Video Article

Imaging Calcium Responses in GFP-tagged Neurons of Hypothalamic Mouse Brain Slices

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Abstract

Despite an enormous increase in our knowledge about the mechanisms underlying the encoding of information in the brain, a central question concerning the precise molecular steps as well as the activity of specific neurons in multi-functional nuclei of brain areas such as the hypothalamus remain. This problem includes identification of the molecular components involved in the regulation of various neurohormone signal transduction cascades. Elevations of intracellular Ca^{2+} play an important role in regulating the sensitivity of neurons, both at the level of signal transduction and at synaptic sites.

New tools have emerged to help identify neurons in the myriad of brain neurons by expressing green fluorescent protein (GFP) under the control of a particular promoter. To monitor both spatially and temporally stimulus-induced Ca^{2+} responses in GFP-tagged neurons, a non-green fluorescent Ca^{2+} indicator dye needs to be used. In addition, confocal microscopy is a favorite method of imaging individual neurons in tissue slices due to its ability to visualize neurons in distinct planes of depth within the tissue and to limit out-of-focus fluorescence. The ratiometric Ca^{2+} indicator fura-2 has been used in combination with GFP-tagged neurons[1]. However, the dye is excited by ultraviolet (UV) light. The cost of the laser and the limited optical penetration depth of UV light hindered its use in many laboratories. Moreover, GFP fluorescence may interfere with the fura-2 signals[2]. Therefore, we decided to use a red fluorescent Ca^{2+} indicator dye. The huge Strokes shift of fura-red permits multicolor analysis of the red fluorescence in combination with GFP using a single excitation wavelength. We had previously good results using fura-red in combination with GFP-tagged olfactory neurons[3]. The protocols for olfactory tissue slices seemed to work equally well in hypothalamic neurons[4]. Fura-red based Ca^{2+} imaging was also successfully combined with GFP-tagged pancreatic β-cells and GFP-tagged receptors expressed in HEK cells[5,6]. A little quirk of fura-red is that its fluorescence intensity at 650 nm decreases once the indicator binds calcium[7]. Therefore, the fluorescence of resting neurons with low Ca^{2+} concentration has relatively high intensity. It should be noted, that other red Ca^{2+}-indicator dyes exist or are currently being developed, that might give better or improved results in different neurons and brain areas.

Video Link

The video component of this article can be found at http://www.jove.com/video/4213/

Protocol

1. Preparation of Solution and Agarose Gel

1. Prepare extracellular solution according to the table with double distilled water. The pH will be ~7.3 after 10 min aeration with carbogen (95% O_2 / 5% CO_2), the osmolarity 300 mOsm[8]. If a higher osmolarity is required, it can be adjusted by adding more glucose (1 mM equals 1 mOsm). The solution is filtered twice using a 0.2 µm membrane filter to eliminate dust particles and possible bacterial contaminations.

Name of reagent	Abbreviation	Mol. weight	Conc.	Company	Cat. N°
Sodium chloride	NaCl	58.44 g/mol	120 mM	VWR	27810
Sodium hydrogen carbonate	$NaHCO_3$	84.01 g/mol	25 mM	Merck	106329
Potassium chloride	KCl	74.55 g/mol	5 mM	Merck	104936
BES*	$C_6H_{15}NO_5S$	213.25 g/mol	5 mM	Sigma	14853
Magnesium sulfate, anhydrous	$MgSO_4$	120.37 g/mol	1 mM	Sigma	M7506
Calcium chloride dihydrate	$CaCl*2H_2O$	147.02 g/mol	1 mM	Merck	102382
D(+)-Glucose monohydrate	$C_6H_{12}O_8*H_2O$	198.17 g/mol	10 mM	Merck	108342

* N,N-Bis(2-hydroxyethyl)-2-aminoethansulfonic acid

2. The solution is stored at 4 °C, but should be aerated with carbogen (95% O_2 / 5% CO_2) for 10 min before use.
3. At this time, prepare also 1 cm^3 blocks of agar gel to stabilize the brain during the cutting procedure (see step 3). First, dissolve 4% (w/v) agar (Sigma) in double distilled water by heating the solution to approximately 60 °C. The heated dissolved agar solution is poured into a square Petri dish to a height of 1 cm. After cooling and hardening, the 4% agar gel is cut into blocks of 1 cm^3. These blocks can be stored for up to 4 weeks at 4 °C.

