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

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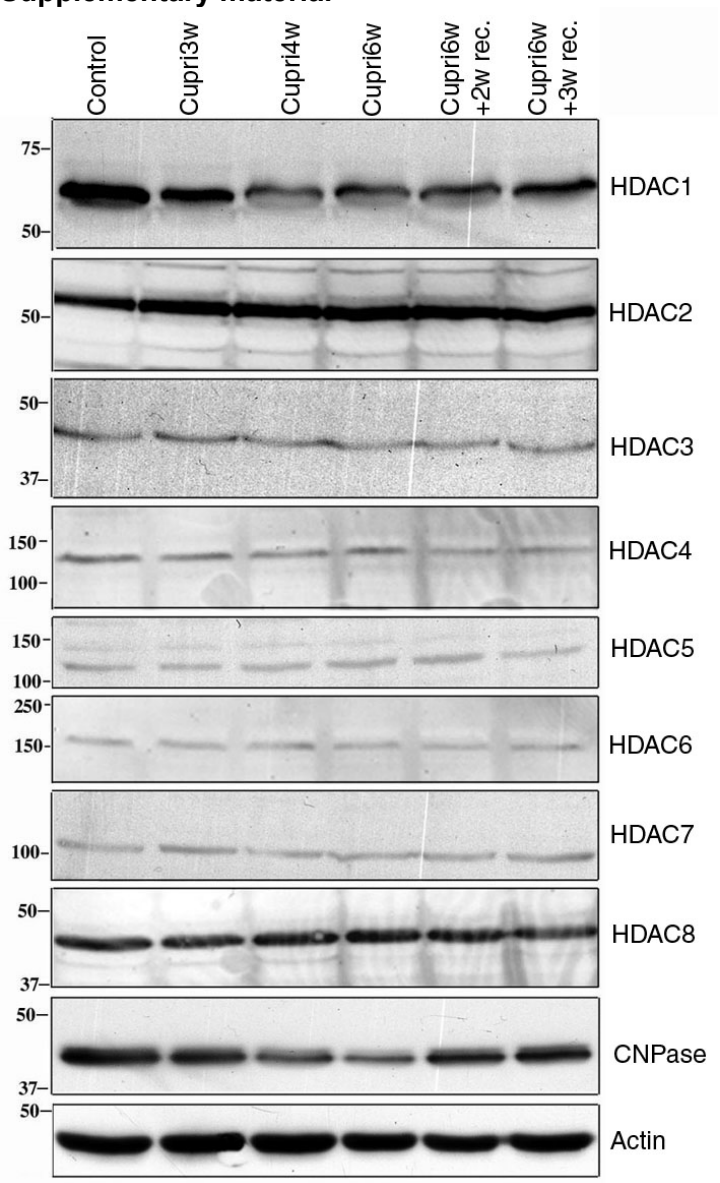
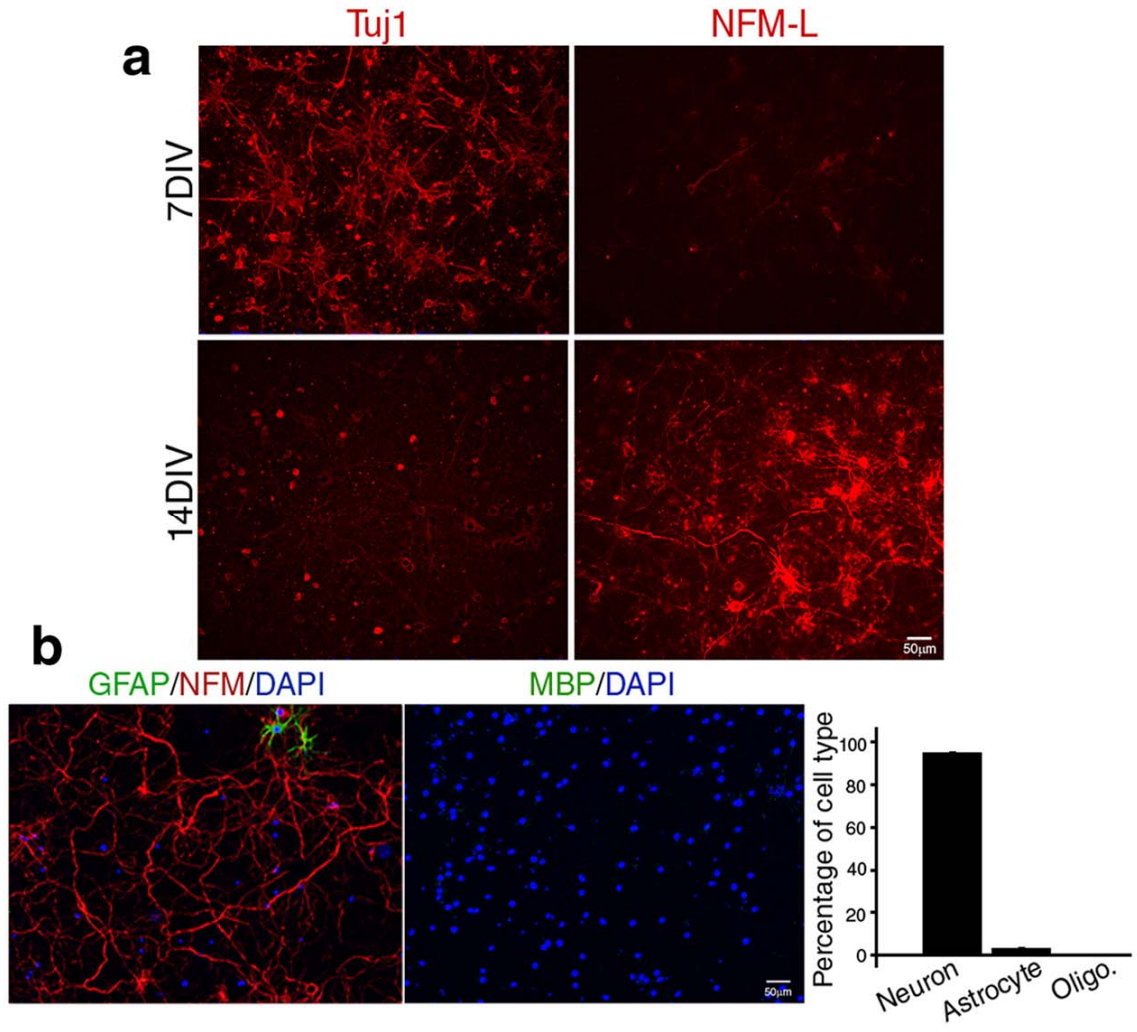
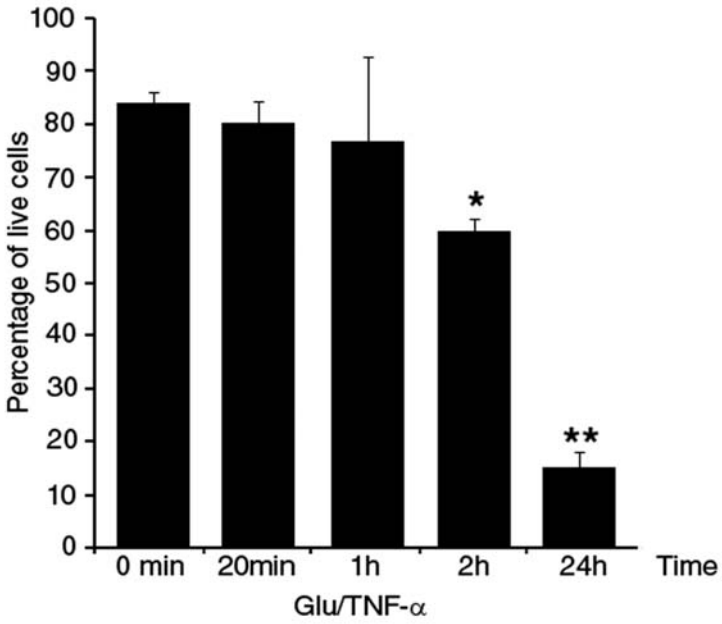
Supplementary material



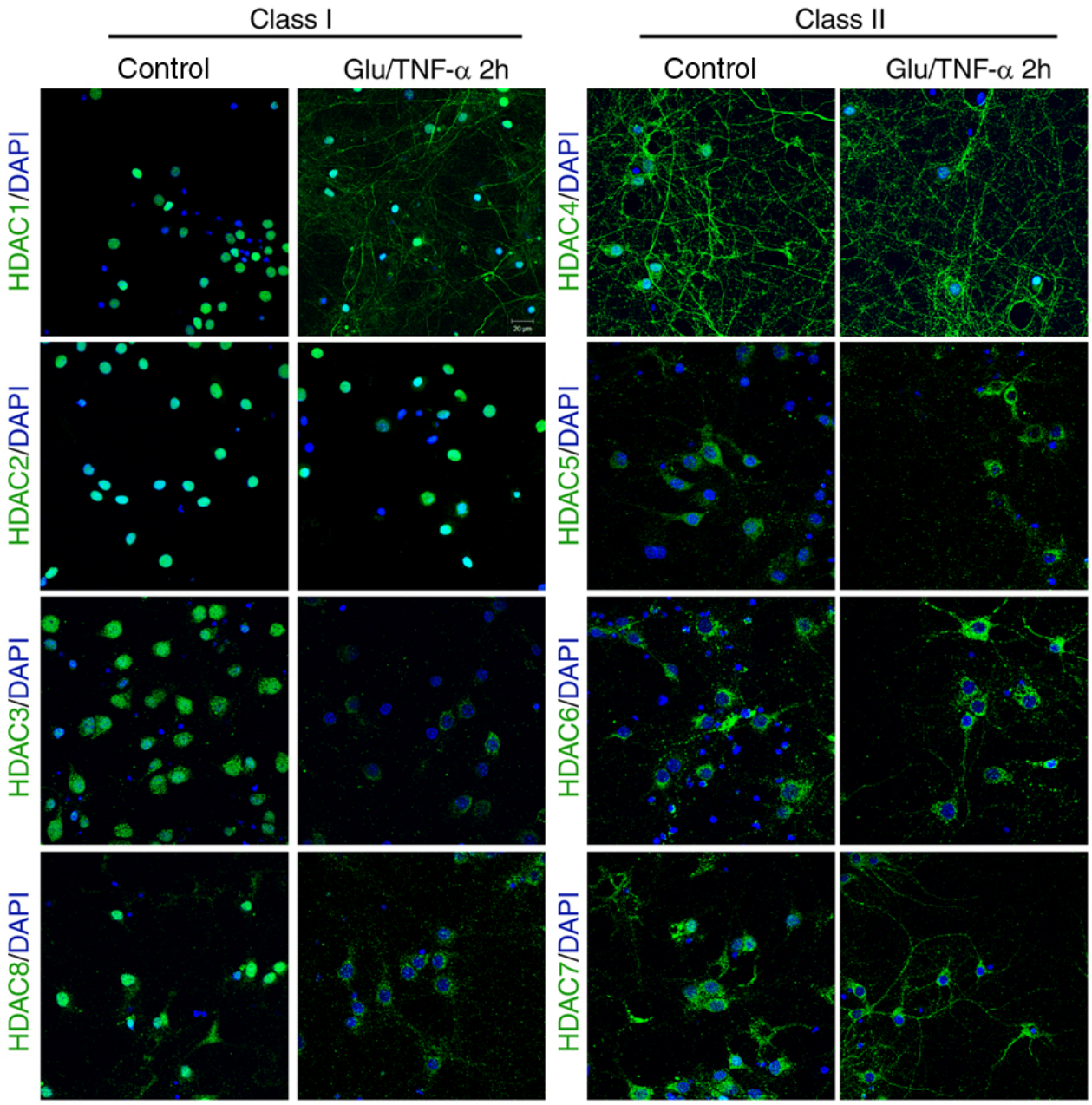
Supplementary Figure 1. Western blot analysis of the levels of multiple HDAC isoforms in callosal extracts from mice fed on a cuprizone diet for the indicated time points. Protein extracts