An Antibody Feeding Approach to Study Glutamate Receptor Trafficking in Dissociated Primary Hippocampal Cultures

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Abstract

Cellular responses to external stimuli heavily rely on the set of receptors expressed at the cell surface at a given moment. Accordingly, the population of surface-expressed receptors is constantly adapting and subject to strict mechanisms of regulation. The paradigmatic example and one of the most studied trafficking events in biology is the regulated control of the synaptic expression of glutamate receptors (GluRs). GluRs mediate the vast majority of excitatory neurotransmission in the central nervous system and control physiological activity-dependent functional and structural changes at the synaptic and neuronal levels (e.g., synaptic plasticity). Modifications in the number, location, and subunit composition of surface expressed GluRs deeply affect neuronal function and, in fact, alterations in these factors are associated with different neuropathies. Presented here is a method to study GluR trafficking in dissociated hippocampal primary neurons. An “antibody-feeding” approach is used to differentially visualize GluR populations expressed at the surface and internal membranes. By labeling surface receptors on live cells and fixing them at different times to allow for receptors endocytosis and/or recycling, these trafficking processes can be evaluated and selectively studied. This is a versatile protocol that can be used in combination with pharmacological approaches or overexpression of altered receptors to gain valuable information about stimuli and molecular mechanisms affecting GluR trafficking. Similarly, it can be easily adapted to study other receptors or surface expressed proteins.

Keywords

Neuroscience; Issue 150; glutamate receptor; surface expression; receptor internalization; receptor recycling; antibody feeding; transfection; neurons

Introduction

Cells utilize the active process of trafficking to mobilize proteins to specific subcellular localizations and exert strict spatiotemporal regulation over their function¹. This process is

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Video Link

The video component of this article can be found at <https://www.jove.com/video/59982/>

Disclosures

The authors have nothing to disclose.

especially important for transmembrane receptors, as cellular responses to different environmental stimuli rely on intracellular cascades triggered by receptor activation. Cells are able to modify these responses by altering the density, localization, and subunit composition of receptors expressed at the cell surface via receptor subcellular trafficking regulation². Insertion of newly synthesized receptors into the plasma membrane, along with endocytosis and recycling of existing receptors are examples of trafficking processes that determine the net pool of surface-expressed receptors². Many molecular mechanisms cooperate to regulate protein trafficking, including protein-protein interactions and posttranslational modifications such as phosphorylation, ubiquitination, or palmitoylation².

Regulation of receptor trafficking is particularly required in strongly polarized cells with highly specialized structures. The paradigmatic example is the control of neuronal function by regulated trafficking of glutamate receptors (GluRs)^{3,4}. Glutamate, the main excitatory neurotransmitter, binds and activates surface-expressed GluRs to control fundamental physiological neuronal functions such as synaptic neurotransmission and synaptic plasticity. The fact that altered GluR trafficking has been observed in a broad spectrum of neuropathies, ranging from neurodevelopmental disorders to neurodegenerative diseases, highlights the importance of this process⁵. Thus, understanding the molecular events that control GluR trafficking is of interest in many areas of research.

In this protocol, an antibody-feeding based method is used to quantify the level of surface-expressed GluRs in primary hippocampal neurons as well as evaluate how changes in internalization and recycling result in the observed net surface expression. The use of pharmacology and/or overexpression of exogenous receptors harboring specific mutations makes this protocol a particularly powerful approach for studying molecular mechanisms underlying neuronal adaptation to different environmental stimuli. A final example of the utility of this protocol is studying how multifactorial changes in the environment (such as in a disease models) affects GluR trafficking through the examination of surface expression in such models.

Using specific examples, it is initially demonstrated how a pharmacologic manipulation mimicking physiological synaptic stimulation [chemical LTP (cLTP)] increases the surface expression of the endogenous GluA1 subunit of the AMPA-type of GluRs (AMPA receptors)⁶. The trafficking of an overexpressed phospho-mimetic form of the GluN2B subunit of NMDA-type of GluRs (NMDARs) is also analyzed to exemplify how this protocol can be used to study the regulation of GluR trafficking by specific posttranslational modifications. Though these specific examples are used, this protocol can easily be applied to other GluRs and other receptors and proteins that possess antigenic extracellular domains. In the case that there are no antibodies available for extracellular domains, overexpression of extracellular epitope-tagged (e.g., Flag-, Myc-, GFP-tagged, etc.) proteins can assist in protein labeling.

