Titles: Both systemic Calcitonin Gene Related Peptide (CGRP) and a vestibular challenge promote anxiety-related behaviors and dynamic imbalance in mice

Abbreviated Title: CGRP and a vestibular challenge promote anxiety-related behaviors and dynamic imbalance

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Abstract

Motion-induced anxiety and agoraphobia are more frequent symptoms in patients with vestibular migraine than migraine without vertigo. The neuropeptide calcitonin gene-related peptide (CGRP) is a therapeutic target for migraine and vestibular migraine, but the link between motion hypersensitivity, anxiety, and CGRP is relatively unexplored, especially in preclinical mouse models. To further examine this link, we tested the effects of systemic CGRP and off-vertical axis rotation (OVAR) on elevated plus maze (EPM) and rotarod performance in male and female C57BL/6J mice. Rotarod ability was assessed using two different dowel diameters: mouse dowel (r = 1.5 cm) versus rat dowel (r = 3.5 cm). EPM results indicate CGRP increased anxiety indexes and time spent in the closed arms in females but not males, while OVAR increased anxiety indexes and time spent in the closed arms in both sexes. The combination of CGRP and OVAR elicited even greater anxiety-like behavior. On the rotarod, CGRP reduced performance in both sexes on a mouse dowel but had no effect on a rat dowel, whereas OVAR had a significant effect on the rat dowel. Rotarod performance is influenced by dowel diameter, with larger dowels presenting greater challenges on balance function. These results suggest that both CGRP and vestibular stimulation induce anxiety-like behavior and that CGRP affects dynamic balance function in mice depending on the type of challenge presented. Findings highlight the potential translation of anti-CGRP receptor signaling therapeutics for treating motion hypersensitivity and motion-induced anxiety that manifests in vestibular migraine.

Significance statement

Anxiety is very common in patients with dizziness and vestibular migraine (VM). Elevated CGRP levels have been linked to migraine symptoms of increased light and touch sensitivity in mice and humans and we wondered if a systemic injection of CGRP into mice would increase anxiety and imbalance; and if mice further exposed to a vestibular stimulus would have their anxiety measures sharpened. We observed a female preponderance in both CGRP and motion-induced anxiety-like

behaviors, suggesting that the role of CGRP in migraine's anxiety symptoms can be recapitulated in the mouse. Our findings suggest that CGRP signaling has a pertinent role in motion-induced anxiety and dynamic imbalance, and warrants the potential use of anti-CGRP therapies for the treatment of these symptoms.

Introduction

Anxiety is a common burden that arises with persisting dizziness in patients with vestibular disorders (Balci and Akdal 2020; Bigelow et al. 2016; Huang, Wang, and Kheradmand 2020). Patients with vestibular migraine (VM) have been reported to exhibit severe anxiety and neuroticism towards their condition based on outcomes from the Dizziness Handicap Inventory, Beck depression and anxiety scales, and Short Form health surveys (Ak et al. 2022). Elevated levels of anxiety are also associated with a 53% increased likelihood of falls based on a meta-analysis of eighteen studies linking anxiety as a risk factor for falls (Hallford et al. 2017). Fear of falling is largely detrimental to balance performance in older adults and vestibular patients.

The neuropeptide Calcitonin Gene-Related Peptide (CGRP) is involved in migraine and VM and is known to cause pain, various hypersensitivities, and anxiety (Hoskin and Fife 2021; Russo and Hay 2022). Recent studies have shown CGRP to induce light-aversion, tactile sensitivity, and nociceptive squinting behaviors (Wang et al. 2022; Wang et al. 2021). However, motion sensitivity due to CGRP is relatively unexplored in mouse models, even though motion-induced anxiety and agoraphobia – a fear of one's surroundings – are more frequent symptoms in patients with vestibular migraine than migraine without vertigo (Kutay et al. 2017). Thus, this paper aims to study sex-specific changes in motion-induced anxiety and balance behavior in the C57BL/6J mice that are treated with systemically delivered CGRP and a challenging vestibular stimulus.

We used off-vertical axis rotation (OVAR) as the vestibular stimulus, since OVAR can be disorienting and can promote motion sickness and nausea in rodents and human healthy controls (Furman, Schor, and Schumann 1992; Idoux, Tagliabue, and Beraneck 2018; Zhang et al. 2022).

The elevated plus maze is a behavioral test extensively used to assess anxiety-like behavior in rodent models (Pellow et al. 1985). Measurements of the time spent in open versus closed arms and the number of entries (bouts) into each arm can be used to calculate an anxiety index, an indication of anxiety-like behavior that increases with a mouse's greater presence in the closed arms (Cohen, Matar, and Joseph 2013). This test is used in preclinical research to evaluate anxiety-like behaviors caused by anxiogenic drugs like the known headache-inducing agent sodium nitroprusside (Al-Humaidhie, Thiab, and Hassen 2021).

The rotarod assay is commonly used to assess a mouse's gait and balance on a rotating rod, and the mouse's time (latency) to fall off the rod can provide behavior insights attributed to central and peripheral neurological dysfunction (Deacon 2013). Mice that lack αCGRP have been shown to perform poorly on rotarod (Jones et al. 2018).

To date, there are limited studies that have assessed CGRP-specific changes in mouse behavior that relies on vestibular cues (Zhang et al. 2020), and if CGRP-induced anxiety is exacerbated by vestibular provocation. Thus, this study's objectives are to examine the role of intraperitoneally delivered CGRP and a vestibular stimulus in anxiety and dynamic balance behavior in the wildtype C57BL/6J mouse.