were obtained from the corpus callosum of mice, kept on a 0.2% cuprizone diet for the indicated time points. After separation, western blot analysis was performed with the antibodies listed on the right side. Demyelination was assessed by the decreased levels of the myelin gene CNPase (detected at 4 and 6 weeks). Actin was used as loading control.



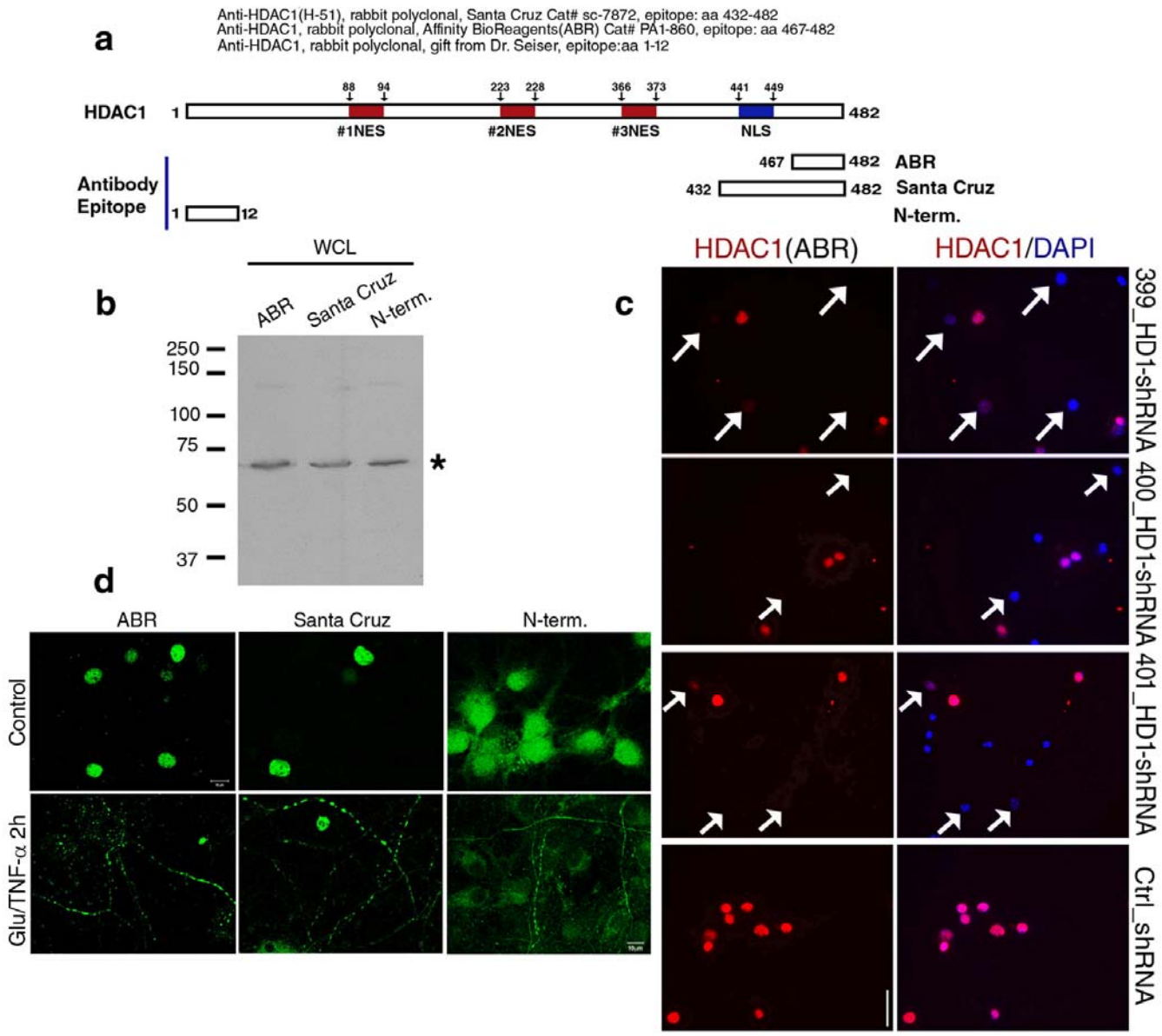
Supplementary Figure 2. Characterization of primary neuronal cultures (a) Immunocytochemistry