Video Article

Physiological, Morphological and Neurochemical Characterization of Neurons Modulated by Movement

Dean Dessem[1]

[1]Department of Neural and Pain Sciences, University of Maryland

Correspondence to: Dean Dessem at ddessem@umaryland.edu

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Abstract

The role of individual neurons and their function in neuronal circuits is fundamental to understanding the neuronal mechanisms of sensory and motor functions. Most investigations of sensorimotor mechanisms rely on either examination of neurons while an animal is static[1,2] or record extracellular neuronal activity during a movement.[3,4] While these studies have provided the fundamental background for sensorimotor function, they either do not evaluate functional information which occurs during a movement or are limited in their ability to fully characterize the anatomy, physiology and neurochemical phenotype of the neuron. A technique is shown here which allows extensive characterization of individual neurons during an *in vivo* movement. This technique can be used not only to study primary afferent neurons but also to characterize motoneurons and sensorimotor interneurons. Initially the response of a single neuron is recorded using electrophysiological methods during various movements of the mandible followed by determination of the receptive field for the neuron. A neuronal tracer is then intracellularly injected into the neuron and the brain is processed so that the neuron can be visualized with light, electron or confocal microscopy (Fig. 1). The detailed morphology of the characterized neuron is then reconstructed so that neuronal morphology can be correlated with the physiological response of the neuron (Figs. 2,3). In this communication important key details and tips for successful implementation of this technique are provided. Valuable additional information can be determined for the neuron under study by combining this method with other techniques. Retrograde neuronal labeling can be used to determine neurons with which the labeled neuron synapses; thus allowing detailed determination of neuronal circuitry. Immunocytochemistry can be combined with this method to examine neurotransmitters within the labeled neuron and to determine the chemical phenotypes of neurons with which the labeled neuron synapses. The labeled neuron can also be processed for electron microscopy to determine the ultrastructural features and microcircuitry of the labeled neuron. Overall this technique is a powerful method to thoroughly characterize neurons during *in vivo* movement thus allowing substantial insight into the role of the neuron in sensorimotor function.

Video Link

The video component of this article can be found at https://www.jove.com/video/2650/

Protocol

1. Animal Preparation

1. Anesthetize rat with sodium pentobarbital (50mg/kg IP) and place on a heating pad. Shave the skin overlying the posterior skull with animal clippers. Check the animal to assure that a surgical level of level of anesthesia has been obtained by testing for the absence of a withdrawal reflex and vocalization when the toes are pinched as well as the absence of a palpebral reflex. Check the level of anesthesia every 15 minutes and maintain a surgical level of anesthetisa by injections of sodium pentobarbital 15mg/kg every 45 minutes.
2. Use aseptic technique and make an incision in the inguinal region just distal to the crease formed by the abdomen and inner thigh and insert a cannula (1mm diameter, Clay Adams) into the femoral vein, and femoral artery. Make a midline incision in the submandibular region, reflect the infrahyoid muscles. Make a small incision in the trachea and insert a cannula (2mm diameter) to allow ventilation. Tye a suture around the trachael cannual to secure it in place.Monitor systemic diastolic and systolic blood pressure via the arterial cannula. In addition to monitoring the withdrawal and palpebral reflex now monitor blood pressure to assess the level of anesthesia. Administer additional anesthesia when needed via the venous cannula.
3. Place the rat into a stereotaxic frame and perform a craniotomy to expose the cerebellum. Attach the tracheal cannula to a rodent ventilator. Ventilate the animal with a volume of 2 cm^3 at a rate of 100/min with humidified air. A positive end expiratory pressure of 1 cm H_2O should be maintained to prevent lung collapse. Every 15 minutes hyperinflate the lungs to prevent atelectasis. Then cover the surface of the brain with warmed mineral oil (30°C). Be careful to avoid the large venous sinus located directly under the junction between the parietal and interparietal bones.
4. Apply cyanoacrylate glue to a rod coupled to an electromagnetic vibrator and attach the rod in the diastema of the jaw. Move the mandible using command signals to the vibrator from either a A/D computer output or a signal generator.
5. Place a siliver-silver chloride grounding electrode under the skin adjacent to the craniotomy.

