

# Behavioral testing and preliminary analysis of the hamster visual system

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**The dependence of visual orienting ability in hamsters on the axonal projections from retina to midbrain tectum provides experimenters with a good model for assessing the functional regeneration of this central nervous system axonal pathway. For reliable testing of this behavior, male animals at least 10–12 weeks old are prepared by regular pretesting, with all procedures carried out during the less active portion of the daily activity cycle. Using a sunflower seed attached to a small black ball held at the end of a stiff wire, and avoiding whisker contact, turning movements toward visual stimuli are video recorded from above. Because at the eye level, the nasal-most 30° of the visual field can be seen by both the eyes, this part of the field is avoided in assessments of a single side. Daily sessions consist of ten presentations per side. Measures are frequency of responding and detailed turning trajectories. Complete assessment of the functional return of behavior in this testing paradigm takes 3–6 months to complete.**

## INTRODUCTION

We were able to successfully use nanotechnology and molecular self-assembly to repair injured brain structures<sup>1</sup>. To achieve axonal regeneration after injury in the central nervous system (CNS), we had to overcome several formidable barriers, such as scar tissue formation after tissue injury, gaps in nervous tissue formed during phagocytosis of dying cells after injury and the failure of many adult neurons to initiate axonal extension. Using the mammalian visual system as a model, we used a designed self-assembling peptide nanofiber scaffold to create a permissive environment for axons to regenerate through the site of an acute injury and to knit the brain tissue together. In experiments using a severed optic tract in the hamster, we showed that regenerated axons reconnect to target tissues with sufficient density to promote functional return of vision, as evidenced by visually elicited orienting behavior.

The experiments utilized five separate protocols to be published separately: (i) handling and injection of nanoscale self-assembling materials in the brain of mammals; (ii) neurosurgical procedures for the transection of the optic tract in young and adult hamsters; (iii) behavioral testing and preliminary analysis of the hamster visual system; (iv) anatomical tracing and assessment of CNS regeneration in the visual system of rodents; and (v) in-depth behavioral analysis.

Here, we present the third protocol, developed for using animal behavior to test functional regeneration in the CNS. We followed this protocol information in our recently published experiments showing return of visual function with regeneration of the optic tract in Syrian hamsters<sup>1</sup>. The details are based on the experiments described in that report; the behavioral assessments in this study followed a series of earlier studies published between 1967 and 1992 using the Syrian or golden hamster, *Mesocricetus auratus*<sup>2,4-6,8,9,11,12,14</sup>.

In our recent experiments<sup>1</sup>, we assessed, in terms of frequency of positive responses, whether an animal responded visually, or did not respond, within 3 s following presentation of a lure (sunflower seed) in the visual field. We did not attempt to map the entire visual field for responsive and blind areas. This protocol will provide the

basics of animal behavior and the best location and time to test Syrian hamsters for visually guided behavior.

Although this protocol is used for testing behavioral function after a transection of the brachium of the superior colliculus, it could also be used to measure (i) behavioral responses to multiple stimuli and acuity testing; (ii) behavioral testing for treatments for macular degeneration, glaucoma or retinitis pigmentosa; (iii) survival of the retina and its components; and (iv) the behavior of genetically modified animals that have undergone loss of specific cones, testing for differential color perception and using orienting as an output.

This model is very robust and can be used for any indication of vision loss or restoration of vision in any of the aforementioned visual diseases. In addition, you could follow the progression of these diseases and then after treatment measure the change in the slope of the decline, or the increase, in the restoration. The motor readout from this model is very powerful and allows for the measurement of small changes in function over a wide visual field as well as a range of illumination.

## Advantages of using hamsters as experimental subjects

Assessing visually guided behavior has been well worked out for the Syrian or golden hamster<sup>1-14</sup>. Because of their high motivation to hoard seeds, which they put into cheek pouches, they require no food deprivation. The testing is straightforward if one understands the guidelines and procedures. This encouraged the choice of hamsters as the experimental subjects in our work on CNS regeneration. We have found that similar assessments of vision in mice and rats are more difficult, and may require many modifications to the procedures described below (RGE-B and GES, unpublished work).

## Specification of directions in reference to the animal

To make the directions uniform, we are using the following indications of direction. Rostral is toward the nose of the animal,



**Figure 1** | Figure showing the animal from the dorsal view. Right and left are from the perspective of the animal. Rostral (toward the nose) and caudal (toward the tail) are shown. When the seed is presented, it is usually presented on the right or left from caudal to rostral positions.

caudal is toward the tail of the animal and the right and left sides are always from the perspective of the animal (Fig. 1).

