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TITLE:

Investigating Migraine-Like Behavior using Light Aversion in Mice

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SUMMARY:

Rodents are not able to report migraine symptoms. Here, we describe a manageable test paradigm (light/dark and open field assays) to measure light aversion, one of the most common and bothersome symptoms in patients with migraines.

ABSTRACT:

Migraine is a complex neurological disorder characterized by headache and sensory abnormalities, such as hypersensitivity to light, observed as photophobia. Whilst it is impossible to confirm that a mouse is experiencing migraine, light aversion can be used as a behavioral surrogate for the migraine symptom of photophobia. To test for light aversion, we utilize the light/dark assay to measure the time mice freely choose to spend in either a light or dark environment. The assay has been refined by introducing two critical modifications: pre-exposures to the chamber prior to running the test procedure and adjustable chamber lighting, permitting the use of a range of light intensities from 55 lux to 27,000 lux. Because the choice to spend more time in the dark is also indicative of anxiety, we also utilize a light-independent anxiety test, the open field assay, to distinguish anxiety from light aversive behavior. Here, we describe a modified test paradigm for the light/dark and open field assays. The application of these assays is described for intraperitoneal injection of calcitonin gene-related peptide (CGRP) in two mouse strains and for optogenetic brain stimulation studies.

INTRODUCTION:

Migraine is a prevalent neurological disease, affecting approximately 17% of Americans[1] and is the second leading cause of disability globally[2,3]. Patients experience headache that lasts 4-72 hours accompanied with at least one of the following symptoms: nausea and/or vomiting, or photophobia and phonophobia[4]. Recent advances in the development of calcitonin gene-related peptide (CGRP) antibodies that are now FDA approved have begun a new era for migraine treatment[5-7]. These antibodies block either CGRP or its receptor and prevent migraine symptoms in approximately 50% of migraine patients[7]. Within the past year, two small-molecule antagonists of the CGRP receptor have also been FDA approved for abortive treatment of migraines, and two more are in the pipeline[8]. Despite this therapeutic progress, mechanisms by which migraine attacks occur still remain elusive. For example, the sites of CGRP action are not known. The efficacy of therapeutic antibodies that do not appreciably cross the blood-brain barrier suggests that CGRP acts at peripheral sites, such as the meninges and/or trigeminal ganglia. However, we cannot rule out central actions at circumventricular organs, which lack a blood-brain barrier[9]. At least for photophobia, we think this is less likely given our results with light aversion using transgenic nestin/hRAMP1 mice in which hRAMP1 is overexpressed in the nervous tissue[10]. Understanding mechanisms of migraine pathophysiology will provide new avenues to the development of migraine therapeutics.

Preclinical animal models are critical to understanding disease mechanisms and the development of new drugs. However, migraine assessment in animals is challenging since animals cannot verbally report their sensations of pain. Given the fact that 80-90% of migraine patients exhibit photophobia[11], light aversion is considered to be an indicator of migraine in animal models. This led to the need to develop an assay to assess light aversion in mice.

The light/dark assay contains a light zone and a dark zone. It is widely used for measuring anxiety in mice based on their spontaneous exploration of novel environments that is countered by their innate aversion to light[12]. Some studies set 1/3 of the chamber as the dark zone, while others set 1/2 of the chamber as the dark zone. The former setting is often used to detect anxiety[13]. While we initially chose equally sized light/dark chambers, we have not compared the two relative sizes. We can comment that the overall size of both chambers is not a major factor since the initial testing box[14] was considerably larger than the subsequent apparatus[15], yet results were essentially the same.

Two critical modifications to this light/dark assay to assess light aversion were: the testing condition and the light intensity (**Figure 1**). First, mice are pre-exposed to the light/dark chamber to reduce exploratory drive[16] (**Figure 1A**). The necessity and times of pre-exposures depend on mouse strains and models. Wildtype C57BL/6J mice usually require two pre-exposures[10], while only one pre-exposure for CD1 mice is sufficient[17]. In this manner, light aversive behavior can be unmasked in these two mouse strains. Second, the chamber lighting has been adapted to include an adjustable range of light intensities from dim (55 lux) to bright (27,000 lux) where 55 lux is comparable to a dark overcast day, and 27,000 lux is comparable to a bright sunny day in the shade[10]. We have found that the required light intensity varies with the strain and genetic model. For this reason, individuals should first assess the minimum light

intensity for their experimental paradigm.

Even with these modifications to the assay, which can reveal a light-aversive phenotype, it is necessary to test anxiety-like behavior to distinguish between light aversion due to light alone versus due to anxiety. The open field assay is a traditional way to measure anxiety based on the spontaneous exploration of novel environments. It differs from the light/dark assay in that the exploratory drive is countered by the innate aversion to unprotected open spaces. Both the center and edges of the chamber are in the light, so the open field assay is a light-independent anxiety assay. Thus, the combination of the light/dark and open field assays enables us to distinguish between light aversion due to an avoidance of light versus an overall increase in anxiety.

CGRP is a multifunctional neuropeptide that regulates vasodilation, nociception, and inflammation[18]. It is widely expressed in the peripheral and central nervous systems. It plays an important role in migraine pathophysiology[18]. However, the mechanism underlying CGRP action in migraine is unclear. By utilizing the light/dark and open field assays with this modified test paradigm, we were able to identify light-aversive behavior in mice following peripheral[10,16] (**Figure 2**) and central[14-16,19] CGRP administration. In addition to neuropeptides, the identification of brain regions involved in light aversion is also important in understanding migraine pathophysiology. The posterior thalamic nuclei are an integrative brain region for pain and light processing[19], and the thalamus is activated during migraine[20]. Thus, we targeted posterior thalamic nuclei by injecting adeno-associated virus (AAV) containing channelrhodopsin-2 (ChR2) or eYFP into this region. By combining this optogenetic approach with these two assays, we demonstrated that optical stimulation of ChR2-expressing neurons in the posterior thalamic nuclei induced light aversion[19] (**Figure 3**). In this experiment, given the dramatic effect on the evoked light aversion in these optogenetically manipulated mice, pre-exposures to the chamber were skipped.

PROTOCOL:

Animal procedures were approved by the University of Iowa Animal Care and Use Committee and performed in compliance with the standards set by the National Institutes of Health.

1. Light/dark assay

1.1 Light/dark chamber apparatus (see **Table of Materials**) setup. All the equipment in this section is commercially available.

1.1.1 On a shelf, place the sound-attenuating cubicle (interior: 59.7 x 38 x 35.6 cm in W x H x D) containing a pull-out drawer for easy access to the chamber and dark insert.

1.1.2 Connect the DC power supply and a DC-regulated power supply to the sound-attenuating cubicle.

1.1.3 Place the transparent seamless open field chamber (27.31 x 27.31 x 20.32 cm in L x W x H)

on the pull-out drawer of the cubicle.

1.1.4 Place the black, infrared (IR)-transparent plastic dark insert (28.7 X 15 X 20.6 cm in L x W x H) in the open field chamber. Ensure that the chamber is divided into two zones of an equal size: a dark zone and a light zone.

1.1.5 Connect three sets of 16-beam IR arrays on the X, Y and Z axes of the open field chamber to the IR USB controller via cables.

1.1.6 Connect the IR USB controller to a computer.

1.1.7 Install the tracking software on the computer which can record and collect mouse location and activity.

1.1.8 For the light panel setup, first remove the light-emitting diode (LED) light panel (27.70 x 27.70 cm in L x W; 360 LEDs, daylight-balanced color, 5600K, 60° flood beam spread) from its original housing.

1.1.9 Assemble the light panel with the LED driver, the heat sink, and the power supply. Multiple LED light panels can be connected to one power supply, heat sink, and LED driver to achieve uniform light panel control.

1.1.10 Construct a customized acrylic platform (29.77 x 27.70 x 8.10 cm in L x W x H) comprising of 7 identical shelves at 0.53 cm intervals (**Figure 1B**). Permanently affix the customized acrylic shelf to the ceiling inside the cubicle above the chamber.

