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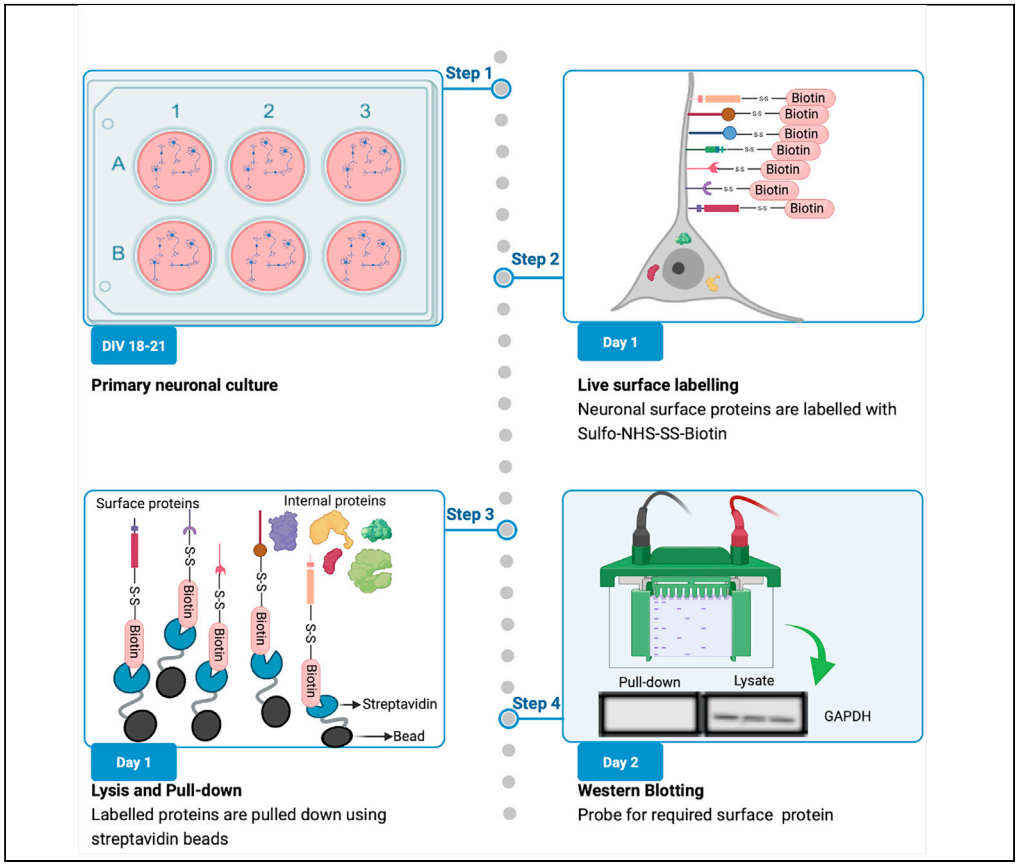
Surface biotinylation of primary neurons to monitor changes in AMPA receptor surface expression in response to kainate receptor stimulation



Here we detail a surface biotinylation technique used to label surface-expressed proteins in primary neuronal cultures. Surface proteins are labeled with membrane-impermeant Sulfo-NHS-SS-biotin, and isolated by pull-down with streptavidin beads followed by western blotting to measure levels of surface expression of the protein of interest under different conditions. We have used this approach extensively to monitor activity-dependent changes in α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and kainate receptor (KAR) subunits. However, this protocol can be used to investigate any surface-expressed protein.

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Highlights

Protocol for biotin labeling of surface-expressed proteins in cultured neurons

Isolation of labeled proteins allows quantification of surface protein levels

AMPA, kainate, and NMDA receptor subunits are used as examples

Allows analysis of activity-dependant and/or stress-evoked changes

Nair et al., STAR Protocols 2, 100992
December 17, 2021
https://doi.org/10.1016/j.xpro.2021.100992

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Surface biotinylation of primary neurons to monitor changes in AMPA receptor surface expression in response to kainate receptor stimulation

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https://doi.org/10.1016/j.xpro.2021.100992

SUMMARY

Here, we detail a surface biotinylation technique used to label surface-expressed proteins in primary neuronal cultures. Surface proteins are labeled with membrane-impermeant Sulfo-NHS-SS-biotin, and isolated by pull-down with streptavidin beads followed by western blotting to measure levels of surface expression of the protein of interest under different conditions. We have used this approach extensively to monitor activity-dependent changes in α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and kainate receptor (KAR) subunits. However, this protocol can be used to investigate any surface-expressed protein.

For complete details on the use and execution of this protocol, please refer to Nair et al. (2021).

BEFORE YOU BEGIN

Ethical approval

All the animal experiments and procedures were performed in compliance with the UK Animal Scientific Procedures act (1986) and were guided by the Home Office Licensing Team at the University of Bristol. All animal procedures relating to this study were approved by the Animal Welfare and Ethics Review Board at the University of Bristol (approval number UIN/18/004).