of primary neuronal cultures kept either 7 (7DIV) or 14 days in vitro (14DIV) and stained with antibodies for Tuj1 (red) to label neuroblasts or NFM-L (red) to label differentiated neurons. Scale bar 50µm. (b) The presence of other cell types was assessed in 14DIV cultures by immunocytochemistry for NFM (red) and GFAP to label astrocytes (green), or MBP (green) to identify oligodendrocytes. DAPI (blue) was used as nuclear counterstain. The quantification of the results is shown on the right panel.



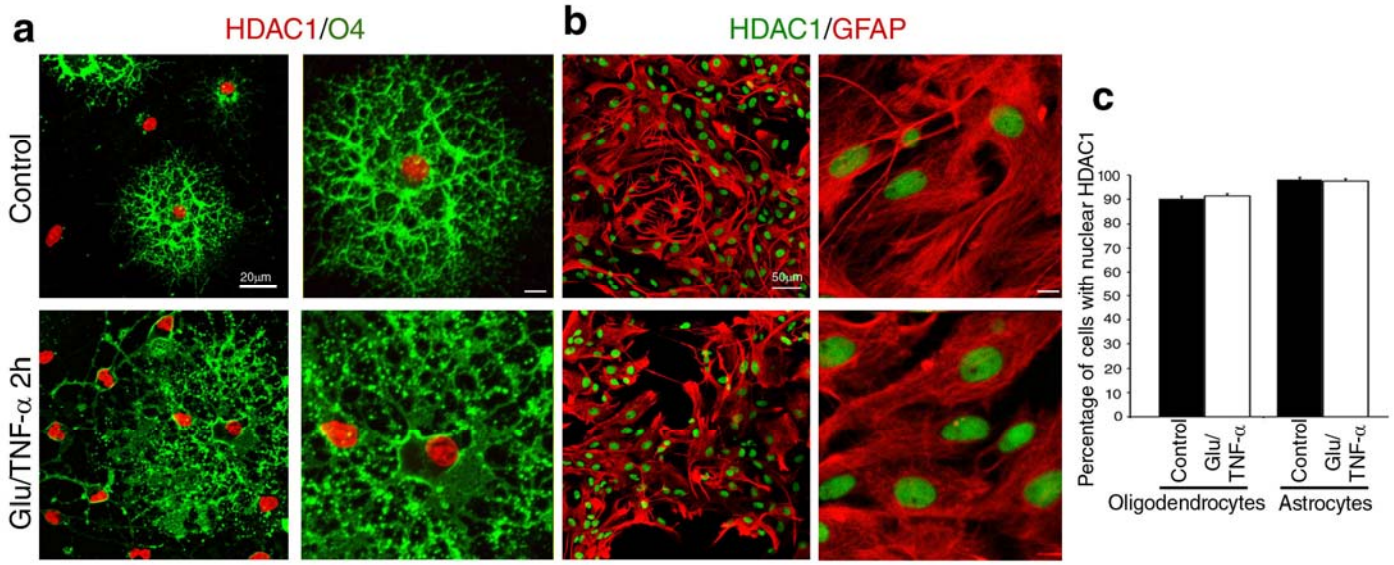
Supplementary Figure 3. Decreased cell survival is detected only after prolonged exposure to glutamate and TNF-α. Live-dead cell tests were performed in neurons after exposure to 50μM glutamate/200ng/ml TNF-α for the indicated time periods. Bar graphs indicate the percentage of live cells relative to the total DAPI+ nuclei at 24 hour after treatment; error bars represent standard deviation. * $P < 0.05$, ** $P < 0.01$



