Human Endogenous Retrovirus K Envelope in Spinal Fluid of Amyotrophic Lateral Sclerosis Is Toxic

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Objective: Human endogenous retroviruses have been implicated in neurodegenerative diseases including amyotrophic lateral sclerosis (ALS). Expression of human endogenous retrovirus K (HERV-K) subtype HML-2 envelope (Env) in human neuronal cultures and in transgenic mice results in neurotoxicity and neurodegeneration, and mice expressing HML-2 Env display behavioral and neuromuscular characteristics resembling ALS. This study aims to characterize the neurotoxic properties of HML-2 Env.
Methods: Env neurotoxicity was detected in ALS cerebrospinal fluid and confirmed using recombinant Env protein in a cell-based assay and a mouse model. The mechanism of neurotoxicity was assessed with immunoprecipitation followed by mass spectrometry and Western blot, and by screening a panel of inhibitors.
Results: We observed that recombinant HML-2 Env protein caused neurotoxicity resulting in neuronal cell death, retraction of neurites, and decreased neuronal electrical activity. Injection of the Env protein into the brains of mice also resulted in neuronal cell death. HML-2 Env protein was also found in the cerebrospinal fluid of patients with sporadic ALS. The neurotoxic properties of the Env and the cerebrospinal fluid could be rescued with the anti-Env antibody. The Env was found to bind to CD98HC complexed to β1 integrin on the neuronal cell surface. Using a panel of compounds to screen for their ability to block Env-induced neurotoxicity, we found that several compounds were protective and are linked to the β1 integrin pathway.
Interpretation: HERV-K Env is released extracellularly in ALS and causes neurotoxicity via a novel mechanism. Present results pave the way for new treatment strategies in sporadic ALS.

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Approximately 8% of the human genome is composed of retroviral sequences that have been integrated through repeated germline infections during evolution. Most of these retroviral elements either lack complete open reading frames (ORFs) or are silenced through epigenetic modifications, but few may be expressed and have been associated with a variety of diseases.[1,2] Human endogenous retrovirus-K (HERV-K) belongs to the

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beta-retrovirus-like supergroup of viruses. It was first described in 1985, when it was isolated as a human DNA sequence clone (clone HML-2) with similarities to the murine mammary tumor virus class of endogenous retroviruses.[3] Although most HML-2 proviruses do not possess complete ORFs, a few are transcriptionally active with low levels of expression in healthy tissues such as brain, heart, peripheral blood mononuclear cells, lung, liver, and breast,[4,5] as well as in synovial tissue.[6] HERV-K expression is especially prominent during the earliest stages of embryonic development[7–9] and in certain cancers.[10,11]

HERVs have been implicated in autoimmune, neurodegenerative, and chronic inflammatory diseases and cancer.[1,2] Among these diseases, abnormal activation and subsequent expression of HERV proteins have been reported in multiple sclerosis and amyotrophic lateral sclerosis (ALS).[12-15] ALS is a neurodegenerative disease characterized by loss of both upper and lower motor neurons. Most ALS cases are sporadic, but 10 to 20% patients show familial disposition. The symptoms usually begin in one anatomical region and then spread, causing motor paralysis and dysphagia, and ultimately affect respiratory function. In some patients, involvement of the prefrontal cortex leads to cognitive dysfunction. Several studies have found evidence of reverse transcriptase activity in brain, cerebrospinal fluid (CSF), and blood of patients with ALS (reviewed in Alfahad and Nath[16]), which was identified to be encoded by the *pol* gene of HML-2 in brains of patients with sporadic ALS.[17] Although two groups did not see a difference between HERV-K gene expression between ALS and controls,[18-20] we have identified activation of specific loci in ALS brain with ORFs for HML-2 envelope (Env).[17,21] Additionally, HML-2 Env expression was detected in cortical and anterior horn cells of the spinal cord of ALS patients, and a transgenic mouse model expressing the HML-2 Env protein recapitulated many of the phenotypic and pathological features seen in ALS.[21]

Although there is considerable evidence linking HML-2 to ALS, very little is known about the mechanisms by which it may cause observed neurotoxicity. In this study, we have demonstrated that Env can be released extracellularly and detected in ALS CSF. We then characterized the neurotoxic properties of the Env protein to determine the underlying pathophysiological mechanisms and identify potential therapeutic targets.

Materials and Methods

Cell Culture and Reagents

Human neural stem cell (NSC)-derived neuronal cultures were prepared as previously described.[22] Briefly, NSCs at 80% confluency were differentiated into neurons with neuronal differentiation medium (DMEM/F12 with GlutaMax, 1.8% bovine serum albumin, 1× StemPro hESC supplement [A1000701; Thermo Fisher Scientific, Waltham, MA], 10ng/ml brain-derived neurotrophic factor [450-02; Peprotech, Huissen, the Netherlands], and glial cell-line derived neurotrophic factor [450–10, Peprotech]). Neurons at 7 to 10 days in vitro were used in various assays. The recombinant HERV-K (HML-2) Env protein from MyBioSource (San Diego, CA; #MBS1391552) utilized in all experiments contained an intact surface unit domain and a transmembrane domain (amino acids 90–632 of consensus sequence).

Cerebrospinal Fluid

CSF was obtained from patients with ALS and healthy controls following informed consent and approval by the institutional review board of the National Institutes of Health (NIH). Diagnosis of ALS was made using the El Escorial criteria.[23] None of the patients had a family history of ALS. CSF was centrifuged within 30 minutes of the lumbar puncture, and cell-free CSF was aliquoted and stored at −80°C until used. A fresh aliquot of CSF was used for each assay without undergoing freeze thaw cycles.

Development and Characterization of Monoclonal Antibody to HML-2 Env Protein

A monoclonal antibody (K01) to the Env protein of HML-2 was developed by GeNeuro (Plan-les-Ouates, Switzerland). Female OF mice (Charles River, Wilmington, MA) were immunized with HML-2-Env (MyBioSource, #MBS1391552). Hybridomas were generated as described previously,[24] and clones were screened for production of high-affinity monoclonal antibody that selectively bound to a highly conserved epitope on the surface domain of the HML-2 Env protein. K01 is a full-length antibody of the IgG2b/ kappa subclass. Specificity and sensitivity were confirmed using enzyme-linked immunosorbent assay and Western blotting immunoassays. It recognizes both native and denatured HML-2 Env protein as well as nonglycosylated and glycosylated forms. For visualization of the antibody-targeted epitope of HML-2, the predicted structure for endogenous retrovirus group K member 113 Env polyprotein was retrieved from the AlphaFold Protein Structure Database.[25] The molecular graphics and epitope visualization were performed using UCSF Chimera, a molecular modeling system developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco with support from NIH P41-GM103311.[26]

Neurotoxicity Assay

Human neuronal cultures (20,000 cells/well), stably expressing tdTomato fluorescent protein to label the cells and processes, were seeded into 96-well plates and maintained at 37°C with 5% CO_2. Neurons were treated with recombinant HML-2 Env protein at varying concentrations. For all subsequent experiments, Env was used at 100nM concentration. Neurons were incubated with K01 or control mouse monoclonal IgG2b (MA1-10418, Thermo Fisher Scientific) antibodies (3μg/ml) for 60 minutes, and Env protein (100nM) was then added, unless otherwise noted. Heat-inactivated Env (95°C for 15 minutes, cooled to 4°C, and warmed to 37°C prior to adding to neurons)

was used as a control. The cells were imaged with the GE (Milwaukee, WI) INCell Analyzer 2000 BioImager or Molecular Devices (Sunnyvale, CA) ImageXpress Micro Laser Confocal imager (4 images/well) at 24, 48, 72, and 96 hours post-treatment. In a separate set of experiments, CSF (#9) was applied to neurons in the presence and absence of K01 or control IgG, and images were captured at 48, 60, 72, and 84 hours postexposure on the NanoEntek (Seoul, South Korea) JuLI Stage or BioTek (Winooski, VT) Cytation5/BioSpa bioimager. In another set of experiments, Env protein (100nM) was applied to the neurons in the presence or absence of K01 (3μg/ml), anti-CD98 (PIPA581032; Invitrogen, Carlsbad, CA; 3μg/ml), and control IgG (3 or 6μg/ml). High content imaging/analysis was achieved with GE Investigator 1.93 analysis software and MetaMorph (Molecular Devices). Neuronal viability and neurite length were quantitated and plotted with GraphPad Software (San Diego, CA) Prism 7.02.

Electrophysiological Analysis Using Microelectrode Array

Human neurons (200,000 cells/well) were seeded into a 48-well microelectrode array (MEA) plates (M768-tMEA-48B; Axion BioSystems, Atlanta, GA) containing 16 active recording electrodes/well. Electrophysiological activity, noted by increased spike rate in the wells, increased significantly by 21 days in vitro and was monitored by recording spontaneous electrical activity in all wells for 5 minutes per day to establish a baseline. Neurons were preincubated with K01 or control nonimmune IgG (3μg/ml) for 60 minutes and then treated with Env protein (100nM). One sample of K01 (3μg/ml) was preincubated with Env (100nM) for 30 minutes, and then added together to the neurons. In separate experiments, CSF samples were thawed on ice, diluted 100-fold in differentiation media, and added to the neurons. Spontaneous electrical activity was recorded daily, beginning at 24 hours post-treatment. Quantitation of electrical activity (number of spikes, mean firing rate, and number of bursts) was completed with Axion BioSystems AxIS software.

In Vivo Toxicity of HML-2 Env

Animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH. Three-month-old adult C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME).

The Env protein was diluted to a final injection dosage of 160ng per mouse. Tris–ethylenediaminetetraacetic acid (EDTA) buffer with 10% glycerol was prepared as a vehicle control. K01 antibody and mouse IgG2b isotype control monoclonal antibody were diluted to 480ng per mouse. Env protein or Tris-EDTA glycerol buffer was incubated with isotype control antibody or K01 at room temperature for 1 hour prior to injection.

