Video Article

In Vivo Two-photon Imaging Of Experience-dependent Molecular Changes In Cortical Neurons

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Abstract

The brain's ability to change in response to experience is essential for healthy brain function, and abnormalities in this process contribute to a variety of brain disorders[1,2]. To better understand the mechanisms by which brain circuits react to an animal's experience requires the ability to monitor the experience-dependent molecular changes in a given set of neurons, over a prolonged period of time, in the live animal. While experience and associated neural activity is known to trigger gene expression changes in neurons[1,2,] most of the methods to detect such changes do not allow repeated observation of the same neurons over multiple days or do not have sufficient resolution to observe individual neurons[3,4]. Here, we describe a method that combines *in vivo* two-photon microscopy with a genetically encoded fluorescent reporter to track experience-dependent gene expression changes in individual cortical neurons over the course of day-to-day experience.

One of the well-established experience-dependent genes is Activity-regulated cytoskeletal associated protein (Arc)[5,6]. The transcription of Arc is rapidly and highly induced by intensified neuronal activity[3,] and its protein product regulates the endocytosis of glutamate receptors and long-term synaptic plasticity[7]. The expression of Arc has been widely used as a molecular marker to map neuronal circuits involved in specific behaviors[3]. In most of those studies, Arc expression was detected by *in situ* hybridization or immunohistochemistry in fixed brain sections. Although those methods revealed that the expression of Arc was localized to a subset of excitatory neurons after behavioral experience, how the cellular patterns of Arc expression might change with multiple episodes of repeated or distinctive experiences over days was not investigated.

In vivo two-photon microscopy offers a powerful way to examine experience-dependent cellular changes in the living brain[8,9]. To enable the examination of Arc expression in live neurons by two-photon microscopy, we previously generated a knock-in mouse line in which a GFP reporter is placed under the control of the endogenous Arc promoter[10]. This protocol describes the surgical preparations and imaging procedures for tracking experience-dependent Arc-GFP expression patterns in neuronal ensembles in the live animal. In this method, chronic cranial windows were first implanted in Arc-GFP mice over the cortical regions of interest. Those animals were then repeatedly imaged by two-photon microscopy after desired behavioral paradigms over the course of several days. This method may be generally applicable to animals carrying other fluorescent reporters of experience-dependent molecular changes[4].

Video Link

The video component of this article can be found at https://www.jove.com/video/50148/

Protocol

The experimental procedures described below were approved by the National Institute of Mental Health Animal Care and Use Committee and were in accordance with *the National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

1. Pre-operative Preparation

1. Clean all tools in a hot bead sterilizer before aseptic surgery, clean the surgery site with 70% ethanol, and put down clean drop cloths. Wear sterile gloves. Anesthetize the animal with freshly prepared 1.2% avertin solution, given at 0.02 ml/g, intraperitonially. Alternatively, anesthetize with isoflurane gas through a nose cone, 5% for induction, 1.5% for maintenance, with a passive scavenger circuit. Check the anesthesia level using tail or toe pinches to ensure full sedation.

2. Cover the animal's eyes with sterile ophthalmic ointment, and inject freshly prepared dexamethasone (0.2 mg/kg) and carprofen (5 mg/kg) subcutaneously to prevent brain swelling and inflammation[11]. Shave the hair over the skull between the ears, and sterilize the skin with betadine scrub and three alternating swabs of 70% ethanol.
3. Mount the animal in a stereotaxic surgery stage with earbars, with a water circulation heating pad below the animal set at 37 °C. Regularly check the animal's anesthesia levels, and supplement the original anesthetic dose as needed.
4. Inject 200 µl of 0.5% Marcaine under the scalp skin to numb the area. Incise the skin and remove the skin flap over the skull. Remove the periosteum and dry the area with clean cotton swabs.

