Video Article

Acute Dissociation of Lamprey Reticulospinal Axons to Enable Recording from the Release Face Membrane of Individual Functional Presynaptic Terminals

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URL: https://www.jove.com/video/51925
DOI: doi:10.3791/51925

Keywords: Neuroscience, Issue 92, reticulospinal synapse, reticulospinal axons, presynaptic terminal, presynaptic calcium, voltage-gated calcium channels, vesicle fusion, synaptic transmission, neurotransmitter release, spinal cord, lamprey, synaptic vesicles, acute dissociation

Date Published: 10/1/2014

Citation: Ramachandran, S., Alford, S. Acute Dissociation of Lamprey Reticulospinal Axons to Enable Recording from the Release Face Membrane of Individual Functional Presynaptic Terminals. *J. Vis. Exp.* (92), e51925, doi:10.3791/51925 (2014).

Abstract

Synaptic transmission is an extremely rapid process. Action potential driven influx of Ca^{2+} into the presynaptic terminal, through voltage-gated calcium channels (VGCCs) located in the release face membrane, is the trigger for vesicle fusion and neurotransmitter release. Crucial to the rapidity of synaptic transmission is the spatial and temporal synchrony between the arrival of the action potential, VGCCs and the neurotransmitter release machinery. The ability to directly record Ca^{2+} currents from the release face membrane of individual presynaptic terminals is imperative for a precise understanding of the relationship between presynaptic Ca^{2+} and neurotransmitter release. Access to the presynaptic release face membrane for electrophysiological recording is not available in most preparations and presynaptic Ca^{2+} entry has been characterized using imaging techniques and macroscopic current measurements – techniques that do not have sufficient temporal resolution to visualize Ca^{2+} entry. The characterization of VGCCs directly at single presynaptic terminals has not been possible in central synapses and has thus far been successfully achieved only in the calyx-type synapse of the chick ciliary ganglion and in rat calyces. We have successfully addressed this problem in the giant reticulospinal synapse of the lamprey spinal cord by developing an acutely dissociated preparation of the spinal cord that yields isolated reticulospinal axons with functional presynaptic terminals devoid of postsynaptic structures. We can fluorescently label and identify individual presynaptic terminals and target them for recording. Using this preparation, we have characterized VGCCs directly at the release face of individual presynaptic terminals using immunohistochemistry and electrophysiology approaches. Ca^{2+} currents have been recorded directly at the release face membrane of individual presynaptic terminals, the first such recording to be carried out at central synapses.

Video Link

The video component of this article can be found at https://www.jove.com/video/51925/

Introduction

Synaptic transmission is an extremely rapid and precise process. Action potential invasion of the presynaptic terminal leads to opening of VGCCs located in the release face membrane, the resulting increase in presynaptic Ca^{2+} acting as the trigger for vesicle fusion and neurotransmitter release[1]. All of these steps occur within hundreds of microseconds[2], and hence require tight spatial coupling of VGCCs to the vesicle fusion machinery[3]. Presynaptic Ca^{2+} fluxes have been primarily characterized through imaging approaches using Ca^{2+} sensitive dyes[4]. Incorporating Ca^{2+} buffers that modulate Ca^{2+} in presynaptic neurons has been used to indirectly characterize the relationship between presynaptic calcium and neurotransmission[3]. In addition, modulating the presynaptic free Ca^{2+} concentration by uncaging Ca^{2+} [5] or recording macroscopic Ca^{2+} currents have been used in conjunction with measures of vesicle fusion and/or release; such as capacitance measurements[6] or postsynaptic responses[2] to address the same question. However, characterizing Ca^{2+} currents directly at the release face, the specialized section of the presynaptic membrane where membrane depolarization is translated into Ca^{2+} currents triggering synaptic vesicle fusion and neurotransmitter release, is integral to obtaining a precise measure of the Ca^{2+} requirement for synaptic vesicle fusion. In addition, the ability to directly characterize Ca^{2+} currents at individual presynaptic terminals, coupled with accurate simultaneous measurements of vesicle fusion and release allows a precise elucidation of the timing relationship between the time course of the action potential, presynaptic Ca^{2+} current, vesicle fusion and release. Access to the release face membrane is not available in the majority of presynaptic terminals due to close apposition by the postsynaptic dendrites. This inaccessibility has been a major obstacle in the characterization of VGCCs since it prevents direct measurements of current at individual presynaptic terminals. Direct characterization of presynaptic Ca^{2+} currents at individual presynaptic terminals has thus far not been possible in central synapses and has only been achieved in two calyceal type presynaptic terminals; calyx-type synapse of the chick ciliary ganglion[7-10] and rat calyces[11,12]. In all other presynaptic terminals including the giant reticulospinal synapse in the lamprey spinal cord[13], the lack of access to the presynaptic release face membrane has necessitated the use of indirect approaches such as Ca^{2+} imaging to study presynaptic Ca^{2+} fluxes.