### Marking and labeling of individuals

It is important to mark animals individually to avoid mix-ups; marks are made while the animal is anesthetized during surgery by clipping the ears in the pattern shown in Figure 2. However, you can also use implantable electronic animal tags. These are small capsules that are inserted under the skin of the animal and read electronically with a handheld scanner.

### Groups

How do you decide how many animals are needed in each group? The total number of animals we used for the time course of recovery will be covered in another protocol. As this is the behavioral protocol, we will only give the numbers necessary to ensure validity and significance of results. The experimental group that receives a treatment should contain ten animals and the control groups should have at least five animals for each control used.

Depending on the type of operation and the part of the visual system being operated, additional animals may be necessary to get relevant statistics and show a valid result.

The animals were distributed in the following manner:

- Ten animals were normal controls, which received no operation.
- Ten animals were operated on and received the treatment.
- Ten animals were operated on and received the vehicle control; in this case, it was saline.

**Figure 2** | Ear marking is a way to identify the animal after surgery, as well as if there are multiple animals in the same cage. The ear cuts are made while the animals are anesthetized. All references to animal orientation are from the animal's perspective, i.e., A2 is a left ear cut even though it appears on the right of the picture.

- Five animals had one eye removed to determine spontaneous turning rates.

Also note that, because surgery was unilateral, the unaffected side of operated animals provided control data.

### Group formation: “blind” testing

Experimental and control animals are given identification numbers that provide no clue to group membership to the persons testing their behavior. The appearance of the animals should likewise not make group membership obvious. When some of them look different because of surgical procedures, some or all control animals are made to look similar, so as to exclude bias and to obtain a fair and reliable outcome.

### Understanding the visual fields

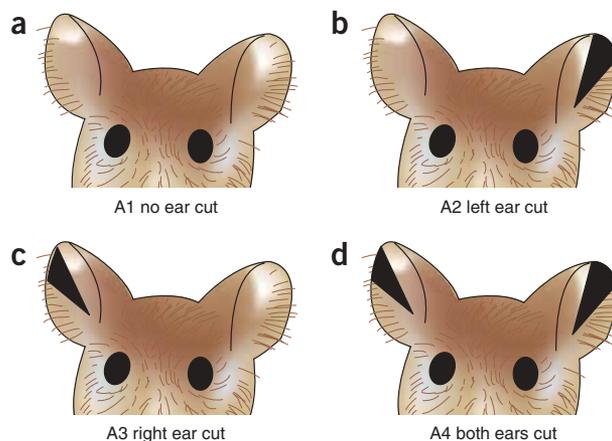
The target lure (sunflower seed) should be presented to the animal within its visual field on one side, which, at the level of the animal's eyes, extends from straight ahead to 135° laterally (Fig. 3). The temporal portion of this field extends from 60 to 135°. (The center of the eye, projected into the visual field, looks at a point 60° from the straight ahead position and 30° elevated from the eye level.) The field of one eye extends to as far as 30° into the opposite side (−30°); thus, the central 60° should be considered to be binocular (Fig. 3). Unless the untested eye is covered or blinded (enucleated), a turning response may be the result of the untested eye seeing the seed, which could give a false response. Moreover, the nasal-most 30° of an eye projects to both sides of the brain. It is therefore crucial to present the lure from the posterior approach (unless this is a probe trial). Furthermore, the lure should be presented less than 30° above or below eye level, to remain well outside the visual field of the untested eye (Fig. 4).

To maximize visibility and avoid whisker contact, the lure should generally be presented 15–30 cm from the head.

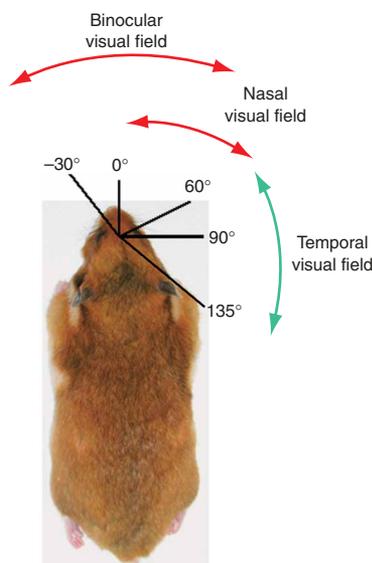
If it is important to do a more detailed mapping of the visual field, refer to the published description and accompanying photograph of an apparatus constructed for that purpose<sup>11</sup>.

