Using the visual cliff assay to assess binocular deficits in amblyopic mice

Authors: Héctor De Jesús-Cortés[1], Daniel A. Bowen[1,2], Francis Reilly-Andújar[1], Sophie Lu[1], Eric D. Gaier[1,2,3], Mark F. Bear[1]

Affiliations: [1]Picower Institute for Learning and Memory, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA, USA
[2]Department of Ophthalmology, Boston Children's Hospital, Boston, MA, USA
[3]Department of Ophthalmology, Harvard Medical School, Boston, MA, USA

Correspondence: Mark F Bear PhD
Picower Institute for Learning and Memory
43 Vassar St
Cambridge, MA 02139
Phone: 617-324-7002
Email: mbear@mit.edu

Funding: MFB: NIH R01 EY029245, RPB #42894, Picower Neurological Disorder Research Fund
EDG: NIH K08 EY030164

Conflict of interest: MFB: Luminopia, Inc. (scientific advisor, equity)
EDG: Luminopia, Inc. (scientific advisor, equity, patent), Stoke Therapeutics Inc. (consultant)

Key words: Visual cliff assay, stereopsis, binocular vision, monocular deprivation, amblyopia

Abstract
The visual cliff assay (VCA) has been used in multiple animal models including humans to assess stereopsis, the pinnacle percept of binocular function that is markedly disrupted in amblyopia. Amblyopia is a neurodevelopmental disorder of the visual system caused by refractive error, strabismus, or obscuration of the visual axis during infancy and early childhood. While the current standard of care can improve visual acuity in the amblyopic eye, gains are infrequently associated with improvements in stereoscopic depth perception and typically only achieved when treatment is initiated in early childhood. The mouse model of amblyopia induced by monocular deprivation (MD) is a powerful tool to study the mechanistic pathophysiology of this disorder and test novel therapeutics. However, to date, only one study has used the VCA to assess the effects of MD in mice in a limited capacity. Advantages of the VCA include no need for operant conditioning or training, completion in minutes, and minimal equipment. We comprehensively characterize and validate fundamental aspects of the VCA including test-retest reliability and contributions of binocularity using multiple monocular and binocular manipulations with clinically relevant paradigms. After long-term (3 weeks) MD, mice exhibit reduced predilection for the safe side, decreased latency to cross to the cliff side, and more crosses to the cliff side compared to sham littermate controls. Our data demonstrate that the VCA can detect binocular deficits in amblyopia but with important limitations that guide how the VCA should be applied to read out binocular function in both mechanistic and therapeutic studies in mice.

Introduction

The visual cliff assay (VCA) was developed by Eleanor J. Gibson and Richard D. Walk as a developmental psychological tool to test stereoscopic depth perception of infants (Gibson and Walk, 1960). The VCA is comprised of a clear surface place atop a patterned floor with a sharp drop off. Subjects with functional stereoscopic vision are inherently averse to the "cliff" side and tends to spend more time on the "safe" side. Adapted for use in several animal species ranging from humans to mice (Cornwell et al., 1976; Kaufman, 1976; Morrison, 1982; Green et al., 1993; Witherington et al., 2005; Baroncelli et al., 2013; Rodkey, 2015), the VCA is considered a classic experimental paradigm that relies on instinctive aversion to efficiently assess perception of stereoscopic depth. Among rodents, the VCA has primarily been applied in rats to study the development of depth perception and the role of early-life visual experience (Kaufman, 1976; Morrison, 1982; Baroncelli et al., 2013; Rodkey, 2015). Whether mice possess binocular vision has been debated for more than a century. Initially, mice were deemed incapable of visually perceiving depth (Waugh, 1910). Since then, emerging evidence has demonstrated that mice are able to discriminate stereoscopic surfaces despite having only 40 degrees of binocular visual field (Drager, 1978; Heesy, 2004; Scholl et al., 2013; Samonds et al., 2019; Williams et al., 2021). Genetic manipulability and the array of uniquely applicable experimental tools make mice an attractive model to study the neural basis for stereopsis (Boone et al., 2021) and clinical conditions that disrupt it.

Amblyopia ("lazy eye") is a prevalent neurodevelopmental visual disorder stemming from abnormal visual experience early in life (Bradfield, 2013; McConaghy and McGuirk, 2019). Deficits in stereoacuity are a key feature among many that comprise amblyopia (Webber and Wood, 2005; Levi, 2006; Maurer and McKee, 2018). The current treatment standard involves occlusion (patching) of the unaffected, fellow eye, but this approach is only effective during a critical period of visual development that ends at approximately age 8. Though most amblyopic children experience visual acuity gains in their amblyopic eye with patching, stereopsis rarely recovers (Wallace et al., 2011; Birch, 2013; Stewart et al., 2013; Levi et al., 2015).

Monocular deprivation (MD) via unilateral eyelid suture during the critical period is the predominant mode to model and study amblyopia (Wiesel and Hubel, 1963; Li et al., 2014). MD >4 days causes durable electrophysiologic and behavioral deficits that can be observed for ≥1 month after opening the amblyopic eye (Prusky et al., 2000; Fong et al., 2016; Stephany et al., 2018; Fong et al., 2021). MD has been applied in mice to reveal that the underlying pathophysiology of amblyopia relies on mechanisms of long-term depression at thalamocortical synapses (Frenkel and Bear, 2004; Crozier et al., 2007; Blais et al., 2008; Linden et al., 2009; Smith et al., 2009; Yoon et al., 2009; Cooper and Bear, 2012; Cooke and Bear, 2014). MD has also been applied in rodents to study novel treatment approaches such as environmental enrichment (Sale et al., 2007), dark exposure (Montey and Quinlan, 2011), pharmacologic retinal silencing (Fong et al., 2016; Fong et al., 2021) and ketamine (Grieco et al., 2020). The VCA has been used successfully to study amblyopia after MD in rats (Baroncelli et al., 2013; Sansevero et al., 2019). However, only one study to date has employed the VCA in mice to study stereoscopic vision in the context of MD with limited detail (Mazziotti et al., 2017).

Recognizing the need for a reliable behavioral assay to study stereoscopic deficits in mice, we aimed to systematically characterize the VCA, probe the effects of clinically relevant manipulations of binocularity on the VCA, and define amblyopic deficits using the VCA in mice. We hypothesized that the VCA can detect binocular deficits in mice secondary to early-life, long-term MD. Through our comprehensive approach, we assess the feasible of using the VCA behavioral paradigm in studies of amblyopic stereoacuity deficits in mice.

