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Studying protein degradation pathways *in vivo* using a cranial window-based approach

Vivek K. Unni^a, Darius Ebrahimi-Fakhari^{a,b}, Charles R. Vanderburg^c, Pamela J. McLean^a, and Bradley T. Hyman^{a,*}

^aAlzheimer's Research Unit, MassGeneral Institute for Neurodegenerative Disease, MGH Harvard Medical School, CNY114, 16th St., Charlestown, MA, 02129, USA

^bInstitute of Anatomy & Cell Biology, University of Heidelberg, INF 307, 69120, Heidelberg, Germany

^cHarvard Neurodiscovery Center, MGH Harvard Medical School, CNY114, 16th St., Charlestown, MA, 02129, USA

Abstract

Understanding how specific proteins are degraded by neurons in living animals is a fundamental question with relevance to many neurodegenerative diseases. Dysfunction in the ubiquitin-proteasome system (UPS) specifically has been implicated in several important neurodegenerative diseases including Alzheimer's Disease, Parkinson's Disease, and amyotrophic lateral sclerosis. Research in this area has been limited by the fact that many inhibitors of the UPS given systemically do not cross the blood-brain barrier (BBB) in appreciable levels. This limits the ability to easily test *in vivo* specific hypotheses generated in reduced systems, like brain slice or dissociated cell culture, about whether the UPS may degrade a particular protein of interest. Although several techniques including intracerebral application via direct syringe injection, catheter-pump systems and drug-eluting beads are available to introduce BBB-impermeant drugs into brain they each have certain limitations and new approaches could provide further insights into this problem. In order to test the role of the UPS in protein degradation *in vivo* we have developed a strategy to treat mouse cortex with the UPS inhibitor clasto-lactacystin betalactone (CLBL) via a "cranial window" and recover the treated tissue for immunoblot analysis. This approach can be used in several different cranial window configurations including single window and double hemi-window arrangements that are tailored for different applications. We have also developed two different strategies for recovering treated cortical tissue including a vibratome/Laser Capture Microscopy (LCM)-based and a vibratome only-based approach, each with its own specific advantages. We have documented UPS inhibition >600 μ m deep into the cortex with this strategy. This set of techniques in the living mammalian brain is complementary to previously developed approaches and extends the repertoire of tools that can be used to the study protein degradation pathways relevant to neurodegenerative disease.

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*Corresponding Author: Bradley T. Hyman, Rm 2-2009, CNY114, 16th St., Charlestown, MA 02129, bhyman@partners.org, telephone: 011 617 726 2299, fax: 011 617 724 1480.

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Keywords

cranial window; *in vivo*; protein degradation; ubiquitin-proteasome system; mouse; cortex

1. Introduction

Protein degradation is a crucial mechanism used by post-mitotic cells like neurons in the central nervous system for the removal of abnormally aggregated or modified toxic proteins and understanding how this process works in the living brain is a fundamental problem in neurodegeneration research today. Because of the constant threat of protein misfolding and the consequent risk of toxic protein accumulation neurons use complex degradation machineries like the ubiquitin-proteasome system (UPS, [1]) and the autophagy-lysosome pathway [2] to maintain protein homeostasis. Abnormalities in the UPS pathway, specifically, have been implicated in many important neurodegenerative diseases including Alzheimer's Disease [3], Parkinson's Disease (PD, [4,5]), and amyotrophic lateral sclerosis [6]. However, work primarily from reduced preparations like cell culture is often conflicting with regards to the importance of the UPS in the degradation of pathogenic proteins. For instance, there is evidence both for [7–9] and against [10] the UPS degrading the PD-related protein alpha-synuclein in cell culture. The cause for this discrepancy between studies is not clear and may be due to specific differences in cell types or culture conditions used. Because of this, new experimental paradigms that allow blood-brain barrier (BBB)-impermeant UPS inhibitors to be applied to mammalian brain *in vivo* and reach their targets hold the promise of advancing our understanding of how specific proteins are degraded in a relevant system.