Methods

Animals: C57BL/6J mice were obtained from Jackson Laboratories (JAX 664) and Mice were housed under a 12 to 12 day/night cycle at the University of Rochester 's Vivarium under the care of the University of Rochester's Veterinary Services personnel. They were kept in cages of up to 5 mice per cage and had ad libitum access to food, water, and bedding material. Equal numbers of male (M) and female (F) mice were tested, with a total of 76 mice (38M/38F) used in these studies. All animal procedures were approved by the University of Rochester's IACUC committee and performed in accordance with NIH standards.

The studies were designed to have sufficient power to detect male/female differences. Before beginning the experiments each day, mice were acclimated to the testing room, which was maintained at a temperature of 22-23 degrees Celsius, for at least 30 minutes. They remained in this room until the experiments were completed. Injections were given after the acclimation period. Mice were tested at ages between 2.3 and 6 months for both the elevated plus maze and rotarod tests.

Injections: Injections were given intraperitoneally (IP) using a 33-gauge insulin syringe and behavioral testing was carried out ~20 minutes post-injection. PBS was used as the diluent and control injection. Rat α-Calcitonin Gene-Related Peptide (CGRP) (Sigma) was injected at a dose of 0.1 mg/kg.

Elevated Plus Maze (EPM): EPM measures anxiety by utilizing the inherent conflict between the exploration of a novel area and avoidance of its aversive features. The mouse is placed in the middle of the maze, which is an open square (5 x 5 cm) in the center of four arms (30 cm long x 5 cm wide with 2 enclosed and 2 open). The maze consists of opaque plexiglass panels and is mounted on a stand 30 cm high. A video camera records for 30 wutes of the mouse exploring the maze in regular, room lighting conditions. Mice have four five-minute sessions collected in two days. Mice were injected with either vehicle control (day 1) or CGRP (day 3) twenty minutes prior to the first EPM test. The mice would then be subjected to the vestibular challenge (see Vestibular

124 Challenge – Off-Vertical Axis Rotation (OVAR) for methods) and would then be tested again for
125 EPM activity. The session is scored to determine the animal's tendency for exploring enclosed
126 versus open arms, and the time spent in each area and the number of bouts into each arm are
127 recorded. An anxiety index per animal is computed per experimental condition (**Fig. 1A**). Following
128 the recording, the mouse is returned to its home cage. The maze is cleaned between subjects using
129 an animal disinfectant (Rescue).

130 Rotarod: Dynamic balance was assessed with the Rotarod (Columbus Instruments) configured with
131 either a mouse dowel (r = 1.5 cm) or a rat dowel (r = 3.5 cm). Mice were tasked to maintain balance
132 and gait on the different dowels rotating from 5 to 44 rpm at an acceleration step of 2.4 rpm every 4
133 seconds. Latency to fall (LTF) is measured when mice fall from the dowel. Three days of rotarod
134 testing were performed. The first day was a training day where mice were tested for 6 to 8 trials. On
135 the second day, mice were briefly trained and were then injected with vehicle control. Twenty
136 minutes after the injection, mice were tested for 3 trials (pre-VC) and were then stimulated with
137 OVAR for 30 seconds. Mice were then immediately tested for 3 trials on the rotarod after the
138 challenge (post-VC). Approximately 10-30 seconds pass in between subsequent trials during pre-VC
139 and post-VC tests. On day 3, the same mice were re-tested with the same methods but were instead
140 injected with CGRP (**Fig. 2A**).

141 Vestibular Challenge – Off-Vertical Axis Rotation (OVAR): In this study, a two-cage rotator (cage
142 dimensions: 12.7 cm x 8.9 cm) was built to impose off-vertical axis rotation (60 rpm, 45° tilt from the
143 vertical for 30 seconds) as a vestibular stimulus to mice during elevated plus maze and rotarod
144 experiments. The mice are secured 22 cm from the rotational axis, and the device can rotate two
145 mice at a time.

146 Data Analysis and Statistics: Elevated plus maze data were analyzed by assessing time spent in
147 closed arms as a percentage, the number of bouts through the center platform, and a calculated
148 anxiety index (equation found in **Fig. 1A**). For rotarod assessment, the max latency to fall (LTF) of
149 the three trials per testing condition was determined per mouse and indicated the mouse's best

150 effort for that testing condition. A group average of the max LTFs and standard error (mean + SEM)
151 was computed for comparisons. Statistical analyses were performed using GraphPad Prism 9.5.
152 Two-way repeated measures analysis of variance (RM-ANOVA) were primarily computed for
153 females or males across elevated plus maze and rotarod testing, with the following factors: IP CGRP
154 vs IP vehicle, and before versus after the vestibular challenge. Tukey multiple comparisons test was
155 the preferred post hoc analysis for RM-ANOVAs. Unpaired t-tests were only used when comparing
156 differences in rotarod ability due to dowel lengths after IP vehicle treatment. Statistical significance
157 was set at $p \leq 0.05$ for all analyses. F-values and p-statistics for RM-ANOVAs across behavioral
158 outcomes can be found In **Table 1**.