The current protocol provides instructions for quantifying specific GluR subtype density and trafficking using specific antibodies. This protocol can be utilized to study 1) total GluR surface expression, 2) GluR internalization, and 3) GluR recycling. To study each process individually, it is advised to begin with sections 1 and 2 and continue with either section 3, 4, or 5. In all cases, finish with sections 6 and 8 (Figure 1).

Protocol

Work pertaining to hippocampal primary culture preparation was reviewed and approved by the Northwestern University Animal Care and Use Committee (protocol #IS00001151).

1. Preparation Before Labeling

1. Preparation and maintenance of primary hippocampal cultures

1. Prepare primary hippocampal cultures at a density of 150,000 cells plated on poly-D-lysine-coated (0.1 mg/mL) 18 mm cover glasses. Excellent guides for dissociated neuronal culture preparation are available^{7,8}.

NOTE: If required, the cultures may be treated with cytosine arabinoside (Ara-C, 10 mM from DIV1) to avoid glial proliferation in the preparation.

NOTE: Alternative coating reagents such as fibronectin (1 mg/mL) or laminin (5 mg/mL) may be used instead of poly-D-lysine.

2. Maintain cultures in a cell incubator at 37 °C and 5% CO₂ in 2 mL/well of neurobasal media supplemented with B27 and 2 mM L-glutamine.

NOTE: Substitutes for L-glutamine (e.g., Glutamax) can be used, if desired.

3. On weekly-basis, remove half the volume of media and replace with the same volume of supplemented neurobasal media.

2. OPTIONAL: Transfection of mutated and/or epitope-tagged receptors

NOTE: Neurons should be transfected at least 3–4 days prior to the analysis time point to allow for receptor expression. The use of young neurons [Days in vitro 6–9 (DIV6–9)] results in better transfection efficiency than older (DIV15–20) neurons, but a sufficient number of transfected cells (>20) can be achieved regardless of the DIV employed.

1. For each well of a 12-well plate, dilute 1.5 µg of plasmid containing the construct of interest in 100 µL of fresh neurobasal media without B27 or glutamine supplementation in a microcentrifuge tube and mix by vortexing quickly.

NOTE: For successful transfection, it is critical that the neurobasal media used is as fresh as possible, ideally less than 1 week after bottle opening.

2. In a second microcentrifuge tube, mix 1 µL of an appropriate lipofection reagent in 100 µL of fresh neurobasal media and mix gently.

NOTE: Do not vortex the lipofection reagent mixture. Use of fresh lipofection reagents can improve transfection efficiency.

3. Incubate the tubes for 5 min at room temperature (RT).
4. Add the lipofection reagent mixture dropwise to the DNA mixture, mix gently, and incubate for 20 min at RT.
5. Adjust the volume of media in each well to 1 mL of conditioned media.
6. Add the lipofection reagent -DNA mixture dropwise to the well.
7. Return cells to the incubator and allow at least 3–4 days for protein expression.

NOTE: For the purposes of the internalization and recycling protocols outlined below, hippocampal neurons were transfected at DIV11–12 with constructs expressing GluN2B tagged with GFP in the extracellular domain (GFP-GluN2B) and imaged at DIV15–16.

3. OPTIONAL: Incubation of cells with drugs (chronically or acutely) in the conditioned media until fixation.

NOTE: For acute treatment, begin treating cells before labeling. Depending on the drug treatment protocol used, cells can be maintained in drug-containing media during section 2. In our example, DIV21 cells were subject to a cLTP protocol to increase surface-expressed AMPAR⁹.

1. Exchange conditioned media for extracellular solution (ECS).
2. Treat cells with 300 μ M glycine in ECS for 3 min at RT. As a control, treat a sister coverslip with ECS (without glycine).
3. Wash cells 3x with 37 °C ECS and return the cells in ECS (without glycine) to the cell incubator for 20 min prior to continuing with section 2.

NOTE: ECS (in mM): 150 NaCl, 2 CaCl₂, 5 KCl, 10 HEPES, 30 Glucose, 0.001 TTX, 0.01 strychnine, and 0.03 picrotoxin at pH 7.4.

2. Live Labeling of Surface-expressed Receptors

1. Prepare coverslips for labeling
 1. To save reagents and facilitate manipulation, transfer coverslips cell side up to a paraffin film-covered tray.

NOTE: It is critical to never let the samples dry out.