2. Chronic Cranial Window Surgery

1. Use a high-speed dental drill with a 0.5 mm burr to gently outline a 3-5 mm diameter circle over the brain region of interest. Periodically wet the drilling site with sterile 0.9% saline and clear away bone dust with clean cotton swabs. If the bone bleeds, use gelfoam presoaked with sterile saline to blot the bleed and wait for it to stop.
2. When the last bone layer is reached, lift and remove the bone island with fine-tipped forceps. Dura attachments to the bottom shelf of the bone may be encountered if the window crosses a bone suture. These should be gently removed as the bone flap is lifted.
3. Roll moistened gel foam gently over the exposed dura to clean its surface and wait for any dural bleeding to stop. **Critical step:** do not proceed until all dural bleeding has stopped. Blood that becomes trapped inside the cranial window usually leads to an opaque window.
4. If the region of interest is under a location where the dura is particularly thick, removing the dura above it may be necessary. If this is the case, gently separate the dura from the pia below with very fine forceps, make a small incision in the lifted dura with another pair of fine forceps, then grasp the cut edges and gently split the dura. The dura is thin but strong, so be careful not to slice the brain with the intact edges of the dura.
5. Rinse the area with sterile ACSF or sterile saline.
6. Lay a sterile glass coverslip (3-5 mm) over the dura or pia. **Critical step:** If the surrounding skull curves significantly, use Kwiksil[12] adhesive to fill in spaces where the dura or pia would not make close contact with the coverslip in regions that will not be imaged.
7. Use cyanoacrylate gel to cover the elastomer adhesive and the edges of the glass coverslip. Cover the entire exposed skull with cyanoacrylate gel, or a krazyglue and dental cement mixture. **Critical step**: Do not allow any cyanoacrylate gel to touch the brain surface.
8. Embed a custom-made metal head fixation bar at the opposite end of the skull.
9. Return the animal to a warm recovery chamber, after an intraperitonial injection of ketoprofen, 5 mg/kg, for pain management. Continue the analgesic for two more days post-operatively.
10. After two weeks of post-operative recovery, anesthetize the animal with isoflurane (5% for induction, 1.5% for maintenance), mount it in a custom-made microscope stage with a head-fixation frame, and check the cranial window for optical clarity under illumination with blue light. Brain surface blood vessel clarity is highly indicative of cranial window quality. If the blood vessel edges are sharply defined, the window is likely to be useable. The two-week post-operative recovery time is recommended to allow sufficient time for the animal to recover from surgery. Cranial windows typically clear and improve during this time, and remain optically transparent for additional weeks to months until regrowth of skull or thickening of the dura degrades window quality.

3. Behavioral Protocol and Laser Scanning Two-photon Microscopy

1. Animals with clear cranial windows will be exposed to an environmental stimulus or subjected to a behavioral training session, and then imaged afterwards at the time of maximal Arc-GFP expression. Before beginning a behavioral protocol, the animal should be kept in a consistent home cage environment to minimize day-to-day variation in baseline Arc-GFP expression levels.
2. Begin behavioral training or environmental stimulation paradigms, depending on the brain region of interest. For example, animals can be exposed to different visual environments over consecutive days, and imaged each day after visual stimulation to identify stimulus-specific responses in the visual cortex[10].
3. Arc-GFP fluorescence in neurons typically reaches its peak level two hours after stimulation. The experimental timeline may be optimized to facilitate the detection of Arc-GFP expression in neurons at their peak fluorescence levels.
4. After a behavioral training session or environmental stimulation is completed, anesthetize the animal with isoflurane (5% for induction, 1.5% for maintenance) and mount the animal in the custom-made microscope stage with head-fixation frame under the two-photon microscope.
5. The animal's head position is fixed to the head-fixation frame that connects directly to the stage, using the implanted metal bar on the skull. Isoflurane and oxygen are continuously supplied to the mouse through a nose cone. Body temperature is maintained using a heating pad.
6. Ensure that the microscope detectors are protected from ambient light by conducting two-photon laser scanning in a dark room. Use a 20x or 25x (1.05 numerical aperture) water immersion lens for imaging. First, under epi-fluorescence illumination, acquire an image of the surface blood vessel patterns over the brain region of interest with a CCD camera for future image alignment.
7. Start two-photon laser scanning to acquire a 3-D image stack. An Olympus FV1000MPE multi-photon microscope is used in our setup. The excitation wave-length of the two-photon laser is set at 920 nm, and the power of the laser emitted from the objective is set at approximately 50 mW. Emitted fluorescence is simultaneously detected in green and red channels (dichroic mirror at 570 nm, barrier filters at 495-540 nm and 570-625 nm), using an external two-channel photomutiplier detection system placed close to the specimen. Arc-GFP fluorescence only appears in the green channel, whereas tissue auto-fluorescence appears in both channels[13, 14].
8. Typical image stacks have dimensions of approximately 320x320x100 µm (width x length x depth), a horizontal resolution of 0.5 µm/pixel, and a vertical resolution of 3 µm/pixel.
9. After acquiring the image stack, return the animal to its home cage. Do not disturb the animal until the next behavioral and imaging session. Repeat the behavior and imaging procedure across days as desired. Use the previously acquired brain surface blood vessel image to orient back to the same imaging location.

