Video Article

Quantifying Subcellular Ubiquitin-Proteasome Activity in the Rodent Brain

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Abstract

The ubiquitin-proteasome system is a key regulator of protein degradation and a variety of other cellular processes in eukaryotes. In the brain, increases in ubiquitin-proteasome activity are critical for synaptic plasticity and memory formation and aberrant changes in this system are associated with a variety of neurological, neurodegenerative and psychiatric disorders. One of the issues in studying ubiquitin-proteasome functioning in the brain is that it is present in all cellular compartments, in which the protein targets, functional role and mechanisms of regulation can vary widely. As a result, the ability to directly compare brain ubiquitin protein targeting and proteasome catalytic activity in different subcellular compartments within the same animal is critical for fully understanding how the UPS contributes to synaptic plasticity, memory and disease. The method described here allows collection of nuclear, cytoplasmic and crude synaptic fractions from the same rodent (rat) brain, followed by simultaneous quantification of proteasome catalytic activity (indirectly, providing activity of the proteasome core only) and linkage-specific ubiquitin protein tagging. Thus, the method can be used to directly compare subcellular changes in ubiquitin-proteasome activity in different brain regions in the same animal during synaptic plasticity, memory formation and different disease states. This method can also be used to assess the subcellular distribution and function of other proteins within the same animal.

Video Link

The video component of this article can be found at https://www.jove.com/video/59695/

Introduction

The ubiquitin-proteasome system (UPS) is a complex network of interconnected protein structures and ligases that controls the degradation of most short-lived proteins in cells[1]. In this system, proteins are marked for degradation or other cellular processes/fates by the small modifier ubiquitin. A target protein can acquire 1-7 ubiquitin modifications, which can link together at one of seven lysine (K) sites (K6, K11, K27, K29, K33, K48 and K63) or the N-terminal methionine (M1; as known as linear) in the previous ubiquitin[2]. Some of these polyubiquitin tags are degradation-specific (K48)[3], while others are largely independent of the protein degradation process (M1)[4,5,6]. Thus, the protein ubiquitination process is incredibly complex and the ability to quantify changes in a specific polyubiquitin tag is critical for ultimately understanding the role of that given modification in cellular functioning. Further complicating the study of this system, the proteasome, which is the catalytic structure of the UPS[7], both degrades proteins but can also be involved in other non-proteolytic processes[8,9]. Not surprisingly then, since its initial discovery, normal and aberrant ubiquitin-proteasome activity has been implicated in long-term memory formation and a variety of disease states, including many neurological, neurodegenerative and psychiatric disorders[10,11]. As a result, methods which can effectively and efficiently quantify UPS activity in the brain are critical for ultimately understanding how this system is dysregulated in disease states and the eventual development of treatment options targeting ubiquitin and/or proteasome functioning.

There are a number of issues in quantifying ubiquitin-proteasome activity in brain tissue from rats and mice, which are the most common model systems used to study UPS function, including 1) the diversity of ubiquitin modifications, and 2) distribution and differential regulation of UPS functioning across subcellular compartments[12,13,14]. For example, many of the early demonstrations of ubiquitin-proteasome function in the brain during memory formation used whole cell lysates and indicated time-dependent increases in both protein ubiquitination and proteasome activity[15,16,17,18,19,20]. However, we recently found that ubiquitin-proteasome activity varied widely across subcellular compartments in response to learning, with simultaneous increases in some regions and decreases in others, a pattern that differs significantly from what was previously reported in whole cell lysates[21]. This is consistent with the limitation of a whole cell approach, as it cannot dissociate the contribution of changes in UPS activity across different subcellular compartments. Though more recent studies have employed synaptic fraction protocols to study the UPS specifically at synapses in response to learning[22,23,24], the methods used occlude the ability to measure nuclear and cytoplasmic ubiquitin-proteasome changes in the same animal. This results in an unnecessary need to repeat experiments multiple times, collecting a different subcellular fraction in each. Not only does this result in a greater loss of animal lives, but it eliminates the ability to directly compare UPS activity across different subcellular compartments in response to a given event or during a specific disease state. Considering that protein targets of ubiquitin and the proteasome vary widely throughout the cell, understanding how ubiquitin-proteasome signaling differs in distinct subcellular compartments is critical for identifying the functional role of the UPS in the brain during memory formation and neurological, neurodegenerative and psychiatric disorders.

To address this need, we recently developed a procedure in which nuclear, cytoplasmic and synaptic fractions could be collected for a given brain region from the same animal[21]. Additionally, to account for the limited amount of protein that can be obtained from collecting multiple subcellular fractions from the same sample, we optimized previously established protocols to assay in vitro proteasome activity and linkage-specific protein ubiquitination in lysed cells collected from rodent brain tissue. Using this protocol, we were able to collect and directly compare learning-dependent changes in proteasome activity, K48, K63, M1 and overall protein polyubiquitination levels in the nucleus and cytoplasm and at synapses in the lateral amygdala of rats. Here, we describe in detail our procedure (**Figure 1**), which could significantly improve our understanding of how the UPS is involved in long-term memory formation and various disease states. However, it should be noted that the in vitro proteasome activity discussed in our protocol, while widely used, does not directly measure the activity of complete 26S proteasome complexes. Rather, this assay measures the activity of the 20S core, meaning it can only serve as a proxy to understand the activity of the core itself as opposed to the entire 26S proteasome complex.

Protocol

All procedures including animal subjects have been approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (IACUC).

1. Collection and dissection of rodent brain tissue

NOTE: This protocol can be applied to a variety of brain regions and used with various tissue collection procedures. Below is the procedure used in our lab for subcellular of rat brain tissue, using 8-9 week old male Sprague Dawley rats. In order to process all cellular compartments in the same animal, section 1.3. must be followed regardless of the tissue collection procedure used.