159 **Results**

160 CGRP and OVAR's impact on elevated plus maze activity: In sixteen (8M/8F) C57BL/6J mice, we
161 evaluated differences in anxiety indexes (**Fig. 1B**), time spent in closed arms (**Fig. 1C**), and the
162 number of bouts through the center (**Fig. 1D**) by examining the effects of IP CGRP and OVAR as
163 the vestibular challenge (VC). OVAR significantly increased anxiety in all IP vehicle-treated mice (p
164 $= 0.002$). Anxiety indexes also increased from IP vehicle to IP CGRP prior to the OVAR ($p = 0.02$),
165 and the OVAR further increases anxiety in CGRP-treated mice ($p = 0.003$) (**Fig. 1B**). These changes
166 in anxiety indexes strongly correlate with time spent in the closed arms (**Fig. 1C**). Notably, all mice -
167 on average - spend ~52% of their time in the closed arms, but this percentage increases to ~70%
168 due to IP CGRP, ~78% due to OVAR (IP vehicle injection), and ~90% when mice are treated with IP
169 CGRP and OVAR. When examining if sex-specific differences were present in anxiety outcomes, we
170 observed that OVAR caused an increase in anxiety indexes ($p = 0.03$) for females and a trending
171 effect in males ($p = 0.09$) (**Fig. 1B**). OVAR-induced anxiety correlated with an increased percentage
172 of time spent in the closed arms for females ($p < 0.001$) and males ($p = 0.02$). Before OVAR, IP
173 CGRP caused significant increases in female anxiety indexes ($p = 0.01$) and correlated with
174 increased time in the closed arms ($p = 0.002$) but these observations were not seen in males (**Fig.**
175 **1C**). Additionally, CGRP/Post-OVAR anxiety indexes were higher than only CGRP-induced anxiety
176 in females ($p = 0.02$) and correlated with increased time spent in the closed arms ($p = 0.007$). While

177 the analyses on anxiety indexes and time spent in closed arms were robust in females, we did
178 observe CGRP-treated males to spend increased time in the closed arms after the OVAR ($p = 0.04$),
179 though this observation did not translate to a significant increase in male anxiety indexes.
180 Additionally, we quantified the number of bouts through the center platform as an indicator of
181 movement, because each bout indicates one instance where a mouse moved from an open to a
182 closed arm and vice versa. In **Fig. 1D**, when sexes were pooled, IP vehicle-treated mice
183 experienced a 2.8x fold reduction in center bouts after OVAR stimulation ($p < 0.001$). Likewise, a
184 3.2x reduction in center bouts was observed after OVAR when mice were treated with IP CGRP ($p <$
185 0.001). More specifically in a particular sex, OVAR strong affected the number of center bouts
186 detected in females ($p < 0.001$) and in males ($p < 0.001$) during IP vehicle testing. After the OVAR in
187 IP CGRP-treated mice, females exhibited significantly reduced center bout activity ($p = 0.009$) and
188 so did males ($p < 0.001$). Even without OVAR, IP CGRP affected center bouts in females ($p = 0.01$)
189 and this was a trend seen in males ($p = 0.06$). These results show CGRP and a vestibular challenge
190 can independently elicit anxiety-like behavior in mice with a female preponderance in observed
191 outcomes, but the use of both stressors is additive and elicits even stronger anxiety in mice.

192

193 <u>Differences in C57B6/J's rotarod ability tested on different dowel diameters</u>:

194 While OVAR Is used as the primary vestibular challenge, we wanted to observe how balance
195 function differs when mice perform rotarod on different dowel lengths (1.5 cm vs 3.5 cm radius). We
196 hypothesized that mice would be further challenged on the rat dowel, because to maintain balance
197 on a larger diameter dowel rotating at the same parameters (in rpm) as a smaller dowel, a greater
198 distance of the larger dowel's circumference must be displaced per second. We calculated that the
199 mouse had to travel ~2.3x more distance on the rat dowel per second of rotation at the lowest (5
200 rpm) and the highest (44 rpm) speeds, ultimately becoming more of a challenge. This difficulty was
201 reflected in the data. We observed IP vehicle-treated mice on the mouse dowel exhibited higher max
202 LTFs ($n = 20$, 42.62 ± 1.93 s) compared to mice tested on the rat dowel ($n = 39$, 30.75 ± 1.33 s),

highlighting a difference in best performance by -11.87 ± 2.31 seconds when naïve mice are challenged to do rotarod on a larger dowel diameter (unpaired t-test, $t = 5.13$, $df = 57$, $p < 0.0001$). Due to this disparity in best performance between dowel lengths, we attempted to calibrate the rat dowel so that instead of matching the mouse dowel's rpm, it would match the displaced distance per second. We dedicated twenty naïve, age-matched mice (10M/10F) to this task, and observed mice to exhibit significantly higher max LTFs (greater than 90 seconds on average per trial) when rat dowel parameters were altered (data not shown). We surmised that mouse gait cannot be easily tuned by modulating the displaced distance of the dowel and that other factors must be at play (e.g., attention, position of paws in respect to curved dowel surface), and thus did not explore this calibration further.

<u>CGRP and OVAR's effects on mouse dowel-rotarod:</u>

Twenty mice (10M/10F) were assessed for rotarod ability on the mouse dowel, before and after OVAR as the vestibular challenge (VC). In all mice prior to the OVAR stimulus (pre-OVAR), IP CGRP significantly reduced rotarod performance with a mean reduction of 11.01 ± 2.07 s ($p = 0.0002$) compared to IP vehicle (**Fig. 2B-D**). This impaired performance due to systemic CGRP was still observed after OVAR, as post-OVAR outcomes showed a decrease from IP vehicle to IP CGRP ($p = 0.03$). A disrupted pre-VC rotarod ability due to CGRP is observed in both sexes, with a mean reduction of 9.02 ± 2.47 s ($p = 0.02$) in females and of 13.00 ± 3.32 ($p = 0.02$) in males. When comparing post-OVAR outcomes, only males indicated a significant difference between IP vehicle and IP CGRP ($p = 0.009$).

<u>CGRP and OVAR's effects on rat dowel-rotarod:</u>

Thirty-nine mice (20M/19F) were used to assess CGRP and OVAR's effects on rotarod configured with a rat dowel ($r = 3.5$ cm). When sexes are pooled, IP CGRP had no effect but OVAR significantly affected performance in all mice (**Fig. 2E-G**). OVAR reduce rotarod ability in mice treated with IP vehicle by 5.92 ± 1.44 s ($p = 0.001$) and when treated with IP CGRP by 4.92 ± 1.72 s ($p = 0.03$). In

229 female mice specifically, OVAR impacted performance after IP vehicle by 7.51 ± 1.87 s ($p = 0.004$).
230 OVAR did not significantly impact male rotarod ability on the rat dowel after IP vehicle, nor were
231 post-OVAR outcomes strongly different after IP CGRP testing.

