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Surface Trafficking of Sodium Channels in Cells and in Hippocampal Slices

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Abstract

The voltage-gated sodium channel (Na_v1) plays an important role in initiating and propagating action potentials in neuronal cells. We and others have recently found that the Alzheimer's disease-related secretases BACE1 and presenilin (PS)/ γ -secretase regulate Na_v1 function by cleaving auxiliary subunits of the channel complex. We have also shown that elevated BACE1 activity significantly decreases sodium current densities in neuroblastoma cells and acutely dissociated adult hippocampal neurons. For detailed molecular studies of sodium channel regulation, biochemical methods are now complementing classical electrophysiology. To understand how BACE1 regulates sodium current densities in our studies, we setup conditions to analyze surface levels of the pore-forming Na_v1 α -subunits. By using a cell surface biotinylation protocol, we found that elevated BACE1 activity significantly decreases surface Na_v1 α -subunit levels in both neuroblastoma cells and acutely prepared hippocampal slices. This finding would explain the decreased sodium currents shown by standard electrophysiological methods. The biochemical methods used in our studies would be applicable to analyses of surface expression levels of other ion channels as well as Na_v1 in cells and adult hippocampal neurons.

Keywords

Voltage-gated sodium channel; BACE1; Presenilin; γ -Secretase; Cell surface biotinylation; Neuroblastoma; Hippocampal neurons; Adult hippocampal slices

1. Introduction

The voltage-gated sodium channel (Na_v1) is one of the major ion channels regulating electrical signaling in excitable cells (1). In neuronal cells, Na_v1 plays an important role in generating and propagating the action potential and therefore, regulates neuronal excitability (1, 2). While standard electrophysiological methods are used to study Na_v1 activity, biochemical studies are required to understand the detailed molecular mechanisms of channel regulation. Na_v1 is composed of a single pore-forming α -subunit and one or two auxiliary β -subunits (3, 4). Ten α - and four β -subunits are currently known (4, 5). The β -subunits are type I membrane proteins that modulate the total/surface expression and inactivation of the α -subunits by direct interaction (2, 6–11). In neuronal cells, the majority of mature Na_v1 α -subunits is retained inside the cells while only a small portion gets to the cell surface and mediates sodium currents (6, 12). Therefore, it is very important to study how the surface trafficking of Na_v1 α -subunits is regulated.

We and others have recently reported that two specific proteases, BACE1 and presenilin (PS)/ γ -secretase, cleave auxiliary subunits of the Na_v1 and thereby regulate Na_v1 function (13–16). Both proteases also mediate the generation of the Alzheimer's disease-related

amyloid β -peptide (A β) and elevated BACE1 activity is associated with sporadic forms of the disease (17–21). We found that elevated BACE1 activity in neuroblastoma cells and acutely dissociated adult hippocampal neurons significantly decreases sodium current densities (15). To study the molecular mechanism underlying BACE1-mediated sodium current regulation, we set up conditions to analyze the surface expression levels of Na_v1 α -subunits, the major pore-forming Na_v1 subunits in neuronal cells. To analyze surface Na_v1 α -subunit levels in conditions similar to those used for sodium current measurements, we modified a protocol developed by Thomas-Crusells et al. (22). By applying this protocol, we were able to detect a significant decrease of surface Na_v1 α -subunit levels in both neuroblastoma cells and acutely prepared hippocampal slices from BACE1 transgenic animals. This would explain the decreased sodium currents found by standard electrophysiological methods.

2. Materials

2.1. Cell Culture

1. B104 neuroblastoma medium: Dulbecco's modified Eagle's medium (DMEM, Lonza/BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, Utah, USA), penicillin/streptomycin (Lonza/BioWhittaker), and 2 mM L-glutamine (Lonza/BioWhittaker).
2. Dulbecco's phosphate buffered saline (DPBS, Lonza/BioWhittaker).
3. Trypsin-EDTA solution (Lonza/BioWhittaker).
4. 100 and 150 mm cell culture plates.