1. Extract the rat brain and place into a cryogenic cup pre-chilled on dry ice. Flash-freeze the brain on dry ice, or use liquid nitrogen if available, and transfer to a -80 °C freezer. Brains can be dissected the same day or at a later time.
2. Remove the frozen brain from the cryogenic cup and place into a rat brain matrix chilled with dry ice. Each slot on the matrix corresponds to 0.5 mm, which can be used to determine the approximate location of the region of interest (ROI).
    1. Using a Rat Brain Atlas, insert a razor blade into the matrix immediately in front (anterior) of the predicted start of ROI. Next, insert a razor blade immediately at the predicted end (posterior) of ROI.
    2. Remove the slice between the razor blades using a scalpel and place onto a microscope slide chilled on dry ice. Typically, sections are 2-3 mm thick but this varies based on the ROI.
3. Using a scalpel, dissect out the ROI one hemisphere at a time. Place each hemisphere into separate 1.5 mL microcentrifuge tubes pre-chilled on dry ice. Frozen tissue can immediately be used for subcellular fractionation or processed at a later time if stored at -80 °C.

2. Nuclear and cytoplasmic extraction

NOTE: This protocol uses premade stock solutions of common lab chemicals, including 0.1 M HEPES, 1 M $MgCl_2$, 1 M Dithiothreitol (DTT), 0.5 M ethylenediaminetetraacetic acid (EDTA), 5 M NaCl, 10% NP-40 (IGEPAL) and 50% glycerol. If endpoint is used in proteasome activity assays, glycerol and ATP can be added to all buffers to help prevent disassembly of proteasome complexes during lysis.

1. Prepare the Lysis Buffer. Add 8.63 mL of ultrapure (deionized and distilled) water to a sterile 15 mL conical tube.
    1. To the conical tube, add 1,000 µL of 0.1 M HEPES, 15 µL of 1 M MgCl, 100 µL of 1 M DTT and 50 µL of 10% NP-40.
    2. Add 100 µL of protease inhibitor cocktail and 100 µL of phosphatase inhibitor cocktail. Briefly vortex the solution to mix and place on wet ice to chill. Note that the solution may have a yellow tint from the phosphatase inhibitor.
    NOTE: The use of protease inhibitors is essential to preserve protein and ensure the specificity of the in vitro proteasome activity assay. However, these inhibitors may also result in a slight reduction in proteasome activity, meaning that the activity measured on the in vitro assay may be an underestimate of actual activity levels.
2. Prepare the Extraction Buffer. Add 5.925 mL of ultrapure (deionized and distilled) water to a sterile 15 mL conical tube.
    1. To the conical tube, add 2,000 µL of 0.1 M HEPES, 1250 µL of 50% glycerol, 15 µL of 1 M $MgCl_2$, 5 µL of 1 M DTT, 5 µL of 0.5 M EDTA and 600 µL of 5 M NaCl.
    2. Add 100 µL of protease inhibitor cocktail and 100 µL of phosphatase inhibitor cocktail. Briefly vortex the solution to mix and place on wet ice to chill. Note that the solution may have a yellow tint from the phosphatase inhibitor.
3. Remove the 1.5 mL centrifuge microtube containing one hemisphere of the ROI from the -80 °C freezer. Ensure that the hemisphere used is counterbalanced across extraction conditions for each experimental group. For example, in a two-group experiment, use the left hemisphere for the first two animals in each group and the right hemisphere for the next two samples, etc.
4. Using a scalpel, transfer the frozen brain tissue to a 2 mL glass Teflon homogenizer. Add 500 µL of Lysis buffer to the Teflon tube.
    1. Using pestle B, homogenize the same tissue using 15 strokes until no visible amount of solid material are present. Use a turning motion (clockwise or counter-clockwise) during each stroke.
5. Using a 1,000 µL pipette, transfer the homogenized sample to a new 1.5 mL microcentrifuge tube. Place the tube on wet ice and incubate for 30 min.
6. Place tube in microcentrifuge and spin for 10 min at 845 x *g* and 4 °C. After completion, carefully remove the supernatant by pipetting and place into a new 1.5 mL microcentrifuge tube; this is the Cytoplasmic Fraction, which can be stored on ice or at 4 °C until Section 2.9.
7. Add 50 µL of Extraction buffer to the resulting pellet and resuspend by pipetting. Do not vortex the pellet. Place the tube containing the resuspended pellet on ice and incubate for 30 min.
8. Place the tube in microcentrifuge and spin for 20 min at 21,456 x *g*, 4 °C. After completion, carefully remove the supernatant by pipetting and place into a new 1.5 mL microcentrifuge tube; this is the Nuclear Fraction. The pellet can now be discarded.

9. Measure protein concentration of the Cytoplasmic and Nuclear extractions using the Dc protein assay (according to the manufacturer's instructions), or any equivalent assay for samples collected using non-ionic detergents. Proceed immediately to proteasome activity assay (Section 4) or Western blotting (Section 5). Alternatively, samples can be stored at -80 °C until needed.

3. Synaptic fraction collection

NOTE: This protocol uses premade stock solutions of common lab chemicals, including 1 M Tris (pH 7.5), 0.5 M EDTA, 5 M NaCl and 10% SDS. If endpoint is used in proteasome activity assays, glycerol and ATP can be added to all buffers to help prevent disassembly of proteasome complexes during lysis

1. Prepare the TEVP buffer with 320 mM sucrose. Add 60 mL of ultrapure (deionized and distilled) water to a clean 100 mL beaker.
    1. To the beaker, add 1,300 µL of 1 M Tris (pH 7.5), 260 µL of 0.5 M EDTA, 100 µL of protease inhibitor cocktail, 100 µL of phosphatase inhibitor cocktail and 10.944 g of sucrose. Mix with a stir bar until sucrose is completely dissolved.
    2. Bring pH to ~7.4 by adding hydrochloric acid (HCl) dropwise to the solution using a pipette.
    3. Transfer the solution to a 100 mL volumetric flask. Bring volume to 100 mL by adding ultrapure water. Chill the final solution on ice until needed.