1.1.11 Insert the LED light panel into the slot between the bottom two shelves. Adjust the light panel to different heights (**Figure 1B,C**), if necessary (e.g., if using optogenetic mice. Details are discussed in Section 3).

1.1.12 Turn on the heat sink, LED driver, and power supply. Confirm that the LED driver can dictate the LED light intensity by measuring the light intensity on the chamber floor and confirm that the floor is lit evenly.

1.2 Behavioral test procedure

NOTE: Mice are housed on a 12 h light cycle. All behavioral experiments are performed during the light cycle. Mice, including both males and females, aged 10-20 weeks old, are used. In this protocol, naïve wildtype CD1 and C57BL/6J mice experience two pre-exposures to the light/dark chamber followed by exposure with treatment and a post-treatment exposure. There is a three-day interval between each exposure to allow mice to recover (Day 1, 4, 7 and 10 as described below and **Figure 1A**). However, CD1 mice do not require the 2nd pre-exposure and can be tested in dim light.

1.2.1 On day 1 (Pretreatment 1), turn the light/dark assay apparatus on and set the light intensity to 27,000 lux.

1.2.2 Open the tracking software and set up a new protocol. In the **New Protocol** setting, set the **Duration** to 30 min. In the **New Analysis** setting, set **Data Bins by Duration** to 300 s.

1.2.3 In the **New Zone** setting, choose **Pre-Defined Zones**. Choose **2** and then **Horizontal**. Check if the chamber is divided into two equal-size zones horizontally for recording.

1.2.4 Habituate mice to the testing room for 1 h prior to the testing. During habituation, keep the room light on to not disrupt the mouse's circadian rhythm. Make sure all the equipment for the light/dark assay is turned on, allowing the mice to fully acclimate to the testing room environment.

1.2.5 Select **Acquire Data**. Enter mouse IDs. Start the protocol

1.2.6 Pull the drawer outside of the sound-attenuating cubicle to access the light/dark chamber and the dark insert. Gently place a mouse in the light zone of the chamber and push the drawer inside of the cubicle. Ensure that the software detects the mouse immediately and begins to record activity.

1.2.7 Wait for the recording to automatically stop after 30 min. Return the mouse to its home cage.

1.2.8 Clean the chamber and dark insert using alcohol-odor germicidal disposable wipes containing 55.0% isopropyl alcohol, 0.25% alkyl C12-18 dimethyl ethylbenzyl ammonium, and 0.25% alkyl C12-18 dimethyl benzyl ammonium chloride as anti-microbial active ingredients to eradicate any olfactory cues left by the previous mouse.

1.2.9 On day 4 (Pretreatment 2), repeat steps 1.2.1 to 1.2.8.

1.2.10 On day 7 (the treatment day), repeat step 1.2.1 and 1.2.4. After the habituation, administer mice with CGRP (0.1 mg/kg, intraperitoneal injection (i.p.)). Return mice to their home cages.

1.2.11 After 30 min, start the protocol and run the mouse in the light zone as mentioned in steps 1.2.5 to 1.2.7. The recovery time in home cages after injections can be shortened or lengthened depending on the treatment[21].

1.2.12 Clean the chamber and dark insert as described in step 1.2.8. The experiment can be paused at step 1.2.12 before starting the open field assay.

1.2.13 On day 10 (post-treatment day), repeat steps 1.2.1 to 1.2.8.

2. Open field assay

2.1 The apparatus setup

2.1.1 Open field chamber setup: Use the same sound-attenuating cubicle and open field chamber used in the light/dark assay, without using the dark insert.

2.1.2 Light panel setup: Use the same setup used in the light/dark assay. Ensure that the light intensity is the same as used in the light/dark assay.

2.2 Behavioral test procedure

2.2.1 Turn the apparatus on. Set the light intensity to 27,000 lux.

2.2.2 Open the tracking software.

2.2.3 Set up a new protocol, the same as is used in the light/dark assay except for the **New Zone** settings. Choose **1** followed by the **Center** in the **New Zone** settings. Set the periphery as 3.97 cm from the perimeter and the center as 19.05 × 19.05 cm.

2.2.4 Habituate mice to the testing room as described in step 1.2.4.

2.2.5 Administer CGRP (0.1 mg/kg, i.p.) to the mice. Return the mice to their home cages.

2.2.6 After 30 min, start the protocol. Pull the pull-out drawer outside of the sound-attenuating cubicle and gently place a mouse in the middle of the open field chamber. Push the drawer inside of the cubicle.

2.2.7 Track behavior for 30 min. Then return mice to their home cages.

2.2.8 Clean the apparatus as described in step 1.2.8.

3. Modified light/dark assay for optogenetic mice

3.1 The apparatus setup

3.1.1 Make two modifications to the dark insert.

3.1.1.1 Modify the opening of the dark insert to 5.08 x 5.08 cm (W x H) with a small slit 0.95 x 10.16 cm (W X H) between the top and the opening of the dark insert (**Figure 1D** top left).

NOTE: This modification allows a mouse to go to the dark zone without difficulty when the fiber-optic cannula on the mouse head is attached to the patch cord.

3.1.1.2 Extend the top of the dark insert over the light area as a triangular porch (H=6.5 cm) (**Figure 1D** top right and bottom left). Cut a circular hole (D=1.7 cm) out of the porch and insert a holder into the hole to place and stabilize the rotary joint, which connects the laser and the fiber-optic patch cords (**Figure 1D** top left and bottom left).

NOTE: The modifications result in small change in the light intensity reaching the floor of the dark zone (17 lux with modifications vs 14 lux without modifications, measured on the back-right corner of the dark zone under 27,000 lux).

3.1.2 Insert the rotary joint into the holder on the dark insert.

3.1.3 Connect the 30.5 cm fiber-optic patch cord to the rotary joint. Confirm that the rotary joint can rotate smoothly so that the patch cord can rotate without difficulty as the mouse traverses the chamber.

3.1.4 For the rest of the setup, use the same apparatus setup as used in section 1 (light/dark assay).

3.2 Behavioral test procedure

NOTE: Unlike the wildtype mice, the optogenetic mice do not receive pre-exposures (Pretreatment 1 and 2).

3.2.1 On the test day, insert the LED light panel into the second lowest slot (28.23 cm from the floor of the camber) to allow space for connecting the patch cord. Turn the light/dark assay apparatus on and set the light intensity to 55 lux.

3.2.2 Use the same protocol setup as that in 1.2.2 and 1.2.3 except that **Data Bins By Duration** is set to 60 s in the **New Analysis** setting to be congruent with the laser stimulation protocol in Step 3.2.3.

3.2.3 Turn the laser power button on. Set the laser pulse controller to stimulate for 1 min followed by 1 min without stimulation over 30 min.

3.2.4 Habituate mice to the testing room with the light on for 1 h prior to the testing.

3.2.5 Start the protocol. Pull the pull-out drawer outside of the sound-attenuating cubicle to access the light/dark chamber and the dark insert.

3.2.6 Gently restrain the mouse and couple the optic-fiber cannula on the mouse head to the fiber-optic patch cord via a mating sleeve (**Figure 1D** bottom right). Place the mouse gently in the light zone and push the drawer inside of the cubicle. Make sure that the protocol will begin to record mouse behavior automatically.

3.2.7 At 1 min, switch on the pulse controller and then turn the failsafe key to **ON**. Make sure laser stimulation of the targeted brain region is occurring every other minute.

3.2.8 After 30 min when the protocol stops automatically, turn the failsafe key to **OFF**. Then turn the pulse controller off.

3.2.9 Uncouple the mouse and the fiber-optic patch cord. Return the mouse to the home cage.

3.2.10 Clean the chamber and dark insert as described in step 1.2.8.

4. Modified open field assay for optogenetic mice

4.2 The apparatus setup

4.2.1 Stabilize the rotary joint above the chamber using a stand and a clamp (**Figure 1E**).

4.2.2 Connect the fiber-optic patch cord with a length of 50 cm to the rotary joint. Check if the rotary joint can rotate smoothly.

4.2.3 Set the rotary joint to the appropriate height on the stand: ensure that the fiber-optic patch cord can only just reach every corner of the chamber, which will help avoid any interference with mouse movement.

