Title: Role of Calcitonin Gene-Related Peptide (CGRP) in Auditory, Static, and Dynamic Imbalance Behaviors: Implications for Vestibular Migraine

Abbreviated Title: CGRP in auditory and imbalance of VM

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Abstract:

About 42% of people with migraine have a vestibular component causing balance problems and dizziness. In fact, VM is a major cause of vertigo in dizziness clinics and is estimated to affect 1% of the overall population. As migraine increases light, and sound sensitivities, it also increases sensitivities to movement or perceived movement in VM Patients with migraine, and especially VM, exhibit a heightened sense of sound, or phonophobia. Phonophobia is also related to hyperacusis (extreme sensitivity to sound). Behavioral evidence of hyperacusis and phonophobia in mice can be inferred using the acoustic startle reflex (ASR). The most common symptoms of VM were unsteadiness, balance disturbances, and "light headedness". When balance disturbances were quantified, VM patients swayed more than migraine-only or healthy controls, when challenged with competing stimulus. In addition to static imbalance, VM patients also showed dynamic imbalances such as gait disturbances. In this study we tested wildtype C57B6/J mice to determine if mice exhibited increased sound (measured by acoustic startle), static imbalance (measured by postural sway), and dynamic imbalance (measured by rotarod), when challenged with systemic CGRP (IP) 0. 1 mg/kg. We found both sexes of mice were affected by systemic CGRP in sound and dynamic imbalance testing, yet only female mice showed increased postural sway after systemic CGRP, recapitulating the higher incidence of VM in females. These non-invasive assays of VM pave the way to further explore mechanisms of CGRP signaling in VM, and test effectiveness of migraine therapeutics in VM.

Significance Statement: About 42% of people with migraine have a vestibular component causing balance problems and dizziness, called vestibular migraine (VM). VM is a major cause of vertigo in dizziness clinics and is estimated to affect 1% of the overall population. While there are preclinical models of the light and touch sensitivities of migraine, a mouse model for VM is not available. Here we show robust assays of sound and motion sensitivities in wildtype mice, paving the way to test effectiveness of new migraine therapeutics for VM.

Introduction:

Migraine is a debilitating chronic neurological disorder that is listed by the World Health Organization

(WHO) as the most debilitating neurological disorder worldwide and is estimated to affect 18% of women and 6% of men (Goadsby PJ et al., 2002;Lipton RB et al., 2007). Migraine is characterized by recurrent attacks of debilitating headaches and nausea, with heightened sensory sensitivities, such as light (photophobia), sound (phonophobia), touch (allodynia) and movement (vertigo). About 42% have a vestibular component causing balance problems and dizziness (Kuritzky A et al., 1981;Neuhauser H et al., 2001;Neuhauser HK, 2007;Neuhauser HK, 2009). This form of migraine, termed vestibular migraine (VM), has been recently defined as having at least five episodes of moderate or severe vertigo, history of migraine, either phonophobia, photophobia, or visual aura, and vertigo that cannot be caused by other pathologies (Lempert T et al., 2022;Lempert T et al., 2012). In fact, VM is a major cause of vertigo in dizziness clinics (Kayan A and Hood JD, 1984), yet despite migraine and VM's prevalence, the pathophysiology remains uncertain.

Patients with migraine, and especially VM, exhibit a heightened sense of sound, (Ashkenazi A et al., 2009;Lopez-Escamez JA et al., 2014) or *phonophobia* (fear of sound). *Phonophobia* is also related to *hyperacusis* (extreme sensitivity to sound) (Katzenell U and Segal S, 2001;Liberman MC et al., 2016;Schaaf H et al., 2003). Existence of *hyperacusis* and *phonophobia* behaviors in mice can be inferred using the acoustic startle reflex (ASR) and pre-pulse inhibition (PPI) of ASR (Allen PD and Ison JR, 2010;Allen PD and Ison JR, 2012;Ison JR and Allen PD, 2012;Ison JR et al., 2017;Xiong B et al., 2017). As migraine increases light (Chanda ML et al., 2013;Girotra P et al., 2017;Rossi HL et al., 2016;Russo AF, 2015), and sound sensitivities, it also increases sensitivities to movement or perceived movement in VM (King S et al., 2014;Lewis RF et al., 2011;Lewis RF et al., 2011;Wang J and Lewis RF, 2016;Wang J and Lewis RF, 2016). VM patients have both higher perceptual sensitivities to dynamic tilts, to tilts during off-vertical axis centrifugation (OVAR), and motion perception (King S,Wang J,Priesol AJ and Lewis RF, 2014;Lewis RF,Priesol AJ,Nicoucar K,Lim K and Merfeld DM, 2011;Lewis RF,Priesol AJ,Nicoucar K,Lim K and Merfeld DM, 2011;Wang J and Lewis RF, 2016). Interestingly, many VM patients suffer from non-vestibular migraine symptoms for ~8 years before the onset of VM (Cohen JM et al., 2011). The most common symptoms of VM were unsteadiness, balance disturbances, and "light headedness" (Cohen JM,Bigal ME and Newman LC, 2011;Teggi R et al., 2016). When balance disturbances were quantified, VM patients swayed more than migraine-only or

healthy controls when challenged with optic flow, and after optokinetic (OKN) stimulation (with eyes closed) than did either healthy controls or migraine-only patients (Furman JM et al., 2005;Furman JM and Whitney SL, 2005;Panichi R et al., 2015). Interestingly, when migraine symptoms between migraine-only and VM were compared, the VM group more often experienced auras and phonophobia during migraine attacks (Akdal G et al., 2013;Lopez-Escamez JA,Dlugaiczyk J,Jacobs J,Lempert T,Teggi R,von Brevern M and Bisdorff A, 2014).

Calcitonin gene-related peptide (CGRP) is hypothesized to play a role in sensory hypersensitivity in migraine (Russo AF, 2015). CGRP levels are elevated during migraine and normalization of CGRP levels is associated with the relief of pain by triptan anti-migraine drugs (Goadsby PJ,Lipton RB and Ferrari MD, 2002;Juhasz G et al., 2005;Lassen LH et al., 2002). To test the role of CGRP in VM symptoms of sound sensitivity and imbalance, we assessed the acoustic startle response, postural sway, and rotarod ability of C57B6/J mice after systemic (intraperitoneal) administrations of vehicle control followed by CGRP one week later. We found that systemic CGRP caused severe phonophobia, increased postural sway in females, and impaired rotarod performance. These findings suggest that CGRP is acting either at the brainstem, midbrain, or periphery, and that we can recapitulate VM symptoms in wildtype C57B6/J mice, allowing for this preclinical model to be used for assessment of therapeutics and to better understand pathophysiology of VM.

Methods:

Animals: C57B6/J mice were obtained from Jackson Laboratories (JAX #664) and were housed under a 12 to 12 day/night cycle under the care of the University Committee on Animal Resources (UCAR) at the University of Rochester. Mice are housed together in cages (max 5 mice per cage) with *ad libitum* access to food, water, and bedding material. Equal numbers of male and female mice were tested and a total of 79 mice (39 M/ 40F) were used for these studies, and studies were powered sufficiently to detect male/female differences. :

Before experimentation per testing day, mice were equilibrated in the testing room controlled for an ambient temperature between 22-23°C for at least 30 minutes, and remained in this room until completion of the experiment. Injections occurred after the equilibration period in the testing room. Mice were tested between 2.3 - 6 months of age for both postural sway and rotarod testing. This age range

in mice correlates to 20-30 years in humans, which is within the age range that patients with migraine develop symptoms (Lessem SE, 2018). While mice were age-matched, different cohorts of mice were used for either postural way or rotarod. In this study, all animals were compared to themselves and to group comparisons, and none of the assays required anesthesia, which can alter behavior.