2.2. Cell Surface Biotinylation of Cultured Neuroblastoma Cells

1. 10 and 50 mL Falcon tubes.
2. Hank's balanced salt solution (HBSS) w/Ca²⁺/Mg²⁺ buffer (pH 7.4): To make 1 L buffer, mix 100 mL 10×HBSS (Invitrogen/GIBCO, Grand Island, NY, USA), 0.47 mL 7.5% sodium bicarbonate (Invitrogen/GIBCO), and 899.5 mL HPLC water. Make it ice-cold before use (see Note 1).
3. Biotinylation solution: Dissolve 7.5 mg of sulfo-NHS-LC-biotin (Thermo/Pierce, Rockford, IL, USA) in 0.75 mL HPLC water and mix well (10 mg/mL, 20× biotinylation stock); add 0.75 mL of 20× biotinylation stock into 14.25 mL HBSS with Ca²⁺/Mg²⁺ buffer (see Note 2).
4. DPBS without Ca²⁺/Mg²⁺ (Invitrogen/GIBCO).
5. Biotinylation stopping solution: HBSS with 200 mM lysine (Sigma, St. Louis, MO, USA).
6. BCA protein assay kit (Thermo/Pierce).
7. Disposable cell scraper (Corning incorporated, Corning, NY, USA).

2.3. Preparation of Acute Brain Slices and Cell Surface Biotinylation

1. 6-well cell culture plates for washing and other general purposes.
2. Tissue culture filter insert (Millipore, Billerica, MA, USA).

¹ Alternatively, DPBS with Ca²⁺ and Mg²⁺ (Invitrogen/GIBCO, Cat # 14040–133) can be used instead of HBSS.

² Prepared the biotin solution fresh just before the experiments.

3. Superglue (Elmer's products Inc, Columbus, OH, USA).
4. Vibratome (model DTK-1500E, Ted Pella Inc, Redding, CA, USA).
5. Slice incubation chamber (*for a picture, see Fig. 1a*).
6. Carbogen gas (95% O₂ and 5% CO₂).
7. Dissection buffer (pH 7.4): 15 mM HEPES, 250 mM sucrose, 2.5 mM KCl, 0.1 mM CaCl₂, 1 mM Na₂HPO₄, 4 mM MgSO₄·7H₂O, and 180 mM glucose (see Note 3).
8. Low calcium buffer (pH 7.4): 15 mM HEPES, 140 mM Isethionate, 2 mM KCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 23 mM d-glucose (see Note 3).
9. NaHCO₃-buffered Earl's balanced salt solution (EBSS), Sigma (see Note 3).
10. 2× biotinylation solution: Dissolve 3.6 mg of sulfo-NHS-LC-biotin (Thermo/Pierce) in 0.6 mL HPLC water and mix well (6 mg/mL, 20× biotin stock); add 0.6 mL biotin stock into 5.4 mL NaHCO₃-buffered EBSS (see Note 2).
11. Biotinylation stopping solution: EBSS with 200 mM lysine.

2.4. Affinity-Purification of Biotinylated Proteins

1. Complete protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA).
2. Extraction buffer (pH 7.4): 10 mM Tris-HCl, pH 6.8, 1 mM EDTA, 150 mM NaCl, 0.2% SDS, 0.5% sodium deoxycholate (Sigma), 1% Triton X-100 (Roche), complete inhibitor cocktail tablet (1 tablet per 10 mL), 1 mM PMSF (Sigma), and 1 mM 1,10-phenanthroline (PNT, ICN Biochemicals Inc, Aurora, OH, USA).
3. Sonicator (Ultrasonic cleaner, Branson Ultrasonic Corp, Danbury, CT, USA).
4. NeutrAvidin (Thermo/Pierce): Wash three times with extraction buffer before use.

2.5. SDS-Polyacrylamide Gel Electrophoresis

1. 4× LDS sample loading buffer (Invitrogen) with 8% (v/v) β-mercaptoethanol.
2. Tris-Acetate gel running buffer (1×): Dilute the 4× Tris-acetate running buffer (Invitrogen) with three volumes of deionized water.
3. NuPage 3–8% Tris-Acetate gel (1.0 mm thickness, 10 well, Invitrogen).
4. Prestained molecular weight markers: Rainbow full-range markers (GE healthcare, UK).

2.6. Western Blotting Analysis for Sodium Channel α-Subunits and Control Proteins

1. Transfer buffer (1×): Dilute 1 volume of 20× NuPAGE transfer buffer (Invitrogen) with 17 volumes of deionized water and 2 volumes of HPLC grade methanol (Merck, Fair Lawn, NJ, USA) and store in a close capped plastic bottle at 4°C.
2. Immuno-Blot PVDF membranes (for protein blotting, 0.2 μm, Bio-Rad, Hercules, CA, USA).
3. Extra thick filter paper (Bio-Rad).
4. Hoefer semidry gel transfer unit (Hoefer, Holliston, MA, USA).