With this focus in mind we have developed a set of “cranial window”-based *in vivo* pharmacologic treatment strategies in mice and paired them with different tissue recovery methods to apply either the BBB-impermeant UPS inhibitor clasto-lactacystin betalactone (CLBL) or vehicle to the neocortex, recover treated cells, isolate proteins and validate target enzyme inhibition. Cranial window techniques have been used in many systems including mouse [11], rat [12], rabbits [13], cats [14], dogs [14] and primates [15] to image a variety of processes including aspects of the cerebral microcirculation, intrinsic optical signals and fluorescent probes. We have adapted an approach previously published by our group [16] in several ways to optimize pharmacologic treatment of neocortical cells in mice and to create multiple, isolated chambers to treat different hemispheres with different agents. In addition, we have also developed two different methods to recover treated tissue including both a vibratome/laser capture microscopy (LCM)-based and a vibratome only-based strategy, each with different and complementing advantages. Finally, we have validated this approach by applying CLBL to inhibit proteasomal degradation in mouse brain, recovering treated tissue and demonstrating UPS inhibition up to several hundred microns deep into the cortex. The set of strategies we have developed should be useful to study UPS-mediated protein degradation in the living brain and can potentially be extended to other important biochemical pathways where specific pharmacologic agents are known and assays are available to verify the appropriate controls.

2. Materials & Methods

2.1 Cranial window surgery and drug application

The general technique we use for making cranial windows for *in vivo* multiphoton imaging have been published previously [16]. In our study we have taken advantage of this approach and modified the procedure to optimize the application of BBB-impermeant agents to cortical areas. This protocol allows for reliable drug application chronically over time. BDF-1 strain mice (Charles River Laboratories) were anesthetized with 1–2% inspired

isoflurane (Aerrane, Baxter) and buprenorphine given for analgesia (0.05 mg/kg, Bedford Laboratories) via intraperitoneal injection. After firmly securing the head in a custom-built stereotaxic frame, an electric razor was used to prepare the surgical field and a protective ointment (Puralube) was applied to the eyes. To maintain body temperature the stereotaxic frame was placed on a homeothermic blanket and a rectal probe (Harvard Apparatus) used for internal temperature monitoring (target 33–37°C). The surgical field was sterilized using alternating swipes of 70% isopropanol and povidone-iodine and a subcutaneous local anesthetic (Lidocaine 2%, 0.1 ml, Hospira Inc.) was administered before removing scalp and exposing the skull (Fig. 1A). After removing the periosteum with a cotton applicator swab, a wooden toothpick was used to apply a mixture of dental acrylic cement (Stoelting Co.) and cyanoacrylate glue (KrazyGlue) to the skull and create a chamber ~2–3 mm high, ~10 mm in diameter covering the region ~3 mm anterior of bregma to ~1 mm posterior to lambda (Fig. 1B). Then the cement-glue mixture was allowed to harden for ~5–10 min. Next a dental drill was used to carefully remove a thin layer of bone in a circular pattern inside the well. This was repeated to remove successive layers of bone until the skull was penetrated. Care was taken not to damage underlying cortex during this step. This resulted in a circular bone flap that was gently removed by forceps. After gently detaching the flap, sterile phosphate buffered-saline (PBS)-saturated gel foam was employed to halt any bleeding, maintain a moist environment and to prevent the cortex from drying out. Using fine forceps the dura mater was carefully reflected by peeling it slowly towards the midline. Removal of the dura is crucial for pharmacologic treatment and is done carefully to prevent damage to exposed cortex (Fig. 1C). Next ~100–200 μ L of PBS (containing the drug of choice) was applied to the well to cover the exposed cortex and the chamber sealed with a 12 mm glass cover slip using the cement-glue mixture (Fig. 1D). After hardening of the dental acrylic cement (~5–10 min) the animal was allowed to recover from anesthesia.

To create a two chamber system for acute drug applications that do not require the animal to recover from anesthesia a similar technique to that described above was used except that a bone bridge was preserved above the midline (Fig. 2A). Next, after removing the dura over each hemi-window, molten paraffin wax was applied with a wooden applicator to create two isolated chambers (Fig. 2B). A PBS solution containing dye (Fluoro-Ruby 10k MW dextran, 1%, Invitrogen or India Ink 1%) was used in one chamber and solution without dye in the other to test the integrity of the two wells (Fig. 2C). No leakage from one well to the other should be observed. Next different PBS solutions containing different pharmacologic agents (e.g. active agent and vehicle) can be applied to each well as required (Fig. 2D). Creating a two chamber system for longer drug applications that require the animal to recover from anesthesia can be done using a modification of the technique outlined in Figure 1. In this case the skull is prepared and a ring-shaped well is created in a similar manner (Fig. 3A–B). Next a mid-sagittal wall is created between the two hemispheres using the cement-glue mixture (Fig. 3C). Then two symmetric hemi-bone flaps are removed along with underlying dura (Fig. 3D). We have tested the integrity of the two wells in the same manner as is used with wax wells (with colored solution), but in our experience the chance of leakage from one well to the other using this approach is very low (Fig. 3E). Next a half-circular glass cover slip can be used to seal each compartment with different drug containing solutions as required (Fig. 3F).

