summation of the gain and phase of the 2 unimodal reflexes, but is also affected by other
497 factors. In fact, a subset of the IDPN individuals showed severely degraded bimodal
498 responses, so that their combination of VOR and OKR is not only sub-optimal but also less
499 effective than the unimodal (OKR) reflex. A sweep-based analysis suggested that in these
500 individuals, the VOR unimodal responses were not reliable, i.e the low reproducibility
501 indicates that the same stimuli are unfaithfully encoded into eye movements. We interpret
502 this low reproducibility as a sign of noisiness within the VOR pathway that would not only
503 preclude the optimal integration of both sensory signals, but also disturb the use of retinal
504 information to stabilize the gaze. According to statistical optimality theories, as the Maximum
505 Likelihood principle, the decrease in VOR reliability should lead to a reduction in its relative
506 sensory weight. This filtering-out of a degraded signal has been shown during visuo-vestibular
507 integration for monkey heading perception (Fetsch et al., 2010), or for human object
508 discrimination using visual and haptic senses (Ernst and Banks, 2002). Our results suggest that
509 the gaze stabilizing system is not able to optimally adapt to the degradation by filtering-out
510 vestibular signals during combined visuo-vestibular combination.

511 The integration of visual and vestibular inputs occurs in several structures, including in the
512 cerebellar flocculi (Jang et al., 2020) and brainstem (Carcaud et al., 2017) (see review De
513 Zeeuw et al., 2021). In the case of vestibular loss, most of the defects are expected to concern
514 the central vestibular neurons involved in the VOR, which also integrate visual inputs (ES, or
515 eye-sensitive neurons; Beraneck and Cullen, 2007). After unilateral neurectomy, vestibular

516 neurons in the Deiters nuclei responded to higher frequencies during visual stimulation (Cat:
517 Zennou-Azogui et al., 1994), a change compatible with the hypothesis that OKR gain increase
518 partly takes place in the vestibular nuclei. It was shown that inhibitory floccular inputs (part
519 of the OKR indirect pathway) and excitatory vestibular inputs often colocalized on the
520 dendrites of central vestibular neurons. One possibility is therefore that the massive
521 disorganization of vestibular periphery inputs led in the long term to synaptic and intrinsic
522 changes at the level of central vestibular neurons (Beraneck and Idoux 2012; Carcaud et al.
523 2017), thus impairing their capacity to optimally integrate both signals.

524

525 **Conclusions**

526 Balance dysfunction occurs frequently in aging people (85% prevalence above 80; Agrawal et
527 al., 2013) but also in younger people (prevalence of 35% in the 40 year-old). While animal
528 models often use permanent vestibular lesions (Simon et al., 2020), many diseases in fact
529 consist in a gradual, transient and/or partial loss of vestibular function. For instance Menière's
530 disease, which represents ~9% of all vestibular pathologies in the adult and occurs in the <60
531 years old, is characterized by recurrent episodes with brief (<24h) fluctuating symptoms and
532 otherwise normal vestibular function before long-term deterioration arises (Lopez-Escamez
533 et al., 2015). Transitory vestibular symptoms are also commonly reported in vestibular
534 neuritis where, although symptoms tend to rapidly disappear due to vestibular
535 compensation, vestibular function can recuperate up to a year after the initial loss
536 (Welgampola et al., 2019). Gradual and partial vestibular loss is also encountered as a side-
537 effect of some ototoxic anti-cancer treatments (prevalence in treated patients ~40%;
538 cisplatin; Paken et al., 2016; Prayuenyong et al., 2018). Here, we took advantage of a
539 subchronic ototoxic protocol to determine how animals adapt to partial and transitory loss of
540 vestibular hair cells. We show that the loss, and then the partial recovery of the VOR, is
541 correlated with the integrity of the type I HC, demonstrating for the first time their essential
542 role in the VOR, whether of canalar or otolithic origin. We show that sensory (visual)
543 substitution would theoretically compensate for vestibular loss, but that injured mice have
544 suboptimal responses when combining visual and vestibular information. Finally, we show
545 that this impairment in multisensory integration would be linked to the loss of "reliability" of
546 the vestibular signal, degraded by ototoxicity. Overall, these results suggest that transitory

547 peripheral infraction have long term consequences, and that the capacity of central vestibular
548 structures to cope (vestibular compensation; Cullen et al., 2009; Beraneck and Idoux,
549 2012;(Lacour et al., 2016) with the sensorineural loss might critically depend on the integrity
550 of the neural elements involved. Future studies should aim at obtaining information about
551 the degradation of signal transmission following IDPN treatment and characterize the amount
552 of peripheral population-coding necessary to preserve optimal vestibular function.

553 **Material & Methods**

554 **Ethics**

555 A total of 56 C57/BL6J mice in an equal partition of males and females were used for the
556 protocol. Animals were used in accordance with the European Communities Council Directive
557 2010/63/EU. All efforts were made to minimize suffering and reduce the number of animals
558 included in this study. All procedures were approved by the ethical committee for animal
559 research of the Université Paris Cité.

560

561 **Headpost implantation surgery and animal care**

562 Surgical procedures, postoperative care, device fixation and animal surveillance during the
563 protocol were performed as described previously in França de Barros et al., 2019, 2020.
564 Briefly, 6-weeks-old mice anaesthetized with isoflurane gas had their heads shaved with small
565 clippers. Then, lidocaine hydrochloride (2%; 2mg/Kg) was injected locally before a
566 longitudinal incision of 1.5 cm was made to expose the skull. Just anterior to the lambda
567 landmark, a small custom-built headpost (3x3x5mm; poly lactic acid) was cemented (C&B
568 Metabond; Parkell Inc., Edgewood, NY) and laterally covered with resin (Heraeus) for
569 protection. Animals were fully recovered 30 min after the end of the surgery, yet
570 buprenorphine (0.05mg/kg) was provided for postoperative analgesia and they were closely
571 monitored for the following 48 hours.

572

573 **Subchronic ototoxic exposure**

574 28 mice were treated with 3,3'-iminodipropionitrile (IDPN, Sigma Aldrich, 317306, 90%) for
575 6 weeks (IDPN group) at 30mM concentration of IDPN in their drinking water at least 72h
576 after the surgery. After 6 weeks of treatment, a washout period of 6 weeks followed. Previous
577 experiments (Greguske et al., 2021) had demonstrated that at these concentrations, ototoxic
578 lesions produced by IDPN are largely reversible. The SHAM group was tested the exact same
579 way but was not exposed to IDPN in their drinking water. Video-oculography tests were
580 performed before the beginning of the treatment and once every two weeks until week 12
581 (W12), for a total of 7 sessions of tests for each mouse.