2. Save and maintain conditioned media at 37 °C for incubation and washing steps.

NOTE: For an 18 mm coverslip, incubation with 75–100 μ L of media for antibody labeling and 120–150 μ L for internalization/recycling are recommended.

2. Labeling of surface receptors with primary antibody

1. Incubate cells with primary antibody diluted in conditioned media for 15 min at RT.
NOTE: For GFP-tagged receptors, rabbit anti-GFP antibody at a dilution of 1:1000 was used. For endogenous GluA1, mouse anti-GluA1 at a 1:200 dilution was used.
2. Carefully aspirate off the antibody-containing media using a vacuum pipette and wash cells three times with conditioned media.
NOTE: If conditioned media is unavailable, all washing steps may be performed using PBS+ [phosphate buffered saline (PBS) containing 1 mM MgCl₂ and 0.1 mM CaCl₂]. Manual aspiration using a micropipette may be performed if gentle vacuum aspiration is not available.

3. Surface Expression (Figure 2)

1. Secondary antibody labeling of surface-expressed receptors
 1. Wash once with PBS+.
 2. Fix cells by incubating with 4% paraformaldehyde (PFA) and 4% sucrose in PBS for 7–8 min.
NOTE: Unlike other fixation methods such as methanol incubation, PFA does not permeabilize the plasma membrane and is therefore suitable for surface-expression analysis. For optimal results, use freshly prepared PFA. Short-term storage of PFA at 4 °C or long-term (up to 30 days) storage at –20 °C is permissive for adequate fixation.
CAUTION: PFA is a known carcinogen. Use proper personal protective equipment and a safety hood when handling.
 3. Wash cells three times with regular PBS.
NOTE: Alternatively, 0.1 M glycine can be used for washing PFA instead of PBS, as glycine will quench any remaining fixative that may increase the background in the preparation.
 4. Block nonspecific binding sites by incubating with 10% normal goat serum (NGS) in PBS for 30 min at RT.
NOTE: Blocking time can be extended without adverse effects on labeling.
 5. Incubate with fluorescently-tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT to label primary antibody-labeled receptors (i.e., surface-expressed).
NOTE: In these examples, a 1:500 dilution of Alexa 555-conjugated secondary antibodies:goat anti-rabbit for GFP-labeled receptors and goat anti-mouse for GluA1 was used.

6. Wash cells with PBS 3x.
2. Labeling of intracellular receptors
 1. Permeabilize cells with 0.25% Triton X-100 in PBS for 5–10 min at RT.
NOTE: To check that the initial round of antibody labeling occupies all surface epitopes, this permeabilization step can be skipped in a sister culture. In this case, no signal for intracellular receptors should be obtained. Additionally, to check that no internal receptors have been labeled in the previous section 2 (i.e., showing the integrity of the plasma membranes in culture), the permeabilization step can be skipped in a sister culture, and a primary antibody against an intracellular protein (e.g., PSD-95 or MAP2) can be utilized in step 2.2.1. No signal should be obtained from this primary under these conditions. In this case, a rabbit anti-PSD-95 antibody (1:500) was used.
 2. Block with 10% NGS in PBS for 30 min at RT.
 3. Label intracellular receptors by incubating permeabilized cells with the same primary antibody used in section 2.2 diluted in 3% NGS in PBS for 1 h at RT.
NOTE: The antibody dilution for labeling intracellular receptors may be different than that required for labeling surface-expressed receptors. In the example of GluA1, the same antibody dilution (1:200) was used.
 4. Wash cells 3x with PBS.
 5. Label with second fluorescently-tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT.
NOTE: In these examples, a 1:500 dilution of goat anti-mouse Alexa 647-conjugated secondary antibody (for GluA1) was used.
 6. Wash cells 3x with PBS.

4. Internalization (Figure 3)

1. Internalization of antibody-labeled surface receptors
 1. After labeling of surface-expressed receptors and antibody washing (section 2.2), maintain cells in conditioned media without antibody and return them to the incubator (37 °C) to allow for internalization.
NOTE: For NMDA receptors, 30 min for internalization is recommended. As a control, a sister culture may be maintained with conditioned media at 4 °C during the internalization process. Minimal receptor internalization should occur under these conditions.
2. Labeling of surface receptors
 1. Wash cells once with PBS+.