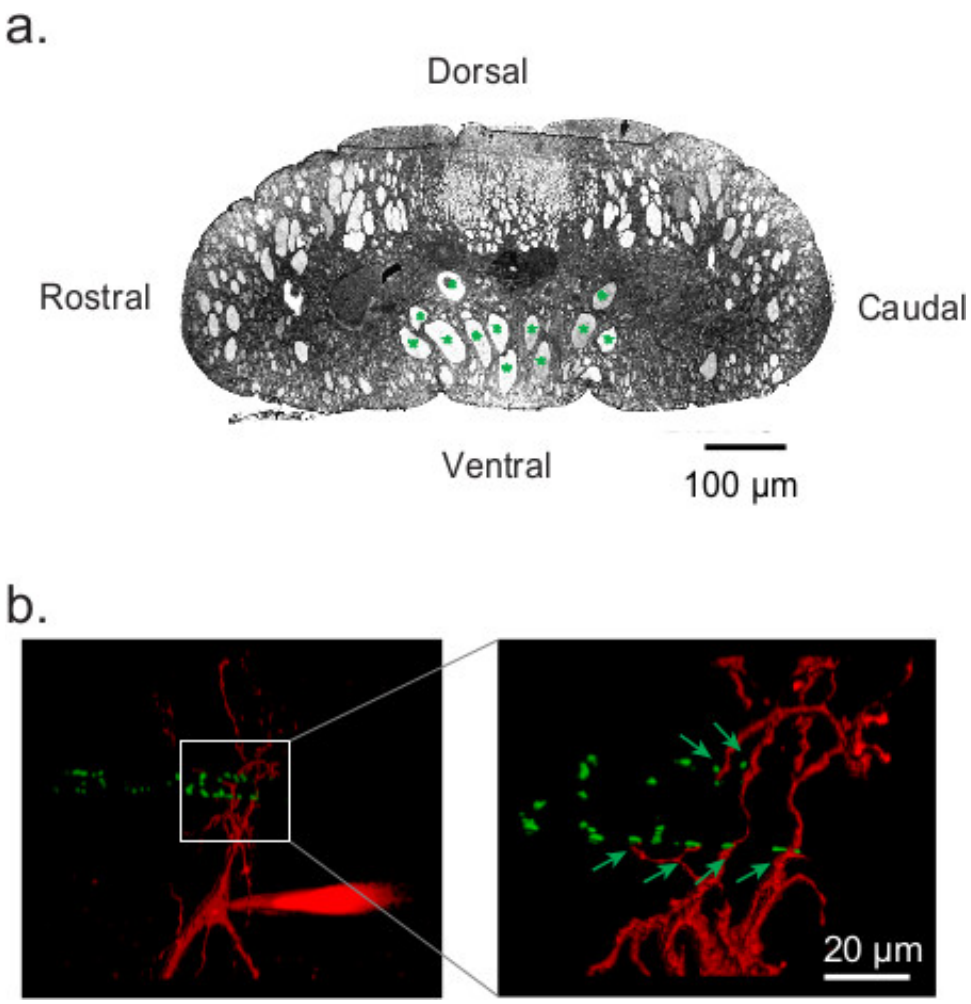

Figure 1. Lamprey giant reticulospinal synapse. (a) Cross-section of lamprey spinal cord indicating dorso-ventral orientation. Reticulospinal axons are marked with green asterix. **(b)** 3-D reconstruction of the reticulospinal synapse in the lamprey spinal cord showing presynaptic reticulospinal axon making numerous en passant contacts (marked by green arrows) onto the postsynaptic neuron[13]. Presynaptic terminals have been labeled with Alexa Fluor 488 hydrazide conjugated phalloidin (green), while the postsynaptic neuron has been filled with Alexa Fluor 568 hydrazide (red).

Lamprey giant reticulospinal axons, located in the ventral region of the spinal cord parallel to the rostral-caudal axis **Figure 1a**, form multiple *enpassant* synaptic contacts onto neurons of the spinal ventral horn[14] **Figure 1b**[13]. Macroscopic whole-cell Ca^{2+} currents have been recorded from reticulospinal axons in the intact spinal cord[13,15]. However, previous blind attempts at direct measurement of Ca^{2+} currents in reticulospinal axons in the intact lamprey spinal cord using cell-attached patch clamp technique have proven unsuccessful[13] due to lack of access to the presynaptic release face membrane owing to the opposing postsynaptic processes **Figure 1b**. The release face membrane has been previously made accessible by removal of the postsynaptic neuron[11], mechanical perturbation of the synapse prior to recording[12] or enzymatic treatment coupled with mechanical dissociation[16]. Given the complex organization of the spinal cord, it would prove extremely difficult to identify the postsynaptic neuron and retract it mechanically or perturb the synapse. Hence, we decided to use enzymatic treatment[17] followed by mechanical dissociation.

Using this approach, we have developed an acutely dissociated preparation of the lamprey spinal cord that yields viable isolated reticulospinal axons with functional presynaptic terminals devoid of any postsynaptic processes, thereby providing unrestricted access to individual presynaptic terminals. In conjunction with a standard inverted microscope and fluorescence imaging, it enables us to identify and target individual fluorescently-identified presynaptic terminals, with a patch pipette containing a recording solution that isolates Ca^{2+} currents **FIgure 4c** and **Figure 4d**, for recording using cell-attached voltage-clamp technique. Ca^{2+} currents have been recorded directly at the presynaptic release face membrane of individual presynaptic terminals **Figure 4f**. This is a significant breakthrough in the field of synaptic transmission since it is the first such recording to be carried out at central synapses.

Protocol

1. Preparation of Poly-D-lysine Hydrobromide

1. Prepare 1 mg/ml poly-D-lysine hydrobromide in 0.1 M borate buffer (pH 8.5).

2. Aliquot and store at -20 °C.

2. Poly-lysine Coating of Coverslips

Note: Carry out all cleaning and coating steps in a laminar flow chamber.

1. Place coverslips in a Petri dish containing 1 N Hydrochloric acid (HCl) for 2 hr.
2. Aspirate all HCl and rinse with 70% Ethanol (EtOH) 2-3x.
3. Leave in 70% EtOH for 1 hr.
4. Aspirate all 70% EtOH and rinse with 100% EtOH 2-3x.
5. Leave in 100% EtOH for 2 hr. Aspirate all EtOH.
6. Blot dry with filter paper and air dry for a few seconds.
7. Place O/N in 1 mg/ml poly-lysine solution.
8. Rinse coverslips next day with Millipore water 4-5x.
9. Air dry poly-lysine coated coverslips on glass rollers in a clean Petri dish.
10. For immunohistochemistry, use 35 x 10 mm Petri dish lids. Prepare sylgard (polydimethylsilioxane, PDMS) lined dishes by pouring PDMS (elastomer and curing agent 10:1 by weight) into the dish to a thickness of 0.3 cm and allow to set at 30 °C O/N. Once this has set, cut a 2 cm by 1 cm inset into the PDMS. Clean and coat the inset with poly-lysine following the same steps as mentioned above for coverslips.
11. Store poly-lysine coated coverslips and dishes covered inside the laminar flow chamber until use (up to 2 weeks, but achieve best adhesive results by preparing periodically every 3-4 days).
12. Prepare a PDMS block, to pin the spinal cord in the vibrating tissue slicer. Mix Elastomer A and Elastomer B 1:1 by weight. Pour into a 100 x 15 mm Petri dish and allow to set O/N at 30 °C. Cut a rectangular piece of the PMDS and glue it to the slicing base plate using epoxy glue. Allow the glue to set fixing the PDMS in place and slice multiple thin sections off the surface to obtain a flat surface to pin the tissue on.