582

583 **Experimental groups**

584 3 different batches of mice were used, each composed of both IDPN-treated mice and SHAM.
585 The mice of the first one (n=19) were subjected only to vestibular stimulations, to test the
586 effect of IDPN on the canal-related or otolith-related VOR responses during the 12 weeks of
587 protocol. The second group (n=24), designed to compare the dynamic of VOR and OKR
588 changes was tested with angular VOR stimulations, otolithic test, optokinetic (visual)
589 stimulations, and combined vestibular and visual stimulation (VOR in light; referred to as
590 Combined Gaze Response or CGR). The third group (n=11) designed to correlate ototoxic
591 effects of IDPN on vestibular hair cells (HC) and VOR function was tested for canal-related or
592 otolith-related VOR responses, before treatment and after 6 weeks of treatment and
593 immediately used for immunohistochemical assessment of the vestibular sensory epithelia
594 (see below). A subset of the second group (n=12 out of 24) was also used for
595 immunohistochemical assessment at the end of the 12 weeks of protocol.

596

597 **Video oculography recording sessions**

598 The recording procedure was similar to the one presented in Carcaud et al. 2017 & França de
599 Barros et al. 2020. The animals were head-fixed in a custom build Plexiglas tube, and their
600 head was oriented in a 30° nose down position to align the horizontal canals to the yaw plane.
601 Their left eye movements were recorded using a non-invasive video oculography system (ETL
602 -200 Iscan, acquisition rate 120Hz, Stahl et al 2000). Eye, head, and virtual drum (OKR
603 stimulation) position signals were digitally recorded (CED mk3 power 1401, acquisition rate
604 1kHz) with Spike 2 software. Signals were analysed offline in Matlab (Matlab, the MathWorks,
605 Natick, MA, USA; RRID, SCR:001622) programming environment. The restraining apparatus
606 was fixed on a rotation platform on top of an extended rig with a servo-controlled motor.
607 Single VOR or OKR recording sessions lasted no longer than 45 minutes in total.

608

609 **Vestibular stimulations and analysis**

610 All vestibular-specific tests were performed in a temperature-controlled room with all sources
611 of light turned off except for computer screens. The turntable was further surrounded with a
612 closed box to isolate the animal from remaining light, with a final luminance inside the box
613 <0.02 lux. Myosis was induced with topical 2% pilocarpine applied 10 minutes before
614 experimentation.

615 *Vestibulo ocular reflex in dark (VORd) tests* were performed in the dark with the mouse
616 surrounded by an opaque black dome (Fig1.A). Sinusoidal angular rotations around the
617 vertical axis were used to record the horizontal angular vestibulo-ocular reflex (aVOR), at
618 different frequencies: 0.2, 0.5, 0.8; 1 and 2Hz at a peak velocity of 30°/s.

619
620 *Angular vestibulo-ocular reflex* analysis was similar to the one described in Carcaud et al.
621 2017. Segments of data with saccades were excluded from VOR slow-phase analysis. For
622 horizontal sinusoidal rotations, at least 10 cycles were analyzed for each frequency. VOR gain
623 and phase were determined by the least-squares optimization of the equation:

$$EH_v(t) = g \cdot \{[HH_v \cdot (t - t_d)] + C^{te}\}$$

624
625
626
627 where $EH_v(t)$ is eye horizontal velocity, g (gain) is a constant value, $HH_v(t)$ is head horizontal
628 velocity, t_d is the dynamic lag time (in msec) of the eye movement with respect to the head
629 movement, and C^{te} is an offset. The t_d was used to calculate the corresponding phase (ϕ°) of
630 eye velocity relative to head velocity. The Variance-Accounted-For (VAF) of each fit was
631 computed as

$$1 - \left[\frac{\text{var}(est - EH_v)}{\text{var}(EH_v)} \right]$$

632
633
634 where var represents variance, est represents the modeled eye velocity, and EH_v represents
635 the actual eye horizontal velocity. VAF values for VOR measures were typically between 0.70
636 – 1 (>95% of recordings), where a VAF of 1 indicates a perfect fit to the data. For IDPN-treated
637 mice, abnormally low (<0.10) values of gain associated with VAF<0.5 were nevertheless
638 included in the gain statistical analysis but specifically reported in grey (Figure 2 & Figure 1
639 supplement). Corresponding phase values were not included in the statistical analysis of the
640 aVOR phase.

641
642 *Static Ocular Counter-roll reflex tests* were performed as described in (Simon et al., 2021) by
643 measuring the vertical eye movement generated in response to different roll angles. The table
644 was moved from left to right in incremental steps of 10° (from 0 to 40°), with static periods of
645 at least 10s between oscillations (Fig1.D) to record the stabilized eye elevation and

646 declination. The OCR gain corresponds to the slope of the linear regression of the vertical eye
647 angle vs. the head tilt angles (Oommen and Stahl, 2008).

648 *Off vertical axis rotation (OVAR) tests* were performed with the vestibular turntable tilted with
649 a 17° off-axis angle following the methodology described in Beraneck et al., 2012 & Idoux et
650 al., 2018. 50°/s continuous stimulations were performed in a counter-clockwise and then
651 clockwise direction. OVAR generates a continuous change in the head tilt angle, compensated
652 through a maculo-ocular reflex (MOR) by the generation of a horizontal nystagmus
653 compensating for the table constant rotation. This oculomotor response was quantified
654 following the methodology described in Idoux et al 2018. First, quick-phases were identified
655 and removed. During rotations, the velocity of horizontal slow phases is modulated
656 (modulation, μ) around a constant bias (β). Both parameters (μ and β) were calculated from
657 the sinusoidal fit of eye horizontal slow-phase velocity using the least-squares optimization
658 of the equation:

$$659 \quad SP(t) = \beta + \mu \cdot \sin[2\pi \cdot f_0 \cdot (t + t_d)]$$

660

661 where $SP(t)$ is slow-phase velocity, β is the steady-state bias slow phase velocity, μ is the
662 modulation of eye velocity, f_0 is the frequency of table rotation, t_d is the dynamic lag time (in
663 msec) of the eye movement with respect to the head movement. The bias (Maculo-ocular
664 reflex Bias) is reported here as the main index of the MOR response (Hess and Dieringer, 1990;
665 Beraneck et al., 2012).

666

667 **Optokinetic reflex tests and analysis**

668 Horizontal optokinetic stimulations were performed as previously described in França de
669 Barros et al. 2020. The mice were placed under a semi-opaque plastic dome and all sources
670 of light were turned off. The projected stimulation consisted of a randomly distributed white
671 dots pattern on a black background image (250000 white dots, max width 0.075°). The
672 optokinetic sinusoidal stimulations were tested at 0.1, 0.2, 0.33, 0.5 and 1Hz at a peak velocity
673 of 10°/s. The gain and phase were obtained by the same least-squares optimization method
674 described above for the aVOR. To prevent putative cross effects between visual and vestibular
675 stimulations, VOR and OKR test sessions were performed on separate days.

676

677 **Combined visual and vestibular stimulations**

678 Combined visual and vestibular stimulations measuring the combined gaze reflex (CGR)
679 consisted of horizontal vestibular stimulations while projecting the fixed dotted pattern used
680 for OKR on the surrounding dome. Horizontal angular sinusoidal rotations were performed at
681 frequencies of 0.2, 0.5 and 1Hz with a peak velocity of 30°/s. The CGR gain and phase
682 quantifications were performed following the same methodology as for aVOR. To avoid
683 interference, these were performed on n=8 SHAM and n=12 IDPN after the end of the VOR
684 and OKR test sessions on W12. To test the effects at W6, a specific group of mice (n= 4 SHAM
685 and n=7 IDPN) was tested at W0 and W6.