### Order and frequency of testing

The order in which animals are tested on a given day is varied. This is because responsiveness of an animal can change with time of day in ways that can vary for unknown reasons among animals. During



## PROTOCOL

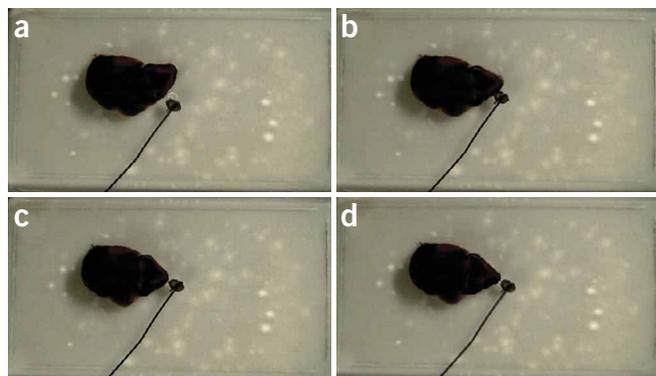


**Figure 3** | The visual field of a hamster from above. The hamster eye has a visual field of 165° when viewed dorsally. The nasal most 45° should be avoided (allowing an extra margin) because the animal will be able to see the stimulus with either the opposite eye or the ipsilateral projection. Most of the stimuli are presented in the area from 60 to 135°. The stimulus enters the field from behind.

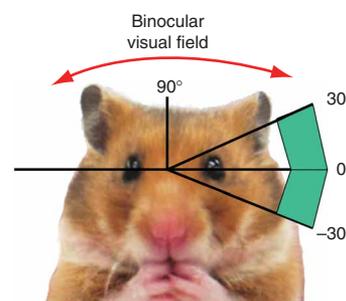
some sessions, one or more of the animals may obviously be ignoring the seeds, showing little or no motivation to get them. Such animals seem to be more interested in jumping down from the elevated testing surface than in obtaining the seeds. The entire session is aborted in such cases and repeated on a later date, at a different time of day. Some animals show much variation in responsiveness from day to day. Generally, animals are tested once every other day to monitor their progress.

### Duration of each testing session

Duration varies depending on the responsiveness of the animal. Twenty trials are sufficient on a good run if the animal is cooperative. If an animal is not responsive, even on the unaffected side, after repeated trials (~10×) over the entire visual field, then the animal is considered uncooperative. In addition, we can test the animal's motivation by touching the whiskers, which will elicit a head turning response. If this does not result in the taking of a seed and the animal continues to be unresponsive on the normal side,



**Figure 4** | The visual field of the animal from the front. When the stimulus is presented, it is important to stay below 30° from the horizontal. This will ensure that only the affected area of the eye will be used for behavior. If the stimulus reaches too high above the horizontal plane, the animal may see the stimulus with the opposite eye.



then it should be returned to the cage and tested later. (Because the animals only have a transection of one brachium of the superior colliculus, one side should be unaffected.)

### Time-of-day effects

It is best not to test the hamster during the time of greatest activity – from 2 h before to 4 h after the onset of darkness. In nature, this is the time of foraging, centered on the twilight period when the animal is most likely to be subject to predation. An anxious hamster will usually exhibit scanning and agitated movements when on the platform. It may be hyper-responsive due to an odor or noise.

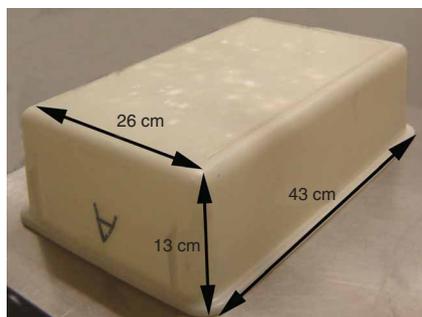
Notice the duration between frames in the illustrations and the amount of movement the animal performs from frame to frame (see **Supplementary Video 1** online). Freezing is a typical anti-predator behavior and could be caused by the time of day the animal is being tested. However, sometimes, it can be an initial sign that vision is returning to the animal (see **Supplementary Video 2** online). In our experience, the behavioral testing of this animal can be conducted 2 or 3 h after the onset of the light cycle (around 9 o'clock if the lighting is on between 06:00 and 18:00, i.e., during office hours, etc.) and testing should not be continued after 16:00.

### Orienting to vibrissal stimuli

Touch of the vibrissae (whiskers) is the most reliable stimulus for eliciting turning of the head. The experimenter must be aware of the length of the vibrissae so any visual stimulus is kept well beyond their range. However, when you want the hamster to respond to the presentation of a seed and it is too wary to respond visually, you can elicit a turning response by touching the vibrissae after the visual presentation. In **Figure 5**, the stimulus is touching the tips of the whiskers. This is done when testing begins to get the animal used to turning when the seed is presented. At first, the animal does not respond to the seed. After the animal is trained, it will readily orient toward the seed and take it. As testing progresses, whisker stimulation is used on the blind side of an animal to

**Figure 5** | The stimulus is touching the tips of the whiskers. This is done when testing begins to get the animal used to turning when the seed is presented. At first, the animal is too cautious to move toward the seed. After the animal is trained, it will orient toward the seed and take it. As testing progresses, whisker stimulation is used on the blind side of the animal to prevent a turning preference (see **Supplementary Video 3**). Each frame is 0.16 s from the previous; this is a very fast action elicited by touching the whiskers and not the side of the animal.