3. Acute Dissociation of Lamprey Spinal Cord to Yield Isolated Reticulospinal Axons

1. Anesthetize an ammoecoete or adult lamprey (*Petromyzon marinus*) with tricaine methanesulphonate (MS-222; 100 mg/L). Add anesthetic into the water, in a plastic cup covered by a lid, containing the lamprey to be sacrificed.
2. Decapitate lamprey in cold (4 °C) Ringer's solution of the following composition (in mM): 130 NaCl, 2.1 KCl, 2.6 $CaCl_2$, 1.8 $MgCl_2$, 4 HEPES, 4 dextrose (pH 7.6, osmolarity 270 mOsm) and remove body wall muscles to expose dorsal surface of spinal cord.
3. Remove meninx primitiva from the dorsal surface of the spinal cord using fine forceps. Do not remove ventral meninx primitiva at this stage.
4. Cut spinal cord into 1 cm long pieces.
5. Pin a spinal cord piece using fine insect pins, dorsal side facing up, on a PDMS lined slicing base plate (see 2.12) in a vibrating tissue slicer chamber containing ice-cold Ringer's solution.
6. Remove a central section of the dorsal column of the spinal cord by slicing along the rostro-caudal axis with the blade, leaving behind intact dorsal column sections on the rostral and caudal ends to serve as handles during the dissociation process **Figure 2a** and **Figure 2b**. Use slowest speed setting that permits slicing and a depth setting that removes only the dorsal column leaving the underlying reticulospinal axon column undamaged **Figure 2b**.
7. Incubate sliced spinal cord pieces at RT for 45 min in a cocktail of 1 mg/ml protease (Type XIV from Streptomyces griseus) and 1 mg/ml collagenase prepared (Type IA from Clostridium histolyticum) in Ringer's solution (adapted from El Manira & Bussières, 1997 17).
8. Pin enzyme-treated spinal cord pieces using fine pins in a PDMS lined Petri dish containing cold (4 °C) Ringer's solution.
9. Remove ventral meninx primitiva with fine forceps.
10. Cut lateral tracts of spinal cord with a scalpel blade at the midpoint of the sliced dorsal section leaving the central column of reticulospinal axons intact in the spinal cord **Figure 2d**.
11. Place a drop of immersion oil on the lens.
12. Place the poly-lysine coated coverslip (**Figure 3a**, top piece, red rectangle) in the slot of the recording chamber **Figure 3** and apply vacuum grease to all edges (space between red and blue rectangles in **Figure 3a**, to facilitate a seal. Screw the top into place. Place the chamber in the inset on the recording rig.
13. Add Ringer's solution into the recording chamber using a Pasteur pipette.
14. Connect the outflow tubing of the pressure bottle containing antifreeze solution to the thermoelectric cooling device input tubing. Connect the output tubing of the thermoelectric cooling device to the input end of the outer cooling jacket and the output end to the reservoir **Figure 3b**.
15. Place one piece of spinal cord at a time in the recording chamber and gently separate the spinal cord maintaining it along the coverslip at all times using Teflon coated forceps until axons are isolated **Figure 2e** and **Figure 2f**.
16. Bring the recording solution temperature to 10 °C by passing the pressurized (nitrogen gas is pushed into the bottle) antifreeze solution (by displacement), via the thermoelectric cooling device, through the outer cooling jacket of the recording chamber **Figure 3b**.
17. Allow axons to recover for 1 hr post dissociation at 10 °C.

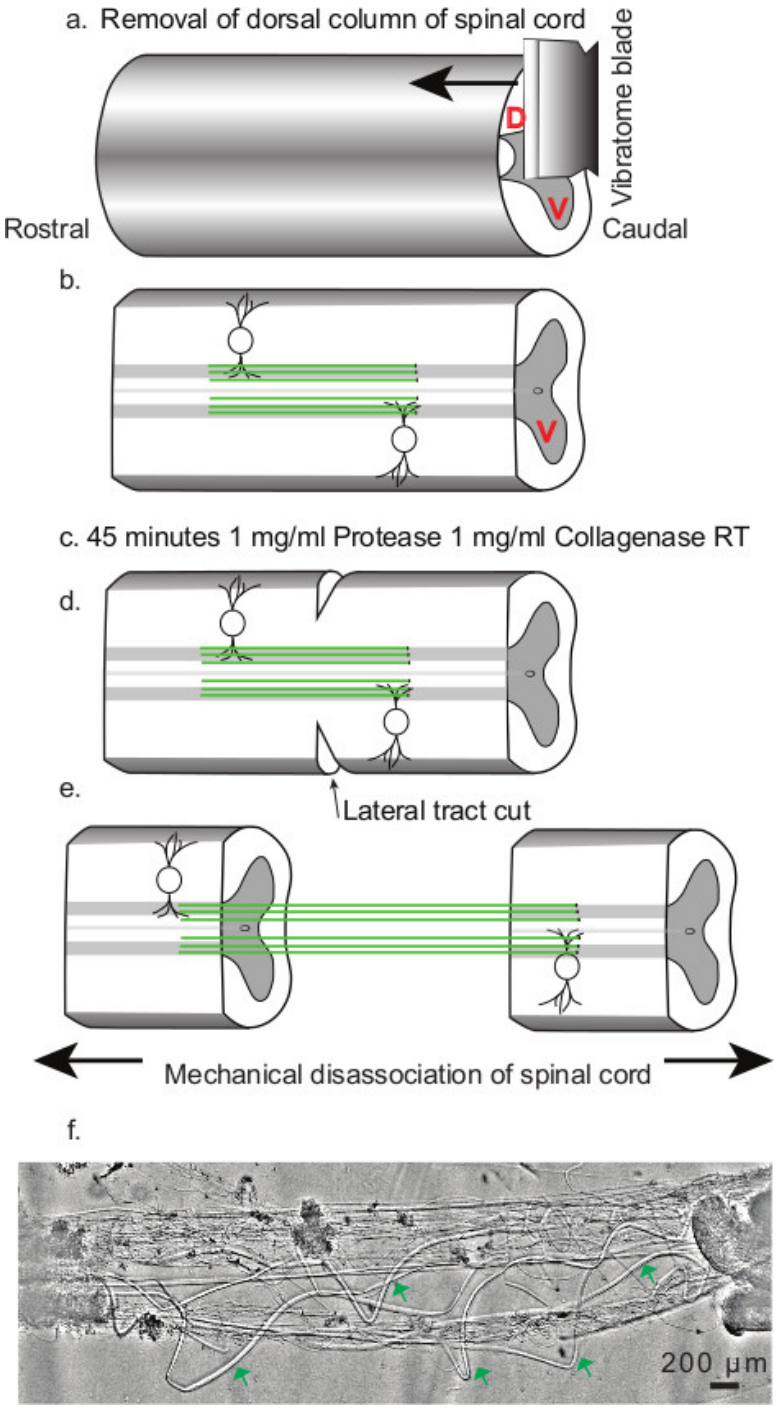

Figure 2. Schematic description of dissociation protocol for isolation of reticulospinal axons. (a) Removal of dorsal column. The arrow indicates the direction of slicing of the tissue. The dorsal horns are marked by the alphabet D (red font color), while the ventral horns by the alphabet V (red font color). **(b)** Dorsal column removed in the central portion of the spinal cord exposing the reticulospinal axons (green lines). The ventral horns, which remain intact after the slicing process, are marked by the alphabet V (red font color). **(c)** 45 min treatment with protease and collagenase enzymes cocktail (1 mg/ml). **(d)** Cutting of lateral tracts of the spinal cord; indicating position, direction and extent of lateral cut. **(e)** Mechanical dissociation of spinal cord. Arrows indicate position of forceps and direction of separation force during dissociation. **(f)** Representative example of dissociated reticulospinal axon preparation. Green arrows mark regions of acutely dissociated reticulospinal axons without any postsynaptic processes.