2. Prepare the Homogenization Buffer. Add 60 mL of ultrapure (deionized and distilled) water to a clean 100 mL beaker.
    1. To the beaker, add 5,000 µL of 1 M Tris (pH 7.5), 3,000 µL of 5 M NaCl, 100 µL of protease inhibitor cocktail, 100 µL of phosphatase inhibitor cocktail and 2,000 µL of 10% SDS. Mix with a stir bar.
    NOTE: Ionic detergents can interfere with proteasome activity assays by resulting in denaturing of the proteasome complex. The volume of SDS could be lowered to help preserve proteasome function if desired.
    2. Bring pH to ~7.4 by adding HCl dropwise to the solution using a pipette.
    3. Transfer the solution to a 100 mL volumetric flask. Bring the volume to 100 mL by adding ultrapure water. Store the final solution at room temperature; do not chill as SDS will precipitate out of solution.

3. Remove the 1.5 mL centrifuge containing one hemisphere of the ROI from the -80 °C freezer. Ensure that the hemisphere used is counterbalanced across extraction conditions for each experimental group. For example, in a two-group experiment, use the left hemisphere for the first two animals in each group and the right hemisphere for the next two samples, etc.
4. Using a scalpel, transfer the frozen brain tissue to a 2 mL glass Teflon homogenizer. Add 500 µL of TEVP buffer to the Teflon tube.
    1. Using pestle B, homogenize the same tissue using 15 strokes until no visible transfers of solid material are present. Use a turning motion (clockwise or counter-clockwise) during each stroke.

5. Using a 1,000 µL pipette, transfer the homogenized sample to a new 1.5 mL microcentrifuge tube. Centrifuge the sample at 1,000 x *g* for 10 min, 4 °C.
6. Collect the supernatant and transfer to a new 1.5 mL microcentrifuge tube using a 1,000 µL pipette. Centrifuge the sample at 10,000 x *g* for 10 min, 4 °C. The original pellet (P1) contains nuclei and the large debris and can be discarded.
7. Transfer the supernatant to a new 1.5 mL microcentrifuge tube. This is a Cytosolic Fraction. Add 50 µL of Homogenization buffer to the pellet (P2) and resuspend by pipetting until no solid material is visible.
8. Centrifuge the sample at 20,000 x *g* for 10 min, 4 °C. Transfer the supernatant to a new 1.5 mL microcentrifuge tube; this is the Crude Synaptosomal Membrane (Synaptic) Fraction. The pellet can be discarded.
9. Measure the protein concentration of the Synaptic fraction using the Dc protein assay (according to the manufacturer's instructions), or any equivalent assay for samples collected using ionic detergents. Proceed immediately to proteasome activity assay (Section 4) or Western blotting (Section 5). Alternatively, samples can be stored at -80 °C until needed.

4. Proteasome activity assay

NOTE: Proteasome activity can be measured in homogenized brain tissue using a slightly modified version of the 20S Proteasome Activity Kit. This assay does not directly measure the activity of complete 26S proteasome complexes. Rather, it measures the activity of the 20S core, meaning it can only serve as a proxy to understand the activity of the core itself as opposed to the entire 26S proteasome complex. The success of this assay declines with repeated freeze-thaw cycles and/or increasing levels of detergents, particularly ionic, and requires the use of a plate reader with a 360/460 (excitation/emission) filter set and heating capabilities up to 37 °C.

1. Plate reader settings: Pre-warm to 37 °C and hold through the run.
    1. Set excitation to 360 and emission to 460. If the 96 well plate used is clear, set the optics position to **Bottom**. If a dark/black 96 well plate is used, set optics position to **Top**.
    2. Set older plate reader models to **Auto-Gain** under the fluorescent read options; newer models are preset for this. Program a kinetic run with a time of 2 h, scanning (reading) every 30 min.

2. Reconstitute the 10x assay buffer provided in the kit with 13.5 mL of ultrapure water. Add 14 µL of 100 mM ATP to the now 1x buffer; this significantly enhances proteasome activity in the samples and improves assay reliability. The final 20S Assay buffer can be stored on ice or at 4 °C until needed and is stable for several months.
3. Reconstitute the AMC standard provided in kit with 100 µL of DMSO. Perform this step in the dark or under low light conditions, as the standard is light sensitive.
4. Create a stand curve of AMC using the reconstituted standard, in the dark or under low light conditions.
    1. In separate 0.5 mL microcentrifuge tubes, add 16, 8, 6.4, 3.2, 1.6, 0.8, 0.4 and 0 µL of the AMC standard, which corresponds to 20, 10, 8, 4, 2, 1, 0.5 and 0 µM AMC concentration.

2. To these tubes, in the same order, add 84, 92, 93.6, 96.8, 98.4, 99.2, 99.6 and 100 µL of 20S Assay buffer. This creates a series of high to low AMC concentrations, which will be used for plate reader calibration and analysis of proteasome activity in the homogenized samples.
3. Store all diluted standards on ice in the dark until needed.