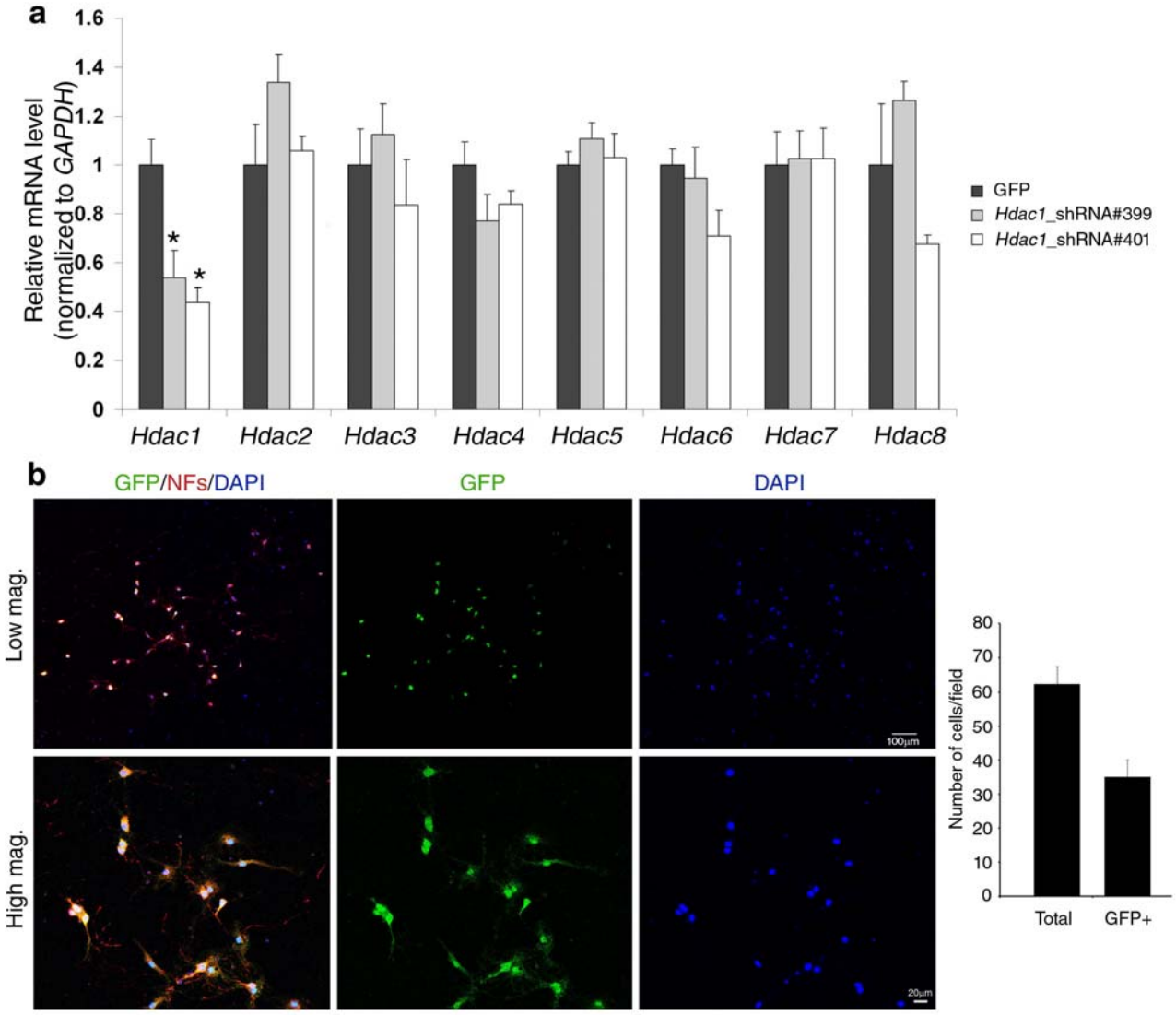
Supplementary Figure 4. Export of HDAC1 from the nucleus to the neurites of cultured neurons is induced by treatment with glutamate and TNF-α. Confocal images of cultured neurons either untreated (Control) or exposed to 50μM glutamate/ 200ng/ml TNF-α for two hours followed by staining with antibodies against class I (a) or class II (b) HDAC isoforms (green) and DAPI (blue) as nuclear counterstain. Scale bar 10μm, 63x objective.