**Figure 6** | The cage bottom makes a great platform for behavioral testing. It measures 26 cm × 43 cm. This size is the minimum size that allows the animals to move freely over the area and still have enough room to present the stimulus. If the platform is too large, it is difficult to present the seed to the animal without moving, or waiting for the animal to come to a specific location. The cage is at the minimum height (13 cm) because the animal will want to climb down if it is any lower.



prevent the formation of a turning preference (see **Supplementary Video 3** online).

### Orienting to auditory stimuli

Novel auditory stimuli will often produce a surveying movement, in which the hamster stops locomotion (freezes), sits up

and moves the head back and forth as it attempts to locate the source of the sound. During periods of greater wariness of predators, the sound may lead to a rapid running response instead of the freezing response. The animal soon habituates to the ordinary sounds of the laboratory, so these auditory effects are not usually a problem.

### Place of testing

The best location for testing hamsters is the room where they are housed, because the background odor is most familiar to the animals. If this is not possible, the testing should be carried out in a room that is not contaminated by odors of animals other than hamsters, or by people not conducting hamster experiments.

### Preparation before testing

Behavioral testing will be more difficult with animals that have undergone recent traumatic experiences. These may include changing of cages and any loud noises while other cages were changed in the neighboring rooms. Also make sure you wait at least 2 weeks after any operation or procedure that required the animals to be anesthetized. These disturbances and procedures can cause aberrant behavior such as hyperactivity or sluggishness.

## MATERIALS

### ANIMALS NEEDED

Syrian or golden hamster, *M. auratus*

### ANIMAL SETUP

**How to select the hamsters** In hamsters, the skull and brain develop to their full size at age 12 weeks; behavioral assessment is easiest when animals are at least 10–12 weeks old. The animals reach sexual maturity as early as 5 weeks (females) or 6 weeks (males), but the adolescent animals are too hyperactive for easy testing. However, animals that are handled during this early period are more cooperative when testing begins at a later age. Both males and females can be tested. However, males are usually chosen, as in our recent experiments<sup>1</sup>, for two reasons: first, the females show a 4-day cycle of sexual receptivity, which can affect their behavior; second, if both females and males are used, special procedures are required because of the powerful effects of the female odors on male behavior. If the male detects female odor on the testing platform, he will be distracted by the odor and will decrease his responsiveness to other stimuli.

**Human handling** It is necessary to get the animals accustomed to the experimenters and to the testing situation and procedures before beginning the formal data collection. This is easiest if the animals have been handled during their development. However, with patience, it can be accomplished even for animals raised in greater isolation.

- Begin handling the hamsters when they are between 5–6 and 10–12 weeks in age, at least during cage cleaning times.

**Seed acceptance** Sunflower seeds are a favorite food of hamsters, and under the right conditions they will respond to visual presentations of a seed, turn toward it, take it into their mouth and transfer it into a cheek pouch. When an animal is responsive, this can be repeated many times in rapid succession, 30–50 times or even more, depending on the size of the seeds and the size of the hamster. Before a hamster readily makes such responses or even takes a seed presented to it by a human hand, it must become accustomed to the situation so that anti-predator responses have disappeared. To accomplish this:

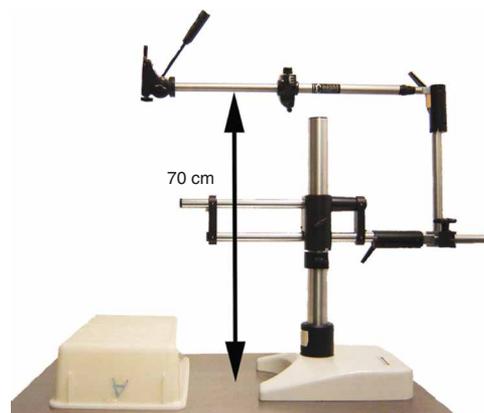
- Present seeds to the animal for 5 min daily, stimulating the whiskers. Begin by presenting seeds through the openings in the cage top. When this is

successful, remove the cage top and present seeds by hand. When this is successful, put the animal on the cage top and continue the procedure. For greater speed, do two sessions per day separated by at least a few hours.