Buffers and reagents

Timing: 0.5–1 h

1. On the day of experiment, prepare buffers and other reagents as per the requirement. See materials and equipment for a list of recipe tables.
2. Prepare 500 mL fresh Earle's buffer (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 25 mM HEPES, 5 mM glucose) and adjust the pH to 7.4 with Tris-Base.
3. After adjusting the pH transfer 5 mL of Earle's buffer to a bijou container or a falcon tube and add 5 μL of Tetradotoxin (TTX) (1 mM stock; 1 μM final concentration) and 20 μL of GYKI53655 (10 mM stock; 40 μM final concentration).
4. Mix the solution thoroughly and transfer 2.5 mL of the solution to a fresh bijou and add 2.5 μL of kainate (10 mM stock; 10 μM final concentration).
5. Leave both bijous (control and kainate solutions) in a 37°C water bath for at least 20 min to pre-warm the buffers.
6. Prepare 10 mL of 0.3 mg/mL Sulfo-NHS-SS-biotin in Earle's buffer.

7. Prepare 20 mL of 100 mM NH_4Cl solution in Earle's buffer.
8. Prepare the wash buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS).
9. Prepare 20× protease inhibitor cocktail; 20× solution is made by dissolving 1 protease inhibitor cocktail tablet in 2 mL of dH_2O.
10. Prepare neuronal lysis buffer. Add 20× protease inhibitor cocktail to the wash buffer to a final concentration of 1× (for example, 0.5 mL of 20× protease inhibitor cocktail in a final volume of 10 mL lysis buffer).
11. All the buffers mentioned in steps 2 and 6–10 should be left on ice for at least 30 min prior to the start of the experiment.
12. Prepare a flat surface of ice in an ice box to incubate the dishes of neurons on for the duration of the surface biotinylation procedure.

Note: It is important that all the steps in the surface biotinylation with Sulfo-NHS-SS-biotin are performed on ice unless specifically otherwise stated. In addition to protease inhibitors, we recommend using a phosphatase inhibitor cocktail if the phosphorylation status of the protein(s) under investigation are of interest.

Note: Sulfo-NHS-LC-biotin can be used as an alternative to cleavable Sulfo-NHS-SS-biotin. In this protocol, the SS linkage is not cleaved.

Note: The kainate stimulation described below is performed in the presence of TTX, to prevent confounding issues resulting from action potential firing, and GYKI53655 to prevent direct activation of AMPARs by kainate.

Primary hippocampal neuronal culture

Timing: 3 days

Coating the wells with poly-L-lysine (PLL)

13. Day 1 – Coat the wells of a 6 well plate with 1 mL of 1 mg/mL Poly-L-Lysine (PLL) in borate buffer (50 mM boric acid and 10 mM sodium tetraborate in dH_2O).
14. Leave the plates in a 37°C/5% CO_2 cell culture incubator.
15. Day 2 – Wash the plates 3 times with 2 mL dH_2O.
16. Add 1.5 mL of plating media (Neurobasal® (Gibco) supplemented with 5% Horse Serum, 2% B27, 1% glutamax, and 1% penicillin-streptomycin) and leave the plates in a 37°C/5% CO_2 cell culture incubator for 12–24 h.
17. Day 3 (dissection day) – Exchange the plating media for 2 mL of fresh plating media and return the plates to the incubator until the neurons are ready for plating.

Note: Leave 50 mL of plating media and the required amount of Hank's balanced salt solution (HBSS) at 37°C before starting the dissection.

Note: All the steps (except dissection of tissues) must be performed in sterile conditions under a laminar air-flow chamber

Preparation of hippocampal cultures

18. After dissection of hippocampi from E17-18 rat embryos, transfer the hippocampal tissue to a laminar air-flow chamber.
19. Rinse the hippocampus 3 times with 10 mL of HBSS.
20. Trypsinise the tissue for 9 min with 0.005% Trypsin/EDTA in HBSS at 37°C with occasional swirling.

21. Aspirate the trypsin and rinse the hippocampus 3 times with 10 mL HBSS
22. Add 1 mL of fresh plating media (to stop enzymatic action of trypsin).
23. Aspirate the plating media and replace with 1 mL of fresh plating media.
24. Triturate the hippocampus with a P1000 pipette by holding the tip of the pipette against the bottom of the falcon tube (~12–15 strokes).

⚠ CRITICAL: Be gentle with the trituration to minimize damage to the neurons. Care should be taken to avoid bubbles.

25. Count the cells using a haemocytometer.
26. Plate the cells at a density of 500,000 cells per 35 mm dish for biotinylation experiments by adding cells to the prepared dishes.
27. Place the cells in a 37°C/5% CO_2 incubator and allow the cells to adhere for 2 h.
28. Aspirate the plating media and replace with 2 mL serum-free feeding media (Neurobasal® medium supplemented with 2% B27, 1% glutamax and 1% penicillin-streptomycin) and replace cells in a 37°C/5% CO_2 incubator.
29. After 7 days *in vitro* (DIV), add a further 1 mL feeding media to the neurons.

Note: Neuronal surface biotinylation is performed at DIV 17–21 for optimal surface expression of ionotropic glutamate receptors (iGluRs).

⚠ CRITICAL: Care should be taken not to dislodge the adherent cells while aspirating the media from the wells.