Results

The VCA requires visual input

To characterize the VCA, we first quantified the inherent tendency of mice to stay on the "safe" side under normal lighting conditions. Female and male mice (P45-58, n=7) were placed on the middle of a clear square box (Fig. 1A) facing the "safe" side and allowed to freely explore the arena for 5 minutes as previously described (Mazziotti et al., 2017). Sessions were video recorded from above and behaviors were scored by percent time spent on the safe side by each minute (Fig. 1B), the time to first zone transition (Fig 1C), and the number transitions between zones by minute (Fig. 1D). On average, animals spent 96.0±4.8% (95% CI) of the first minute on the safe side. A non-parametric repeated Friedman test showed a main effect of test minute on the proportion of time spent on the safe side over the 5-minute test (p =.0060). Pairwise comparisons revealed that time spent on the safe side decreased significantly to 60.0±22.2% (Δ=37.5%; p=.0162) and 57.0±22.2% (Δ=40.6%; p=.0021) seconds during the third and fourth minutes, respectively (Fig. 1B). On average, animals made the first zone transition to the cliff side at 66.0±15.9 seconds (Fig. 1C). Repeated Friedman test showed a main effect of test minute on the number of zone transitions (p=.0088). We observed a significant increase in the number of zone transitions by the third (1st min: 0.6 ± 0.7, 3rd min: 4.1 ± 2.5; p=.0211), fourth (4.1±1.7; p=.0162), and fifth minutes (3.9±1.8; p=.0211) (Fig. 1D). The exploration index (EI; difference between time on safe and cliff sides divided by the total time), a standard single measure of VCA performance, was 0.44±0.23 (Fig 1E).

To determine the contribution of vision, we tested VCA performance in complete darkness using night vision goggles in a separate cohort of animals (P45-58, n=7) (Fig. 1F). On average, animals spent 58.6±8.2% of the first minute on the safe side (Fig 1G), significantly less than in the light (p=.0006; Mann Whitney test). Repeated measures ANOVA showed no effect of test minute on the proportion of time spent on the safe side (p=.3765) (Fig 1G), that ranged between 49.3–59.6% over the 5-minute test. On average, animals made the first zone transition to the cliff side at 10.1±7.9 seconds (Fig. 1H), significantly sooner than in the light (p<.0001, unpaired t test). Repeated measures ANOVA showed no effect of test minute on the number of zone transitions (p=.8995) (Fig 1I). EI was significantly decreased in the dark (0.11±0.09) compared to the light (p=.0006, unpaired t test) (Fig 1E vs J). Taken together, these data unambiguously indicate that vision is required for normal performance on the VCA in mice, and that visual cues deter zone transitions in the first 2 minutes of the test.

Test-retest stability of VCA performance

Recognizing that experiments involving a treatment or intervention may benefit from multiple runs in the VCA, we next examined test-retest reliability. We modified the assay to only test animals for two minutes to avoid habituation to the cliff side in the first trial (see Fig. 1). A new cohort of female and male mice (P48-68, n=20) was run 4 times at 1-week intervals. Repeated measures Friedman test showed a borderline, but ultimately statistically non-significant effect of trial number on percent of time spent on the safe side (p=.0589) (Fig 2A). Given the marginal effect of trial number and our objective to assess repeatability over weeks, we examined pairwise comparisons. Between week 0 and week 1, there was no change in time spent on the safe side (p=.0825). There was a main effect of trial number on the time to first zone transition (p=.0248) that was carried by a decreased between the first and second trial (Fig. 2B, p=.0212). There was a main effect of trial number and the number of zone transitions (p=.0218) with no pairwise differences with the first trial (Fig. 2C, p values>.1501). Thus, VCA performance remains relatively stable over weeks.

Disruptions of binocularity impair VCA performance

Knowing that VCA performance requires vision, we next tested the contribution of binocularity in the 2-minute test. VCA performance has previously been shown to drop under monocular conditions (with one eye sutured closed) in both rats (Baroncelli et al., 2013) and mice (Mazziotti et al., 2017), so we began by replicating this result. Male and female mice (P42-60, n=11 per group) had one or both eyes sutured closed under isoflurane anesthesia 1-3 hours prior to VCA testing. Control (sham) animals underwent the same manipulations, but the eyes were immediately re-opened. Eye closure decreased performance in a dose-dependent manner across all metrics (Fig. 3A–D). ANOVA showed a main effect of eye closure on percent time spent on the safe side (p<.0001). Time spent on the safe side decreased by 14.0% with one eye closed (p=0.0082) and 29.7% with both eyes closed (p<.0001) (Fig. 3A). There was a main effect of eye closure on time to first zone transition (p=.0013) with a decrease of 38.5±8.8 seconds with one eye closed (p=.0068) and 11.2±6.7 seconds with both eyes closed (p=.0025) (Fig. 3B). There was a main effect of eye closure on the number of zone transition (p=.0006) (Fig. 3C) with an increase of 5.3±2.5 with one eye closed (p=.0085) and 6.4±2.1 with both eyes closed (p=.0019). The dose-dependence of eye closure on VCA performance corroborates the dependence of the VCA on vision (Fig. 1), but also suggests a contribution of binocular function. Considering that differences in movement may confound VCA performance comparisons between groups, we used open-source pose estimation software (EzTrack (Pennington et al., 2021)) to quantify the total distance traveled during the 2-minute test. There was a main effect of eye closure on the total distance traveled (p=.0006) (Fig 3D). Pairwise comparisons revealed a significant increase of 501 cm (45%) in total distance traveled when both eyes were closed (p=.0009), but no significant change when only one eye was closed (248 cm; p=.0990). Therefore, we can at least partially attribute VCA performance deficits under monocular conditions to a lack of binocularity.

Monocular occlusion significantly narrows the visual field particularly in mice and could influence VCA performance, which is globally dependent on vision (Fig. 1). To better delineate whether VCA performance is dependent on balanced binocular inputs rather than a narrowing of the visual field, we next tested the influence of optical manipulations. Spherical aberration degrades spatial acuity and stereopsis, particularly with interocular disparity (Jimenez et al.,

2008). The optics of the murine eye impart significant susceptibility to optical aberration as a function of pupil size (Gomes et al., 2023), likely owing to the relative sphericity of the lens. We applied atropine to dilate the pupil of one or both eyes and tested animals for 2 minutes in the VCA ≥60 minutes later (P38-42, n=12–13 per group, Fig. 3E–H). ANOVA showed a main effect of atropine on time spent on the safe side (p=.0160). Pairwise comparisons revealed that monocular atropine decreased the percent time spent on the safe side by 11.5% (p=.0083) whereas binocular atropine imparted no significant change (p=.1464) (Fig. 3E). Atropine imparted no main effect on the time to first zone transition (p=.3551) (Fig. 3F) or the number of zone transitions (p=.1826) (Fig. 3G). In contrast to eye closure, atropine imparted no change in total distance traveled during the test (p=.5630) (Fig. 3H). Therefore, the effect of atropine on time spent on the safe side can be attributed to binocular imbalance. These data demonstrate that disruption of balanced binocular input is sufficient to impair VCA performance.

Amblyopic mice demonstrate binocular dysfunction in the VCA

We next tested the effects of amblyopic visual deficits via MD in the VCA. Female and male mice underwent long-term (3 weeks) MD or sham eyelid closure/opening (Sham control n=44, MD n=52) from P21–P42 and were tested in the VCA 1-3 hours after eye opening (Fig. 4A). A subset of animals (n=19 each group) was retested 2 weeks later.

Amblyopic mice showed impaired performance across all VCA metrics (Fig. 4B–D). MD decreased time spent on the safe side by 7.5% compared to control animals (p=.0070) (Fig. 4B). MD decreased the time to first zone transition by 16.5 seconds (20.6%) (p=.0243) (Fig. 4C) and increased the number of zone transitions (p=.0393) (Fig. 4D). MD had no effect on total distance traveled (p=.6218) (Fig. 4E), suggesting that amblyopia does not increase ambulatory movement as occlusion does.