- To place a hamster on the cage top or anywhere else, remove it from its cage by scooping it up in a disposable coffee cup. Another method is to insert the edge of the cage top into the cage and coax the animal to walk on it, and then move it back to the top of the cage with the animal on it. **! CAUTION** Do not reach into the hamster's cage and grab the animal. Remember that you are invading its home and this can elicit strong defensive reactions, even biting attacks, sometimes even by a tame animal. Once the animal is walking outside the home area, it is easier to handle. It will strongly resist being placed in the cup or on the cage top only at the beginning, and you can reduce or avoid such resistance by following the above procedures. By housing animals in groups, this type of behavior is greatly reduced.

**Effects of odors** Novel odors will reliably interrupt the hamster's movements. Problems for testing are generally caused only by the effects of female odors on males or by predator odors. **▲ CRITICAL** For successful testing of the hamsters, certain basic rules should be followed to avoid the disruptive effects of odors on their behavior. Investigators should not wear any perfumes. (The most disruptive are the more expensive perfumes containing substances derived from odor glands of animals.) Clothing that has contacted pet animals, especially dogs and ferrets, should be removed. Investigators should wear clean lab gowns and gloves. Special care must be taken to isolate hamsters from ferret colonies; odors clinging to

**Figure 7** | The cage bottom is on the left and the camera holder is on the right. We used a microscope stand and part of a tripod from a camera setup to make this apparatus. In other experiments, we have used a boom stand. The camera is approximately 70 cm from the top of the table. This gives the maximum view from the camera to enable recording of the turns with enough resolution using a standard lens. If the camera is too far away, the image will be grainy when blown up.



## PROTOCOL

**Figure 8** | The seed presentation device is a long wire with a small stopper that has been cut down and notched, so a sunflower seed can be attached each time. (a) The handle is 12 cm long attached to a 38-cm wire. (b) The close-up of the stopper and wire with a seed inserted. The wire is a coat hanger that is coated with plastic. Gradations are in cm.

the lab coats of personnel who have been in a ferret room can be very disruptive to hamsters. This is easily handled by the use of disposable lab coats. If these are not available, then washing the lab coat between testing sessions is fine.

**Right-left differences and group differences** The surgery in our experiment was a unilateral transection of the optic tract in hamsters. As the spared optic tract represents the visual field on the opposite side, and only the nasal-most portion of the field on the same side, we were able to use each animal as its own control by comparing responses to stimuli on the two sides, avoiding stimulation of the field within 45° of the midline. The nasal-most visual field is represented on both sides of the midbrain tectum; in behavioral testing of one-eyed animals, we see responses to stimuli no more than 30° into the nasal part of the opposite field.

In addition to the right-left comparisons, we included unoperated and sham-operated animals in control groups. We also included animals with unilateral eye removal, so that we could find the rate of spontaneous turning toward the blind side when a stimulus was presented there (Fig. 10). In the past, we had found such turning to occur in 12% of presentations. In the recent work, this baseline rate varied from 0 to 20% in different weeks, except during 2 earlier weeks of testing when it was 32 and 27%.

**Pretesting helpful hints** Before testing the animals, ensure that the labels are in place and the tester does not know anything about the animals. For best results, have two different testers who test the animals at different times.

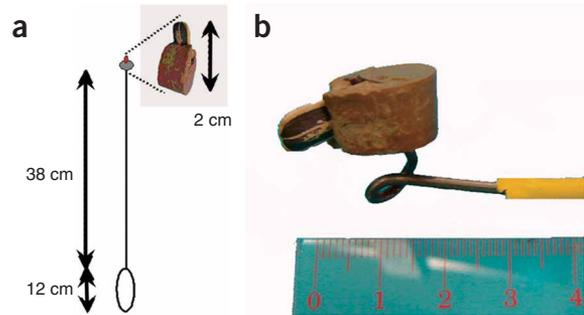
**Probe trials** During the first week, to get the animals ready to be tested, conduct a series of probe trials, which may last over 2 weeks. During this period, the seed is presented initially in front of the animal to get them interested and then is gradually moved to other parts of the visual field (Fig. 10). We began the trials at 6 weeks after surgery because this was the time we saw recovery in our earlier work. However, we have since started to test 3 weeks after surgery. It always takes longer to test the animals in the early stages of axonal regeneration.

### EQUIPMENT

Cages  
Video recording equipment  
Seed/stimulus device  
Sunflower seeds  
Lab coats, gloves, etc.

### EQUIPMENT SETUP

**Cages** Hamsters are housed in standard laboratory cages during the testing periods. Housing them individually in a cage is best, but two to four animals can be housed together if they do not fight after they recover from surgery. This is often possible if the animals are littermates and have been together since birth.