2. Dissection of the Mouse Brain

Please make sure that all animal experimental procedures are performed in accordance with the guidelines established by the animal welfare committees of the respective institutions.

1. Before sacrificing the animal, make sure the agar gel blocks are ready for use.
2. Anesthetize the mouse with isoflurane (1-4% isoflurane in oxygen using a precision vaporizer for approximately 1 min). It is important to prevent hypoxia and therefore neuronal damage. A disadvantage of isoflurane is the cost and logistics of using precision vaporizers. A fatal overdose in an open system could be an alternative, since the mouse will be sacrificed. Occupational safety is in this case a serious concern. The isoflurane must be directly vented out of the room. Therefore, an overdose in an open system should be performed in a chemical fume hood.
3. Euthanize the mouse by decapitation after monitoring the depth of anesthesia by testing the rear foot reflex. This pedal or paw pinch reflex is performed by firmly pinching a paw or toe between one's fingers to elicit a withdrawal response by the animal. An animal that shows a reflex is not at a surgical level of anesthesia and definitely not in a state to euthanize.
4. After decapitation, cut the scalp with a single edge razorblade centrally in sagittal direction, from the frontal bone to the external occipital protuberance. Move the two parts of the scalp, first from the medial caudal end in rostral lateral direction, then down in ventral direction (**Figure 1A-C**).
5. Make two lateral and one rostral cut in the foramen magnum with a small spring scissor (see for cutting direction the black dashed arrows in **Figure 1C**). Carefully cleave off the cranium from mediocaudal to lateral rostral with blunt forceps (see broad grey arrows in **Figure 1C**). In older animals a small cut at the sagittal suture is helpful and often necessary to avoid damaging cortical layers of the brain.
6. At this moment the occipital, interparietal and parietal bones should be detached. If still present, the dura mater should be carefully removed using forceps to prevent damage to the brain in the next step.
7. Take away the squamosal bone, the anterior ethmoidal and frontal foramen (**Figure 1D**).
8. The brain can now easily be removed using an inverted micro spoon spatula and severing the cranial nerves (**Figure 1E**).

3. Slicing Coronal Hypothalamic Sections of the Mouse Brain

1. Place the brain on its ventral side on a cut-resistant surface and remove the cerebellum with a single edge razor blade (**Figure 2A**). This straight cut surface will be the basis for mounting the brain on a plate to enable slicing of coronal brain sections.
2. Glue the brain with the cut section on the baseplate of the microtome (Zeiss, Hyrax V50) using low amounts of a fast curing high performance cyanoacrylate adhesive superglue (Loctite 406; **Figure 1A**). In addition, glue a 1 cm^3 agar gel block (see 1.5) at the ventral side of the brain (see 'v' in **Figure 2B**) onto the baseplate of the microtome (Zeiss, Hyrax V50) to support and fix the brain while slicing. Make sure not to use too much glue, to evade that glue traverses between brain and gel block causing problems in removing tissue slices after the cut. The gel block will be located at the opposite side of the cutting blade from the microtome (**Figure 2B**).
3. After a few seconds the bond has dried, put the plate into the bath of your microtome filled with 6 °C cold oxygenated (95% O_2 / 5% CO_2) extracellular solution (see 1.1).
4. Use appropriate low slicing velocity and cut 300 µm thick slices. In case of our microtome, we use a frequency of 60 Hz, an amplitude of 0.8 mm and a velocity of 0.8 mm/s (**Figure 2C**). The coronal brain slices are either collected directly after each cut or can be left in the bath until the whole brain has been sectioned. The coronal brain slices are carefully transferred to a beaker with cold oxygenated extracellular solution. Various tools have been designed to perform the transfer (*e.g.* cut broad plastic or glass Pasteur pipettes or broad spoon spatulas). It depends on the experimentator which is being preferred. Most importantly, the slices should be handled appropriately to minimize damage.