4.2.4 For the rest of the setup, use the same apparatus setup as used in section 1 (Light/dark assay), but without the dark insert.

4.3 Behavioral test procedure

4.3.1 Turn the light/dark assay apparatus on and set the light intensity to 55 lux.

4.3.2 Use the same protocol setup as that in the modified light/dark assay (section 3) except for the **New Zone** settings. Choose **1** following by **Center** in **New Zone** settings. Set the periphery as 3.97 cm from the perimeter and the center as 19.05 × 19.05 cm.

4.3.3 Turn the laser power button on. Set laser pulse controller to stimulate for 1 min followed by 1 min without stimulation over 30 min.

4.3.4 Perform habituation and the rest of the test as described in steps 3.2.4 to 3.2.10 except for two changes to step 3.2.6: place the mouse gently in the middle of the chamber instead of the light zone; keep the pull-out drawer outside of the cubicle due to the patch cord connecting to the mouse's head.

REPRESENTATIVE RESULTS:

This behavioral test paradigm is designed to test light aversive behavior. It can be performed

using both naïve wildtype mice and optogenetic mice to investigate light aversion in real time during the stimulation of a targeted neuronal population.

This procedure has been used to study the effect of peripheral CGRP treatment in CD1 and C57BL/6J mice[10,16] and optical stimulation of neurons in the posterior thalamic nuclei in C57BL/6J mice[19] on light-aversive behavior. Mice, including both males and females, aged 10-20 weeks old, were used in the experiments (**Figure 2A**, **Figure 2B-D,** and **Figure 3**). The results revealed that i.p. injection of CGRP significantly decreased the duration of time spent in the light zone in the light/dark assay in CD1 (**Figure 2A**) and C57BL/6J (**Figure 2B**) mice, but did not affect the time mice spent in the center in the open field assay in CD1 (data not shown) and C57BL/6J mice (**Figure 2D**)[10,16]. This suggests that peripheral CGRP induces light aversion but not general anxiety. Treatment with CGRP also increased the amount of time mice rested in the dark zone but not in the light zone in both CD1 (data not shown) and C57BL/6J mice (**Figure 2C**).

For the optogenetic protocol, we targeted calmodulin kinase II alpha (CaMKIIa)-expressing neurons in the posterior thalamic nuclei by injecting AAV2-CaMKIIa-hChR2(E123A)-eYFP or the control virus AAV2-CaMKIIa-eYFP[19]. At the same time, a fiber-optic cannula was implanted in the posterior thalamic nuclei. Three weeks following injection to allow sufficient time for ChR2 expression, we performed optical stimulation of neurons in the posterior thalamic nuclei and noted a corresponding decrease in the duration mice spent in the light zone in the light/dark assay in ChR2-injected mice compared to control virus-injected mice (eYFP) (**Figure 3A**). There was no noted difference in the time in center in the open field assay between ChR2 and control eYFP mice (**Figure 3C**), indicative of a light-aversive response that was not solely driven by anxiety[19]. Furthermore, an increase in the resting time in the dark zone, but not in the light zone, was also noted (**Figure 3B**). The same results were obtained when using 55 lux and 27,000 lux (**Figure 3**). The 55-lux procedure was included because migraine patients are sensitive even to dim light.

FIGURE AND TABLE LEGENDS:

Figure 1: The light/dark assay timeline and apparatus. (**A**) Timeline of the testing paradigm: After two pre-exposures to the light/dark chamber (Pre 1 and Pre 2), mice are administered CGRP (0.1 mg/kg, i.p.) followed by a post-treatment measurement (Post). At least one day after the light/dark assay, mice are given CGRP (0.1 mg/kg, i.p.) again and are run in the open field assay. (**B**) The LED panel is held at the top of the chamber by an acrylic shelf and illuminates the test area. The height of the light panel can be adjusted by using slots at different heights. (**C**) The light/dark chamber contains a dark insert with a small opening. A LED light panel is above the chamber. (**D**) Front, side, and top views of the modified dark insert. The opening in the dark insert is extended with a small slit for the movement of the patch cord (top left). The top of the dark insert extends over the light area as a triangular porch with a holder for the rotatory joint (top right and bottom left). The optic-fiber patch cord is connected to the fiber-optic cannula via a mating sleeve (bottom right). (**E**) The modified open field assay. The stand and clamp hold the rotatory joint. The chamber is pulled out to the front of the cubicle with the doors left open to allow the free movement of the mouse with the patch cord attached to the mouse head.

Figure 2: Peripheral CGRP administration evokes light aversion in bright light in two strains of wildtype mice. CD1 and C57/BL6J mice were tested according to the timeline described in Figure 1A. (**A**) The time CD1 mice spent in the light zone per 5 min interval over 30 min (27,000 lux). Time in light data is shown over time during the test (left panel) and as the average time per 5 min interval for individual mice (right panel). Comparisons were made between vehicle and CGRP at each time point, and between Tx and Pre2 or Post as indicated by brackets**.** (Veh, n=19; 0.1 mg/kg CGRP, n=19) (**B**) Time C57BL/6J mice spent in the light zone per 5 min interval over 30 min (27,000 lux). Time in light data are shown over time during the test (left panel) and as the average time per 5 min interval for individual mice (right panel) (Veh, n=42; 0.1 mg/kg CGRP, n=44). (**C**) The mice from panel B were also analyzed for resting behavior in the dark and light zones during the light/dark assay. (**D**) The mice from panel B were subsequently tested in the open field assay. The percentage of time spent in the center of the chamber per 5 min interval over 30 min after treatment with vehicle or CGRP (0.1 mg/kg, i.p.) (Veh, n=9; 0.1 mg/kg CGRP, n=9). The percentage of time in the center data is shown over time during the test (left panel) and as the average percentage of the time in the center per 5 min interval for individual mice (right panel). For all panels, mean±SEM is shown, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. This figure is modified from Mason et al. 2017[10].

Figure 3: Optical stimulation of CaMKIIa-expressing neurons in the posterior thalamic nuclei induces light aversion in both dim and bright light. (**A**) Posterior thalamic nuclei of C57BL/6J mice injected with AAV encoding either ChR2 or eYFP (at 55 lux: eYFP n = 8, ChR2 n = 11; at 27,000 lux: eYFP n = 12, ChR2 n = 18) were stimulated by blue laser (473 nm, 20 Hz, 5 ms pulse width, 10 mW/mm^2). Left panel shows the time mice spent in the light zone per 5 min interval over 30 min at 55 or 27,000 lux. Comparisons were made between eYFP and ChR2 groups at each time point. Right panel shows the average time per 5 min interval for individual mice. (**B**) The mice from panel A were also analyzed for resting behavior in the light (left panel) and dark (right panel) zones during the light/dark assay. (**C**) The mice from panel A were subsequently tested in the open field assay. Average percentage of the time spent in the center of the open field chamber per 5 min interval over 30 min (Laser: 473 nm, 20 Hz, 5 ms, 10 mW/mm^2). (eYFP n = 8, ChR2 n = 9). For all panels, mean±SEM is shown, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. This figure is modified from Sowers et al. 2020[19].

DISCUSSION:

The light/dark assay is widely used to assess anxiety-like behavior[12]. The assay relies on the innate aversion of mice to light and their drive to explore when placed into a novel environment (light zone). However, as we report here, this assay can also be used to assess light-aversive behavior as well.