Three mice per group were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg). Using a stereotaxic apparatus, layer 5 of the motor cortex of the left hemisphere was injected with 2μl of each solution at a rate of 0.1μl/min using a microinjector. Brain tissues were collected at 1 week post-injection, fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline (PBS), and cryopreserved in 10% sucrose in PBS and frozen until sectioning. The brain was cut into 30μm coronal sections containing the primary motor cortex. Sections ranging 1.7mm from the bregma to 0.1mm from the bregma at 200- to 300μm intervals were selected for staining with Cresyl violet solution (PS102-01; Fd Neurotechnologies, Columbia, MD). Lesion volume was estimated using Image J software. Apoptotic neurons were detected by In Situ Apoptosis detection kits (S7100; Millipore, Billerica, MA). Sections were counterstained with hematoxylin (Dako, Glostrup, Denmark). Images were processed using an Aperio whole slide scanner (Leica Biosystems, Nussloch, Germany). Deoxyuridine triphosphate nick-end labeling (TUNEL)-positive (apoptotic) cells were quantified.

Detection of HML-2 Env Protein in CSF by Immunocapillary Western Blot

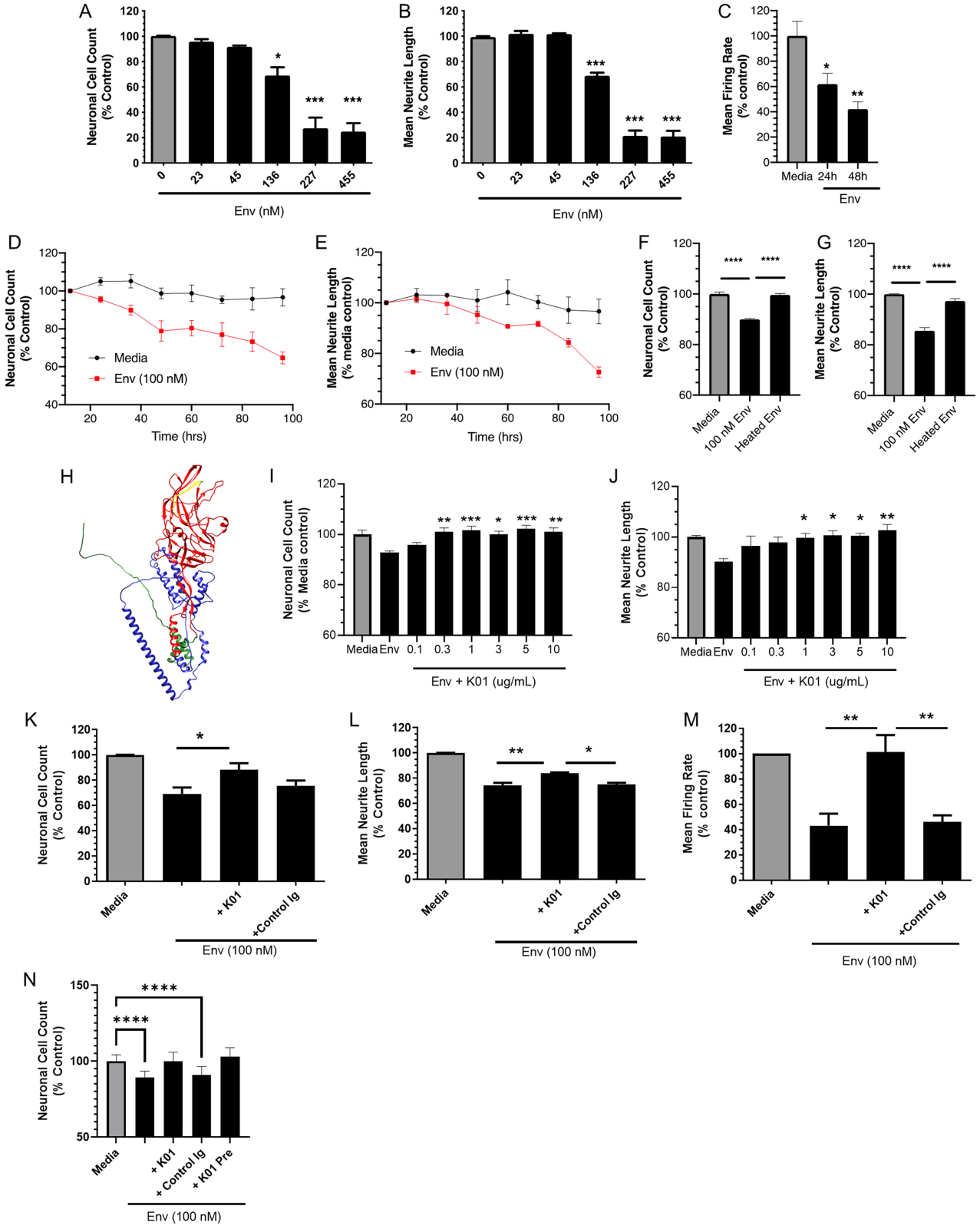
HERV-K ENV antigen detection was performed on a Wes device for Western blot analysis using an automated capillary-based size sorting and immunolabeling system (ProteinSimple, San Jose, CA). Four microliters of nondiluted CSF, HML-2 Env recombinant protein (1μg), or 1× PBS were loaded in each capillary of the 12- to 230kDa separation matrix, and K01 (30μg/ml) was used as primary antibody. Detection was performed using the antimouse detection module horse radish peroxidase (ready to use, ProteinSimple). The chemiluminescent signal was quantified by the Wes platform and was returned under electropherograms or digital Western blots representation using Compass software (ProteinSimple). A standard curve was plotted using recombinant Env (5–2,000ng/ml). The concentration of the Env in the CSF samples was determined by measuring area under the curve and then comparing it to the standard curve.

Quantification of HERV-K Env RNA in CSF Samples by Digital Polymerase Chain Reaction

CSF samples were centrifuged at 300 × *g* for 10 minutes to remove cells. Total nucleic acids were extracted from 400μl of the supernatants with an EZ1 Advance XL device (QIAGEN, Hilden, Germany) and the EZ1 Virus Mini Kit v2.0 (QIAGEN). Extracted nucleic acids were eluted in 60μl of RNAse-free water and treated with the Turbo DNA-free kit (Thermo Fisher Scientific). Reverse transcription was performed with the SuperScript III First-Strand Synthesis SuperMix kit (Thermo Fisher Scientific), with no reverse transcriptase controls for each sample. The reaction was performed in duplicate with the AutoDG Droplet Digital polymerase chain reaction (PCR) system (Bio-Rad Laboratories, Hercules, CA) with a set of primers and a probe specific to HML-2 *env*[27] and cellular RPP30 primers as a control. PCR was performed in a T100 Thermal cycler (Bio-Rad Laboratories) at 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds and 60°C for 1 minutes, and 95°C for 10 minutes. The number of copies was determined in a QX200 Digital PCR reader (Bio-Rad Laboratories).

Immunoprecipitation and Western Blot Analysis

A total of 293 T cells were transfected with CD98 (OriGene, Rockville, MD; RC200178) using Lipofectamine 3000. Cells were incubated for 48 hours and treated with 100nM Env protein for 1 hour at 37°C. One million cells/immunoprecipitation were lysed using radioimmunoprecipitation assay buffer and



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immunoprecipitated overnight at 4°C using anti-IgG (Cell Signaling Technology, Danvers, MA; 5412S), anti-6XHis (Abcam, Cambridge, UK; 9108), or anti-CD98 (Invitrogen, PA5-81032). Complexes were precipitated using Protein A/G beads (Pierce, Rockford, IL) and washed 2× with TNE300 + 0.1% NP-40, 2× with TNE50 + 0.1% NP-40, and 2× with PBS + 0.05% Triton X-100 (PBS-T). Beads were resuspended in 30µl Tris-Glycine Sample Buffer supplemented with 2-mercaptoethanol, incubated at 95°C for 1 hour, and run on a 4 to 20% Tris-Glycine gel (Invitrogen). The gel was transferred on to a polyvinylidene difluoride membrane using an iBlot2 (Thermo Fisher Scientific, IB24001), blocked for 1 hour with 5% milk in PBS-T, then incubated overnight with anti-CD98 (Abcam, 108300). The membrane was incubated with appropriate secondary antibody for 1 hour at room temperature and visualized.

Immunocytochemistry

Neurons were seeded into a glass bottom dish (300,000 cells/dish), treated with 100nM of Env protein, and incubated at 37°C for 24 hours. Neurons were then fixed with 4% PFA for 30 minutes and permeabilized and blocked with PBS-T + 5% normal goat serum for 30 minutes at room temperature. K01 (1:100) and CD98 primary antibodies (Abcam, 108300; 1:100) were incubated overnight at 4°C. Cells were incubated with α-mouse-488 and α-rabbit-594 (1:500) for 1 hour at room temperature and then stained with 4,6-diamidino-2-phenylindole.

Statistical Analyses

Statistical analyses were conducted with analysis of variance using Prism. When an overall difference of $p < 0.05$ was obtained, group differences were calculated by post hoc analysis using Tukey multiple comparisons test. A simple linear regression was used to determine a correlation between the concentration of the Env in the CSF and the Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS).

Results

HML-2 Env Causes Neurotoxicity

We have shown that HML-2 Env expression can cause toxicity in human neurons in vitro and contribute to neurodegeneration in vivo.[21] To further investigate this, neurons were treated with increasing concentrations of recombinant HML-2 Env protein and assessed for neurotoxicity, in terms of neuronal cell counts and mean neurite length (Fig 1A,B). Neurons treated with 136nM Env protein displayed 35 to 40% loss of neurons and shortening of their neurites, and higher concentrations showed >80% neurotoxicity.

To observe changes in electrophysiology and progressive morphological changes in neurons in response to HML-2 Env exposure, time course experiments were conducted (see Fig 1). Human neurons were seeded into a 48-well MEA plates for 28 days, and spontaneous electrical activity was recorded to establish baseline data prior to treatment. Neurons were then treated with Env protein (100nM) or fresh differentiation media, and spontaneous electrical activity was recorded beginning at 24 hours post-treatment. The mean firing rate decreased at 24 and 48 hours postexposure, by 40% and 59%, respectively. Similarly, treatment of neurons with 100nM Env protein caused significant decreases in cell count and mean neurite length by 24 hours postexposure, with progressive toxicity over 96 hours. Heat-treated Env (100nM) had no effect on cell number and mean neurite length.