To assess the stability of these deficits, VCA performance metrics were compared between timepoints (Fig. 4F–H). Comparisons between control and MD animals at the 2-week timepoint were under-powered, so we focused our analysis on within animal stability in the MD group. There was no change in time spent on the safe side (p=.8006) (Fig. 4F). Time to first zone transition decreased by 19.1±16.6 seconds on average (p=.0236) (Fig 4I), but there was no change in the number of zone transitions (p=.1626) (Fig. 4J). Follow-up performance largely fell below that of control animals (Fig. 4F–H, gray bars). MD had no effect on total distance traveled at the 2-week timepoint (p=.8667) (Fig. 4I). Taken together, these data demonstrate that MD imparts a stable amblyopic deficit in binocular function in mice to which multiple VCA metrics are sensitive.

While observing animal behavior over the course of the test, we noticed that many animals approach the cliff side, extend their nose, and then retract. Suspecting this behavior may represent visual detection of the cliff, we counted the number of retractions over the 2-minute test. MD decreased the mean number of retractions from 3.4±1.0 (control) to 2.0±0.6 (p=.0208) (Fig. 5A). The number of retractions by amblyopic animals remained stable (p=.0979) and below

the range for control animals on retesting 2 weeks later (Fig. 5B). Thus, retraction behavior is also sensitive to amblyopic deficits assessed through the VCA.

Materials and methods

Mouse Studies

All experimental procedures followed the guidelines of the National Institutes of Health and the Association for Assessment and Accreditation of Laboratory Animal Care International and adhered to protocols approved by the Committee on Animal Care at MIT. Female and male C57BL/6 mice were housed in groups of 2-5 same-sex littermates beginning at P21 after being bred in the MIT animal colony. Mice had access to food and water *ad libitum* and were kept in a temperature-controlled room and maintained on a 12-hour light/dark cycle. Animals with any apparent congenital ocular abnormalities were excluded.

Eyelid Suture

For monocular deprivation experiments, eyelid suture (or sham eyelid suture) occurred at p21. Mice were anesthetized via inhaled isoflurane (1-3% in oxygen). Both eyes were inspected for abnormalities and kept lubricated using single-use artificial tears. The right eyelid was closed with two horizontal mattress sutures using 7-0 Prolene (Ethicon, polypropylene, 8648 G). Toesnails on the ipsilateral forepaw and hind paw were trimmed. Sham littermate controls had their eyes reopened immediately after being sutured shut. For reopening of the eye following long-term MD, mice were anesthetized and maintained as prior. The sutures were cut and removed from the deprived eye and adhesions were lysed. Sham controls were kept under isoflurane for an equivalent amount of time. Mice whose eyelids were not fully shut at the time of eye reopening or demonstrated any apparent ocular injury were excluded.

Atropine Administration

Mice were anesthetized and maintained as described above. Atropine Sulfate Ophthalmic Ointment 1% from Bausch and Lomb was administered to the right eye of mice in the monocular atropine condition and both eyes in the binocular atropine condition. For control and untreated eyes were administered bacitracin, neomycin, and polymyxin ophthalmic ointment.

Whisker Trimming

All mice had their whiskers trimmed 1 hour before performing the VCA to reduce use of non-visual cues during the test. Monocularly deprived mice and their sham littermate controls had their whiskers trimmed using small clippers just prior to turning off anesthesia following the suture removal as previously described. Mice in the atropine experiment had their whiskers trimmed following administration of ointment.

Visual Cliff Assay

All visual cliff experiments were conducted in a quiet, temperature-controlled room during the 12-hour light cycle. The apparatus consisted of an open field behavioral box with transparent plexiglass wall and floor (42cm x 42cm x 30.5cm) was placed on the edge of a bench with half the box extended out with a separate black box placed along the walls of the apparatus. A black and white stripe cover was placed on the bench top and dropped down vertically to the floor to create the illusion of a drop off, or "cliff", with a petri dish placed in the middle of the box as seen in Fig. 1A. A 1080P wide angle ELP 100fps USB camera with infrared capability was kept 52cm above the plexiglass floor of the box and was connected to a computer to record each session.

Immediately following whisker removal, mice were brought to the same room as the apparatus and habituated for at least 30 minutes in their home cage before beginning each trial. Mice were placed on the petri dish facing the safe side in all cases. The trial ended 2–5 minutes after the animal stepped off the petri dish. The surface of the plexiglass chamber was cleaned with Peroxigard in between all trials. In all test-retest experiments, the chamber was rotated between trials for each animal to mitigate contributions by visual cues not related to the cliff.

Visual cliff performance was scored manually using a stopwatch to calculate the percent time the mouse spent on the safe side, the number of zone transitions between sides, and the time of the first transition to the cliff side by an experimenter blinded to condition. A zone transition was defined as all four limbs of the mouse crossing to the next zone. Manually scored metrics were confirmed using the open-source pose estimation software ezTrack (https://eztrack.studio), which was also used to measure total distance traveled and locomotion for each animal. Fidelity of automated tracking was poor for experiments recorded in the dark using the infrared setting, so total distance traveled could not be reliably measured and compared for this condition.

Experimental design and statistical tests

Pilot experiments were run for each study using 3-5 animals (not included in final data), and necessary sample sizes were calculated using the G* power (Faul et al., 2007). All other statistical testing was performed using GraphPad (Prism version 9.0 for Mac, GraphPad Software, San Diego, California USA). Normality of all datasets and groups were tested using the normality test Shapiro-Wilk. Datasets containing non-normally distributed data were tested using non-parametric tests (Kruskal-Wallis, Friedman or Wilcoxon tests). Normally distributed data were analyzed by ANOVA or *t* tests. $p<.05$, adjusted for multiple comparisons, was considered statistically significant in all cases.

Discussion

We systematically characterized the VCA as a method to study binocular perceptual deficits in mice. Our results corroborate a diverse history of VCA application in various species from rodents to humans (Gibson and Walk, 1960; Fox, 1965; Baroncelli et al., 2013). Mice are the preferred species to study experience-dependent neuroplasticity of visual processing despite important differences from humans with respect to neuroanatomy and circuitry of the visual

system (Hooks and Chen, 2020). Mechanistic studies in mice are best validated through behavioral studies that demonstrate functional relevance. Here, we provide clear evidence that the VCA relies on balanced binocular inputs for optimal performance in mice. The VCA is an attractive paradigm with considerable advantages including relatively fast execution (2 minutes), no need for training, repeatability, and readout through several simple metrics (one of which, the retraction index, we report here for the first time). However, the assay also has important limitations, as evidenced through our results, that should be weighed carefully when considering its use.