5. Reconstitute the proteasome substrate (Suc-LLVY-AMC) provided in kit with 65 µL of DMSO. Perform this step in the dark or under low light conditions, as the substrate is light sensitive. Create a 1:20 dilution of the proteasome substrate in a new 1.5 mL microcentrifuge tube using 20S Assay buffer. Store the diluted substrate on ice in the dark until needed.
   NOTE: For example, if the plate will have 10 samples and 1 blank, you will need enough diluted substrate for 22 wells (with duplicates) at 10 µL per well. This equates to 220 µL need + 30 µL for pipetting errors, requiring 12.5 µL of substrate and 237.5 µL of 20S Assay buffer.
6. Thaw desired samples (if frozen) and add a normalized amount to a 96 well plate. Run each sample in duplicates. The amount of sample needed varies based on tissue preparation. Generally, 10-20 µg is sufficient for any subcellular fraction.
7. Bring the sample well volume to 80 µL with ultrapure water. The amount added depends on the volume of sample added. For example, if sample 1 was 4.5 µL of protein and sample 2 was 8.7 µL, then the amount of water needed will be 75.5 µL and 71.3 µL, respectively. In two separate wells, add 80 µL of water alone; these will be the Assay Blanks.
   NOTE: In order to limit changes in protein volume due to pipetting errors, protein concentrations can be normalized to the least concentrated sample. This will allow use of the same sample volume in all conditions.
8. Add 10 µL of 20S Assay buffer to each well, including Assay Blanks. A repeater/automated pipette is recommended here to ensure consistent assay volume across wells.
9. **Optional:** At this stage, introduce in vitro manipulations if desired; this will require that each sample has an additional 2 wells per treatment, including the vehicle. If so, add 5-10 µL of drug/compound of interest to 2 of the sample wells and an equivalent volume of control/vehicle to another 2 wells. Place the plate onto the pre-warmed plate reader or into a 37 °C incubator for 30 min.
10. Turn off the lights or enter a dark room. Add all 100 µL of diluted AMC standards to a new well; each standard will have a single well.
11. In the dark, add 10 µL of diluted proteasome substrate to wells containing sample and Assay Blanks, but not to the AMC standard. A repeater/automated pipette is recommended here to ensure consistent assay volume across wells.
12. Place the plate into the plate reader and start the kinetic run.
    NOTE: The plate does not need to be under constant agitation during the kinetic run, however, the user can choose to if desired
13. At end of the kinetic run, export raw 360/460 fluorescent values to Microsoft Excel.
    1. Average together duplicate wells for each standard, each sample and the Assay Blank for all 5 scans. Raw fluorescent values should increase across scans for the samples but remain stable (or decrease slightly) for standards and Assay Blanks.
    2. Take the highest AMC standard well average and divide by the known concentration (20 µM). Divide this value by the sample concentration used in the assay to get standardized AMC value. For each sample and Assay Blank average, divide by the standardized AMC value.
    3. Take this final value and divide by the sample concentration used in the assay to get the normalized value for each sample and the Assay Blank. Do this for all 5 scans.
    4. Subtract the normalized Assay Blank value from each normalized sample value for all 5 scans.

5. Quantification of linkage-specific protein ubiquitination

1. Quantification of diverse polyubiquitin tags in different subcellular fractions collected from rodent brain tissue should be done using a variety of standard Western blotting protocols in combination with unique, linkage-specific polyubiquitin antibodies.
   1. For denaturing, mix the normalized samples with an equal volume of Laemmli sample buffer supplemented with β-mercaptoethanol to a percentage of 5% by volume, per the manufacturer's instruction.
   2. For quantification of all monoubiquitination and polyubiquitination modifications regardless of linkage, use a pan-ubiquitin.
   3. To recognize all polyubiquitinated proteins, use an ubiquitin antibody that does not cross-react with monoubiquitination.
   4. For linkage-specific polyubiquitination, use antibodies that can detect Lysine-27, Lysine-48, Lysine-63 and linear (M1) polyubiquitination.

2. To prevent cross-contamination between developments, which could result in a false positive or interfere with imaging of a different ubiquitin modification, strip membranes between developments using 0.1 M NaOH for 10 min.
   1. Wash the membranes in TBS with 0.1% tween twice for 10 min and reblock (using whatever blocking agent is preferred in the Western blot protocol used).
   2. Incubate the membrane with the secondary antibody and redevelop in order to confirm that the membrane was stripped of primary and secondary antibodies properly.
   3. **Recommended:** For successful development of different ubiquitin antibodies without contamination, use fluorescent or near-infrared imaging systems. This often times can prevent carryover between antibodies.

3. Some ubiquitin Western blot images will provide columns of distinct bands (such as M1) while others produce a smear-like pattern with few or no clear lines (common with K48). For quantification of imaged ubiquitin Western blots, draw a box around the column that extends the entire molecular standards ladder.
   1. Adjust the box up (or down) if ubiquitin staining does extend through the entire ladder; this is common for Lysine-48 modifications and varies widely across subcellular compartments.
   2. Subtract out the background, which is calculated as the mean optical density of the background immediately surrounding the column on all sides.

Representative Results

Using the procedure described here, nuclear, cytoplasmic and synaptic fractions were collected from the lateral amygdala of the rat brain (**Figure 1**). Purity of the individual fractions were confirmed via Western blotting, probing with antibodies against proteins that should be enriched or depleted in the lysate. In the first hemisphere where a crude synaptic fraction was collected, postsynaptic density protein 95 (PSD95) was present in the synaptic, but not nuclear, fraction, with lower levels in the cytoplasm (**Figure 2A**). This is consistent with previous work demonstrating that the synaptic fraction preparation isolates both presynaptic and postsynaptic components[25]. Conversely, the nuclear protein histone H3 was present in the nuclear, but not the synaptic, fraction, with lower levels in the cytoplasm (**Figure 2B**). The presence of PSD95 and H3 in the cytoplasm is consistent with their cytoplasmic translation. The cytoplasmic protein β-tubulin was present in our cytoplasmic fraction, but was largely absent from the nuclear lysate (**Figure 2C**), with lower levels in the synaptic region. This suggests that we were able to produce a nuclear fraction that was largely absent of cytoplasmic proteins. The presence of tubulin in the synaptic region is consistent with previous studies[26]. All three fractions showed similar levels of the housing keeping protein β-actin (**Figure 2D**), which was used as a loading control. Collectively, these results confirm that the purity of the nuclear, cytoplasmic and synaptic fractions collected from a single rat lateral amygdala.