Neurotoxicity of HML-2 Env Can Be Blocked by a Monoclonal Antibody

We developed a monoclonal antibody (K01) to a conserved epitope that maps to the surface unit HML-2 Env protein. This conserved epitope (SLDKHKHKKLQSFYP) is predicted to be on a highly accessible portion of the HML-2 protein (see Fig 1). Given the sensitivity and specificity of K01, neurons were treated with 100nM Env protein alone or in the presence of K01 (0.1–10µg/ml) for 96 hours. Protection against HML-2 Env-induced cell loss and reduction in neurite length was noted in a dose-dependent manner, with complete protection at

FIGURE 1: Neurotoxic properties of HML-2 envelope (Env). (A, B) Neurons were exposed to different concentrations of recombinant Env protein and analyzed at 96 hours post-treatment for (A) neuronal cell count and (B) mean neurite length. (C) Neurons were treated with 100nM Env protein, and spontaneous electrical activity was recorded at 24 hours and 48 hours post-treatment. (D, E) Neurons were treated with media or 100nM Env protein and imaged every 12 hours for 4 days. (F, G) Env or heat-treated Env protein (100nM) was added to the neurons and incubated for 96 hours and analyzed for (F) neuronal cell counts and (G) mean neurite length. (H) K01 epitope visualized on predicted structure of Env protein generated by AlphaFold. The signal peptide (green), transmembrane domain (blue), surface unit (red), and antibody-binding epitope (yellow) are shown. (I, J) Neurons were preincubated with K01 monoclonal antibody for 1 hour followed by incubation with 100nM Env protein and analyzed for (I) neuronal cell count and (J) mean neurite length. (K, L) Neurons were preincubated with K01 monoclonal antibody or a nonimmune control antibody (3µg/ml) for 1 hour and then treated with 100nM Env protein for 96 hours and analyzed for (K) neuronal cell count and (L) mean neurite length. (M) Spontaneous electrical activity was recorded at 24 hours after Env treatment. (N) Neurons were incubated with either K01 monoclonal antibody or nonimmune control antibody (3µg/ml) for 1 hour and then treated with 100nM Env protein, or Env was preincubated with K01 antibody for 1 hour at 37°C prior to addition of the complex to neurons at the same concentrations for 96 hours. Neurons were imaged, and neuronal cell counts were analyzed. These data represent mean + standard error of the mean from 3 separate determinants of 4 to 8 replicates, which were analyzed by analysis of variance and pairwise analysis with Tukey multiple comparison test. *$p < 0.5$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

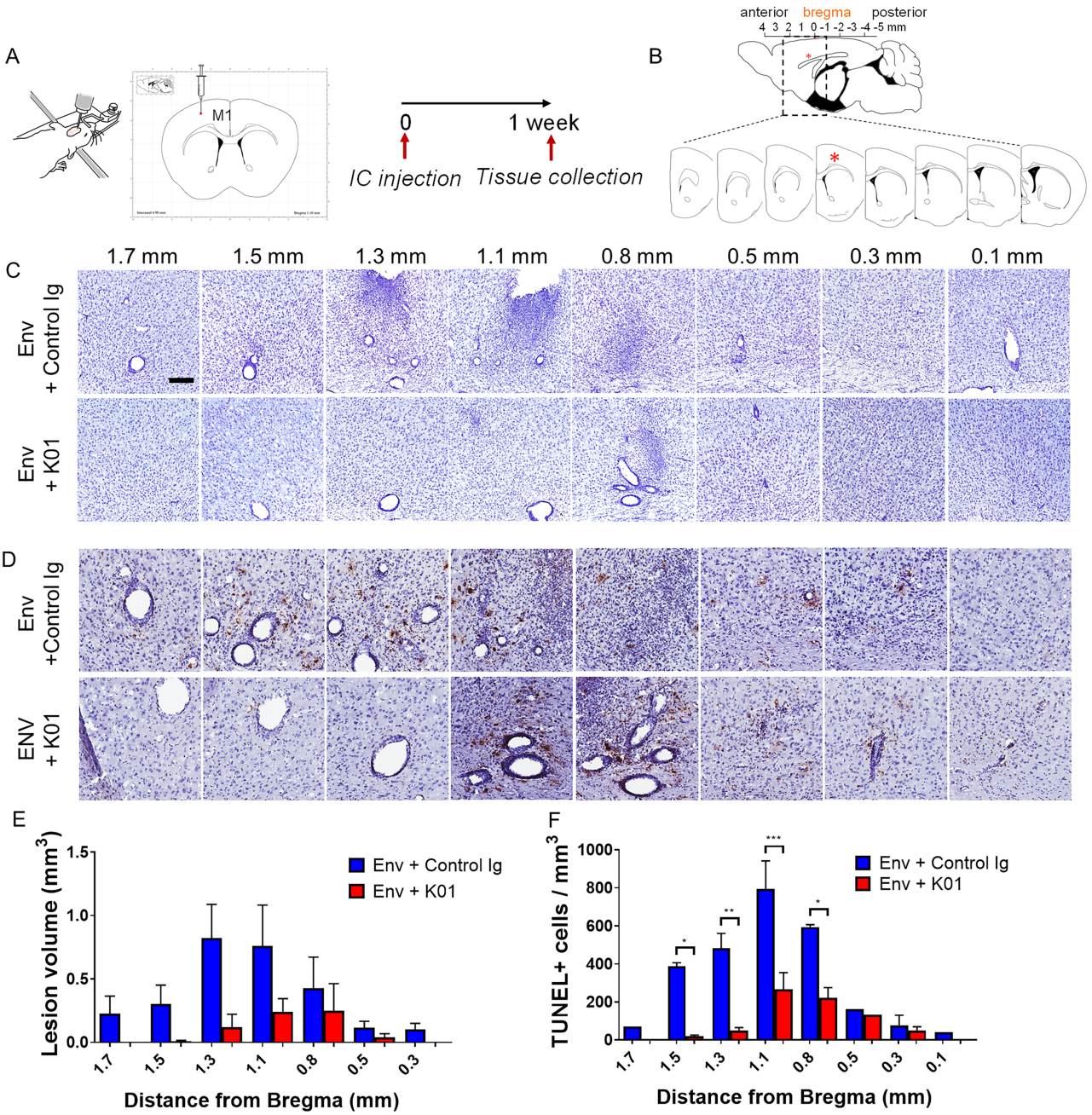
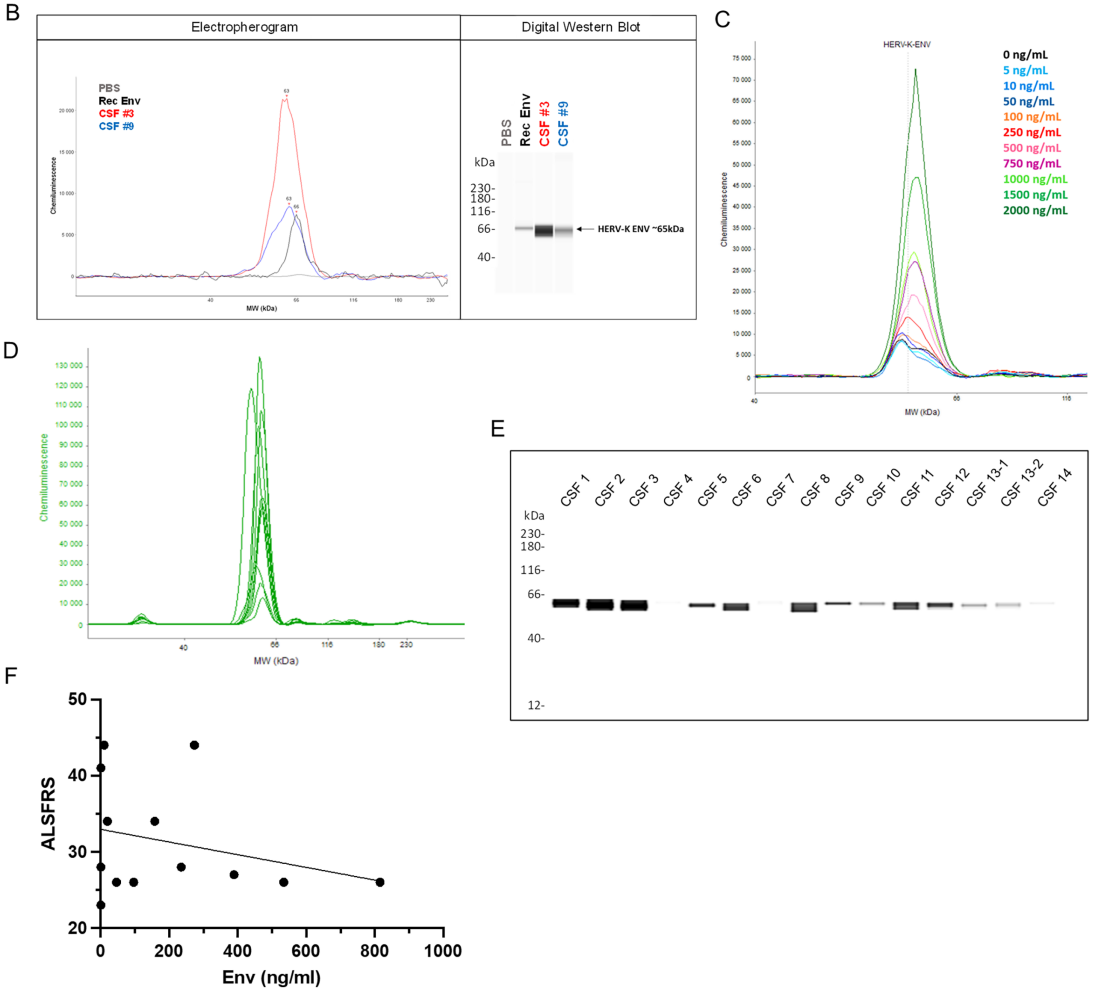


FIGURE 2: HML-2 envelope (Env)-induced neurotoxicity in vivo in mouse brain. (A) Experimental approach showing stereotactic injection of Env protein and K01 antibody or Env protein and control Ig into the primary motor cortex of 3-month-old C57BL/6 mice. Brain tissues were collected 1 week after the injection. IC = intracerebral. (B) Schematic representation of the reference sections used for analysis. Coronal sections at 200- 300μm intervals were used for staining. The asterisk represents injection coordinates to target the primary motor cortex. (C, D) Representative photomicrographs of Cresyl violet (C) and deoxyuridine triphosphate nick-end labeling (TUNEL; D) stained motor cortex. The numbers above each image indicate the distance between the section plane and bregma. (E, F) Bar graphs represent lesion volume (E) and number of TUNEL-positive cells (F). Results were analyzed by analysis of variance and pairwise analysis with Tukey multiple comparison test. *$p < 0.5$, **$p < 0.01$, ***$p < 0.001$.