2. Electrode preparation

1. Fabricate microelectrodes from quartz or aluminosilicate glass using a horizontal microelectrode puller.
2. Fill the microelectrode with 5-10% biotinamide dissolved in 0.25M KCl and 0.5M Tris HCl buffer (pH 7.6) or 2% tetramethlyrhodamine in saline (pH 6). Check electrode impedance with an electrode tester. To record from large diameter axons make electrodes with an impedance of 60-80M Ω, for small axons and interneurons make electrodes with impedance of 80-150MΩ.
3. Place the microelectrode into the head stage of the electrometer. Visualize the electrode through a small telescope (20X with an enclosed reticle) which is fixed in position behind the animal's head. Using the telescope is important because it allows electrodes to be stereotaxically positioned with great precision.

3. Electrophysiological recording and intracellular staining

1. Make a small opening in the pia mater and overcompensate the capacitance feedback so that when the electrode touches the brain a feedback signal is produced. This allows accurate location of the surface of the brain.
2. Produce repeated mandibular displacement and advance the electrode into the brain with a stepping motor.
3. Recognize neuronal impalements by sudden drops in DC potential and identify intrasomatic neuronal impalements by ongoing synaptic activity. Buzzing the electrode with overcompensation of capacitance or tapping rarely produce a successful cell penetration. Because of the high impedance of the electrodes, neuronal responses are rarely observed prior to penetration.
4. After you impale a neuron and the penetration is deemed stable, characterize the neuronal response using ramp and hold and sinusoidal jaw movement.[5] Map the receptive field of the neuron by probing the skin around the head and intra oral cavity with a non-conductive probe such as a wooden stick. If relevant to the study, examine the response of the neuron to other functionally relevant stimuli such as muscle contraction and noxious stimuli.[6] Primary afferent nociceptive neuronal receptors respond to nociceptive stimuli. General anesthesetics such as sodium pentobarbital do not block axonal conduction in primary afferent neurons but rather depress synaptic transmission. Thus axonal conduction in the primary afferent neuronal axon is preserved.
5. Inject current (DC, 1-4nA) for a total injection time of 15-70nA minutes. Monitor electrode penetration during current injection and discontinue if membrane potential becomes more positive than -30mV.

4. Tissue processing

1. Euthanize the animal by overdose of pentobarbital (140mg/kg IV) and perfuse with a vascular rinse (0.9% NaCl 38°C containing 500 units of heparin and 1ml 2% xylocaine followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4).
2. Remove the brain and section it at 50-100µm using a vibratome in either the frontal, sagittal or horizontal plane. Secions are collected free floating in 25°C PBS.
3. Process the brain for DAB by incubating in 1-2% normal goat serum and 1% Triton X-100 in 0.01M PBS followed by incubation in avidin biotin complex (1:50 Elite Vectastain). React sections using nickel-DAB with H_2O_2. To process for Texas Red, incubate sections in avidin-biotin complex (1:50) in PBS overnight at 4°C and then incubate in 4% Texas Red avidin DCS in PBST at 4°C overnight. Counterstain sections with a fluorescent Nissl stain (NeuroTrace) 20min at 23°C.
4. If neuron was injected with rhodamine, visualize the injected neuron directly with a fluorescent microscope (Ex 545nm).

5. Combining method with retrograde labeling, immunocytochemistry, confocal imaging, quantitative colocalization analysis

The success of combining this technique with other methods is largely dependent upon good intracellular labeling.

1. The method visualized here can easily be combined with retrograde neuronal labeling.[7-10] To do this, anesthetize the animal and using aseptic technique, inject a neuronal tracer such as horseradish peroxidase (20% horseradish peroxidase. Sigma VI, Sigma) and 1% wheat-germ aggultinated horseradish peroxidase (Sigma) into peripheral target regions such as the masseter muscle. This neuronal tracer will be taken up into peripheral axons and transported via axonal transport to the motoneuron somata. For the masseter muscle, 15µl of tracer is injected into the muscle using a sterile 10 microliter microsyringe. Animals are then placed on a heating pad and monitored until recovered from anesthesia and then placed in their cages for recovery. After 24h, anesthetize animals and physiologically characterize neurons and intracellularly stain them. Then perfuse the animal as described above and process tissue sections for the presence of HRP using tetramethylbenzidine (TMB) as the chromagen and sodium tungstate as the stabilizer and intensified with cobalt. Place sections in 1% sodium tungstate dissolved in 0.1M PBS (pH 6.5) and 0.0007% TMB dissolved in absolute ethanol and acetone at 15°C for 20 min. Then react tissue sections by adding 1.0 ml of 0.3% H_2O_2 per 100ml of incubation solution for 60 min. Rinse tissue in 0.1M PBS (pH 6.5) and place in 0.05% diaminobenzidine (pH 7.4), 0.02% cobalt, and 0.01% H_2O_2 in 0.1M PBS (pH 7.4) for 10 min. at 37°C. Process the tissue for visualization of the neuron injected with biotinamide as described and visualize the relationships between the labeled sensory neuron and a variety of motoneurons.
2. The technique shown here can also readily be combined with immnocytochemistry.[6] As an example, immunocytochemically process the brain for synaptophysin to accurately locate synapses within labeled neurons (**Fig. 3**). To do this, incubate brain sections in mouse anti-synaptophysin antibody (1:10,000) for 2 days at 4°C and then incubate in anti-mouse FITC (1:400) for 1h at 23°C.
3. Neurons labeled with fluorescent markers or processed for fluorescent imaging are ideal for confocal imaging. Figure 3 is an example of an optical section obtained with confocal microscopy through a axon bouton. (**Fig. 3**). Generate animations of labeled neurons by acquiring multiple optical sections through the labeled neuron (**Fig. 4**).
4. Neurons labeled with this method can be used for quantitative colocalization analysis. To do this, use a publicly available software macro in conjunction with NIH Image (found at http://phy.ucsf.edu/°idl/colocalization.htm). Localize synaptophysin within single axon terminals by combining intracellular labeling with synaptophysin immunocytochemistry.[6]