232

233 **Discussion**

234 Preclinical research in migraine – via rodent models – has provided evidence that biological sex can
235 drive the occurrence of certain mouse surrogate behaviors that resemble clinically reported
236 symptoms, known to arise more often in women than men. In this study through the elevated plus
237 maze (EPM) test, we show that intraperitoneally delivered (IP) CGRP causes a majority of female
238 mice to spend increased time spent in the closed arms and exhibit significantly higher anxiety
239 indexes compared to their baseline, but this change in anxiety indexes was not significantly seen in
240 males. Consistent with our results, a prior study indicated that central, intracerebroventricular (i.c.v.)
241 administration of CGRP from 250 ng to 1 ug decreases open-field activity of adult male Wister rats in
242 a dose-dependent manner (Kovács et al. 1999). A previous study on mutant mice, with an existing
243 severe vestibular deficit showed increased anxiety when tested on the elevated plus maze and also
244 were impaired on the mouse dowel rotarod. Interestingly, daily vestibular training improved both
245 anxiety and rotarod behaviors (Shefer et al. 2015) in these mutant mice.

246

247 Motion-induced anxiety was more pronounced in female mice when were tested with the EPM after
248 being stimulated with a30 second off-vertical axis rotation (OVAR) as a vestibular challenge.
249 Understandably, both sexes experience a significant increase in motion-induced anxiety (a near
250 38% increase in anxiety-indexes) due to CGRP and OVAR when compared to their normal baseline
251 anxiety - measured after vehicle administration (no vestibular challenge). We also observed reduced
252 movement in the EPM when we measured a mouse's bouts through the center, as mice treated with
253 OVAR were not as active as their pre-OVAR state after IP vehicle or IP CGRP injections. This
254 motion-induced anxiety and reduced activity observed during EPM is of clinical importance, as a

255 clear, positive correlation between anxiety, depression, and vertigo is observed in patients with
256 vestibular disorders (Omara et al. 2022), and so studying this link in mouse models provides insight
257 into this correlation. The EPM is also a suitable assay as a surrogate behavior for agoraphobia in
258 mouse models for migraine, as mice spend more time in the closed arms when they exhibit anxiety-
259 like behaviors, and mirrors patients' tendencies to avoid open spaces during vestibular migraine
260 attacks (Kutay et al. 2017).

261

262 Our EPM data is consistent with previous reports in rodents that suggest a female preponderance
263 towards anxiety. Experiments using the open field assay and unpredictable, sound stress highlighted
264 a female likelihood to anxiety-like behaviors (Viero et al. 2022; Wang et al. 2022). Outside of anxiety
265 but still related to the CGRP's sexually dimorphic effects, administration of CGRP to the spinal cord
266 (intra-thecal) caused a dose-dependent, longer-lasting hind paw allodynia in female rodents, as
267 opposed to a more momentary effect in males (Paige et al. 2022).

268

269 The use of OVAR as a vestibular provocation is rarely used in rodent studies, and here we show that
270 even a brief, vestibular stimulus can bring out significant anxiety and balance complications in the
271 wildtype C57BL/6J strain.

272

273 Anxiety is a risk factor for the progression of episodic to chronic migraine, and elevated CGRP levels
274 have been shown to alter neuronal activity that is linked to anxiety behaviors (Durham 2016). The
275 results here further elucidate CGRP's role in motion-induced anxiety and balance function in mouse
276 models, and should be further explored in the presence of CGRP-targeted therapeutics. Peripherally
277 administered CGRP-signaling antagonists have been widely observed to reduce CGRP-induced
278 migraine-like sensitivities in rodents and humans, and therefore would be hypothesized to attenuate
279 anxiety caused by IP CGRP (Araya et al. 2019; Raffaelli, Neeb, and Reuter 2019).

To conclude, our study extends our current understanding of CGRP-induced anxiety by incorporating a motion sensitivity component, and uniquely evaluates mouse balance function to a degree not observed in prior rotarod studies. We observed a female preponderance in both CGRP and motion-induced anxiety-like behaviors, suggesting that the role of CGRP in migraine's anxiety symptoms can be recapitulated in preclinical models. The results of this study provide support to claims that CGRP has a pertinent role in motion-induced anxiety and dynamic imbalance, and warrants the potential use of anti-CGRP therapies for the treatment of these symptoms.