3μg/ml. Subsequently, neurons were treated with 3μg/ml of either nonimmune IgG control antibody or the K01 antibody. Cells treated with Env alone and cells treated with Env and nonimmune antibody showed decreased numbers of cells with clumping of neurons and loss of neurites, whereas K01-treated cells showed no change in cell number or morphology. These effects were maintained at 96 hours post-treatment. Similar effects were also seen on spontaneous electrical activity of the neurons. In neurons treated with Env alone, the mean firing rate decreased ~60%, similar to neurons treated with nonimmune IgG. Neuronal cultures pretreated with K01

A

ALS	Age	Sex	Race	Duration of symptoms	Onset location	ALSFRS	AUC	HML-Env (ng/ml)
CSF 1	53	M	W	10 yrs	Limb	26	2.83E+05	534.30
CSF 2	69	M	W	2 yrs	Limb	unknown	4.09E+05	920.82
CSF 3	55	M	W (H)	2 yrs	Limb	26	3.75E+05	815.25
CSF 4	59	F	W	2 yrs	Bulbar	28	6.38E+04	<10
CSF 5	63	M	W	3 yrs	Limb	26	1.41E+05	96.60
CSF 6	47	M	W	1 yr	Bulbar	44	1.98E+05	273.38
CSF 7	65	F	W	4 yrs	Limb	23	7.98E+04	<10
CSF 8	59	M	W	3 yrs	Limb	27	2.36E+05	389.09
CSF 9	49	F	W	6 mths	Limb	34	1.16E+05	19.58
CSF 10	36	M	W	1 yr	Limb	44	1.13E+05	10.20
CSF 11	55	F	W	3 yrs	Limb	28	1.86E+05	235.39
CSF 12	46	M	W	2 yrs	Limb	34	1.61E+05	158.16
CSF 13	50	F	W	3 yrs	Limb	26	1.24E+05	46.17
CSF 14	55	F	W	2 yrs	Limb	41	6.60E+04	<10
Control 1	47	M	B	-	-	-	7.69E+04	<10
Control 2	59	M	B	-	-	-	8.22E+04	<10
Control 3	68	M	W	-	-	-	9.09E+04	<10
Control 4	74	M	W	-	-	-	9.34E+04	<10
Control 5	62	M	W	-	-	-	8.06E+04	<10
Control 6	56	M	W	-	-	-	8.86E+04	<10
Control 7	53	M	W	-	-	-	4.92E+04	<10
Control 8	49	M	B	-	-	-	1.07E+05	<10
Control 9	55	F	W	-	-	-	6.37E+04	<10
Control 10	55	F	B	-	-	-	1.32E+05	69.33



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presented spontaneous mean firing rates that were comparable to media-treated cultures, suggesting complete protection from Env-mediated decreases in function. Preincubation of recombinant Env with K01 provided the same protective effect as treatment of the neurons with K01 prior to addition of recombinant Env.

In Vivo HML-2 Env-Mediated Neurotoxicity

To further validate our in vitro findings, 3-month-old C57BL/6 mice were stereotactically injected with Env protein and either the K01 monoclonal antibody or an isotype control monoclonal antibody (Fig 2). At 1 week postinjection, brain tissues were collected and coronal sections at 200- to 300μm intervals from the bregma were selected for downstream staining. Cresyl Violet staining was performed to assess lesion volume and TUNEL staining was performed to investigate apoptotic neurotoxicity. Cresyl violet staining showed reduced cell viability in those animals treated with Env and control antibody and significant protective effects of the K01 antibody. Staining at 0.8, 1.1, 1.3, and 1.5mm from bregma showed TUNEL-positive apoptotic cells in the Env and control antibody group, whereas those treated with Env and K01 antibody showed significantly reduced levels of TUNEL-positive staining. These findings suggest that the K01 antibody is specific for HML-2 Env and has protective effects against the Env-induced apoptotic cell death of cortical neurons in the primary motor cortex.

Detection of HML-2 Env Protein and RNA in CSF of Patients with ALS

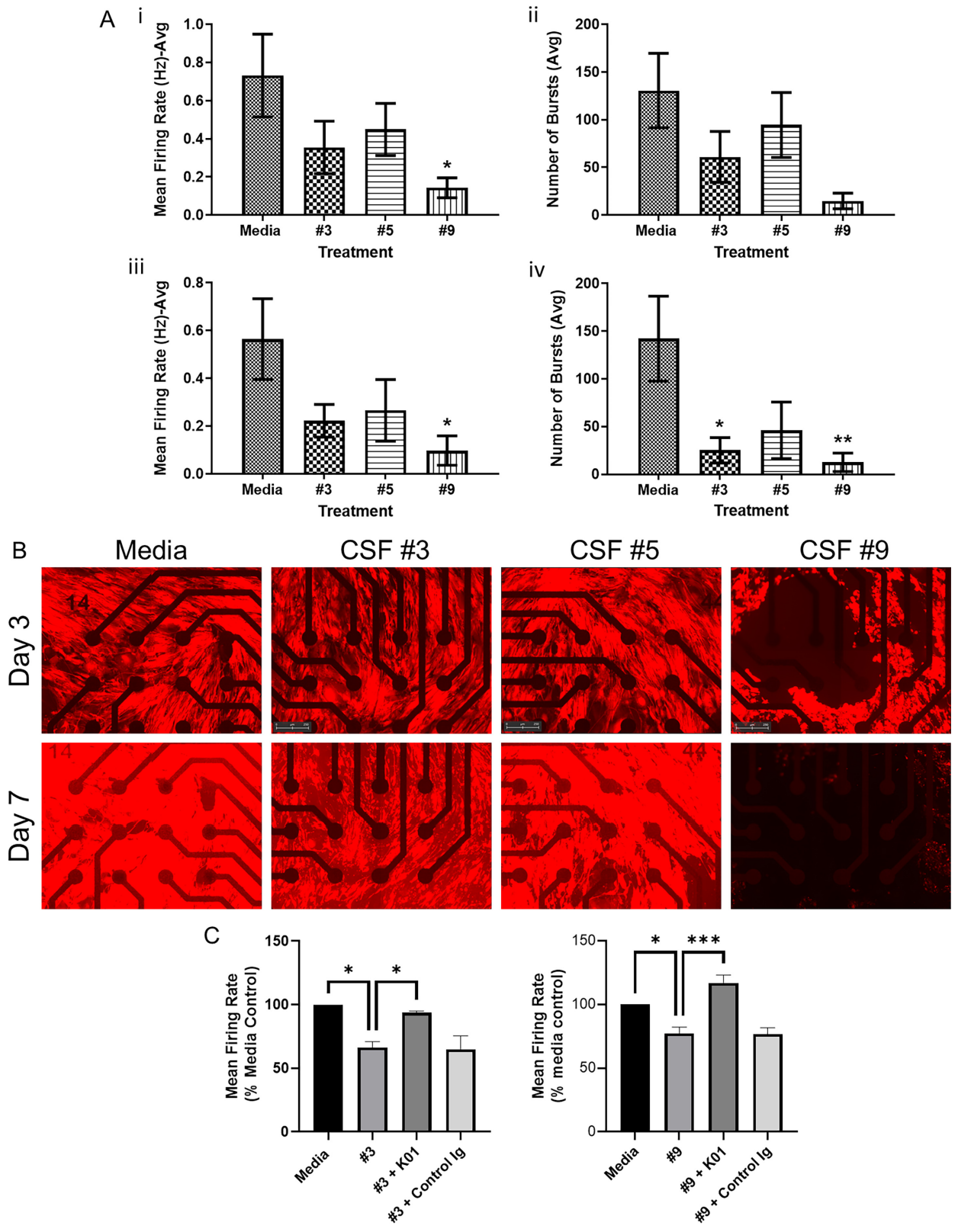
We have shown that the HML-2 Env protein could be detected by immunostaining in neurons in the brain of patients with ALS.[21] Here, we determined whether Env protein could also be detected in the CSF of these patients. All CSF samples (14 individuals with sporadic ALS and 10 healthy volunteers) were analyzed by digital Western blot assay using K01 antibody. This assay was validated using recombinant Env protein, which showed a single band in the CSF at ~65kDa (Fig 3). A standard curve was generated using the recombinant Env. This was used to compare the intensity of the bands, and the area under the curve was obtained from each of the CSF samples. Env was detectable in 11 of 14 ALS patients, but in only one of the healthy volunteers. The amount of protein ranged from 10.20 to 920.82ng/ml. Patient #13 had 2 CSF samples obtained at an interval of 4 months. Both samples showed similar amounts of Env protein (46.17 and 49.13ng/ml). There was no significant correlation between the concentration of the Env in the CSF and the ALSFSR. Digital droplet PCR of the ALS CSF samples showed the presence of HERV-K *env* RNA in 4 of 15 samples from 14 patients (CSF #3, #9, #11, and #12) and in 1 of 10 control samples (Control #9).

Neurotoxic Properties of CSF from ALS Patients and Blockage with Antibody to HERV-K Env

All 15 CSF samples from 14 ALS patients (see Fig 3A) were screened to determine toxic effects on human neuronal cultures. Neurotoxicity screening assay optimization using CSF from a healthy human volunteer found that 100-fold diluted CSF had no toxic effects on cell counts or neurite process length, and thus the ALS patient CSF samples were all diluted by 100-fold in the assay. The most significant neurotoxicity was observed with ALS CSF #3, #5, and #9 and therefore were used for subsequent studies. No neurotoxicity was observed with any control CSF samples. The effect of these CSF samples on spontaneous electrical activity in neurons was investigated. By 3 days, the mean firing rates decreased to ~50%, 60%, and 20% compared to the media-treated control wells when treated with CSF samples #3, #5, and #9, respectively (Fig 4). A similar trend was noted for the number of electrical bursts, with the most significant reduction with CSF #9. Further progressive decreases in these neuronal functional parameters were noted at 7 days post-treatment, with a significant decrease in electrical bursts with both CSF #3 and #9. The CSF-treated cells showed loss of neuritic processes and clustering of neurons with neuronal loss in a time-dependent manner. Spontaneous electrical activity was also measured in the presence of the K01 and control antibody (3μg/ml). CSF #3 and #9 showed a significant reduction in mean firing rate in a time-dependent manner that was mitigated by the addition of the K01 antibody.