2. Fix cells with 4% PFA and 4% sucrose in PBS for 7–8 min.
CAUTION: Use proper personal protective equipment and a safety hood when handling PFA.
 3. Wash cells 3x with regular PBS.
 4. Block with 10% NGS in PBS for 30 min at RT to prevent nonspecific binding.
 5. Incubate samples with fluorescently-tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT to label primary antibody-labeled receptors (i.e., surface-expressed receptors which were not internalized).
NOTE: For this example, Alexa 555-conjugated goat anti-rabbit secondary antibody (1:500) was used for labeling.
 6. Wash cells 3x with PBS.
3. Labeling of internalized receptors
 1. Permeabilize cells with 0.25% Triton X-100 in PBS for 5–10 min.
 2. Block nonspecific binding by incubation with 10% NGS in PBS for 30 min at RT.
 3. Incubate samples with fluorescently tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT to label internalized antibody-labeled receptors.
NOTE: For this example, Alexa 647-conjugated goat anti-rabbit secondary antibody (1:500) is used for labeling.
 4. Wash cells 3x with PBS.

5. Recycling (Figure 4)

1. Internalization of antibody-labeled surface receptors
 1. After labeling of surface-expressed receptors and antibody washing (section 2.2), maintain cells in conditioned media without antibody and return them to the incubator (37 °C) to allow for internalization.
NOTE: For NMDA receptors, 30 min for internalization is recommended.
2. Blocking of stable surface expressed receptors
 1. To block the epitopes on the primary antibody attached to surface-expressed receptors that have not been internalized, incubate cells with unconjugated Fab anti-IgG (H+L) antibody fragments (against the primary used in section 2.2) diluted in conditioned media (20 µg/mL) for 20 min at RT. This treatment prevents future interaction with secondary antibodies.

NOTE: For this example, Goat anti-rabbit Fab fragments were used.

NOTE: Control experiment: to ensure that complete blocking of surface-expressed receptors has occurred, sister coverslips can be incubated with and without Fab. Cultures should be fixed immediately after Fab treatment, and both cultures are incubated with Alexa 555-conjugated secondary antibody. No Alexa 555 signal in the Fab-incubated cells indicates proper antibody blocking.

2. Wash cells 3x with conditioned media.
3. Incubate cells with conditioned media containing 80 μ M dynasore to prevent further internalization and return cells to the incubator (37 °C) to allow for recycling of internalized receptors. Dynasore is a GTPase inhibitor that inhibits dynamin and therefore prevents internalization.

NOTE: 45 min for NMDAR recycling is recommended. Note that Dynasore exclusively blocks the dynamin-dependent internalization process (e.g., NMDARs internalization). However, internalization of other synaptic protein (dynamin-independent) can still occur in the presence of Dynasore.

3. Labeling of recycled receptors

1. Wash cells once with PBS+.
2. Fix cells with 4% PFA and 4% sucrose in PBS for 7–8 min.

CAUTION: Use proper personal protective equipment and a safety hood when handling PFA.

3. Wash cells 3x with PBS.
4. Block with 10% NGS in PBS for 30 min at RT to prevent nonspecific binding.
5. Label cells with first fluorescently-tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT.

NOTE: For this example, Alexa 555-conjugated goat anti-rabbit antibody (1:500) was used for labeling.

6. Wash cells 3x with PBS.

NOTE: Longer washes with PBS (5–10 min) may help to reduce background in the preparation.

4. Labeling of internalized receptors

1. Permeabilize cells with 0.25% Triton X-100 in PBS for 5–10 min.
2. Block with 10% NGS in PBS for 30 min at RT.
3. Label with second fluorescently-tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT.

NOTE: For this example, Alexa 647-conjugated goat anti-rabbit antibody (1:500) was used for labeling.

4. Wash cells 3x with PBS.

6. Mounting and Imaging of Samples

1. Mount cells by gently placing the coverslips cell side down on 12–15 mL of the appropriate mounting media.

NOTE: Aspiration of excess mounting media will improve the quality of images.

2. Image cells on an appropriate confocal microscope.

NOTE: It is recommended to image a z-stack at 60x magnification with 0.35 μm steps, encompassing the entire thickness of the neuron.

7. Time Considerations

1. This is a long protocol that can be stopped at several points. If desired, perform blocking and primary antibody incubation steps overnight at 4 °C in a humid chamber.
2. Alternatively, if desired, use a microwave tissue processor to vastly speed up post-fixation incubation times. For all steps, use 150 W at 30 °C for “On” settings.
 1. To block, run the processor at 2 min “On,” 1 min “Off,” and 2 min “On.”
 2. For primary and secondary antibody incubation steps, run the processor at 3 min “On,” 2 min “Off,” and 3 min “On.”

