# Molecular mechanisms of delayed axonal damage in traumatic brain injury (Banner index #507157)

#### Year1 1, 2 and 3 (no cost extension): 2008 - 2010

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- 2. Name of Organization: UMDNJ-Robert Wood Johnson Medical School
- 3. Grant Title: Molecular mechanisms of delayed axonal damage in traumatic brain injury (Banner index #507157)
- 4. Grant Number: 07-3203-BIR-0
- 5. Grant Period Covered: 4/1/08-3/30/10
- 6. Date submission of report: 6/30/10

#### Aims of the Project:

Following traumatic brain injury (TBI) there is a slow progression of cell death and breakage of nerve cell processes that continues to silently plague the victim for months and years after the original injury. Every year there are least 225,000 patients nationwide and 12,000 patients in the State of New Jersey who suffer brain injury and its deleterious aftereffects. Although this progressive damage cannot be easily detected by conventional imaging methods, its clinical manifestations are commonly observed and may range, depending on the severity of the injury, from altered personality traits and memory loss to severe neurological signs of functional loss that include both cognitive and sensorimotor skills. Despite the potentially devastating nature of brain injury on the individual and the enormous emotional and financial burden that TBI may have on the patient's family, very little is known about the signaling events responsible for this ongoing "silent" damage that continues to accumulate in the neural cells of brain-injured patients. As a result of this lack of knowledge there no therapy is available for the prevention of these delayed clinical consequences. This contributes to the increasing financial costs for society for long-term care with little prospect of ameliorating the progression of TBI effects.

# **Project Successes: Year 1 -2:**

During The first year and a half of this grant the project was led Dr. Patrizia Casaccia-Bonnefil, M.D. Ph.D. She left the institution in June 2009. In this section, I will attempt to summarize her findings as previously reported to the commission.

During this first year Dr. Cassaccia-Bonnefil's group worked to define the mechanisms responsible for the silent and persistent killing of nerve cells that occur for a long time after the original accident. Preliminary data obtained from their laboratory and from her collaborators at the Center for Brain Injury and Repair at U Penn, had suggested a possible cause for the damage to neural cells, by analogy with the neuropathological findings detected in demyelinating disorders. They confirmed that some of the changes detected in degenerating axons during the days following TBI are similar to those observed in demyelinating disorders such as multiple sclerosis (MS). They analyzed the functional relevance of these changes in neurons cultured in a dish and exposed to the same toxins that are present in the brain after TBI.

The short-term goal is to thoroughly understand the mechanisms leading to axonal loss and the long-term goal is to find ways to prevent these degenerating changes and translate the basic research findings into therapeutic strategies.

One of the most important observations that Dr. Casaccia-Bonnefil's group have made in animal models of demyelination is the nuclear-cytoplasmic shuttle of an enzyme called HDAC1, prior to the onset of axonal damage. They hypothesized that this event could be upstream of a signaling cascade linking myelin cells to axons. In broad terms, they hypothesized that the progressive neuropathology observed in white matter tracts of TBI patients results from a cascade of events that is initiated by the initial damage to myelin and/or oligodendrocytes. The proposed model implied that the neuronal changes leading to axonal degeneration are initiated by this loss of myelin components and, in turn, promote a wave of delayed oligodendrocytic death, leading to white matter atrophy. The model predicted that the initial damage to myelin and oligodendrocytes caused by the inflammatory response to the physical trauma, induced changes in the subcellular localization of specific isoforms of histone deacetylases (HDACs) that initiate a cascade of events terminating with axonal degeneration and delayed oligodendrocytic death. In physiological condition, HDAC class I are nuclear, and contribute to the transcriptional repression of specific sodium channel subunits (Nav1.2).

The first experiments of this grant proposal were aimed at addressing the validity of this hypothesis. The Casaccia-Bonnefil laboratory first tested sections from control pig brain to verify the nuclear localization of HDAC1. In agreement with the predicted nuclear localization of the enzyme, they detected immunoreactivity in the nuclei of cells within the gray (Fig. 1) and white matter (Fig. 2) region of the brain.

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Fig 2. Nuclear localization of HDAC1 in the gray matter of the uninjured cortex in the brain of a miniatureswine.Immunohistochemistry with antineurofilament antibodies (green), HDAC1 antibodies (red) and DAPI as nuclear counterstain. Note the colocalization of HDAC1 and DAPI.



Fig. 2. Nuclear localization of HDAC1 in the white matter of the uninjured cortex in the brain of a miniature swine.

Immunohistochemistry with anti-neurofilament antibodies (green), HDAC1 antibodies (red) and DAPI as nuclear counterstain. Note the colocalization of HDAC1 and DAPI



Fig. 3. Cytosoliclocalization of HDAC1 in the cortex of a miniature swine three days after traumatic brain injury.Immunohistochemistry with anti-neurofilament antibodies (green), HDAC1 antibodies (red) and DAPI (blue) as nuclear counterstain



Fig. 4. Localization of HDAC1 in the axonal swellings in the brain of miniature swines three days after TBI. Immunohistochemistry with anti-neurofilament antibodies (green), HDAC1 antibodies (red) and DAPI (blue) as nuclear counterstain. The white arrows point at regions of axonal enlargements where we detected co-localization of HDAC1 and neurofilaments/



Fig. 5. Cytosolic localization of HDAC1 after exposure of cultured cortical neurons to glutamate or oxidative stress precedes the onset of axonal swellings. Confocal image of neurons treated with oxidative stress or glutamate to induce axonal damage, as indicated by the presence of axonal swellings. Cultures were stained with antibodies against HDAC1 (green), and neurofilament (red). Note that the localization of HDAC1 in the axoplasm precedes the onset of axonal damage

Dr. Casaccia-Bonnefil and her group then repeated the experiment in sections from animals with TBI. The first assessed the presence of immunoreactivity in the nuclei of cells around the lesion



Fig. 6. Lack of transcriptional response regulating the distinct sodium channel subunits. Quantitative real time PCR of RNA samples isolated from the cortex of miniature swines undergoing TBI. The relative levels were normalized to GAPDH and the results are presented in terms of relative level over time after injury.

(Fig. 3) and we detected a clear cytoplasmic localization of the enzyme in neurons surrounding the regions of hemorrhage and edema. To further characterize the localization of HDAC in the region of axonal damage, they focused on the detection of axonal swellings, called ovoids or end-bulbs and also in this case. We detected co-localization of the exported enzymes and neurofilaments (Fig. 4).

In an attempt to understand whether HDAC localization to the axonal compartment was the cause or the consequence of axonal damage, Dr. Casaccia-Bonnefil and her colleagues adopted a reductionist approach and performed experiments on neurons that were cultured from neonatal rats and exposed to neurotoxins characteristically found in the site of lesion. It was reasoned that a time course of HDAC accumulation in the axonal compartment would provide some critical information regarding the potential role of HDAC as cause or effect. For this reason, cultured neurons were exposed to glutamate or oxidative stress and the cells were stained for HDAC1 and neurofilaments (Fig. 5).

Further experiments have shown that silencing of HDAC1 prevents the onset of axonal damage in neurons exposed to the same toxins, thereby uncovering a very exciting and novel potential mechanism of regulation of neurodegeneration. Future investigation will be needed to further characterize the role of HDAC1 in the axoplasm, given its potential importance for therapeutic applications.

#### **Problems encountered:**

Dr. Casaccia-Bonnefil's team were unable to detect changes in the mRNA levels of specific sodium channel subunits in the brain of miniature swine with TBI (Fig. 6).

Because the results shown in Fig. 5 do not support the hypothesized transcriptional increase of specific sodium channel subunits, Dr. Casaccia-Bonnefile felt that there should be some changes in focus for the project. One change was that that there should be an analysis of the

definition of the axoplasmic role of HDAC1. Preliminary experiments have shown that silencing of this molecule prevents axonal damage in cultured neurons. It is now imperative to understand the molecular and cellular basis for this event because it could lead to the development of important therapeutic strategies.

#### Additional Project Successes: Year 2 - No Cost Extension: Established a mouse model of traumatic brain injury at UMDNJ-Robert Wood Johnson Medical School. Since my taking over of the grant from Dr. Patrizia Casaccia-Bonnefil, MD, PhD, who left the institution in June 2009, I have established a mouse model at the medical school to study the epigenetic consequences traumatic brain injury (TBI).

This is a change in focus form the original grant's research plan (see below). Following discussions with Dr. Casaccia-Bonnefil, we decided a change of focus was necessary since she *was unable to confirm her original hypothesis* with the pig brain material.

We have set-up a laboratory within the RWJMS vivarium devoted to applying the Lateral Fluid Percussion Injury (LFPI) model to mice. The instrumentation for this investigation was funded by the commission in grant number 08.3205-RIR-E-1 (PI S. Thakker-Varia). My role in this latter grant is a collaborator, in which my task is to do the surgery and injuries. The fluid percussion injury device was manufactured by the Custom Design & Fabrication Department of Radiology at Virginia Commonwealth University, Richmond, VA. I had previously been trained to use this equipment by Dr. Jonathan Lifshitz in his laboratory at the University of Kentucky, Lexington, KY. The equipment, custom-built did not arrive on campus until Fall 2008 and it was not until January 2009 that most of the equipment was in place and testing was begun.

As a result of the time it took to establish the mouse model at RWJMS, I asked for a nocost extension of the grant. Since this time we have injured in excess of 50 - 60 mice (this does not include the 40 plus animals that I have injured under Dr. Thakker-Varia's grant) with post-injury survival times of 1 - 30 days. We have been able "tweak" the model to have a survival rate in excess of 85%. We are currently engaged in histological analysis looking for any alterations in expression of in a number of epigenetic markers (presently we are focusing on HDAC1, HDAC2 and Sirt2. In doing so we have had to establish immunohistochemical protocols appropriate for the mice.

As a result of the generous funding from the commission, I have been able to mentor 2 medical students and 5 undergraduate students in brain injury research. As a result of their experiences, these students have expressed interest in doing work on problems centered on traumatic injury to the central nervous system.

Some of our findings are to be reported to the Society for Neuroscience in Chicago (October 2009). Preliminary finding were also reported locally to a summer medical student research conference held on July 9, 2009 and a poster presented by the medical students to the local research community will be held on October 6, 2009. Further we have begun a series of promising collaborations with both Rutgers and UMDNJ faculty to further understand the molecular consequences to traumatic injury to the central nervous system (see below).

#### **Results of Mouse Studies:**

#### Established and Applied the Lateral Fluid Percussion Model of Traumatic Brain Injury to the Study of the Epigenetic Alterations Resulting from Injury.

We have successfully applied LFPI to the study of the consequences of TBI on alterations in the epigenetic factors resulting from injury. We have at present injured in excess of 50 - 60 mice. We have been able to fine-tune the injury so that we have survival rate of about 85%. When death occurs it, does so within minutes of the injury. As such we feel that established model does have considerable validity in our search to understand and treat human brain injury.

We are currently analyzing brains from mice that survived the injury form 1 - 30 days. The analysis is still ongoing and much of our material still has not been examined.

#### The Liquid Percussion Injury Methods

A summary of the methods is provided here since they were not described in the original proposal.