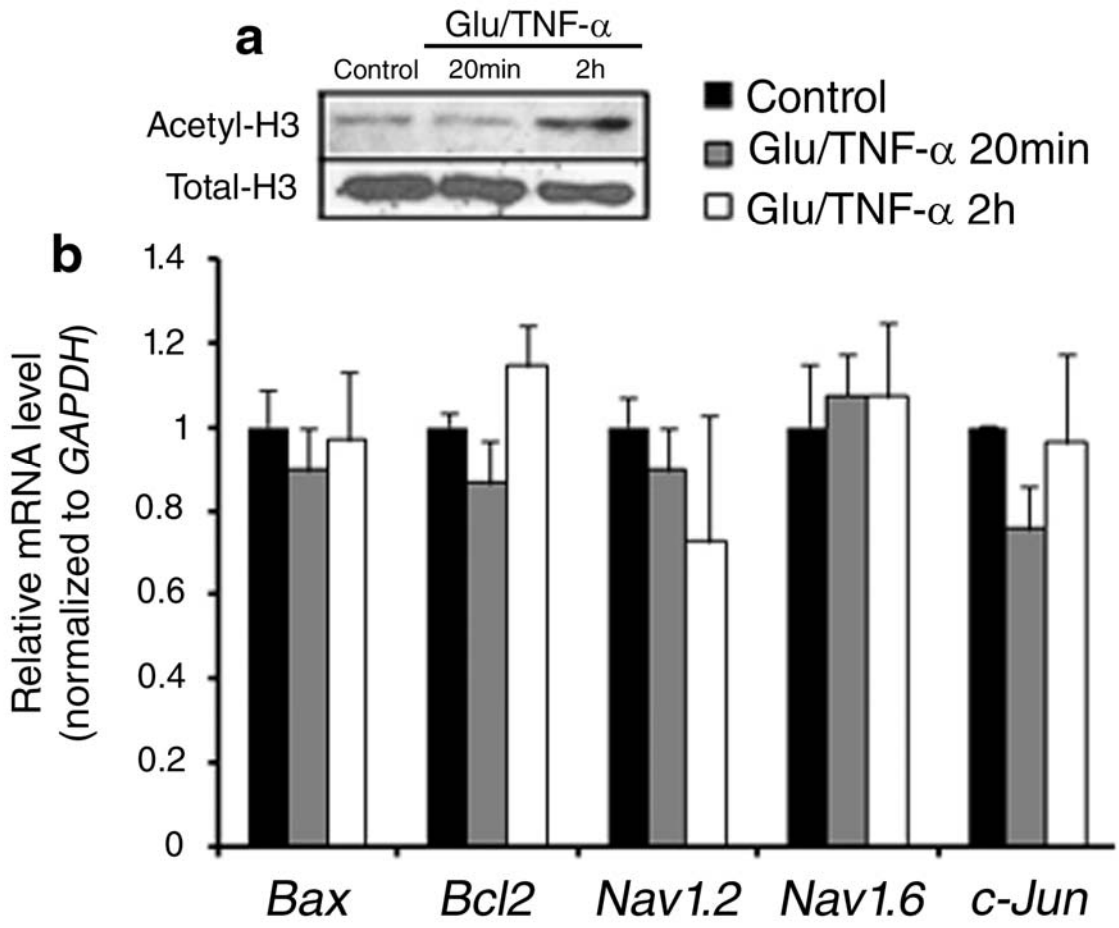
Supplementary Figure 5. Specificity of antibodies for HDAC1 (a) A schematic diagram of HDAC1 protein structures and relative epitopes of commercially available antibodies from Affinity BioReagents (ABR) and Santa Cruz Biotechnology (Santa Cruz) or generated by Dr. Seiser, University of Vienna (N-term.). Blue box indicates the nuclear localization sequence (NLS). (b) To examine the specificity of HDAC1 antibody, first, whole cell lysate (WCL) of corpus callosum from C57BL/6J was immunoblotted with anti-HDAC1 antibodies. * indicates the band immunoreactive with HDAC1 antibodies. (c) For silencing of *Hdac1*, cultured murine neurons were infected with either control (Ctrl_shRNA) or three different lentiviral particles (399_HD1-shRNA, 400_HD1-shRNA, and 401_HD1-shRNA) against *Hdac1* (arrow). 72 hours after infection, cultures were processed for immunocytochemistry using antibodies specific for HDAC1 (ABR, red) antibody. DAPI (blue) was used as nuclear counterstain. Scale bar 50 μm. (d) Confocal images of cultured neurons either untreated (Control) or exposed to toxic stimuli for two hours followed by staining with HDAC1 antibodies from different sources (Santa Cruz, ABR, and N-term.). Note that the cytosolic localization upon exposure to glutamate and cytokines was detected with all three reagents.