Representative Results

This protocol describes a method to track experience-dependent molecular changes in individual cortical neurons in live animals. A chronic cranial window is first created over a cortical region of interest in a mouse carrying a fluorescent reporter of gene expression. Two-photon

microscopy can then be coupled with various behavioral paradigms to observe behaviorally induced molecular changes in individual neurons and track such changes in the same sets of neurons over multiple days (**Figure 1**).

In Arc-GFP mice, experience-dependent changes in Arc gene expression can be reliably imaged in individual cortical neurons for multiple days using this protocol. The transgenic Arc-GFP knock-in mouse expresses a destabilized green fluorescent protein (d2EGFP, protein half-life approximately two hours) under the control of the endogenous promoter of the immediate early gene Arc (**Figure 2**).

Past protocols describing chronic cranial windows have outlined critical steps in conducting successful cranial window surgeries[11]. This protocol provides additional guidance in removing the dura if needed, which increases the likelihood of obtaining clear optical windows when such windows are situated over skull sutures (see Step 2.4).

After recovery from cranial window surgery, animals are mounted in a custom-made head-fixation frame and stage. **Figure 3** depicts the headbar and head-fixation frame used during two-photon imaging. Maintaining a consistent two-photon microscope imaging stage position and setup will facilitate day-to-day realignment of images when returning to a previously imaged brain region, and increase experimental efficiency when multiple animals are imaged on the same day (**Figure 4**).

During behavioral training or environmental stimulation, it is advisable to house animals singly, and in an environmentally consistent home cage location, to minimize day-to-day fluctuations in background Arc-GFP activation levels (**Figure 1**).

A critical sign of a stable, clear cranial window is the crisp pattern of blood vessels under epi-fluorescent illumination. This blood vessel pattern should not change significantly across multiple days of imaging (**Figure 5**).

Figure 6 illustrates the expression patterns of Arc-GFP in frontal cortical neurons under a baseline home cage condition (A) and after performance of a new motor behavior (B). Layer II/III cortical neurons in the same brain region in the same animal can be reliably imaged, and the same neurons should be able to be identified over multiple days of imaging. It is typical to collect a 3-D image stack to sample hundreds of neurons. Neuron edges should be sharp and clear throughout the image stack. Tissue auto-fluorescence in the red channel also provides an index of window clarity. If the imaged region becomes drastically murkier over days, the cranial window quality is likely deteriorated. A typical cranial window will remain optically clear for at least a week. After several weeks to months, the regrowth of skull from the edges of the cranial window and thickening of the dura will eventually prevent further imaging experiments.