It is critical to consider the number and necessity of pre-exposures prior to testing. This depends on the mouse strain or model. For example, in our light/dark assay protocol, naïve wildtype CD1 and C57BL/6J mice are pre-exposed to the light/dark chamber twice prior to undergoing the treatment test procedure, while optogenetic mice do not undergo pre-exposure. A recent publication reported that one pre-exposure is sufficient for CD1 mice to display light aversion after i.p. CGRP administration[17]. Consequently, the significance of the

novelty parameter will have lessened upon arrival of the treatment day[10,16]. Pre-exposures can unmask light-aversive phenotypes by reducing the exploratory drive and thus altering the balance between exploration and aversion. In some cases, pre-exposure is not necessary. For example, with genetically altered mice with increased CGRP receptors in the nervous system, pre-exposure was not necessary[14]. Likewise, with optogenetically manipulated mice, in which CaMKIIa-expressing neurons in the posterior thalamic nuclei were targeted for optical stimulation, pre-exposure was not necessary, presumably because the light-aversive response was so robust upon direct stimulation of the brain[19]. Thus, the number and necessity of pre-exposures to the chamber must be carefully considered when using different mouse strains or models. Indeed, overexposure of mice to the chamber may reduce exploratory behavior. This will lead to the mice preferentially occupying the dark zone, regardless of treatment, therein reducing the ability to observe a light-aversive response. Conversely, insufficient pre-exposure to the assay may lead to exploratory behavior masking potential light-aversive behavior.

A post-treatment exposure serves to identify whether a mouse has fully recovered from the CGRP injection administered 2 days prior. This is essential prior to running the open field assay or any other assay to confirm that no prolonged treatment effect is present that will affect future behavioral tests.

We opted for a 30-min protocol duration based on previous observations[10]. We have tested mice in the light/dark assay for 10 min[15], 20 min[16] and 30 min[10] separately. CGRP decreased the amount of time mice spent in the light between 0-30 min, but past 30 min the control mice preferred to spend more time in the dark compared to 0-30 min, hence leading to the decision to test for 30 min. In a similar fashion, the testing duration can be adjusted with reference to the time-response curve for different mouse models. It should be noted that lengthening the exposure time to the light/dark chamber may reduce motivation to explore the light zone.

We analyzed many different parameters to assess the animal behavior. One essential feature of the light/dark assay is a measurement of the time a mouse spends in the light zone, directly reflecting light aversion. Percentage of time spent resting, the number of vertical beam breaks (to measure rearing activity) in light or dark zones, and the number of transitions between the two zones are used to assess motility. Resting time and vertical beam breaks are normalized to the time spent in each zone in order to avoid false conclusions regarding movement. We include all mice in the analyses except: mice that remain in the light zone for the entire 30 min of testing, mice that spend over 90% of time resting in total (both light and dark zones), and statistical outliers (>3 SDs from the mean). The number of mice that are excluded is generally less than 1%. For the open field assay, the percentage of time in center is the main measurement used to assess anxiety-like behavior.

In the modified light/dark assay, the positioning of the fiber-optic cannula at some brain regions can greatly restrict mouse movement and, in some instances, prevent the mouse from reaching the dark zone. Consequently, entry into the dark zone will be negatively reinforced and, after multiple attempts, the mouse may show a learnt preference for the light, even remaining in the light zone during the entire testing period. This can be rectified by modifying the size and shape

of the opening in the dark insert. As an example, when fiber-optic cannulae were installed in the cerebellum of wildtype C57BL/6J mice, the mice had difficulty crossing the opening of the dark insert. After altering the width of the opening to 6.10 cm instead of 5.08 cm, the mice were able to traverse the opening freely.

A 30.5 cm fiber-optic patch cord is used in the modified light/dark assay, based on the size of the open field chamber, allowing the mouse to move freely. A shorter cord length will prevent a mouse from moving to the corners, while a longer cable may tangle and hinder movement. The length of the fiber-optic patch cord used for the modified open field assay is 50 cm. The length is not as strict as that in the light/dark assay since the height of the rotary joint can be adjusted according to the length of the fiber-optic patch cable, ensuring that the mouse is able to just reach the corners of the chamber.

Based on power analyses, 10-12 mice per group are needed for CD1 and C57BL/6J mice with i.p. CGRP, and for optogenetic C57BL/6J mice to detect significant light aversion. However, the C57BL/6J group size was considerably larger than the CD1 group size (**Figure 2A,B**) because the C57BL/6J mice were unresponsive to CGRP in a subset of the tests[10], meaning multiple tests were conducted to account for this high variability in light-aversive behavior in these mice. Specifically, two experiments were combined for the CD1 mice and four experiments were combined for C57BL/6J mice with i.p. CGRP (**Figure 2A,B**)[10]. The reason for this variability is not known, but humans also show variability in their responses to CGRP and light. Intravenous (i.v.) injection of CGRP induced migraine attacks in around 63~75% of migraine patients, with 70~90% of patients who displayed migraine attacks exhibiting photophobia[22-25]. Altogether, the assay has considerable variability and in addition to the number of mice, it is essential to do at least two and preferably three fully independent experiments with different cohorts of mice.

Bedding is not required in the light/dark chamber and the experimenter is not required to pre-handle or habituate the mice. As a precautionary measure the two pre-exposure procedures serve the purpose of acclimating the mice to the olfactory and physical cues of the experimenter; however, Ueno H. et al. demonstrated that there is no difference in time in light in the light/dark assay or time in center in the open field assay between mice after repeated handling and mice with no handling[26].

The open field assay can assess the contribution of anxiety towards a light-aversive phenotype. There are other well-validated anxiety-related assays, such as the elevated zero maze and the elevated plus maze[27]; however, the open field assay is the most procedurally relevant control to the light/dark protocol since the same testing chamber is used for both assays. Even so, an assessment of anxiety can be strengthened by utilizing multiple assays or by measuring multiple parameters in a single test given that anxiety is a complicated and multifaceted behavior. Importantly, even if there is no anxiety phenotype in the open field assay, this does not rule out an anxiety component to the light-aversive phenotype. For example, light might be triggering an anxiety response. The open field test only indicates that anxiety alone is not driving the response to light. While an anxiolytic drug, such as benzodiazepame, might be used in this assay, such an approach would have complications, e.g., anxiolytic drugs affect locomotion.

Instead, we opted to use clinical anti-migraine medications, including sumatriptan, to validate the migraine-like status of the light-aversive phenotype. Sumatriptan successfully reversed CGRP-induced light aversion in both CD1 and C57BL/6J mice[10].

Unlike the modified light/dark assay, the chamber on the pull-out drawer is outside of the cubicle with cubicle doors open in the modified open field assay due to the patch cord connecting to the mouse's head. Instead of 55 lux, the room light reaches the floor of the chamber at ~1000 lux. Even though the light intensity is different, the open field assay is a light-independent test. In detail, increasing the light intensity from 55 to 27,000 lux in the open field assay resulted in a trend of a decrease in time in the center in C57BL/6J mice, suggesting that the light intensity may influence mouse behavior[28]. However, the difference between the control and experimental groups was not significant under neither 55 nor 27,000 lux[28]. Additionally, the difference in light intensity between 55 and 1000 lux is far more subtle than between 55 and 27,000 lux. Wireless optogenetics can solve this problem as there would be no patch cord, allowing the open field chamber to be pushed inside of the sound-attenuating cubicle.

In addition, the patch cord still limits mouse movement despite selecting an optimal length. In the future, wireless optogenetics will offer a non-invasive alternative to cable-based optogenetic techniques.

It should be noted that we used acute injection of CGRP, which only replicates in part the prolonged CGRP release that accompanies migraine attacks. While we injected CGRP into mice to model migraine based on the premise that plasma CGRP levels were increased[29] and that i.v CGRP induced migraine attacks in migraine patients[22-25,30], this will not replicate the condition in the patient where CGRP is maintained at high levels for a relatively long time (patient measurements were taken at a median 3 hours after the migraine started[29]), nor does it replicate chronic migraine where levels are reported to be elevated even between attacks[31]. Moreover, other pain-induced mediators have not been tested in our paradigm.

When performed in conjunction with the open field assay, the light/dark assay is effective in assessing light-aversive behavior in mice.