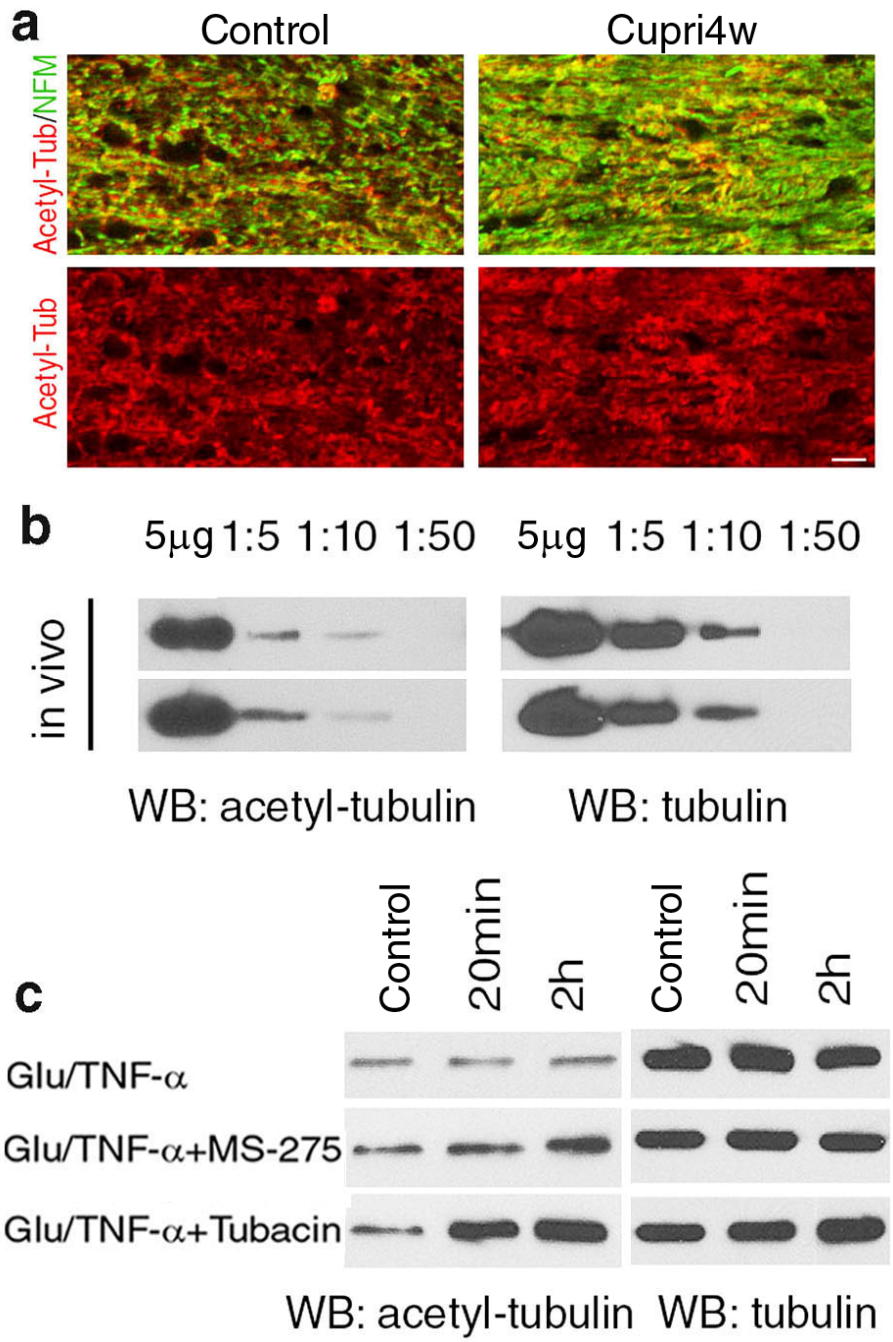
Supplementary Figure 6. The inducible nuclear export of HDAC1 is cell-specific and does not occur in glial subtypes. Primary oligodendrocytes (a) and astrocytes (b) were exposed to 50μm glutamate/200ng/ml TNF-α for 2 hours and then processed for immunocytochemistry. (a) Oligodendrocytes were stained with anti-HDAC1 (red) and O4 (green) antibodies. Scale bar 20 μm in left panel and 5 μm in right panel. (b) Astrocytes either untreated (Control) or treated with toxic stimuli were stained with anti-HDAC1 (green) and GFAP (red). Scale bar 50 μm in left panel and 10 μm in right panel. (c) Quantification of the percentage of cells with nuclear HDAC1 showed that the same treatments responsible for HDAC1 nuclear export in neurons did not affect the subcellular localization of the enzyme in glial cells.