Next, all lysates were confirmed for functional proteasome activity using the described modified version of the in vitro 20S proteasome activity assay. In all lysates, success of the assay was defined as an increase in raw fluorescent units (RFU) detected from the first scan (0 min) to the fifth/final scan (120 min). For all of these analyses, 10 μM AMC was used as the highest standard for normalization of the raw fluorescent units (RFU). In the crude synaptic fraction, RFU peaked at scan 5 (**Figure 3A**), resulting in a normalized RFU of just under 0.1 (**Figure 3B**). In the cytoplasmic fraction, RFU increased across scans (**Figure 3C**) with a final normalized RFU of ~1.6 (**Figure 3D**). The nuclear fraction also displayed time-dependent changes in RFU (**Figure 3E**), with a final normalized RFU of 0.3 (**Figure 3F**). The differences in proteasome activity across compartments likely reflects the availability of proteasomes in the fraction, which are generally most abundant in the cytoplasm and nucleus[27], compartments which have the highest level of activity in our preparation. The lowest level of activity in the synaptic region is consistent with it being the only lysate that was collected using ionic detergents, which can reduce proteasome activity due to the harsher denaturing condition. Importantly, RFU did not increase across time in the Assay Blanks or in lysates (synaptic) incubated with the highly specific and potent proteasome inhibitor clasto-lactacystin-β-lactone (**Figure 3G**), displaying final normalized RFU levels of 0.01 and 0.001, respectively (**Figure 3H**). This suggests that the observed change in RFU was due specifically to activity of the proteasome and not other proteases. Furthermore, when analyzed across experimental conditions, there was an increase in nuclear, but not cytoplasmic, proteasome activity in the lateral amygdala following learning, which occurred simultaneously with a decrease in synaptic proteasome activity in comparison to control animals (**Figure 4**). Collectively, these results confirm that proteasome activity could be accurately measured in all three subcellular fractions collected from a single rat lateral amygdala.

One of the advantages of the proteasome activity assay is that in vitro manipulations can be introduced to the samples immediately prior to addition of the proteasome substrate, which allows identification of specific molecules which can regulate the proteasome in that particular subcellular fraction. As an example of this, the role of CaMKII (calcium/calmodulin dependent protein kinase II) was assessed in the synaptic fraction, the harshest denatured lysate collected, since CaMKII is thought to regulate proteasome function at synapses. A 30 min incubation with the CaMKII inhibitor myr-AIP (myristolayted autocamtide-2 related inhibitory peptide) resulted in a significantly diminished increase in proteasome activity on the assay, which only reached levels that were ~61% of the untreated controls (**Figure 5A**). Conversely, the same manipulation applied to the cytoplasmic did not result in a change in proteasome activity from vehicle treated wells (**Figure 5B**). These results confirm that proteasome activity can be further manipulated in vitro and that this manipulation can have different effects depending on the subcellular fraction collected.

In addition to quantifying proteasome activity, the described protocol can be used to measure subcellular differences in diverse ubiquitin modifications using Western blot procedures. It is important to note that the ubiquitin tags that can be detected are limited by the availability of linkage-specific antibodies, which currently include K48, K63 and M1 for rats (note: a K27 antibody is available, though did not produce a detectable image in any lateral amygdala fraction or whole cell lysates under a variety of conditions). Overall polyubiquitination, degradation-independent linear/M1 and K63 ubiquitination and degradation-specific K48 ubiquitination were detected in all subcellular fractions. Importantly, when analyzed across different experimental conditions, there was an increase in overall (**Figure 6A**), linear (**Figure 6B**), K63 (**Figure 6C**) and K48 (**Figure 6D**) polyubiquitination in the lateral amygdala nuclear fraction following learning in comparison to control animals. At the same time, in the cytoplasmic region, overall polyubiquitination decreased and K48 ubiquitination increased following learning, while synaptic K63 ubiquitination was reduced. Collectively, these results indicate that within the same animal subcellular differences in linkage-specific protein ubiquitination can be accurately detected.