Drug administration: Injections are performed intraperitoneally (IP) with a 0.33 x 12.7 insulin syringe. Dulbecco PBS served as the vehicle control. IP CGRP is prepared at 0.1 mg/kg (rat α-CGRP, Sigma). Injection volumes were calibrated so that each animal received 1 injection per testing day at ~100 ul. After injection, animals are placed in a separate cage from their home cage to recuperate from injection stress. Mice were tested 25 minutes after IP delivery of either vehicle or CGRP. Animals were gently handled so anesthesia is not needed during injections. Animal procedures were approved by the University of Rochester's IACUC committee, performed in accordance with the standards set by the NIH.

Behavioral Tests of Sound Hypersensitivity:

To assess behavioral hyperacusis, or heightened sound sensitivity, we made use of the acoustic startle response (ASR) and the ability of prepulses to inhibit the ASR [i.e., prepulse inhibition (PPI)]. PPI can be tested in quiet or with pulses embedded in masking noise (Bogart LJ et al., 2011)]. Experiments were conducted within a sound-attenuating room (IAC, Bronx, NY) with Sonex foam lined walls. Mice were placed individually in a wire-mesh cage, 5 cm wide, 7 cm long, and 4 cm high, having free sound penetration. The testing cage was oriented so that the mouse's head faced a Tucker Davis ES2 electrostatic speaker (Prepulse Speaker) located 46 cm directly in front of the mouse's head (prepulses 30-80dB). A second ES2 speaker (Masker Speaker) was positioned 7 degrees to the left of the Prepulse Speaker, and this broadcast the masker signal when it was present (60dB). The startle speaker was a Radio Shack tweeter that was suspended 15 cm above the mouse (130 dB). The acoustic startle speaker and its supports, the pedestal, acrylic shelf, and the table on which the apparatus was placed were all covered with echo absorbing foam and carpeting. Force of the acoustic startle response (ASR) was transduced by an accelerometer and amplitude scored as integrated root mean square voltage for 100 ms after delivery of the ASR. Experimental stimuli were controlled, and responses were recorded using custom MATLAB (MathWorks) software. To avoid potential skewing

effects of outliers on means, we used a nonparametric measure of the prepulse effect rather than the simple ratio of mean response magnitudes in prepulse and control conditions, typically reported as %PPI. PPI (A') is the area under the receiver operating characteristic curve and is a measure of overlap of two distributions; it is insensitive to the precise nature of their underlying distributions. Scores ranged from 50% for completely overlapping distributions of prepulse and control conditions to 100% for completely non-overlapping distributions. Each animal was only tested for 45 min/day (one series of acoustic startle/day. We tested acoustic startle series in quiet and in noise, There was no prepulse in these sessions, which were designed to characterize the startle response of each mouse. There were 12 conditions in this session with startle stimuli ranging from 80 to 130 dB SPL, and delivered in silence or in a continuous background noise, which, when present, was on for the duration of the inter-trial interval and broadcast from the Masker Speaker. We also tested PPI in quiet, in this instance the prepulse was a 40 ms duration broadband noise burst with 15ms linear-gated rise-fall time, broadcast from the Prepulse Speaker. The interstimulus interval was 100ms and the startle level was 120 dB SPL. There were 12 conditions in this session; prepulses 30 to 75 dB SPL in 5 dB steps, and two no-prepulse control conditions. We also tested PPI in noise, and in this instance the prepulse was a 40ms duration broadband noise burst with 15ms linear-gated rise-fall time, broadcast from the Prepulse Speaker. A continuous broadband 60 dB SPL masker was played from the Masker Speaker for the duration of the session. The interstimulus interval was 100ms and the startle level was 120 dB SPL. There were 12 conditions in this session; prepulses 48 to 75 dB SPL in 3 dB steps (-12 to +15 dB S/N), and two no-prepulse control conditions.

Vestibular Challenge - Off-Vertical Axis Rotation (OVAR): For rotarod testing, off-vertical axis rotation (OVAR) was used as the vestibular challenge. The use of OVAR as a vestibular stimulus is evidence based; prior human and rodent studies have used OVAR to test the otolith-ocular reflex and assess semicircular canal-otolith interaction (Beraneck M et al., 2012;Furman JM et al., 1992;Hess BJM and Dieringer N, 1990). Constant velocity OVAR at a tilt can be disorienting and promote motion sickness in human participants (Dai M et al., 2003;Dai M et al., 2010), and further studies in mice have shown that provocative rotation leads to kaolin consumption – a behavioral marker for illness - and observations of urination, piloerection, and tremor (Idoux E et al., 2018). In

this study, a two-cage rotator (cage dimensions: 12.7 cm x 8.9 cm) was built to impose off-vertical axis rotation (60 rpm, 45° tilt from the vertical) as a vestibular stimulus onto mice during motion-sickness testing. The rotator tests two mice at a time, and mice are secured 22 cm from the axis of rotation. When one mouse is tested, an object of equivalent weight is placed into the other cage for balancing before rotation.

Vestibular Challenge – Orbital Rotation: A steady orbital rotation was used as the vestibular challenge during postural sway testing. Mice were placed in a cage fastened to the surface of an orbital shaker with a built-in orbital radius of 2 cm. During testing, animals were rotated at a constant 125 rpm for 5 minutes. Based on previous studies, this stimulus is believed to mainly activate the semicircular canals – with minimal involvement of the otoliths – due to similar studies in mice where rotation along a vertical axis at constant frequency causes eye movements evoked by angular stimulation of the canals (Idoux E et al., 2018).

Rotarod testing for balance: Dynamic balance was assessed with the Rotarod (Columbus Instruments) configured with a mouse dowel (radius = 3 cm). The machine is designed by the manufacturer to test four mice at a given time. Mice were tasked to maintain balance on the rotating dowel that accelerated from 5 to 44 rpm at an acceleration step of 2.4 rpm every 4 seconds. Latency to fall (LTF) is measured when mice fall off the rotating dowel. Prior to testing, animals are given a day to train on the rotarod. The training period requires the animals to undergo 6-8 trials, with the expectation that the animal learns to stay on the rod longer as the dowel rotates to the maximum set speed. On days of testing, mice were first trained 2-4 times to reinforce training memory. Mice were then given 20 minutes rest before we tested the mice for three trials to represent the animal's baseline LTFs. The mice then experienced off-vertical axis rotation (OVAR) as a vestibular challenge for 30 seconds, and another three measurements were taken after OVAR sequentially. Approximately 15-30 seconds pass after OVAR stimulation and before each mice conducted its first trial after the vestibular challenge.

Rotarod Experimental Design: Mice were trained and assessed for effects of CGRP and vestibular challenge on rotarod ability. For this study, off-vertical axis rotation (60 rpm at 45°tilt) was used as the vestibular challenge. No mice were excluded in this assay.