2.2 Treated tissue recovery via vibratome and then laser capture microscopy

After appropriate topical exposure of cortex to applied solution animals were deeply anesthetized by intraperitoneal injection with a ketamine (10 mg/mL, Phoenix Pharmaceuticals Inc.) + xylazine (1 mg/mL Lloyd Laboratories) solution, 0.1–0.2 mL/10 g body weight. Next animals were quickly decapitated and the brain removed. Olfactory bulb and cerebellum were removed and a mid-sagittal cut was made to separate the two

hemispheres. Each hemisphere was mounted onto a vibratome stage (VT1000S, Leica) cut (medial) surface down with cyanoacrylate glue (Krazy Glue). Next ice-cold PBS was used to cover the brain and 100 μ m thick parasagittal sections were made. Each section was mounted onto PEN membrane frame slides (MDS Analytical Technologies) and dehydrated in 75% ethanol for 2 min. After that sections were allowed to dry in a negative pressure fumehood for ~5 min. Freshly prepared sections were mounted in a Veritas Laser Capture Microscope (Arcturus) and CapSure LCM Macro caps (Molecular Devices) loaded. Following calibration of capture and cutting lasers, ribbons of treated cortex as determined by Fluoro-Ruby staining (Fig. 4A) were welded to Macro caps and cut from PEN membrane frame slides. Approximately 5–6 ribbons were removed from each brain section and approximately 5–7 sections worth of tissue could be welded to one Macro cap (Fig. 4B).

2.3 Treated tissue recovery via vibratome only

After appropriate topical exposure of the prepared cortical area animals were deeply anesthetized as described in Section 2.2. Following decapitation the brain was quickly removed and mounted onto a vibratome stage (VT1000S, Leica) ventral surface down in ice-cold PBS with additional oxygen supply (Fig. 5A). Serial horizontal sections of the treated cortex, each 100 μ m thick, were made (Fig. 5B) and the tissue was transferred to the appropriate buffer-solution for homogenization and immunoblotting (see Section 2.4). Using this approach only treated cortical tissue can be selectively removed (Fig. 5C).

2.4 Immunoblot analysis

Clasto-lactacystin betalactone (CLBL)-induced proteasome inhibition was assessed by immunoblot analysis. Mice were treated with either CLBL (20 μ M, Cayman Chemical) or DMSO vehicle (1%, Sigma) solution for 24hr (elapsed duration between starting topical application and sacrificing animal) using the single widow approach (Fig. 1). Tissue was recovered using either the LCM-based (Fig. 4) or vibratome-based (Fig. 5) approach. Recovered tissue was homogenized in Tris-HCl (50 mM, pH7.5) containing 5 M guanidinium (Fisher Scientific) and protease-inhibitor cocktail (Roche) and agitated overnight, followed by centrifugation (13000 $\times$ g) for 5 min. The supernatant was collected and dialyzed using membrane filters (MF-Millipore, 0.025 μ m) against urea (6 M, Fisher Scientific) for 1 hour. Total protein concentration was determined in the dialysate using a BCA protein assay. The NuPAGE gel system (Invitrogen) was utilized for protein separation using a 10% Bis-Tris gel and 3-(N-morpholino)propanesulfonic acid (MOPS) buffer. Samples (10 μ g) were solubilized in lithium dodecyl sulphate (LDS) buffer under reducing conditions. Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Perkin Elmer) followed by blocking in blocking buffer (Li-Cor Biosciences) for 1 hour at room temperature. Ubiquitin immunoreactivity was determined using a rabbit polyclonal antibody (Dako, Z0458, 1:1000, overnight at 4C), GAPDH with a mouse monoclonal antibody (Millipore, MAB374, 1:10000, overnight at 4C) and α -tubulin with a mouse monoclonal antibody (Sigma, DM1A, 1:4000, overnight at 4C) with infrared fluorescent-labeled secondary antibodies (Li-Cor Biosciences, IRDye 800 anti-rabbit, and IRDye 700 anti-mouse, both 1:2000, 1 hour at room temperature). Quantification was done using the Odyssey infrared-imaging system (Li-Cor Biosciences).