Monocular occlusion, pupillary dilation (optical aberration), and amblyopia degraded performance in the VCA, supporting the role of binocular sensory integration as required for the task. That monocular pupillary dilation significantly reduced % time spent on the safe side whereas bilateral atropine did not (Fig. 3E) highlights the need for balanced visual input in binocular integration. Monocular blur more significantly degrades stereopsis as compared with binocular blur (Westheimer and McKee, 1980; Legge and Gu, 1989). However, the manipulations that disrupted binocular function did not drive VCA performance to levels we observed running animals in the dark (Fig. 1G–J) or with both eyes closed (Fig. 3A–C). Manipulations of binocular function in our studies, such as unilateral pupillary dilation and amblyopia after MD, may only partially disrupt binocular function. This cannot be the sole explanation for this disparity as we demonstrated a graded effect with eye closure that unambiguously precludes binocular function. Another mode by which animals can detect depth is by discerning changes in monocular cues with movement, called motion parallax (Ellard et al., 1984; Ellard et al., 1986). Motion parallax may account for the partial effects of complete disruption of binocularity on VCA performance and is an unavoidable potential confounder in the VCA.

Only one prior study applied the VCA following monocular deprivation in mice (Mazziotti et al., 2017). The duration of MD was short (3 days beginning at P24), and animals were tested immediately after eye opening. It is well-established that the visual responses following <6 days of MD during the critical period spontaneously recover with restoration of binocular experience alone (Hofer et al., 2006; Kaneko et al., 2008). Reversible axial length shortening that accompanies eyelid suture may exacerbate hyperopia of the murine eye and contribute to the visual deficits observed immediately after eye opening and the transitory nature of the MD effect. This raises concern that the binocular deficits revealed immediately after eye opening may reflect (possibly overlayed) non-amblyopic visual changes. By contrast, long-term (>6 days) MD reliably produces a stable ocular dominance shift, and our data indicated stability of binocular deficits behaviorally over at least 2 weeks (Figs. 4,5).

Strengths of our study include the systematic approach to fully characterize VCA performance in mice and its dependence on binocular vision. Our primary experiments are well-powered and thus provide a guide for future experiments seeking to employ this assay. The pattern of behavior was consistent across experiments in our study. Manipulations that decreased the time spent on the safe side, thereby reducing the exploration index, were frequently associated with changes in the latency to and frequency of zone transitioning. Comparisons across timepoints (both within and between trials, unique to our study) and cross-correlation between multiple variables beyond

time spent on the safe side to include metrics on zone transitioning and retraction behavior serve to validate our results using the VCA and its utility in studies of binocular function in mice.

The main limitation of the VCA assay is the relatively small effect sizes (7-14%), which we found consistently across experiments and VCA metrics. This necessitated the use of >40 animals to make reliable comparisons across groups of mice to reveal a meaningful binocular deficit with at least 80% power after MD (Fig. 4). Our effect size for binocular requirement in the VCA is consistent with those reported previously; unilateral eye closure reduces time on the safe side by ~15% in mice (Mazziotti et al., 2017). The effect is larger in rats at 31% (Baroncelli et al., 2013). However, larger effects were reported after MD for both mice (~20%) (Mazziotti et al., 2017) and rats (15%) (Sansevero et al., 2020). Modest effect sizes limit the utility of the assay to demonstrate group differences and partial within animal (e.g. recovery after treatment) effects. Based on our observations in amblyopia (Fig. 4), to achieve 80% power, hypothetical treatments restoring 100%, 75%, 50% binocular function would require 42, 73, and 165 animals per group to statistically demonstrate an effect. Therapies aimed at restoring binocular function in mice evaluated using the VCA should not be discounted if adequate numbers are not employed. We made multiple iterations of and adjustments to the VCA to address this limitation without success. Re-structuring the assay to better employ the wider superior visual field in mice, promote a forced-choice, and reduce the impact of chance led to the pole-decent task to measure depth discrimination in mice which showed a more favorable 62.5% effect size (Boone et al., 2021). Systematic characterization of this task and testing of the effect of MD have yet to be reported but would be of great interest.

In conclusion, application of the VCA in mice can detect binocular dysfunction with high clinical relevance. We provide a strong foundation on which to base future study design employing the VCA in mice including anticipated reliability of test-retest variance and effect size following MD. While our results demonstrate the necessity of balanced binocular inputs for optimal VCA performance, the VCA has major limitations. VCA performance is variable in mice and potentially confounded by monocular cues. Small effect sizes across VCA metrics limit the power and utility of the assay to detect partial effects. Investigators should consider and account for these limitations when seeking to employ the VCA as a tool to measure binocular function in mice.

References

Baroncelli L, Braschi C, Maffei L (2013) Visual depth perception in normal and deprived rats: effects of environmental enrichment. Neuroscience 236:313-319.

Birch EE (2013) Amblyopia and binocular vision. Progress in retinal and eye research 33:67-84.

Blais BS, Frenkel MY, Kuindersma SR, Muhammad R, Shouval HZ, Cooper LN, Bear MF (2008) Recovery from monocular deprivation using binocular deprivation. J Neurophysiol 100:2217-2224.

Boone HC, Samonds JM, Crouse EC, Barr C, Priebe NJ, McGee AW (2021) Natural binocular depth discrimination behavior in mice explained by visual cortical activity. Curr Biol 31:2191-2198 e2193.

Bradfield YS (2013) Identification and treatment of amblyopia. Am Fam Physician 87:348-352.
Cooke SF, Bear MF (2014) How the mechanisms of long-term synaptic potentiation and depression serve experience-dependent plasticity in primary visual cortex. Philosophical transactions of the Royal Society of London Series B, Biological sciences 369:20130284.
Cooper LN, Bear MF (2012) The BCM theory of synapse modification at 30: interaction of theory with experiment. Nat Rev Neurosci 13:798-810.
Cornwell P, Overman W, Levitsky C, Shipley J, Lezynski B (1976) Performance on the visual cliff by cats with marginal gyrus lesions. J Comp Physiol Psychol 90:996-1010.
Crozier RA, Wang Y, Liu CH, Bear MF (2007) Deprivation-induced synaptic depression by distinct mechanisms in different layers of mouse visual cortex. Proc Natl Acad Sci U S A 104:1383-1388.
Drager UC (1978) Observations on monocular deprivation in mice. J Neurophysiol 41:28-42.
Ellard CG, Goodale MA, Timney B (1984) Distance estimation in the Mongolian gerbil: the role of dynamic depth cues. Behav Brain Res 14:29-39.
Ellard CG, Goodale MA, Scorfield DM, Lawrence C (1986) Visual cortical lesions abolish the use of motion parallax in the Mongolian gerbil. Exp Brain Res 64:599-602.
Faul F, Erdfelder E, Lang AG, Buchner A (2007) G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 39:175-191.
Fong MF, Mitchell DE, Duffy KR, Bear MF (2016) Rapid recovery from the effects of early monocular deprivation is enabled by temporary inactivation of the retinas. Proc Natl Acad Sci U S A 113:14139-14144.
Fong MF, Duffy KR, Leet MP, Candler CT, Bear MF (2021) Correction of amblyopia in cats and mice after the critical period. Elife 10.
Fox MW (1965) The visual cliff test for the study of visual depth perception in the mouse. Anim Behav 13:232-233.
Frenkel MY, Bear MF (2004) How monocular deprivation shifts ocular dominance in visual cortex of young mice. Neuron 44:917-923.
Gibson EJ, Walk RD (1960) The "Visual Cliff". Scientific American 202:64-71.
Gomes J, Sapkota K, Franco S (2023) Central and Peripheral Ocular High-Order Aberrations and Their Relationship with Accommodation and Refractive Error: A Review. Vision (Basel) 7.
Green PR, Davies IB, Davies MN (1993) Interaction of visual and tactile information in the control of chicks' locomotion in the visual cliff. Perception 22:1319-1331.
Grieco SF, Qiao X, Zheng X, Liu Y, Chen L, Zhang H, Yu Z, Gavornik JP, Lai C, Gandhi SP, Holmes TC, Xu X (2020) Subanesthetic Ketamine Reactivates Adult Cortical Plasticity to Restore Vision from Amblyopia. Curr Biol 30:3591-3603 e3598.
Heesy CP (2004) On the relationship between orbit orientation and binocular visual field overlap in mammals. Anat Rec A Discov Mol Cell Evol Biol 281:1104-1110.
Hofer SB, Mrsic-Flogel TD, Bonhoeffer T, Hubener M (2006) Prior experience enhances plasticity in adult visual cortex. Nat Neurosci 9:127-132.
Hooks BM, Chen C (2020) Circuitry Underlying Experience-Dependent Plasticity in the Mouse Visual System. Neuron 106:21-36.
Jimenez JR, Castro JJ, Jimenez R, Hita E (2008) Interocular differences in higher-order aberrations on binocular visual performance. Optometry and vision science : official publication of the American Academy of Optometry 85:174-179.