Training: Prior to assessing the effects of CGRP and the vestibular challenge on rotarod ability, mice (10M/10F) were given a day to exclusively train on rotarod. Mice were tested for 8 training trials. After a 15 minutes rest, mice were then assessed again for three test trials to enforce memory.

Vestibular challenge (VC) assessment: One day after training, mice were evaluated for the effects of the VC – off-vertical axis rotation (OVAR) - after treatment of vehicle control. Mice were first briefly trained for 2 to 4 trials to regain memory. Mice were then tested for 3 trials (vehicle/pre-VC) approximately 20 minutes after administration of IP vehicle. After obtaining pre-VC measurements, mice were challenged with OVAR for 30 seconds and were then placed on the rotarod for 3 additional trials (vehicle/post-VC).

Effects of CGRP and VC: A Day after vehicle control testing, the same mice were assessed for their rotarod ability after i) systemic delivery of CGRP and ii) the effects of VC after CGRP delivery. Mice were briefly trained and were then administered IP CGRP. After CGRP administration, mice were tested for three trials prior to the VC (CGRP/pre-VC). Mice were then challenged with OVAR and were then assessed on the rotarod for 3 additional trials (CGRP/post-VC). Testing occurred the day after vehicle control experiments.

<u>Center of Pressure (CoP) Testing for Postural Sway:</u> Postural sway was measured as a correlate for static balance. Mice were weighed and placed on a force plate designed to measure a mouse's forces in the X, Y, Z and its moments in the XY, YZ, and XZ directions. We used the AMTI Biomechanics Force platform (model HEX6x6) that comes with AMTI automated acquisition software. During experimentation of a given mouse, the CoP area was calculated from the relative output of four vertical sensors, and changes in CoP can be used to measure postural sway of the mouse. A modified MATLAB code was used to analyze CoP areas, and the code generated a 95% confidence ellipse enclosing 95% of the CoP trajectory values recorded in a single trial. The computed 95% confidence ellipse's area indicated the amount of sway exhibited by a mouse on a particular trial. The CoP test shows high sensitivity and high reliability when testing the same animal repeatedly.

During a given test, a mouse was first allowed to freely maneuver on the force plate for 2 minutes to acclimate to the novel environment. An accessory plexiglass cover is placed over the force plate to prevent animals from moving off the test plate. After acclimation, 8 to 10 baseline measurements were

taken of the mouse with each measurement indicating a CoP area (resolution per CoP measurement: 300 samples per second). These measurements were taken when the mouse showed no movement and its four paws were touching the surface of the force plate, as we were not focused on studying postural changes when the animal was standing, grooming, or doing additional behaviors. The mouse was then subjected to the VC for 5 minutes and was then placed back onto the AMTI Force platform. Five minutes after placement of the mouse, an additional 10 measurements are recorded of the mouse's sway while it was still and not moving.

Postural Sway Experimental Design: A group of mice were used to assess test-retest differences in center of pressure (CoP) area without the vestibular challenge (VC). A different group of mice were used to assess the effect of CGRP and vestibular challenge on changes in postural sway. We analyzed three aspects of postural sway in mice treated with CGRP and the VC: i) the CoP area (cm^2), ii) the major axis (cm) which corresponds to anteroposterior changes, and iii) the minor axis (cm) which corresponds to mediolateral changes. The VC used in this assay was the orbital rotation at 125 rpm for 5 minutes. Prior to mouse testing, we measured the CoP area of a dead weight (toy mouse) for comparison with live mice. **Test-retest**: Mice (10M/10F) were evaluated for test-retest differences in CoP area after systemic administration of vehicle control. Mice were administered IP vehicle 25 minutes prior to postural sway testing. Testing occurred 4 days apart. **CGRP and VC's effects**: A total of 19 mice (9M/10F) were used to assess the effects of CGRP and VC on postural sway. Mice were first tested after systemic delivery of IP vehicle, and postural sway was measured before and after the vestibular challenge. Four days after vehicle control testing, mice were treated with IP CGRP and were tested again 20 minutes after IP administration. In certain testing conditions, mice exhibited unreasonably large detected CoP values (> 15 cm^2) that suggested that they were either moving during the time of measurement or that they were at the edge of the force plate. When mice were at the edge of the force plate, they tended to place their tail in between the force plate and the accessory plexiglass cage and would lean on the plexiglass cage; these behaviors and subsequent movements can over-exaggerate the recorded CoP areas. While we tried to minimize this behavior, certain mice gravitated to the corners as an anxiety behavior and this behavior could not be readily changed during testing. As such, we excluded any mice that exhibited these behaviors during

each testing condition. Two females with IP vehicle (pre and post vestibular challenge), two males with IP vehicle (pre and post vestibular challenge), and 2 males with IP CGRP (post vestibular challenge), were excluded from the study's analysis.

Power Analysis:

The number of each sex needed is predicted to be 4 animals based on power analysis using an alpha of 0.05, with 95% power and effect size d=10.58; based on findings from n=10 mice with ASR/ PPI testing (Bogart LJ,Levy AD,Gladstone M,Allen PD,Zettel M,Ison JR,Luebke AE and Majewska AK, 2011). The number per sex for postural sway was predicted to be three based on power analysis using an alpha of 0.05, with 95% power (Cohen d's effect size =5.0); based on findings from n=10 mice with CoP testing (46). The number per sex needed for rotarod is four based on power analysis using an alpha of 0.05, with 95% power and Cohen d's effect size =2.68 (based on findings from n=10 control mice with rotarod testing (31)). For postural sway and rotarod testing, we were overpowered.

Statistical Analysis:

For acoustic startle response (ASR) testing, startle stimulus was measured in response to brief noise bursts, delivered in quiet or in background noise. We determined a startle threshold and an optimum level for maximal startle reflex for both vehicle and CGRP injected animals. All animals showed prepulse inhibition of the startle response (PPI) when prepulses were presented in quiet or in the presence of a continuous 60dB SPL broadband masker. We converted the raw startle data to an A' metric where 0.5 indicates no inhibition of startle and 1.0 is complete inhibition. 50%. For PPI testing, we determined target reception thresholds (signal-to-noise ratio (SNR) that gives A'=. 75 or 50% inhibition).

For postural sway testing, minimums of 8 different 95% ellipse areas were measured per mouse and an average ellipse area was computed per mouse. Then data from each mouse was grouped based on testing condition, and a group average 95% ellipse area ± standard error of the mean (SE) was determined. Rotarod data was analyzed by first determining – in each animal - the MAX latency to fall (LTF) value from the three trials assessed per testing condition. Then max LTF values were grouped and an average max LTF ± SE was computed. Statistical analyses were conducted in GraphPad Prism 9.0. Repeated measure ANOVA (RM-ANOVA) was the primary statistical tool, but

mixed-effects (ME) models were the statistical alternative when comparisons included groups with excluded mice. Greenhouse-Geisser corrections were applied to ME models where sphericity could not be assumed. Bonferroni and Tukey multiple comparisons test were used to assess for differences between treatment groups for rotarod and postural sway respectively. For post-hoc comparisons, the mean ± standard error of the difference is represented as an absolute value, and is indicated by mean ± SE (absolute difference). Significance was set at $p < 0.05$ for all analyses and exact p-values are reported except for comparisons where $p < 0.0001$ or $p > 0.999$.