#### Anesthesia

For surgery, animals will be anesthetized with 4-5% isoflurane in 100% 02, delivered through a small induction chamber. Once anesthetized, the hair over the scalp will be shaved. At the designated surgery location, animals are placed in a stereotaxic frame, equipped with a specialized nose cone for continuous inhalation of isoflurane (2.5%). Prior to brain injury, animals will be anesthetized with 4-5% isoflurane in 100% 02, delivered through a small induction chamber. **Perisurgical Analgesia** To minimize the possibility of pain, Marcaine is applied topically to the wound area. We routinely administer Buprenorphine (Buprenex) 0.1 mg/Kg 30 min pre-operatively.

#### **Craniotomy and Implantation of Luer Hub**

The animal is placed in a mouse stereotaxic frame (Kopf). The scalp is reflected with a single incision and the fascia scraped from the skull. A thin plastic disc, with the outer diameter equal to the inner diameter of a trephine, is glued with Vetbond (3M, St. Paul, MN) onto the skull between Lambda and Bregma, and between the sagittal suture and the lateral ridge over the right hemisphere. Using a trephine (3mm outer diameter), the craniectomy is performed, keeping the dura intact.

A rigid Luer-loc needle hub (3 mm inside diameter) is secured to the skull over the opening with cyanoacrylate adhesive and dental acrylic. The skull sutures were sealed with the cyanoacrylate during this process to ensure that the fluid bolus from the injury remained within cranial cavity. The hub is filled with saline. The animal is sutured, placed on a heating pad, and returned to the home cage once ambulatory.



Figure 7: From Thompson et al 2005



Figure 8: The Fluid Percussion Injury Setup at UMDNJ-RWJMS. A: The fluid percussion injury (FPI) device. B: Test mouse undergoing craniotomy. C: The attachment of the Luer-loc hub. D: Mouse recovering from surgery within a heated cage. E: Mouse attached to the FPI device via a fluid-filled catheter in preparation for injury. F: sample trace in which the nature of the injury is monitored

Fluid percussion brain injury involves the rapid displacement of neural tissue by a rapid fluid pulse to the brain. After a 30-60 min period of recovery from surgery, the

animals will be re-anesthetized. The recovery period enhances the face validity of the brain injury model by returning the mice to a condition that more closely equates the human condition (in which brain injury occurs in the absence of anesthesia). Once the animal reaches a surgical plane of anesthesia (one respiration per two seconds), the animal is promptly connected to the fluid percussion injury device through the Luer-loc fitting of the hub. Once a normal breathing pattern resumes, before sensitivity to stimulation, a 1.2 – 1.5 ATM pulse will be generated to move through the device and the hub to strike the intact dura, creating an elastic decompression (20-25 msec) of the brain. Immediately after injury, the hub, and dental acrylic are removed en bloc. The scalp incision is then sutured and topical Marcaine applied. The time elapsed until the animal spontaneously righted is recorded as an acute neurological assessment, and defined as the righting reflex time. Upon return of righting reflex (approximately 10 minutes), the animals are returned to normal housing conditions. At the moderate level of injury, 20-25% of animals will die as a result of the injury within the acute posttraumatic period (15 min), generally from respiratory failure and pulmonary edema. However, out experience this summer has been a survival rate close to 85%. This is a normal and desired feature of the TBI model because it mimics human TBI. According to the experimental outline, animals will be behaviorally evaluated and euthanatized at varying time points after injury (1 day to 56 days). One group of animals will serve as an uninjured control (sham), where the surgical procedures are performed, animals are attached to the injury device, but the injury is not administered. In addition we are studying intact naïve mice that will not be exposed to any surgical trauma to serve as a baseline.

#### **Tissue Harvest**

Under deep anesthesia (Na Pentobarbital > 100 mg/Kg BW) the chest cavity is opened exposing the heart. A needle, through which the perfusates will be administered, is placed into the left ventricle and the right atrium is cut allowing for fluid outflow. We first perfuse 20 ml of phosphate-buffered saline followed by 4% pararformaldehyde (100 to 200 ml).

#### Analysis

The brains are frozen and sectioned on a cryostat (20 um sections) and subjected to double immunohistochemical analysis. We localize various isoforms of HDAC (currently focusing on HDAC1) and acetylated histone 3 (H3) with specific antibodies and cell types distinguished by antibodies directed against cell-specific antigens (e.g., NeuN for neurons; APC/CC1 for oligodendrocytes; GFAP, for astrocytes; MAC1 for microglia).

#### Some Preliminary Findings:

Some of our preliminary findings presented below. We have determined that HDAC1 is often associated with neurons within the cortex. However, HDAC1+ cortical oligodendrocytes are observable (see below). Immunostaining for HDAC1 appears to be most intense in the injured regions of the brain during the first week post injury.

Cortex Contralateral to LFPI



Figure 9 (Contralateral to Injury) Most HDAC1 positive cells are also immunopositive for NeuN (1day post injury) **A.** HDAC immunoreactive neurons in the contralateral or intact cortex (arrows point several immunopositive cells). **B:** NeuN immunoreactivity (arrows) **C:** Nissl stain. **D.**: Merge A + B to reveal double staining for HDAC1 and NeuN. Cortex Ipsilateral to LFPI



Figure 10 (Ispilateral to injury) HDAC1 immunoreactivity within the ipsilateral or injured cortex. The mouse was sacrificed 1 day after being subjected to LFPI. A: HDAC1 positive cells (arrows). B: NeuN Immunostaining to reveal surviving neurons (arrows). C: NeuroTrace blue staining (Nissl). D: A and B merged revealing double labeled cells (arrows).

# **Naïve Control Cortex**



**Figure 11**. HDAC1 immunostaining in a naïve control mouse cortex. **A**: HDAC1 immunostaining. **B**: NeuroTrace Blue (Nissl) staining. Note tight nuclear staining.

# LFP elicits increased HDAC1 staining and inflammation in the injured cortex.

Within a week following injury, there was a strong inflammatory response within the cortex,



Figure 12: LFPI leads to an increase in MAC1 immunoreactivity and HDAC1 staining within the Ipsilateral to the cortex injury as compared to the contralateral cortex. A,D: MAC! Immunostaining. **B**.E: HDAC1 immunoreactivity. C.F: NeuoTrace (Nissl) staining. 6 days post injury



Figure 13: LFPI may lead to inflammatory a heightened response in both intact and injured cortices with increased HDAC1 immunoreactivity being evident in the injured cortex. A, B: MAC1 Immunostaining to reveal microglia/ macrophages. C, D: HDAC1 positive cells. E.F: Neurotrace blue staining. 10 days post injury.

particularly in areas associated with increased HDAC1 staining (Figures 12 - 13).

# HDAC1 Immunoreactivity is increased in neurites following injury

As predicted by Dr. Patrizia Casaccia-Bonefil's model, LFPI elicted a strong HDAC1 presence in neurites. What is novel here is that HDAC1 response appears to be in both axons *and* dendrites (See Figure 14). We shall actively pursue this question in the future.



**Figure 14:** Increased HDAC1 immunoreactive fibers within the damaged cortex ipsilateral to LFPI, 30 days post injury. **A,B, and C: ipsilateral to injury. D, E, and F: Contralateral cortex. A, D:** HDAC1 immunostaining. **B, E:** SMI32 (non phosphorylated neurofilament, heavy chain) immunopositive neurites. **C:** Merge A + B. **F:** Merge D + E.

# HDAC1 in axons may be associated with B-APP immunoreactivity following injury

Following brain injury we observed a possible association between increased HDAC1 staining in axons and the expression of B-amyloid precursor protein (B-APP) staining. B-APP generally associated with axonal pathology, characteristic of traumatic brain injury and neurodegenerative disease such as Alzheimer's Disease .



**Figure 13:** HDAC1 immunoreactivity may associated with  $\beta$  -amyloid precursor protein following a stab wound to the cortex. **A:** HDAC1 immunoreactivity. **C:** Merge A + B. Arrows point a close association between HDAC1 reactivity and  $\beta$ -APP expression. 30 days post injury.



**Figure 14:** Regions of intense HDAC1 Staining is often associated with Beta-Amyloid Precursor Protein ( $\beta$ -APP) immunoreactivity. One month following LFP; ipsilateral to injury. **A:** HDAC immunopositive fibers within the external capsule (EC) (arrow). **B:**  $\beta$ -APP immunoreactive fibers found within the EC. **C:** The merging of images A and B.

The findings thus far indicate that the lateral fluid percussion injury model is suitable to address many of the

questions originally raised by this project implicating epigenetic factors in neuronal pathology following traumatic injury to the brain. The finding with the mouse are, in general, consistent with Dr. Patrizia Casaccia-Bonnefil's earlier findings both with the pig model and in culture. Initially there appears to be a increase in HDAC1 immunoreactivity within the first week following injury observable within the cell body. Later there appears to be a strong association between increased HDAC1 staining within axons and B-APP, a maker that has long been associated with neuronal pathology. However, our data also suggests that here may be additional complications with the original model: Within the cortex, we see intense HDAC1 immunostaining with the dendrites as well as axons following injury. Second, in intact, uninjured mouse cortical neurons we can find HDAC1 within their processes. This suggests



**Figure 15**: A sham operate cortex ipsilateral to surgery immunostained for HDAC1 (A) and for the oligodendrocyte marker, APC (B). Arrows point to double labeled cells. A: HDAC1. B: APC. C: Merge A+B. D: NeuroTrace Blue. The majority of HDAC1+ cells are not positive for the APC, even though several examples may be found.

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that there is a role for HDAC1 in these process in non-pathological states that needs to be explained. Also, this raises the question: Is the role of HDAC1 the same in the axon as it is in the dendrite?



**Figure 16:** Sham operate corpus callosum, ipsilateral to the surgery immunostained for HDAC1 (A) and APC (B). White arrows indicated double-labelled cells. Yellow arrow points to an APC+ oligodendrocyte that is negative for HDAC1. White-dotted lines in D indicate the approximate dorsal and ventral borders of the corpus callosum (CC). Within the CC both HDAC1+ and HDAC1 - cells were observed.

We have also attempted to characterize HDAC1 responses if any to injury in oligodendrocytes since HDAC1 has been shown to play important roles in oligodendrocyte development and differentiation. Above, we have indicated that most cells within the cortex that are HDAC1+ are also positive for the neuronal marker NeuN. However, using the oligodendrocyte marker, APC



**Figure 17:** Cortices of an animal that underwent FPI. Left contralateral to the injury; right ipsilateral to the injury, White dotes in B indicate the focal region of the injury. A and B HDAC2. C and D DAPI staining. Note the apparent increase in HDAC2 immunoreactivity ipsilateral to the injury. =



**Figure 18:** High power, confocal image of the the cortex ipsilateral to FPI immunostained for HDAC2 (A) NeuN (B). C: Merged image of A and B. Note that all NeuN+ cells are also positive for HDAC2.

molecular consequences of TBI.

we were able to find some double labelled cells within the cortex (Figure 15). Within the corpus callosum we observed both HDAC1 and HDAC1- oligodendrocytes. We have not as, yet observed any obvious change in expression within the oligodendrocytes associated with injury. We intend to pursue this further.

Finally we have also looked HDAC2, which has been reported to function with HDAC1, to see what role it might play in mediating the effects of traumatic injury. Some of our initial findings have suggested that there is an increase in HDAC2 immunoreactivity in the cortex ipsilateral to the injury. This intense immunostaining for HDAC2 appears to be associated with neurons (see Figure 18.)