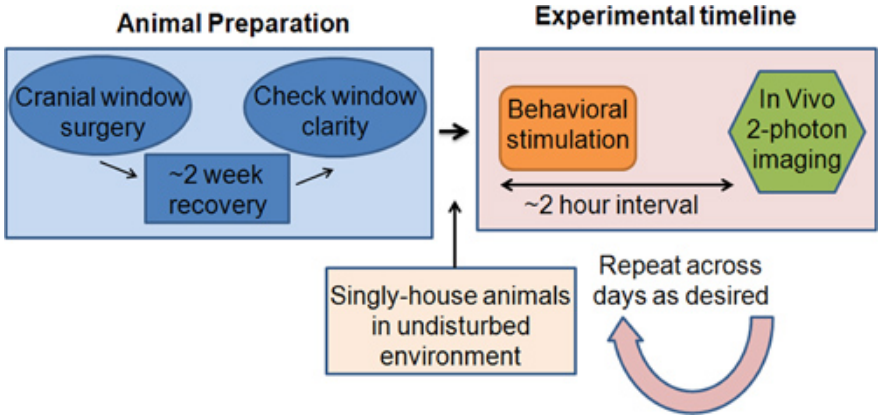

Figure 1. A schema outlining the experimental steps. During the Animal Preparation phase, cranial window surgeries are performed on Arc-GFP mice. The animals are given about two weeks to recover from the surgery in their home cage. The clarity of the cranial windows is then checked using epi-fluorescence microscopy. If the windows are ready for imaging, the animals are singly housed in a quiet, environmentally consistent home cage environment, whence the Experimental Timeline phase can begin. The behavioral stimulation protocol and the imaging interval may be tailored to detect Arc-GFP at its peak fluorescence, which typically occurs two hours after activation. After the behavioral stimulation is complete, the animal is anesthetized and the cortical region of interest is imaged through the cranial window using two-photon microscopy. The animal is then returned to the home cage. The behavioral and imaging sessions can be repeated across days as desired.

Figure 2. A diagram illustrating the construction of the Arc-GFP knock-in mouse line, in which a gene encoding destabilized green fluorescent protein replaced the coding part of the immediate early gene Arc. In this strain of mouse, GFP expression is under the control of the endogenous Arc promoter. This diagram is redrawn based on the description in Reference 10.

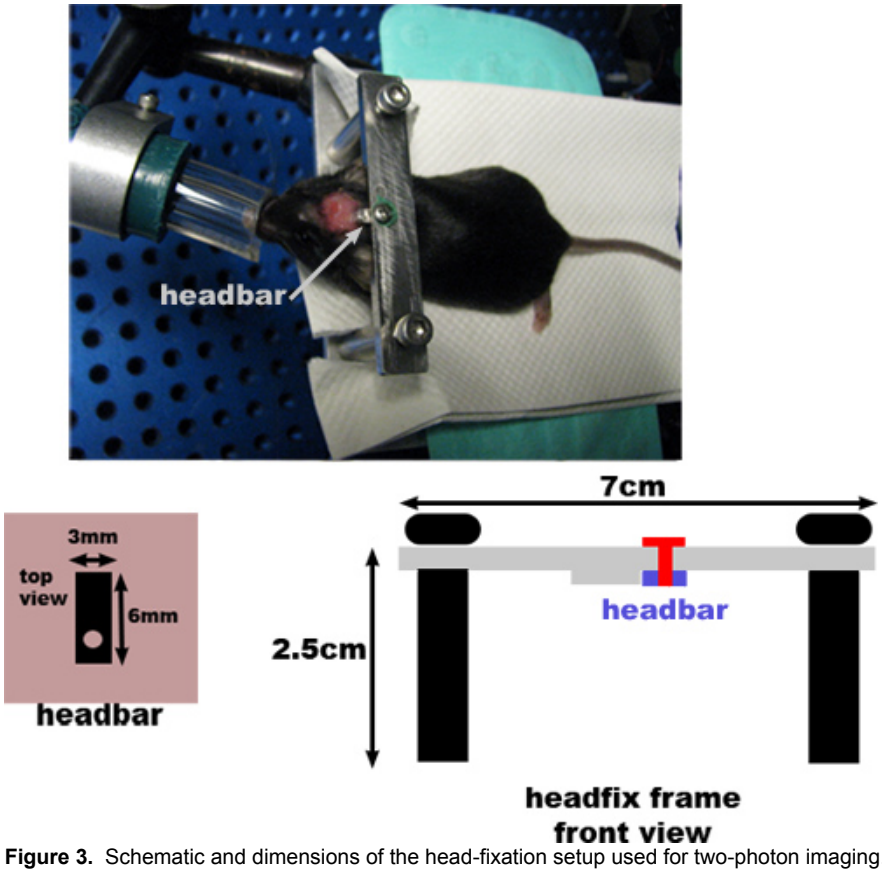

Figure 3. Schematic and dimensions of the head-fixation setup used for two-photon imaging. The solid half of the headbar is glued to the back of the skull (Section 2.8), and the end with the screw hole is used to attach the anesthetized animal's head to the frame. The head-fixation frame consists of a thin metal plate mounted atop two poles with screws.