NOTE: We observe no difference in quality by making the above alterations to the protocol.

8. Image Analysis

1. It is recommended to use FIJI <<https://fiji.sc/>> to conduct image analysis, as it is compatible with multiple file formats. For our data, images in the Nikon ND2 file format were acquired.
2. A macro script is provided for easy batch quantification of different parameters pre-selected by FIJI. The following steps are included in the macro:

NOTE: For these examples, “integrated intensity” was measured.
3. Open the image files in FIJI and separate channels.
4. Z-project each channel stack as a maximum intensity projection.
5. Set a lower threshold for each channel.

NOTE: Thresholds should be empirically determined for each experimental data set. While each channel can have a separate lower threshold value, it crucial that

channel threshold values are consistently maintained for all images of the same data set.

6. Select three to five secondary or tertiary dendrites and save them as regions of interest (ROIs).
7. Measure the integrated density of each ROI in surface and intracellular channels.
8. Normalize the signal for each ROI by dividing the integrated density value of the surface channel by the intracellular channel.
9. Repeat the measurements for all control and experimental images and normalize experimental values to control values (e.g., GluN2B WT or no-glycine conditions).

Representative Results

This protocol to study glutamate receptor trafficking is based on differential labeling of receptors expressed at the cell surface and those expressed in internal membranes. Segregation is achieved by the labeling the receptors before and after membrane permeabilization, using the same primary antibody but a secondary antibody conjugated to a different fluorophore. As outlined by the optional steps included the protocol, this is a very versatile method for interrogating different receptor trafficking processes, such as internalization and recycling, and can be easily adapted to the investigator's needs (Figure 1).

First, a method is provided for the quantification of receptors expressed at the neuronal surface. This protocol allows for quantification of basal surface expression as well as studying the molecular mechanisms by which different drugs or mutations alter basal levels of surface-expressed receptors. Surface-expressed receptors were first labeled by incubating with both primary and secondary antibody prior to permeabilization. After permeabilization with 0.25% Triton X-100, the intracellular pool of receptors is accessible for antibody labeling. To illustrate this protocol, surface vs. intracellular staining of AMPA receptors (AMPA receptors) in control cultures and after induction of cLTP was conducted. Specifically, live cells were labeled using an antibody against an extracellular epitope in the GluA1 subunit of the AMPAR, and cells were fixed then labeled with Alexa 555-conjugated secondary. The cells were then permeabilized and labeled with same anti-AMPA antibody and incubated with a secondary antibody conjugated to Alexa 647. This dual labeling allows clear visualization of the two GluA1 populations.

After acquiring confocal images, the fluorescent signal can be easily quantified to show an increase in surface expression, relative to the intracellular population, following cLTP (Figure 2A,B). As a control, the permeabilization step was skipped (incubation with 0.25% Triton X-100) in a sister coverslip and a primary antibody was used against PSD-95, a standard intracellular marker for excitatory synapses. As shown in Figure 2C, no signal for PSD-95 can be obtained in non-permeabilized cells, demonstrating the integrity of the plasma membrane. This indicates that the signal obtained for "surface GluA1" indeed corresponds to surface-expressed receptors (i.e., signal for surface-expressed GluA1 does

not include intracellular receptors). Importantly, a minimal signal for “internalized GluA1” can be observed under non-permeabilization conditions, showing that all surface epitopes are occupied by the initial round of antibody labeling (i.e., signal for intracellular GluA1 does not include surface-expressed receptors).

A second process that can be examined utilizing this protocol is the internalization of surface-expressed receptors. Specifically, surface receptors are labeled on a live cell, and neurons are returned to the incubator for a given time to undergo receptor internalization by clathrin-mediated endocytosis. Following this step, cells are fixed to preserve the spatial expression of primary antibody-labeled receptors (i.e., surface-expressed and internalized receptors). Then, surface receptors (i.e., those which have not been internalized in the time period of interest), are labeled with a secondary antibody prior to permeabilization. Following permeabilization, receptors that have been internalized are labeled by a secondary antibody with a different fluorophore.