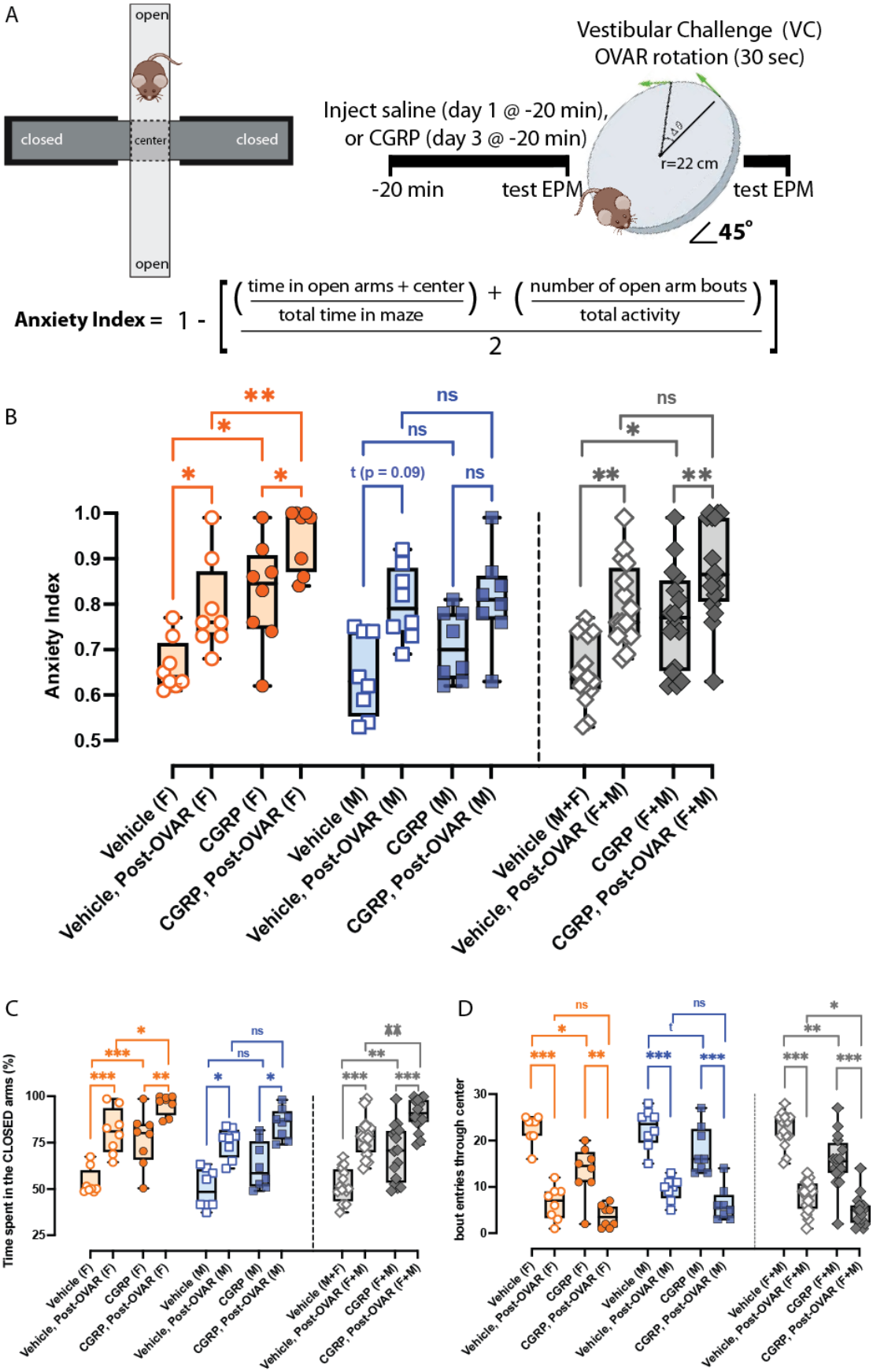
A
open
closed
center
closed
open
Vestibular Challenge (VC)
OVAR rotation (30 sec)
Inject saline (day 1 @ -20 min),
or CGRP (day 3 @ -20 min)
-20 min
test EPM
r=22 cm
test EPM
∠45°
Anxiety Index = 1 - [(time in open arms + center / total time in maze) + (number of open arm bouts / total activity) / 2]
B
Anxiety Index
t (p = 0.09)
ns
Vehicle (F)
Vehicle, Post-OVAR (F)
CGRP (F)
CGRP, Post-OVAR (F)
Vehicle (M)
Vehicle, Post-OVAR (M)
CGRP (M)
CGRP, Post-OVAR (M)
Vehicle (M+F)
Vehicle, Post-OVAR (F+M)
CGRP (F+M)
CGRP, Post-OVAR (F+M)
C
Time spent in the CLOSED arms (%)
D
bout entries through center