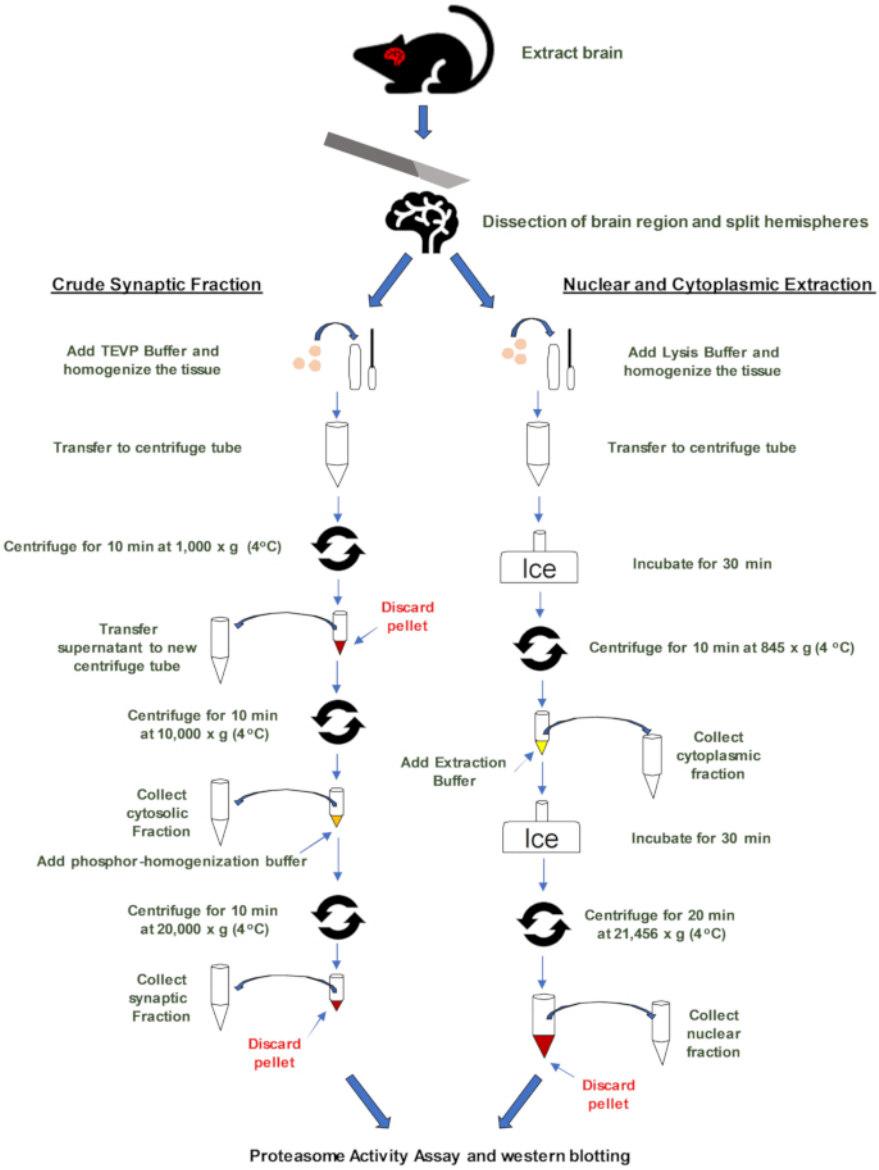

Figure 1. Schematic for subcellular fractionation of rat brain tissue. Rodent brain is extracted, brain region dissected and hemispheres split. Using a series of buffers and centrifuging steps, nuclear and cytoplasmic fractions are collected from one hemisphere while a crude synaptic fraction is collected from the other. Both fractions are then used for proteasome activity assays and Western blotting, to assay protein polyubiquitination levels. Please click here to view a larger version of this figure.

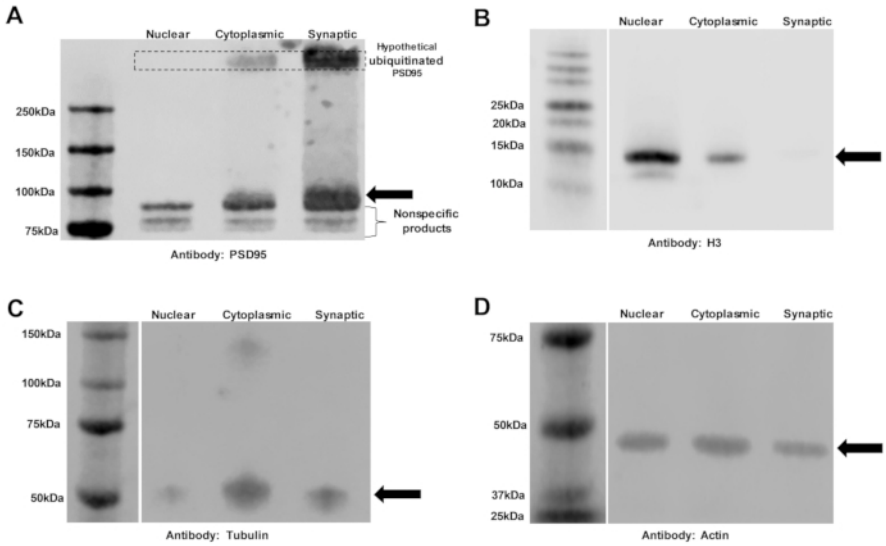

Figure 2. Confirmation of crude synaptic, nuclear and cytoplasmic fraction purity. (**A**) The synaptic protein postsynaptic density protein 95 (PSD95; 1:1000) was present in the synaptic, but not nuclear fraction with lower levels in the cytoplasm. (**B**) Histone H3 (1:500) was present in the nuclear, but not synaptic, fraction with lower expression in the cytoplasm. (**C**) β-Tubulin (1:1000) was present in the cytoplasmic, but largely absent from the nuclear, fraction with lower expression in the synaptic lysate. (**D**) Housekeeping protein β-actin (1:1000) was present in all subcellular compartments. Areas indicate the expected size of the target protein. This figure has been modified from Orsi, S. A.*et al.*[21]. Please click here to view a larger version of this figure.