# **Project Challenges**

Because the results shown in Fig. 6 do not support the hypothesized transcriptional increase of specific sodium channel subunits, wanted to focus her analysis at the definition of the axoplasmic role of HDAC1. Thus, the original hypothesis is in need of alteration. In addition, many of the reagents that were available did not lend themselves to pig tissue. Discussions between the original PI and myself suggested that a a change in directions was warranted. A better characterization of how HDACs might function in the injured brain might be defined. Since, we were establishing a mouse model for TBI here at

UMDNJ-RWJMS it might more effective to do the work in house in the mouse. This would allow us to more easily vary the experimental conditions and examine such factors as inflammation. The mouse, because of its size and low cost, would allow future studies in which pharmacological agents may be employed in a cost-effective manner. In addition, mouse genetics is well known and can be easily manipulated which offers additional tools to study the

With the departure of the original PI and the need to develop and establish a new animal model, a considerable amount of time was lost. In addition, 2 technicians and a graduate student who where supported by the grant left with her so it was up to me to continue the work and train new personnel. Thus, I asked for a no cost extension to continue the work. Finally, there were protracted discussions between my self and the commission over the change in focus and rebudgeting of the project-this also led to lost time during the no cost extension year of several months.

## Notwithstanding these challenges, we have made considerable progress:

- 1. Established moue model of LFPI to study TBI and its epigenetic consequences.
- 2. Performed and are continuing to perform histological analyses and established immunohistochemical protocols for mice.
- 3. Mentored 2 medical students and 5 undergraduate students in TBI research.
- 4. Submitted abstract to Society for Neuroscience Meeting in Chicago, 2009.
- 5. Acquired a treasure trove material to be analyzed--much of it is still frozen an in need of processing.
- 6. Established new collaborations with Rutgers University Faculty.
- 7. Established new collaborations within the Department of Neuroscience and Cell Biology, UMDNJ-RWJMS.
- 8. In the process of developing some new hypothesis concerning HDACs role in mediating the deleterious consequences of central nervous system injury.

A word needs to said about original hypothesis that Hypothetical Model Revision: underlies the rationale for this project. The data collected thus far suggests that the First, as mentioned above in Figure 6 the predicted model needs to be revised. changes in sodium channels were not detected in the pig material. Further, Figure 14 presents evidence that suggests that intense HDAC1 immunoreactivity associated with the injury was found in axons and dendrites and we have observed detectable levels of HDAC1 in the neurites of uninjured neurons. Finally, the model was based on an analogy with multiple sclerosis, which suggests that the loss of oligodendrocytes would set up conditions that induced the translocation of HDAC1 form the nucleus to the axon, leading to axon pathology. In our mouse model, we did not see any appreciable demyelination and yet there were increases in HDAC1 expression in many neurons in This suggests that there may be other triggers that need to be explored, injured brain. Just what specific role HDAC1 might play in the pathological such as inflammation. process not clear.

# Implications for future research and clinical treatment

As stated at the outset, following traumatic brain injury (TBI) there is a slow progression of cell death and breakage of nerve cell processes that continues to silently plague the victim for months and years after the original injury. These ongoing degenerative effects often leads to a worsening of functional losses (including memory, cognition and sensorimotor). As of now there is no Effective therapies will only occur when we understand the molecular effective treatment. substrate for these degenerative changes. There are numerous findings form the published literature as well as generated by this project (e.g., Dr. Casaccia-Bonnefil's findings on silencing HDAC1) that suggest that HDACs may be a potentially important target for pharmacological therapies. Inhibiting HDACs have often been shown to be neuroprotective in a variety of models, ranging from trauma, to stroke, to neurodegenerative disease. As of now our tools are rather "crude" with general HDAC inhibitors, which may have serious side effects: Neurons may be protected and oligodendrocyte precursors might be prevented form differentiating. These are

empirical questions that need to be explored. Thus, detailed studies must be conducted to define among other things, which cell types are involved, the timing of the alterations, the HDAC1 and HDAC2 binding partners in the cytoplasm, the upstream triggers for changes in HDAC expression during pathological conditions. etc. I plan to peruse many of these and related questions in the future.

# **Plans for Future Research**

First, my immediate plans is to continue to process and analyze stored mouse brain material. I plan to try to further identify the cell types with altered expression of HDAC1 and HDAC2 as well as subcellular localization. I have brains that represent both short-term and long-term survival. In addition to the qualitative analysis that has been performed, we plan to devise quantification protocols to analyze are results. We have been hampered in quantification because of the distortions and severe degeneration that occur in in the brain that result form the injury.

**Collaborations:** The funding of this project has also allowed me to establish new collaborations that will allow me to investigate some of the unanswered questions raised by these studies.

Traumatic Brian Injury and Inflammation: One common factor associated with neurodegeneration whether it is induced by trauma, stroke or a disease process in the inflammatory response. Controlling this response central nervous system injury has proved neuroprotective following trauma in both animal models and human patients. We which to examine how modulating the inflammatory response might affect HDAC1 and HDAC2 expression and determine whether this modulation in HDAC1 and HDAC2 expression might lead to better prognosis following brain trauma. To examine the effects of inflammation, I have begun a collaboration with Dr. Melitta Schachner an her students (Department of Cell Biology and Neuroscience, Rutgers University) in which we are injuring both wild-type and RAG2deficient mice, which are immune deficient. We have run a pilot study to determine the feasibility of the project last fall and are now engaged performing the experiments. Dr Schachner has perviously shown that, following spinal cord injury RAG2 mice have better outcomes than their wild-type conterparts. In addition to this I have also begun to examine RAG1-deficient mice with hope of making specific comparisons. At present i have several brains awaiting processing. Depending on the outcomes of these studies, Dr. Schachner and I will seek extramural funding.

**Possible role of HDAC1 and HDAC2 in mediating the injury signal.** Although HDAC1 and HDAC2 have important roles in suppressing gene expression by de-acetylation of histones found within the chromatin, their role in the cytoplasm is less clear. Damaged axons communicate their distress to the cell by an "injury signal". A major component of this signal is carried by a class of proteins known as importins. Working with a colleague, Dr. Mladen-Roko Rasin, (Department of Neuroscience and Cell Biology, RWJMS-UMDNJ) we have observed significant alterations in cortical expression of one of importins with indications of significant alterations in dendritic structure following spinal cord injury. We are presently attempting to confirm these findings. It turns out some of the binding partners of these

importins also are binding partners of HDAC1 within the cytoplasm. Some important ground work for this has been provided by Kim et al (2010) (see publications below) I plan to extend these findings to our LFPI injury model, looking to see how inflammation may influence the "injury signal.. To further elucidate the injury signal Dr. Rasin and I plan to submit an RO1 in the fall. This project will focus on alterations in gene expression and importin signaling mechanisms.

# **Publications and Presentations**

**Crockett**, D.P., Kim, J., Casaccia-Bonnefil, P. Epigenetic consequences of traumatic brain injury (TBI) Program No. 245.8. 2009 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2009. Online

Kim, J., Shen, S. Dietz, K., He, Y., Reynolds, R., Casaccia, P. HDAC1 nuclear export induced by pathological conditions is essential for axonal damage. Nat. Neuroscie 2010 Feb:13(2) 180-189.

# HDAC1 nuclear export induced by pathological conditions is essential for the onset of axonal damage

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Histone deacetylase 1 (HDAC1) is a nuclear enzyme involved in transcriptional repression. We detected cytosolic HDAC1 in damaged axons in brains of humans with multiple sclerosis and of mice with cuprizone-induced demyelination, in *ex vivo* models of demyelination and in cultured neurons exposed to glutamate and tumor necrosis factor- $\alpha$ . Nuclear export of HDAC1 was mediated by the interaction with the nuclear receptor CRM-1 and led to impaired mitochondrial transport. The formation of complexes between exported HDAC1 and members of the kinesin family of motor proteins hindered the interaction with cargo molecules, thereby inhibiting mitochondrial movement and inducing localized beading. This effect was prevented by inhibiting HDAC1 nuclear export with leptomycin B, treating neurons with pharmacological inhibitors of HDAC activity or silencing HDAC1 but not other HDAC isoforms. Together these data identify nuclear export of HDAC1 as a critical event for impaired mitochondrial transport in damaged neurons.

Axonal damage has been described in several neurodegenerative disorders<sup>1</sup> and in inflammatory demyelination, such as that of multiple sclerosis<sup>2–4</sup>. The morphological changes associated with axonal damage include the presence of localized axonal swellings, characterized by the succession of enlargements and constrictions along the axon ('beads-on-a-string' appearance) and the detection of 'ovoids' or 'end bulbs', resembling the terminal stumps of transected axons<sup>3,4</sup>. Axonal transections have been considered a hallmark of irreversible axonal degeneration<sup>3,5</sup>, and the presence of ovoids in the proximal part of the axons is typically associated with rapid retrograde degeneration<sup>6</sup> and neuronal death<sup>7,8</sup>. These neuropathological findings can be detected in distinct neurodegenerative disorders, which thereby suggests that these pathological conditions might share a similar mechanism of induction of axonal damage.

A marker of axonal damage is immunoreactivity with antibodies for the hypophosphorylated form of neurofilament heavy chain (SMI32). Neurofilaments, axonal cytoskeletal molecules, consist of three subunits classified on the basis of molecular weight: 200-kDa heavy (NFH), 150-kDa medium (NFM) and 68-kDa light (NFL) chains. In physiological conditions, these subunits are phosphorylated and the degree of phosphorylation correlates with axonal caliber and speed of axonal transport<sup>9</sup>, possibly by affecting the association of neurofilaments with the motor protein kinesin<sup>10</sup>. Hypophosphorylated neurofilaments, in contrast, are characterized by enhanced susceptibility to protease digestion<sup>11</sup>, greater tendency to self-aggregate<sup>12</sup> and colocalization with tumor necrosis factor (TNF)- $\alpha$  immunoreactivity<sup>13</sup>, and they are typically detected in the brains of animals used as models of demyelination<sup>14</sup> and of humans with multiple sclerosis<sup>15</sup>. Although the molecular mechanism linking axonal transport and neuropathology is not well characterized, many studies have reported that disruption of axonal transport<sup>16</sup> results in the rapid accumulation of proteins at the sites of swelling<sup>17</sup>. High concentrations of glutamate in cultured neurons impair neurofilament transport and induce cytoskeletal protein accumulation at the sites of axonal swelling<sup>18</sup>, thereby suggesting a causal relationship between localized swellings and local disruption of axonal transport<sup>19</sup>. Impaired axonal transport is likely to eventually trigger Wallerian degeneration of distal axons, and therefore it can be considered one of the first signs of damage that is associated with localized swelling and ultimately leads to transection.