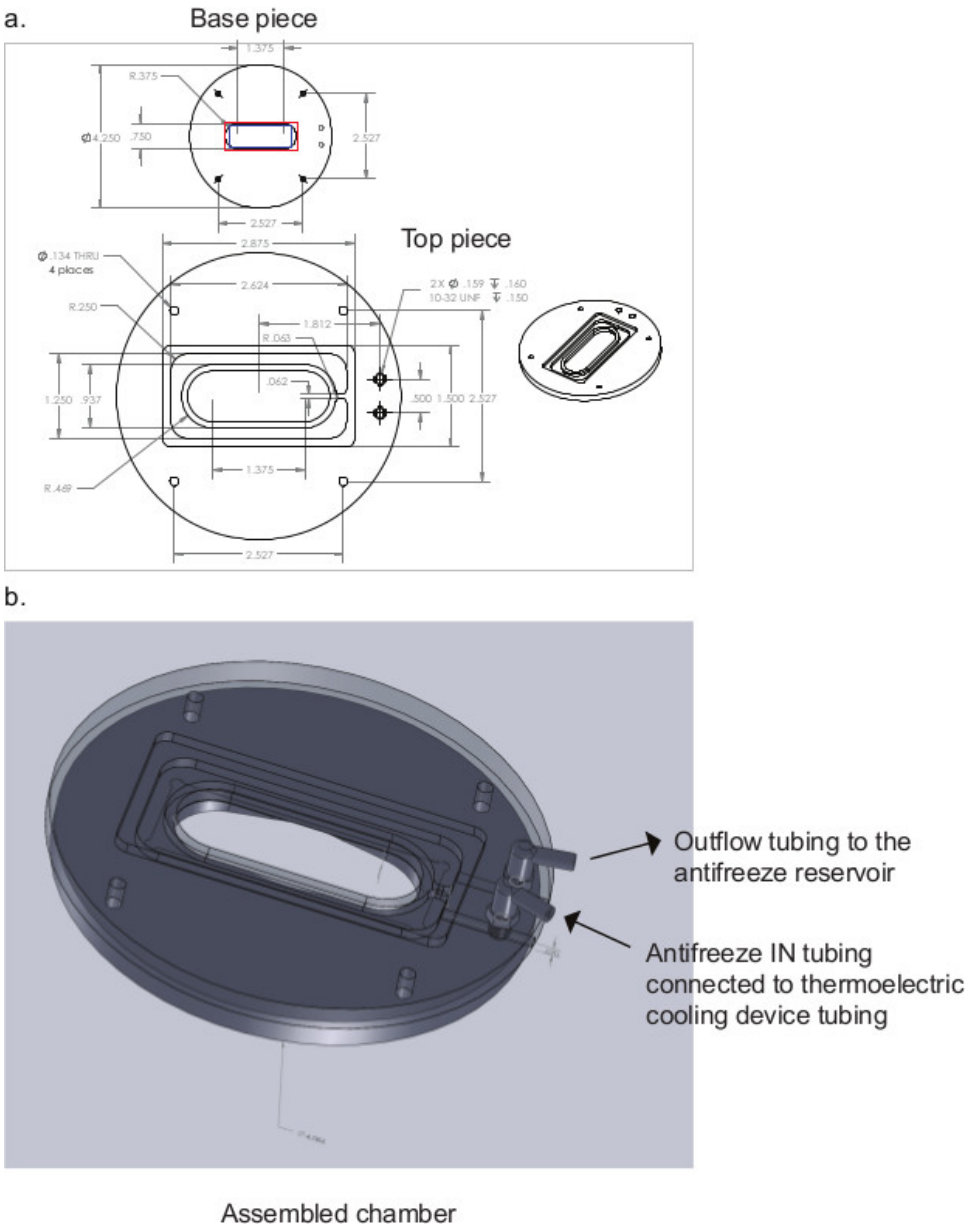

Figure 3. Schematic of recording chamber for electrophysiology experiments. Dimensions provided are in inches. Red rectangle shown in basepiece diagram **(a)** indicates positioning of coverslip in the coverslip groove in the basepiece. The region between the red and blue rectangle, shown in the basepiece diagram **(a)** is where the high vacuum grease is applied. **(b)** shows the assembled recording chamber.

4. Labeling and Identification of Presynaptic Terminals with FM 1-43

1. Label presynaptic terminals, by incorporating FM 1-43 into vesicles during synaptic exo-endocytosis during high K+ depolarization. Perfuse preparation with 5 µM FM 1-43 (in 5 ml Ringer's solution containing 30 mM KCl).
2. Perfuse preparation with 1 mg/ml Advasep-7 (in 5 ml Ringer's solution) to remove excess FM dye)[18].
3. Perfuse preparation with Ringer's solution for 15 min to washout any remant dye and the Advasep.
4. Image with 100 X oil immersion lens (NA 1.25) on an inverted fluorescence microscope, in conjunction with a digital CCD camera and image acquisition software Micromanager, using standard fluorescence imaging protocols.
5. Identify fluorescently labeled presynaptic terminals to target for recording **Figure 4c** and **Figure 4d**.

5. Immunohistochemistry of Isolated Reticulospinal Axons

1. Fill dish inset (Protocol section 2.10.) with divalent-ion (Ca^{2+} and Mg^{2+}) free Ringer's solution.
2. Fabricate patch pipettes in a P-87 micropipette puller. Place a small amount of suture glue at one end of the poly-lysine inset using a patch pipette (1.5 mm outer diameter glass) and suction (using a silicone tubing 0.89 mm inner diameter with a micropipette tip attached to the suction end).