3. Results

We have adapted the general cranial window surgery procedure previously published by our group [16] to study protein degradation pathways *in vivo* and to maximize topical CLBL exposure to the treated region of cortex (see Material & Methods Section 2.1 for specific details). Briefly, after removing overlying scalp and cleaning of the skull (Fig. 1A), we use a mixture of dental cement and cyanoacrylate glue to create a ring-shaped well (Fig. 1B).

Next, a circular flap of bone overlying sensory-motor cortex and the underlying dura mater are removed. Removing the dura is critical since this membrane is relatively impermeable to many pharmacologic agents including proteasome inhibitors (Fig. 1C). Next a CLBL-containing solution is placed into the well covering the exposed cortex and a cover slip is used to seal the well (Fig. 1D). We find that this general approach exposes the CLBL-containing solution to a sufficient area of cortex for subsequent recovery and immunoblot analysis (see below). The use of the well also improves CLBL solution-cortex contact since some amount of brain edema can occur causing the cortex to contact the cover slip when no well is made. Use of the well lifts the cover slip several mm above the craniotomy and ensures that the CLBL solution adequately covers the exposed cortex.

We have also developed modified versions of this surgical strategy that can be used in different situations (see Material & Methods Section 2.1 for specific details). One variant involves making two symmetric hemi-cranial windows, one each over the right and left hemispheres, with a mid-sagittal bone bridge left between them (Fig. 2A). If short CLBL application times (minutes to hours) are required this can be done by removing the two bone flaps and underlying dura, then using paraffin wax to create two symmetric wells (Fig. 2B). We find it helpful at this stage to place a colored dye-containing solution in one chamber and a clear solution in the other to test the integrity of the two wells and to confirm that there is no leakage of solution from one chamber to the other (Fig. 2C). Next different solutions can be placed in each of the two different wells (e.g. CLBL and vehicle) for the required period of time. If the treated tissue will be recovered using a LCM-based approach we add a fluorescent dye to each solution to aid in tissue recovery during processing (Fig. 2D, see below).

Another set of modifications of this general strategy can be used if longer drug applications are required; long enough that the animal needs to recover from anesthesia (see Material & Methods Section 2.1 for specific details). This procedure is similar to the one above except that a cement-glue mixture is used instead of wax to create a ring shaped well (Fig. 3A–B) and the mid-sagittal wall dividing the two compartments (Fig. 3C). Then the two bone flaps and dura are removed (Fig. 3D) and CLBL solution is placed over one hemisphere followed by a half-circle shaped glass cover slip to seal this well (Fig. 3D) from the outside environment. We have used a colored dye to test for communication between the two wells, however in our experience when the cement-glue mixture is used communication is rarely if ever observed. Finally, a second vehicle containing solution is applied to the second well and this chamber sealed (Fig. 3E).

We have developed two different techniques for recovering treated cortex after topical treatment to investigate the effect of proteasome inhibition as described above. The first uses a combined vibratome/LCM-based approach to harvest tissue (see Material & Methods Section 2.2 for specific details). Briefly, after the animal has been treated for the required amount of time and the brain removed, para-sagittal sections are made using a vibratome. Sections are then mounted on LCM-compatible slides, dehydrated, dried and processed using LCM. When processing tissue in this way, we find that using a fluorescent dye in the drug-containing solution like Fluoro-Ruby dextran conjugates is critical for defining the region exposed to drug (Fig. 4A1–2). This also allows for matching of dextran conjugate molecular weight (MW) with drug MW when large MW drugs are used. With the vibratome/LCM-based approach the treated region can be selectively removed (Fig. 4A3). After LCM, a cap is produced that has treated sections of cortex adherent to it that can be used for a variety of analyses (Fig. 4B), including quantitative PCR, microarray and protein biochemical assays. Recovery of treated tissue in this manner routinely yields ~50–100 μ g of total protein per brain that we used for further analysis with immunoblotting (Fig. 6A).