6. Representative Results:

An overview of the representative results that can be obtained using this method are illustrated in **Figure 1**. This single brainstem neuron was electrophysiologically recorded during movement of the mandible and, as can clearly be seen, the response of this neuron (**Figure 1** lower left, light blue) was modulated during movement. This neuron was injected with biotinamide after electrophysiological characterization and subsequently processed for visualization. The reconstructed neuron (**Figure 1** middle, green) can be related to an anatomical landmark, in this case the trigeminal motor nucleus designated (red outline). Based upon the neuronal response during movement and reconstruction this neuron can be identified as a secondary muscle spindle afferent neuron. **Figure 2** illustrates a representative example of the physiological response of a neuron during jaw displacement. The response of the neuron is represented as instantaneous firing frequency. Note that the neuronal response closely mimics mandibular displacement indicating that this particular neuron provides sensory feedback related to mandibular position. **Figure 3** is a high magnification image of an intracellularly stained axon combined with staining for synaptophysin and a Nissl stain. Note the colocalization of synaptophysin (yellow) within the axon bouton. **Figure 4** is an animation of a single, physiologically characterized and intracellularly labeled neuron.

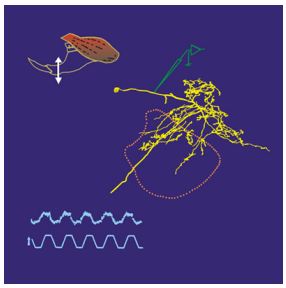

Figure 1. Overview of method. Upper left: mandibular displacement. Middle Intracellular recording (green) from single neuron (yellow). The morphology of this neuron was reconstructed after intracellular recording and injection. Red outline indicates location of the trigeminal motor nucleus. Lower left: physiological response of this neuron during jaw movement.

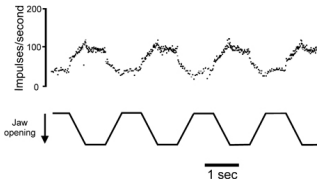

Figure 2. Representative physiological response of a single muscle sensory neuron recorded *in vivo* during movement of the mandible. Note the similarity of the neuronal response with jaw displacement.

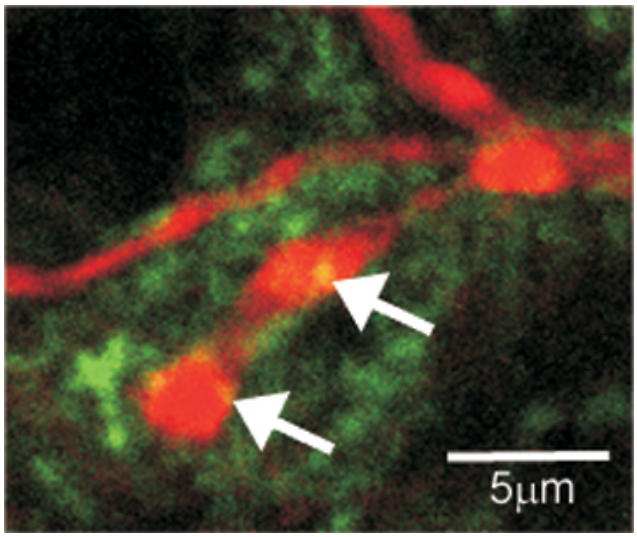

Figure 3. Terminal axonal arborization with synaptic boutons (red swellings) of an intracellularly-stained sensory neuron which responded during muscle probing. Subsequent immunocytochemical processing for synaptophysin shows localization of synaptophysin within the axonal bouton (yellow). Green is a fluorescent Nissl stain.