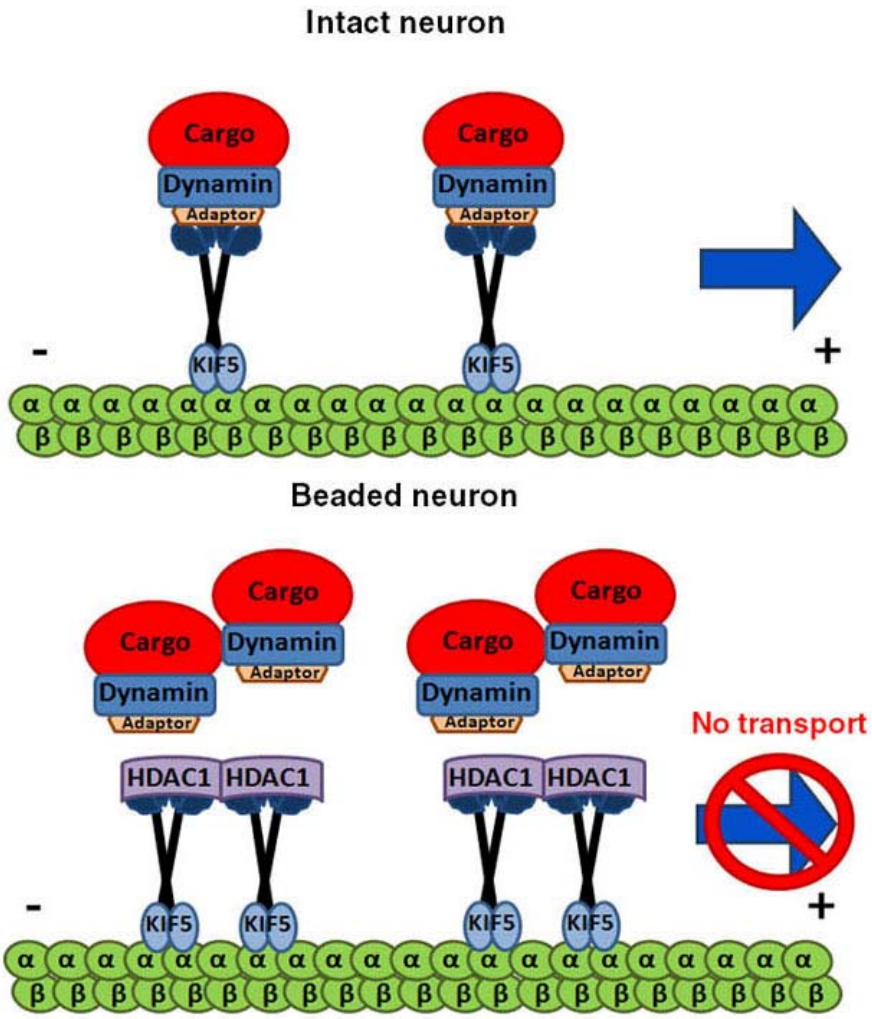
Supplementary Figure 7. Specificity and efficiency of HDAC1 silencing. (a) Quantitative PCR of RNA samples isolated from cells were infected with either control shRNA (i.e. GFP turbo) or two different *Hdac1* constructs in lentiviral vectors (shRNA#399 and shRNA# 401 from Sigma). 72 hours after infection, RNA was extracted from infected cells and processed for quantitative PCR using primers for the indicated *Hdac* isoforms. Bar graphs represent the average value transcript levels, relative to *GAPDH*. *$P < 0.05$ (b) Immunocytochemistry for GFP (green) and neurofilament (red), to verify the efficiency of gene transfer mediated by lentiviral particles. DAPI (blue) was used as nuclear counterstain. Scale bar 100µm upper panel and 20µm lower panel. The bar graphs indicate the number of transduced cells.



Supplementary Figure 8. HDAC1 nuclear export does not affect gene expression prior to the onset of neuritic swelling. (a) The levels of acetyl-H3 were examined using western blot analysis with antibodies specific for acetyl-H3 and total H3. Protein extracts from neurons either untreated (Control) or treated with 50μm glutamate/200ng/ml TNF-α for 20min or 2h were processed for western blot. Note that acetyl-H3 starts to increase only after two hours. (b) Quantitative PCR of RNA isolated from neurons either untreated (Control, black bar) or treated with glutamate and TNF-α for 20 minutes (Glu/TNF-α 20min, gray bar) or for two hours (Glu/TNF-α 2 hours, white bar). The transcript levels of *Bax, Bcl2, Nav1.2, Nav1.6* and *c-Jun* were normalized to the levels of *GAPDH*. Note that the expression levels of transcripts related to apoptosis or axonal damage were not affected by the two-hour period of glutamate and TNF-α treatment.



Supplementary Figure 9. The acetylation of α-tubulin in neurons is not affected by inflammatory stimuli. (a) Confocal images of callosal axons from untreated mice (Control) and from cuprizone-treated mice (Cupri4w). Coronal sections of brain were processed for immunohistochemistry with antibodies specific for NFM (green) and acetylated-α-tubulin (red). Scale bar 10µm (b) Serial dilutions of protein extracts from mice either untreated (Control) or kept for 4 weeks on a 0.2% cuprizone diet (Cupri4w) were separated by SDS-PAGE and then processed for western blot analysis using antibodies for total α-tubulin (right) and for acetyl-tubulin (left). No difference was detected between untreated and treated animals (c) Whole cell lysates (WCL) from primary neurons treated with only 50uM glutamate/200ng/ml TNF-α or with toxic stimuli and each HDAC inhibitor were processed for immunoblot analysis using specific antibodies against to acetylated-α-tubulin (left) and α-tubulin (right). No difference in acetylation level of α-tubulin was detected between untreated and treated cultures.



Supplementary Figure 10. Schematic model of the mechanism relating HDAC1 to impaired axonal transport in response to glutamate and cytokines. Top panel (Intact neuron): axonal transport (blue arrow) of cargo (red) occurs in physiological condition because of the interaction between dynamin and adaptor proteins (orange) and anchorage to motor proteins (KIF5, light blue circles with black bar) thereby allowing for movement along the microtubules (green). Lower panel (beaded neuron): impaired transport in neurons exposed to inflammatory stimuli, due to competition between HDAC1 and the adaptor proteins for binding to the motor proteins. In these conditions, the dynamin on cargo is unable to form functional interactions with the motors and therefore axonal transport is blocked.