**Cage bottom** The cage bottom is a convenient and useful test platform, because the odors are very familiar to the animal. The cages we used have a bottom measuring 26 cm × 43 cm (Fig. 6).

**Platform** An alternative to the cage bottom as the test arena is an elevated platform placed on a cage or the equivalent, made of wood or plastic. A wooden platform is preferred by the hamsters. Wood may be more difficult to clean, but even with thorough cleaning of plastic, it is difficult to remove all traces of animal odors. It is important to use different platforms for males and females because of the distractions caused by odors of the opposite sex; males are most susceptible to this. Separate platforms for each individual would be ideal, but not essential.

**Video recorder** A video camera is mounted directly above the test platform so that accurate measures of azimuthal positions of the stimulus can be made (Fig. 7). If it is desirable to measure elevations as well, at least one other camera is necessary. We used only an overhead camera in our experiments.

**Lighting** The normal room lighting is acceptable. Reduced lighting can be very adequate and can result in more active animals. Bright lights should be avoided, and are not necessary for modern video recording.

**Visual background** High contrast for the visual stimulus is provided by the plain gray laboratory walls and ceilings.

**Seed/stimulus device** For visual stimulus presentations, attach a small black rubber ball or block, 1–1.5 cm in diameter, to the end of a 50-cm white wire, stiff enough to hold the ball without much bending. Coat-hanger wire works well. In the ball, cut a slot that will hold a sunflower seed by its edge (Fig. 8). The ball can be cut from the rubber stopper of a water bottle.

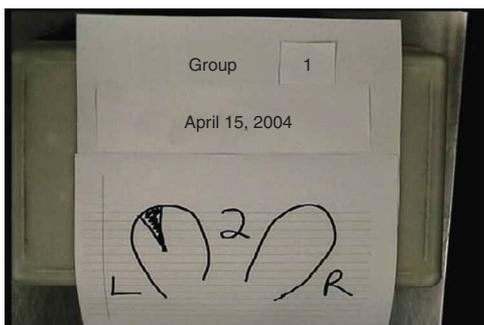
**Attire** Every animal facility has its own regulations for the attire that must be worn by the investigators when working with animals. We believe that every time an animal is touched, the investigator should always be wearing the same type of gloves. This will ensure that the animal will not react differently to different investigators when they are being handled and tested. When moving from one hamster to another, gloves only need to be changed if moving from male to female animals and vice versa.

## PROCEDURE

### Visual presentation procedure

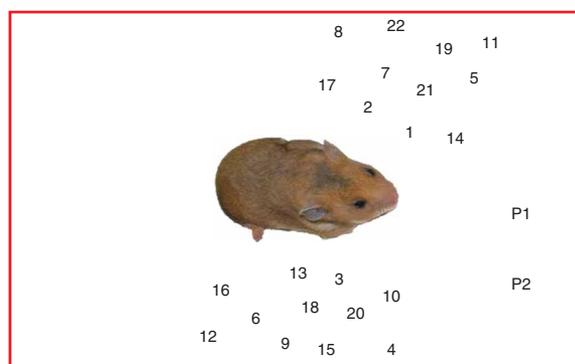
1 | Turn the video recorder on, before presentations begin.

**! CAUTION** There is no pretesting performed on the animals before surgery. We have carried out extensive testing in the past and began to see recovery at approximately 6 weeks after surgery and treatment. As testing progresses, the animals



**Figure 9** | Before the trial is started, the animal should be identified by recording the label for 15–20 s. We prepared templates to easily assemble the label with the ear marking, group number and surgery date. Each tape has two labels: one is on the outside of the cassette for the date and time of the recording; the other is recorded on the tape. In some cases, you may want to have a day label in the frame while testing the animal. You can also rely on the time stamp of the recorder, but care should be taken to check it each time you record. The preferred way is to record the label and turn off the date and time function of the recorder because it may mask the image of the movement of the animal if detailed analysis of the responses is required at a later stage.

**Figure 10** | The order of the stimulus presentation on a typical day. The two probe trials (P1 and P2) are presentations in front of the animal and are carried out to get the animal ready for the trials. P1 and P2 are in the binocular visual field and the seed can be seen by both eyes. The rest of the presentations are numbered 1–22 where the seed is presented in the temporal visual field of the animal and only the tested eye can see the seed. Note the randomness of positioning the seed to avoid development of bias in the response.



that have regeneration become faster and more responsive, reaching the seed more quickly as the testing progresses.

2| Record the label of the animal from the premade labels that are big enough to see from the camera. Record the label for 15–30 s (Fig. 9).