5. If the microtome can be programmed to cut slices automatically, you can start to prepare the Ca^{2+} indicator dye loading solution at this moment. Otherwise, it is recommended that the preparation of this solution is performed before the brain slicing has ended to minimize delays for measuring Ca^{2+} responses in the neurons, namely before starting step 2.

4. Preparation of the Ca^{2+} Indicator Dye Loading Solution

A critical step in loading neurons remains often the health of the cells which depends on the amount of damage induced by and the speed of the dissection procedure. Another essential step seems to be the use of fresh Pluronic F-127 solution (see 4.1). It is being recommended to make this solution in the laboratory and not to use a premade solution from a vendor. Depending on the temperature, the humidity and the shelf lifetime of the Pluronic F-127 solution, we noted degradation of olfactory and brain neurons during the Ca^{2+} loading procedure.

1. Prepare the 20% (w/v) Pluronic F-127 (Sigma) in dimethyl sulfoxide (DMSO) by adding the Pluronic F-127 powder on top of the DMSO solution. Directly sonicate this solution without prior vortex or mixing. Within 2 min sonication the Pluronic F-127 will be dissolved. 100 µl Pluronic F-127 solutions are prepared fresh weekly.
2. Take one tube of 50 µg cell-permeable fura-red/AM (Invitrogen; AM, acetoxymethyl ester) and add 5 µl 20% Pluronic F-127 solution. Mix the solution using the tip of your pipette.
3. Add 45 µl extracellular solution (see 1.1) to the mix and vortex it shortly.
4. Add an additional 325 µl extracellular solution and sonicate the tube for 3 min.
5. After sonication add 1.156 ml oxygenated (95% O_2 / 5% CO_2) extracellular solution to get your final Ca^{2+} *indicator dye loading solution* (30 µM fura-red/AM, 0.33% DMSO and 0.065% Pluronic F-127). Store the tube in a dark place until use (see 4.8).
6. Transfer the coronal brain slices to a 6-well cell culture plate (BD Falcon) filled with oxygenated (95% O_2 / 5% CO_2) extracellular solution (up to six slices per well).
7. Suck off the oxygenated extracellular solution from the chambers of the 6-well plate taking care not to damage the brain slices.
8. Pipet directly 750 µl of the Ca^{2+} indicator dye loading solution to every well. The brain slices should be covered by the solution containing fura-red/AM (immersion loading).
9. Incubate the slices in an O_2/CO_2 cell culture incubator (O_2: 23.5%; CO_2: 5%) for 45 to 60 min at 37 °C.
10. At the end of the incubation time, replace the Ca^{2+} indicator dye loading solution by fresh oxygenated extracellular solution to prevent overloading the cells with fura-red and influencing in a subtle way the Ca^{2+} measurement via the chelating action of the dye. The slices are then being kept in the O_2/CO_2 incubator (see previous point for settings) until use and are viable for up to 3-6 hr.

5. Microscopy and Analysis

In this protocol, the fluorescence intensity of GFP, which identifies the cell of interest, and of the Ca^{2+} indicator dye will be measured simultaneously in brain slices. Thus, the confocal microscope should be equipped with the correct laser, filters and two photomultiplier tubes to collect the two emission signals. GFP and the change in fluorescence intensity of fura-red can be measured using a single excitation wavelength of 488 nm. Emission fluorescence from the fluorophores can be collected using a 522/DF35 nm filter for GFP and a long-pass filter for wavelengths greater than 600 nm for fura-red.