³All the solutions for brains slices must be cooled and saturated with carbogen gas (95% O₂ and 5% CO₂) by bubbling for more than 30 min before use.

5. Tris-buffered saline with Tween-20 (1× TBST): Prepare 20× stock with 1.37 M NaCl, 27 mM KCl, 250 mM Tris-HCl, pH 7.4, 0.3% (v/v) Tween-20; dilute 100 mL with 900 mL water before use.
6. Blocking buffer: 5% (w/v) nonfat dry milk in TBST.
7. Primary antibody dilution buffer: TBST supplemented with 1% (w/v) bovine serum albumen (BSA, Sigma).
8. Primary antibody solutions used in our study: Anti-pan α -subunit antibody (1:500, Sigma), anti-Nav1.1 α -subunit antibody (1:500, Sigma), anti-Nav1.2 α -subunit antibody (1:500, Alomone, Israel), anti-GAPDH antibody (BD bioscience), anti-N-cadherin antibody (BD bioscience/transduction laboratory).
9. Secondary antibody solution: Anti-mouse IgG conjugated to horseradish peroxidase (HRP) (1:6,000) in TBST with 1% skim milk or anti-rabbit IgG conjugated to HRP in TBST (1:6,000, GE healthcare).
10. Enhanced chemiluminescent (ECL) reagents (GE healthcare) and SuperSignal (Thermo/Pierce).
11. VersaDoc imaging system (Biorad).
12. Bio-Max ML film (Kodak, Rochester, NY).
13. Stripping buffer: 60 mM Tris-HCl (pH 6.8) buffer with 2% (w/v) SDS.

3. Methods

We made several changes to the surface biotinylation protocol originally developed by Thomas-Crusells et al. (22) to optimally analyze Nav1 α -subunits. First, we used strong ionic detergents, such as sodium deoxycholate and SDS together with a nonionic detergent to extract biotinylated proteins. Since Nav1 α -subunits are extremely hydrophobic membrane proteins with high molecular weight, strong detergent conditions are required to efficiently solubilize them from the plasma membrane. In the same detergent conditions, we confirmed that NeutrAvidin effectively captures the biotinylated proteins by using a total biotin staining protocol (see Fig. 1b). Second, we tested and optimized a combination of protease inhibitors that can effectively block the nonspecific degradation of Nav1 α -subunits, even with 4°C overnight incubation. Third, for adult hippocampal slice preparation, we used the exact same conditions and buffer systems that had been used to measure the sodium currents in our previous electrophysiological studies (15). These conditions allow for direct comparison of the electrophysiology and surface expression data. To analyze Nav1 α -subunits in neuroblastoma cells, here we used B104 cells stably expressing human Nav1 β 2-subunit. Since most neuroblastoma cells lack endogenous expression of the β 2-subunit, we made this cell line to study Nav1 regulation specifically by the β 2-subunit. The methods described here may also be applicable to analyze the surface expression of hydrophobic α -subunits of other ion channels in addition to Nav1.

3.1. Cell Surface Biotinylation of Cultured Neuroblastoma Cells

1. B104 rat neuroblastoma cells stably expressing human Nav1 β 2-subunit are maintained in B104 neuroblastoma medium supplemented with 0.2 mg/mL zeocin (Invitrogen).
2. Detach the cells from the plates with the trypsin-EDTA solution when the cells are approaching full confluence.
3. Plate the trypsinized cells into 100 or 150 mm cell culture dishes in 1:3 dilutions.

4. When the plated cells approach to 50–70% confluency (it generally takes 1–2 days), wash cells three times with 15 mL of ice-cold HBSS w/ Ca^{2+} and Mg^{2+} buffer.
5. Place the cell plates on ice and add 15 mL of ice-cold biotinylation solution.
6. Incubate the cells for 1 h on ice in a dark place with gentle rocking (see Notes 4 and 5).
7. Wash the cells two times with ice-cold biotinylation stopping solution (5 min incubation each).
8. Wash the cells two times with 15 mL ice-cold HBSS w/ Ca^{2+} and Mg^{2+} buffer followed by a DPBS (without Ca^{2+} / Mg^{2+}) washing.
9. Immediately add 400 μL extraction buffer (see Note 6).
10. Incubate on ice for 5 min.
11. Scrape the cells from the plates with disposable cell scraper and transfer the cell extracts to 1.5 mL tubes on ice.
12. Disrupt the cell clumps with gentle pipetting.
13. Incubate on ice for 10 min.
14. Centrifuge the cell extracts at $12,000 \times g$ (tabletop centrifuge), 4°C for 15 min.
15. Transfer the supernatant to new tubes on ice (see Note 7).
16. Measure protein concentration by BCA protein assay kit.