The Mogil group modified the elevated plus maze to measure light aversion in mice, with the closed arms being illuminated by bright light and the open arms remaining dark[32]. The standard elevated plus maze has often been used to detect anxiety-related behavior in animals. This assay is based on the conflict between a mouse's innate desire to explore a novel environment and being placed in a compromising position in the open unprotected maze arms. In the modified protocol, mice are forced to select between the closed arms, which are illuminated with bright light, and the open unprotected arms, which are dark. The preference to the former suggests anxiety overrules light aversion while the preference to the latter suggests light aversion takes precedence over anxiety. The Mogil group also conducted a standard elevated plus maze to evaluate anxiety-like behavior[32]. The purpose is the same as conducting the open field assay in our protocol. *Cacna1a* mutant mice, a familial hemiplegic migraine model, showed

photophobia when the closed arms were bright. In contrast, anxiety-like behavior was not detected when the standard elevated plus maze was conducted[32]. In rats, by using both the modified elevated plus maze and the light/dark assay, it was demonstrated that nitroglycerin (NTG) was able to induce photophobia[33,34], which was rescued by sumatriptan[34]. In the standard elevated plus maze setting where light is absent within the closed arms, NTG induced anxiety-like behavior in rats[34], suggesting that NTG-induced light aversion is accompanied with anxiety. To our knowledge, there are no publications using the light/dark assay and the modified elevated maze in the same mouse model. All in all, both the modified elevated plus maze and the light/dark assay proposed in this protocol have been demonstrated as effective measures of light-aversive behavior in mice.

We use the daylight LED panel with a daylight-balanced color (5600K), with a 60° flood beam spread, yielding no shadowing at a height of ~30 cm from the floor of chamber at either 55 lux or 27,000 lux. Other studies investigating light aversion have utilized the light/dark assay with varying modifications. For example, studies have used different light intensities for the light zone, ranging from hundreds to thousands of lux[35-37]; used light at different wavelengths (e.g. blue and yellow)[38]; or used different temperatures of light (cold and warm)[39]. Caution should be taken for the heat produced by the light since it can affect the temperature of the dark and light zones and interfere with the mice's behavior, potentially causing a preference to a specific zone. Besides, it is also important to use the light with a good viewing angle to avoid shadow on the floor of the chamber. Light intensity is important for the test too. 25,000 -27,000 lux is approximately equivalent to bright daylight. By conducting the light/dark assay at such a high light intensity, it is possible to amplify the treatment effect; however, it is essential to consider the retinal damage[40] and the negative effect of such a high light intensity on a mouse's willingness to go into light. Some studies reported that mouse eyes exposed to direct light[41] and mice exposed to bright light for several hours (e.g. 30,000 lux for 4 hrs[42]) experienced retinal damage. In the light/dark assay, there is a dark zone for the mouse to escape from the bright light if the mouse desires. In addition, previous studies found that mice in the control group (C57BL/6J mice) spent a similar amount of time in the light zone under 55, 1000 and 27,000 lux[28]. For CD1 mice, the control group spent about 1/3 of the time in the light under 27,000 lux[10] and unpublished data show similar results at 55 lux. It suggests that 27,000 lux light on its own does not make CD1 and C57BL/6J mice distressed. Nonetheless, caution should be taken when opting for a higher light intensity.

Alongside differences in light setting, researchers have opted for a variety of approaches in analyzing the light/dark data. When assessing light aversion, the amount of time spent in the light zone with the light switched off (or with red light illumination of the light zone, given that mice eyes are less receptive to red light) are included in the calculation. For example, aversion index= (time in $light_{0\ lux}$-time in $light_{test}$)/ time in $light_{0\ lux}$ was used by the Gorin group to assess light aversion[43]. Here, the 'light off' or 'red light' conditions are included to confirm that the avoidance of the light zone is conditional on light being present in opposed to simple place preference. We conducted this procedure with i.p. injection of CGRP and found that mice receiving CGRP did not have a place preference with light off in the light zone, confirming that CGRP-induced aversion is light-dependent[16]. Lastly, the Gorin group used the time mice spent in

the periphery of the light zone in the light/dark assay as a measure of anxiety[36]. We utilize a traditional test for anxiety, the open field assay. No matter which analysis method is chosen, it should be noted that the contribution of anxiety to light aversion cannot be ignored. This protocol attempts to partition out anxiety-like and light-aversive behavior by utilizing the light/dark and open field assays in tandem.

This protocol addresses the use of the light/dark and open field assays for the detection of light-aversive behavior in mice. This provides a useful tool to identify the mechanisms of neural circuits and brain regions driving photophobia. The test paradigm can be migraine-specific or can be expanded into other disorders involving photophobia. With respect to migraine, we have tested two other neuropeptides associated with migraine pathogenesis: pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP). PACAP and VIP were demonstrated to induce light aversion in CD1 mice[17,21]. In addition to migraine, photophobia is also a symptom of many other disorders, including bradyopsia, acute ocular injury or inflammation, traumatic brain syndromes, Lyme disease, albinism and cone dystrophy[36]. Thus, this test paradigm provides a tool to investigate mechanisms underlying photophobia-related disorders. Moreover, the pairing of optogenetic methods with conventional pharmacological approaches will undoubtedly assist in the development of novel therapeutics for photophobia-related disorders.

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DISCLOSURES:
The authors have no conflicts of interest to report.