686 A simple model has been developed to predict for each individual and for three stimulation
687 frequencies (0.2, 0.5 and 1Hz) the theoretical CGR response gain and phase were given the
688 same two parameters observed for their unimodal VOR and OKR responses. The experimental
689 VOR and OKR gain and phase are used to compute the sinusoid representing the two
690 unimodal responses. The gain and phase of the CGR sinusoid resulting from adding the VOR
691 and OKR sinusoids are quantified by measuring its peak-to-peak amplitude and by identifying
692 the time-lag that maximizes its cross-correlation with the sinusoidal stimulus (note that the
693 sum of two sinusoids with the same period, even if with different phases, is always a sinusoid).
694 Independently of the mouse, the unimodal VOR and OKR sinusoids are shifted in time before
695 their summation, in order to improve, on average, the fit between predicted and observed
696 CGR responses for SHAM mice. The two optimal shift parameters, one for VOR and one for
697 OKR, obtained for each frequency, are then used also for all IDPN-treated mice.

698 To identify subpopulations of IDPN-treated mice, we computed an agglomerative hierarchical
699 cluster tree on a dataset composed of individual aVOR gain and Δ (calculated as CGR-OKR
700 gain). The classification method used unweighted average Euclidean distance between
701 clusters.

702 To describe the mice VOR responses without any assumption about their sinusoidal nature
703 the two following characteristics have been quantified: the reproducibility of the ocular
704 response to the vestibular stimulation and the amplitude of the raw eye movements
705 generated by the vestibular stimulation. These analyses were performed on the slow phases
706 horizontal eye trajectories, $EHp(t)$, recorded during each sweep of stimulation.

707 The reproducibility index, Rep , was obtained by computing the matrix of correlation
708 coefficient, $R_{i,j}$, between each couple (i,j) , of the N selected sweeps, and then computing the
709 mean of all above-diagonal elements of the matrix as reported in the following equation (this

710 choice aims at considering only once each $R_{i,j}$, since $R_{j,i}=R_{i,j}$ and to exclude diagonal elements,
711 $R_{i,i}=1$).

$$712 \quad Rep = \frac{\sum_{i=1}^{N-1} \sum_{j=i+1}^N R_{i,j}}{N(N-1)/2}$$

713 The amplitude, Amp , of the raw eye movements was quantified by computing for each sweep
714 how much on average, during a stimulation cycle, the eye moved horizontally away from its
715 average position (σ_{EHP}). Before averaging this parameter over all the sweep, it had to be
716 squared. Finally, to have a parameter value in degrees, the root mean squared of the mean
717 was computed.

$$718 \quad Amp = \sqrt{\frac{\sum_{i=1}^N \sigma_{EHP}^2}{N}}$$

719

720 **Immunolabelling of the vestibular HC**

721 Two groups of mice (n=11 at W6 and n=12 at W12) were used to perform
722 immunofluorescence analysis on hair cells in the vestibular endorgans. Mice were
723 anaesthetized with an overdose of intraperitoneal injection of ketamine hydrochloride
724 (10%)/xylazine (5%) and decapitated. The histology was done following the protocol
725 established by Lysakowski et al. (2011), as described previously (Maroto et al., 2021). The
726 vestibular epithelia (one horizontal canal and one utricle) were dissected and fixed for 1h in
727 a 4% solution of paraformaldehyde (PFA). PFA was washed twice with phosphate-buffered
728 saline (PBS) and the samples were then embedded in a cryoprotective solution (34.5%
729 glycerol, 30% ethylene glycol, 20% PBS, 15.5% distilled water) to be stored at -20° . Before the
730 immunochemistry, samples were put at room temperature and rinsed twice in PBS. While
731 under slow agitation, the samples were incubated twice, first for 1h with a 4% Triton X-100
732 (Sigma Aldrich) in PBS to permeabilise and a second time for 1h in 0.5% Triton X-100 1% fish
733 gelatin (CAS #9000-70-8, Sigma-Aldrich) in PBS to block. The incubation with the primary
734 antibodies was then performed in 0.1M Triton X-100, 1% fish gelatin in PBS at 4° for 24h. After
735 rinsing, the second antibodies were incubated in the same conditions. The 2nd antibodies
736 were rinsed and the vestibular epithelia were mounted on slides with fluoromount (F4680,
737 Sigma-Aldrich) and were visualised with a confocal microscope Zeiss LSM880 (with an
738 objective of 63x NA:1.4). To properly analyse the whole vestibular epithelium, Z-stacks of 0.5
739 μm were obtained and observed with ImageJ (National Institute of Mental Health, Bethesda,

740 Malyland, USA). The primary antibodies used were rabbit anti-Myosin VIIa (Myo7a) (Proteus
741 Biosciences, #25-6790), mouse anti-contactin-associated protein (CASPR1) (Neuromab #75-
742 001), guinea pig anti calretinin (Synaptic Systems #214-104) and goat anti-osteopontin (Spp1)
743 (R&D Systems #AF08). Their respective secondary antibodies were Dylight 405 donkey anti-
744 rabbit igG H+L (Jackson Immuno Research #711-475-152, Alexa Fluor 488 donkey anti-mouse
745 IgG H+L (Life Technologies #A21202), Alexa Fluor donkey anti-guinea-pig IgG H+L (Jackson
746 ImmunoResearch #706-605-148) and Alexa Fluor 555 donkey anti-goat IgG H+L (Invitrogen
747 #A21432).

748 HC counts were obtained from square areas (67.5 x 67.5 μm) of the central and peripheral
749 parts of two vestibular organs (horizontal semi-circular canal and utricle) in the W6 group and
750 the W12 group. The areas for counting were obtained from the same location within the
751 epithelium in all animals. The global number of HC was assessed with the cytoplasmic labelling
752 of the anti-Myo7a antibody (Hasson et al., 1997). Type I HC were labelled using two different
753 antibodies: anti-Spp1 in the neck of the type I HC (McInturff et al., 2018) and the presence of
754 anti-CASPR1 in the calyceal junctions of the calyx (Sousa et al., 2009 p.20). Type II HC were
755 distinguished by the colocalization of Myo7a and calretinin (Maroto et al, 2021).

756

757 **Statistical analysis**

758 For both OKR and VOR stimulations, the effect of the protocol on the gain and phase were
759 statistically tested by performing repeated measures ANOVA. The *treatment* (SHAM or IDPN)
760 was considered as between individual independent factor with the *Weeks* (W0, W2, W4, W6,
761 W8, W10, W12) and the *Frequencies* (0.1, 0.2, 0.33, 0.5 and 1Hz for the OKR; 0.2, 0.5, 0.8, 1
762 and 2Hz for the VOR) were considered as within individual independent factors. The main
763 effects of those factors and their interactions were tested and reported. In the case of the
764 OCR and OVAR, mix-model ANOVA was used with only *Weeks* considered as within factors.
765 For the CGR, measured at W6 and W12, mix-model ANOVA was used with the *Frequencies*
766 and *Model* (Measured or Model CGR) used as within factors. For the comparison between the
767 OKR and VOR Δ gain, a repeated measures ANOVA was applied on the Δ gain (Wx-W0) with
768 the *stimulation modality* (OKR or VOR) as between individual independent factor and the
769 *Weeks* as within individual independent factor. For the correlation between OCR and VOR, as
770 well as the measured and theoretical CGR gain a linear regression model was fitted.