1. To start monitoring stimulus-induced changes in the fluorescence signal, which are a measure of the intracellular Ca^{2+} concentration, one of the fura-red loaded brain slices is transferred to a recording chamber (*i.e.* Warner Instruments RC-27 Open Bath Chamber) that can be mounted on the confocal microscope setup (**Figure 3A,B**).
2. Secure the brain slice with a harp (**Figure 3C**) to prevent the slice to move due to the perfusion speed of the bath solution (oxygenated extracellular solution; see step 1.1). The harp (slice holder) is made of a parallel array of nylon threads (separated from each other by ~1 mm) strung on a U shape silver or platinum frame. The temperature of the bath solution at this step should be at least room temperature. If higher temperatures are required, appropriate care has to be taken to prevent condensation on microscope lenses and movement of the plane of focus due to shifting parts in the microscope.
3. Perfuse the slice for 10 min with oxygenated extracellular solution to remove any surplus of extracellular Ca^{2+} indicator dye. The flow rate of the perfusion should be adjusted to ~100 µl/s (tips regarding an appropriate perfusion system see [9]).
4. Look at the slice through the microscope at low magnification, make a note of the orientation of the slice for your records and find your area of interest in the slice, in our case the hypothalamic area in the brain.
5. Change to high magnification and find the cell of interest in the slice by collecting GFP images and simultaneously checking the fluorescence intensity of the fura-red signal (**Figure 4A-C**). Cells near the surface could be damaged or dead. Therefore, cells located at a depth of more than 10 µm should be selected for imaging. We could reliably measure cells up to a depth of 40-50 µm, whereafter the signal strength started to drop. Remember that the fura-red signal of cells at rest with low Ca^{2+} concentration have relative high fluorescence intensities. This high fluorescence intensity is in this case not a sign of dead cells. The GFP signal can be used to detect any drift or movement of the tissue slice or as an indicator for changes in intracellular pH[10].
6. Adjust the laser power to a value that allows measurements in the change of fura-red fluorescence intensity and prevents bleaching of the two fluorophores. Therefore, start with the lowest laser power and adjust to obtain a sufficient signal-to-noise ratio by changing black level (offset), detector aperture, gain and laser neutral density filters. Except for the laser power, the same should be done for the GFP signal.
7. Start to acquire images at rates between 0.5 - 2 Hz to collect the fura-red and GFP signals. The acquisition rate should be optimized to the expected rate of the Ca^{2+} signal. Voltage-dependent Ca^{2+} spikes may cause quicker and shorter transients than activation of some signal transduction cascades requiring activation of various second messengers. The length of the image acquisition should be appropriate for the purpose of the experiment. The GFP signal over time will help determine if any movement of the brain slice has occurred.

8. During the acquisition and the experiment, all scanning head settings have to be held constant to record reliable results that can be compared.
9. The changes in fluorescence over time can be analyzed using different mathematical programs, *i.e.* ImageJ (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/), Igor Pro (Wavemetrics) or MatLab (The MathWorks). By encircling the somata of a GFP-tagged neuron indicating the region of interest (ROI), this exact same region can be analyzed for changes in Ca^{2+} using the fura-red fluorescence signal over time.

Ca^{2+} signals can be presented as arbitrary fluorescence units or as values (ΔF/F) of the relative change in fluorescence intensity (ΔF) normalized to the baseline fluorescence (F). This procedure results in a negative deflection when the intracellular Ca^{2+} concentration increases using fura-red as the Ca^{2+} indicator dye. To ease the interpretation of the results, we recommend multiplying the ΔF/F values with -1 to obtain positive fluorescence signals to display a rise in Ca^{2+} (**Figure 4C**).

To compare results between neurons the amplitude and frequency of the Ca^{2+} signals are usually analyzed. Yet, some Ca^{2+} signals do not occur with a regular period or comparable amplitudes. Some signals might be strongly influenced by stochastic processes within the cell. Thus, to quantify the total change in Ca^{2+} in a given cell and to enable comparison of Ca^{2+} responses between neurons in different brain regions, analysis of the area-under-the-curve (AUC) is more appropriate. This measure for the amount of Ca^{2+} encompasses any initial Ca^{2+} transient, second phases and sustained elevated Ca^{2+} responses and oscillations. In this case care should be taken to analyze the same time period to enable the comparison between neurons.