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Fig 1

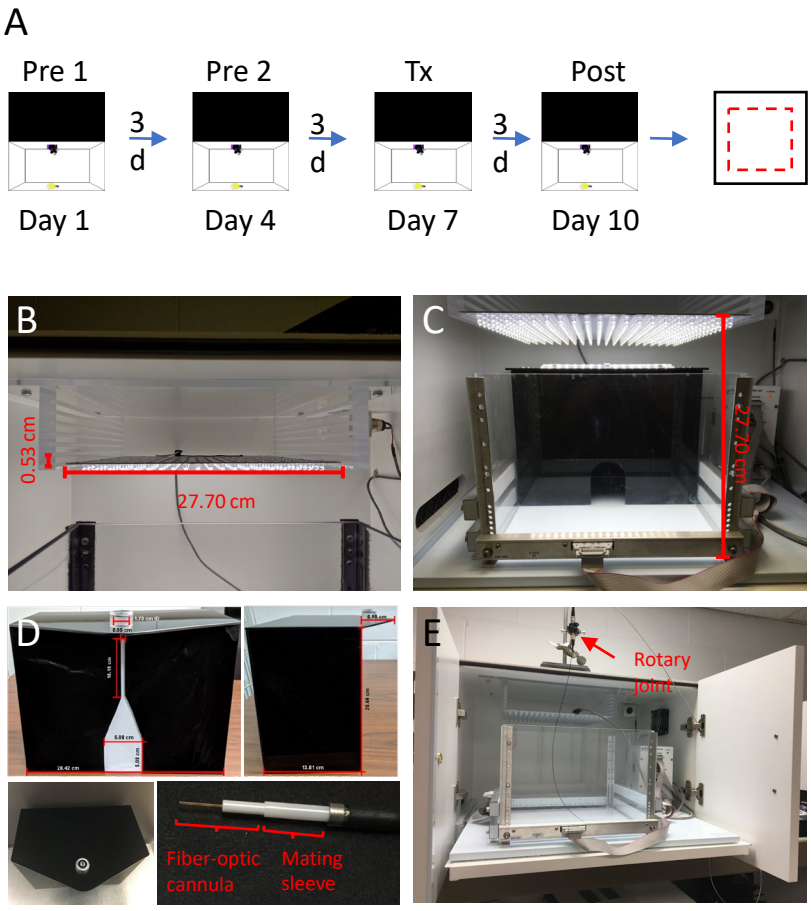


Fig 2

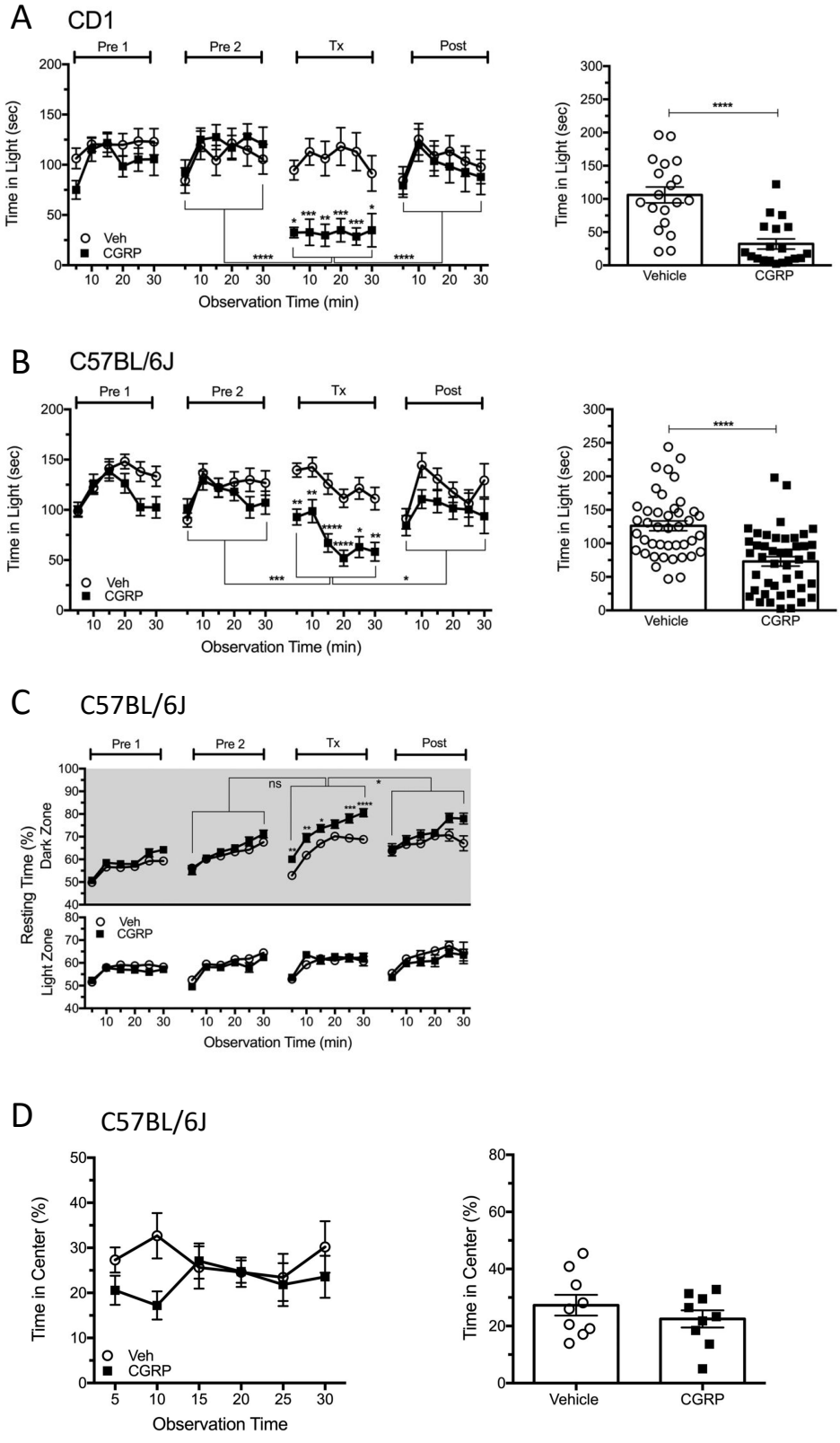
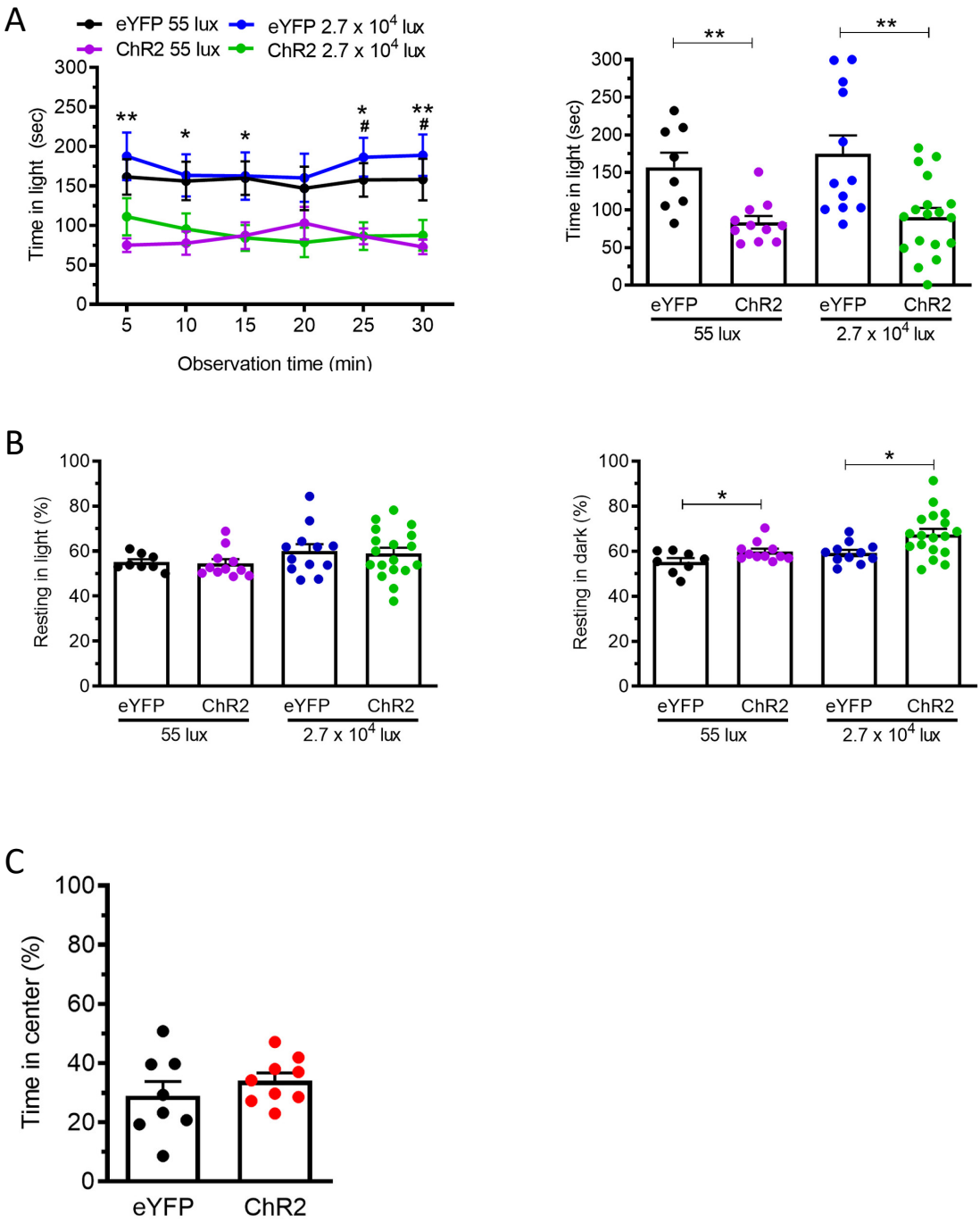


Fig 3



We thank the editor and reviewers for their insightful comments that have greatly improved the quality of this manuscript. We have addressed all the concerns. Responses are detailed below and all modifications are indicated in red in the manuscript.

Reviewer #1:

Manuscript Summary:

The manuscript by Russo et al. described a test paradigm that allows the evaluation of light aversion in two mice strain. Since photophobia is a frequent symptom of migraine patients, addressing light aversion in the experimental setting may contribute to a better understanding of this symptom and its management.

Major Concerns:

There are some reports of anxiety like behavior in migraine models, including induced by CGRP injection in rodents. Would it be possible to dissociate light aversion form anxiety in you test paradigm by using pharmacological tools (i.e. anxiolytic drugs)?

Response: We thank the reviewer for the suggestion. We have added this point to the discussion, page 11. In brief, we have considered this, but realized that the most commonly used anxiolytic drugs (benzodiazepams) have side effects, such as decreased motility, which could be a confounder. While we recognize that there is no perfect solution, the pharmacological approach we chose was to test anti-migraine medications to validate the migraine-like aspect of the light aversion phenotype.

The test seems to present high variability. Please comment on this and add the number of animals used in each experimental protocol in the figure's legends.