Kaneko M, Hanover JL, England PM, Stryker MP (2008) TrkB kinase is required for recovery, but not loss, of cortical responses following monocular deprivation. Nat Neurosci 11:497-504.
Kaufman LW (1976) Duration of early visual experience and visual cliff behavior of chicks. Dev Psychobiol 9:1-4.
Legge GE, Gu YC (1989) Stereopsis and contrast. Vision Res 29:989-1004.
Levi DM (2006) Visual processing in amblyopia: human studies. Strabismus 14:11-19.
Levi DM, Knill DC, Bavelier D (2015) Stereopsis and amblyopia: A mini-review. Vision Res 114:17-30.
Li YT, Chou XL, Tao HW (2014) Monocular Deprivation in Mice. Bio Protoc 4.
Linden ML, Heynen AJ, Haslinger RH, Bear MF (2009) Thalamic activity that drives visual cortical plasticity. Nat Neurosci 12:390-392.
Maurer D, McKee SP (2018) Classification and diversity of amblyopia. Visual neuroscience 35:E012.
Mazziotti R, Baroncelli L, Ceglia N, Chelini G, Sala GD, Magnan C, Napoli D, Putignano E, Silingardi D, Tola J, Tognini P, Arthur JSC, Baldi P, Pizzorusso T (2017) Mir-132/212 is required for maturation of binocular matching of orientation preference and depth perception. Nat Commun 8:15488.
McConaghy JR, McGuirk R (2019) Amblyopia: Detection and Treatment. Am Fam Physician 100:745-750.
Montey KL, Quinlan EM (2011) Recovery from chronic monocular deprivation following reactivation of thalamocortical plasticity by dark exposure. Nature communications 2:317.
Morrison PR (1982) Distance cues and depth avoidance on the visual cliff. Percept Mot Skills 54:1195-1198.
Pennington ZT, Diego KS, Francisco TR, LaBanca AR, Lamsifer SI, Liobimova O, Shuman T, Cai DJ (2021) ezTrack-A Step-by-Step Guide to Behavior Tracking. Curr Protoc 1:e255.
Prusky GT, West PW, Douglas RM (2000) Behavioral assessment of visual acuity in mice and rats. Vision Res 40:2201-2209.
Rodkey EN (2015) The visual cliff's forgotten menagerie: rats, goats, babies, and myth-making in the history of psychology. J Hist Behav Sci 51:113-140.
Sale A, Maya Vetencourt JF, Medini P, Cenni MC, Baroncelli L, De Pasquale R, Maffei L (2007) Environmental enrichment in adulthood promotes amblyopia recovery through a reduction of intracortical inhibition. Nat Neurosci 10:679-681.
Samonds JM, Choi V, Priebe NJ (2019) Mice Discriminate Stereoscopic Surfaces Without Fixating in Depth. J Neurosci 39:8024-8037.
Sansevero G, Baroncelli L, Scali M, Sale A (2019) Intranasal BDNF administration promotes visual function recovery in adult amblyopic rats. Neuropharmacology 145:114-122.
Scholl B, Burge J, Priebe NJ (2013) Binocular integration and disparity selectivity in mouse primary visual cortex. J Neurophysiol 109:3013-3024.
Smith GB, Heynen AJ, Bear MF (2009) Bidirectional synaptic mechanisms of ocular dominance plasticity in visual cortex. Philosophical transactions of the Royal Society of London Series B, Biological sciences 364:357-367.
Stephany CE, Ma X, Dorton HM, Wu J, Solomon AM, Frantz MG, Qiu S, McGee AW (2018) Distinct Circuits for Recovery of Eye Dominance and Acuity in Murine Amblyopia. Curr Biol 28:1914-1923 e1915.

Stewart CE, Wallace MP, Stephens DA, Fielder AR, Moseley MJ (2013) The effect of amblyopia treatment on stereoacuity. Journal of AAPOS : the official publication of the American Association for Pediatric Ophthalmology and Strabismus / American Association for Pediatric Ophthalmology and Strabismus 17:166-173.
Wallace DK, Lazar EL, Melia M, Birch EE, Holmes JM, Hopkins KB, Kraker RT, Kulp MT, Pang Y, Repka MX, Tamkins SM, Weise KK (2011) Stereoacuity in children with anisometropic amblyopia. Journal of AAPOS : the official publication of the American Association for Pediatric Ophthalmology and Strabismus / American Association for Pediatric Ophthalmology and Strabismus 15:455-461.
Waugh KT (1910) The rôle of vision in the mental life of the mouse. Journal of Comparative Neurology and Psychology 20:549-599.
Webber AL, Wood J (2005) Amblyopia: prevalence, natural history, functional effects and treatment. Clin Exp Optom 88:365-375.
Westheimer G, McKee SP (1980) Stereogram design for testing local stereopsis. Invest Ophthalmol Vis Sci 19:802-809.
Wiesel TN, Hubel DH (1963) Single-Cell Responses in Striate Cortex of Kittens Deprived of Vision in One Eye. J Neurophysiol 26:1003-1017.
Williams B, Del Rosario J, Muzzu T, Peelman K, Coletta S, Bichler EK, Speed A, Meyer-Baese L, Saleem AB, Haider B (2021) Spatial modulation of dark versus bright stimulus responses in the mouse visual system. Curr Biol 31:4172-4179 e4176.
Witherington DC, Campos JJ, Anderson DI, Lejeune L, Seah E (2005) Avoidance of Heights on the Visual Cliff in Newly Walking Infants. Infancy 7:285-298.
Yoon BJ, Smith GB, Heynen AJ, Neve RL, Bear MF (2009) Essential role for a long-term depression mechanism in ocular dominance plasticity. Proc Natl Acad Sci U S A 106:9860-9865.