6. Representative Results

To start characterizing gonadotropin releasing hormone receptor (GnRHR) expressing neurons in the hypothalamus we made use of transgenic mice that express GFP after Cre-mediated excision in GnRHR-expressing neurons[4,11]. GFP fluorescent neurons were identified in various brain areas, including the hypothalamus. To investigate the physiological properties of these GnRHR neurons, we first recorded Ca^{2+} signals in hypothalamic slices using a confocal microscope. First, we obtained coronal brain slices from these mice using the above-described protocol. **Figure 1** illustrates the necessary tools, material and steps for excising a mouse brain. The coronal hypothalamic brain slices were cut (**Figure 2**) and then loaded according to the steps in point 4 of the protocol. Single brain slice of the appropriate area are placed in a recording chamber, secured with a harp (**Figure 3**) and then imaged using a confocal microscope (see steps 5.1-5.9). **Figure 4** shows an example of two individual coronal brain slices identifying single GnRHR-τGFP cell bodies, the fluorescence at rest after loading the brain slice with fura-red/AM and the merged confocal image indicating that the GFP neuron had taken up fura-red sufficiently to enable investigation of stimulus-induced Ca^{2+} signals in these cells. Using our protocol we initially tested whether GnRHR neurons utilize similar Ca^{2+} signals for stimulus detection in different areas of the hypothalamus in response to direct activation with GnRH (**Figure 4E**). However, these signals differed in their waveform depending on stimulus strength and brain area[4]. To quantify the change in the dynamics of the Ca^{2+} responses, the area-under-the-curve (AUC) can be calculated as a measure for the increase in intracellular Ca^{2+} (**Figure 4E**)[4]. Studies are currently underway to investigate the molecular basis underlying the Ca^{2+} waves and oscillations, their dependence on sex and hormonal status of the animal, and whether they can be modulated by other natural stimuli.

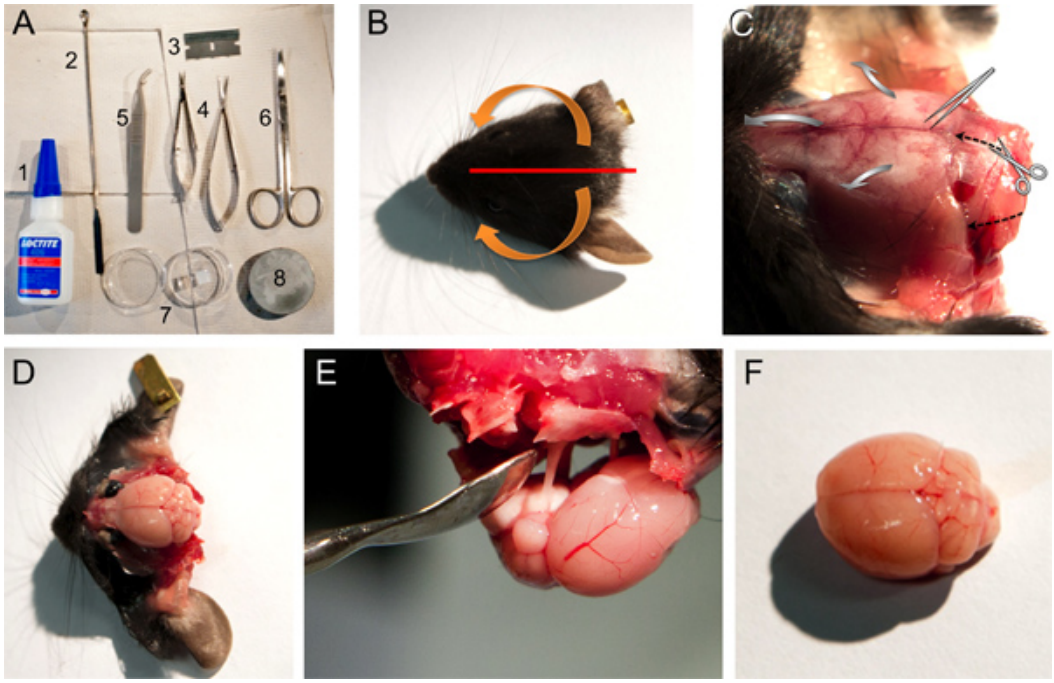

Figure 1. Tools, material and steps for excising a mouse brain. A. Tools and materials used for brain dissection: 1, Loctite 406 superglue; 2, micro spoon spatula; 3, single edge razor blade; 4, small and medium spring scissors; 5, blunt forceps; 6, scissors; 7, Petri dish containing agar