3.2. Cell Surface Biotinylation of Acute Adult Brain Slices

1. Saturate the dissection buffer, low calcium buffer, and EBSS buffer with carbogen gas (95% O_2 /5% CO_2) by bubbling the buffers with the gas for more than 30 min.
2. Add 100 mL dissection buffer into glass beaker and store at -80°C for 15–20 min until the buffer reaches 50% frozen ice/water status.
3. Sacrifice the mice by deep isoflurane anesthesia/cervical dislocation and quickly decapitate.
4. Rapidly open the skull and pour 5 mL ice-cold dissection buffer directly into the skull, remove the brain, and transfer the brains to ice-cold dissection buffer (see Note 8).
5. Trim the top and bottom of the brain and fix it with superglue to the cooled metal stage.
6. After fixing the brains with the cooled metal stage, cut the brain slices (300–400 μm) by using the vibratome (see Note 9).

⁴It is very important to use a gentle rocker to avoid stressing the cells. Alternatively, we found that the biotinylation works well without rocking if the plates are leveled to ensure even distribution of the biotinylation buffer.

⁵Biotinylation should be done in dark conditions. We generally cover each whole plate with aluminum foil.

⁶It is important to add the extraction buffer drop by drop to cover the entire cell culture plate.

⁷After the protein assay, the extracted samples are directly used for affinity purification of biotinylated proteins or can also be frozen and used later.

⁸The speed is the key to the successful experiment. Brains should be removed and kept on ice-cold dissection buffer within 5 min after sacrificing the mice.

⁹We used 12.5° cutting angle and the lowest speed for making coronal sections. We generally obtained five to six brain slices containing the hippocampal region. Dissection buffers in the vibratome should be continuously bubbled with carbogen gas (95% O_2 /5% CO_2).

7. Transfer the slices to ice-cold low-calcium buffer with wide-open glass pipettes and wash three times with the same buffer.
8. Transfer the washed slices onto the top of the tissue culture filter insert in a slice incubation chamber that is submerged in EBSS, continuously bubbled with carbogen gas (95% CO₂/5% CO₂).
9. Incubate the slices for more than 1 h at room temperature.
10. Briefly wash the slices three times with ice-cold low-calcium buffer.
11. Transfer the slices to another slice incubation chamber with 3 mL ice-cold EBSS in each well.
12. Incubate the slices on ice for 10 min.
13. Add 3 mL of 2× biotin solution (0.3 mg/mL final) to the wells of the slice incubation chamber.
14. Incubate on ice for 45 min (see Note 5).
15. Briefly wash the slices three times with ice-cold low-calcium buffer.
16. Transfer the slices to another incubation chamber containing biotinylation stopping solution and incubate for 5 min.
17. Repeat step 16 two times.
18. Briefly wash the slices three times with ice-cold low-calcium buffer
19. Dissect the individual sections under a surgical microscope and transfer hippocampal, cortical, and striatal regions to dry-ice frozen 1.5 mL tubes (see Note 10).
20. For extracting the hippocampal sections, 100 µL of extraction buffer is added per hippocampal section.
21. Disrupt the hippocampal slices by pipetting, followed by 5 min sonication on a water-bath type sonicator with maximum energy.
22. Incubate samples on ice for additional 10 min.
23. Centrifuge the slice extracts at 12,000 × *g* (tabletop centrifuge), 4°C for 15 min.
24. Transfer the supernatant to new tubes on ice.
25. Measure protein concentration by the BCA method.

3.3. Affinity Purification of Biotinylated Proteins

1. Dilute the extracted samples with the extraction buffer for a final total protein concentration of 1 µg/µL.
2. For total protein analysis, 25–30 µg samples are mixed with 10 µL 4× LDS sample loading buffer with 8% (v/v) β-mercaptoethanol, quickly frozen on dry ice, and stored at –20°C until SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Frozen samples will be heated at 95°C for 5 min before gel loading.
3. To capture the biotinylated proteins, 100–300 µg of samples (100 µg total for hippocampal slices; 300 µg for neuroblastoma cells) are mixed with 40 µL of NeutrAvidin beads (see Note 11).