3. Carry out dissociations using the same procedure as mentioned in protocol section 3 **Figure 2**. Place one end of the spinal cord on the suture glue and press gently with forceps to adhere it strongly to the surface. Place another drop of suture glue at the other end of the inset and gently stretch the spinal cord until axons are dissociated and adhere the free spinal cord end by dragging it gently over the suture glue. Fix in place by gentle pressing down with the forceps.
4. Exchange divalent free Ringer's solution with regular Ringer's solution by perfusion.
5. Allow axons to recover for 20 min post dissociation at 10 °C.
6. Fix dissociated axons in 4% paraformaldehyde (PFA) {prepared in Phosphate Buffer Saline (PBS, (mM) NaCl 137, KCl 2.7, Na_2HPO_4 10, KH_2PO_4 1.8, pH 7.4)} for 20 min. Filter PFA solution prior to use by passing through a 0.2 µM syringe filter.
7. Wash out the PFA by perfusing 0.1 M glycine (in PBS) for 10 min.
8. Incubate in 0.1% Triton-X (in PBS) for 10 min.
9. Wash by perfusing PBS for 20 min.
10. Block with 5% non-fat milk (in PBS) for 6 hr at 4 °C.
11. Add in primary antibody to VGCC of interest (1:200 dilution in PBS) and incubate for 20 hr at 4 °C.
12. Wash by perfusing PBS for 20 min.
13. Block with 5% non-fat milk (in PBS) for 10 min at 4 °C.
14. Add in secondary antibody (1:400 dilution in PBS) and incubate in dark for 2 hr at 4 °C.
15. Wash by perfusing with PBS for 20 min.
16. Block with 1% Bovine Serum Albumin (in PBS) for 20 min.
17. Add in Alexa Fluor 488 phalloidin (5 Units/µl working concentration; stock prepared in methanol 200 Units/ml).
18. Wash by perfusing with PBS for 20 min.
19. Image using 100X water immersion lens on confocal microscope.

6. Electrophysiological Recording

1. Fabricate aluminosilicate glass patch pipettes (pipette resistance 2-5 MΩ) in a P-87 micropipette puller. Design patch pipette such that pipette tip encompasses entire presynaptic terminal diameter.
2. PDMS coat patch pipettes by dipping the patch pipette under 50-60 psi pressure into PDMS[23] (attach a silicone tubing, 0.89 mm inner diameter, connected to a nitrogen gas cylinder, to the back end of the patch pipette) and dry using a heat gun. Alternately, manually coat the pipette with PDMS, applying coating as close to the tip as possible, under a compound microscope.
3. Fire polish patch pipettes using a microforge (a custom built platinum filament fitted onto the stage of a compound microscope).
4. Identify isolated axons demonstrating labeled presynaptic terminals by fluorescence microscopy.
5. Fill recording solution (designed to isolate Ca^{2+} currents; 10 mM $CaCl_2$ or 90 mM $BaCl_2$ as charge carrier, HEPES buffered pH 7.6, osmolarity 270 mOsm) into the patch pipette using a syringe.
6. Insert patch pipette into the pipette holder and position above bath using a motorized manipulator MP225. Gently lower the patch pipette into the bath and position against the face of a fluorescently identified presynaptic terminal.
7. Advance the patch pipette slowly until contact is made with the membrane. At this point, achieve a gigaohm seal by gentle mouth suction through a tube attached to the pipette holder.
8. To achieve the extremely low background noise levels required for recording single channel Ca^{2+} currents, use an Axopatch 200B with a cooled headstage for the recordings. Sample data at 20-50 kHz and filter using a 5 kHz Bessel filter. Carry out data acquisition using an Axograph X.
9. Use a standard step protocol, in increments of 10 mV as stimulus. Incorporate a pre-pulse in the protocol, preceding the step protocol, to ensure maximal activation of Ca^{2+} channels. Incorporate a 10 mV leak step into the step protocol for post-analysis subtraction of leak currents.

Representative Results

This dissociation protocol yields healthy and functional isolated reticulospinal axons devoid of postsynaptic projections **Figure 2f**, but which nevertheless retain functional presynaptic terminals capable of evoked synaptic vesicle exo-and endocytosis **Figure 4c** and **Figure 4d**. Sections of the isolated regions of the reticulospinal axons can be clearly identified under light microscopy to be clear of any other neuronal processes allowing unrestricted access to the reticulospinal axon membrane **Figure 2f**. The axons retain structural integrity after dissociation. Isolated axons were patched in whole cell configuration and filled with Alexa Fluor 488 hydrazide. The dye distributed uniformly within the axon and no dye leakage was observed from the axon **Figure 4a**.