Several pathological stimuli can impair axonal transport, including accumulation of mutant proteins, cytoskeletal disorganization, excitotoxicity and altered histone deacetylase (HDAC) activity<sup>17,20</sup>. HDACs are a family of enzymes originally named after their ability to remove acetyl groups from lysine residues located within the N-terminal tails of histones, causing compaction of chromatin and repression of transcription<sup>21,22</sup>. On the basis of their primary structure, HDACs can be further classified as class I (HDAC1, 2, 3 and 8), class II (HDAC 4, 5, 6, 7 and 9), class III (SIRT1-SIRT7) and class IV (HDAC11)<sup>21</sup>. It has now been shown that HDACs also modulate the activity of nonhistone proteins such as YY1 (ref. 23) and NF-KB (ref. 24). In addition, class II HDACs are cytosolic enzymes that remove acetyl groups from the epsilon positions of lysine residues of cytosolic proteins, including  $\alpha$ -tubulin<sup>25</sup>. The class II HDACs HDAC4 and HDAC5 shuttle in and out of the nucleus. In physiological conditions, they are detected in the cytoplasm<sup>21</sup>. In pathological conditions (for example, in Huntington's disease), however, HDAC5 is detected in the nucleus, where it is thought to repress gene expression<sup>26</sup>. Acetylation of  $\alpha$ -tubulin regulated by a

Received 21 August; accepted 18 November; published online 27 December 2009; doi:10.1038/nn.2471

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microtubule-associated deacetylase, HDAC6 (ref. 25), negatively affects axonal transport by removing acetyl groups from  $\alpha$ -tubulin, thereby impairing its ability to recruit the motor proteins kinesin-1 and dynein to microtubules. In agreement with the negative effect of HDAC6 in vesicular transport, this molecule has been noted as a component of Lewy bodies in Parkinson's disease<sup>27</sup>, and in Huntington's disease it has been associated with defective release of neurotrophic factors<sup>28</sup>. The data from these studies suggest that axonal transport is negatively regulated by HDAC6-dependent deacetylation of  $\alpha$ -tubulin in neuro-degenerative disorders.

Impaired axonal transport has also been correlated with cytoskeletal disorganization caused by the proteolytic degradation of cytoskeletal proteins induced by calcium activated proteases<sup>29</sup>. It has been proposed that excess Ca<sup>2+</sup> activates Ca<sup>2+</sup>-dependent proteases such as calpain and caspases, which act on several substrates, most of which are cytoskeletal proteins<sup>29</sup>. However it is unclear whether

calcium entry and HDAC activity are independent mechanisms associated with early and late stages of axonal damage.

This study describes the neuron-specific and calcium-dependent nuclear export of HDAC1 in demyelinating conditions and in neurons exposed to excitotoxic amino acids and cytokines, which we used to mimic the inflammatory environment characteristic of the demyelinated brain<sup>30</sup>. We identify molecular events responsible for morphological changes

Figure 1 Cytosolic HDAC1 is detected in animal models of demyelination, in the brains of humans with multiple sclerosis and in demyelinated slice cultures. (a) Confocal micrographs of the corpus callosum of control and 4-week cuprizone-fed mice (Cupri4w), stained with antibodies specific for class I (HDAC1, HDAC2, HDAC3, HDAC8) and class II (HDAC4, HDAC5, HDAC6 and HDAC7) HDAC isoforms, indicated on the left (green), and with antibody to neurofilament medium chain (NFs, red). Scale bar,  $10 \mu m$ ; ×63 objective. (b) Confocal image of the colocalization (arrows) of hypophosphorylated neurofilament heavy chain (SMI32, red) and HDAC1 (green) in damaged axons in the corpus callosum of cuprizone-fed mice. Scale bar, 5  $\mu$ m. (c) Luxol fast blue plus periodic acid Schiff (LFB, PAS) staining of human brain sections (MS001 block 1 and block 2) identifying areas of white matter demyelination (arrows; scale bar, 200 µm). (d) Serial sections of the same lesions, showing the presence of HDAC1<sup>+</sup> end bulbs (green) in neurofilament-positive (red) axons (white arrows). Scale bar, 10 µm. (e) Adjacent sections processed for immunohistochemistry with antibodies for SMI32 (red) and HDAC1 (green) in white-matter lesions. DAPI (blue) was used as nuclear counterstain. Scale bar. 10 um. (f) Confocal images of cerebellar slice cultures either untreated (control) or treated with lysolecithin and LPS to induce demyelination (Lyso/LPS 17 h + LPS 24 h). Cultures were stained with antibodies specific for myelin basic protein (MBP, red) and NFs (green) antibodies. (g) The same cultures were then stained for HDAC1 (green) and SMI32 (red).

of neurites that were independent of HDAC6-mediated tubulin deacetylation and dependent on a cytotoxic function of cytosolic HDAC1.

#### RESULTS

#### HDAC1 nuclear export in damaged axons

Previous studies reported that the axonal changes detected in humans brains affected by multiple sclerosis can also be observed in the corpus callosum of mice with cuprizone-induced demyelination<sup>14,31</sup>. Therefore, we processed brain sections from C57BL/6J mice maintained on a 0.2% cuprizone diet for 4 (n = 9) or 6 weeks (n = 6) or on a regular diet (n = 15). To test the hypothesis that histone deacetylases could be involved in axonal damage induced by neuroinflammation, we focused our analysis on callosal axons in the 4-week group, because at this time point we can detect demyelination associated with extensive microglial infiltration and axonal damage<sup>31,32</sup>. We used antibodies specific for class I and class II HDACs



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and for neurofilament medium chain to identify callosal axons. We detected no difference in the subcellular localization of class I and II HDAC isoforms in cuprizone-treated and control mice (Fig. 1a), with the exception of HDAC1, which showed a more prominent cytosolic localization in the corpus callosum of cuprizone-treated mice, especially in areas associated with microglial infiltration (Fig. 1a) and axonal damage (Fig. 1b). The protein levels for the distinct HDAC isoforms remained unchanged throughout the duration of the cuprizone treatment (Supplementary Fig. 1, n = 3 mice per time point). Cytoplasmic HDAC1 was also present in sections from brain blocks that were selected from four people with neuropathologically confirmed multiple sclerosis. Tissues were sampled from the superior frontal gyrus by selecting those blocks containing macroscopically visible lesions. We screened the areas of interest for the presence of demyelination, using Luxol fast blue plus periodic acid Schiff staining (Fig. 1c), and then evaluated the immunoreactivity of SMI32, an antibody specific for the hypophosphorylated form of neurofilaments heavy chain, in 29 areas of interest within the demyelinated regions. The number of HDAC1<sup>+</sup> and SMI32<sup>+</sup>HDAC1<sup>+</sup> profiles was quantified per ×400 microscopic field (0.29 mm<sup>2</sup>). No HDAC1<sup>+</sup>SMI32<sup>-</sup> profiles were noted. The average number of HDAC1<sup>+</sup> processes per mm<sup>2</sup> was  $7.55 \pm 1.8$  (mean  $\pm$  s.e.m.), and the average number of SMI32<sup>+</sup> neurites was  $29.8 \pm 6.1$  (mean  $\pm$  s.e.m.). Thus, we detected colocalization in 25.3% of damaged axons identified by immunoreactivity for SMI32 (Fig. 1d,e).

We also detected HDAC1 translocation in an *ex vivo* model of inflammatory demyelination, induced by exposure of cerebellar slice cultures to lysolecithin and lipopolysaccharide (LPS)<sup>33</sup>. Using

**Figure 2** Glutamate and TNF-α treatment of primary neuronal cultures induces neuritic beading followed by transections. (a) Confocal image of cultured neurons either untreated (control) or treated with 50 μM glutamate and 200 ng ml<sup>-1</sup> TNF-α for 2 or 24 h. Cells were stained with antibodies for NFM (green) to visualize neurites and SMI32 (red) to identify damaged neurites. Scale bar, 10 μm in low (left and middle columns) and 2 μm in high (right column) magnification. (b) Bar graphs showing the percentage of SMI32<sup>+</sup> beaded neurites relative to the total number of NFM<sup>+</sup> neurites (mean ± s.d., *n* = 83 ± 12 neurites counted in each condition. \**P* < 0.05, \*\*\**P* < 0.001, two-tailed Student's *t*-test). (c) Time-lapse video microscopy of cultured neurons exposed to 50 μM glutamate and 200 ng ml<sup>-1</sup> TNF-α. Images were captured every 30 min for 12 h. Scale bar, 5 μm. (d) Graph of the percentage of normal-appearing, beaded and transected neurites, relative to the total, at each time point. Error bars, s.d.

immunohistochemistry with antibodies specific for NFM-L and for myelin basic protein (MBP) to identify demyelination, we detected multiple beaded axons in demyelinated white matter regions in the presence of activated microglia (**Fig. 1f**). These axons were also immunoreactive for HDAC1 and SMI32 (**Fig. 1g**), thereby confirming the cytoplasmic localization of HDAC1 at the early stages of axonal damage after demyelination and microglial activation.

#### HDAC1 nuclear export precedes the onset of axonal damage

It has been previously proposed that glutamate and cytokines produced by microglial cells can be neurotoxic, and therefore we exposed primary neuronal cultures to glutamate and cytokines in order to best mimic the inflammatory environment in the human brain affected by multiple sclerosis<sup>30</sup>. Immunocytochemistry was used to characterize the cellular composition of the neuronal cultures (Supplementary Fig. 2a,b). Increasing concentrations of glutamate together with a constant amount of cytokine (200 ng ml<sup>-1</sup> TNF- $\alpha$ ) were tested for the ability to induce neurite pathology. We chose the lowest concentration (50  $\mu$ M glutamate and 200 ng ml<sup>-1</sup> TNF- $\alpha$ ) that induced neuritic beading within 2 h of exposure to perform a time-course experiment of axonal damage, using immunoreactivity for SMI32 and morphological appearance of beaded neurites as detection criteria. In agreement with the progressive nature of damage, after 2 h, 42.88  $\pm$  8.06% of the neurites showed the characteristic beaded morphology and 20.6  $\pm$  9.46% were also SMI32<sup>+</sup>; after 24 h, 88.07  $\pm$ 1.98% showed beading and  $61.58 \pm 2.23\%$  were SMI32<sup>+</sup> (Fig. 2a,b). To confirm the relationship between localized beading and later signs of damage, we conducted live imaging on neurons before and after treatment with glutamate and TNF- $\alpha$  and followed the morphological changes occurring during the next 12 h, using time-lapse video microscopy. During the first 2 h, we detected a progressive increase in the percentage of beaded neurites, which was later replaced by the detection of transections, a hallmark of late stages of axonal damage (Fig. 2c,d), and the latter was associated with decreased neuronal viability (Supplementary Fig. 3).

The progressive nature of the morphological changes detected in the neurites after treatment with glutamate and TNF- $\alpha$  correlated with the progressive changes of HDAC1 subcellular localization. Before exposure to excitotoxic amino acids and cytokines, we detected HDAC1 within the nuclear compartment (**Fig. 3a**). However, within the first 5 min of treatment we observed it in a perinuclear location (**Fig. 3a**), by 20 min it was evenly distributed along the entire length of the neurites, and at 2 h it formed aggregates within the enlargements of the beaded neurites (**Fig. 3a,b**). On the basis of these results, we hypothesized that nuclear export of HDAC1 was part of the mechanism leading to neuronal damage. Previous evidence had suggested that localized enlargements and beading were associated Figure 3 Cytosolic localization of HDAC1 precedes the onset of localized beading and impaired mitochondrial transport. (a) Confocal images of neurons before and after exposure to 50  $\mu M$  glutamate and 200 ng ml^-1 TNF- $\alpha$  for the indicated time periods. Control and treated cultures were stained with antibodies specific for HDAC1 (green) and for neurofilament medium and light chains (NFs, red). Top row scale bars, 5  $\mu$ m or 2  $\mu$ m; bottom rows, 10  $\mu$ m. (b) Bar graphs indicating the percentage of beaded neurites relative to the total NFs+ population at the indicated time points; error bars, s.d.; \*P < 0.05. (c) Live cell images of moving mitochondria, labeled with MitoTracker, in neurons exposed to 50 µM glutamate and 200 ng ml<sup>-1</sup> TNF- $\alpha$ . The position of each mitochondrion was recorded every 5 s for 5 min, and are shown at the indicated time points with the tracks of mitochondrial movement pseudocolored. Scale bar, 5 µm. (d) Bar graphs indicating the average speed of moving mitochondria, with s.d.; \*\* P < 0.01.



with disrupted axonal transport and consequent accumulation of proteins and organelles<sup>16</sup>. Labeling of treated primary cultured neurons with the cell-permeable mitochondrial dye MitoTracker Green FM (Invitrogen) followed by time-lapse video microscopy allowed us to visualize all of moving mitochondria (**Fig. 3c**). The average speed of mitochondrial transport in untreated primary neurons was  $0.23 \pm 0.04 \,\mu m \, s^{-1}$ . After 5 min of treatment, when HDAC1 was detected in perisomatic locations but not yet in the neurites, mitochondrial transport was still  $0.21 \pm 0.07 \,\mu m \, s^{-1}$ . At 20 min, however, when HDAC1 reached the neurites, mitochondrial transport was slower ( $0.089 \pm 0.012 \,\mu m \, s^{-1}$ ). By 2 h, the average speed had decreased even more ( $0.038 \pm 0.029 \,\mu m \, s^{-1}$ ), and this correlated with the detection of localized neurite swellings (**Fig. 3d**). Thus, impaired mitochondrial transport preceded the appearance of localized beading.