Note: Poly-D-lysine can also be used as an alternative to PLL. This should be determined by the experimenter depending upon the availability of resources.

Note: The day of the surface biotinylation experiment must be determined by analyzing the expression profile of the receptor or protein of interest.

Note: While B27 contains biotin, this is not an issue – the biotin used to label surface proteins is Sulfo-NHS-SS-biotin that contains an active group attached that allows it to react with primary amines on surface-expressed proteins. Unmodified biotin in media does not have this active group, so it doesn't label proteins when present in the media.

Note: The same protocol can be used for dissociating and plating cortical neurons.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit Polyclonal Anti-Glutamate receptor 1 (working dilution 1:1000)	Merck Millipore	AB1504
Rabbit Monoclonal Anti-GluR6/7, clone NL9 (working dilution 1:1000)	Merck Millipore	04-921
Mouse Monoclonal Anti-Glutamate Receptor (GluA2 antibody) (working dilution 1:1000)	BD Pharmingen	556341
Mouse Monoclonal Anti-GAPDH (working dilution 1:10000)	Abcam	Ab8245
Chemicals, peptides, and recombinant proteins		
EZ-Link™ Sulfo-NHS-SS-biotin	Thermo Fisher Scientific	21331
EZ-Link™ Sulfo-NHS-LC-biotin	Thermo Fisher Scientific	21335
Streptavidin-Agarose from *Streptomyces Avidinii*	Merck	S1638-5ML
cOmplete™ Protease Inhibitor Cocktail	Merck	CO-RO
Dulbecco's phosphate-buffered saline (DPBS)	Thermo Fisher Scientific	14200-059

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Poly-L-lysine	Merck	P4707
Boric acid	Merck	B0394
Sodium tetraborate (Borax)	Merck	221732
HBSS	Gibco™	24020117
Neurobasal medium	Gibco™	21103049
Trypsin-EDTA	Gibco™	25300096
GlutaMAX™	Gibco™	35050061
Horse Serum	Gibco™	26050088
B27™ Supplement	Gibco™	17504044
Penicillin-Streptomycin	Gibco™	15070063
Kainic acid	Tocris Bioscience	0222
Tetrodotoxin citrate	Tocris Bioscience	1069
GYKI53655	Tocris Bioscience	2555
D-Glucose	Merck	158968
HEPES	Merck	H4034
KCl	Merck	P5405
$CaCl_2$	Merck	746495
$MgCl_2$	Merck	208337
$MgSO_4$	Merck	M7506
Triton X-100	Thermo Fisher Scientific	28313
Tris-Base	Merck	77-86-1
Sodium dodecyl sulfate (SDS)	Merck	L3771
Glycerol	Merck	G5516
Bromophenol blue	Merck	B8026
β-mercaptoethanol	Scientific Laboratory Supplies	97622
Experimental models: Cell lines		
Primary rat hippocampal neuronal cultures	Fletcher-Jones et al. (2019) and Konopacki et al. (2011)	N/A
Experimental models: Organisms/strains		
Wistar rat	University of Bristol Animal Services	N/A
Other		
6 well cell culture plate	Greiner Cellstar	GD657160
Sonicator	Misonix Microsonultrasonic cell disruptor	NA
Haemocytometer	Improved Neubauer	NA

MATERIALS AND EQUIPMENT

- Earle's buffer

Reagent	Final concentration (mM)	Amount (g)
NaCl	140	4.0908
KCl	5	0.1863
$CaCl_2$	1.8	0.0998
$MgCl_2$	0.8	0.0380
HEPES	25	2.978
D-Glucose	5	0.4503
dH_2O		Make upto 500 mL

Note: pH should be adjusted to 7.4 with Tris-Base. The solution should then be left on ice for at least 30 min prior to biotinylation. Prepare fresh buffer on the day of the experiment.

Alternatives to Earle's buffer

In this protocol, we use Earle's buffer as described throughout. However, depending on the particular experimental requirements, HEPES-buffered saline (HBS) or Dulbecco's phosphate-buffered saline (DPBS) can be used in place of Earle's buffer.

- HEPES-buffered saline (HBS)

Reagent	Final concentration (mM)	Amount (g)
NaCl	137	4.003
D-Glucose	5	0.4503
KCl	15	0.5591
HEPES	25	2.978
$CaCl_2$	1.5	0.0832
$MgSO_4$	1.5	0.0902
dH_2O		Make upto 500 mL

Note: pH should be adjusted to 7.4 with Tris-Base. The solution should be left on ice for at least 30 min prior to biotinylation. Prepare fresh buffer on the day of the experiment.

- Dulbecco's phosphate-buffered saline (DPBS).

Make the required amount of 1× DPBS by diluting 10× DBPS (Thermo Fisher) in dH_2O.

Note: We recommend making all the buffers (Earle's buffer, HBS and DPBS) fresh on the experiment day.

Other reagents

- Wash buffer – Lysis buffer without protease inhibitor cocktail

Reagent	Final concentration (mM)	Amount
Tris-pH 7.4	50	10 mL of 1 M Tris-PH 7.4
NaCl	150	1.7532 g
Triton X-100	1%	2 mL
SDS	0.1%	0.2 g
dH_2O		Make upto 200 mL

Note: Wash buffer can be stored at 4°C for up to 1 month.