3| Place the animal on the test platform and let it walk around. In this case, we used only males. Remember, do not test males and females on the same platform. When males smell a female, they are distracted.

4| Holding the other end of the wire, thrust the ball-with-seed into the visual field from the rear or from below the platform, moving it slightly to increase visibility, holding it 15–30 cm from the head in the visual field on one side. See **Supplementary Video 4** online. This is the first day of testing a treated animal that later regained vision. Also note that this animal is being tested on the unaffected side.

5| To maximize the chance of positive responses, move the stimulus initially through the more peripheral part of the field (80–100° from the straight ahead position) into the center of the field of the eye (60° from straight ahead). The position is varied because of movement of the animal, but we never intentionally move nasal to the 45° position. Present the seed at heights varying from eye level to about 30° elevated.

6| After about 3 s, remove the stimulus from the visual field or reward the animal with the seed by moving it into the whisker field. We used this method of stimulus presentation in daily sessions of at least ten presentations per side, varying the side of presentation (Fig. 10). Results were recorded on a sheet by another person who was not testing (Fig. 11). However, if the animal was unresponsive, but cooperative, we tested up to 40 presentations. Most of the time, 20 presentations, randomly changing sides, are sufficient.

**▲ CRITICAL STEP** A two-person team coordinates this activity by assigning one person to be the animal tester and the other person as data recorder, writing whether the animal has responded or not on the data sheet. As the animal is presented with a stimulus, the tester will verbally tell the recorder whether the animal responded, and the robustness of that response, or if the animal failed to respond at all. This is just preliminary data and must be followed up by the video analysis.

7| After the animal shows a turning response, move the seed into the whisker field, so that the animal can take it from the ball. See **Supplementary Videos 5** and **6** online. This treated adult animal turns toward the stimulus in the affected right visual



Group (Surgery date--), Animal X ( ear cut)

**Figure 11** | Data sheet is used for each of the animals and the individual trials are marked in each box. Time and date are recorded at the top of each set of boxes and the trial is marked with a minus (–) sign for a negative response or with a plus (+) sign for a positive response in each box. For animals that had a response that was strong, we gave the animal (+++), for a medium response (++) , weak response (+) and for no response (–). If the whiskers were touched, then we would mark (W) in the field. The sheets were used only after the video tape was analyzed for comparison. It is very important to write down the time of day the test is performed. For normal controls, a surgery date is noted and placed on the cage. This is needed owing to animal care requirements, as we cannot remove the surgical date for the other animals.

Test date:		Time:	
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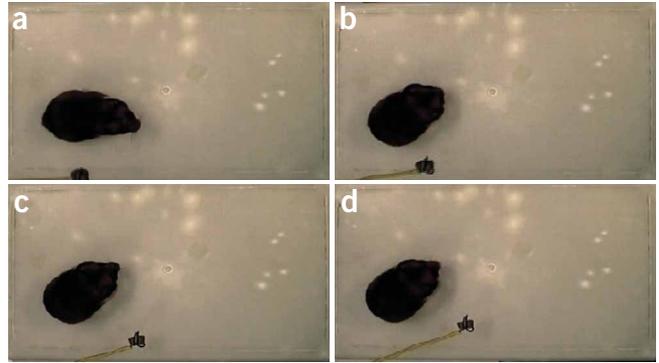
Test date:		Time:	
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## PROTOCOL

**Figure 12** | This blind control animal received a unilateral transection of the left brachium with saline injected into the cut. The stimulus is placed in the right visual field in the first frame; times are 0.00 s (a), 0.48 s (b), 0.96 s (c) and 1.34 s (d). The animal turned away from the stimulus even as the stimulus was continually moved into the field of view. Although this animal could see on the left, when the stimulus was placed in the right visual field, the animal rarely turned toward it (see **Supplementary Video 7**).



field in small steps, prolonged here by movements of the stimulus away from the animal. The recording was made 12 weeks after surgery and treatment, when the animal showed a robust response.

**8** | When the animal does not respond to a visual presentation (**Fig. 12**), touch the whiskers on about half of the trials and give the seed to the animal when he turns toward it. See **Supplementary Video 7** online, which shows a blind control animal that received a unilateral transection of the left brachium with saline injected into the cut. Although this animal could see on the left, when the stimulus was placed in the right visual field, the animal rarely turned toward it. As we do this on both right and left sides, we prevent the formation of turning biases.

**9** | For a presentation trial to be counted as positive or negative for a turning response, the animal must show evidence of motivation to take the seeds. This is best assessed from the normal side control presentations (see **Supplementary Video 8** online). Using daily sessions in our recent experiments, it was not possible to test all the animals every day. We tried to test each of them every second day.