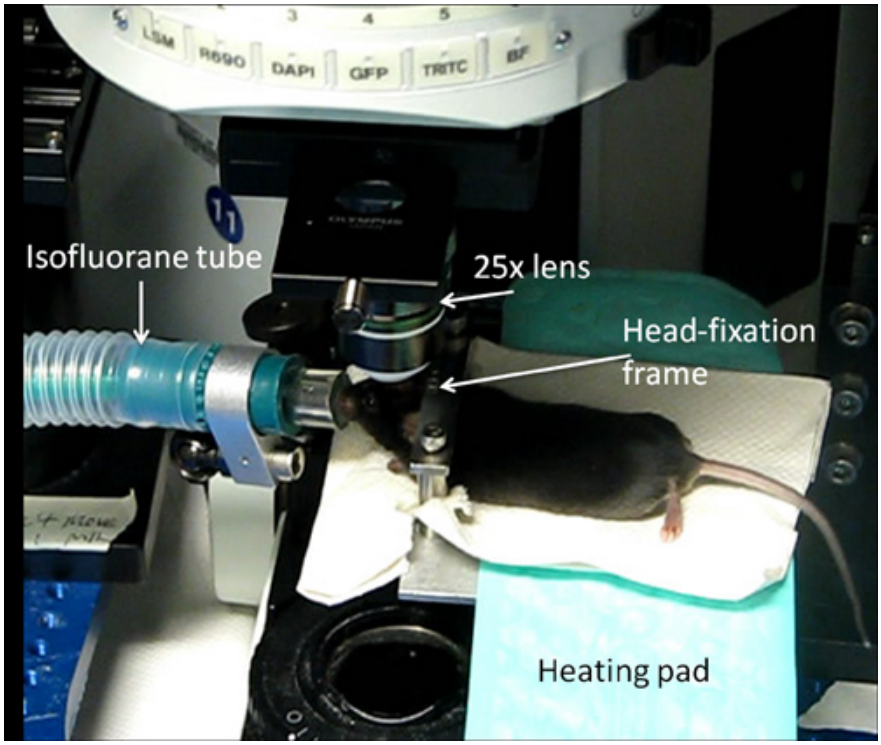

Figure 4. Example of a laser scanning two-photon microscope setup for Arc-GFP mice imaging. Note the 25x water immersion lens, heating pad and custom microscope stage with a head-fixation frame. The anesthetized Arc-GFP mouse shown here is ready for *in vivo* imaging.

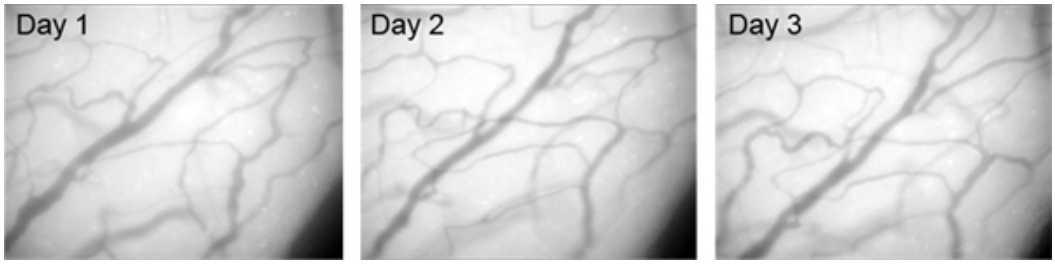

Figure 5. Images of brain surface blood vessels in a chronic cranial window over multiple days, showing the clarity and stability of blood vessel patterns over time. Brain surface was illuminated with blue light, and the images were taken through a 25x water immersion lens by a CCD camera mounted on the microscope.