Figure legends:

Figure 1. VCA performance requires visual input. A. Graphical design of the VCA with measurements of the box and table that were used. Animals were placed on a petri dish (2cm height) in the middle of the platform facing the safe side and allowed to explore the arena for 5 minutes. **B.** Percent time spent on safe side (per minute) plotted as a function of test minute. Dotted line (y=50%) represents chance performance. **C.** Time to first zone transition from safe to cliff side plotted as a function of test minute. **D.** The total number of transitions between zones plotted as a function of test minute. **E.** Exploration index ([time on safe – time on cliff]/ total time) over the 5-minutes test. **F.** Graphical design of the VCA in the dark using a different cohort of animals. **G.** Percent time spent on safe side in the dark (per minute) plotted as a function of test minute. Dotted line (y=50%) represents chance performance. **H.** Time to first zone transition from safe to cliff side in the dark plotted as a function of test minute. **I.** The total number of transitions between zones in the dark plotted as a function of test minute. **J.** Exploration index over the 5-minutes test in the dark. Mean values are represented as bars for each test minute in each condition. Individual animals are represented as grey lines. Dunn's multiple comparison representations of statistical significance compared to the first minute in each condition: * <.05, ** <.01.

Figure 2. Test-retest stability of VCA performance. A. Percent time spent on safe side (average over each 2-minute trial) plotted as a function of testing week (1-week trial interval). Dotted line (y=50%) represents chance performance. **B.** Time to first zone transition from safe to cliff side plotted as a function of testing week. **C**. The total number of transitions between zones plotted as a function of testing week. Mean values are represented as bars for each test week. Individual animals are represented as grey lines. Dunn's multiple comparison representations of statistical significance compared to week 0: * <.05.

Figure 3. Disruptions of binocularity impair VCA performance. (**A–D**) Animals performed the 2-minute VCA with neither (sham), one or both eyes sutured shut. **A.** Percent time spent on safe side plotted for each eye closure group. Dotted line (y=50%) represents chance performance. **B.** Time to first zone transition from safe to cliff side plotted for each eye closure group. **C**. The total number of transitions between zones plotted for each eye closure group. **D.** Total distance traveled during the test plotted for each eye closure group. (**E–H**) Animals performed the 2-minute VCA after neither, unilateral or bilateral pupillary dilation via atropine ointment administration. **E.** Percent time spent on safe side plotted for each atropine group. Dotted line (y=50%) represents chance performance. **F.** Time to first zone transition from safe to cliff side plotted for each eye atropine group. **G**. The total number of transitions between zones plotted for each atropine group. **H.** Total distance traveled during the test plotted for each atropine group. Individual animals are represented as circles and mean ±SEM as black horizontal and vertical lines. Dunn's multiple comparison representations of statistical significance between groups: * <.05, ** <.01, *** <.001, **** <.0001.

Figure 4. MD causes stable binocular dysfunction in the VCA. A. Experimental timeline showing MD (or sham closure) performed from P21-P42. VCA was performed 1-3hrs after eye opening (**B–E**). **B.** Percent time spent on safe side plotted for each group. Dotted line (y=50%) represents chance performance. **C.** Time to first zone transition from safe to cliff side plotted for each group. **D**. The total number of transitions between zones plotted for each group. **E.** Total distance traveled during the test plotted for each group. (**F–I**) VCA was retested in a subset of animals 2 weeks later. **F.** Percent time spent on safe side plotted for the MD subset at test and retest. Dotted line (y=50%) represents chance performance. **G.** Time to first zone transition from safe to cliff side plotted for the MD subset at test and retest. **H**. The total number of transitions between zones plotted for the MD subset at test and retest. **I.** Total distance traveled during the test plotted for the MD subset at test and retest. Individual animals are represented as circles and mean ±SEM as black horizontal and vertical lines. Sham range of SEM is depicted as gray shaded region. Comparison representations of statistical significance between groups (Mann-Whitney) or between tests (paired Wilcoxon): * <.05, ** <.01.

Figure 5. MD decreases retraction behaviors during the VCA. A. Number of retractions over the 2-minute test plotted for each group. **B.** Number of retractions over the 2-minute test plotted for the MD subset at test and retest. Individual animals are represented as circles and mean ±SEM as black horizontal and vertical lines. Sham range of SEM is depicted as gray shaded region. Comparison representations of statistical significance between groups (Mann-Whitney): * <.05.