In this protocol, it was examined how a particular phosphorylation within the PDZ ligand of the GluN2B subunit of NMDARs (at S1480) induces NMDAR internalization. To do so, primary cultures were transfected with a phospho-mimetic receptor, in which serine (S1480) had been substituted by glutamate (E). The resultant mutant, GluN2B S1480E, acts as a “constitutively-phosphorylated” form of GluN2B. To ease labeling of GluN2B and identify the phospho-mimetic mutant, GFP was used as an epitope tag on the extracellular side of GluN2B (GFP-GluN2B S1480E). Surface receptors on live cells were labeled with an anti-GFP antibody for 15 min at RT. Next, the excess antibody was washed with conditioned media and returned the cells to 37 °C for 30 min to allow for endocytosis. Then, cells were fixed to freeze receptor movement. Receptors that remained on the surface were then labeled with Alexa 555-conjugated secondary antibody prior to permeabilization.

To identify receptors that had been internalized during the 30 min incubation period, cells were permeabilized, and the internalized receptors (already labeled with a primary antibody) were then labeled with Alexa 647-conjugated antibody. Again, this dual-labeling strategy allows quantification of the proportion of internalized receptors. This example highlights that GluN2B phosphorylation at S1480 promotes receptor internalization, as the phospho-mimetic mutant S1480E displayed a much higher internalization ratio compared to WT receptors (Figure 3A,B). As a control, a sister culture at 4 °C was maintained in conditioned media during internalization to strongly slow the process. As expected, no signal was obtained for “internalized” receptors under these conditions (Figure 3C).

Lastly, this protocol can be utilized to examine the recycling of previously internalized receptors. This protocol variation is a continuation of the internalization protocol, by following these receptors back to the cell surface. There are two crucial components to this variation. Firstly, receptors which remained stably expressed at the surface during the entire protocol (i.e., receptors that were not internalized or recycled) must be “blocked” so that they are not mistaken for recycled receptors. To do so, before performing the recycling step, live neurons are incubated with high concentrations of Fab that interact with primary antibody-labeled receptors expressed at the surface to prevent further binding of the fluorophore-conjugated secondary antibody. Second, internalization should be prevented

during the recycling phase, so that recycled receptors are not repeat internalized. This was done by adding dynasore to the media during recycling as this drug blocks processes reliant on dynamin such as clathrin-mediated endocytosis, such as NMDAR internalization. In this protocol, studying the trafficking of the phospho-mimetic mutant GluN2B S1480E was performed as well as surface labeling of GFP-GluN2B on live cells, which allowed for internalization during a 30 min period as explained before.

Following this, surface receptors that were not internalized during this period were blocked by incubating the cells with Fab for 20 min at RT. Next, cells were again incubated at 37 °C for 45 min to allow for previously internalized GluN2B to be recycled back to the cell surface. Dynasore was present in the media during the recycling step. Fixation of the cells following this step allows for the identification of recycled receptors (i.e., those that are unblocked and expressed on the cell surface) and internalized receptors (i.e., those that are primary antibody labeled and intracellular). By 1) labeling recycled receptors with an Alexa 555-conjugated secondary antibody prior to permeabilization and 2) labeling internalized, but not recycled, receptors with Alexa 647-conjugated secondary antibody, a recycling ratio was generated to show that GluN2B S1480E does not have any effect on NMDAR recycling (Figure 4A,B). As a control, it was ensured that complete blocking of surface-expressed receptors occurred by incubating sister coverslips in the presence or absence of Fab, followed with PFA fixation. As shown in Figure 4C, a strong signal can be observed for surface-expressed GluN2B in the absence of Fab blocking. This signal disappears in Fab-treated cultures, demonstrating that the blocking protocol is sufficient to completely block the surface-expressed epitopes and that the surface signals observed after recycling indeed correspond to receptors trafficked back to the plasma membrane.

Discussion

The interaction between a cell and its environment (e.g., communication with other cells, response to different stimuli, etc.), heavily relies on the correct expression of receptors at the cell surface. The rapid and fine-tuned regulation in surface-expressed receptor content enables proper cellular response to a constantly changing environment. In the particular case of neurons, alterations in the number, localization, and subunit composition of synaptically expressed receptors heavily influences synaptic communication, synaptic plasticity, synaptogenesis, and synaptic pruning^{3,5,10}. Therefore, accurate analysis of receptor surface expression and the mechanism underlying its regulation is an important topic of research.

A number of modalities exist by which receptor surface expression can be studied. In general, these fall under three main categories: (i) biochemical isolation of the surface expressed receptor population, (ii) imaging techniques to visualize receptors at the surface, and (iii) functional techniques to monitor the consequences of receptor activation. These approaches are complementary and can be utilized in conjunction to answer specific questions about surface expression of receptors.