771 The effect of IDPN exposure on the HC count was reported with a Kruskal Wallis test. For all
772 analyses the significance threshold was set at $p < 0.05$ and Newman Keuls post hoc test was
773 performed if the main effect or an interaction was found significant.

774 **FIGURE LEGENDS**

775 Figure 1: Effects of subchronic IDPN on canal- and otolithic- dependent VOR.

776 **A)** Illustration of the angular horizontal vestibulo-ocular reflex (aVOR) set-up. All tests are
777 performed in complete dark. **B)** Examples of velocity aVOR traces in response to table
778 rotations (1Hz at $30^\circ/s$) recorded in the dark in an IDPN mouse before (W0, corresponding
779 gain of 0.785; VAF of 0.98), after 6 weeks of treatment (W6, gain: 0.14 ; VAF: 0.61) and 6
780 weeks of washout (W12, gain:0.48; VAF: 0.92). Right movement is represented up. **C)** Mean
781 aVOR gain of SHAM (n=22) and IDPN (n=21) mice during the protocol. **D)** Illustration of
782 the ocular- counter roll (OCR) set-up. **E)** Examples of raw OCR traces at W0, W6 and W12 in a IDPN
783 mouse recorded in the dark. Tilt to the right is represented up (positive values). (Left) eye elevation is
784 represented up. **F)** Mean OCR gain of SHAM (n=14) and IDPN (n=13) mice. We note that there
785 was a significant difference between SHAM and IDPN during the initial measurements at
786 W0. However, at this time point mice were not yet separated into different groups. This
787 incidental difference completely disappeared on the measurement performed at W2.
788 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Error bars represent \pm SEM.

789

790 Figure 2: Comparison of the dynamics of canal and otolithic loss of function.

791 **A)** Evolution of the Mean Gain of the aVOR (1Hz) and OCR responses of IDPN mice (n=13)
792 during the treatment and washout periods. **B)** Individual gains for 1Hz aVOR (left) and OCR
793 (right) of the 13 IDPN mice, identified with similar symbols. The grey symbols in the left panel
794 correspond to aVOR gain values associated with VAF<0.5. **C, D)** Individual Δ aVOR gains as a
795 function of individual Δ OCR gains at W6 (C) and W12 (D) compared to W0, for SHAM (n=14)
796 and IDPN (n=13) mice. The linear regression corresponds to IDPN values is represented, as
797 well as the 50% confidence interval of each group (shaded areas). The symbols for each
798 animal are the same in panels B, C and D. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Error bars
799 represent \pm SEM.

800 Figure 3: Immuno-labelling of HC in the central regions of the horizontal SCC ampulla and
801 striolar region of the utricle Macula.

802 **A, C** Immunolabelling of type I HC (Spp1+ and CASPR1+), type II HC (Calre +), or all HC (Myo7a)
803 for the SHAM, IDPN W6 and IDPN W12 groups in the central ampulla of the horizontal canal
804 (A1) and central utricular maculae (C1). Cell count at W6 and W12 in the central horizontal
805 ampulla (A2 and A3) and central utricular maculae (C2 and C3) for individual mice. **B1, D1**
806 Individual number of central Spp1+ type I HC, CASPR1 + type I HC and Calre+ type II HC, or all
807 HC(Myo7a) as a function of the aVOR gain (B1) or OCR gain (D1) at W6 (circle) and W12
808 (squares) groups. The linear regressions correspond to all individuals (n=23 mice). **B2, D2**
809 Comparison of the number of CASPR1 type I HC as a function of the aVOR (B2) gain or OCR
810 gain (D2) at W6 (black and circle) and at W12 (pink and square) for each IDPN mice of the
811 W12 group (n=8). Note that all points are shifted toward the regression line (redrawn from
812 respectively B1 and D1), indicating that the number of cells at W12 better correlates with the
813 recovered aVOR. (*p< 0.05; **p < 0.01; ***p < 0.001). Error bars represent \pm SEM.

814

815 Figure 4: Effects of subchronic IDPN treatment on the OKR.

816 **A**) Illustration of the optokinetic reflex (OKR) set-up. **B**) Example of raw traces of an OKR
817 recorded in response to stimulation at 0.5Hz at a peak velocity of 10°/s before (W0), after 4
818 weeks of IDPN treatment (W4), 2 weeks (W8) and 6 weeks of washout (W12). All traces are
819 from the same individual. **C**) Mean OKR gain of IDPN (n=12) and SHAM (n=12) mice. **D**) OKR
820 gain and **E**) phase for IDPN (n=12) and SHAM (n=12) for each frequency at W0, W6, W8 and
821 W12. (*p< 0.05; **p < 0.01; ***p < 0.001). Error bars represent \pm SEM.

822

823 Figure 5: Comparison of the IDPN treatment on OKR and aVOR.

824 **A**) Mean Δ aVOR and Δ Gain OKR for 0.2, 0.5 and 1Hz for IDPN (n=12) and SHAM (n=12) mice.
825 **B**) Individual Δ OKR gains as a function of individual Δ aVOR gains. The 50% confidence interval
826 of each group is represented in the shaded areas. **C**) aVOR and OKR gains of IDPN mice (n=12)
827 at W0 and W8 for frequencies of 0.2, 0.5 and 1Hz. **D**) Percentage of the individual vestibular
828 weight (inset), as a function of the percentage of the individual OKR gains change for IDPN
829 (n=12). The linear regression corresponds to all values (n=36). (*p< 0.05; **p < 0.01; ***p <
830 0.001). Error bars represent \pm SEM.