- 4× sample buffer – without β-mercaptoethanol

Reagent	Final concentration (mM)	Amount
Tris pH 6.8	240	24 mL
SDS	8%	8 g
Glycerol	40%	40 mL
Bromophenol blue	0.009%	0.009 g
dH_2O		Make upto 100 mL

- 2× sample buffer

Reagent	Amount (μL)
4× sample buffer	500
β-mercaptoethanol	100
dH_2O	400

Note: 4× sample buffer is stable for long periods at room temperature (18°C–25°C); however, once β-mercaptoethanol has been added, sample buffer should be used within 1 week.

- Borate buffer

Filter and sterilize the buffer using a vacuum filtration system into a fresh and sterile 500 mL glass

Reagent	Final concentration (mM)	Amount
Sodium tetraborate (Borax)	10 mM	1.0061 g
Boric acid	50 mM	1.5458 g
dH_2O		Make upto 500 mL

bottle using a 0.2 μm pore membrane. The sterile borate buffer can be stored at room temperature (18°C–25°C) for 1–2 months.

- Plating media

Reagent	Final concentration (mM)	Amount
Neurobasal medium	NA	455 mL
Horse serum	5%	25 mL
B27 supplement	2%	10 mL
Glutamax	1%	5 mL
Penicillin-streptomycin	1%	5 mL

- Feeding media

Reagent	Final concentration (mM)	Amount
Neurobasal medium	NA	480 mL
B27 supplement	2%	10 mL
Glutamax	1%	5 mL
Penicillin-streptomycin	1%	5 mL

Note: Plating and feeding media should be prepared in a laminar air-flow chamber and sterile conditions should be maintained. The media can be stored at 4°C for 1–2 months.

STEP-BY-STEP METHOD DETAILS

Stimulation of neurons with kainate

Timing: 50 min

Stimulation of primary hippocampal neurons with 10 μM kainate for 20 min to investigate kainate-induced changes in the surface expression of AMPA and kainate receptors (Figure 1)

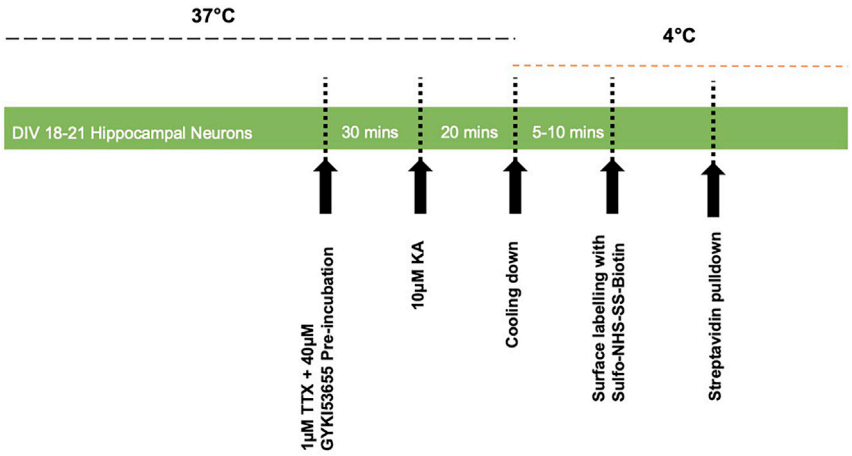

Figure 1. Timeline for sustained KA stimulation

Note: This protocol is optimized for neurons plated in a 6 well dish (35 mm per well) with a plating density of 500,000 cells. The same protocol can also be used for neurons plated in independent dishes of different sizes with adequate scaling. For optimal consistency, we do not recommend labeling more than 6–7 wells at a time.

Note: While, as an example, we outline the procedure to perform kainate simulation of primary hippocampal neurons, this basic procedure can be modified to perform stimulation of neurons with any drug of choice.

Note: As outlined above, kainate stimulation is carried out in the presence of TTX and GYKI53655 to prevent action potential firing and direct activation of AMPARs by kainate, respectively.

1. Reduce the media in each well to 2 mL.

Note: If required, the media in each well can be reduced from 2 mL to 1 mL dependent on resource availability. However, it is important to ensure that the neurons are always covered with media to prevent drying out.

2. Transfer 500 μL of media from each well to new Eppendorf tubes.
3. Add 2 μL of TTX (1 mM stock) and 8 μL GYKI53655 (10 mM stock).
4. Mix well by pipetting up and down and carefully add the solutions back into the repective wells, giving a final concentration in the media of 1 μM TTX and 40 μM GYKI53655.

⚠ CRITICAL: Care should be taken not to dislodge the neurons while adding media.

5. Leave the plates in a 37°C incubator for 30 min.
6. Aspirate the drug-containing media and replace it with prewarmed Earle's buffer (Control and kainate).
7. Leave the plates in the incubator for 20 min.

Note: Constant levels of media must be maintained in each well to ensure that the final concentration of drugs in each well is consistent.