Figure 4. Animation of muscle spindle primary afferent neuron axon whose physiological response was recorded *in vivo* during mandibular movement, The axon was then intracellularly stained and processed for visualization.
Download a high resolution video of Figure 4 here
Download a medium resolution video of Figure 4 here

Discussion

The method illustrated here is a powerful technique which provides important insight into the function of single neurons and how the response of individual neurons contributes to neuronal circuits.[9] This knowledge is fundamental to understanding sensorimotor function. The greatest strength of this technique is that is allows determination of a large number of parameters about a neuron including physiology, morphology and synaptic morphology and distribution. When combined with other techniques such as retrograde neuronal labeling additional information such as neuronal circuitry can be characterized.[7,8] Another advantage of this method is that it can be learned in steps. For instance, intracellular recording can be conducted initially, followed by intracellular staining with immunocytochemistry or retrograde neuronal labeling added after mastery of the initial method. Perhaps the greatest limitation of the technique is that only a small number of neurons can be labeled in any one experiment. Typically two to three neurons of a particular physiological type are initially labeled. Once the potential relationship between physiology and morphology is formulated, additional experiments are used to carefully check this relationship in experiments in which only a single neuron is labeled.

The most crucial step for the success of this method is maintaining electrophysiological intracellular recording stability. Recording stability will vary greatly depending upon the location within the brain of the neuron under study but a number of manipulations can be used to increase recording stability. A pneumothorax can be performed and the animal artificially ventilated to reduce respiratory pulsations. Stability can be increased further by applying a positive end expiratory pressure of about 1 cm H_2O. When the region of interest within the brain is reached, stability can be enhanced by hyperventilating the animal by decreasing respiratory volume and increasing respiratory rate. Some electrophysiological studies have applied warm agar over the brain and cannulated the bladder; these procedures have not been effective in increasing neuronal recording stability in the brainstem. It is important to point out that injection times do not need to be long. Good results can be obtained with injection times of about 5 minutes. Due to the small tip size of the microelectrodes, breakage of the electrode within the brain typically does not produce a large release of tracer. Therefore the electrode can be replaced and neurons successfully recorded and stained within several hundred microns of the location of electrode breakage. If the electrode is clogged or the recording solution does not fill the tip of the electrode adequately, noise will be greatly increased and the electrode should be replaced. Testing electrode impedance prior to insertion into the brain greatly reduces unproductive electrode tracts and saves time. If you are attempting to record from a small region of the brain stereotaxic positioning is paramount. I use a telescope attached to the recording table to maintain a fixed stereotaxic zero. The telescope is very useful because the electrode can be placed into the electrode holder attached to the recording table and then viewed under magnification. This allows very accurate placement of the microelectrode and zeroing of replacement electrodes.

A number of recent studies have used juxtacellular neuronal labeling.[11,12] With this method an electrode is placed in proximity to a neuron based upon the characteristics of the neuronal recording and a neuronal tracer is ejected. An obvious potential problem with this method is spurious labeling since tracer can be incorporated into the dendrites and axons of other neurons in the vicinity of the electrode. In addition, input-output relationships of the neuron cannot be determined because extracellularly recorded action potentials can be generated not only by synaptic input to the neuron but by intrinsic properties of the neuron. With the method reported here, neurons are only labeled while the microelectrode is actually within the neuron and thus there is no ambiguity concerning the attribution of neuronal activity to the stained neuron. This is particularly important when labeling axons because movement of the microelectrode by a few microns results in spurious labeling. Additionally, subthreshold events including synaptic potentials can be recorded from the impaled neuron.

Future studies could combine this method with evoked movements. For instance cortical stimulation can evoke masticatory movements and the recording stability within the brainstem should allow intracellular recording and staining of neurons during these evoked movements. Since this technique can be done with minimal surgical intervention, it may also be possible to use this method to inject substances which alter gene expression *in vivo*.

Disclosures

Experiments on animals were performed in accordance with the guidlines and regulation set forth in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, revised 1985) and the University of Maryland Animal Care and Use Committee.

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