gel block; 8, base plate for mounting brain into microtome. B-F. Images of some steps being described in point 2 of the protocol. B. Photograph of a mouse head indicating the cutting position of the scalp (red line) and arrows (orange) indicating the direction the skin should be pulled away from the bone (see step 2.4). C. Photograph of the mouse head after the skin is pulled away showing the bone structures (see step 2.4). Cutting direction of the scissor and the direction for breaking open the cranium with the blunt forceps is indicated with either the black dashed arrows or grey broad arrows, respectively. D. Photograph of the mouse brain after eliminating the various bone structures (see step 2.5 - 2.7). E. Photograph of the removal of the brain still connected to the skull via the cranial nerves. F. Photograph of a relatively undamaged mouse brain.

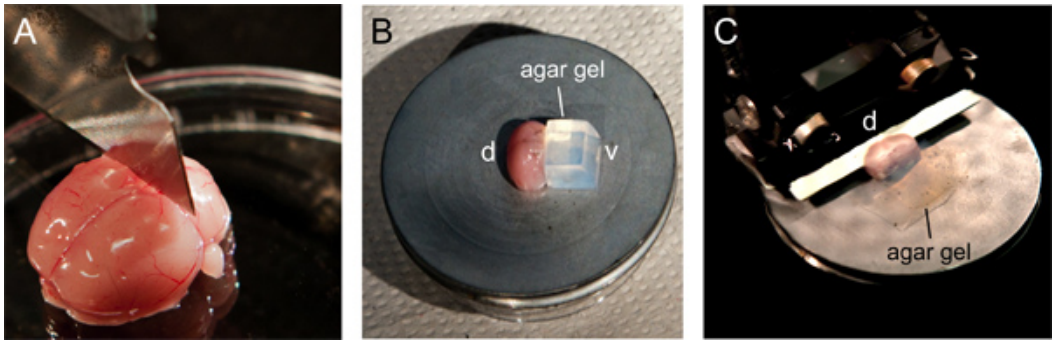

Figure 2. Slicing of coronal hypothalamic sections of the mouse brain. A. Photograph indicating the position of the single edge razor blade for eliminating the cerebellum (see step 3.1). B. Position of agar gel block in relation to the brain glued onto the baseplate of the microtome (see step 3.2). C. Cutting of coronal brain slice (note here the location of brain and gel block positions in regard to the cutting blade of the microtome; see step. 3.4). d, dorsal; v, ventral.

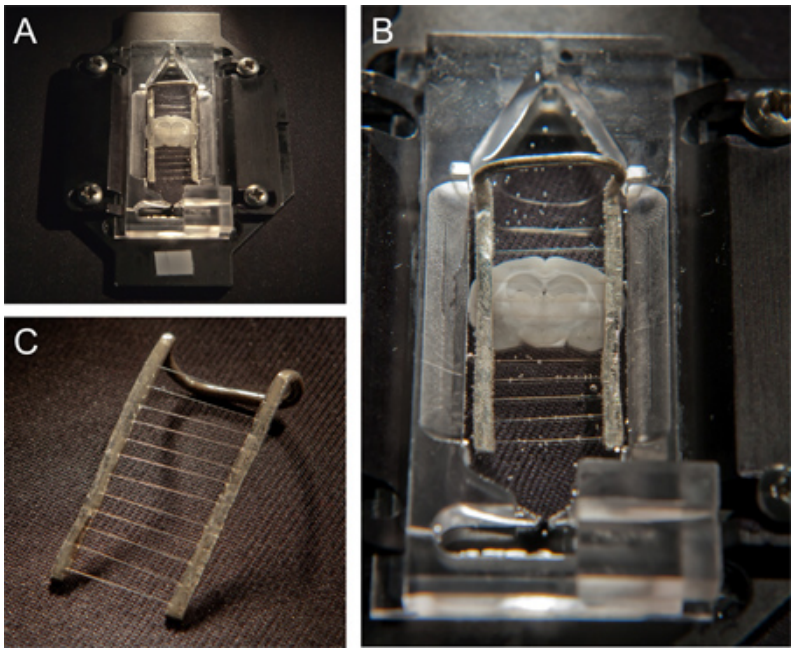

Figure 3. Brain slice positioned in recording chamber. A,B. Overview (A) and higher magnification (B) of a Warner Instruments RC-27 open bath recording chamber giving broad access to the hypothalamic areas of the coronal brain slice (see step 5.1). C. U-shaped metal harp containing parallel arrays of nylon threads which will hold the slice in position in the recording chamber (see step 5.2).