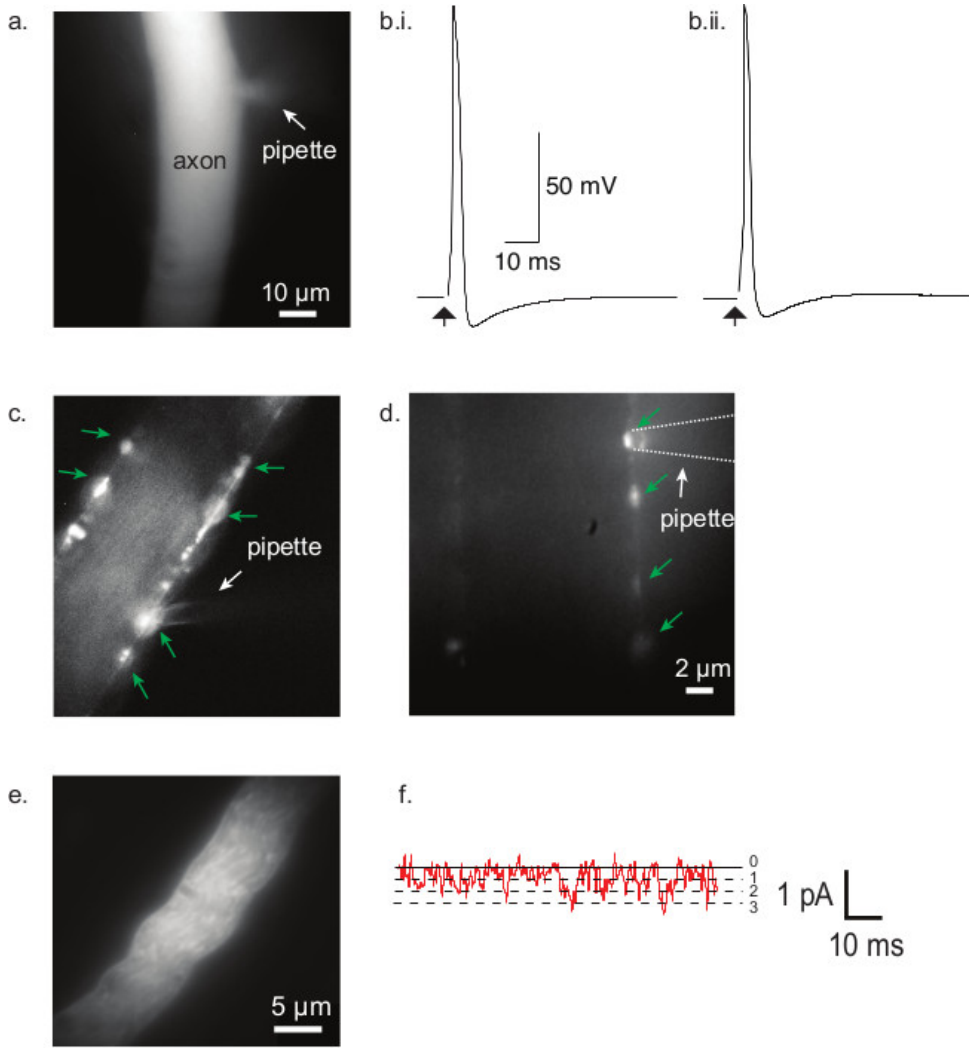

Figure 4. Representative recordings from an isolated reticulospinal axon. (a) Isolated reticulospinal axon filled with Alexa Fluor 488 hydrazide with a patch pipette in whole cell configuration. Pipette position is indicated with a white arrow. **(b)** i. Representative action potential fired in an isolated reticulospinal axon. ii. Representative action potential fired in a reticulospinal axon in the intact spinal cord. Black arrow indicates time point of stimulation. **(c)** and **(d)** Two images of an isolated reticulospinal axon showing punctate labeling of presynaptic terminals with FM 1-43 and targeting of an individual presynaptic terminal with patch pipette for cell-attached patch recording. Green arrows mark individual presynaptic terminals while white arrow marks the patch pipette. **(e)** Dead isolated reticulospinal axon showing indiscriminate incorporation of FM 1-43 throughout the axon. 4f. Representative example showing cell-attached recording of Ca^{2+} currents (10 mM $[Ca^{2+}]_{external}$ in the recording solution in the patch pipette) from the presynaptic release face membrane demonstrating the opening of multiple Ca^{2+} channels. Solid line marked 0 indicates closed state of channel, while dashed lines indicate the Gaussian peaks for channel openings.

Acutely dissociated axons retain electrical properties. Resting membrane potential (RMP) can be measured by impaling the disassociated axon with a microelectrode or by patching the axon in whole cell configuration in current clamp mode. Similar RMPs are recorded in both methods, with a mean of -57.94 ± 1.63 mV (n = 17 axons). In addition, dissociated axons can be stimulated to fire action potentials **Figure 4b**, with the shape and time course of the action potential being comparable to one fired in a reticulospinal axon in the intact spinal cord.

Vesicle fusion and recycling persist in dissociated axons. Recycling vesicle clusters can be labeled with the styryl dye FM 1-43 during high potassium (30 mM KCl) stimulation[19]. Upon dye clearance, distinct punctate labeling of presynaptic terminals can be observed **Figure 4c** and **Figure 4d**, with a distribution similar to reticulospinal axons in the intact spinal cord[13]. FM 1-43 staining provides a clear distinction between healthy and unhealthy axons as unhealthy axons show indiscriminate FM dye entry throughout the axon **Figure 4e**. The ability to clearly identify individual presynaptic terminals allows us to target them with a patch pipette for recording **Figure 4c** and **Figure 4d**. Utilizing this ability, we have recorded Ca^{2+} currents directly from the release face membrane of single presynaptic terminals **Figure 4f**.

The acutely isolated reticulospinal axon preparation also offers distinct advantages in comparison to the intact spinal cord for immunohistochemistry of single presynaptic terminals. The lack of any background autofluorescence and the absence of staining on postsynaptic structures or glia allows for unambiguous identification of antibody labeling at identified presynaptic terminals (**Figure 5b**, i). Coupled with a presynaptic marker such as Alexa-488 conjugated phalloidin (**Figure 5a**, ii) and (**Figure 5b**, ii), which labels presynaptic actin[20], localization of immunohistochemical labeling to presynaptic terminals can be clearly demonstrated (**Figure 5a**, iii). This approach has been used

in conjunction with confocal microscopy to carry out immunohistochemical characterization of the different VGCC subtypes showing their specific localization to presynaptic terminals **Figure 5a**.

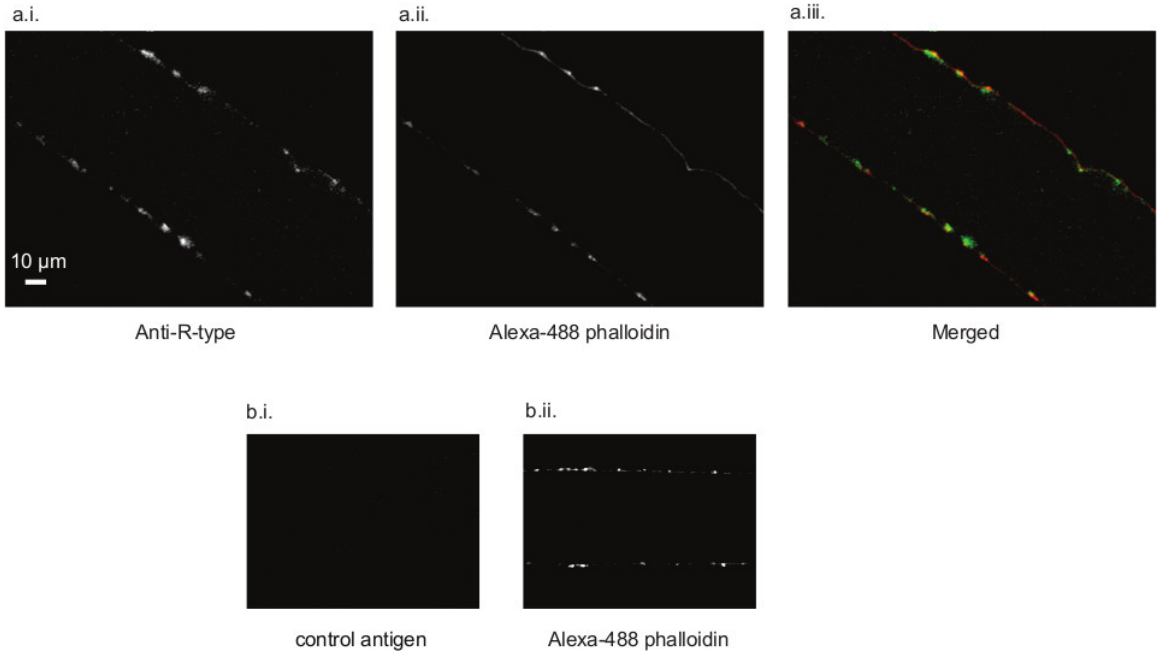

Figure 5. Immunostaining of an isolated reticulospinal axon. (a) Immunohistochemical characterization of R-type (CaV2.3) calcium channel at individual presynaptic terminals. i. A polyclonal primary antibody was used to label an epitope in the intracellular loop between domains II and III of the R-type calcium channel (host - rabbit). Secondary antibody was Alexa Fluor 633 hydrazide conjugated goat anti-rabbit IgG. ii. Presynaptic terminals identified by Alexa Fluor 488 phalloidin labeling of presynaptic actin. iii. Colocalization between R-type antibody labeling (red) and Alexa-488 phalloidin (green) shown in a merged overlay. **(b)** i. Preincubation of anti R-type calcium channel antibody with control antigen does not yield any specific labeling of calcium channels. ii. Alexa Fluor 488 phalloidin labeling of presynaptic terminals for the same axon showing discrete punctate labeling.