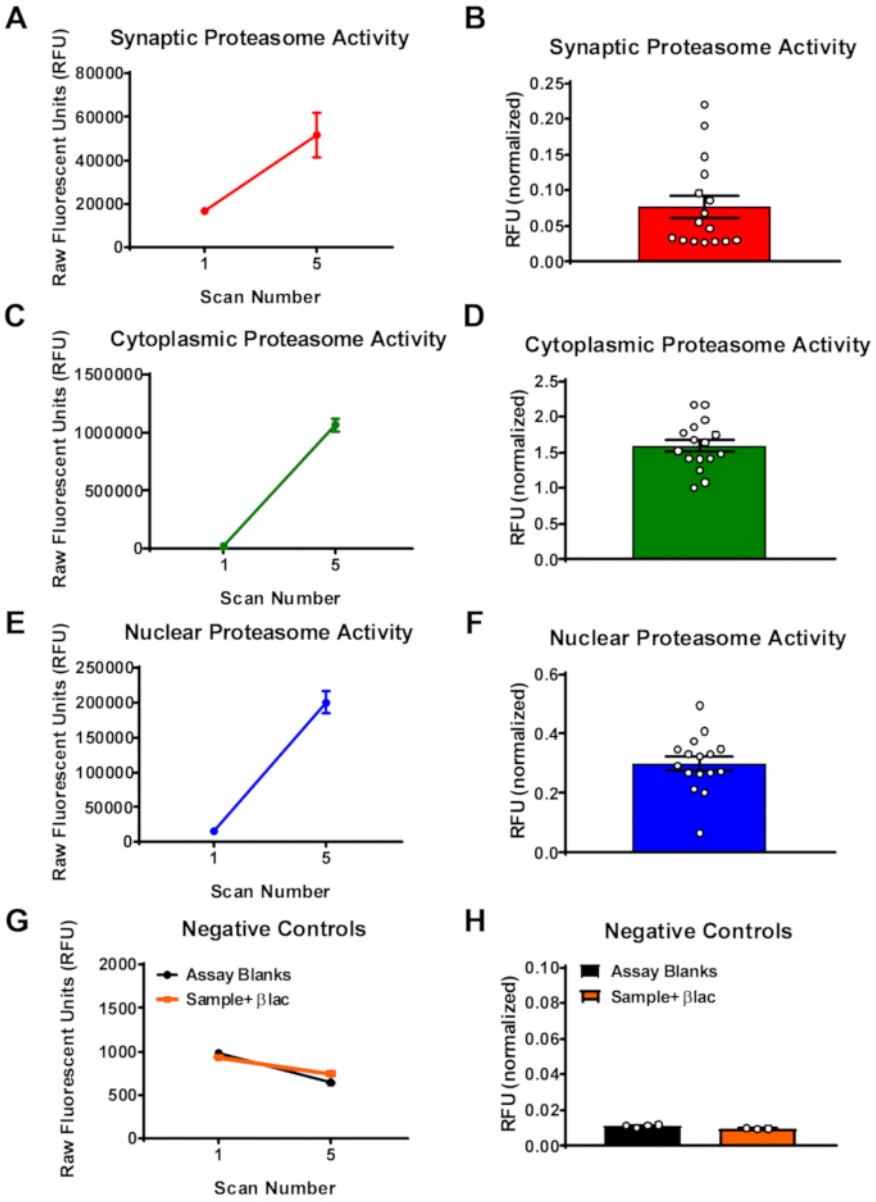

Figure 3. Quantification of proteasome activity in nuclear, cytoplasmic and synaptic fractions collected from the lateral amygdala of the same animal. During the in vitro proteasome activity assay, relative fluorescent units (RFU) detected increased from the beginning (Scan 1) to the end (Scan 5) of the assay in the synaptic (**A**), Cytoplasmic (**C**) and nuclear (**E**) fractions. Quantification of this change from baseline indicated a normalized RFU value (relative to 10 μM AMC) of 0.1 in the synaptic (**B**), 1.6 in the cytoplasmic (**D**) and 0.3 in the nuclear (**F**) fractions. The proteasome inhibitor βlac prevented RFUs from changing across the session (**G-H**). This figure has been modified from Orsi, S. A.*et al.*[21]. Please click here to view a larger version of this figure.

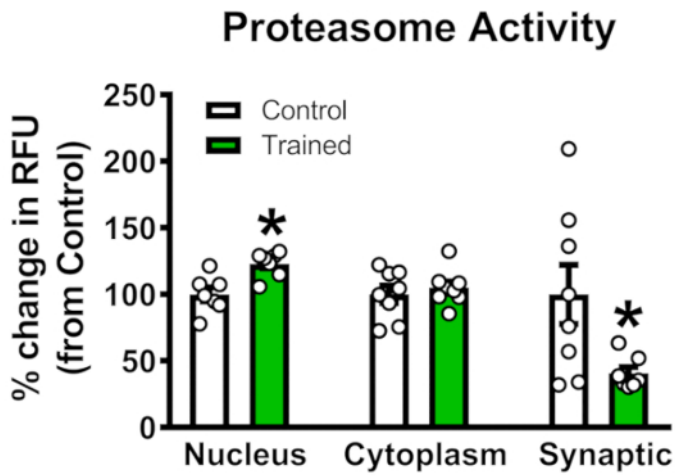

Figure 4. Subcellular differences in proteasome activity in the lateral amygdala of the same animal. An increase in nuclear proteasome activity was detected in trained (fear conditioned) animals relative to controls, which corresponded to a decrease in activity within the synaptic fraction. Cytoplasmic proteasome activity remained at baseline. *$P < 0.05$ from Control. This figure has been modified from Orsi, S. A.*et al.*[21]. Please click here to view a larger version of this figure.

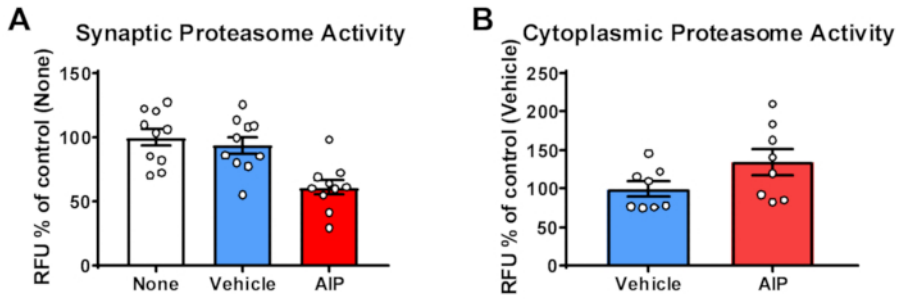

Figure 5. In vitro manipulation of proteasome activity in collected synaptic and cytoplasmic fractions. In vitro manipulation of CaMKII signaling via the inhibitor AIP reduced proteasome activity in the synaptic *(***A**), but not the cytoplasmic (**B**), fraction from the rat lateral amygdala. This figure has been modified from Jarome, T. J. et al.[23]. Please click here to view a larger version of this figure.