### Preliminary analysis of behavioral data

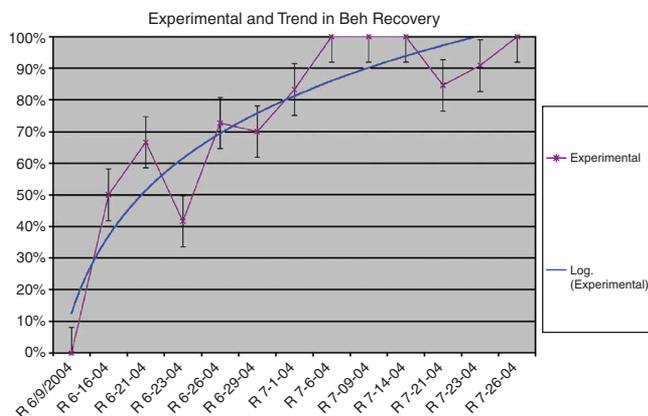
**10** | *Response frequency verification.* As the number of positive responses and no-response trials are written down as they occur, the frequencies of responding to the left and right sides of the visual field are known without looking at the video recordings. However, it is important to check all responses in slow motion viewing. In such analysis, the azimuthal position of the stimulus at the onset of a turn is noted, and trials are rejected when the stimulus is found to be in the nasal 45°. Initially, only + or – responses are scored and plotted, sufficient to prove that the animals are responding. Other parameters can also be considered, for example, the direction of presentation, angle of presentation relative to the head and duration of head turning versus the angle of presentation. All of these can be considered at an advanced level of analysis to be presented in the detailed analysis of head turning. However, the guidelines for the preliminary review are described here. We compared the video review with the responses recorded during the tests; the comparisons in most cases match with less than 5% variation.

**▲ CRITICAL STEP** It is important when the video tape is reviewed that it should be by two different individuals, or the tapes need to be reviewed three times, again without any prior knowledge of what the labels are until the experiment is unblinded.

**11** | *Response latencies.* The longer the latency of turning after the initial appearance of the stimulus in the visual field, the more likely that it is merely a spontaneous turn. Therefore, if a response does not begin within 3 s of initial presentation, it is discounted. If the animal is frozen in place, he may be responding to the stimulus but not making a turn. Sessions where this is frequent are discounted.

**12** | *Statistical analysis.* For statistical analysis of the preliminary data, we add the total number of valid presentations for each animal and then calculate the percentage of turns. To calculate this, first determine if the presentation is valid and then divide the number of turning responses by the number of valid presentations of stimulus.

We usually graph results for both sides (eyes) to determine if the animal is not responding to the stimulus. However, the illustrated graph just shows the percent of correct responses by the affected eye (**Fig. 13**). This is done for each day because the animal will perform better on some days than others; do not be concerned with 1 or 2 days that are outliers.



**Figure 13** | The graph shows the percent of positive responses that animal performed each day. There is some variation from day to day, which is expected. The blue line is the overall trend. It appears that the animal is recovering after the surgery and treatment. Percent is on the y-axis and the test dates are on the x-axis.

When you look at the entire group of animals, this tends to be insignificant. There are several tests we perform: some are nonparametric and others are standard ANOVA.

## ? TROUBLESHOOTING

If the stimulus inadvertently touches the vibrissae, or if it is uncertain whether it was too close to the head and may have touched them, discount the presentation and repeat. This judgment can be made by the presenter or independently by another observer. In our testing, two people were present during the testing sessions.

## ● TIMING

Complete testing of the animals will take 6 months from the date of surgery. However, in some cases, you may want to allow for longer term survival and this can extend 8 months or longer from the date of the operation.

## ANTICIPATED RESULTS

Normal unoperated animals will respond on average 85–95% of the time in the direction toward the stimulus. For blind control animals, you can expect to see spontaneous turning at a rate usually below 25%. As testing continues, this response rate will decrease. However, in treated animals that are being tested 6 weeks after surgery and function is starting to return, the animals will respond at 40–70%. As the recovery continues, you will see 70–95% responses in the direction toward the presented stimulus. If one of your treatments progressively damages the retina, initially the animal may start to respond robustly but then the animal becomes unable to see, even if there is restoration of connections. This can happen when multiple eye injections are made.

It is useful for all observers to conduct preliminary testing of both normal and blind animals to become familiar with the behavior. Older animals become slower with some variation in speed during a turn when the animal moves its feet; this is a result of aging and not regeneration.

*Note: Supplementary information is available via the HTML version of this article.*

**COMPETING INTERESTS STATEMENT** The authors declare that they have no competing financial interests.

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