Morphological changes with CSF #9 treatment were also monitored in the presence of the K01 antibody and isotype control (3μg/ml) over 84 hours. Photomicrographs taken at 12-hour intervals showed progressive loss of neurons and neurites with clustering that was prevented by K01 but not the isotype control antibody

FIGURE 3: Detection of HML-2 in cerebrospinal fluid (CSF) samples from amyotrophic lateral sclerosis (ALS) patients. (A) ALS patient demographics including age, sex, race, duration of symptoms, onset location, Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS) score, and concentration of envelope (Env) in CSF as area under the curve (AUC) and ng/ml. B = Black; F = female; H = Hispanic; M = male; W = White. (B) Recombinant (Rec) HML-2 Env, undiluted CSF, and phosphate-buffered saline (PBS) were analyzed by electrophoresis separation matrix in independent capillaries. HML-2 Env was detected using the K01 antibody. The signals are represented on electropherograms and digital Western blots. A very clean signal is observed at 65kDa with the HML-2 Env. A similar signal is seen with CSF Samples #3 and #9 from patients with ALS. HERV-K = human endogenous retrovirus-K. MW = molecular weight. (C) Standard curve with recombinant Env. (D) Signal intensity of the bands from each ALS patient. (E) Western blots for detection of HML-2 Env are shown for CSF Samples #1–#14 from patients with ALS. (F) Correlation between the HML-2 Env concentration and the ALSFRS.



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(Fig 5). Quantitation showed that CSF #9 exposure resulted in a time-dependent decrease in cell number and mean neurite length over 84 hours. Cultures treated with the isotype control antibody plus CSF displayed fewer cells and shorter processes, whereas neurons exposed to K01 (3μg/ml) maintained more neurons and longer

processes compared to media-treated wells. The protective effects of K01 were sustained over the entire observation period as evident by 84 hours post-treatment, but only the effects on neurite length were statistically significant, as the magnitude of effects was much greater on neurite length compared to cell death. The same neurotoxic trend was observed in a subsequent experiment demonstrating that heat inactivation of the Env protein provided the same level of protective effect as treatment with K01.

Identification of Cell Membrane Proteins That Bind HML-2 Env Protein

To characterize the mechanism by which HML-2 Env protein causes toxicity, human neurons were treated with recombinant Env protein, immunoprecipitated with a 6X-His antibody, and analyzed by mass spectrometry to identify protein targets of HML-2 Env. The following membrane proteins were identified: CD98HC and β1 integrin, which have been previously identified as HML-2 Env receptors in pluripotent stem cells.[8] The CD98HC target was validated using Western blot for CD98 (Fig 6). The expression of CD98HC and colocalization with recombinant Env protein was also confirmed by immunostaining. The role of CD98HC in Env protein-mediated toxicity was further confirmed by utilizing an antibody to this receptor to block neurotoxicity.

Identification of Pathways Involved in HML-2 Env Protein-Mediated Neurotoxicity

To identify signaling molecules involved in mediating the neurotoxicity of the HML-2 Env protein, 40 compounds were screened for their neuroprotective efficacy against the HML-2 Env (Fig 7A). Neurons were preincubated with each of the compounds (10μM), followed by exposure to Env protein. Of the screened compounds, retinoic acid receptor (RAR) agonists, GSK3β inhibitors, cyclic adenosine monophosphate (cAMP) pathway activators, and some flavonoids were effective in preventing the Env-mediated neurotoxicity. Conversely, various receptor tyrosine kinase inhibitors, immune mediators, and N-methyl-D-aspartate receptor antagonists were ineffective at preventing toxicity. Based on these screening data, two compounds, the GSK3β inhibitor, BIO, and the RAR agonist, AM580 (see Fig 7B,C) were selected for further analysis. Both BIO and AM580 compounds blocked neurotoxicity in a concentration-dependent manner, with effective concentrations of 0.03 to 1μM and 0.03 to 10μM, respectively.

Discussion

These data demonstrate a novel pathogenic mechanism of HERV-K subtype HML-2 on neurons mediated by its extracellular Env protein. This work has identified HML-2 Env receptors on the cell surface and the associated intracellular signaling pathways. Direct neurotoxicity in human neuronal cultures was investigated with the recombinant Env protein, and similar effects were recapitulated with ALS CSF, in which Env protein could be detected. HML-2 Env could be detected in the CSF of 12 of 15 ALS patients by digital Western blot, confirming its release and circulation in patients' body fluids with relevance to the central nervous system. We were unable to detect HML-2 Env in the CSF of healthy controls. However, previous studies have shown that expression of HML-2 is not specific for ALS and can be found in a number of cancers and other diseases.[1] Although its activation in neurological diseases has not been well studied, HML-2 RNA has been detected in CSF of patients with Alzheimer disease.[28]

Although the toxicity of the recombinant protein was concentration dependent, progressed over time, and was associated with functional deficits observed by electrophysiological abnormalities, the toxicity of the CSF did not parallel the concentrations of the Env. The bioactive envelope protein is probably a fraction of the total protein, which is likely a mixture of denatured, polymerized, and bioactive proteins. Further viral proteins are often complexed to other proteins that may modulate their neurotoxic properties. The functional assay provides a measure of the bioactive Env. Thus, the present study shows that the observed neurotoxicity was specific to Env, as it was blocked by a monoclonal antibody specific to the Env protein. Furthermore, the Env-mediated neurotoxicity was sensitive to heat inactivation of the protein, suggesting

FIGURE 4: Amyotrophic lateral sclerosis (ALS) patient cerebrospinal fluid (CSF) impairs neuronal functioning. (A) CSF #3, #5, and #9 were diluted 100-fold in neuronal differentiation media and applied to human neuronal cultures. Spontaneous electrical activity (i and iii) and number of electrical bursts (ii and iv) were quantitated every 24 hours. Data are shown at 3 days (i and ii) and 7 days (iii and iv) postexposure. (B) Four images (2 × 2) at ×10 magnification stitched together from the recording wells show neurons on the electrodes that correspond to Panel A above at 3 (top) and 7 (bottom) days. (C) Neurons were treated with media, K01 (3μg/ml), and control Ig (3μg/ml) prior to exposure to ALS CSF #3 or #9 (100-fold dilution) in triplicate. At 4 days after CSF treatment, spontaneous electrical activity and mean firing rate were analyzed. Data in A and C represent the mean and standard error of the mean of 10 replicates per condition and were analyzed by analysis of variance and pairwise analysis with Tukey multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

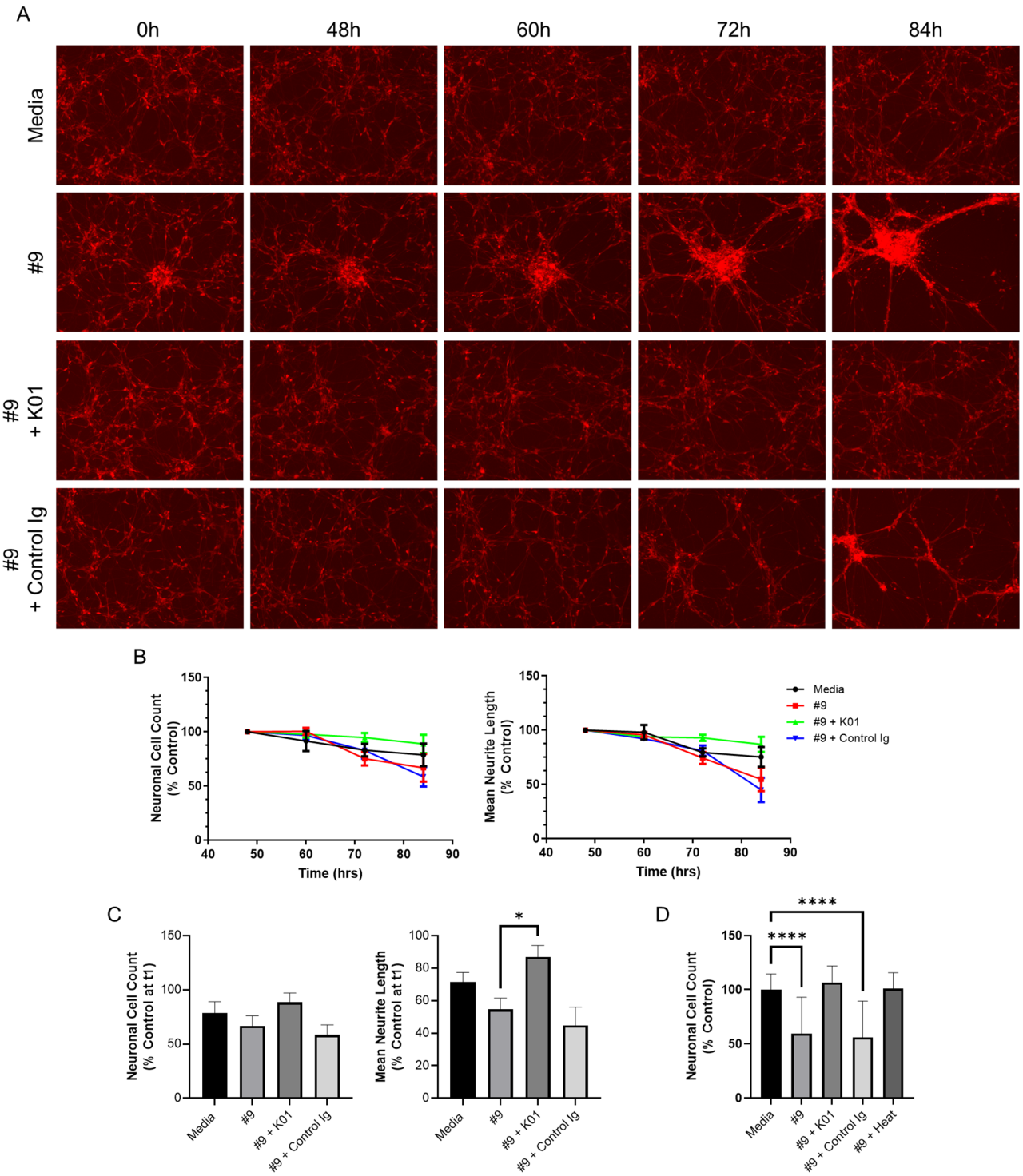
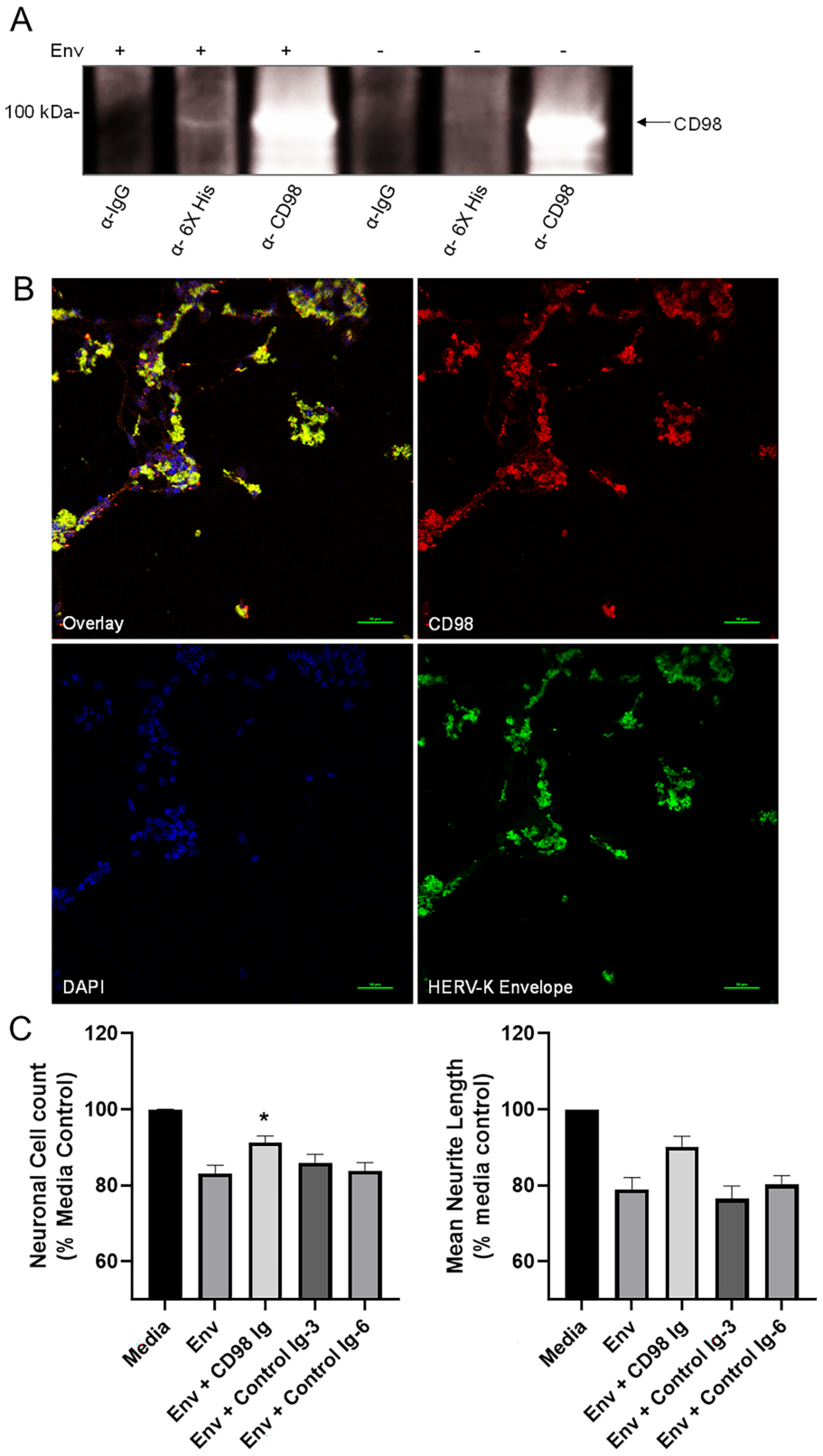