831

832 Figure 6: Visuo-Vestibular interactions following IDPN treatment.

833 **A)** aVOR, OKR, CGR data (CGR) and predicted CGR (MODEL) gains (left panel), and CGR phase
834 (data and model, right panel), for the SHAM mice (n=12) at W6 and W12. **B)** aVOR, OKR, CGR
835 data (CGR) and predicted CGR (MODEL) gains for the IDPN mice (n=19) at W6 and W12. **C)**
836 Comparison of the predicted versus measured CGR for all frequencies tested. The dotted line
837 at 45° represents a perfect match between prediction and data (optimal CGR). **D)** aVOR, OKR,
838 and CGR values of the IDPN mice (n=19) (left panel). Clustering analysis (right panel)
839 distinguish 2 groups based on Delta (CGR-OKR), and a subgroup based on VOR. The horizontal
840 coordinate of each cluster (vertical lines) represents the distance between two connected
841 clusters. **E)** Δ (CGR-OKR) as a function of the VOR Sweep Amplitude of the $\Delta+$ (green triangle),
842 $\Delta+(-)$ (green diamonds) and $\Delta-$ (orange) IDPN mice. The inset panel represents typical raw
843 cycles representative of the 3 subgroups. **F)** Delta (CGR-OKR) as a function of the VOR
844 reproducibility of the $\Delta+$ (green triangle), $\Delta+(-)$ (green diamonds) and $\Delta-$ (orange) IDPN mice.
845 Regression line correspond to all values (n=19)

846

847 Figure 1 supplement: Effects of subchronic IDPN on canal- and otolith-dependent VOR.

848 **A, B)** aVOR **(A)** Gain and **(B)** Phase across all frequencies and weeks tested for IDPN (n=21)
849 and SHAM (n=22) mice. The grey symbols in the left panel correspond to aVOR gain values
850 associated with VAF<0.5. **C)** OVAR MOR bias for IDPN (n=13) and SHAM (n=14) mice during the
851 protocol. (*p< 0.05; **p < 0.01; ***p < 0.001). Error bars represent \pm SEM.

852

853 Figure 3 supplement: Effects of the IDPN on the number of HC in the peripheral regions of the
854 horizontal SCC ampulla and extrastricular utricle Macula.

855 **A)** Individual cell count of peripheral horizontal semi-circular canal type I and type II HC for
856 W6 (1) and W12 (2). **B)** Cell count of peripheral utricular type I and type II HC for W6 (1) and
857 W12 (2). **C)** Individual number of peripheral Spp1+ type I HC (1), CASPR1 + type I HC (2) and
858 Calre+ type II HC (3) as a function of the aVOR gain for W6 (circle) and W12 (squares). The
859 linear regression of both groups (n=23 mice) and the goodness of fit is represented. **D)**
860 Individual number of peripheral Spp1+ type I HC (1), CASPR1 + type I HC (2) and Calre+ type II
861 HC (3) as a function of the OCR gain for W6 (circle) and W12 (squares). The linear regression

862 of both groups (n=23 mice) and the goodness of fit is represented. (*p< 0.05; **p < 0.01; ***p
863 < 0.001). Error bars represent \pm SEM.

864

865 Figure 4 supplement: Effect of the IDPN on optokinetic reflex amplitude and timing.

866 **A)** OKR Gain across all frequencies and weeks tested **B)** OKR Phase across all frequencies and
867 weeks tested. (*p< 0.05; **p < 0.01; ***p < 0.001). Error bars represent \pm SEM.

TABLES

Table 1 Statistics table of the aVOR gain for the IDPN treated group.

W0	W2	W4	W6	W8	W10	W12	
	ns	***	***	***	***	***	W0
ns		***	***	***	***	***	W2
***	***		***	***	ns	ns	W4
***	***	***		*	***	***	W6
***	***	***	*		***	***	W8
***	***	ns	***	***		ns	W10
***	***	ns	***	***	ns		W12

Table 2: Statistics table of the OKR gain for the IDPN treated group.

W0	W2	W4	W6	W8	W10	W12	
	ns	ns	ns	***	ns	ns	W0
ns		ns	ns	***	**	ns	W2
ns	ns		ns	ns	ns	ns	W4
ns	ns	ns		*	ns	ns	W6
***	***	ns	*		ns	ns	W8
ns	**	ns	ns	ns		ns	W10
ns	ns	ns	ns	ns	ns		W12

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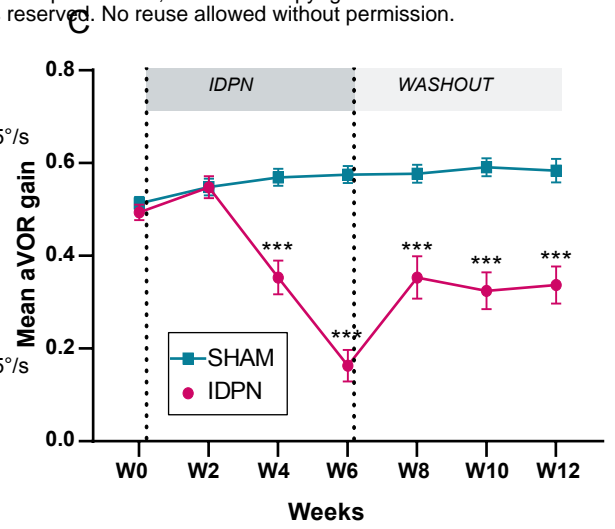
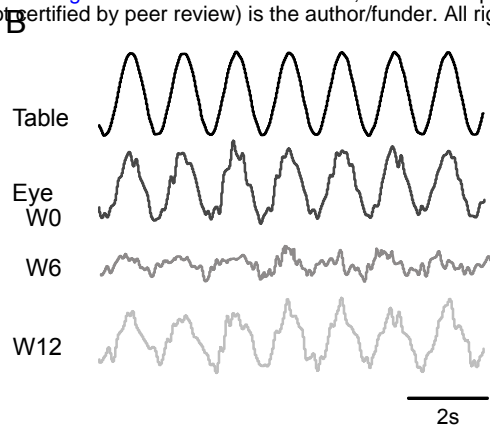
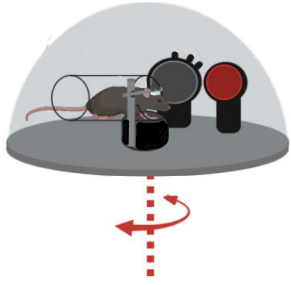
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A Angular Vestibulo-Ocular Reflex in the dark test



D Ocular Counter Roll Test

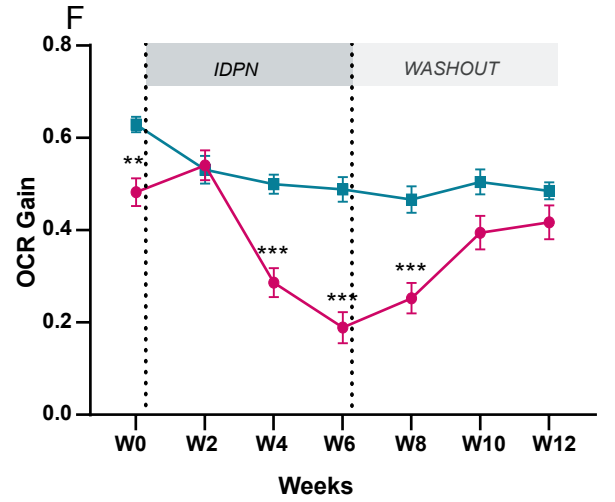
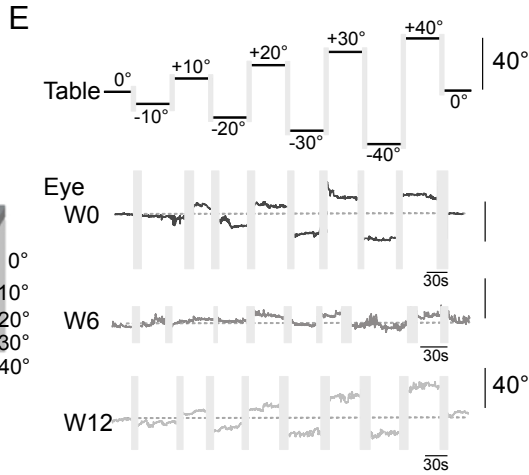
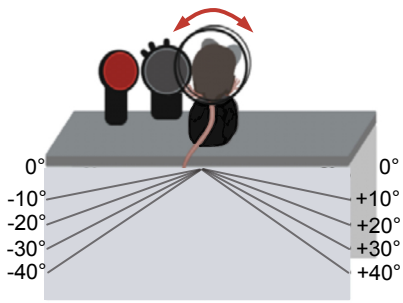


Figure 1: Effects of subchronic IDPN on canal- and otolithic-dependent VOR.