Results:

CGRP's effect on sound sensitivity

Our sound sensitivity measure in the mouse model makes use of the acoustic startle response (ASR) in quiet and in background noise, and the ability of prepulses to inhibit the ASR [ie, prepulse inhibition (PPI)] to assess the mouse's ability to detect prepulse signals presented in quiet. Mice were assessed for the effects of IP CGRP as compared to IP vehicle on their baseline ASR as outlined in **Fig. 1 A** (quiet) and **Fig. 1E** (background noise 60 dB). In both vehicle and CGRP injected animals there was an increase in ASR with increasing loudness of the startle stimulus (ES). Performing such a startle series in quiet and masking noise with vehicle injections can also serve as an exclusion criterion as the startle response should increase as the ES level increases, and if this is not the case then the animal does not have a robust startle response and should be excluded. All animals tested exhibited robust startle responses. When ASR was tested after systemic CGRP injection and compared to vehicle injected mice, all animals exhibited reduced ASR thresholds showing that CGRP significantly increased sound sensitivity to the startle stimulus in quiet (**Fig. 1 B,C,D**) and in background noise (**Fig. 1 F,G,H**).

We also assayed the animals' ability to use a brief prepulse of broadband noise (BBN, 2–100 kHz) to inhibit the acoustic startle (120 dB BBN) when mice were injected with vehicle or CGRP. These tests were performed in quiet (schematized in Fig. 2A) and each animal's inhibition of the ASR was tested over 20 times in each test condition. A non-parametric measure A′ was defined as 1.0 if the PPI is completely separable from the no-prepulse control condition and varied to 0.50 which is defined as not separable from the control condition. Converting the ASR values to A′ measures allowed for

normalization across animals. All animals with vehicle injection were able to inhibit ASR to at least 50% (A′ = 0.75) with BBN prepulses, and this sensitivity increased (reduced PPI thresholds-50% PPI level (A′ = 0.75)) when mice were injected with CGRP, as shown in Fig. 2 B,C,D. Female mice thresholds were more affected by CGRP (10dB), as shown in Fig. 2B as compared with 3dB increased sound sensitivity observed for male mice (Fig. 2C) difference for male mice, with sexes combined shown in Fig. 2D (5dB difference). Because the 50% PPI measure is a logarithmic measure (dB) a difference of 3-10 dB is a significant threshold difference.

CGRP and VC's effects on rotarod ability

Mice were assessed for the effects of IP CGRP and the vestibular challenge (VC) on rotarod ability after the training day. The vestibular challenge used in this assay was off-vertical axis rotation (OVAR) and involved rotating mice at 60 rpm at a 45° tilt for 30 seconds in a two-cage rotator **(Fig. 3A).** Cages were placed 22 cm from the axis of rotation. As a measure of a mouse's best trial per testing condition, the maximum latency to fall (LTF) of the three trials conducted per mouse was selected, and group max LTFs were analyzed. Repeated-measures ANOVA (RM-ANOVA) was used to assess the factors of sex and IP CGRP compared to IP vehicle (saline) in-group max LTFs (**Table 1**). While a significant difference was seen with IP CGRP's effects ($F = 28.3$, $p < 0.0001$), biological sex was not observed to impact rotarod ability. A before-after plot was used to depict changes in group max LTFs due to IP CGRP during pre-VC testing, and Bonferroni multiple comparisons tests showed that IP CGRP impacted rotarod ability by reducing max LTFs in females (mean ± $SE_{\text{abs. difference}} = 9.02 \pm 2.93$ s, adj. $p = 0.01$) and in males (mean ± $SE_{\text{abs. difference}} = 12.99 \pm 2.93$ s, adj. $p = 0.005$) **(Fig. 3B)**. When males and females were pooled together, the RM-ANOVA showed that IP CGRP's effects impacted rotarod ability ($F = 25.2$, $p < 0.0001$) but the VC did not have an impact. A violin plot was used to show the effects of IP CGRP on rotarod testing in all mice before and after the use of OVAR as a vestibular challenge (Fig. 3C). Bonferroni post-hoc analysis showed that compared to vehicle control, IP CGRP greatly impacted rotarod ability by reducing MAX LTFs during pre-VC (mean ± $SEM_{\text{abs. difference}} = 11.01 \pm 2.07$ s, adj. . $p = 0.0002$) and post-VC (mean ± $SEM_{\text{abs. difference}} = 8.41 \pm 2.62$ s, adj. $p = 0.03$) testing.

CGRP's and VC's effects on Postural Sway

Mice were assessed for center of pressure (CoP) 95% ellipse areas on a force platform and the experimental timeline is shown in **Fig. 4A**. Twenty minutes after delivery of IP vehicle and without a vestibular challenge (VC), group averages of 95% ellipse areas were computed to be 0.5 ± 0.06 cm^2 for females and 0.4 ± 0.04 cm^2 for males **(Fig. 4B and C).** For comparison, a deadweight (toy mouse) was also measured on the force platform, and its average 95% ellipse area was computed to be 0.02 ± 0.0005 cm^2 **(Fig. 4D).** A different cohort of male and female mice was assessed for a test-retest of 95% ellipse areas after IP vehicle delivery, and testing occurred four days apart. No differences were seen in either sex during the test-retest. A mixed-effect model was used to assess the factors of sex, CGRP, and vestibular challenge on 95% ellipse areas and is graphically depicted via a violin plot **(Table 2, Fig. 4E).** Significant differences were seen across all three factors: sex ($F = 22.5$, $p = 0.002$), CGRP's effects ($F = 7.1$, $p = 0.02$), and VC's effects ($F = 5.1$, $p = 0.04$). Tukey post-hoc analysis was used to observe differences in specific comparisons across the three factors. No changes in 95% ellipse areas were observed due to the vestibular challenge in either treatment. However, in females when compared to their vehicle control, a significant increase in the 95% ellipse area was observed in pre-VC values after IP CGRP injection (mean ± $SE_{abs.\ difference}$ = 0.94 ± 0.23 cm^2, adj. $p = 0.046$) as seen in the before-after plot **(Fig. 4F).** This observation did not occur in male mice. When looking at sex differences, sex differences were not observed after delivery of IP vehicle. However, after IP CGRP, females had higher 95% ellipse areas compared to males during the pre-VC test (mean ± $SE_{abs.\ difference}$ = 1.01 ± 0.19 cm^2, adj. $p = 0.006$), and this observation was also seen in the post-VC test (mean ± $SE_{abs.\ difference =}$ 1.50 ± 0.37 cm^2, adj. $p = 0.03$).

Major and Minor Axes Changes

The major and minor axes of 95% ellipse areas were also analyzed with mixed-effects models and post-hoc analysis through Tukey's multiple comparisons test **(Fig. 4G and H).** The major and minor axes act as correlates for anteroposterior and mediolateral sway directions, respectively, in this mouse model. In females prior to the VC, major axis lengths increased after IP CGRP compared to their vehicle control response (mean ± $SE_{abs.\ difference}$ = 0.60 + 0.13 cm, adj. $p = 0.03$). Additionally, female mice exhibited higher major axes lengths during pre-VC testing compared to males tested with IP

CGRP (mean ± SE $_{abs.\ difference}$ = 0.57 + 0.12 cm, adj. p = 0.003). When observing minor axes, females exhibited higher minor axes lengths than males during pre-VC testing (mean ± SE $_{abs.\ difference}$ = 0.13 + 0.03 cm, adj. p = 0.03) and post-VC testing (mean ± SE $_{abs.\ difference}$ = 0.21 + 0.05 cm, adj. p = 0.008). No other significant changes were observed due to CGRP or the vestibular challenge on major and minor axes.