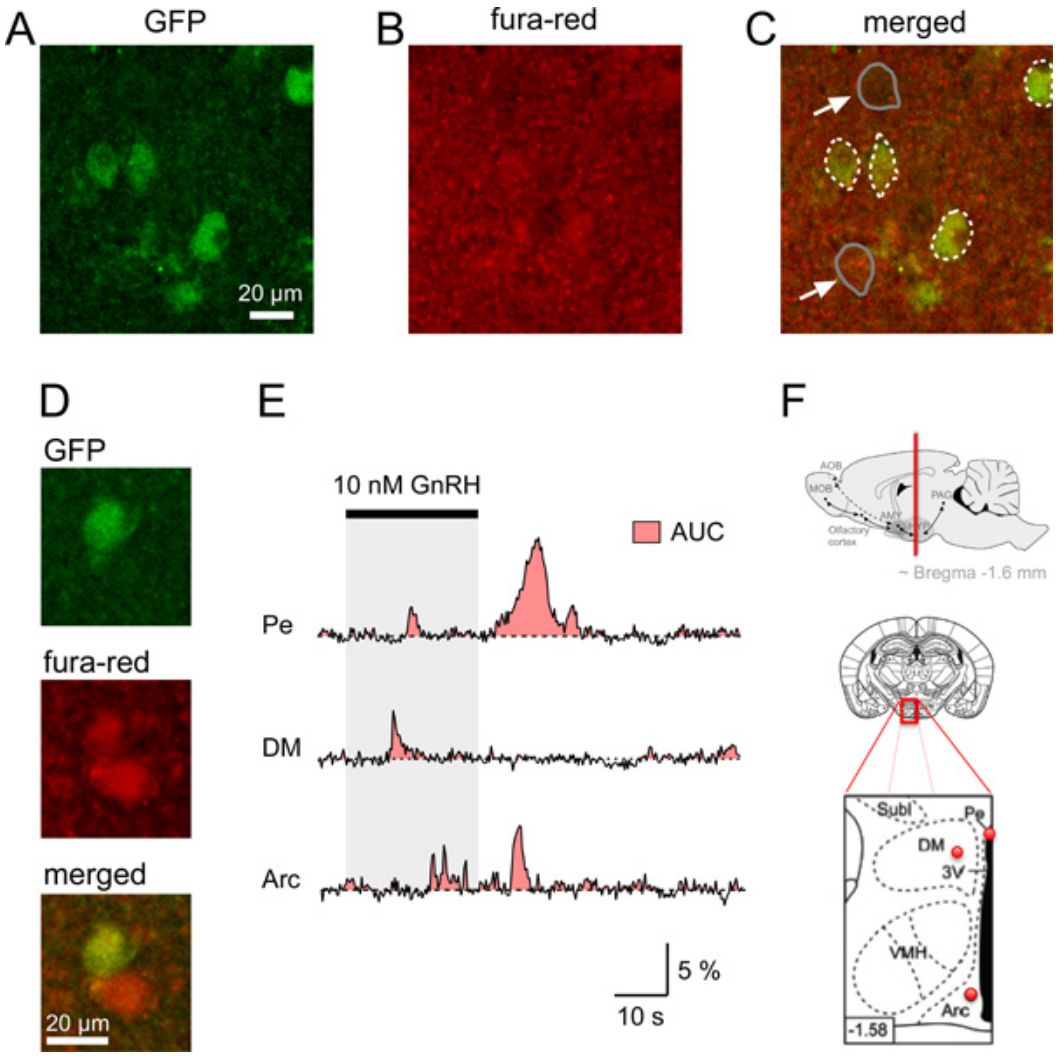

Figure 4. Ca^{2+} signals in τGFP neurons of mouse hypothalamic brain slices. A-D. Identification of a GFP neuron and simultaneous acquisition of the fura-red fluorescence in coronal mouse brain slices. A. Confocal image of a coronal brain slice identifying GnRHR-τGFP neurons (*green*). B. Relatively uniform fluorescence signal (red) of the brain area shown in A observed after loading the brain slice with fura-red/AM. C. Merged image showing the neurons depicted in A loaded with the Ca^{2+} indicator dye (*yellowish*). Boundaries of GFP neuron somata are indicated in dashed white lines, whereas examples of two non-GFP somata (arrows) are indicated in grey lines. D. Example of a GFP and non-GFP neuron with higher amounts of red fluorescence compared to the background. E. Examples of somatic stimulus-induced Ca^{2+} responses from individual GFP-tagged neurons in different hypothalamic brain areas (Pe, periventricular nucleus; DM, dorsomedial hypothalamus; Arc, arcuate nucleus). Area-under-the-curve (AUC) is depicted by the red area. The distinct Ca^{2+} signals between GnRHR-expressing neurons from different hypothalamic nuclei can be compared using the AUC as an estimate for the total change in Ca^{2+} in a given cell during the same period. F. Schematic drawings and diagram indicating the location of the GFP-tagged neurons analyzed in A-E. *Upper panel*: location of a coronal brain section containing hypothalamic brain regions (red line). *Middle and lower panel*: Schematic drawing of a brain slice (*middle*) and magnification of its red boxed area (*lower panel*) indicating with red dots the approximate position of the recorded GFP-tagged neurons from the Pe, DM and Arc shown in E; black area in lower panel scheme: 3rd ventricle. Lower two diagrams are adapted from Paxinos and Franklin[12]. Lower left corner number indicates the distance (mm) from Bregma.

Discussion