Fig. 1

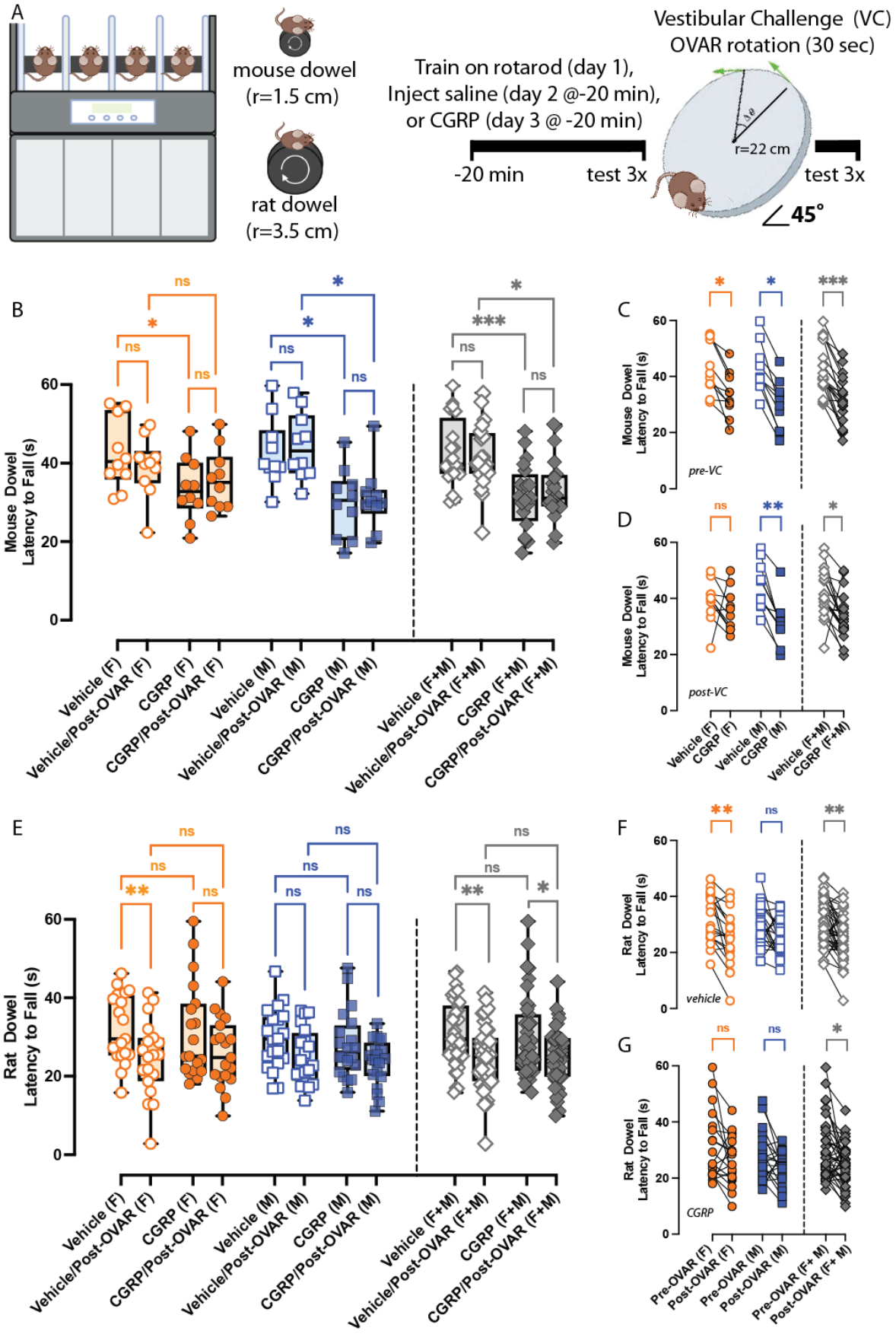
A
mouse dowel
(r=1.5 cm)
rat dowel
(r=3.5 cm)
Train on rotarod (day 1),
Inject saline (day 2 @-20 min),
or CGRP (day 3 @ -20 min)
-20 min
test 3x
Vestibular Challenge (VC)
OVAR rotation (30 sec)
r=22 cm
test 3x
45°
B
Mouse Dowel
Latency to Fall (s)
Vehicle (F)
Vehicle/Post-OVAR (F)
CGRP (F)
CGRP/Post-OVAR (F)
Vehicle (M)
Vehicle/Post-OVAR (M)
CGRP (M)
CGRP/Post-OVAR (M)
Vehicle (F+M)
Vehicle/Post-OVAR (F+M)
CGRP (F+M)
CGRP/Post-OVAR (F+M)
C
pre-VC
D
post-VC
E
Rat Dowel
Latency to Fall (s)
F
vehicle
G
CGRP
Pre-OVAR (F)
Post-OVAR (F)
Pre-OVAR (M)
Post-OVAR (M)
Pre-OVAR (F+ M)
Post-OVAR (F+ M)

Fig. 2

293 **Table 1**

Biological Sex	Behavioral Outcome	F (DF_n, DF_d)	P-val
Female	Anxiety Index	CGRP: F (1.00, 7.00) = 67.11,	$p < 0.001$
		OVAR: F (1.00, 7.00) = 53.97,	$p < 0.001$
	Time in Closed Arm (%)	CGRP: F (1.00, 7.00) = 31.59,	$p < 0.001$
		OVAR: F (1.00, 7.00) = 129.1,	$p < 0.001$
	Bouts Through Center Arm	CGRP: F (1.00, 7.00) = 14.88,	$p = 0.006$
		OVAR: F (1.00, 7.00) = 380.8,	$p < 0.001$
	Rotarod- mouse dowel	CGRP: F (1.00, 9.00) = 9.71,	$p = 0.01$
		OVAR: F (1.00, 9.00) = 0.12,	$p = 0.73$
	Rotarod - rat dowel	CGRP: F (1.00, 18.00) = 0.02	$p = 0.9$
		OVAR: F (1.00, 18.00) = 14.34	$p = 0.001$
Male	Anxiety Index	CGRP: F (1.00, 7.00) = 2.77,	$p = 0.14$
		OVAR: F (1.00, 7.00) = 30.09,	$p < 0.001$
	Time in Closed Arm (%)	CGRP: F (1.00, 7.00) = 16.38,	$p = 0.005$
		OVAR: F (1.00, 7.00) = 76.02,	$p < 0.001$
	Bouts Through Center Arm	CGRP: F (1.00, 7.00) = 7.63,	$p = 0.03$
		OVAR: F (1.00, 7.00) = 135.3,	$p < 0.001$
	Rotarod- mouse dowel	CGRP: F (1.00, 9.00) = 19.40,	$p = 0.002$
		OVAR: F (1.00, 9.00) = 1.74,	$p = 0.22$
	Rotarod - rat dowel	CGRP: F (1.00, 19.00) = 1.21	$p = 0.29$
		OVAR: F (1.00, 19.00) = 7.13	$p = 0.01$
Female + Male	Anxiety Index	CGRP: F(1.00, 15.00) = 21.38,	$p < 0.001$
		OVAR: F(1.00, 15.00) = 82.01,	$p < 0.001$
	Time in Closed Arm (%)	CGRP: F(1.00,15.00) = 42.38,	$p < 0.001$
		OVAR: F(1.00,15.00) = 202.8,	$p < 0.001$
	Bouts Through Center Arm	CGRP: F (1.00, 15.0) = 22.30,	$p < 0.001$
		OVAR: F (1.00, 15.00) = 431.0,	$p < 0.001$
	Rotarod- mouse dowel	CGRP: F(1.00,19.00) = 25.20,	$p < 0.0001$
		OVAR: F(1.00,19.00) = 0.17,	$p = 0.69$
	Rotarod - rat dowel	CGRP: F(1.00,38.00) = 0.19	$p = 0.67$
		OVAR: F(1.00,38.00) = 20.98	$p < 0.0001$