Live surface biotinylation of neurons

Timing: 3 h

Labeling of neuronal surface proteins with Sulfo-NHS-SS-biotin for subsequent isolation using streptavidin beads

Note: It is recommended that neurons are left on ice for 5–10 min after being removed from the incubator especially while performing acute experiments that may alter the trafficking of surface receptors. Cooling down the neurons to 4°C prevents trafficking of the receptors and avoids cold shocking the neurons by immediate addition of an ice-cold buffer (Figure 2).

Note: Any of the above-mentioned buffers (Earle's buffer, HBS, DPBS) can be used, but for pharmacological stimulations, either pre-warmed Earle's buffer or HBS is preferred, and the same buffer (cold) is advised for washing. DPBS is ideal for biotinylation without prior stimulations.

8. Leave dishes containing neurons on ice for 5–10 min.
9. Aspirate the neuronal media and wash the cells 2 times with 2 mL of cold Earle's buffer.

⚠ CRITICAL: Washing should be performed with caution to prevent dislodging of the neurons from the plates.

10. Aspirate the buffer and add 1.5 mL of buffer containing 0.3 mg/mL Sulfo-NHS-SS-biotin and incubate on ice for 10 min with gentle swirling every 2.5 min.
11. After labeling the surface proteins with biotin, wash the cells 3 times with 2 mL of cold Earle's buffer.
12. Aspirate the buffer, add 2 mL of 100 mM NH_4Cl to each well, and incubate on ice for 1 min to scavenge unbound Sulfo-NHS-SS-biotin.
13. After 1 min, aspirate the buffer containing NH_4Cl and gently wash once with cold Earle's buffer.
14. Remove the buffer and add 200 μL of neuronal lysis buffer.

Note: Although we recommend using 200 μl of neuronal lysis buffer to lyse the cells, we advise the experimenter to scale this according to the abundance of their protein of interest or depending on the quality of antibody used for detection by Western blot.

15. Gently rotate the plate, and scrape off the cells from all sides of the well using a cell scraper.
16. Transfer the lysate to a 1.5 mL Eppendorf tube.
17. Sonicate the lysate briefly (3 pulses at 2.5 amplitude for ~2 s).
18. Leave the lysate on ice for 30 min.

Washing streptavidin beads

Timing: 10 min

Washing of streptavidin beads with lysis buffer to remove any unwanted components in the storage buffer

Note: This protocol assumes 6 different wells of neurons are being labeled. For more or fewer conditions, the volume of beads washed should be scaled up/down accordingly (Figure 3).

19. *Add 150 μL of beads to a 1.5 mL Eppendorf tube containing 1 mL of wash buffer.