FIGURE 5: Monoclonal antibody against HML-2 envelope blocks amyotrophic lateral sclerosis (ALS) cerebrospinal fluid (CSF)-mediated neurotoxicity. Human neurons were pretreated with K01 or control Ig at 3μg/ml for 1 hour and then exposed to ALS patient CSF #9 (100-fold final dilution). (A) Images of the culture were captured at time of treatment to establish baseline and at times indicated after exposure to CSF. (B) Neuronal cell count and mean neurite length were quantified from 6 wells per condition and 4 images per well. Data represent the mean ± standard error of the mean (SEM) as a percent of media control at 48 hours. (C) Data show the mean ± SEM at 84 hour postexposure. (D) Human neurons were pretreated with K01 or control Ig at 3μg/ml for 1 hour and then exposed to freshly thawed ALS patient CSF #9 (100-fold final dilution). CSF #9 was also heat inactivated prior to addition to the cells at a 100-fold final dilution. Data show the mean neuronal cell count as percent of control ± SEM at 96 hours postexposure. Data represent 4–8 replicates per condition and were analyzed by analysis of variance and pairwise analysis with Tukey multiple comparison test. *$p < 0.05$, ****$p < 0.0001$.



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that the toxicity was likely due to a conformationally dependent epitope of Env. The neurotoxicity of the Env protein is not unique to HML-2. We mapped the binding site of a neutralizing antibody to identify the region of the protein responsible for the neurotoxicity, which is located at amino acids Ser298–Pro312. An extensive body of

literature exists on the neurotoxic properties of the Env protein of the human immunodeficiency virus (HIV) and its Tat protein, which is spliced from the *env* gene (reviewed in Nath[29]). HML-2 does not produce a protein equivalent of Tat, but has several loci in the human genome that can produce an intact Env protein.[30] Similar to the HIV Env protein, the HML-2 Env neurotoxicity is mediated by a conformationally dependent epitope.

The mechanism by which HML-2 Env causes neurotoxicity is distinct from that of the HIV Env protein (Fig 8). We identified the binding partner for HML-2 Env protein on the neuronal cell surface, CD98HC, and its involvement in neurotoxicity was validated using antibodies targeted against it. CD98 is a heterodimer composed of a heavy chain termed CD98HC, SLC3A2, or 4F2HC, which is covalently linked to one of several light chains involved in selective transport of various amino acids that are hence essential for cell survival.[31] The heavy chain can bind to 1 of 6 subunits (LAT1, LAT2, xCT, y^+ LAT1, y^+ LAT2, and asc1), which provide substrate specificity for amino acid transport.[32] By controlling localization to the plasma membrane and coupling amino acid transport activities, CD98HC confers protection against oxidative and nutritional stresses and thus provides cells with a survival advantage.[33,34] The cytoplasmic tail of CD98HC can interact with β1 integrin to regulate its signaling and function. These interactions are independent of the amino acid transporter function and are mediated by different domains of the protein.[35] Both functions, amino acid transport and integrin signaling, act in synergy promoting cell survival,[33] and knockdown of CD98HC can lead to apoptosis.[36] We previously identified CD98HC as a receptor for HML-2 Env in pluripotent stem cells. In these cells, CD98HC is linked to the mammalian target of rapamycin signaling pathway and is critical in maintaining stemness.[8] In agreement with our previous studies, we found that CD98HC and β1 integrin immunoprecipitated with HML-2 Env. Immunostaining and Western blot confirmed the interaction of these proteins with HML-2 Env. Interestingly, Env-mediated neurotoxicity could be mitigated using an antibody to CD98, suggesting that interactions at the neuronal surface between these two proteins could potentially block the downstream cell survival functions of the receptor. This indicates a differential response in proliferating stem cells versus terminally differentiated neurons.

To determine the subcellular pathways involved in HML-2 Env-mediated neurotoxicity, we used a panel to compounds to screen for molecules that could block its effect on neurons. We identified the GSK3β, retinoic acid receptor, cAMP, and oxidative stress pathways as important in mediating these effects. Although further studies are needed to determine how these pathways and protein targets are linked, it is possible that GSK3β is linked to β1 integrin via AKT (protein kinase B) such that inhibition of β1 integrin would lead to activation of GSK3β and thereby contribute to decreased neuronal survival.

CD98HC forms a heterodimer with xCT, a transporter that mediates the sodium-independent exchange of L-cysteine and L-glutamic acid.[37] Knockdown of CD98HC leads to decrease in transport of L-cysteine and accumulation of reactive oxygen species.[33] Cysteine is an important constituent of glutathione, conferring antioxidant properties due to the reactive thiol group, which binds reactive oxygen species.[38] It follows that binding of HML-2 Env to CD98 could impair its function, resulting in decreased transport of L-cysteine and eventually an increase in reactive oxygen species. In keeping with this observation, we found that several flavonoids that also bind reactive oxygen species, and thus prevent oxidative stress, blocked the neurotoxic effects of Env.

Several RAR agonists also protected against HML-2-induced neurotoxicity. RARs are present in the nucleus and are members of the nuclear hormone family. They exert their effects by acting as a transcription factor to regulate gene expression and have several cytoprotective functions. We also found that the proteasome inhibitor MG132 significantly exacerbated neurotoxic effects of HML-2 Env, suggesting the role of the proteosome in mediating these effects. It has been shown in cancer cell lines that inhibition of β1 integrin will lead to increased proteasomal activity,[39] which is mediated via AKT and GSK3β.[40] This may have a cytoprotective effect in neurons, where removal of protein aggregates and ubiquitinated proteins is essential for cell function. Thus, blocking proteasome activity may amplify Env-mediated

FIGURE 6: Identification and validation of HML-2 envelope (Env) protein targets. (A) A total of 293 T cells were transfected with a CD98 plasmid, incubated for 2 days, and then treated with 100nM Env protein. Protein targets of HML-2 Env were validated using immunoprecipitation using control Ig, 6X His Ig, and CD98 Ig followed by Western blot for CD98. (B) Immunostaining of neural stem cell-derived neurons treated with exogenous human endogenous retrovirus-K (HERV-K) recombinant protein (100nM) to demonstrate colocalization of CD98 and HML Env protein. (C) All antibodies were preincubated with neurons for 30 minutes before exposure to HML-2 Env. Images were captured at 96 hours after Env exposure. Each experiment utilized 4 fields per well and 6 replicate wells per treatment group. Data represent 7 determinations. For neuronal cell counts, analysis by analysis of variance (ANOVA) revealed $F_{6,42} = 21.20$, $p < 0.0001$, pairwise analysis with Tukey multiple comparison test, $^*p = 0.0164$. For mean neurite length, ANOVA showed $F_{6,42} = 10.81$, $p < 0.0001$, pairwise analysis with Tukey multiple comparison test, $p = 0.005$.