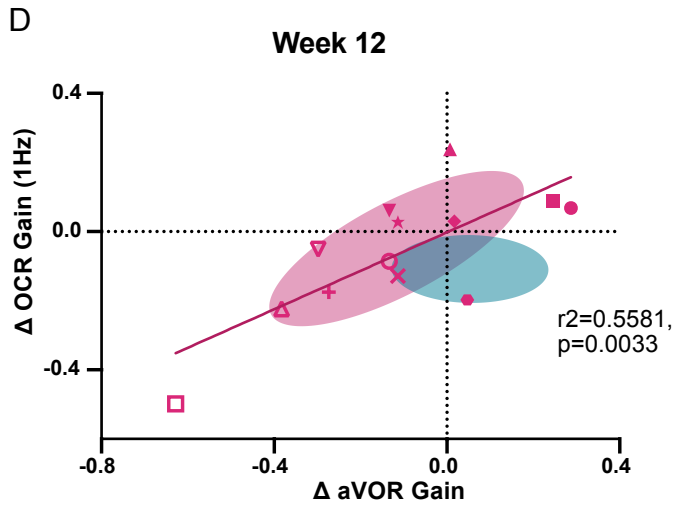
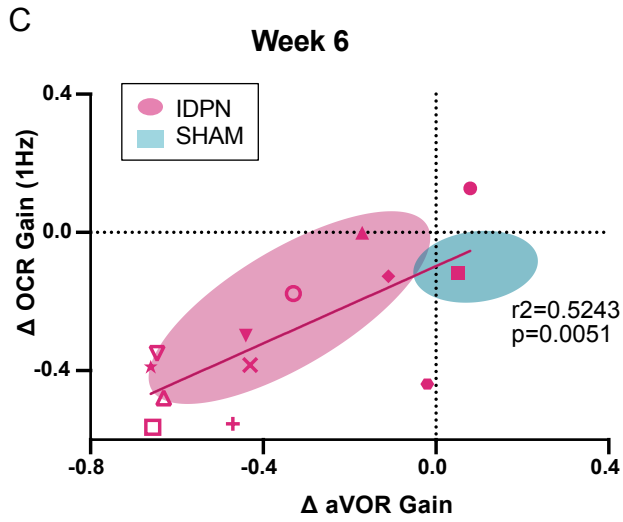
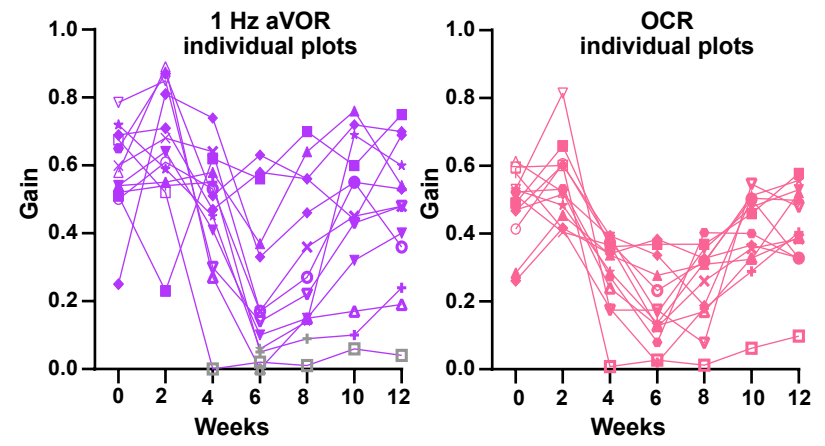
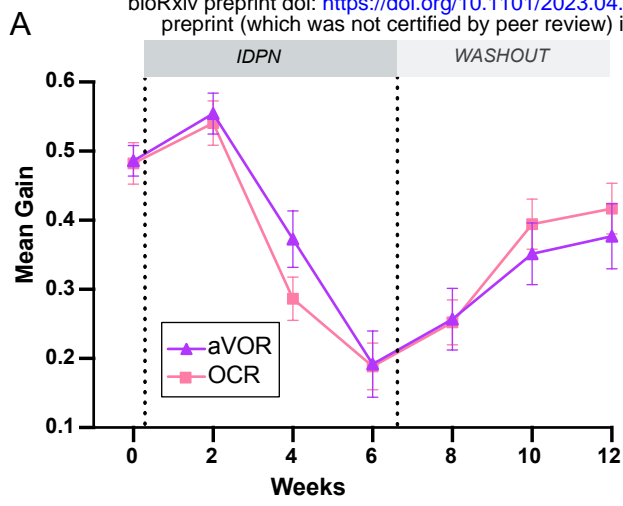


Figure 2: Comparison of the dynamics of canalar and otolith loss of function.