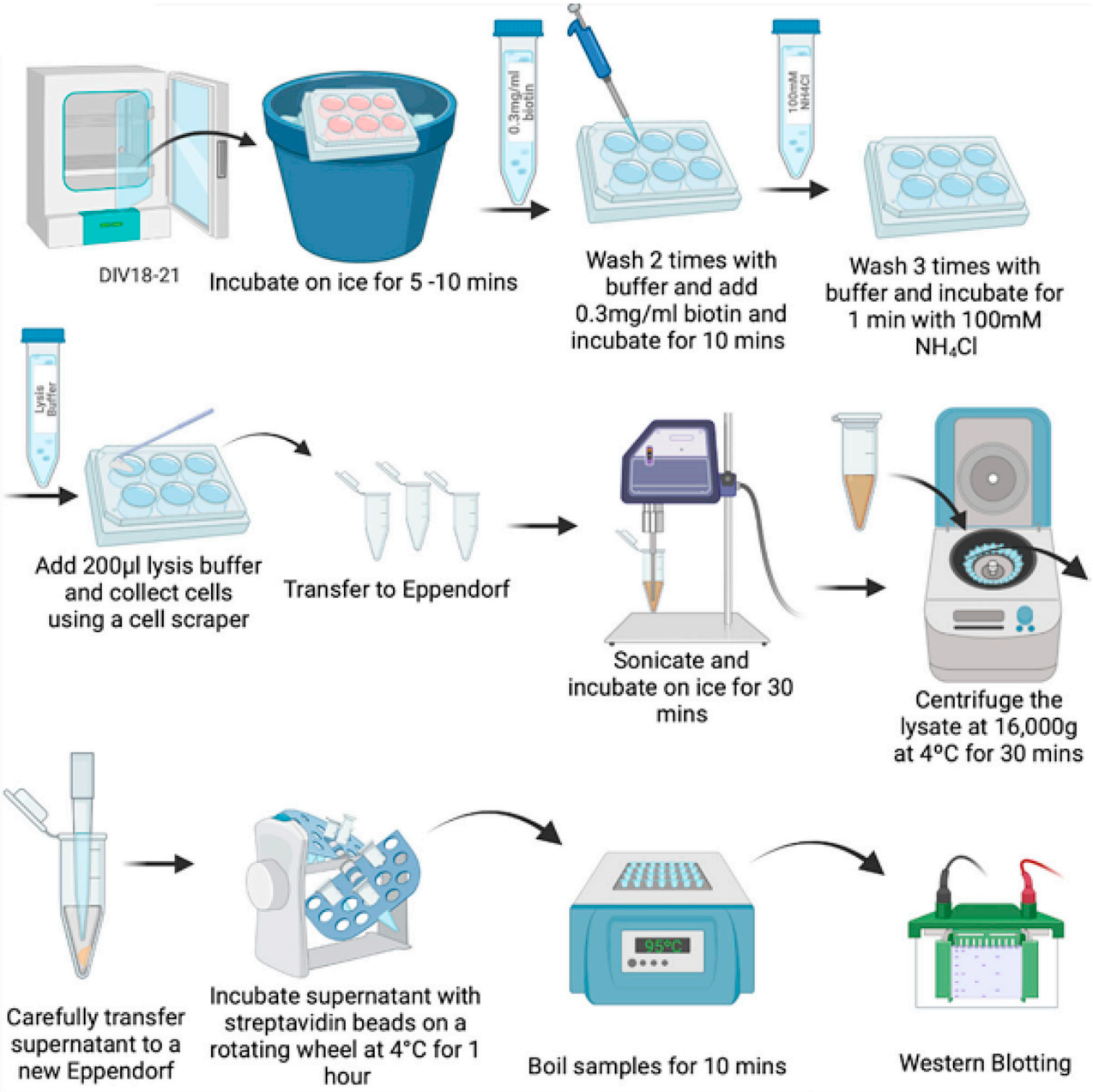

Figure 2. Schematic representation of neuronal surface biotinylation using Sulfo-NHS-SS-biotin

⚠ CRITICAL: When pipetting beads, the tip of the pipette tip must be cut off to prevent damaging the beads as they pass up the narrow tip.

20. Wash the beads twice with the wash buffer by centrifugation at 1500*g* at 4°C for 2 min then remove the supernatant by aspiration.
21. After the second wash, aspirate the supernatant and resuspend the beads to 1 mL in the wash buffer.
22. **Add 150 µL of beads to each of 6 Eppendorf tubes (1.5 mL) with constant inverting to ensure that the beads are mixed properly.

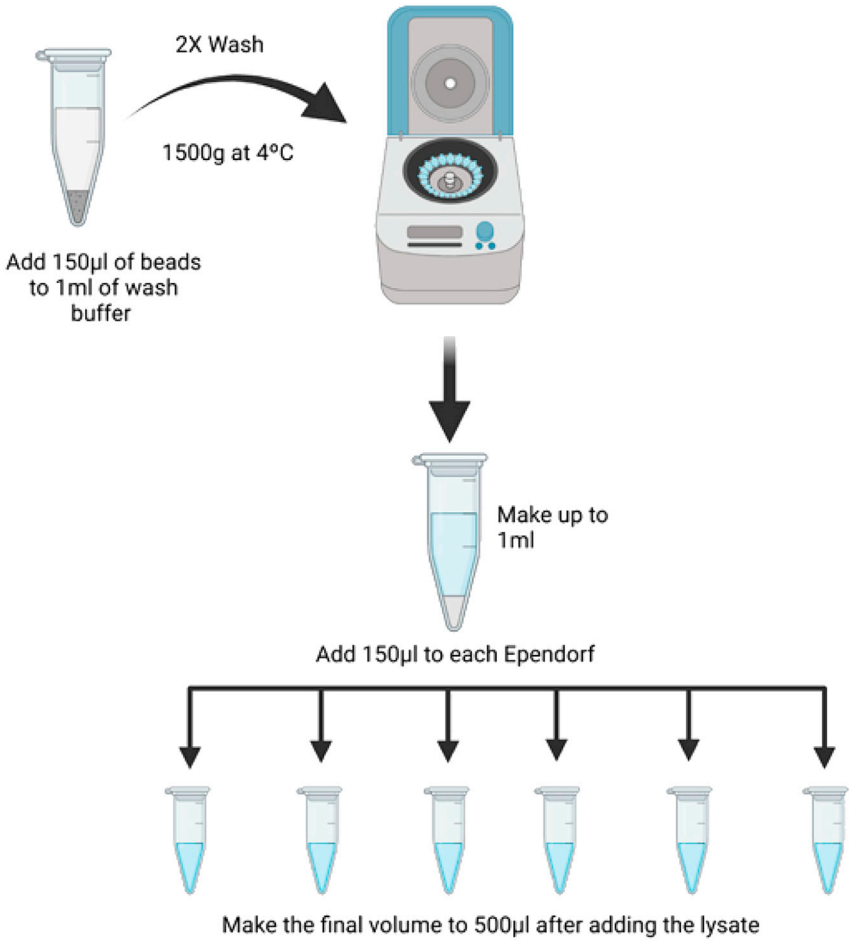

Figure 3. Schematic representation of streptavidin bead washing

23. Leave the beads on ice until the lysate is prepared (30 min–1 h).

Note: *Calculation: 150 µL of beads are used as per the following calculation: Each Eppendorf tube for the pull-down should have 20 µL of beads. So, to allow for pipetting error, 25 µL of beads are washed per condition (25 µL × 6 = 150 µL).

Note: **Calculation: After making up to 1 mL, divide 900 µL by the number of conditions to determine what volume to add to each Eppendorf tube (so, for 6 conditions, 900/6 = 150 µL is added to each well). When calculating, 100 µL is deducted from 1 mL to ensure sufficient beads remain for all conditions after accounting for pipetting error.