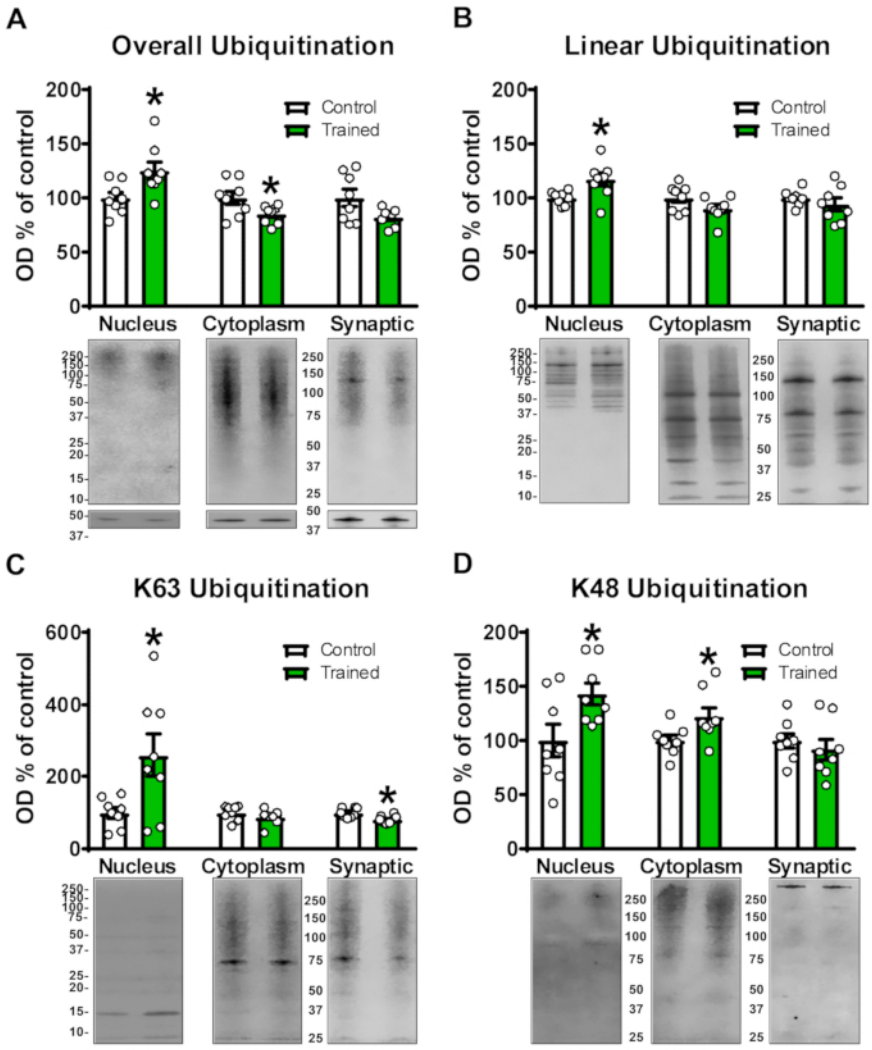

Figure 6. Subcellular differences in linkage-specific protein ubiquitination in the lateral amygdala of the same animal following learning. (**A**)There was an increase in overall ubiquitination in the nuclear fraction following learning, which correlated with a decrease in the cytoplasmic fraction. (**B**) There was an increase in linear ubiquitination in the nuclear, but not cytoplasmic or synaptic, fraction following learning. (**C**) There was an increase in K63 ubiquitination in the nuclear fraction following learning, which correlated with a decrease in the synaptic fraction. (**D**) K48 ubiquitination increased in the nuclear and cytoplasmic, but not synaptic, fraction following learning. *$P < 0.05$ from Control. All obtained ubiquitin optical densities were normalized to β-actin levels (lower representative Western blot images in A), which was used as a loading control. This figure has been modified from Orsi, S. A.*et al.*[21]. Please click here to view a larger version of this figure.

Discussion

Here, we demonstrate an efficient method for quantifying changes in ubiquitin-proteasome activity across different subcellular compartments in the same animal. Currently, most attempts at measuring subcellular changes in activity of the ubiquitin-proteasome system have been limited to a single compartment per sample, resulting in the need to repeat experiments. This leads to significant costs and loss of animal life. Our protocol alleviates this problem by splitting hemispheres, allowing different cellular fractions to be collected from each hemisphere of the same animal. Using this protocol, we were able to show for the first time that in the same animal ubiquitin-proteasome activity differentially changes in nuclear, cytoplasmic and synaptic fractions in response to learning[21].

The main limitation of the protocol described here is it is reliant upon the amount of brain tissue (sample) obtained. For example, as outlined above, this protocol requires splitting hemispheres of a given brain region. However, this may not always be possible, such as in the case of certain areas that are only present in one hemisphere. In these cases, the protocol could be modified by first homogenizing the entire brain region in the TEVP buffer used in the synaptic fraction step (Section 3.1), since this buffer is free of all denaturing agents. The sample can then be split into two equal parts by volume. The first part can be used for the synaptic fraction, following the protocol as described. For the second half of the sample, the non-ionic detergent NP-40 can be added to a final concentration of 0.05%, followed by centrifuging as described in Section 2.6. This will allow the separation of cytoplasmic proteins into the supernatant and nuclear proteins into the pellet, which can be further isolated following the remaining steps in Section 2. Another concern in tissue quantity is that some brain regions are very small in size, such as the prelimbic cortex. However, in these cases, the above protocol can still be used by reducing the volumes of the buffers used, which would

have to be determined empirically based on the size of the brain region collected. Thus, this protocol can be amended to even the more difficult brain regions in which less tissue is available.

One of the major advantages of the protocol we outline here is that it uses common laboratory equipment and reagents that can be found in most facilities, allowing this methodology to be amendable to those even on a restricted budget or with limited resources. Furthermore, while we outline this protocol as a way of measuring subcellular changes in ubiquitin-proteasome signaling, this methodology can also be applied to any other protein or cellular process in which understanding the cellular localization and function is important. Thus, this protocol could have broad applications to understanding the subcellular functions of specific proteins or complexes during learning and memory or different disease states.

Disclosures

The authors have nothing to disclose.

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