A major question in neuroscience is to understand how the brain processes social information. A predominant source of information necessary for social recognition is encoded by olfactory or pheromonal signals. The detection of these signals by neuronal populations in the nose and the recognition of the signals in the brain, especially the hypothalamus, play a key role in many social processes and influence hormones and other neuroendocrine factors[13-16]. An essential obstacle of analyzing neuronal responses in brain areas like the hypothalamus containing multifunctional nuclei with multiple neurons is the identification of specific neurons of interest.

Many transgenic mouse lines have been developed, in which mainly GFP helps to identify neurons. Unfortunately, the fluorescent property of GFP complicates measurements using Ca^{2+} indicator dyes such as fluo-3 and fluo-4[17]. We therefore started to investigate GFP-tagged neurons

using a red-shifted Ca^{2+} indicator dye, like fura-red[3,4]. Fura-red based Ca^{2+} imaging was previously combined with GFP-tagged pancreatic β-cells and GFP-tagged receptors expressed in HEK cells[5,6]. Like fura-2, some cross talk between fura-red and GFP has been reported[2]. Yet, using high quality emission filter sets with specific bandwidths and dichromatic beamsplitter filters can limit the crosstalk/bleed-through between the dyes to some extent. It is wise to confirm the settings of a confocal microscope by measuring both channels (green and red fluorescence) at the same time using (1) brain slices with GFP-tagged neurons, but not loaded with the Ca^{2+} indicator dye, as well as (2) brain slices without GFP-tagged neurons but loaded with the red-shifted Ca^{2+} indicator. Using the same settings on the confocal microscope as with a regular experiment, the investigator can then note if any fluorescence is detected in the disparate channel, which should be avoided.

Some investigators use rhod-2 or recommend the use of X-rhod1 as an alternative red-shifted dye in combination with GFP-tagged cells[2,18,19]. However, rhod AM dyes seem to have a tendency to concentrate in mitochondria[20] and have in many neurons low labeling efficiency[17]. Due to the need of improved red-shifted Ca^{2+}-indicators, investigators and companies are currently developing new probes with hopefully superior performance[21].

Disclosures

No conflicts of interest declared.

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