Response: The reviewer is correct. There is high variability in the average time in light in both CD1 and C57BL/6J mice after i.p. vehicle or CGRP. We have added this to the manuscript along with power analysis data, page 10-11. Based on the power analysis, to see significant light aversion, 10-12 mice per group are needed for CD1 and C57BL/6J mice with i.p. CGRP and for optogenetic C57BL/6J mice. However, our group size for the C57BL/6J mice was considerably larger than the CD1 mice, as presented in Fig. 2A and 2B, because the C57BL/6J mice were unresponsive to CGRP in a subset of the tests, which was stated in the publication (Mason, B.N., et al., J Neurosci, 2017). We still do not know why some cohorts of C57BL/6J were non-responsive and have seen it twice since as well. What it does mean is that multiple cohorts were used to account for this high variability in light-aversive behavior in these mice, ensuring a convincing result. Specifically, two experiments were combined for the CD1 mice and four experiments were combined for C57BL/6J mice with i.p. CGRP (Mason, B.N., et al., J Neurosci, 2017). Such variability also exists in human subjects. This would be an interesting topic to explore in the future. We have also added the number of mice in figure legends.

There is evidence that systemic CGRP does not cross the blood brain barrier. Please comment on this and also on the role of peripheral and central CGRP in inducing light aversion.

Response: The reviewer is correct. We agree that there is no evidence that CGRP can cross the blood brain barrier. We have added this point to the introduction, page 2. While we think that the likely sites of peripheral CGRP action are outside the brain, we acknowledge that we cannot rule out central action at circumventricular organs. CGRP binding and the receptor component, RAMP1 mRNA is distributed in the subfornical organ and area postrema, which lack blood brain barrier characteristics (Eftekhari, S., & Edvinsson, L. Ther Adv Neurol Disord., 2010). But the function of the CGRP receptor in these regions is unknown. Previous studies in our lab showed that i.p. injection of CGRP did not induce light aversion under 55 lux in nestin/hRAMP1 mice in which hRAMP1 is overexpressed in the nervous tissue. Conversely, i.c.v injection of CGRP induced light aversion under 55 lux in the nestin/hRMAP1 mice (Mason, B.N. et al., J Neurosci, 2017). It suggests that peripheral injection of CGRP is unlikely to cross the blood brain barrier in sufficient amounts to exert an effect centrally.

In this light aversion detected in this paradigm observed with other pain-induced mediators?

Response: This is an interesting question. We do not know. We have not tested other pain-induced mediators in our paradigm. We have added this caveat to the discussion, page 12.

C57BL mice showed reduction in the time in center in the OF test 10 min after CGRP injection (Figure 2D). CGRP has a very short half-life and one single systemic injection does not reflect sustained CGRP release that occurs during a migraine crisis. Please comment on this limitation.

Response: We thank the reviewer for bringing up the timing issue. With respect to Figure 2D, while it appears that there is a decrease in time in center, it is not statistically significant and likely reflects normal variation in mouse behavior. Furthermore, that 10 min dip was not seen in other studies (Recober A. et al., J Neurosci, 2009; Kaiser, E.A., et al., J Neurosci, 2012; Mason B.N. et al., Cephalalgia, 2020; Kuburas A. et al., J Neurosci, 2021) and does not correlate with the time in light behavior.

With respect to the duration of CGRP effect and its short half-life in plasma (5-7 min), that is an interesting point. We have added this point to the discussion, page 12. In short, we recognize the caveat that a single acute injection cannot replicate prolonged elevation that is likely experience in episodic and even more so in chronic migraine. This would be an interesting topic to explore in the future, for example with an osmotic pump for prolonged elevation.

Reviewer #2:
Manuscript Summary:

In this study, authors used 1) modified light/dark box with a range of light intensities (55 lux to 27,000 lux) to explore light aversive behaviors induced by CGRP injection, 2) modified open field assay to distinguish anxiety-like behavior from light aversive behavior, in CD1 and C57BL/6J mice strains and for optogenetic stimulation studies. They found that CGRP induces anxiety-free light aversive response in these two strains. Moreover, optical stimulation of CaMKIIa-expressing neurons in the posterior thalamic nuclei induces light aversion in both dim and bright light.

Reviewer #3:

Manuscript Summary:

"Investigating migraine-like behavior using light aversion in mice" is a methods paper, describing the use of the light sensitivity assay in mice, especially in relation to migraine and photophobia. The authors have done an extensive work of validating their light sensitivity assay with two models. The animals are habituated to the test setup and are then put through the light-dark box protocol with a subsequent open field test to distinguish between light sensitive and anxiety-based behavior. The authors provoke light sensitivity in two ways: chemically by using the known migraine-provoking drug CGRP or optogenetically by using laser stimulation of certain brain regions (here AAV2-infected neurons in the posterior thalamic nuclei). Through trial and error, the authors describe how they eventually settled on this protocol incl. modification of light conditions and number of habituations days.

Overall, this is a well-written and detailed paper with a good overview of the protocol and testing setup. Some concerns have been addressed below.

Major Concerns:

1) There are no detailed specifications of the animals used in the model. The authors discuss using two genotypes (C57BL/6J and CD1) but offer no additional information on sex, age or group sizes. Are both sexes used and are they used in the same test chambers? As shown in the attached figures, the group sizes vary quite substantially between the different experiments - that should be addressed. How many animals are needed to have enough power and see a significant effect? If this paper is to serve as a guide for other researchers interested in using these models, supplementary information such as whether bedding is added to the test chambers or whether pre-handling/habituation to the experimenter is necessary, would be nice as well.

Response: We thank the reviewer for pointing out these important factors. We have added all the suggested information to the protocol section, page 4, and result section, page 7. In brief, we used both sexes, aged 10-20 weeks, and tested in the same chamber. We cleaned the chamber between mice to ensure that no olfactory cue was left from the previous mouse. There was no bedding present in the testing chamber. Habituation to the investigator was not necessary.

With respect to the numbers, this is also a good point. The assay has considerable variability and in addition to the number of mice, we also emphasize that it is essential to do at least two and preferably three fully independent experiments with different cohorts of mice. We have added the power analysis and need for independent cohorts to the discussion, page 10-11.

2) My main concern with this specific model is the use of an extremely bright light (27,000 lux), especially in albino mice (CD1). The extreme light intensity causes retinal damage and from an animal welfare standpoint, there are no cause to use this high lux. (If of interest, see this review: "Blinded by the light: retinal phototoxicity in the context of safety studies" by De Vera Mudry et al. (2013) https://pubmed.ncbi.nlm.nih.gov/23271306/). The authors reference other studies using different light intensities (l. 453) - being 700 (ref. 23), 1000 (ref. 24) and 7000 lux (ref. 25), respectively. However, there is still a huge leap from that to 27,000 lux. The authors also discuss the difference between blue and yellow light, but it is not clear which light they use themselves (lines 453-456). The authors do mention that high light intensity might have a negative effect (lines 456-458) but because of the nature of this methods paper (and from an animal welfare standpoint) some argumentation or explanation on the light source and intensity should be added (does not have to be long).

Response: The reviewer has raised a good point and we appreciate the literature recommendation, which we have added to the manuscript. We agree that intense light could cause retinal damage, especially with CD1 albino mice. However, for the light damage exposure paradigm, studies usually exposed mice eyes directly to the light (White D.A., et al., Invest. Ophthalmol. Vis. Sci. 2007) or expose mice to the light for several hours to induce retinal damage (e.g. 30k lux for 4 hrs, Song. D. et al., PLOS ONE, 2017). Moreover, in the light/dark assay, there is a dark zone for the mouse to escape from the bright light. In addition, previous studies in our lab detected the time C57BL/6J mice spent in light using <0.05, 55, 1000 and 27,000 lux and found mice spent similar time in the light zone under 55, 1000 and 27,000 lux (Kuburas A. et al., Invest Ophthalmol Vis Sci. 2014). CD1 mice exhibited similar time in the light at 27,000 lux as the C57BL/6J mice (Mason, B.N., et al., J Neurosci, 2017). In unpublished data, we have seen that CD1 mice showed similar time in the light at baseline (~1/3) under 55 lux and 27,000 lux. This suggests that 27,000 lux does not have a negative effect on the eyes of CD1 and C57BL/6J mice under the conditions of the assay. Nonetheless, we appreciate the animal safety concern and the explanation has been added to the discussion, page 13. We have pointed out that caution should be taken on the choice of light intensity when using different mouse strains.