To address the possibility that the subcellular localization of other HDAC isoforms was similarly affected, we repeated immunocytochemistry in primary neurons, cultured for 2 h in the presence of

glutamate and TNF- $\alpha$ , using antibodies specific for each isoform and DAPI as nuclear counterstain (Supplementary Fig. 4). Of all the HDAC members analyzed, HDAC3, HDAC4, HDAC6 and HDAC8 were also detected in the cytoplasm. However, HDAC1 was the only isoform that was exported from the nucleus and accumulated in the neurites in response to glutamate and TNF- $\alpha$  treatment (**Supplementary Fig. 4**). To further confirm the nuclear export of HDAC1, we repeated the experiment using three distinct HDAC1 antibodies, including two commercially available ones generated against the C terminus and one against the N terminus of the molecule (Supplementary Fig. 5). We obtained the same results with all three reagents. Inducible HDAC1 nuclear export was cell-type specific, as it was detected in primary neurons, but not in astrocytes or oligodendrocytes receiving the same treatment (Supplementary Fig. 6). To further validate the inducible nuclear export of HDAC1 using an alternative approach, we expressed a Flag-tagged HDAC1 molecule in neurons and tracked its subcellular localization in response to glutamate and TNF- $\alpha$  treatment, using



Figure 4 Calcium depletion prevents HDAC1 nuclear export and the onset of neuritic beading induced by treatment with glutamate and TNF- $\alpha$ . (a) Confocal image of primary cultures transfected with Flag-tagged HDAC1 on day 10 and then analyzed 3 d later in the absence (control) or presence of 50  $\mu$ M glutamate and 200 ng mI<sup>-1</sup> TNF- $\alpha$ for 2 h. Transfected cells were detected using antibodies against the Flag tag (green) and NFM (red). Scale bar, 10 µm for low magnification (mag.) and 2  $\mu$ m for high. (b) Confocal images of cultured neurons pretreated with increasing concentrations of the calcium chelator EDTA and stained with antibodies specific for HDAC1 (green) and NFs (red). EDTA prevented neurite beading induced by treatment with 50 µM glutamate and 200 ng ml<sup>-1</sup> TNF- $\alpha$ . Scale bar, 10  $\mu$ m for low magnification and 2  $\mu$ m for high. (c) Bar graph quantifying the results shown in **b** (mean ± s.d.; \*\**P* < 0.01, \*\*\**P* < 0.001).

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Figure 5 Silencing HDAC1, but not other isoforms, prevents neurite beading. (a) Primary neurons infected with Hdac1-shRNA lentiviral particles and then exposed to 50  $\mu$ M glutamate and 200 ng mI<sup>-1</sup> TNF- $\alpha$  for 2 h and processed for immunocytochemistry with antibodies to HDAC1 (green) and NFs (red). DAPI (blue) was used as nuclear counterstain. (b) Cultured neurons infected with class I (Hdac2, Hdac3 and Hdac8) and class II (Hdac4 and Hdac6) Hdac-shRNA lentiviral particles. Cultures were exposed 72 h after infection to 50  $\mu$ M glutamate and 200 ng ml^-1 TNF- $\alpha$  for 2 h and then processed for immunocytochemistry with antibodies specific for HDAC2, HDAC3, HDAC4, HDAC6 or HDAC8 (green) and NFs (red). Scale bars in **a**,**b**, 10 μm for low magnification (mag.) and 2 µm for high. White arrows indicate neuronal nuclei.

an antibody to Flag (**Fig. 4a**). Whereas in the absence of any treatment overexpressed Flag-tagged HDAC1 was detected exclusively in the nuclei of transfected neurons, after treatment with glutamate and TNF- $\alpha$ the Flag-tagged HDAC1 was first detected in perinuclear locations and then distributed all along the neurites (**Fig. 4b**).

Because axonal damage in demyelinating disorders is not limited to areas of acute demyelination<sup>34</sup>, it has been proposed that damage could be caused by calcium entry in response to distinct causes, including myelin loss<sup>5</sup>, activation of glutamate receptors<sup>35</sup> or cytokine-mediated release from intracellular stores<sup>36</sup>. To determine whether HDAC1 nuclear export was downstream of calcium entry, we pretreated neurons with

increasing concentrations of the calcium chelator EDTA for 30 min before exposure to 50  $\mu$ M glutamate and 200 ng ml<sup>-1</sup> TNF- $\alpha$  for 2 h. Cultures were then processed for immunocytochemistry using specific antibodies to HDAC1 and neurofilaments. EDTA pretreatment prevented the onset of neurite beading in a dose-dependent manner, with a maximum effect at a concentration of 10 mM (**Fig. 4c**), which correlated with retention of HDAC1 in the nucleus. Thus, HDAC1 nuclear export in response to glutamate and TNF- $\alpha$ treatment required the presence of calcium.

#### Silencing of HDAC1 prevents neuritic beading

To further define the role of HDAC1 in the induction of neuritic beading, we adopted a silencing approach and tested, for efficiency and specificity of silencing, a minimum of four lentiviral targeting constructs for each HDAC isoform and selected the two most effective ones (**Supplementary Fig. 7**). Primary neurons were transduced with these small hairpin RNAs (shRNAs), and 72 h later they were treated with glutamate and TNF- $\alpha$ . Mock-infected cultures were used as controls (**Fig. 5a**). Silencing of *Hdac1* prevented the appearance of beaded neurites. Whereas the percentage of beaded neurites in mock-infected neurons was 58.1 ± 1.65% ( $n = 35 \pm 2.8$ ), the silencing of *Hdac1* with shRNA decreased the percentage of beaded neurites to 19.4 ± 2.4% ( $n = 34 \pm 2.1$ ).

Silencing of other members of class I (*Hdac2*, *Hdac3* and *Hdac8*) and class II (*Hdac4* and *Hdac6*) histone deacetylases, in contrast, did



not elicit the same effect (**Fig. 5b**). Together these results support a critical role for HDAC1 but not other HDAC isoforms in the onset of neurite beading induced by glutamate and TNF- $\alpha$ .

CRM1-dependent HDAC1 export is essential for axonal damage Because HDAC1 is a nuclear enzyme, we reasoned that it must be exported from the nucleus by a process that is initiated by exposure to pathological stimuli. For class II HDACs, shuttling from the nucleus to the cytoplasm has been attributed to the presence of nuclear export signals (NESs)<sup>21</sup>, which allow binding to the Exportin 1 receptor (CRM1) followed by transport through the nuclear pore. The original consensus sequence for nuclear export and CRM1 binding was identified as LXXXLXXLXL<sup>37</sup>. Amino acid sequence analysis of HDAC1 using the ScanProsite program (Expert Protein Analysis System) revealed the presence of leucinerich motifs (LXXXLXXL or LXXLXL; Fig. 6a), similar to the NES that was originally described in viral proteins<sup>37</sup>. Therefore, we hypothesized that nuclear export of HDAC1 could be mediated by the interaction between CRM1 and specific leucine residues within the putative NES. To test this hypothesis, protein extracts from neuronal cultures exposed to glutamate and TNF- $\alpha$  for 5 and 20 min were immunoprecipitated with HDAC1 antibodies and analyzed by western blot. A complex between CRM1 and HDAC1 (Fig. 6b) was detected during the first 5 min of treatment, but not at later time points (Fig. 6b). To further confirm that this interaction

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Figure 6 CRM1-dependent nuclear export of HDAC1 is essential for the induction of damage by glutamate and TNF- $\alpha$ . (a) Diagram of HDAC1 protein sequence, with potential nuclear export sequences (NESs, red) and nuclear localization domains (NLS, blue). WT, wild type; mut., mutant. (b) Protein extracts from cultured neurons either untreated or treated with toxic stimuli for 5 or 20 min were immunoprecipitated (IP) with antibodies specific for HDAC1 and processed for western blot analysis using CRM1 or HDAC1 antibodies. Whole cell lysates (WCL) were used as controls. (c) WT and NES mutant HDAC1 overexpression, followed by immunoprecipitation with anti-Flag and western blot analysis with anti-CRM1 and anti-Flag. (d) Immunocytochemistry of primary neurons pretreated with increasing concentrations of leptomycin B (LMB) for 30 min, then exposed to 50  $\mu$ M glutamate and 200 ng ml<sup>-1</sup> TNF- $\alpha$  and stained with antibodies for HDAC1 (green) and NFs (red). Scale bar, 10 µm for low magnification (top two rows) and 2 µm for high. (e) Bar graphs indicating the percentage of beaded neurites relative to the total population (mean  $\pm$  s.d.; \*P < 0.05). (f) Pseudocolored image of mitochondrial movement, using time-lapse video microscopy of primary neurons treated with LMB and exposed to glutamate and TNF- $\alpha$ . Untreated cultures were used as controls. Cultures were labeled with Mitotracker and photograms were taken every 5 s within a 5 min time period. LMB treatment ameliorated mitochondrial movement. Scale bar, 5 µm. (g) Bar graphs indicating the average speed of moving mitochondria in each condition; error bars, s.d.; \*P < 0.05.



was mediated by the NES sequence on HDAC1, we generated a C-terminal Flag-tagged point mutant by converting leucine to alanine residues within the putative NES. Overexpression of this mutant HDAC1 interfered with the formation of a complex with CRM1 (**Fig. 6c**), thereby supporting a functional interaction between the putative NES sequence in HDAC1 and CRM1.

The functional significance of HDAC1 nuclear export was then tested with leptomycin B (LMB), a pharmacological inhibitor of CRM1-dependent transport<sup>38</sup>. Pretreatment of neurons with increasing concentrations of LMB for 30 min, before exposure to glutamate and TNF-a, prevented HDAC1 export from the nucleus and inhibited neurite beading in a dose-dependent manner (Fig. 6d,e). Pretreatment of cultured neurons with doses of LMB that prevented HDAC1 nuclear export also affected mitochondrial transport. Whereas an 84% decrease in the speed of mitochondrial transport was detected in cultures exposed to glutamate and TNF- $\alpha$  compared to untreated controls, LMB pretreatment significantly ameliorated the ability of mitochondria to move even in the presence of glutamate and TNF- $\alpha$ , although it was unable to restore the speed to that measured in controls (Fig. 6f,g). Together these results suggest that CRM1-mediated nuclear export of HDAC1 is part of the mechanism that modulates mitochondrial transport in pathological conditions associated with localized neurite swellings and beading.