Discussion

Our dissociation protocol is significant by yielding isolated reticulospinal axons devoid of postsynaptic projections **Figure 2f**, but which nevertheless retain functional presynaptic terminals **Figure 4c** and **Figure 4d**. The absence of postsynaptic processes opposing the presynaptic terminal permits direct recording access to the presynaptic release face membrane at single presynaptic terminals, previously not possible in central synapses and successfully achieved in only two calyceal-type presynaptic terminals; calyx-type synapse of the chick ciliary ganglion[7-10] and rat calyces[11,12]. Individual presynaptic terminals, which at the electron micrographic level have been shown to present simple, single active zones[21], can be identified by labeling recycling vesicle clusters with FM 1-43 and targeted for recording **Figure 4c** and **Figure 4d**. Ca^{2+} currents can be recorded at the release face membrane of individual presynaptic terminals **Figure 4f**. The recording of Ca^{2+} currents directly from the release face membrane of individual presynaptic terminals is significant as this is the first such recording at central synapses. The physiological relevance of the preparation is emphasized by the fact that the axons retain function after dissociation, allowing for recording from presynaptic terminals with similar characteristics to the terminals in the reticulospinal axons in the intact spinal cord. In addition, presynaptic Ca^{2+} transients in reticulospinal axons have been characterized in the intact lamprey spinal cord through calcium imaging[13] providing a reference to validate the results obtained from the acutely dissociated axons.

The key to obtaining isolated reticulospinal axons with functional presynaptic terminals lies in a series of critical sequential steps. Tissue impeding acute dissociation of the giant axons located principally in the dorso-medial region of the spinal cord **Figure 1a** had to be removed first in a vibrating tissue slicer **Figure 2a**. This makes it easier to mechanically dissociate the spinal cord using the least possible force. The slicing of the dorsal column requires the complete removal of the dorsal meninx primitiva since any remaining dorsal meninx primitiva would impede the cutting action of the blade. We find that leaving the ventral meninx primitiva on the spinal cord at the time of slicing helps maintain the shape of the spinal cord and assists in better slicing. Sustaining tension in the spinal cord during the slicing process prevents any lateral movement or impinging of the tissue under the blade that would damage the axons. 1 cm long spinal cord pieces provides a good length for slicing as the tissue can be stretched and pinned down maintaining tension while slicing. Another critical factor to monitor is the depth of the slice; too shallow a slice prevents good separation of the spinal cord while too deep a slice damages the reticulospinal axons. A good slice depth is one where the dorsal column is removed visibly leaving the ventral reticulospinal column undamaged. The depth of the slice has to be gauged during the slicing process given there is variation in spinal cord thickness between animals. The removal of the dorsal column is best carried out in the middle section of the spinal cord piece **Figure 2b** as it allows for the unsliced rostral and caudal ends to serve as handles to grasp the tissue during the dissociation process **Figure 2e**.

After removal of the dorsal column, treating the sliced spinal cord pieces with a mixture of collagenase (Collagenase Type IA from Clostridium histolyticum) and protease (Protease Type XIV from Streptomyces griseus) enzymes (1 mg/ml in Ringer's solution) helps digest the connective tissue and facilitates a gentler dissociation. A 30-45 min digestion at RT is ideal for dissociation. Complete enzymatic dissociation of the lamprey spinal cord has been previously carried out with sequential treatments of collagenase (2 mg/ml, 30 min) and protease (2 mg/ml, 45 min) to

isolate spinal neurons[17]. Sequential treatments versus treatment with a cocktail of protease and collagenase did not yield better results in dissociating reticulospinal axons. We also experimented with different concentrations of the dissociation enzymes. Enzyme concentrations higher than 2 mg/ml or digestion times longer than 45 min resulted in the spinal cord losing its consistency and poor dissociations. The higher enzyme concentrations and prolonged treatment periods might aid in complete dissociation of the spinal cord when yields of smaller neurons are required. Reticulospinal axons, in contrast, have an extended structure and treatments greater than 45 min were counterproductive. Retaining some tension in the spinal cord aided better dissociations.

The next important step is to cut the lateral tracts of the enzyme-treated spinal cord pieces at the midpoint of the sliced region to isolate the final dissociation force to the region encompassing reticulospinal axons. The cuts should extend from the lateral edge of the ventral horn gray matter to the central ventro-medial column of reticulospinal axons leaving them undamaged **Figure 2d**. Cutting the lateral tracts provides a focal point at which forced separation of the spinal cord will occur during mechanical dissociation and facilitates a smoother separation of the tissue. Sustained tension in the spinal cord is required during the cutting process and can be achieved by stretching and pinning the caudal and rostral ends of the spinal cord piece. This prevents tissue deformation under the scalpel blade and consequent damage to the axons.

The final step in the dissociation is applying mechanical tension and gently stretching the tissue along the rostro-caudal axis **Figure 2e** using forceps to grip the tissue. This step is performed over poly-lysine coated coverslips to allow adhesion of the separated axons for subsequent recording. In order to achieve greater adhesion for the long extended reticulospinal axons, we have used a high molecular weight (>300,000) poly-D-lysine hydrobromide, which provides greater number of attachment points, to coat the coverslips and petri dish insets. The poly-lysine coated surface provides sufficient adhesion for the spinal cord ends and the acutely isolated reticulospinal axons. Teflon coated forceps are required to grip the spinal cord because it sticks firmly to regular stainless steel forceps impeding dissociations. The tissue must be stretched slowly and gently pulling the axons out of the spinal cord. The best way to get the axons to settle down is to maintain the spinal cord ends along the glass coverslip at all times during the dissociation process by maintaining pressure downward onto the rostral and caudal spinal cord ends with the forceps.