Examples of biochemical isolation of surface expressed receptors include biotinylation protocols in cultured cells or brain slices and subcellular fractionation of cultured cells or tissue¹¹. Surface biotinylation is based on the labeling of surface-expressed receptors with

biotin by incubating the cultures with ester-activated biotin. The ester group reacts with primary amino groups ($-\text{NH}_2$) to form stable amide bonds. Because this reagent is cell-impermeable, only surface-expressed proteins are labeled with biotin. After cellular lysis using detergents, labeled receptor can be recovered by incubating the cell lysate with Agarose beads conjugated to avidin or its variants which have a strong affinity for biotin, then evaluated by immunoblotting. Subcellular fractionation is a biochemical protocol that uses several centrifugation steps to isolate different cellular membranous compartments based on their different densities¹². Both techniques are useful to quantify changes in surface expression of endogenous proteins and can be used in conjunction with pharmacological approaches (both *in vivo* and in culture) to determine how molecular manipulations affect surface expression of receptors. They are particularly useful because changes in multiple endogenous receptors, relative to a standard, can be quantified simultaneously. However, unlike imaging modalities, such as that described in this protocol, spatial resolution is lost through biochemical processing of samples.

The protocol described here belongs to the imaging techniques category. This group of approaches (such as surface labeling and live-cell imaging of fluorescently tagged overexpressed proteins) are useful to give spatiotemporal resolution to surface expression of receptors. For instance, by examining surface vs. intracellular expression of AMPAR, as exemplified previously, one can examine how changes in expression are pronounced in the dendrites (the biological compartment of interest for this particular application). Like biochemical fractionation, drugs can be used with imaging techniques to determine the effects of a drug on surface expression. In addition, transfection of neurons with receptors containing modifications (mutations or tags) further elaborates the possibilities for imaging. Transfection with mutant receptors can delineate the effects of a particular mutation on surface expression.

Another useful approach to visualize receptor trafficking is live imaging microscopy after transfection of primary cultures with fluorescently tagged constructs, such as Superecliptic pHluorin (SEP)- or other fluorophore-tagged constructs¹³. SEP-tagged receptors can be particularly useful for studying surface expressed receptors, as this fluorophore will only fluoresce when exposed to the extracellular medium. Like previous examples, pharmacological approaches can be used, or mutations made on the receptor, to determine if these change the surface expression characteristics of the receptor. In the case of drugs, live-cell imaging can be used to monitor the modifications in surface expression in real-time. A limitation to live imaging is the lack of mechanistic insight into changes in surface expression. For example, a decreased surface expression might be a result of either increased receptor internalization, reduced receptor recycling, or both.

To answer this question, the protocol described above may be used. Another important trafficking event that can be monitored using live imaging approaches is the lateral diffusion of receptors between different compartments in the plasma membrane (e.g., between synaptic and extrasynaptic sites). In this case, the technique of choice is the use of single-particle tracking approaches in which a fluorescence semiconductor nanocrystal [i.e., Quantum Dot (QD)] is attached to an antibody against an extracellular epitope on the protein of interest. QDs are characterized by remarkable stability (allowing much less

photobleaching than traditional fluorescent proteins), strong brightness, and the alternation between on/off status (“blinking”). By using the appropriate microscope settings, it is possible to track the movement of a single QD (and, therefore, a single protein of interest) through the cellular plasma membrane¹⁴.

The current protocol provides an examination of receptors in the surface population, internalized population, and recycled population, allowing for the calculation of ratios to determine how these processes control total surface expression. Furthermore, stopping the internalization or recycling processes at different timepoints adds temporal resolution. This method, therefore, allows for spatiotemporal surface expression studies. Unlike the other imaging techniques, levels of endogenous surface expression can also be studied (e.g., AMPAR), provided there exists an antibody against the extracellular portion of the receptor. However, because this method requires fixation of cells, it is not compatible with live-cell imaging and thus cannot provide real-time information.

Finally, functional approaches like electrophysiology¹⁵ or calcium imaging¹⁶ are powerful, though often indirect, manners to study the surface expression of receptors. These methods are based on the quantification of functional changes in the cell after receptor activation (e.g., change in membrane potential or calcium concentration). Using pharmacology to isolate the response of a given receptor, these methods allow for estimation of the receptors expressed at the cell surface in a precise, spatiotemporal manner. These methods are versatile and allow for the study of cells in culture and in more physiological conditions *ex vivo* (e.g., acute brain slices) and *in vivo*. However, as in the case of the live imaging approaches, mechanistic information is often missed when using functional approaches.