#### Cytosolic binding partners for HDAC1 in damaged axons

Because HDAC1 nuclear export decreases its bioavailability in the nucleus, it is conceivable that impaired mitochondrial transport could be consequent to decreased nuclear function, rather than to acquisition of a cytosolic function. Histone H3 is one of the main

nuclear substrates for HDAC1, and therefore we assessed the effect of glutamate and TNF- $\alpha$  treatment on the levels of acetyl-H3 (**Supplementary Fig. 8a**) and on the transcript levels of genes previously shown to be regulated by HDAC1<sup>39–42</sup>. We did not detect increased acetyl-H3 until 2 h, and we detected no change in the abundance of transcripts involved in apoptosis (*Bax* and *Jun*), survival (*Bcl2*) or axonal damage (*Scn2a1*, also known as *Nav1.2*; *Scn8a*, also known as *Nav1.6*; and *Jun*) (**Supplementary Fig. 8b**). Because the first localized signs of beading were observed as early as 20 min after exposure to glutamate and TNF- $\alpha$ , well before any change in acetyl-H3 could be measured, we concluded that the effect of HDAC1 nuclear export on neuritic damage was likely due to a cytosolic gain of function rather than loss of its nuclear function.

To mechanistically define the role of cytosolic HDAC1 in axonal damage and find potential binding partners in the axons of damaged neurons, we adopted an unbiased approach and performed MALDI-TOF mass spectrometry on protein extracts isolated from callosal axons of control and cuprizone-treated mice and from untreated or glutamate and TNF-α treated neuronal primary cultures, immunoprecipitated with antibodies to HDAC1. The experiment was independently repeated on three distinct mouse groups and three groups of neuronal cultures. Only molecules that were identified as binding partners in all six independent samples (three in vivo experiments and three in vitro experiments) were further processed for validation analysis, using immunoprecipitation and western blot of protein extracts from the corpus callosum of untreated mice (n = 7) and of mice treated with cuprizone for 4 (n = 7) or 5 weeks (n = 4). The results obtained after immunoprecipitation with anti-HDAC1 were compared with those obtained using anti-HDAC4 and anti-HDAC6 (Fig. 7a).





In agreement with previous reports<sup>43</sup>,  $\alpha$ -tubulin was a prominent band immunoprecipitated by HDAC6 antibodies in extracts from both control and treated groups (Fig. 7a). Tubulin was not precipitated by anti-HDAC4 (Fig. 7a). These data identified differential binding for distinct histone deacetylases to proteins involved in mitochondrial transport (Fig. 7a). The inducible interaction between HDAC1 and a protein involved in axonal transport (KIF2A) was detected only in brain regions characterized by microglial infiltration and cytokine

Figure 8 Activity-dependent interaction of HDAC1 with motor proteins impairs mitochondrial transport. (a) Pseudocolored image of time-lapse video microscopy of neuronal cultures treated with 50  $\mu$ M glutamate and 200 ng mI<sup>-1</sup> TNF- $\alpha$  in the presence of HDAC1 inhibitor MS-275 or HDAC6 inhibitor tubacin. Mitochondrial movement was examined using the lipophilic mitochondrial dve MitoTracker. Scale bar.  $5 \mu m.$  (b) Bar graphs indicating the average speed of moving mitochondria (mean  $\pm$  s.d.). \*P < 0.05, \*\*P < 0.01. (c) Confocal image of neurites in cultures treated with 50  $\mu M$  glutamate and 200 ng mI^{-1} TNF-  $\alpha$ for 2 h (Glu/TNF- $\alpha$ ), or in the presence of MS-275 (Glu/TNF- $\alpha$  + MS-275) or tubacin  $(Glu/TNF-\alpha + tubacin)$ . Immunoreactivity for HDAC1 (green) and NFs (red); DAPI (blue) as nuclear counterstain. (d) Bar graphs of the percentage of beaded neurites in c (mean ± s.d.; \*P < 0.05). (e) Protein complexes containing HDAC1 (left) or HDAC6 (right) and motor proteins, identified by immunoprecipitation (IP) and western blot (WB) analysis. Proteins were extracted from cultured neurons treated as indicated in a. (f) Coimmunoprecipitation with anti-dynamin of protein extracts from either untreated or MS-275-pretreated neurons exposed to 50  $\mu$ M glutamate and 200 ng ml<sup>-1</sup> TNF- $\alpha$ for 20 min or 2 h. The association of dynamin from motors was partially restored by

production (that is, corpus callosum), but was not detected in areas that were not demyelinated (that is, spinal cord) (Fig. 7b).

Previous studies related impaired axonal transport in models of Huntington's disease to deacetylation of  $\alpha$ -tubulin mediated by HDAC6, and suggested a positive effect of treatment with HDAC6 inhibitors on axonal transport<sup>28</sup>. In our experimental system, however, the impaired mitochondrial transport was not ameliorated by the presence of a pharmacological inhibitor of HDAC6 (tubacin; Fig. 8a,b), which increased the acetylation of  $\alpha$ -tubulin. These results correlated well with the observation of similar levels of acetyltubulin in control and cuprizone-treated mice (Supplementary Fig. 9a,b) and in neurons untreated or treated with glutamate and TNF- $\alpha$  (Supplementary Fig. 9c). Together, these results support the concept that distinct HDAC isoforms are differentially involved in

the induction of axonal damage caused by different stimuli.

The detection of cytosolic HDAC1, rather than HDAC6, in response to neuronal exposure to glutamate and cytokines suggested the possibility that HDAC1 inhibitors would improve mitochondrial transport. Indeed, mitochondrial transport in neurons treated with glutamate and TNF- $\alpha$  was significantly better in the presence of the inhibitor MS-275 (Fig. 8a,b) than in its absence or in the presence of the HDAC6 inhibitor tubacin (Fig. 8a,b), thereby supporting an



treatment with MS-275 or silencing Hdac1 with shRNAs. Silencing efficiency was confirmed by western blot analysis using HDAC1 antibody. (g) Western blot analysis of whole cell lysates (WCL) from cultures treated as described in e,f.

HDAC1-dependent mechanism of impaired mitochondrial transport. The respective effectiveness of MS-275 and tubacin in modulating mitochondrial transport in neurons treated with glutamate and TNF- $\alpha$  correlated with the protective role of MS-275, but not tubacin, on the induction of neurite beading (**Fig. 8c,d**). Thus, whereas HDAC1 inhibitors improved mitochondrial transport and prevented damage, HDAC6 inhibitors did not protect from damage induced by exposure to both glutamate and TNF- $\alpha$ .

Because the acetylation status of  $\alpha$ -tubulin was not affected by our experimental paradigm, we reasoned that HDAC1 modulation of axonal transport must include the involvement of other binding partners. The most likely candidates were the motor proteins KIF2A and KIF5, which were identified by MALDI-TOF. Therefore, we conducted immunoprecipitation of extracts from neurons, either untreated or treated with glutamate and TNF- $\alpha$ , with antibodies for HDAC1 or HDAC6 and assessed the identity of the binding partners by western blot analysis, using antibodies specific for  $\alpha$ -tubulin, for KIF2A, for KIF5 and for the cargo molecule dynamin (Fig. 8e). As predicted by the in vivo results, the interaction between  $\alpha$ -tubulin and HDAC1 was detected only after 20 min of treatment and was coincident with the first detection of impaired mitochondrial transport, whereas the interaction with HDAC6 was constitutive (Fig. 8e). Notably, there was a switch in the ability of KIF2A to bind distinct isoforms. Whereas in untreated neurons KIF2A was predominantly bound to HDAC6, in treated neurons this interaction was detected primarily with HDAC1 (Fig. 8e). Finally, while HDAC6 was able to bind dynamin, no interaction was detected between this molecule and HDAC1. These interactions required the specific activity of distinct HDAC isoforms, because only treatment with MS-275, not with tubacin, prevented the interaction of HDAC1 with the motor proteins (Fig. 8e). Because HDAC1 interacted with motor proteins, but not with dynamin, we asked whether the ability of this protein to bind KIF2A and KIF5 was inversely correlated with HDAC1 binding. Indeed, in untreated neurons, dynamin was found in complex with KIF2A, KIF5 and α-tubulin (Fig. 8f). Exposure to glutamate and TNF- $\alpha$  disrupted this interaction, despite the constant protein levels of these molecules (Fig. 8g). Cotreatment with the HDAC1 inhibitor MS-275, in contrast, partially rescued complex formation among KIF2A, KIF5, α-tubulin and dynamin (Fig. 8f). HDAC1 silencing also restored binding of dynamin with  $\alpha$ -tubulin, but only partially restored the interaction with motor proteins (Fig. 8f). The lack of complete reversion of the effect could be explained by taking into account the efficiency of silencing in primary neurons (Supplementary Fig. 7a) or by inferring the existence of additional mechanisms modulating the glutamate-dependent effect on axonal transport. On the basis of these results, we conclude that neuroinflammatory stimuli, including excitatory amino acids and cytokines, induce calcium-dependent HDAC1 nuclear export. Cytosolic HDAC1 binds to  $\alpha$ -tubulin and kinesin motors (KIF2A and KIF5) in an activity-dependent fashion, thereby disrupting the formation of functional interactions between these motor proteins and dynamin on cargo (Supplementary Fig. 10). The impaired axonal transport, coincident with the areas of aggregation of HDAC1-motor protein complexes, might contribute to localized areas of swelling that eventually lead to irreversible transections. Therefore, the definition of the molecular interactions among motor proteins, dynamin,  $\alpha$ -tubulin and HDAC1 is likely to provide a better understanding on how to restore mitochondrial transport and axonal function.

#### DISCUSSION

Axonal damage detected in inflammatory demyelination, such as multiple sclerosis<sup>2,3</sup>, has been associated with impaired mitochondrial

function<sup>5</sup> and related to calcium entry due to aberrant activation of sodium channels or activation by excitatory amino acids and cytokine production<sup>17,20</sup>. However the molecular mechanisms linking defective mitochondrial function to calcium entry remained elusive. In this study we identified calcium-mediated nuclear export of HDAC1 as a critical modulator of impaired mitochondrial transport and the induction of axonal damage in inflammatory demyelination. Cytosolic HDAC1 was detected in the brains of humans with multiple sclerosis, in animal models of demyelination characterized by microglial infiltration and cytokine production<sup>14,31</sup>, and in cultured neurons exposed to excitatory amino acids and cytokines to mimic the inflammatory environment of the brain in multiple sclerosis<sup>30</sup>. Previous studies in other neurodegenerative disorders have focused on the neurotoxic effect of nuclear localization of other isoforms of cytosolic HDACs and suggested that neurotoxicity was the result of HDAC-dependent repression of survival genes<sup>44,45</sup>. Our results, in contrast, suggest a cytotoxic role for the exported HDAC1 that is independent of its nuclear function and that is part of the mechanism impairing mitochondrial transport and inducing neurite beading in response to excitatory amino acids and cytokines. The neuroprotective effect of treatment with leptomycin B, a pharmacological blocker of CRM1-mediated nuclear export, suggested that the role of HDAC1 in axonal damage required a cytosolic, rather than nuclear, localization. If nuclear export of HDAC1 impaired mitochondrial transport owing to its effect on the transcription of proapoptotic genes, then acetylation of nuclear substrates and changes in gene expression should have kinetics that precede impaired mitochondrial transport. Acetylation of nuclear histones and altered gene expression, to the contrary, were detected only at later stages. Thus, we conclude that axonal damage is mediated by a gain of cytosolic function for HDAC1 rather than loss of a protective nuclear function. The toxic effect of cytosolic HDAC1 on axonal transport is consequent to its ability to bind to motor proteins (KIF2A and KIF5) and α-tubulin, disrupting their ability to form complexes with cargo proteins (such as dynamin). These complexes were detected in demyelinated brain regions in animal models characterized by cytokine production by microglial cells (corpus callosum) and in neurons exposed to glutamate and cytokines, but not in undemyelinated regions (spinal cord) or in untreated cultures.