294

295

296 **Figure Legends:**

297 **Figure 1**: **Calcitonin-Gene Related peptide (CGRP) and off-vertical axis rotation (OVAR) on**
298 **elevated plus maze (EPM) behaviors. A,** The EPM and its components are shown on the left, an
299 experimental timeline is provided on the right, and an equation for calculating the anxiety index is
300 provided underneath. **B-D**, In 16 mice (8M/8F), two-way repeated measures (RM) ANOVAs with
301 Tukey post hoc analyses were used to evaluate **B**, anxiety indexes, **C**, time in closed arms, and **D**,
302 center bouts due IP CGRP a brief off-vertical axis rotation (OVAR). Symbols are depicted: female
303 (F) – orange circle, male (M) – blue square, combined (F+M) – gray diamond, vehicle – open, CGRP
304 – closed. B, OVAR significantly increased anxiety in all mice treated with IP vehicle ($p = 0.002$). B,
305 OVAR significantly increased anxiety indexes in all mice treated with IP vehicle, and anxiety indexes
306 also increased from vehicle to CGRP. OVAR further increased anxiety in CGRP-treated mice. **C**,
307 Changes in anxiety indexes strongly correlated with time spent in the closed arms. Females were
308 more sensitive to the effects of OVAR and CGRP than males. **D,** OVAR-induced anxiety strongly
309 affected the number of center bouts detected in females and males during IP vehicle testing, and IP
310 CGRP affected center bouts in females even without OVAR. P-values and asterisks are listed: *$p \leq$
311 0.05, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

312

313 **Figure 2**: **Calcitonin-Gene Related peptide (CGRP) and off-vertical axis rotation (OVAR) on**
314 **rotarod configured with two different dowels. A,** Rotarod configured with a mouse or rat dowel is
315 shown, and an experimental timeline highlighting training and experiment days is provided. Twenty
316 mice (10M/10F) were tested with the mouse dowel, and 39 mice (20M/19F) were tested with the rat
317 dowel. Two-way repeated measures ANOVAs with Tukey multiple comparisons test were used to
318 evaluate maximum latency to fall (s). **B-D,** Before the OVAR stimulus (when sexes are pooled),
319 rotarod performance was reduced from IP vehicle to IP CGRP, and this change was also observed
320 when comparing OVAR outcomes. A disrupted pre-VC rotarod ability due to CGRP is observed in
321 both sexes. After the OVAR, only males indicated a significant difference between IP vehicle and IP

322 CGRP. **E-G,** Opposite of mouse dowel studies, rat dowel studies indicated an effect of OVAR but
323 not systemic CGRP. OVAR reduced rotarod ability in mice treated with IP vehicle and when treated
324 with IP CGRP when sexes were pooled. In female mice specifically, max LTFs dropped from vehicle
325 to vehicle/post-OVAR, but this was not seen in males. P-values and asterisks are listed: *$p \leq 0.05$,
326 **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

327

328 **Table Legend:**

329 **Table 1:** Two-way repeated measure ANOVAs were computed with Tukey multiple comparisons test
330 as the post hoc analysis (Tukey adjusted p-values not shown). F values and associated p-values are
331 provided across the behavioral outcomes assessed in this study: anxiety index (au), time in closed
332 arms (%), bouts through center arm (au), rotarod configured with mouse dowel (s), and rotarod
333 configured with rat dowel (s). ANOVAs were computed in females, males, and when sexes were
334 pooled (F+M).

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References

Ak, Ayşın Kısabay, Neşe Çelebisoy, Hüseyin Nezih Özdemir, and Figen Gökçay. 2022. 'Vestibular migraine and persistent postural perceptual dizziness: Handicap, emotional comorbidities, quality of life and personality traits', *Clinical Neurology and Neurosurgery*, 221: 107409.

Al-Humaidhie, Abeer, Afrah Thiab, and Semaa Hassen. 2021. 'The Study of the Effect of Sodium Nitroprusside in Anxiety-Like Behavior in Mice in Comparison With Diazepam Molecular aspects of Staphylococcus aureus View project COVID-19 View project', *Iraqi Journal of Science*, 61: 485-90.

Araya, Erika Ivanna, Joelle de Melo Turnes, Amanda Ribeiro Barroso, and Juliana Geremias Chichorro. 2019. 'Contribution of intraganglionic CGRP to migraine-like responses in male and female rats', *Cephalalgia*, 40: 689-700.

Balci, B., and G. Akdal. 2020. 'Imbalance, motion sensitivity, anxiety and handicap in vestibular migraine and migraine only patients', *Auris Nasus Larynx*, 47: 747-51.

Bigelow, R. T., Y. R. Semenov, S. du Lac, H. J. Hoffman, and Y. Agrawal. 2016. 'Vestibular vertigo and comorbid cognitive and psychiatric impairment: the 2008 National Health Interview Survey', *J Neurol Neurosurg Psychiatry*, 87: 367-72.

Cohen, Hagit, Michael A. Matar, and Zohar Joseph. 2013. 'Animal Models of Post-Traumatic Stress Disorder', *Current Protocols in Neuroscience*, 64: 9.45.1-9.45.18.

Deacon, R. M. 2013. 'Measuring motor coordination in mice', *J Vis Exp*: e2609.