In summary, a combination of techniques to study receptor surface expression can be employed to fully understand how surface expression is controlled. The protocol presented here is particularly powerful as it provides a mechanistic understanding of the spatiotemporal surface expression of receptors in dissociated primary cultures.

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Figure 1: Schematic of protocol variations to study receptor surface expression, receptor internalization (endocytosis), and receptor recycling.

Figure 2: cLTP increases surface expression of GluA1.

Primary hippocampal neurons at DIV21 were subjected to chemical LTP (cLTP) by incubating with glycine-containing ECS. Distinct labeling of surface-expressed (red) vs. intracellular (blue) GluA1 populations reveals the expected increase in surface expression of AMPAR. (A) Single plane and (B) Z-stacked (maximum intensity projection) confocal pictures. Scale bars = 50 μ m (whole cell) or 5 μ m (dendrite). Graph shows the increased surface expression of GluA1 after cLTP protocol. Surface expression index: surface/intracellular receptors ($n = 3$; number of cells: con = 7; cLTP = 7; values represent mean $\pm$ SEM; **** $p < 0.0001$ using Mann-Whitney U test). (C) Control experiment in which the permeabilization step was skipped. In addition to surface and internal GluA1, the intracellular excitatory synaptic marker PSD-95 was evaluated. Scale bars = 50 μ m (whole cell) or 5 μ m (dendrite).

Figure 3: Phosphorylation of GluN2B at S1480 promotes NMDAR internalization.

Primary hippocampal neurons were transfected with either GFP-GluN2B WT or the phospho-mimetic mutant GFP-GluN2B S1480E on DIV11–12. Following 3–4 days of protein expression, surface GFP was labeled on live cells with a rabbit anti-GFP antibody and cells were then returned to 37 °C to allow for receptor internalization by endocytosis. Surface-expressed exogenous receptors were visualized with Alexa 555-conjugated secondary antibody, and the internalized population identified after permeabilization using Alexa 647-conjugated antibody. For clarity, surface GFP-GluN2B is pseudocolored in green and internalized GFP-GluN2B is pseudocolored in white. (A) Single plane and (B) Z-stacked (maximum intensity projection) confocal pictures. Scale bars = 50 μ m (whole cell) or 5 μ m (dendrite). Graph shows the elevated internalization displayed by the phospho-mimetic mutant GluN2B S1480E. Internalization index: internalized receptors/surface-expressed receptors (n = 6; number of cells: WT = 34; S1480E = 28; values represent mean $\pm$ SEM; ***p < 0.001 using Mann-Whitney U test). (C) Control experiment in which the internalization step (Internaliz.) was performed at 4 °C. Scale bars = 50 μ m (whole cell) or 5 μ m (dendrite).

Figure 4: Phosphorylation of GluN2B at S1480 does not modify NMDAR recycling.

Primary hippocampal neurons were transfected with either GFP-GluN2B WT or the phospho-mimetic mutant GFP-GluN2B S1480E on DIV11–12 as shown in Figure 3. Following 3–4 days of protein expression, surface GFP was labeled on live cells with a rabbit anti-GFP antibody and cells were then returned to 37 °C to allow for receptor internalization by endocytosis. Remaining surface-expressed receptors were blocked by Fab incubation and recycling was allowed for 45 min. Available surface expressed exogenous receptors (recycled) were visualized with Alexa 555-conjugated secondary antibody, and the internalized population identified after permeabilization using Alexa 647-conjugated antibody. For clarity, surface GFP-GluN2B is pseudocolored in white and internalized GFP-GluN2B is pseudocolored in green. (A) Single plane and (B) Z-stacked (maximum intensity projection) confocal pictures. Scale bars = 50 μ m (whole cell) or 5 μ m (dendrite). Graph shows the lack of effect the GluN2B S1480 phosphorylation has on recycling. Recycling index: recycled receptors/internalized receptors ($n = 5$; number of cells: WT = 27; S1480E = 24; values represent mean $\pm$ SEM; n.s. = non-significant using Mann-Whitney U test). (C) Control experiment in which the Fab incubation step to block surface-expressed epitopes was skipped. Scale bars = 50 μ m (whole cell) or 5 μ m (dendrite).