We have also developed a second method for tissue recovery using only a vibratome to selectively recover treated cortex (see Material & Methods Section 2.3 for specific details). In this case brain removal is performed in a similar manner to that described for LCM-based recovery. Then the brain is mounted on a vibratome stage ventral surface down (Fig. 5A). In this case a dye-containing treatment solution is usually not necessary since the region of cortex that was exposed can be clearly visualized due to the mild circular indentation of the cortex created by the round craniotomy. However, it is also possible add any vital dye of choice to the drug-containing solution to more clearly define the treated region if necessary. Next serial horizontal sections of cortex are removed (Fig. 5B) and can be used for further biochemical analysis. When mounted in this way it is possible to selectively remove the treated cortex (Fig. 5C). Removal of treated tissue in this manner to recover the top 300 μm of cortex routinely yields ~100–300 μg of total protein per brain that we used for further analysis with immunoblotting (Fig. 6B).

Mice were treated with CLBL or vehicle for 24hr using the single window protocol (Fig. 1). Subsequently, treated cortical tissue was recovered using either the vibratome/LCM-or vibratome only-based method. Next, tissue sections were solubilized in a guanidinium solution to maximize total protein recovery, dialyzed against urea, run on a SDS-polyacrylimide gel, transferred to a PVDF membrane and probed using specific antibodies (Fig. 6A–B, see Material & Methods Section 2.4 for details). Staining for high molecular weight ubiquitinated species provides evidence of UPS inhibition (Fig. 6A), extending >600 μm deep into the cortex (Fig. 6B). When combined with the evidence that large dextran-conjugated fluorescent dyes can penetrate >400 μm deep (Fig. 4A2) into cortex, this demonstrates that the overall set of techniques described above can be an effective way to inhibit UPS function in mammalian brain *in vivo* and recover treated cortex for a potentially wide range of subsequent biochemical analyses.

4. Discussion

We have developed a cranial window-based surgical approach and paired it with vibratome/LCM- or vibratome only-based tissue recovery methods in order to reliably perform *in vivo* inhibition of UPS function in mammalian cortex. This platform can be used in a manner conceptually similar to UPS inhibition experiments in cell culture to test the role of this pathway in protein degradation in the living brain. The main requirements for successfully extending this strategy to other pathways of interest would be specific pharmacologic modulators and reagents for confirming a modulator's action on positive controls, requirements needed for almost any biochemical experiment regardless of the approach being used. The advantage of the strategy we have outlined, however, is that these pathways can be probed in their native context *in vivo*.

Many strategies have been described in the literature to perform experiments in living brain using pharmacologic agents that do not cross the BBB. Some of these include direct injection of agents into brain parenchyma or ventricles with syringe systems [17], implantation of catheters connected to internal or external pump systems [18], placement of drug-eluting beads [19] and superfusion of the cortical surface [12]. We believe the strategy we have outlined extends this repertoire of approaches to the study of protein degradation and provides another tool that may be advantageous in certain circumstances. For instance, the approach outlined above allows the role of the UPS in protein degradation to be probed specifically in cortical neurons *in vivo* without the expense and complexity of drug-eluting beads, syringe or catheter-pump delivery systems. Also the concentration of applied solution can be varied and dose-response experiments can be carried out with relative ease.

Another advantage is that this system can be easily paired with *in vivo* multiphoton imaging of the same population of cells that are being pharmacologically treated. Cranial window surgical techniques readily allow imaging of fluorophores several hundred μm deep into cortex [11,20]. Our placement of a cement-glue well to maximize drug solution-cortex contact does lift the glass cover slip $\sim 2\text{--}3\text{ mm}$ above the plane of the skull but this is not a significant hindrance to imaging given the long working distance of objectives currently being used. Any of the well-developed strategies for labeling cortical structures for *in vivo* imaging (e.g. viral or transgenic expression of fluorescent proteins, systemic or topical application of small molecule fluorophores) will be straightforward to adapt to the surgical techniques we describe. One of the appeals of our approach is that the same cells that are used to collect *in vivo* imaging data can then be used for subsequent biochemical analysis, significantly increasing the power of the experimental paradigm.

Two different methods for imaging cortical structures using cranial windows have been developed; one involves thinning of the overlying skull to the point of optical translucency [21] and the other removal of the overlying skull flap and placement of a glass cover slip [11]. Each has its own specific advantages, including less brain parenchyma inflammation and reactive gliosis with the former and better optical clarity, the ability to do more frequent repeated imaging of the same location and the ability to apply pharmacologic agents with the latter. For the purposes of our approach, to specifically inhibit the UPS in cortical neurons *in vivo*, removal of the skull flap and underlying dura are needed. As has been previously shown this can lead to reactive glial activation that may produce observed results different from those seen with the thin-skull technique [21,22]. It is important to be aware of this possibility and design appropriate controls since skull and dura removal are required to maximize effective drug penetration with this approach.