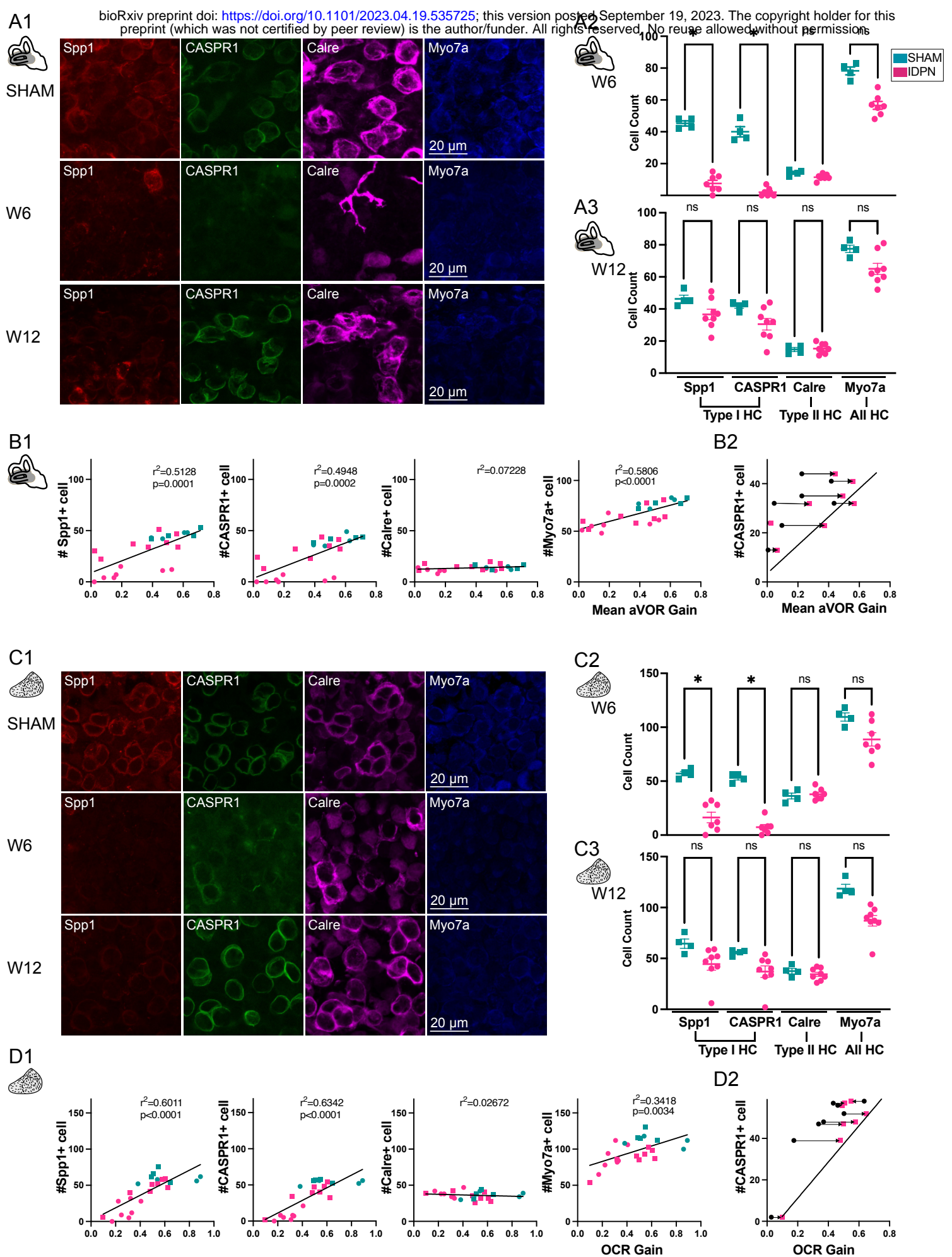


Figure 3: Immuno-labelling of hair cells in the central regions of the horizontal SCC ampulla and striolar cell region of the utricle Macula.

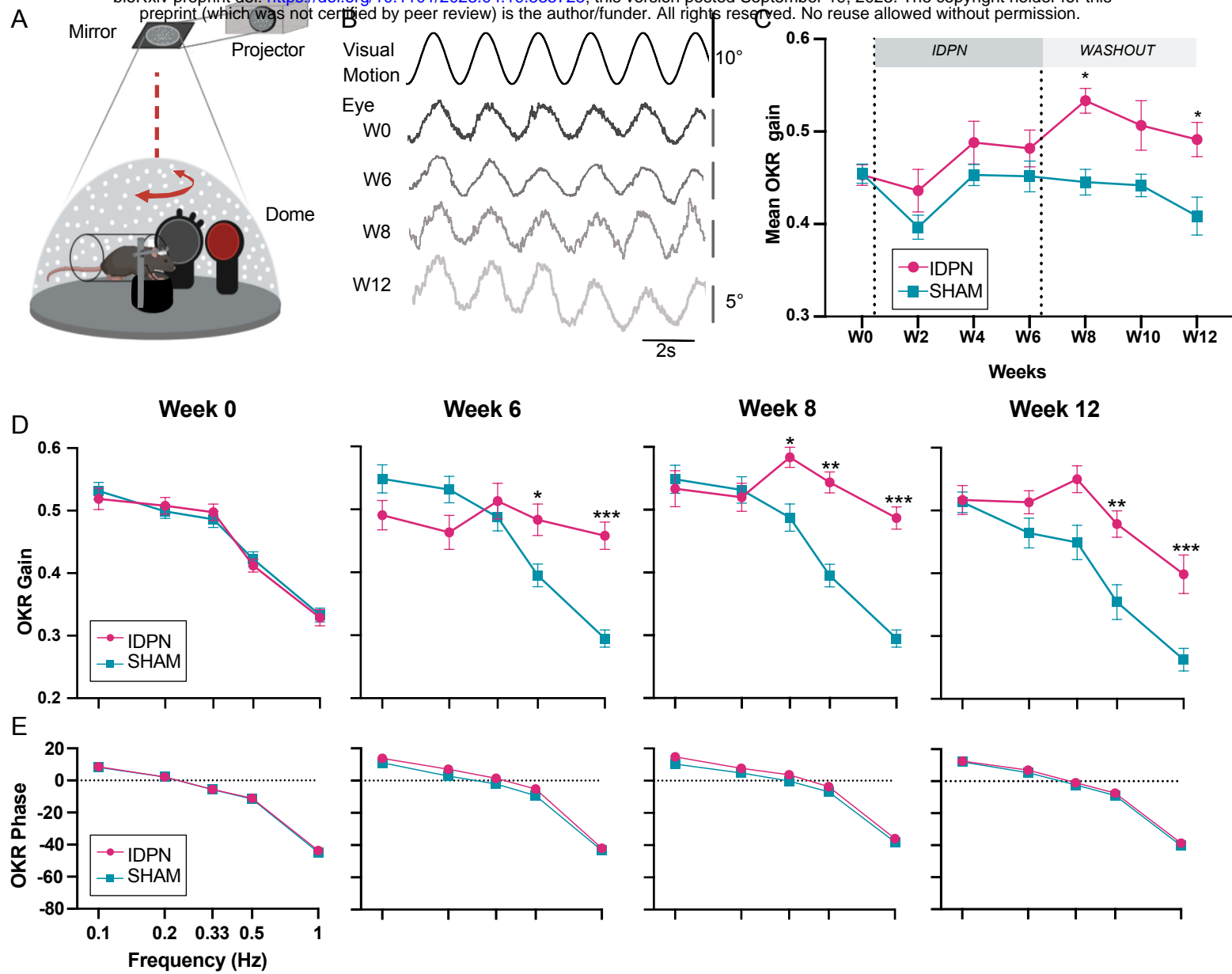


Figure 4: Effects of subchronic IDPN treatment on the OKR.