Discussion:

We found that systemic CGRP injection increased both sound sensitivity (as measured using the acoustic startle reflex (ASR) and pre-pulse inhibition (PPI)) and motion sensitivity (as measured by rotarod and postural sway) in wildtype C57B6/J mice. These assays pave the way to tease out the effects of CGRP signaling on migraine and VM symptoms. Several studies in animal models have provided additional evidence for CGRP's role in sound sensitivity and vestibular dysfunction. Intracerebral-ventricular (ICV) injection of nitroglycerin (NTG) caused impaired balance beam walking, hyperalgesia, and allodynia in rats, and immunohistochemistry detected more CGRP expression in the vestibular nucleus of the NTG rat model than was observed in control rats. The administration of a CGRP-receptor antagonist (BIBN4096BS) was shown to be effective in alleviating these symptoms of vestibular dysfunction and allodynia in this same NTG rat model (Tian R et al., 2022;Zhang Y et al., 2020). In addition, a recent study suggests CGRP has an excitatory role in the auditory nerve response, as CGRP infusion was shown to enhance spontaneous and sound-driven auditory nerve activity in guinea pigs, in comparison to CGRP-null mice where a loss of excitation is observed (Allen PD and Luebke AE, 2017;Le Prell CG et al., 2021). Thus, the auditory nerve may be sensitized to CGRP's effects during a migranous state. Clinical studies on effectiveness of CGRP monoclonal antibodies are underway for VM, using the newly developed VM-PATHI instrument to score outcome measures (Sharon JD et al., 2020). A recent case review has found that 60% of patients with VM have their VM symptoms moderately relieved with CGRP signaling blockers (Hoskin JL and Fife TD, 2022).

We did discover a robust sex difference when assessing for phonophobia of migraine (using PPI) and static imbalance, as female mice showed both increased sensitivity to sound after CGRP injection then their male counterparts; and greater postural sway after CGRP injection and after the

vestibular challenge than observed in male mice. This finding is important and clinically relevant as vestibular migraine (and other vestibular conditions like benign paroxysmal positional vertigo (BPPV), motion sickness, and unspecific vertigo) disproportionally affect more women than men (Dieterich M et al., 2016;Smith PF et al., 2019), and women make up a large majority of clinical trial participants for anti-CGRP treatments (Caronna E et al., 2021;Huang IH et al., 2019). This sexual dimorphism has been explored in other animal assays assessing CGRP's effects in migraine but we are the first to show this striking difference in preclinical correlates for phonophobia and static imbalance (Paige C et al., 2022;Recober A et al., 2009).

Future studies will determine if blocking CGRP receptor signaling by ‘gepants’ or CGRP based antibody treatments can reduce these VM symptoms, and if CGRP signaling blockade as compared to triptan therapy will be effective against sound sensitivities and static and dynamic imbalance sensitivities present in VM.

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Fig. 1:

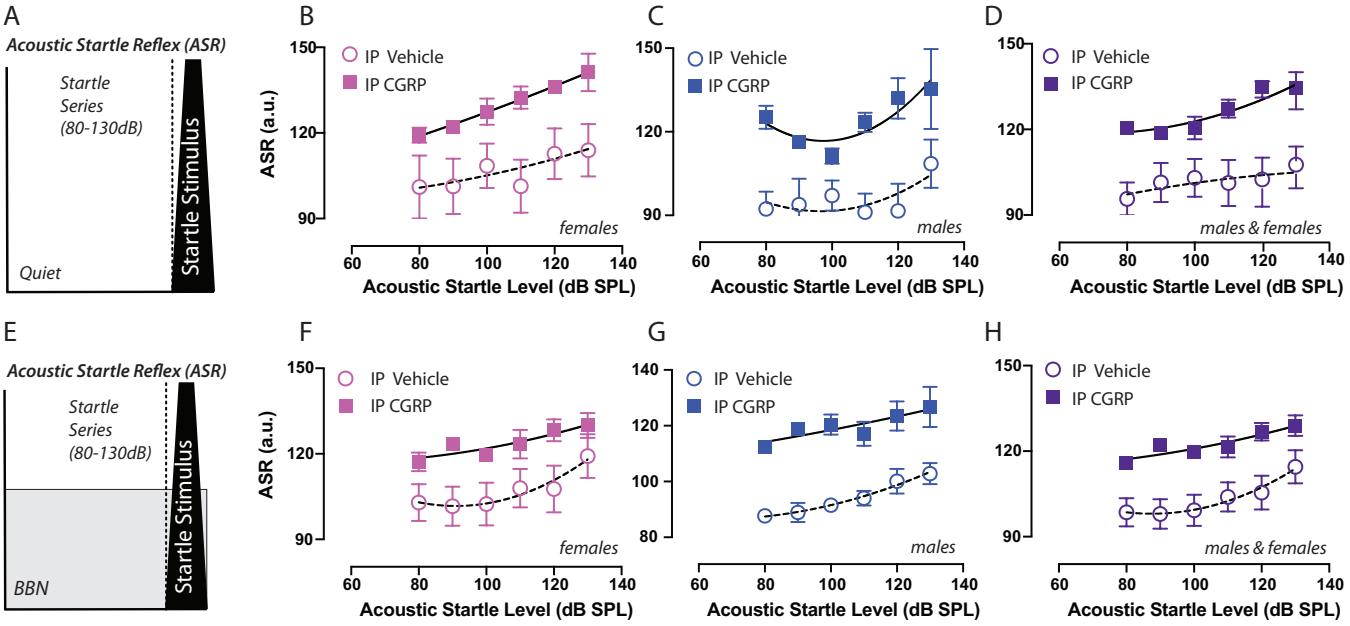
A
Acoustic Startle Reflex (ASR)
Startle Series (80-130dB)
Startle Stimulus
Quiet
B
IP Vehicle
IP CGRP
ASR (a.u.)
150
120
90
females
60
80
100
120
140
Acoustic Startle Level (dB SPL)
C
IP Vehicle
IP CGRP
150
120
90
males
Acoustic Startle Level (dB SPL)
D
IP Vehicle
IP CGRP
150
120
90
males & females
Acoustic Startle Level (dB SPL)
E
Acoustic Startle Reflex (ASR)
Startle Series (80-130dB)
Startle Stimulus
BBN
F
IP Vehicle
IP CGRP
ASR (a.u.)
150
120
90
females
Acoustic Startle Level (dB SPL)
G
IP Vehicle
IP CGRP
140
120
100
80
males
Acoustic Startle Level (dB SPL)
H
IP Vehicle
IP CGRP
150
120
90
males & females
Acoustic Startle Level (dB SPL)

Fig. 2:

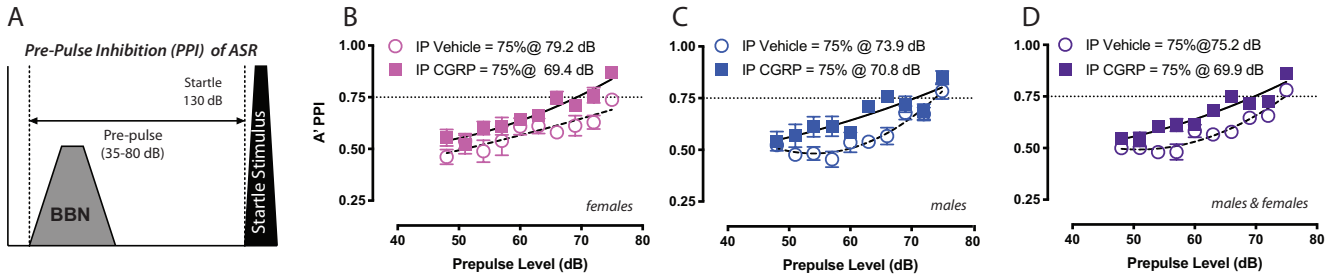
A
Pre-Pulse Inhibition (PPI) of ASR
Startle
130 dB
Pre-pulse
(35-80 dB)
BBN
Startle Stimulus
B
IP Vehicle = 75%@ 79.2 dB
IP CGRP = 75%@ 69.4 dB
A' PPI
1.00
0.75
0.50
0.25
females
40
50
60
70
80
Prepulse Level (dB)
C
IP Vehicle = 75% @ 73.9 dB
IP CGRP = 75% @ 70.8 dB
males
Prepulse Level (dB)
D
IP Vehicle = 75%@75.2 dB
IP CGRP = 75% @ 69.9 dB
males & females
Prepulse Level (dB)

Fig. 3:

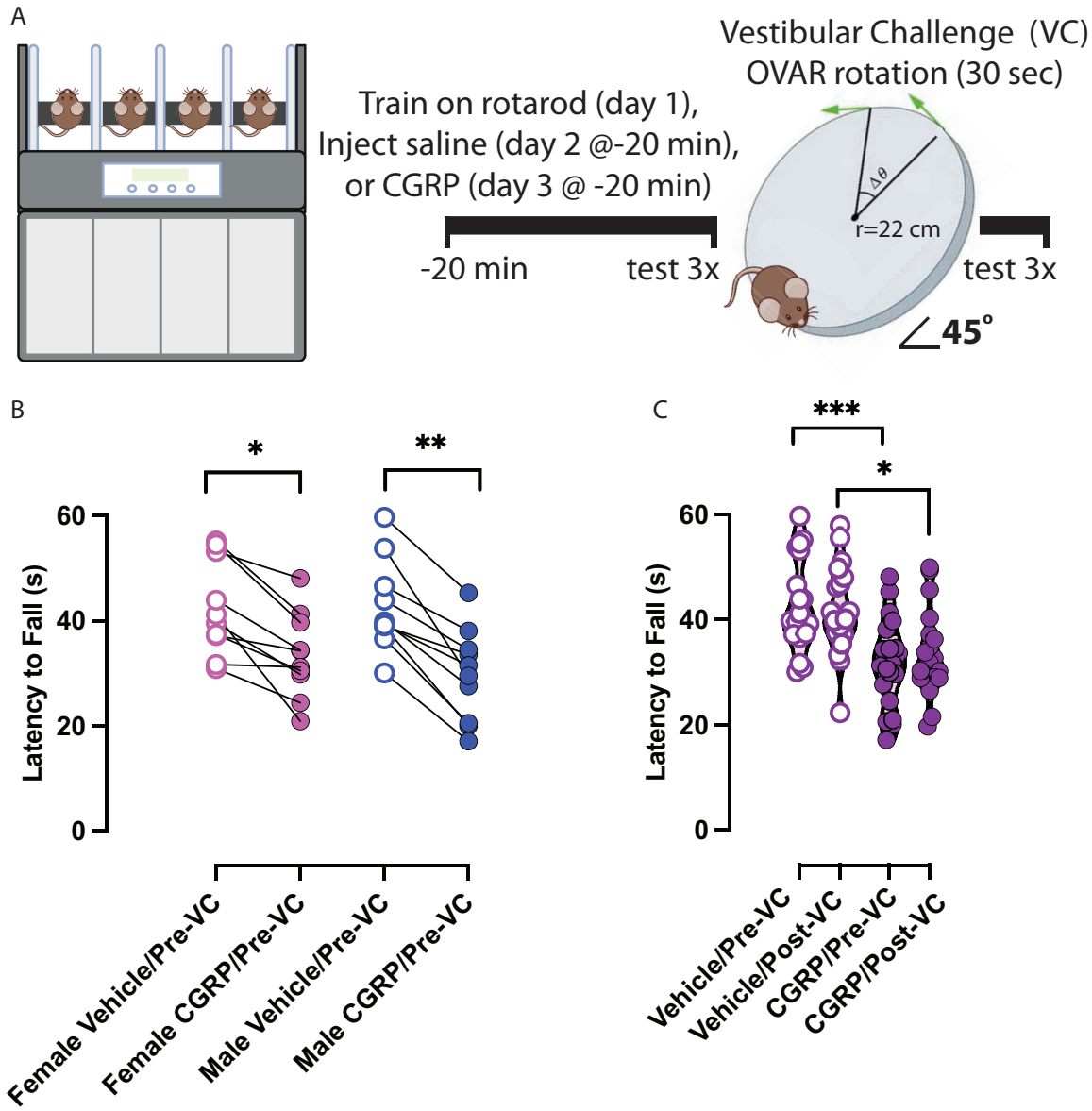
A
Train on rotarod (day 1),
Inject saline (day 2 @-20 min),
or CGRP (day 3 @ -20 min)
-20 min
test 3x
Vestibular Challenge (VC)
OVAR rotation (30 sec)
r=22 cm
test 3x
45°
B
Latency to Fall (s)
60
40
20
0
*
**
Female Vehicle/Pre-VC
Female CGRP/Pre-VC
Male Vehicle/Pre-VC
Male CGRP/Pre-VC
C
Latency to Fall (s)
60
40
20
0
***
*
Vehicle/Pre-VC
Vehicle/Post-VC
CGRP/Pre-VC
CGRP/Post-VC

Fig. 4:

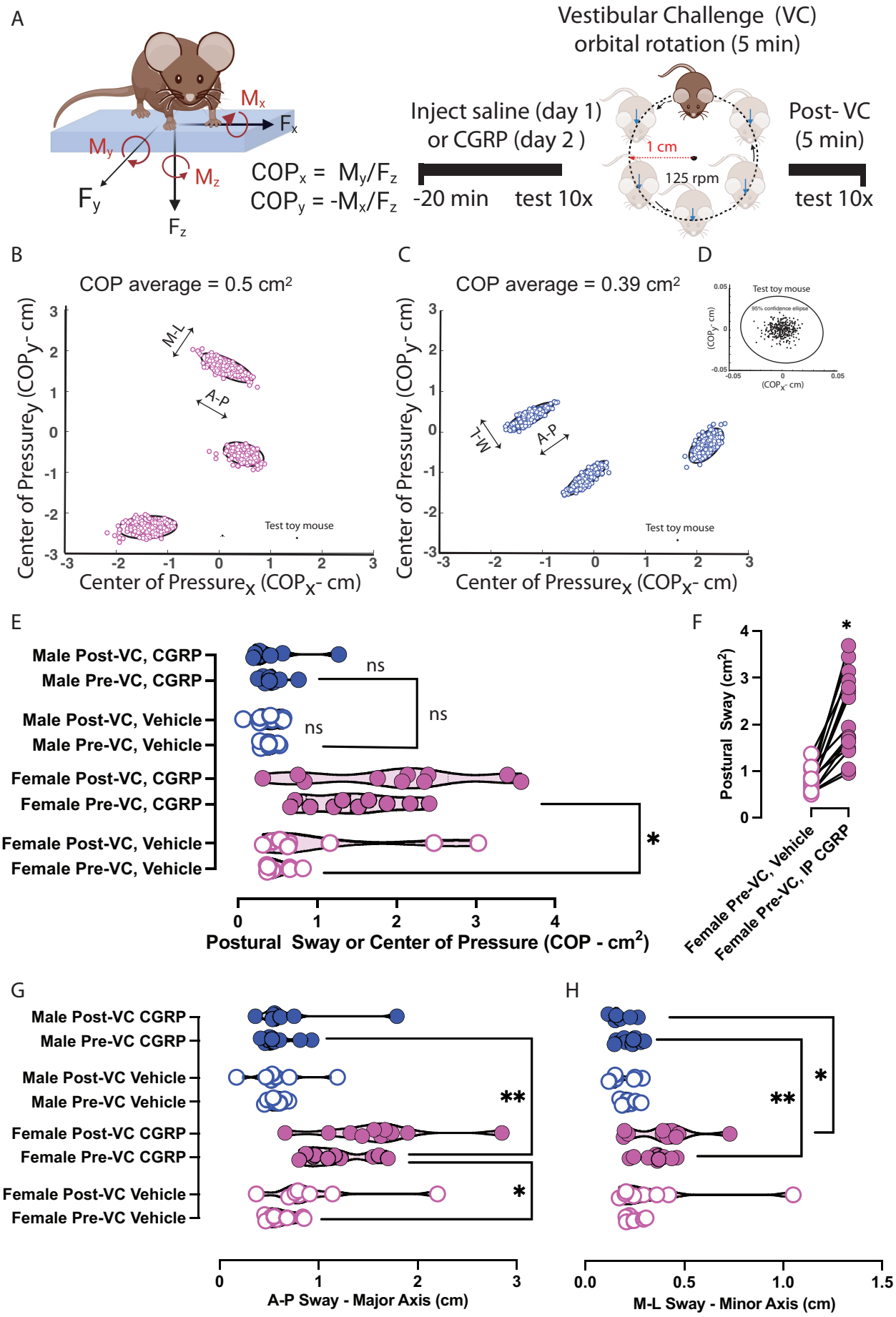
A
Vestibular Challenge (VC)
orbital rotation (5 min)
Inject saline (day 1)
or CGRP (day 2)
-20 min
test 10x
1 cm
125 rpm
Post- VC
(5 min)
test 10x
M_x
F_x
M_y
M_z
F_y
F_z
$COP_x = M_y/F_z$
$COP_y = -M_x/F_z$
B
COP average = 0.5 cm²
M-L
A-P
Test toy mouse
Center of Pressure$_y$ (COP$_y$- cm)
Center of Pressure$_x$ (COP$_x$- cm)
C
COP average = 0.39 cm²
M-L
A-P
Test toy mouse
Center of Pressure$_y$ (COP$_y$- cm)
Center of Pressure$_x$ (COP$_x$- cm)
D
Test toy mouse
95% confidence ellipse
(COP$_y$- cm)
(COP$_x$- cm)
E
Male Post-VC, CGRP
Male Pre-VC, CGRP
Male Post-VC, Vehicle
Male Pre-VC, Vehicle
Female Post-VC, CGRP
Female Pre-VC, CGRP
Female Post-VC, Vehicle
Female Pre-VC, Vehicle
ns
ns
ns
*
Postural Sway or Center of Pressure (COP - cm²)
F
Postural Sway (cm²)
*
Female Pre-VC, Vehicle
Female Pre-VC, IP CGRP
G
Male Post-VC CGRP
Male Pre-VC CGRP
Male Post-VC Vehicle
Male Pre-VC Vehicle
Female Post-VC CGRP
Female Pre-VC CGRP
Female Post-VC Vehicle
Female Pre-VC Vehicle
**
*
A-P Sway - Major Axis (cm)
H
*
**
M-L Sway - Minor Axis (cm)

Figure Legends:

Fig. 1: Systemic CGRP increases Acoustic Startle Responses (ASR) in both quiet and background noise, denoting increased sound sensitivity. A. Schematic of startle stimulus presented in otherwise quiet background conditions. **B.C. D**. For female mice (**B**, n=10), male mice (**C**, n=10) and both sexes combined (**D**, n=20), there were significant differences between vehicle and CGRP ASR measures in quiet conditions. All mice were more sensitive to the same sound level of ASR stimulus after being injected with CGRP. **E.** Schematic of startle stimulus embedded in continuous 60dB SPL broadband background noise. **F.G. H**. Again, ASR embedded in noise was also more sensitive to same sound level after CGRP injection as shown for female mice (F, n=10), male mice (G, n=10) and both sexes combined (H, n=20), there were significant differences between vehicle and CGRP injected animals in ASR in background noise conditions. All mice were more sensitive to the same level of ASR stimulus whether delivered in quiet or in BBN after being injected with CGRP. Moreover, all mice show ASR increases with sound level from 80 to 130dB SPL with near maximal startle is elicited by 130dB SPL stimulus levels. Error bars are SEMs.

Fig. 2: Systemic CGRP reduces Prepulse Inhibition (PPI) thresholds denoting increased sound sensitivity. **A**. Schematic of Prepulse Inhibition (PPI) stimulus presented in otherwise quiet background conditions. **B**. Female mice (n=10) showed a ~10dB threshold difference when injected with CGRP as compared to vehicle condition showing increased sensitivity to prepulse sound with CGRP injection. .**C**. Male mice (n=10) were also affected but to a lesser extent (3.1 dB versus 10 dB) and when both sexes (n=20) are pooled the aggregate difference in prepulse sensitivity is ~ 5dB. In all instances, the threshold for inhibiting their startle response 50% of the time (A'=0.75) is higher at for mice injected with vehicle then for the same mice injected with CGRP. Error bars are SEMs.