Durham, P. L. 2016. 'Diverse Physiological Roles of Calcitonin Gene-Related Peptide in Migraine Pathology: Modulation of Neuronal-Glial-Immune Cells to Promote Peripheral and Central Sensitization', *Curr Pain Headache Rep*, 20: 48.

Furman, J. M., R. H. Schor, and T. L. Schumann. 1992. 'Off-vertical axis rotation: a test of the otolith-ocular reflex', *Ann Otol Rhinol Laryngol*, 101: 643-50.

Hallford, David John, Geoff Nicholson, Kerrie Sanders, and Marita P. McCabe. 2017. 'The Association Between Anxiety and Falls: A Meta-Analysis', *The Journals of Gerontology: Series B*, 72: 729-41.

363 Hoskin, J. L., and T. D. Fife. 2021. 'New Anti-CGRP Medications in the Treatment of Vestibular
364 Migraine', *Front Neurol*, 12: 799002.
365 Huang, T. C., S. J. Wang, and A. Kheradmand. 2020. 'Vestibular migraine: An update on current
366 understanding and future directions', *Cephalalgia*, 40: 107-21.
367 Idoux, Erwin, Michele Tagliabue, and Mathieu Beraneck. 2018. 'No Gain No Pain: Relations
368 Between Vestibulo-Ocular Reflexes and Motion Sickness in Mice', *Frontiers in Neurology*, 9.
369 Jones, Sherri M., Sarath Vijayakumar, Samantha A. Dow, Joseph C. Holt, Paivi M. Jordan, and
370 Anne E. Luebke. 2018. 'Loss of α-Calcitonin Gene-Related Peptide (αCGRP) Reduces
371 Otolith Activation Timing Dynamics and Impairs Balance', *Frontiers in molecular*
372 *neuroscience*, 11: 289-89.
373 Kovács, A., G. Telegdy, G. Tóth, and B. Penke. 1999. 'Neurotransmitter-mediated open-field
374 behavioral action of CGRP', *Life Sci*, 64: 733-40.
375 Kutay, Ö, G. Akdal, P. Keskinoğlu, B. D. Balcı, and T. Alkın. 2017. 'Vestibular migraine patients are
376 more anxious than migraine patients without vestibular symptoms', *J Neurol*, 264: 37-41.
377 Omara, Abir, Eman Mostafa Basiouny, Marwa El Shabrawy, and Reham Rafei El Shafei. 2022. 'The
378 correlation between anxiety, depression, and vertigo: a cross-sectional study', *The Egyptian*
379 *Journal of Otolaryngology*, 38: 143.
380 Paige, C., I. Plasencia-Fernandez, M. Kume, M. Papalampropoulou-Tsiridou, L. E. Lorenzo, E. T.
381 David, L. He, G. L. Mejia, C. Driskill, F. Ferrini, A. L. Feldhaus, L. F. Garcia-Martinez, A. N.
382 Akopian, Y. De Koninck, G. Dussor, and T. J. Price. 2022. 'A Female-Specific Role for
383 Calcitonin Gene-Related Peptide (CGRP) in Rodent Pain Models', *J Neurosci*, 42: 1930-44.
384 Pellow, S., P. Chopin, S. E. File, and M. Briley. 1985. 'Validation of open:closed arm entries in an
385 elevated plus-maze as a measure of anxiety in the rat', *J Neurosci Methods*, 14: 149-67.
386 Raffaelli, B., L. Neeb, and U. Reuter. 2019. 'Monoclonal antibodies for the prevention of migraine',
387 *Expert Opin Biol Ther*, 19: 1307-17.
388 Russo, A. F., and D. L. Hay. 2022. 'CGRP physiology, pharmacology, and therapeutic targets:
389 Migraine and beyond', *Physiol Rev*.

390 Shefer, S., C. Gordon, K. B. Avraham, and M. Mintz. 2015. 'Balance deficit enhances anxiety and
391 balance training decreases anxiety in vestibular mutant mice', *Behav Brain Res*, 276: 76-83.
392 Viero, F. T., P. Rodrigues, J. M. Frare, N. A. R. Da Silva, M. A. Ferreira, A. M. Da Silva, G. C.
393 Pereira, J. Ferreira, M. M. Pillat, G. V. Bocchi, R. Nassini, P. Geppetti, and G. Trevisan.
394 2022. 'Unpredictable Sound Stress Model Causes Migraine-Like Behaviors in Mice With
395 Sexual Dimorphism', *Front Pharmacol*, 13: 911105.
396 Wang, M., T. L. Duong, B. J. Rea, J. S. Waite, M. W. Huebner, H. C. Flinn, A. F. Russo, and L. P.
397 Sowers. 2022. 'CGRP Administration Into the Cerebellum Evokes Light Aversion, Tactile
398 Hypersensitivity, and Nociceptive Squint in Mice', *Front Pain Res (Lausanne)*, 3: 861598.
399 Wang, M., B. N. Mason, L. P. Sowers, A. Kuburas, B. J. Rea, and A. F. Russo. 2021. 'Investigating
400 Migraine-Like Behavior Using Light Aversion in Mice', *J Vis Exp*.
401 Zhang, Y., Y. Zhang, K. Tian, Y. Wang, X. Fan, Q. Pan, G. Qin, D. Zhang, L. Chen, and J. Zhou.
402 2020. 'Calcitonin gene-related peptide facilitates sensitization of the vestibular nucleus in a
403 rat model of chronic migraine', *J Headache Pain*, 21: 72.
404 Zhang, Z. H., L. P. Liu, Y. Fang, X. C. Wang, W. Wang, Y. S. Chan, L. Wang, H. Li, Y. Q. Li, and F.
405 X. Zhang. 2022. 'A New Vestibular Stimulation Mode for Motion Sickness With Emphatic
406 Analysis of Pica', *Front Behav Neurosci*, 16: 882695.

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