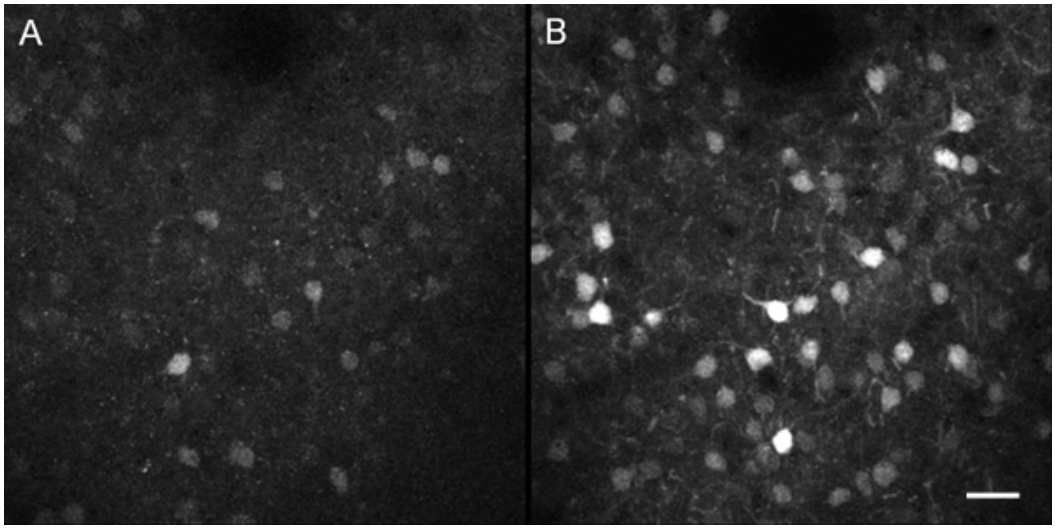

Figure 6. *In vivo* two-photon images of Arc-GFP expression patterns in frontal cortical neurons of a mouse after two different behavioral experiences. (A) The mouse stayed in its home cage before imaging on the first day. (B) The mouse performed a new motor behavior before imaging on the second day. Fluorescent images of the same cortical region were simultaneously acquired in green and red channels. GFP fluorescence only appeared in the green channel, whereas tissue autofluorescence appeared in both channels. Detection parameters were set so that tissue auto-fluorescence levels in both red and green channels had equal readings, and these parameters were maintained throughout experiments. The red channel signal was then subtracted from the green channel signal to remove broad-band tissue autofluorescence during off-line analysis. The resulting green fluorescent signals from an 18 μm thick 3-D image stack were projected by maximum intensity to the horizontal plane, to provide a top-down view of the cellular patterns of Arc-GFP expression. Scale bar, 30 μm.

Discussion

The *in vivo* imaging method described here enables repeated examination of Arc gene expression changes in the same sets of neurons over multiple days in the live animal. It is an efficient and versatile method to obtain information about neural plasticity-related molecular dynamics in individual neurons in response to various behavioral experiences. Standard histochemical methods such as *in situ hybridization* and immunostaining can achieve single-cell resolution[3], but lack the ability to track gene expression changes in the same neurons over multiple days. Bioluminescence, magnetic resonance, or nuclear imaging methods can track gene expression changes in the same animal through appropriate reporters[15], but lack the spatial resolution to distinguish the expression levels in individual neurons. As such, no other existing methods to measure gene expression changes can match both the spatial resolution and the temporal coverage of the imaging method described here.

A critical step in this procedure is the cranial window surgery. It should be performed with extra care to avoid trauma to the brain or leaving blood beneath the glass coverslip, which will cause inflammation and reduce the clarity of cranial windows. For well-conducted cranial surgeries, the window will remain clear for several weeks until thickening of the dura or regrowth of the skull from the edges of the window prevent further imaging. The dura can be removed if necessary, as has been done for chronic optical imaging experiments in rats and monkeys[16,17], to increase the optical clarity of cranial windows.

A limitation for the two-photon microscopy technique used in this protocol is the imaging depth. The maximum imaging depth at which high-quality images can be obtained will depend on the cranial window clarity, the microscope setup, and the brightness of the fluorescent reporters. For Arc-GFP mice, we typically image up to 300 μm below the pial surface. To examine gene expression changes in deep brain regions, fluorescence microendoscopy may be applied to overcome the depth limitation in conventional two-photon microscopy[18].

Potential confounding factors for gene expression changes determined with this *in vivo* imaging method include any lingering effects from cranial surgery or anesthesia that may interfere with behavioral performance or the induction of Arc-GFP. It is important to check the overall extent of Arc-GFP activation in response to specific experiences in fixed brain sections first, and compare this to the extent observed by repeated imaging

using *in vivo* two-photon microscopy. The post-operative recovery period and the repeated imaging intervals can then be adjusted to minimize the potential side effects of surgery and anesthesia on Arc-GFP activation.

The imaging method described here is likely to be generally applicable to transgenic mice carrying fluorescent reporters for other experience-regulated genes[4]. Furthermore, it can be combined with fluorescent markers that highlight the morphology of labeled neurons[8,9,] or indicators that reflect the neurophysiological activities in those neurons[19, 20]. These further applications may provide a more comprehensive view of experience-dependent molecular changes in individual neurons, and relate these molecular changes to the structural and functional modifications that take place in neurons during normal or pathological experiences.

Disclosures

No conflicts of interest declared.

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