The axons are best classified as partially isolated since some portion of the axon remains inside at minimum one end of the spinal cord end depending on the extent of the dissociation. Isolations can be carried out by mechanically separating the two ends of the spinal cord until the axons completely separate from one end of the spinal cord. In this case, one portion of the axon remains inside the spinal cord end from which it emerges while the remaining portion is isolated out of the spinal cord interior. The terminal open end of the axons seals up by a membrane resealing process depending on the health of the axon. Isolations can also be carried out such that the axons emerge out from the spinal cord, but the caudal and rostral ends of the spinal cord remain connected by means of the isolated reticulospinal axons **Figure 2e**. At this point, gently releasing the pressure allows the axons under tension to relax and loop out yielding isolated regions of axons devoid of postsynaptic projections. Both dissociation techniques yield functional axons. The physiological temperature for the lamprey is 10 °C and hence the preparation is allowed to recover at 10 °C for 1 hr to allow for the axons to recover from the acute dissociation. For electrophysiological recordings, the dissociation was carried out on a poly-lysine coated coverslip inserted into a custom chamber **Figure 3**. Lamprey spinal cords have been generally maintained at 10 ± 2 °C by perfusing Ringer's solution, precooled to 10 °C by passing through a thermoelectric cooling device. Exceptionally low background noise is required for resolving single channel Ca^{2+} currents. Perfusion produced artifacts and hence an alternate cooling system had to be designed to maintain the temperature of the dissociated preparation. Recordings were therefore carried out in a still bath containing Ringer's solution. A custom recording chamber was designed for single channel recordings. The chamber had a central bath compartment where the spinal cord was dissociated in Ringer's solution to yield isolated axons. A cooling jacket was designed around the central bath chamber to maintain the temperature of the preparation. The temperature of the bath was maintained by perfusing antifreeze solution, precooled by through a thermoelectric cooling device, through the external cooling jacket by passing nitrogen gas under pressure, bypassing any electromechanical pumping requirement (Protocol section 3.15-3.16).

High K^+ (30 mM KCl) is used to depolarize the axons (section 4.1), so that FM dye is incorporated into vesicles during synaptic exo-endocytosis. It is difficult to retain insertion of a microelectrode in isolated axons for prolonged periods as the slightest drift in axon position causes the electrode to recede from the axon. This makes it difficult to stimulate the axons for prolonged period using a microelectrode. The large axon diameter (20-50 µm) makes it difficult to stimulate using a tungsten stimulation electrode. Hence high K^+ was used to depolarize the axons. The presynaptic terminals in the reticulospinal axons have a diameter of 1-2 µm.

Ca^{2+} currents are localized to the release face membrane at presynaptic terminals and it is therefore crucial to accurately target the presynaptic terminal. Hence, a key requirement to achieve these recordings is being able to manipulate the movement of the patch pipette position in µm increments. Ca^{2+} currents at presynaptic release face membranes have been demonstrated in calyceal synapses to be extremely small in amplitude, less than 0.5 pA. Single channel recordings of Ca^{2+} currents hence require extremely low background noise levels. Low thermal noise was achieved with an Axopatch 200B amplifier and a cooled headstage. Furthermore, contaminating sources of noise need to be systematically identified and eliminated. Patch pipettes were fabricated, from aluminosilicate glass to ensure low noise since the glass has extremely low impurities and high resistivity. The pipettes were created such that the pipette tip diameter was larger than the diameter of the presynaptic terminal thereby completely encompassing the presynaptic terminal, the dimensions of which can be clearly observed by FM 1-43 labeling. This ensured recording from the entire release face membrane. The capacitive noise was reduced by coating the pipette with PDMS as close to the tip as possible. The gigaohm seals at presynaptic terminal membranes are not stable for long periods and recordings typically last a maximum of 2 min. This makes the recordings extremely difficult to obtain since the low success rate necessitates a large number of attempts to obtain successful recordings. Recording solutions have to be designed so that all other known currents in the presynaptic terminal membrane such as voltage-gated Na^+ and K^+ channels and Ca^{2+}-activated K^+ channels are blocked, allowing recording of Ca^{2+} currents.

There are some limitations to using the poly-lysine adhesive surface with this preparation. One is the inability to move the physical location of the chamber containing the preparation because any vibrations disrupted the preparation. Another limitation of the adhesive surface was uncovered during immunohistochemistry experiments, as the rostral and caudal spinal cord end pieces lose adhesion when incubated in 4% paraformaldehyde. We have tried alternative adhesive coatings such as Cell-Tak and encountered similar issues. In order to solve these problems, we used liquid suture glue to affix the spinal cord handles. The specifics regarding the use of the suture glue have been discussed in a previous JoVE article by Chen and Featherstone[22]. The glue sets at a slower rate in solutions without divalent ions and we took advantage of that property and carried out the dissociations in Ringer's solution without Ca^{2+} and Mg^{2+}. This provided us with additional time for manipulation of the tissue during the dissociation process. We adopted a similar approach to Chen and Featherstone[22] using patch pipettes and mouth suction

through a tubing connected to the patch pipette to apply the glue onto the specific target area on the dissociation surface prior to performing the dissociation (Protocol section 5.2). The dissociations were performed as described earlier, with the only difference being that the spinal cord ends were affixed during the dissociation process by gently dragging the handle end over the previously placed glue (Protocol section 5.3). Once the dissociation was completed, the divalent ion free Ringer's solution was exchanged for Ringer's solution containing Ca^{2+} and Mg^{2+} ions by perfusion and the preparation was allowed to recover at 10 °C for 20-30 min on a thermoelectric cooling plate before proceeding to subsequent steps of immunohistochemistry.

The key implication of this technique is a precise understanding of the Ca^{2+} requirement for neurotransmitter release, which has not been completely resolved despite decades of research. A transient increase in presynaptic [Ca^{2+}] has been established as being crucial to triggering release in all synapses. However, consensus is still lacking on the number of Ca^{2+} channels that contribute to the Ca^{2+} domain gating release. Simultaneous measurement of Ca^{2+} currents and capacitance measurements at the release face membrane of individual presynaptic terminals would yield an unambiguous answer to this question. Furthermore, it would enable elucidation of the timing relationship between presynaptic Ca^{2+} entry and vesicle fusion, enabling a precise measure of synaptic delay. The unrestricted accessibility to the release face membrane at individual presynaptic terminals provides the ability to apply a number of different experimental approaches to study various components of neurotransmitter release process. Examples of such approaches include single vesicle imaging using quantum dots to study synaptic vesicle fusion. Vesicle fusion events can also be directly characterized at presynaptic terminals by measuring membrane capacitance changes that underlie vesicle fusion events.

Disclosures

The authors do not have any competing financial interests or other conflicts of interest to disclose.

Acknowledgements

This work has been supported by NINDS, RO1NS52699 and MH84874 to SA.

We would like to thank Dr. Dave Featherstone (Department of Biological Sciences, University for Illinois at Chicago) for providing us with the suture glue used in the immunohistochemistry work. We thank Michael Alpert for his comments and proofreading of the manuscript.

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