A role for cytoplasmic HDAC6 in the inhibition of axonal transport had been previously suggested for pathologies characterized by intracellular accumulation of misfolded proteins<sup>28</sup>. This role was attributed to the ability of HDAC6 to deacetylate  $\alpha$ -tubulin<sup>25</sup>. Because  $\alpha$ -tubulin deacetylation decreases its ability to recruit motor proteins, it was suggested that HDAC6-mediated inhibition of axonal transport decreases the vesicular transport of neurotrophic factors and negatively affects neuronal survival<sup>28</sup>. Our results, however, in a model of axonal damage associated with inflammatory demyelination, suggest a mechanism of impaired axonal transport that is independent of HDAC6. Deacetylation of  $\alpha$ -tubulin was not significantly changed in cultured neurons exposed to glutamate and cytokines or in animal models of demyelination. In addition, only silencing of HDAC1 and treatment with a pharmacological inhibitor of HDAC1 activity (MS-275), but not of HDAC6 activity (tubacin), were able to improve mitochondrial transport, prevent the disruption of complexes between cargos and motor proteins and reduce the formation of axonal swellings. We note that the improvement detected in the presence of pharmacological inhibitors or in response to HDAC1 silencing was not a complete reversion of the toxic effect of glutamate and TNF- $\alpha$  treatment. Previous studies have discussed the importance of phosphorylation of motor proteins in disrupting axonal transport<sup>46</sup>. We cannot formally exclude the possibility that some of

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these mechanisms of impaired axonal transport might still occur in the presence of HDAC inhibitors or HDAC1 silencing and be partially responsible for the incomplete rescue.

We suggest that distinct neurological disorders might share the morphological appearance of damage (such as beaded neurites) by differential involvement of distinct HDAC isoforms. In the case of Huntington's disease, impaired transport consequent to  $\alpha$ -tubulin deacetylation is mediated by HDAC6, whereas in neuroinflammation impaired transport is caused, at least in part, by HDAC1 nuclear export and sequestration of motor proteins into protein complexes that alter mitochondrial transport.

The concept of cytoplasmic retention of HDAC1 in pathways associated with inflammation is supported by studies in other cellular systems. Although most of the literature focuses on the nuclear role of HDAC1, a few studies in neuroblastoma cells<sup>47,48</sup> reported the translocation of HDAC1 to the cytoplasm in response to HSV-1 viral infection. In addition, in 293 and HeLa cells, the NF-KB inhibitor IκBα was shown to interact with HDAC1 and HDAC3 and sequester these proteins in the cytoplasm<sup>49</sup>. We therefore conclude that distinct HDAC isoforms have the ability to negatively regulate axonal transport by interrupting the motor protein-microtubule interaction in a context-specific fashion that is dependent on the cell type and on the pathological stimulus. The identification of HDAC1 nuclear export as an event downstream of calcium entry in response to excitatory amino acids and cytokines treatment defines an important molecular target for pathologies associated with inflammation. Future studies on the molecular characterization of the events leading to altered axonal transport might lead to the identification of new therapeutic targets preventing the irreversibility of axonal transection.

#### METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

#### ACKNOWLEDGMENTS

We thank the University of California Los Angeles Brain Bank and the UK MS Tissue Bank at Imperial College of London for providing the multiple sclerosis tissue samples, C. Seiser (University of Vienna) for providing the antibodies to the N-terminal domain of HDAC1, R. Bansal (University of Connecticut Health Science Center at Farmington) for anti-O4 hybridoma supernatant, X. Pedre and J. Li for rodent handling, S. Schreiber (Broad Institute, Massachusetts Institute of Technology) for tubacin, E. Nestler and H. Covington (Mount Sinai School of Medicine) for MS-275, S. Lagger for sharing unpublished results and critical comments on the manuscript, P. Lobel for discussions regarding MALDI-TOF data, J. Zheng and K. Teng for helpful discussion and R. Rosa for the support of the New Jersey Multiple Sclerosis Research Foundation. The study was supported by NIH-RO1 NS-42925 (P.C.) and NMSS RG-3957. J.Y.K.'s salary was supported in part also by grant no. 07-3203-BIR-E-0 from the New Jersey Commission on Traumatic Brain injury and CB1-0704-2 from the Christopher and Dana Reeve Foundation to P.C.

#### AUTHOR CONTRIBUTIONS

The majority of the experiments in the manuscript were conducted by J.Y.K. S.S. performed the first experiments in the demyelinated mice and the first mass spectrometry. K.D. performed the *in vivo* confocal analysis in demyelinated mouse brains and the SAPK experiments; O.H. and R.R. performed the analysis of the human material; Y.H. performed the qt-PCR experiments. P.C. designed the experimental plan, supervised the project and wrote the manuscript.

Published online at http://www.nature.com/natureneuroscience/.

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#### **ONLINE METHODS**

Antibodies for immunohistochemistry, immunocytochemistry and western blot. We used the following antibodies: neurofilament medium chain (Upstate; Chemicon), 1:300 for immunohistochemistry (IHC) and immunocytochemistry (ICC); neurofilament light chain (Abcam), 1:300 for ICC;  $\alpha$ -tubulin, 1:10,000 for western blot, and acetylated-α-tubulin, 1:10,000 for western blot (both Sigma); CNPase, 1:1,000 for western blot; non-phosphorylated neurofilaments, 1:20,000 for ICC, 1:500 for IHC on human sections; MBP, 1:1,000 for ICC; GFAP, 1:1,000 for ICC (all Sternberger Monoclonal); KIF2A (Abcam), 1:20,000-40,000 for western blot; HDAC1 (Affinity BioReagents), 1:4,000 for ICC and western blot; HDAC1, 1:100 for IHC and ICC, 1:500 for western blot; HDAC2, 1:100 for IHC, 1:1,000 for western blot; anti-HDAC3, 1:200 for ICC, 1:1,000 for western blot; HDAC4, 1:200 for ICC, 1:1,000 for western blot; HDAC5, 1:1,000 for western blot; HDAC6, 1:50 to 1:200 for IHC and 1:200 for western blot; HDAC7, 1:1,000 for western blot; HDAC8, 1:50 for IHC (all Santa Cruz Biotechnology); HDAC3 (Abcam), 1:1,000 for IHC; HDAC4 (Upstate), 1:100 for IHC; HDAC5 (Cell Signaling), 1:100 for IHC; HDAC7 (Cell Signaling), 1:100 for IHC; Flag (Sigma), 1:1,000 for ICC and western blot; CRM1 (BD Transduction Lab), 1:1,000 for western blot; dynamin (Abcam), 1:2,000 for western blot; KIF5 (Abcam), 1:2,000 for western blot; Tuj1 (Covance), 1:1,000 for ICC; histone H3 (Abcam), 1:2,000 for western blot; acetyl-histone H3 (Upstate), 1:5,000 for western blot; O4 hybridoma supernatant, 1:10; GFP (Chemicon), 1:1,000 for ICC.

*In vivo* model of demyelination. For details regarding the cuprizone model of demyelination, please refer to previous papers<sup>31,32</sup>. Mice were maintained in sterile, pathogen-free conditions under the Institutional Animal Care and Use Committee of Robert Wood Johnson Medical School/University of Medicine and Dentistry of New Jersey and Mount Sinai School of Medicine. For immuno-histochemistry, mice were perfused with 4% (wt/vol) paraformaldehyde (PFA) in 0.1 M phosphate buffer and the brains maintained in 30% (wt/vol) sucrose. For RNA or protein extracts, the region of the corpus callosum was dissected and frozen.

Primary cultures. Primary oligodendrocytes and astrocytes were cultured from postnatal day 1 rat pups as described previously<sup>31</sup>. Hippocampi and cortices were dissected from E18 rat or mouse embryos in Hanks' balanced salt solution (HBSS) (Gibco). After digestion with 0.025% trypsin-EDTA for 20 min at 37 °C, cells were incubated with NM10 medium: DMEM (Gibco) with 10% (vol/vol) FBS (Gibco) for 5 min at 25 °C. Cells were then resuspended in neuronal culture medium: Neurobasal supplemented with B27, 10 mM GlutaMax (all Gibco) and 1% penicillin-streptomycin (Sigma). After mechanical dissociation through a pipette, followed by passing into a 22-gauge, 11/2-inch syringe, cells were filtered through a cell strainer (BD Falcon) and maintained at 37 °C in a 5% CO<sub>2</sub> incubator. Three days after plating, 5 nM Ara-C (Sigma) was added to the culture medium to avoid glial contamination. The next day, cells were fed with fresh neuronal medium and incubated 11–12 d more at 37 °C in a 5% CO<sub>2</sub> incubator. Treatments with mouse TNF- $\alpha$  (Chemicon), glutamate (Sigma), EDTA (Sigma), leptomycin B (Sigma), MS-275 (from E. Nestler) and tubacin (a gift from S. Schreiber) were performed at the concentrations indicated in the text. After fixing cells with 1% (wt/vol) PFA for 20 min at 25 °C, cells were postfixed with 100% methanol for 5 min at -20 °C.

**Cerebellar slice cultures.** Slice cultures were obtained by dissecting postnatal day 10 cerebella from C57BL/6J mice and cutting them into 400-µm slices using a tissue chopper (SD Instruments). Slices were placed on collagen-coated Millicell-CM culture inserts (Millipore) and grown in medium containing 50% (vol/vol) basal medium with Earle's salts, 25% (vol/vol) HBSS, 25% (vol/vol) horse serum (all Gibco), 5 mg ml<sup>-1</sup> glucose and 1% (vol/vol) penicillin-streptomycin stock solution (10,000 units ml<sup>-1</sup> and 10,000 µg ml<sup>-1</sup>, respectively), in a 5% CO<sub>2</sub> incubator. Demyelination was induced after 10 d in culture by adding 0.5 mg ml<sup>-1</sup> lysolecithin and 200 ng ml<sup>-1</sup> LPS (both from Sigma) to the medium for 15–17 h. The next day, the medium was replaced with 1% PFA, postfixed in –20 °C 100% methanol and then processed for immunostaining.