A

Class	Compound	Neuronal Cell Counts % Protection	SEM	Mean Neurite Length % Protection	SEM
GSK3b inhibitors	CHIR99021	-29.6	4.8	7.5	7.3
	Tideglusib	51.2	9.9	95.6	11.9
	CHIR98014	34.8	4.1	75.2	6
	BIO	83.3	5.1	89	6.6
	1-Azakenpaullone	-3.7	2.8	26.6	3.8
	AZD2858	-25.3	2.3	5.9	3.6
	IM12	99.4	5.9	87.9	4.8
Retinoic Acid Receptor Ligands	EC23	129	6.8	397.6	5.9
	H47	112.3	6.7	349.8	9.2
	AM80	82.4	14.9	284	23.7
	AM580	128.6	5.6	401	7.2
	Tarazotene	196.3	4.8	464.6	6.8
	H45	84	2.1	219.9	4.3
	BMS-195614	25.1	4.2	47.6	6.2
	AGN193109	-6.8	3.4	-6.6	4
Cyclin Dependent Kinase Ligands	Dinaciclib	-214.6	3.6	-394.2	2.9
	Flavopirodol	-247.5	2.4	-421.4	2
	Roscovitine	13.8	8.6	71.9	10.8
	PHA-793887	-256.1	4.4	-427.7	1.6
	SNS-032	-236.8	2.4	-400.5	3
Flavonoid & Antioxidants	EGCG	78.4	4.7	126.1	8
	Resveratrol	78.6	4.4	150.3	5.6
	Curcumin	7.6	2.5	-16.2	5.6
	C50 Resveratrol	50.8	9	120.2	13.4
	Pterostilbene	12.8	4.1	11.8	8.5
	G138 Resveratol	-1.2	6.3	9.5	7.3
	Epicatechin	-1.7	3.1	19.4	6
	Caffeic acid	27.2	5.1	-94.7	3
NMDA Receptor Antagonists	(+)-MK801	22.3	6.3	42.2	8.5
	(-)-MK801	-8.3	4.5	16.6	8.1
Antibiotics & Antifungals	Doxycycline	19.1	5.1	34.9	5.1
	Minocycline	40.3	6.6	44.6	7.6
	Azithromycin	40.3	4.6	138	4.4
	Fluconazole	5.5	4.8	20.5	7.5
Growth Factor Receptor Antagonists	AG490	-18.6	2.8	-50.6	4.9
	AG1296	-0.6	4.3	32.4	6.4
	Imatinib	12.6	4.8	53	7.2
Proteasome Inhibitors	MG132	-514.5	47.6	-135.7	9.3
cAMP Pathway Activators	Forskolin	106.4	6.9	199.9	9
Others	Clozapine	6.3	2.6	27.8	6.3
	Rapamycin	-7.5	4.1	-15.6	5.4
	Limonin	12.1	5.4	24.1	8.1
	Celecoxicib	2.1	7	41.1	10
	Cyclosporine A	-36.9	4.4	-64.7	6.4
	SC79	6.9	3.7	26.9	5.5
	Everolimus	11.8	3.7	20.6	8.3
	Tacrolimus	34.2	6.6	80.2	5.1

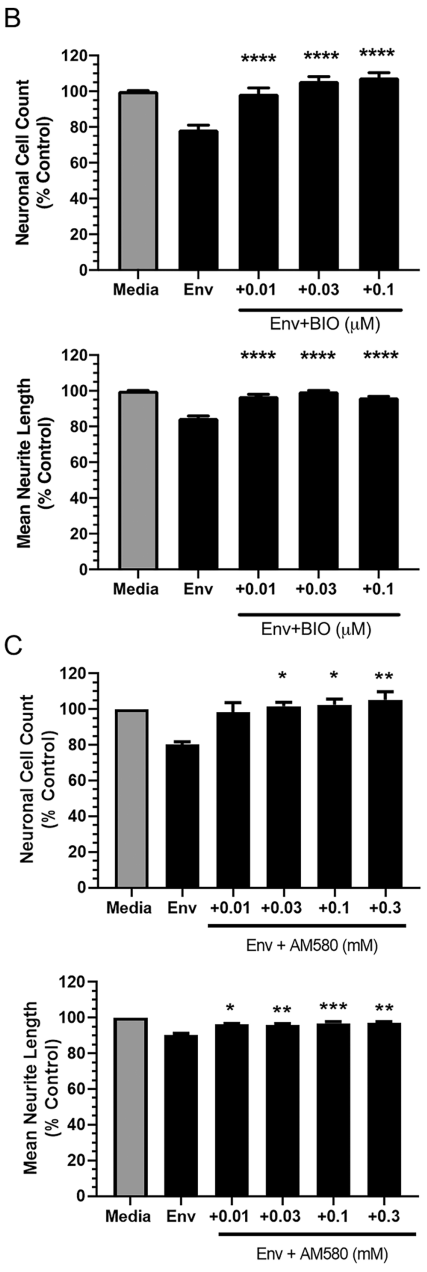

FIGURE 7: GSK3β and retinoic acid receptor play a role in HML-2 envelope (Env)-mediated neurotoxicity. (A) Numerous compounds were screened at 10μM for their ability to alter Env-mediated neurotoxicity. Data are presented as percent protection against Env for both neuronal cell counts and mean neurite length. cAMP = cyclic adenosine monophosphate; NMDA = N-methyl-D-aspartate; SEM = standard error of mean. (B, C) Human neuronal cultures were treated with a GSK3β antagonist, BIO, or a retinoic acid receptor agonist, AM580, for 1 hour prior to adding HML-2 Env (100nM) for 96 hours. Data represent mean + SEM of 3 independent experiments and were analyzed by analysis of variance (ANOVA). (B) Neuronal cell count analysis by ANOVA: $F_{4,10} = 23.49$, $p < 0.0001$. Pairwise analysis with Tukey multiple comparison test: ****$p < 0.0001$ Env versus Env + 0.03μM BIO, and for Env versus Env + 0.1μM BIO. Mean neurite analysis by ANOVA: $F_{4,10} = 79.30$, $p < 0.0001$. Pairwise analysis with Tukey multiple comparison test: ****$p < 0.0001$ Env versus Env + 0.01μM BIO, Env + 0.03μM BIO, and Env + 0.1μM BIO. (C) Neuronal cell count analysis by ANOVA: $F_{5,12} = 7.337$, $p = 0.0023$. Pairwise analysis with Tukey multiple comparison test: *$p = 0.0219$ Env versus Env + 0.01μM AM580, **$p = 0.0067$ Env versus Env + 0.03μM AM580, **$p = 0.0050$ for Env versus Env + 0.1μM AM580, and **$p = 0.0019$ Env versus Env + 0.3μM AM580. AM580 mean neurite analysis by ANOVA: $F_{5,12} = 19.39$, $p < 0.0001$. Pairwise analysis with Tukey multiple comparison test: ***$p = 0.0009$ Env versus Env + 0.01μM AM580, **$p = 0.0014$ Env versus Env + 0.03μM BIO, ***$p = 0.0004$ Env versus Env + 0.1μM AM580, and ***$p = 0.0003$ for Env versus Env + 0.3μM BIO.

β1 integrin effects, which could exacerbate HML-2 Env-mediated neurotoxicity. However, there may be other mechanisms by which HML-2 may cause neuroglial dysfunction. For example, β1 integrin is known to be endocytosed, and several of the compounds shown to block Env-mediated neurotoxicity also acidify the

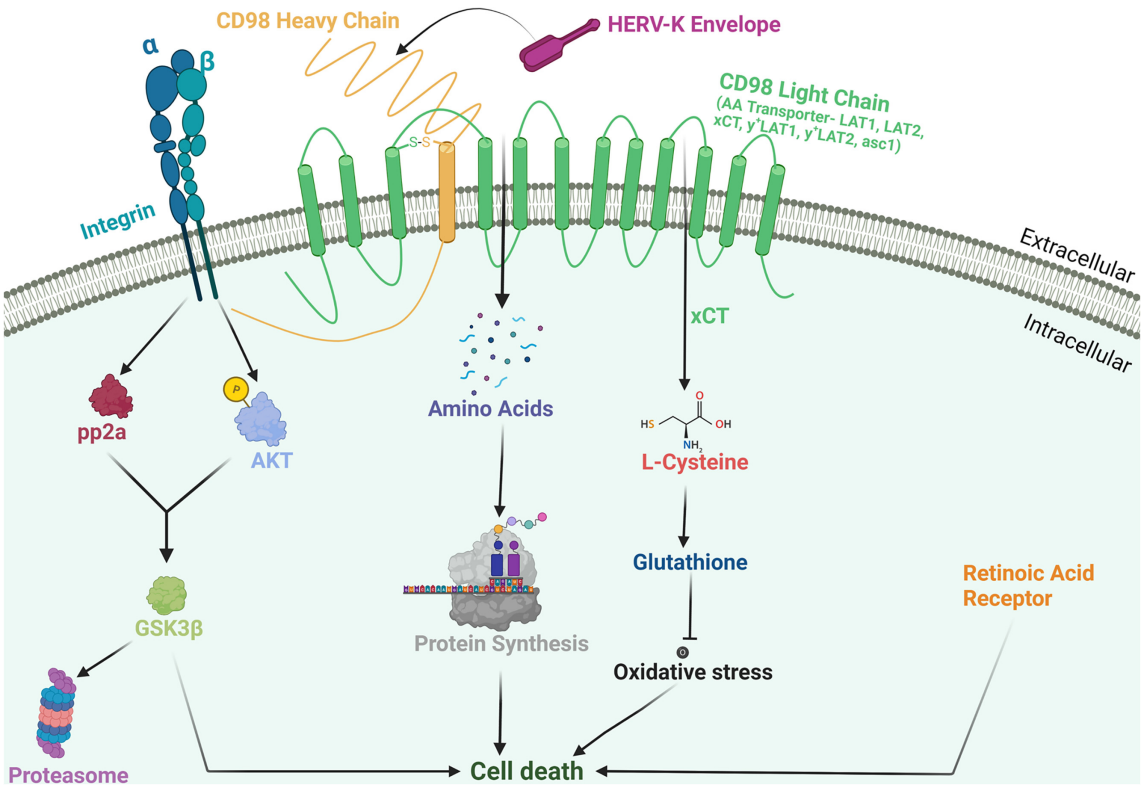

FIGURE 8: Mechanism of HML-2 envelope (Env)-induced neurotoxicity. HML-2 Env binds to CD98HC to inhibit its function. CD98HC is covalently linked to amino acid (AA) transporters. A proper balance of amino acids is necessary for cellular function. One of the transporters, xCT, is specific for cysteine glutamate exchange. Cysteine is an important component of glutathione and thus is critical for preventing oxidative stress. Several flavonoids that bind free radicals were found to be protective against HML-2-induced neurotoxicity. CD98HC also interacts with the cytoplasmic domain of β1 integrin. β1 integrin signals through several kinases that are linked to GSK3β. GSK3β can affect cell function either through interactions with the proteosome or by other mechanisms. GSK3β inhibitor protected against HML-2 Env-induced neurotoxicity, whereas proteasome inhibitor enhanced its toxicity. Retinoic acid receptor agonists also protect against the Env-induced neurotoxicity. HERV-K = human endogenous retrovirus-K.

endolysosome, hence future studies will determine whether these pathways may also be involved. It has also been shown that HML-2 mRNA can interact with toll-like receptor 8 and cause microglial cell activation and neurodegeneration.[28]

Our study has several limitations. An inherent limitation of in vitro assays is that they are optimized to show toxicity over short time periods and may not accurately represent disease progression that occurs over months to years. Thus, concentrations of the Env protein used in these assays or even in the CSF cannot be equated to those in the extracellular space in the brain, which would be most relevant for the exposure to neurons in vivo. Although we were able to block the neurotoxicity of the CSF with a monoclonal antibody to the Env protein, we cannot exclude the possibility that there may be other neurotoxins in the CSF. Detection of viral proteins in CSF samples requires samples collected under very careful conditions. This is particularly important because conformationally dependent epitopes of proteins are very sensitive to suboptimal processing, storage, and freeze–thaw cycles.