¹⁰The dissected slices can be stored at –80°C until the extraction.

4. Incubate for 3 h or overnight at 4°C with rocking (for hippocampal section, 3 h incubation is recommended to avoid a potential protein degradation).
5. Wash four times with the 500 μ L extraction buffer for each 10 min.
6. Add 50 μ L of 1 $\times$ sample loading buffer (4 $\times$ LDS, BME, and Tris-Acetate buffer) and heat at 95°C for 5 min.
7. Briefly centrifuge the beads and collect the supernatant.

3.4. SDS-Polyacrylamide Gel Electrophoresis

1. 3–8% NuPage Tris-Acetate gels are used to resolve Na $_v$ 1 α -subunits with high molecular weight (~260 kDa). The rainbow marker is used as a protein size marker.
2. After loading total and biotin-captured proteins samples, run the gels at 170 V for approximately 60 min.
3. At the end, the gel units are disassembled and the gels are transferred to clean plastic trays containing 1 $\times$ NuPAGE transfer buffer.

3.5. Western Blot Analysis of Total and Cell Surface Na $_v$ 1 α -Subunits and Control Proteins

1. Hoefer semidry gel transfer unit is used to transfer proteins in the NuPAGE gels to PVDF membranes.
2. Once the transfer is complete, PVDF membrane blots are marked and transferred to the blocking buffer.
3. After 1 h incubation at room temperature with gentle rocking, discard the blocking buffer and wash the blots three times for 5 min with TBST.
4. Add primary antibody solution and incubate at 4°C for overnight with gentle rocking.
5. Collect the primary antibody solution and wash three times with TBST for 10 min.
6. After the final wash, place the blots in a clear plastic plate and add 2 mL of ECL plus or SuperSignal.
7. Incubate the blots for 1–3 min and discard the solution.
8. Cover the blots with clean wrap or clear film.
9. The chemiluminescent signal can be captured digitally with an instrument, such as VersaDoc imaging system. Quantification of data is done by attached Quantityone software version 4.6.5 (see Note 12). Example results are shown in Figs. 1c and 2.

3.6. Stripping and Reprobing Blots for Control Proteins

1. After getting the optimal exposure, the blots are stripped and reprobed with other primary antibody solutions, including antibodies for isoform-specific sodium channel antibodies and control proteins, including Na $_v$ 1.1, 1.2, 1.6, *N*-cadherin, and GAPDH.
2. For stripping, the blots are incubated with stripping buffer for 15 min at 65°C.

¹¹To avoid potential pipetting errors, we vortex the beads every time just before transferring NeutrAvidin beads to the samples. In addition, it is strongly recommended to cut the end of pipette tips to avoid disrupting the beads.

¹²Alternatively, Bio-Max ML film can be used to visualize chemiluminescence signals.

3. After washing three times with TBST, reincubate the blots with blocking buffer for 1 h at room temperature.
4. After washing three times again, add another primary antibody solution and incubate for 1 h at room temperature or overnight at 4°C.

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Fig. 1.

Surface-biotinylation analysis of adult brain slices acutely prepared from wild-type control mice. **(a)** The slice incubation chamber. **(b)** Total lysates and NeutrAvidin-captured biotinylated proteins were stained with anti-biotin-horseradish peroxidase (HRP) to measure the efficiency of capturing biotinylated proteins. **(c)** Total lysates (*left panel*) and NeutrAvidin-captured biotinylated proteins (*right panel*) were analyzed by Western blot using antibodies against *N*-cadherin as a membrane protein control, and Actin for a control intracellular protein.

Fig. 2.

BACE1 overexpression reduces the surface levels of Na_v1 α-subunits in adult hippocampal slices as we previously reported (15). Four matching slice pairs from similar locations of the brain were selected from BACE1-transgenic (BACE1-tg) and age-matched wild-type control mice (WT), respectively. Total lysate (1/20th of total input lysate, *left panel*) and NeutrAvidin captured surface biotinylated proteins (*right panel*) were analyzed by Western blot (WB) using antibodies against Na_v1 α-subunits (pan) and control GAPDH.