**Time-lapse video microscopy.** For the time-lapse imaging of fluorescently labeled mitochondria, primary neurons were plated at a density of  $2.5 \times 10^5$ 

into 35-mm coverglass-bottom dishes (Willco Wells) or  $3 \times 10^4$  into eight-well coverglass-bottom slides (Nunc) and maintained for 14 d in neuronal culture medium. Mitochondria were labeled with 20 nM of the lipophilic mitochondrial dye MitoTracker (Invitrogen) for 30 min at 37 °C. The medium was then replaced with fresh neuronal culture medium without phenol red (Gibco) supplemented with 50 µM glutamate and 200 ng ml<sup>-1</sup> TNF- $\alpha$ . At each time point as indicated in the figures, live cell images were captured every 5 s for 5 min using the AxioObserver.Z1 inverted fluorescence microscope equipped with a controlled humidity, temperature and CO<sub>2</sub> incubation system (Carl Zeiss MicroImaging). A ×63 oil immersion objective was used to acquire images. Analysis of mitochondrial movement was performed using ImageJ (US National Institutes of Health) and the AxioVision Rel.4.7 program (Carl Zeiss MicroImaging).

To examine the relationship between morphological changes and progression of axonal damage, we used differential interference contrast imaging. Images were captured every 20–30 min for 12–16 h using AxioObserver.Z1 (Zeiss).

Immunohistochemistry and immunocytochemistry. Immunohistochemistry and immunocytochemistry were performed as described previously<sup>31</sup>. Immunoreactive cells were visualized using confocal microscopy (LSM510 or LSM710 Meta confocal laser scanning microscope; Carl Zeiss MicroImaging). Fixed frozen tissue blocks from three controls and four individuals with secondary progressive multiple sclerosis from the UK multiple sclerosis brain bank were processed for Luxol fast blue plus periodic acid Schiff staining to identify areas of white matter demyelination. The same sections were also stained with antibodies to HDAC1, SMI32 and HDAC6, after citrate buffer microwave retrieval, and directly bound secondary conjugates (Alexa Fluor 488–goat anti-rabbit, Alexa Fluor 546–goat anti-mouse). Twenty-nine areas of interest from five lesion-containing tissue blocks from four individuals with multiple sclerosis were scrutinized for HDAC1, HDAC6 and SMI32 immunoreactivity.

Silencing experiments. For efficiency and specificity of silencing,  $4 \times 10^4$  hippocampal neurons were plated in eight-well chamber slides (Nunc) and cultured for 10–11 d at 37 °C, 5% CO<sub>2</sub> incubator. Infection with the Mission TurboGFP shRNA control transduction particles (Sigma) was conducted at multiplicity of infection = 20, in the presence of 4 µg ml<sup>-1</sup> hexamethrine bromide (Sigma) at 37 °C for 2 h. Cultures were then maintained in medium for 72 h at 37 °C, 5% CO<sub>2</sub> incubator. Infection efficiency was assayed by immunocytochemistry, using antibodies to GFP and NFs. The percentage of infected cells was calculated as the number of GFP<sup>+</sup> cells divided by the total number of DAPI nuclei per field.

For immunocytochemistry (4×10<sup>4</sup> hippocampal neurons in eight-well chamber slides), quantitative PCR  $(3 \times 10^5$  hippocampal neurons in six-well plates) and immunoprecipitation  $(2 \times 10^6 \text{ cortical neurons in 15-cm dishes})$ , primary neurons were cultured for 10-11 d and then infected with lentiviral shRNA constructs from Sigma (Mission shRNA). For each isoform, four shRNA constructs were tested, and only those achieving more than 50% reduction of target are listed below. For Hdac1, shRNA no. 399, 5'-CCGGGGCTTGGGTAATAGCAGC CATTCTCGAGAATGGCTGCTATTACCCAAGCTTTTTG-3'; and shRNA no. 401, 5'-CCGGCCCTACAATGACTACTTTGAACTCGAGTTCAAAGTAGTCA TTGTAGGGTTTTTG-3'. For Hdac2, shRNA no. 396, 5'-CCGGGCTGTGAA ATTAAACCGGCAACTCGAGTTGCCGGTTTAATTTCACAGCTTTTTG-3'. For Hdac3, shRNA no. 389, 5'-CCGGCGTGGCTCTCTGAAACCTTAACTCG AGTTAAGGTTTCAGAGAGCCACGTTTTTG-3'. For Hdac4, shRNA no. 253, 5'-CCGGACTCTCTGATTGAGGCGCAAACTCGAGTTTGCGCCTCAATCAG AGAGTTTTTTG-3'. For Hdac6, shRNA no. 416, 5'-CCGGCCTGGGAAAGAAT CTACTCTACTCGAGTAGAGTAGATTCTTTCC-3'. For Hdac8, shRNA no. 001, 5'-CCGGGTGGATTTGGATCTACACCATCTCGAGATGGTGTAGATCCAAAT CCACTTTTTG-3' (all from Sigma). Neurons were infected with Mission shRNA lentiviral particles (multiplicity of infection = 20) in the presence of 4  $\mu$ g ml<sup>-1</sup> hexamethrine bromide (Sigma). Mock-infected controls consisted of neuronal cultures incubated with 4 µg ml<sup>-1</sup> hexamethrine bromide. Cells were treated with 50  $\mu$ M glutamate and 200 ng ml<sup>-1</sup> TNF- $\alpha$  for 20 min or 2 h and then processed for immunocytochemistry, quantitative PCR or immunoprecipitation.

**Transfection.** Hippocampal neurons (4 × 10<sup>4</sup>) were plated in eight-well chamber slides and cultured in neuronal culture medium for 11–12 d at 37 °C in a 5% CO<sub>2</sub> incubator. The cultures were transfected with C-terminal Flag-tagged HDAC isoforms in the pBJ5 mammalian expression vector (a gift from S. Schreiber) using

the CalPhos mammalian transfection kit (Invitrogen). Cultures were treated 72 h after transfection with 50  $\mu M$  glutamate and 200 ng ml $^{-1}$  TNF- $\alpha$  for 2 h and then fixed for immunocytochemistry.

To examine the potential region of interaction between CRM1 and HDAC1, we generated point mutations within the nuclear export sequence identified in HDAC1 using the QuickChange II SL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instruction. All leucine amino acid residues at positions 158, 161, 163 and 164 of HDAC1 in the pBJ5 vector were replaced with alanine. Wild-type or mutant HDAC1 vectors were then transfected into 293T cells using FuGENE6 (Roche), following the manufacturer's instructions. Protein extracts from transfected cells were processed for immunoprecipitation experiments 24 h later.

MALDI-TOF mass spectrometry. Total proteins of corpus callosum from untreated and 4-week cuprizone treated mice or from untreated and treated neurons were extracted with the same method used for coimmunoprecipitation. Whole protein extracts (5–8 mg) were incubated with 3  $\mu$ g of antibodies to HDAC1 or HDAC6 overnight at 4 °C. After separation by 12% SDS-PAGE (Bio-Rad), proteins were stained with SilverSNAP (Pierce). Bands with a differential pattern between control and cuprizone or untreated and treated cultures were excised and the protein sequences analyzed by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Center for Advanced Proteomics Research; Center for Advanced Biotechnology and Medicine).

**Coimmunoprecipitation and western blot analysis.** Total proteins from tissue or cultured neurons were extracted using a lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM dithiothreitol, 1 mM EDTA, 0.01% phenylmethylsulfonyl fluoride, 1 mM aprotinin and 1 mM leupeptin.

Homogenized tissue or primary neurons were incubated in lysis buffer for 20 min on ice. After sonication on ice (30 s each, three times, cells being kept on ice for 1 min between each pulse), 1.5–2.0 mg of protein extracts were incubated overnight at 4 °C with 2  $\mu$ g of antibody. Immunoprecipitated samples and whole-cell lysates were separated by SDS-PAGE and transferred onto a PVDF (Millipore) membrane using a buffer containing 25 mM Tris base, pH 8.3, 192 mM glycine, 20% (vol/vol) methanol for 1 h at 100 V at 4 °C. Western blot analysis was performed using the appropriate dilution of primary antibodies. Immunoreactive bands were visualized using horseradish peroxidase–conjugated secondary antibodies (Amersham), followed by chemiluminescence with ECL-Plus Western Blotting Detection System (Amersham).

**Quantitative RT-PCR.** Cells were collected in Trizol reagent (Invitrogen) and RNA was isolated following manufacturer's instruction and cleaned using a RNeasy Mini kit (Qiagen). Total RNA 0.5–1  $\mu$ g was used in 20  $\mu$ l of reverse transcription (RT) reaction, using SuperScript RT-PCR kit (Invitrogen). Quantitative RT-PCR was performed using Applied Biosystems SYBR Green PCR master mix in 384-well plate in an ABI 7900HT Sequence Detection PCR system. The PCR was performed in a 20- $\mu$ l reaction mixture containing 0.2  $\mu$ l cDNA as template and 100 nM specific oligonucleotide primer pairs (**Supplementary Table 1**) with 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. The melting curve of each sample was measured to ensure the specificity of the products. Data were normalized to the internal control *Gapdh* and analyzed using Pfaffl  $\Delta\Delta$ Ct method.

**Statistical methods.** Results are expressed as mean  $\pm$  s.d. or s.e.m. and statistically analyzed using two-tailed Student's *t*-tests. *P* < 0.05 was considered to be statistically significant.





Presentation Abstract

Program#/Poster#:	245.8/K25
Title:	Epigenetic consequences of traumatic brain injury (TBI)
Location:	South Hall A
Presentation Time:	Sunday, Oct 18, 2009, 4:00 PM - 5:00 PM
Authors:	* <b>D. P. CROCKETT</b> <sup>1</sup> , J. KIM <sup>2</sup> , P. CASACCIA-BONNEFIL <sup>2</sup> ; <sup>1</sup> Dept Neurosci & Cell Biol, Univ. Med. & Dent., Piscataway, NJ; <sup>2</sup> Depts Neurosci. & Genet., Mount Sinai Sch. of Med., New York, NY
Abstract:	According the CDC, at least 1.4 million people each year suffer from traumatic brain injury with the cost to the economy estimated to be in excess of \$60 billion. The many troubling consequences of TBI include diffuse axonal injury (DAI), secondary cell death (including oligodendrocytes) and inflammation. Many of these pathologies are progressive and are associated with the deep and subcortical white matter. This ongoing pathology probably accounts for the delayed cognitive and behavioral deterioration that is often associated with TBI. Several recent animal studies involving experimental stroke and TBI have suggested that injury may result in significant alteration in histone acetylation and the possible alteration in the subcellular expression (localization) of specific isoforms of histone deacetylases (HDACs) that initiate cascades of events leading to axonal degeneration and delayed neuronal and oligodendrocytic apoptosis. HDACs and histone acetyltransferases (HATs, which are responsible for the acetylation of histones) regulate gene expression by altering the physical characteristics of chromatin. Deacetylation of histones result in gene suppression by compacting the chromatin while acetylation neutralizes the charges on the histones, relaxes the chromatin and promotes gene expression. Pharmacological inhibition of HDACs has led to improved outcomes following injury in animals, perhaps by restoring HDAC-HAT homeostasis disrupted by inflammation. With the hope of

developing novel therapies, we are systematically investigating the effects of TBI on various epigenetic factors. We hope to elucidate the cellular and subcellular alterations in HDAC and histone isoform expression that occurs in the injured brain. Preliminary findings with a penetrating injury model have revealed a significant association between increased HDAC1 expression and B-amyloid precursor protein, a maker of axonal pathology within the subcortical white matter one month after injury.



Disclosures: D.P. Crockett, None; J. Kim, None; P. Casaccia-Bonnefil, None.

Keyword(s): HDAC

Histone

Axonal Damage

Support: New Jersey Commission for Brain Injury Research

[Authors]. [Abstract Title]. Program No. XXX.XX. 2009 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2009. Online.

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