Pull-down of labeled proteins with streptavidin beads

Timing: 1.5–2 h

24. Centrifuge the lysate at 16,000*g* at 4°C for 30 min to pellet cell debris.
25. Transfer the supernatant (the cleared lysate) to a fresh 1.5 mL Eppendorf tube without disturbing the pellet.

Pause point: At this point the supernatant can be stored at −20°C for short-term (~1 month) and −80°C for long-term storage (~6 months).

26. Add 80 μL of the cleared cell lysate to the beads.
27. Add 270 μL of wash buffer to each Eppendorf tube to make the final volume 500 μL (150 μL beads + 80 μL of lysate = 230 μL)
28. Leave the Eppendorf tube on a rotating wheel at 4°C for 1 h.
29. Add 20 μL of the cleared cell lysate to a fresh Eppendorf tube (this serves as a "whole lysate" sample for the subsequent Western blot).

Note: In our experience, ~25% of AMPAR subunits are surface expressed; thus we add 80 μL lysate onto the beads for pull-down and preserve 20 μL of the sample ($1/4^{th}$ of the amount added to the beads) as lysate (total protein). However, the experimenter should scale this amount depending on their protein of interest and its abundance on the cell surface to ensure roughly equal amounts of target protein are present in the pull-down and lysate samples.

30. Wash the beads by adding 500 μL of wash buffer and centrifuging at 1500*g* at 4°C for 2 min.
31. Aspirate the buffer and repeat the wash step another 2 times, for a total of 3 washes.
32. Aspirate the supernatant and add 40 μL of 2× sample buffer to the beads.
33. Add 20 μL of 2× sample buffer to the 20 μL whole lysate sample to make a final concentration of 1× sample buffer.
34. Heat both beads and lysate samples on a heating block for 10 min at 95°C to denature the proteins and to elute the proteins from the beads.

Note: Centrifuge the beads at 1500*g* for 2 min before loading the samples on an SDS-PAGE to ensure beads are settled at the bottom of the Eppendorf tube.

35. Load 20 μL of pull-down and lysate samples in separate wells for Western blotting and detection of the receptor of interest.

Pause point: After boiling, the samples can be stored at −20°C for ≥ 6 months

Note: We strongly recommend blotting for an intracellular protein (e.g., GAPDH) as a negative control to ensure that the neurons remained intact throughout the labeling step and that Sulfo-NHS-SS-biotin, the biotinylation reagent, has not been able to enter the cells and label internal proteins. It is important to emphasize that the Sulfo-NHS-SS-biotin reagent used here is cell impermeant whereas standard biotin present in media is cell permeable.

EXPECTED OUTCOMES

Our protocol allows efficient labeling and isolation of surface proteins from neurons with minimal labeling of internal proteins. Using this method, we recently demonstrated that the kainate stimulation protocol outlined here leads to a reduction in the surface expression of AMPARs and KARs in hippocampal neurons (Figure 4).

For details see Petrovic et al. (2017) and Nair et al. (2021).

LIMITATIONS

Neuronal live surface biotinylation is a powerful technique to label, isolate and quantify levels of surface proteins in neurons. However, for synaptic proteins, a major limitation of this technique is the lack of spatial information, so it is not possible to determine whether the labeled proteins are surface expressed at the synapse, or in the extra-synaptic membrane. As a result, this protocol is usually complemented by imaging studies that can specifically examine changes in the levels of the protein of interest at synapses, or by functional studies using patch-clamp recordings to measure the activity (for example, mini-EPSCs or EPSCs for AMPARs or other iGluRs) of the protein of interest at the synapse.