Regarding the light wavelength, we use the daylight LED panel with a daylight-balanced color (5600K) and a 60° flood beam spread, yielding no shadowing at a height of ~30 cm from the floor of chamber at either 55 lux or 27,000 lux. We have added the explanation on the light source to the discussion, page 12.

Minor Concerns:

1) There are many different protocols to the light-dark box as well as specifications. Perhaps the authors could mention why they use a 50/50 setup with light and dark side when some use 1/3 dark and 2/3 light?

Response: The reviewer raised a good point. We have added the rationale to the introduction, page 2. In brief, we do not know if one size is preferable to another. We have seen similar results with two different chambers that differ in overall size, but both are 50/50 dark and light (Recober, A., et al., Neuropharmacology, 2010).

2) Line 161: are the lights on or off in the testing room when the mice habituate? Although it is mentioned in studies from the same group (ref. 14), ideally it should also be written here as it is a methods paper.

Response: Mice are tested during the light hours. We do not want to alter their circadian rhythm, so lights are on in the testing room when the mice are habituated. We have added it in the protocol, page 4.

3) Regarding the protocol for open field optogenetics (lines 273-275): the lux is measured to 1000 instead of 55 because of the probe although it should be the latter. The authors state "even though the light intensity is different, the open field assay is a light-independent test", however, that does not seem like enough of an argument. In theory, the open field is light-independent but perhaps the shift in light intensity for the optogenic mice may alter the behavior regardless? Have the authors examined whether they see altered behavior or avoidance of the brighter 1000 lux areas compared to the dimmer ones? If possible, this should be addressed.

Response: We thank the reviewer for the insight. We agree that it should be 55 lux. But because of the patch cord, it is impossible to do so. Kuburas et al. (Kuburas A. et al., Invest Ophthalmol Vis Sci. 2014) demonstrated that there was a trend for C57BL/6J mice to spend less time in the center at 27,000 lux compared to 55 lux, suggesting that light intensity may influence the behavior of mice. Despite this trend, there is no significant difference between control and experimental mice at either 55 lux or 27,000 lux, suggesting that light intensity would not affect the difference between control and experimental groups, and thus not affect the interpretation. Thus, we feel confident using 1000 lux in the open field test. Besides, the same light-aversive results were obtained in 55 and 27000 lux for optogenetic mice in the light/dark assay. We have added it to the discussion, page 11, and pointed out that wireless optogenetics would help solve the problem.

~~4)~~ There is a mistake in the lines 290 - 296. Firstly, the authors write that CGRP DECREASES time spent in light. Then it is written that CGRP DECREASED time resting in dark zone. I think the wording should be increased, when discussing time resting? Because in the figure (2D), the

authors have shown that CGRP-treated animals significantly rest more than vehicle animals, when in the dark.

Response: We apologize for the mistake. We have corrected it.

5) The sentence "These phenotypes are congruent with the observation that migraine patients preferentially seek a dark space to rest during an attack." (lines 296-297) is an over-interpretation of the animal model. Although it would be nice, I would argue that one cannot translate the behavior so directly. Migraine patients may prefer to be in the dark and are indeed often resting (whether it be in the dark or not) but it cannot be directly translated to say that the phenotype of resting slightly more in the dark side of the light-dark box is corresponding to patients seeking dark spaces to specifically rest in. Perhaps pain (allodynia) or something else could be responsible for the increased resting time. If possible, this should be rephrased.

Response: We thank reviewer for the suggestion. We agree that it is over-interpreted. We have deleted the sentence.

6) Regarding the optogenetic protocol: the authors mention in lines 308-309 that the same effect was seen at 55 and 27,000 lux. Although it seems obvious, it would be nice if a statement was put in on why 55 lux was then used for further studies.

Response: We thank the reviewer for the suggestion. We have added the reason for testing at 55 lux to the result section, page 8. Migraine patients are sensitive even to a dim light. It led us to test these optogenetic mice at 55 lux. Surprisingly, optogenetic C57BL/6J mice also showed light aversion under 55 lux.

7) In the discussion section (starting at line 429), from line 438 on: the authors compare the modified elevated plus maze model from the Mogil group to their own where mice have to "select between remaining in comfort of the dark chamber or embracing their vulnerability and exploring the light chamber". This statement is a bit too much of a stretch. The modified elevated plus maze with opposing lights is a more complicated model and the behavior advances to risk assessment as well. I would not qualify the light/dark box to measure such complex behavior, it is more of a light sensitivity and anxiety test. Perhaps the authors could rephrase this section.

Response: We thank the reviewer for the suggestion. We agree that the modified elevated maze is a more complex test. We have deleted the sentence “mirrors ours with mice being required to select between remaining in comfort of the dark chamber or embracing their vulnerability and exploring the light chamber” to ensure that we are not attempting to make a direct comparison. We were guilty of becoming a bit too poetic for a scientific paper, which was not appropriate.

8) Regarding statement in lines 441-446: the authors should make clear that these references are from experiments in rats which might not yield the same response as nitroglycerin in mice.

Response: We thank the reviewer for the suggestion. We made the amendment, page 12.

9) Regarding the discussion section starting at line 478: the wording implies that the model is migraine specific. However, I would argue that the model is only migraine-specific when migraine-relevant drugs are used. Light aversion is relevant in many different fields, and the authors' overall protocol and setup could likely be used for other disorders as well. Perhaps a short statement or sentence about how the model is not restricted to only migraine research but can be migraine-specific when using the relevant drugs, could be added.

Response: We thank the reviewer for the suggestion and certainly agree that this assay is applicable beyond migraine. Light aversion is not only involved in migraine, but also other diseases, like bradyopsia, acute ocular injury or inflammation, traumatic brain syndromes, Lyme disease, albinism and cone dystrophy etc. (Thiels, E. Curr. Eye Res., 2008). We have made this point and added other photophobia-related disorders in the discussion, page 13-14.

10) Although it seems obvious, I would like to see one sentence somewhere in the manuscript about the relevance of the post-treatment exposure and why it is used. (This is also mentioned in the authors' other paper (ref. 14) but should also be written in this methods paper)

Response: We thank the reviewer for the suggestion. The purpose of the post-treatment exposure is to identify whether mice have fully recovered from the CGRP injection which was administered 2 days prior. This is essential prior to running the open field assay or any other assay, to make sure that no prolonged treatment effect is present that will influence mice's future behavior. We have added it to the discussion, page 9.

11) I would like to see a short discussion or explanation on the testing time. Why are the animals tested for 30 min? Some of the authors' previous work test the mice for 20 min, for example.

Response: Thanks for the reviewer's insight. The early publications from our lab tested mice in the light/dark assay for 10 min. Mice spent less time in the light at 0-5 min and 5-10 min in the treatment group compared to the control group (Recober, A., et al., Neuropharmacology, 2010), but the second interval showed a greater effect. Based on this observation, along with our effort to reduce exploratory drive to reveal a CGRP effect in wildtype mice, we extended the testing time to 20 min (Kaiser, E.A., et al., J Neurosci, 2012). Finally, we reasoned that a longitudinal analysis might be informative, so we extended the testing time to be 30 min (Mason, B.N., et al., J Neurosci, 2017). In unpublished data, we have found that all mice tend to spend more time in the dark after 30 min, most likely due to diminished exploratory drive over

time. Thus, we chose to use 30 min as the stopping point for our following tests. We have added this point (without the unpublished data) in the discussion, page 10.

12) Overall, the figures are nice, and I appreciate the setup pictures as well. However, the differences between Figure 1C and 1D is not very clear. Either arrows indicating the modifications or better lighting should be added to 1D because it is difficult to see atop the dark side. Alternatively, I would argue that 1E might be enough to show the alteration. Also, if a clearer picture of 1F is possible, that would also be good.

Response: We thank reviewer's suggestions. We have edited the image to make sure it is clear.

Thank you.

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