In conclusion, HML-2 Env protein is released extracellularly into the CSF of patients with ALS and can mediate neurotoxicity via interactions with CD98HC and the associated secondary signaling pathways. Further characterization of these mechanisms may help identify additional therapeutic targets.

Acknowledgments

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Author Contributions

J.P.S., M.-H.L., H.P., and A.N. contributed to conception and design of study. B.S., L.B.R., and A.N. collected

clinical data and patient samples for analysis. J.P.S., M. Ba., N.M., C.D., W.L., K.S., M.-H.L., J.K., M.Bh., T. D.-O., M.G.-M., M.C., B.C., B.S., L.B.R., J.M., J. B., and J.P. contributed to acquisition and analysis of data, and designed and conducted experiments. J.P.S., M.-H.L., N.M., W.L., C.D., H.P., and A.N. contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest

GeNeuro has a joint patent with National Institute of Neurological Disorders and Stroke on the antibody to HERV-K envelope protein (US patent number 10,981,977).

References

1. Garcia-Montojo M, Doucet-O'Hare T, Henderson L, Nath A. Human endogenous retrovirus-K (HML-2): a comprehensive review. Crit Rev Microbiol 2018;44:715–738.
2. Kury P, Nath A, Creange A, et al. Human endogenous retroviruses in neurological diseases. Trends Mol Med 2018;24:379–394.
3. Callahan R, Chiu IM, Wong JF, et al. A new class of endogenous human retroviral genomes. Science 1985;228:1208–1211.
4. Flockerzi A, Ruggieri A, Frank O, et al. Expression patterns of transcribed human endogenous retrovirus HERV-K(HML-2) loci in human tissues and the need for a HERV transcriptome project. BMC Genomics 2008;9:354.
5. Schmitt K, Heyne K, Roemer K, et al. HERV-K(HML-2) rec and np9 transcripts not restricted to disease but present in many normal human tissues. Mobile DNA 2015;6:4.
6. Ehlhardt S, Seifert M, Schneider J, et al. Human endogenous retrovirus HERV-K(HML-2) rec expression and transcriptional activities in normal and rheumatoid arthritis synovia. J Rheumatol 2006;33: 16–23.
7. Fuchs NV, Kraft M, Tondera C, et al. Expression of the human endogenous retrovirus (HERV) group HML-2/HERV-K does not depend on canonical promoter elements but is regulated by transcription factors Sp1 and Sp3. J Virol 2011;85:3436–3448.
8. Wang T, Medynets M, Johnson KR, et al. Regulation of stem cell function and neuronal differentiation by HERV-K via mTOR pathway. Proc Natl Acad Sci U S A 2020;117:17842–17853.
9. Grow EJ, Flynn RA, Chavez SL, et al. Intrinsic retroviral reactivation in human preimplantation embryos and pluripotent cells. Nature 2015; 522:221–225.
10. Buscher K, Hahn S, Hofmann M, et al. Expression of the human endogenous retrovirus-K transmembrane envelope, rec and Np9 proteins in melanomas and melanoma cell lines. Melanoma Res 2006;16:223–234.
11. Lower R, Lower J, Frank H, et al. Human teratocarcinomas cultured in vitro produce unique retrovirus-like viruses. J Gen Virol 1984;65(Pt 5):887–898.
12. Perron H, Lalande B, Gratacap B, et al. Isolation of retrovirus from patients with multiple sclerosis. Lancet 1991;337:862–863.
13. Perron H, Garson JA, Bedin F, et al. Molecular identification of a novel retrovirus repeatedly isolated from patients with multiple sclerosis. The collaborative research group on multiple sclerosis. Proc Natl Acad Sci U S A 1997;94:7583–7588.
14. Kremer D, Gruchot J, Weyers V, et al. pHERV-W envelope protein fuels microglial cell-dependent damage of myelinated axons in multiple sclerosis. Proc Natl Acad Sci U S A 2019;116:15216–15225.
15. Diebold M, Derfuss T. The monoclonal antibody GNbAC1: targeting human endogenous retroviruses in multiple sclerosis. Ther Adv Neurol Disord 2019;12:1756286419833574.
16. Alfahad T, Nath A. Retroviruses and amyotrophic lateral sclerosis. Antiviral Res 2013;99:180–187.
17. Douville R, Liu J, Rothstein J, Nath A. Identification of active loci of a human endogenous retrovirus in neurons of patients with amyotrophic lateral sclerosis. Ann Neurol 2011;69:141–151.
18. Garson JA, Usher L, Al-Chalabi A, et al. Quantitative analysis of human endogenous retrovirus-K transcripts in postmortem premotor cortex fails to confirm elevated expression of HERV-K RNA in amyotrophic lateral sclerosis. Acta Neuropathol Commun 2019;7:45.
19. Mayer J, Harz C, Sanchez L, et al. Transcriptional profiling of HERV-K(HML-2) in amyotrophic lateral sclerosis and potential implications for expression of HML-2 proteins. Mol Neurodegener 2018;13:39.
20. Jones AR, Iacoangeli A, Adey BN, et al. A HML6 endogenous retrovirus on chromosome 3 is upregulated in amyotrophic lateral sclerosis motor cortex. Sci Rep 2021;11:14283.
21. Li W, Lee MH, Henderson L, et al. Human endogenous retrovirus-K contributes to motor neuron disease. Sci Transl Med 2015;7: 307ra153.
22. Efthymiou A, Shaltouki A, Steiner JP, et al. Functional screening assays with neurons generated from pluripotent stem cell-derived neural stem cells. J Biomol Screen 2014;19:32–43.
23. Ludolph A, Drory V, Hardiman O, et al. A revision of the El Escorial criteria—2015. Amyotroph Lateral Scler Frontotemporal Degener 2015;16:291–292.
24. Nath A, Slagle B, Wolinsky JS. Anti-idiotypic antibodies to rubella virus. Arch Virol 1989;107:159–167.
25. Jumper J, Evans R, Pritzel A, et al. Highly accurate protein structure prediction with AlphaFold. Nature 2021;596:583–589.
26. Pettersen EF, Goddard TD, Huang CC, et al. UCSF chimera—a visualization system for exploratory research and analysis. J Comput Chem 2004;25:1605–1612
27. Gold J, Rowe DB, Kiernan MC, et al. Safety and tolerability of Triumeq in amyotrophic lateral sclerosis: the lighthouse trial. Amyotroph Lateral Scler Frontotemporal Degener 2019;20:595–604.
28. Dembny P, Newman AG, Singh M, et al. Human endogenous retrovirus HERV-K(HML-2) RNA causes neurodegeneration through toll-like receptors. JCI Insight 2020;5:e131093.
29. Nath A. Human immunodeficiency virus (HIV) proteins in neuropathogenesis of HIV dementia. J Infect Dis 2002;186:S193–S198.
30. Subramanian RP, Wildschutte JH, Russo C, Coffin JM. Identification, characterization, and comparative genomic distribution of the HERV-K (HML-2) group of human endogenous retroviruses. Retrovirology 2011;8:90.
31. Verrey F, Closs EI, Wagner CA, et al. CATs and HATs: the SLC7 family of amino acid transporters. Pflugers Arch 2004;447:532–542.
32. Alexander SPH, Kelly E, Mathie A, et al. The concise guide to pharmacology 2019/20: transporters. Br J Pharmacol 2019;176:S397–S493.
33. de la Ballina LR, Cano-Crespo S, Gonzalez-Munoz E, et al. Amino acid transport associated to cluster of differentiation 98 heavy chain (CD98hc) is at the cross-road of oxidative stress and amino acid availability. J Biol Chem 2016;291:9700–9711.

34. Cano-Crespo S, Chillaron J, Junza A, et al. CD98hc (SLC3A2) sustains amino acid and nucleotide availability for cell cycle progression. Sci Rep 2019;9:14065.

35. Fenczik CA, Zent R, Dellos M, et al. Distinct domains of CD98hc regulate integrins and amino acid transport. J Biol Chem 2001;276: 8746–8752.

36. Liu C, Li X, Li C, et al. SLC3A2 is a novel endoplasmic reticulum stress-related signaling protein that regulates the unfolded protein response and apoptosis. PLoS One 2018;13:e0208993.

37. Alexander SP, Benson HE, Faccenda E, et al. The concise guide to pharmacology 2013/14: transporters. Br J Pharmacol 2013;170: 1706–1796.

38. van der Reest J, Lilla S, Zheng L, et al. Proteome-wide analysis of cysteine oxidation reveals metabolic sensitivity to redox stress. Nat Commun 2018;9:1581.

39. Sasada M, Iyoda T, Asayama T, et al. Inactivation of beta1 integrin induces proteasomal degradation of Myc oncoproteins. Oncotarget 2019;10:4960–4972.

40. Shimizu H, Seiki T, Asada M, et al. Alpha6beta1 integrin induces proteasome-mediated cleavage of erbB2 in breast cancer cells. Oncogene 2003;22:831–839.