Figure 1

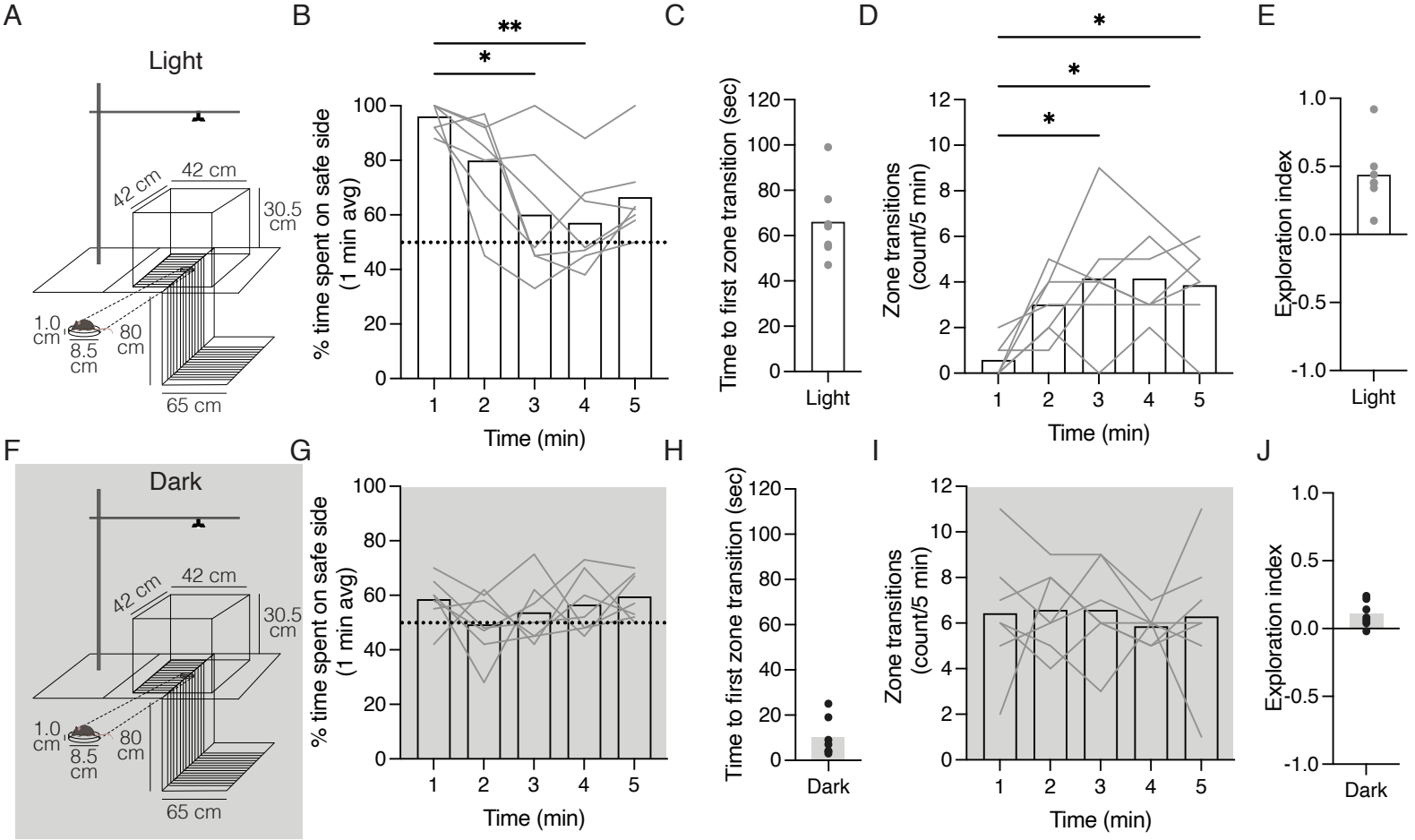

A
Light
42 cm
42 cm
30.5 cm
1.0 cm
8.5 cm
80 cm
65 cm
B
**
*
% time spent on safe side (1 min avg)
Time (min)
C
Time to first zone transition (sec)
Light
D
*
*
*
Zone transitions (count/5 min)
Time (min)
E
Exploration index
Light
F
Dark
42 cm
42 cm
30.5 cm
1.0 cm
8.5 cm
80 cm
65 cm
G
% time spent on safe side (1 min avg)
Time (min)
H
Time to first zone transition (sec)
Dark
I
Zone transitions (count/5 min)
Time (min)
J
Exploration index
Dark

Figure 2

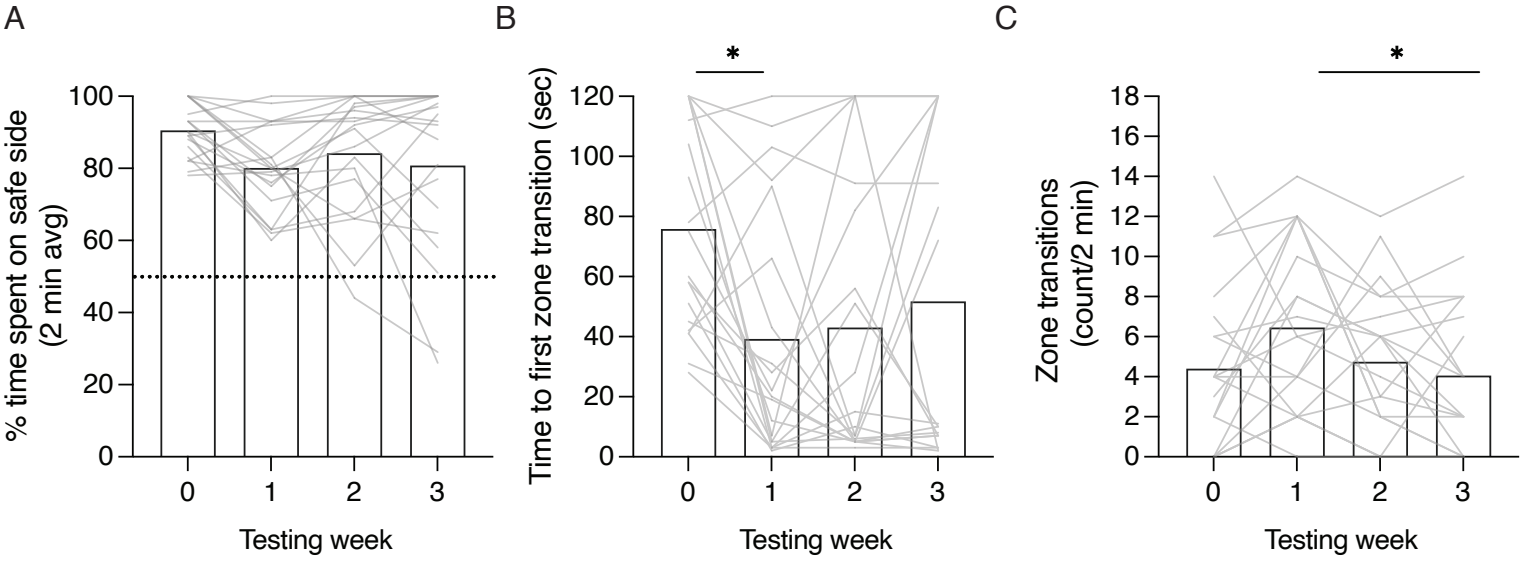

A
% time spent on safe side
(2 min avg)
100
80
60
40
20
0
0
1
2
3
Testing week
B
*
Time to first zone transition (sec)
120
100
80
60
40
20
0
0
1
2
3
Testing week
C
*
Zone transitions
(count/2 min)
18
16
14
12
10
8
6
4
2
0
0
1
2
3
Testing week