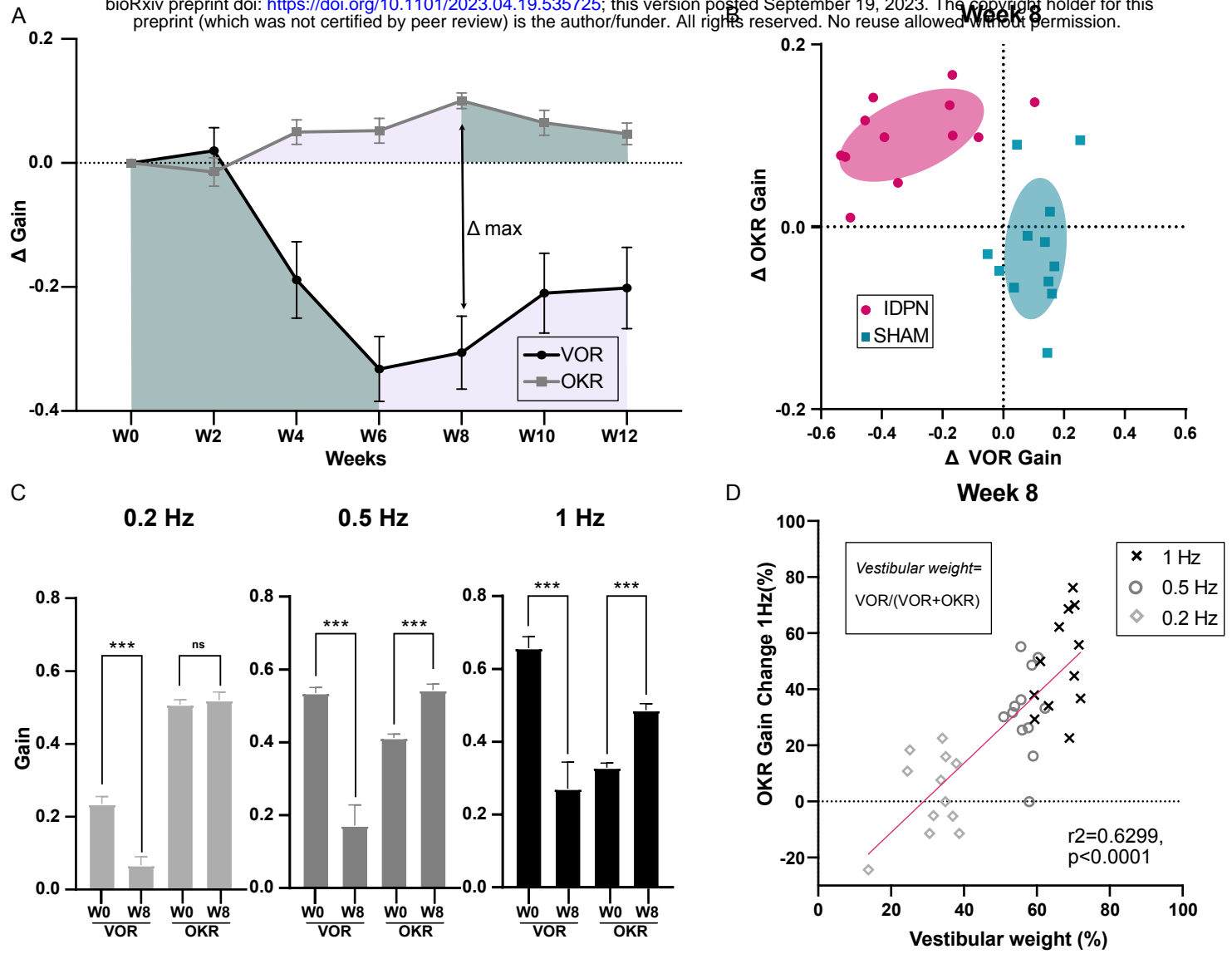


Figure 5: Comparison of the IDPN treatment on OKR and aVOR.

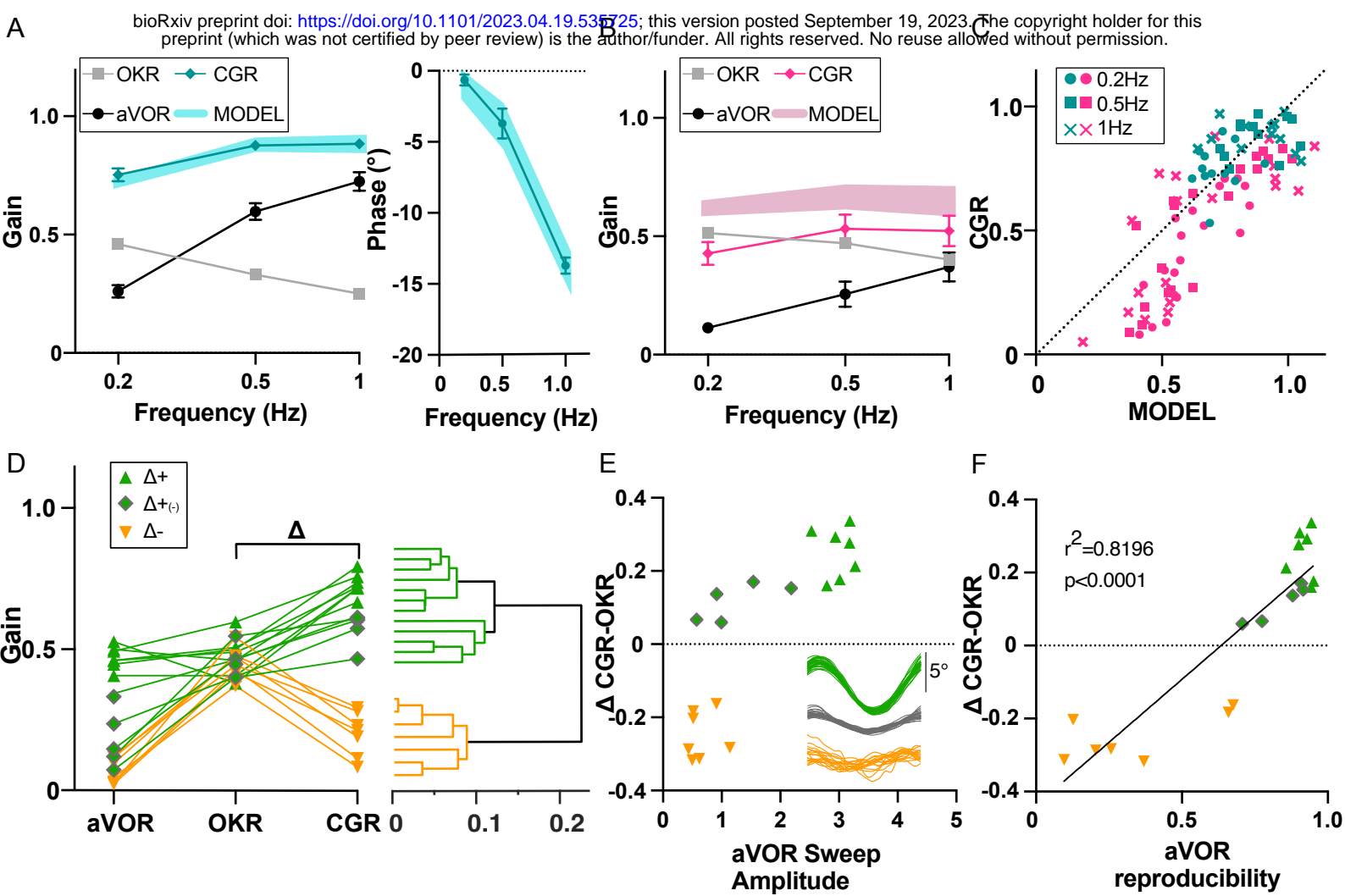


Figure 6: Visuo-vestibular interactions following IDPN treatment.