Fig. 3: CGRP and VC's effects on rotarod. Female (pink) and male (blue) C57B6J mice were tested for rotarod ability after training on rotarod manufactured by Columbus Instruments. Purple is used to highlight comparisons with all mice. Treatments were delivered intraperitoneally (IP) and are graphically depicted in the following manner: vehicle control (open circle), CGRP (closed circle). **(A)** The assay

timeline is illustrated and involved mice being trained on day 1 followed by rotarod testing after delivery of IP vehicle control (saline) or IP CGRP on day 2 or day 3 respectively. Mice were stimulated with off-vertical axis stimulation (OVAR) as the vestibular challenge. **(B)** The before-after plot indicates IP CGRP negatively impacted rotarod ability in females and males during pre-VC testing. Females and males experienced a decrease of 9.02 ± 2.93 s and 12.99-± 2.93 s in max LTF values respectively (adj. p = 0.01 and <0.001). **(C)** The violin plot depicts IP CGRP's effects on rotarod in all mice before and after the use of OVAR. Bonferroni post-hoc analysis showed that compared to vehicle control, IP CGRP greatly impacted rotarod ability by reducing max LTFs by 11.01 ± 2.07 s during pre-VC testing and by 8.41 ± 2.62 s during post-VC testing (adj. p <0.001 and 0.03 respectively).

Fig. 4: CGRP and VC's effects on postural sway. Female (pink) and male (blue) C57B6J mice were tested for postural sway after training on the AMTI Biomechanics Force platform (model HEX6x6). Figures are graphically depicted in the following manner: vehicle control (open circle), CGRP (closed circle). (A) The assay timeline involved mice being assessed for effects of IP vehicle control (saline) or IP CGRP on day 1 or day 2 respectively. A 5-minute orbital rotation (125 rpm for 5 minutes, orbital radius = 1 cm) was used as the vestibular challenge. **(B and C)** Group 95% ellipse areas during pre-VC testing were computed to be 0.50 ± 0.06 cm^2 for females and 0.39 ± 0.04 cm^2 for males. **(D)** A toy mouse was measured to be 0.02 ± 0.0005 cm^2 for comparison with live animals. **(E)** Violin plot graphically depicts the distribution of average 95% ellipse areas across sex and treatment and Tukey post-hoc analysis was used to determine significant differences. **(F)** Before-after plot shows during pre-VC testing, females increased their 95% ellipse areas by 0.94 ± 0.23 cm^2 when treated with IP CGRP compared to their IP vehicle response (adj. p = 0.046). **(G)** During pre-VC testing, females experienced increased major axis lengths by 0.60 ± 0.13 cm after IP CGRP compared to their vehicle control (adj. p = 0.03). Additionally, during pre-VC testing, female mice exhibited 0.57 ± 0.12 cm higher major axes lengths compared to males also given IP CGRP (adj. p = 0.003). **(H)** Minor axes lengths were significantly higher in females than males by 0.13 ± 0.03 cm during pre-VC testing and by 0.21 ± 0.05 cm during post-VC testing (adj. p = 0.03 and 0.008 respectively).

Table 1:

Rotarod, MAX Latency to Fall (s) - Repeated Measures (RM) ANOVA								
Tabular Results				**Multiple Comparisons (Bonferroni)**				
Factors	**P-val**	**F (DF_n, DF_d)**	**$\hat{\varepsilon}$**	**Comparison (T1 vs T2)**	**N_{T1}**	**N_{T2}**	**Mean Diff.**	**Adj. P-val**
CGRP	<0.0001	F (1, 18) = 28.26	NA	Pre-VC:Female Vehicle vs Pre-VC: Female CGRP	10	10	9.02	0.010
				Pre-VC: Male Vehicle vs. Pre-VC: Male CGRP	10	10	12.99	0.005
Sex	0.608	F (1, 18) = 0.27	NA	Pre-VC: Female Vehicle vs. Pre-VC: Male Vehicle	10	10	-0.28	> 0.999
				Pre-VC: Female CGRP vs. Pre-VC: Male CGRP	10	10	3.69	0.690
$CGRP_{SC}$	<0.0001	F (1, 19) = 25.18	1.0	Pre-VC: Vehicle vs. Post-VC: Vehicle	20	20	-0.91	> 0.999
				Pre-VC: Vehicle vs. Pre-VC: CGRP	20	20	-11.01	0.0002
VC_{SC}	0.6856	F (1, 19) = 0.17	1.0	Pre-VC: CGRP vs. Post-VC: CGRP	20	20	1.69	> 0.999
				Post-VC: Vehicle vs. Post-VC: CGRP	20	20	-8.41	0.030

Table 2:

Postural Sway, Center of Pressure (CoP, cm^2) - Mixed Effects (ME) Model								
Tabular Results				**Multiple Comparisons (Tukey)**				
Factors	**P-val**	**F (DF_n, DF_d)**	**$\hat{\varepsilon}$**	**Comparison (T1 vs T2)**	**N_{T1}**	**N_{T2}**	**Mean Diff.**	**Adj. P-val**
CGRP	0.02	F (1, 17) = 7.12	1.0	Pre-VC:Female Vehicle vs. Post-VC:Female Vehicle	8	8	0.65	0.515
				Pre-VC:Female CGRP vs. Post-VC:Female CGRP	10	10	0.51	0.700
				Pre-VC:Female Vehicle vs. Pre-VC:Female CGRP	8	10	0.94	0.046
				Post-VC:Female Vehicle vs. Post-VC:Female CGRP	8	10	0.81	0.734
VC	0.04	F (1, 17) = 5.08	1.0	Pre-VC:Male Vehicle vs. Post-VC:Male Vehicle	7	7	-0.02	> 0.99
				Pre-VC:Male CGRP vs. Post-VC:Male CGRP	9	7	0.02	> 0.99
				Pre-VC:Male Vehicle vs. Pre-VC:Male CGRP	7	9	0.04	0.955
				Post-VC:Male Vehicle vs. Post-VC:Male CGRP	7	7	0.09	> 0.99
Sex	0.002	F (1, 7) = 22.52	NA	Pre-VC:Male Vehicle vs. Pre-VC:Female Vehicle	8	7	0.11	0.768
				Pre-VC:Male CGRP vs Pre-VC:Female CGRP	10	9	1.01	0.006
				Post-VC:Male Vehicle vs Post-VC:Female Vehicle	8	7	0.78	0.485
				Post-VC:Male CGRP vs Post-VC:Female CGRP	10	7	1.50	0.025

Table Legends:

Table 1: A 2-way repeated measures (RM)-ANOVA was computed to assess the effects of calcitonin-gene related peptide (CGRP) and sex on MAX latency to fall (LTF) values prior to the vestibular challenge (VC). A second 2-way RM-ANOVA was computed to assess the effects of CGRP and VC when all mice are pulled together, and factors are titled $CGRP_{SC}$ and VC_{SC} respectively. The Geisser-Greenhouse Correction ($\hat{\varepsilon}$) was applied to the second RM-ANOVA as the two factors CGRP and VC were repeated measures. Bonferroni post-hoc comparisons were made between the first group (T1) versus the second group (T2), and sample sizes are depicted in N_{T1} and N_{T2} respectively. F-values are listed with respect to degrees of freedom (DF_n, DF_d) and p-values are listed accordingly.

Table 2: A 3-way mixed effects (ME)-model was computed to assess the effects of calcitonin-gene related peptide (CGRP), vestibular challenge, and sex on center of pressure (CoP) 95% ellipse areas. The Geisser-Greenhouse Correction ($\hat{\varepsilon}$) was applied to the repeated measure factors CGRP and VC. Tukey post-hoc comparisons were made between the first group (T1) versus the second group (T2), and sample sizes are depicted in N_{T1} and N_{T2} respectively. F-values are listed with respect to degrees of freedom (DF_n, DF_d) and p-values are listed accordingly.