Figure 3

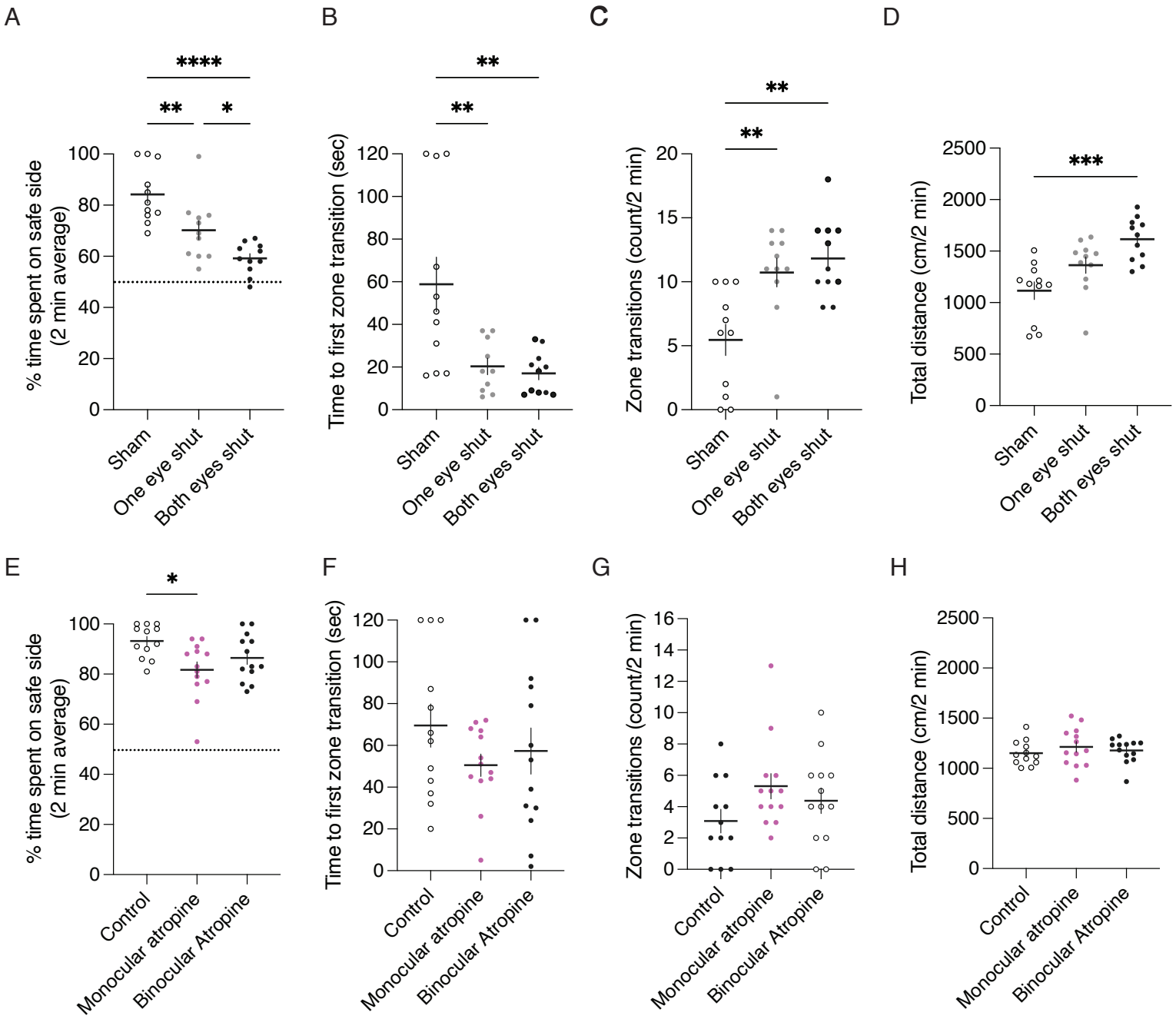

A
B
C
D
E
F
G
H
****
**
*
***
% time spent on safe side (2 min average)
Time to first zone transition (sec)
Zone transitions (count/2 min)
Total distance (cm/2 min)
Sham
One eye shut
Both eyes shut
Control
Monocular atropine
Binocular Atropine

Figure 4

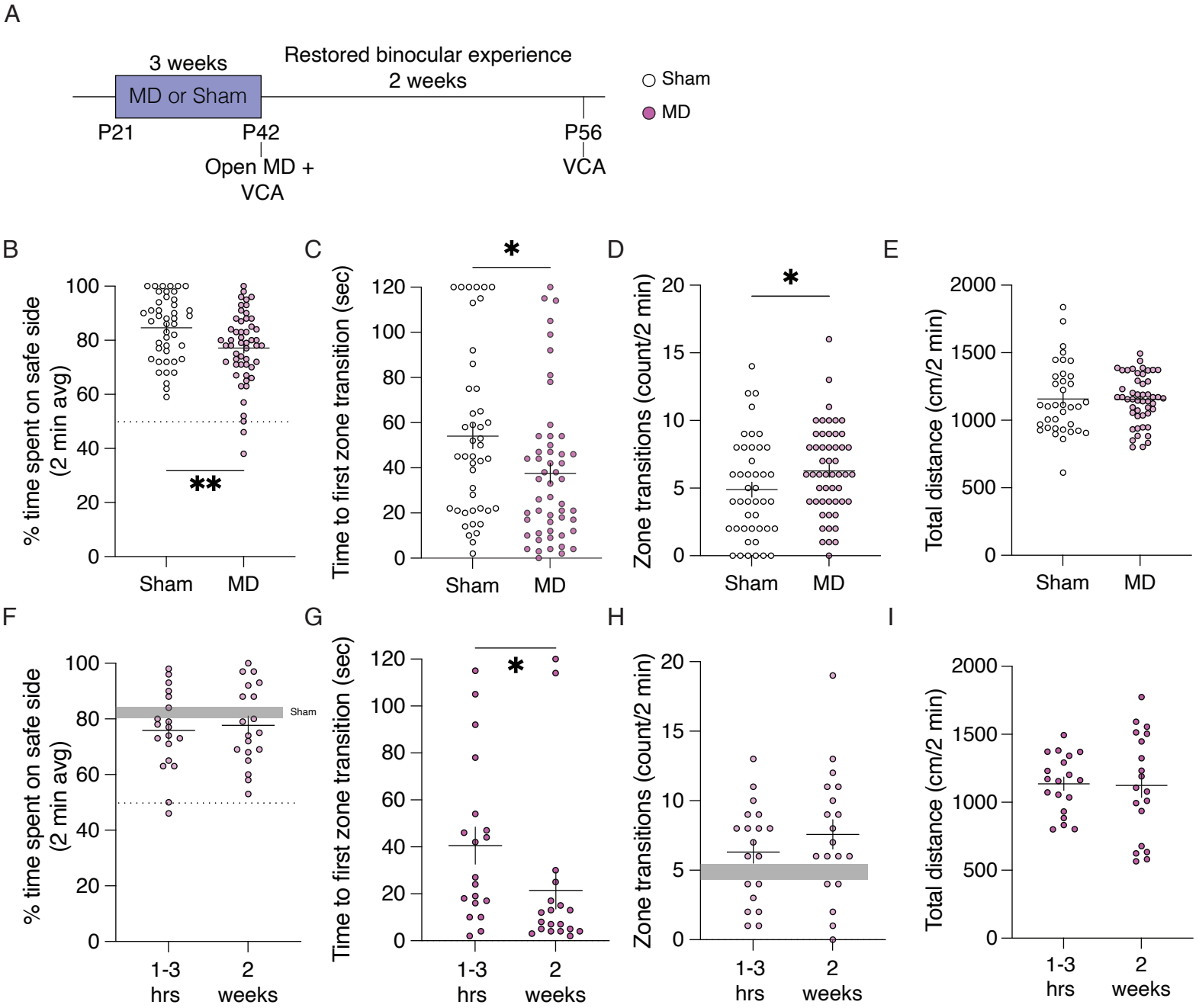
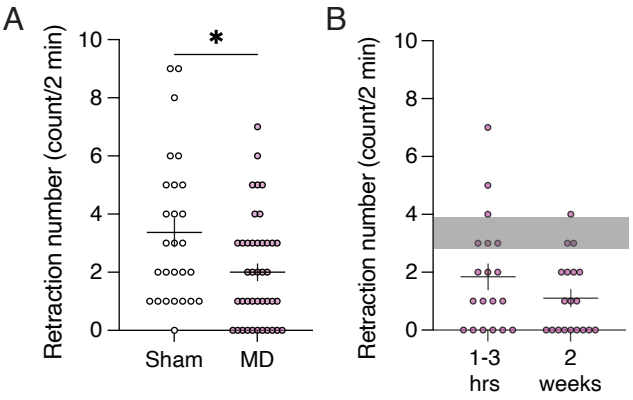

A
3 weeks
MD or Sham
Restored binocular experience
2 weeks
Sham
MD
P21
P42
P56
Open MD +
VCA
VCA
B
% time spent on safe side
(2 min avg)
Sham
MD
C
Time to first zone transition (sec)
D
Zone transitions (count/2 min)
E
Total distance (cm/2 min)
F
G
H
I
1-3
hrs
2
weeks

Figure 5


A
B
Retraction number (count/2 min)
10
8
6
4
2
0
*
Sham
MD
1-3 hrs
2 weeks