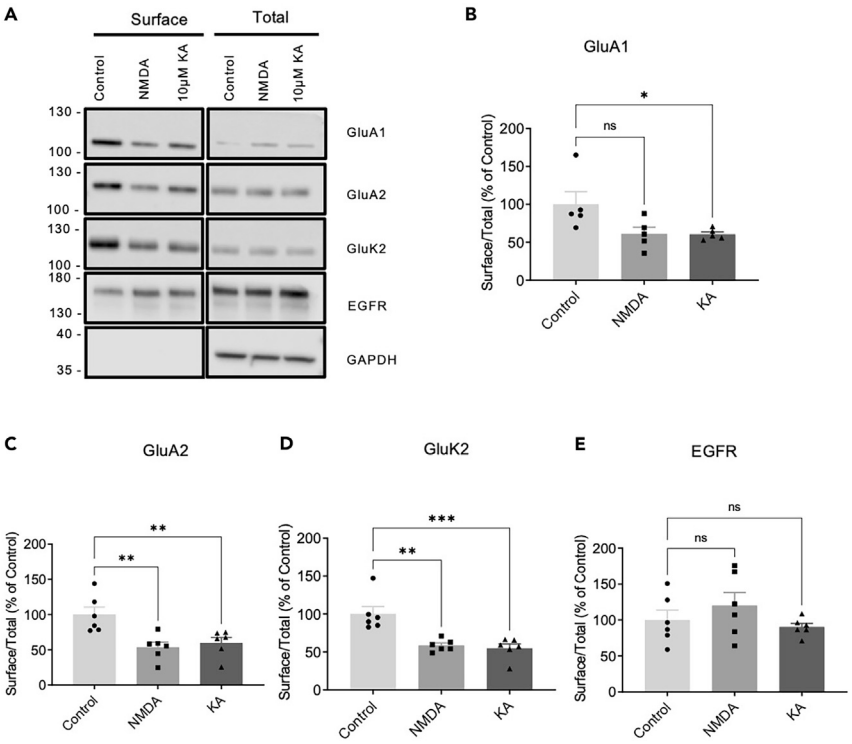

Figure 4. Kainate stimulation reduces surface expression of AMPARs and KARs

DIV 18 cultured hippocampal neurons were pre-treated for 30 min with 1 μM TTX and 40 μM GYKI53655 before treatment with vehicle or 10 μM kainate for 20 min. For NMDA treatment, neurons were pre-treated with 1 μM TTX for 30 min followed by 3 min of treatment with 20 μM NMDA and 20 μM glycine. Surface proteins were biotin labelled with Sulfo-NHS-SS-biotin and isolated by streptavidin pull-down, and lysates and surface fractions Western blotted.

(A) Representative Western blots of surface and total levels of GluA1, GluA2, GluK2, and EGFR. EGFR was used as a non-glutamate receptor expressed on the neuronal surface. GAPDH was used as a control to ensure that the cells contained an intact plasma membrane during exposure to Sulfo-NHS-SS-biotin so that no internal proteins were biotinylated.

(B–E) The surface to total ratio was calculated and expressed as a percentage of the control for (B) GluA1, (C) GluA2, (D) GluK2, and (E) EGFR. N=6 experiments from independent dissections, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; One-way ANOVA with Dunnett's multiple comparisons test, error bars = SEM.

TROUBLESHOOTING

Problem 1

Presence of internal control protein in pull-down lanes (step 34 – step-by-step method details).

One of the potential problems that can be experienced is the presence of the internal control protein such as GAPDH in the pull-down lanes after Western blot. Most likely this is due to disruption of neurons during the surface labeling step or due to endogenously biotin bound proteins like acetyl CoA carboxylase ACC1/2 that can come down in streptavidin pull-downs. The experimenter must be mindful of this and select appropriate internal control proteins.

Potential solution

Neurons are particularly sensitive to changes in osmolarity, which can result in cell lysis. Higher-density cell cultures (500,000) tend to resist small changes in osmolarity. However, if using lower density cultures or if there is a persistent presence of the internal control band in the pull-down, we recommend measuring and adjusting, as necessary, the osmolarity of the buffer (Earle's, HBS or DPBS) using sucrose or dH_2O.

Problem 2

Change in the pH of Earle's buffer or HBS (step 3 - before you begin).

Long-term storage of Earle's buffer or HBS at 18°C–25°C can result in changes in the pH of the buffer. This is often due to the growth of microbes.

Potential solution

Store the stocks at 4°C. Make small aliquots of 10× stocks and store at −20°C for up to 6 months.

Problem 3

Neurons are unresponsive to stimulation (step 35 – step-by-step method details).

While performing kainate or NMDA stimulations prior to surface biotinylation neurons can become less responsive and/or fail to respond to the stimulations. This is most likely due to compromised neuronal health. Therefore, it is essential that biotinylation experiments are carried out on morphologically intact, healthy, viable neurons.

Potential solution

We recommend the experimenter check the neuronal morphology under a DIC microscope prior to chemical stimulations. Neurons lacking well defined arborization, or exhibiting constricted soma, blebbing or bacterial or fungal contaminations should be discarded.

Problem 4

Kainate is ineffective (step 6 – step-by-step method details).

We have found that kainate in solution degrades even when stored frozen at −20 or −80°C.

Potential solution

We do not recommend storage of aliquoted samples of stock kainate solution for more than 3–4 weeks. Best practice is to make fresh stock solutions from powdered kainate and we recommend parallel positive controls, such as NMDA stimulation.

Problem 5

Diversity of neuronal cell types (step 35 – step-by-step method details).

We have noted variations in neuronal responses to the kainate stimulation in cortical versus hippocampal neurons which we attribute to the heterogeneity of the cortical cultures compared to the hippocampal neurons.

Potential solution

The experimenter should select the most appropriate neuronal cell culture type based on the receptor or surface protein of interest. We recommend using hippocampal cultures for studies on synaptic plasticity.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kevin Wilkinson (kevin.wilkinson@bristol.ac.uk).

Materials availability

No new materials are generated for this protocol.

Data and code availability

Data: All data generated using this protocol will be shared by the lead contact upon request.

Code: This protocol has not generated any code.

ACKNOWLEDGMENTS

We are grateful to the BBSRC (BB/R00787X/1), MRC (MR/L003791/1), Leverhulme Trust (RPG-2019-191), and Wellcome Trust (105384/Z/14/A) for financial support. The graphical abstract and figures in this article were made using Biorender.com.

AUTHOR CONTRIBUTIONS

J.D.N. wrote and all authors edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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