We have outlined two different strategies for recovery of treated cortical tissue, one involving a combined vibratome/LCM-based capture and the other vibratome only-based. Each of these approaches has certain strengths depending on the question being answered. The vibratome/LCM-based approach allows recovery of more specific subregions of cortex. For example, it is straightforward with this approach to recover tissue only from specific layers of cortex that might be of interest with high precision. LCM has also been used extensively to recover individual cells from brain tissue and if appropriate markers are available our strategy could be easily modified to recover specific cell types (e.g. layer 5 pyramidal neurons or subclasses of interneurons) that have undergone pharmacologic treatment *in vivo*. The vibratome/LCM-based approach does have the disadvantage that extra time is required to prepare and recover tissue before it is placed in a protease-inhibitor containing solution, potentially decreasing recovery of labile proteins. Vibratome only-based recovery has the advantage that larger sections of treated tissue can be recovered more quickly and without the extra steps required to prepare tissue for LCM, increasing recovery of labile proteins. The vibratome only-based method, however, does not have the selectivity to recover smaller sub-regions of cortex or individual cells as is routinely possible with LCM.

In summary, we describe here a novel approach to study proteasomal degradation pathways living mice by treating cortex with the BBB-impermeant UPS inhibitor CLBL and recovering specific proteins from treated tissue for subsequent immunoblot analysis. We have validated this technique and shown UPS inhibition several hundred microns deep into cortex. These general methods should be widely applicable and useful for studying protein degradation pathways in mouse cortex relevant to neurodegenerative disease.

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Fig. 1. Surgical procedure for cranial window-based pharmacologic treatment of cortex. (A) View of mouse skull after removal of overlying scalp and periosteum. (B) Application of cement-glue mixture to create a circular well. (C) View of cortex after removal of circular bone flap and dura mater. (D) 12 mm circular glass cover slip cemented in place on top of well.

Fig. 2.

Surgical procedure for short-term dual chamber treatment of cortex. (A) View of mouse skull after removal of two symmetric hemi-cranial bone flaps and dura. (B) Application of paraffin wax to create two wells filled with PBS. (C) View of Fluoro-Ruby dextran-containing PBS solution in left well and PBS without dye in right well demonstrates no communication between wells (asterisks in right well shows small collection of blood present before dye-containing solution was applied). (D) Dye-containing solution placed in both wells.

Fig. 3.

Surgical procedure for long-term dual chamber treatment of cortex. (A) View of mouse skull after removal of overlying scalp and periosteum. (B) Application of cement-glue mixture to create a circular well. (C) Application of cement-glue mixture to separate well into two isolated chambers. (D) PBS covering brain after two hemi-cranial bone flaps and dura are removed. (E) India Ink-containing solution in left well with cover slip cemented in place and right well containing PBS solution demonstrates no communication. (F) Dye-containing solution in both wells and both cover slips in place.

Fig. 4. Treated cortex recovery using LCM-based method. (A) Transmitted light (1) and Fluoro-Ruby fluorescence (2) image of sagittal brain section mounted on PEN membrane frame slide before laser capture. (3) Transmitted light image after laser capture shows selective removal of treated cortex. (B) CapSure LCM Macro cap after recovery of treated cortex.

Fig. 5.

Treated cortex recovery using vibratome-based method. (A) View of mounted brain in place on the vibratome stage before sectioning (red circle outlines drug treated area). (B) First 3 pairs of sections (each 100 μm thick) removed from treated cortex. (C) View of mounted brain after sectioning (red circle outlines area where treated cortex was removed).

Fig. 6.

Immunoblot demonstrating proteasome inhibition and depth of drug penetration after cranial window-based CLBL or vehicle treatment for 24 hours. (A) Tissue recovered using the LCM-based approach demonstrates an increase in high molecular weight ubiquitinated species in CLBL-treated cortex versus vehicle-treated. Alpha-tubulin is shown as a loading control. (B) High molecular weight ubiquitinated species, ubiquitin monomer and GAPDH (loading control) staining are shown from CLBL-treated tissue collected using the vibratome-based approach from progressively deeper cortical sections.