Addressing ALS: Corroborating the Methodology of Studying the P2X7R Complex

by
Edward Veytsman

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in BioResource Research
(Honors Associate)

Presented September 15, 2020
Commencement June 2021

AN ABSTRACT OF THE THESIS OF

Edward Veytsman for the degree of Honors Baccalaureate of Science in BioResource Research presented on September 15, 2020. Title: Addressing ALS: Corroborating the Methodology of Studying the P2X7R Complex.

Abstract approved:_____

Alvaro Estevez

Amyotrophic lateral sclerosis (ALS) is characterized by the degeneration of motor neurons in the motor cortex and spinal cord. Recent evidence suggests that the purine receptor P2X7 (P2X7R) plays a central role in the pathology of ALS. Previous research indicates that P2X7R activation by oxidatively modified heat shock protein 90 (Hsp90) or Hsp90 inhibition stimulates motor neuron death. The composition of the P2X7 receptor complex, which includes Hsp90, is cell and species specific and is unknown in motor neurons.

The purpose of this study was to investigate whether post-translational compositional differences in the P2X7R complex between healthy and ALS patients exist. We hypothesized that immunoprecipitation and mass spectrometry would indicate that the P2X7R complex is particularly vulnerable to ligand-independent activation by Hsp90 nitration in ALS patients. Before immunoprecipitation and mass spectrometry could be performed, lentivectors carrying green fluorescent protein under the regulation of the motor neuron promoter Hb9 were produced and were added to motor neurons derived from the inducible neuroprogenitor cells of ALS and healthy patients.

Fluorescence in the motor neurons was observed but documented poorly and will have to be repeated. Additionally, the motor neurons died before immunoprecipitation could be performed and subsequent differentiations failed. Future research will compare mass spectrometry results of the P2X7R complex of healthy and ALS patients. If differences are found, subsequent studies should focus on constructing ligands that occupy ALS-specific P2X7R complex regions and testing their efficacy/safety against the control: healthy patients' P2X7R complex.

Key Words: P2X7 receptor, ALS, motor neurons, Hsp90

Corresponding e-mail address: Veytsmae@oregonstate.edu

Addressing ALS: Corroborating the Methodology of Studying the P2X7R Complex

by
Edward Veytsman

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in BioResource Research
(Honors Associate)

Presented September 15, 2020
Commencement June 2021

Honors Baccalaureate of Science in BioResource Research project of Edward Veytsman presented on September 15, 2020.

APPROVED:

_____

Alvaro Estevez, Mentor, representing Biochemistry and Biophysics Department

_____

Maria Franco, Committee Member, representing Biochemistry and Biophysics Department

_____

Katharine Field, Committee Member, representing BioResource Research

_____

Toni Doolen, Dean, Oregon State University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Edward Veytsman, Author

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease that involves the degeneration of upper and lower motor neurons. Motor neuron death is followed by muscle atrophy, paralysis, and eventual death by respiratory failure. In the United States, ALS affects approximately 30,000 individuals at one time, with 5,000 new cases of ALS diagnosed each year (1). Along with other neurological conditions, such as Parkinson's, nitrative stress has been proposed to play a role in the pathogenesis of ALS (2,3). Nitration of tyrosine residues in the molecular chaperone heat shock protein 90 (Hsp90) is present in spinal motor neurons from ALS patients and stimulates motor neuron death in cell culture (4).

The nitration of free tyrosine or protein tyrosine residues produces 3-nitrotyrosine (2). The presence of nitrotyrosine can be detected by immunohistochemistry, HPLC with spectrophotometric and electrochemical detection, and mass spectrometry, among others (5). Many pathological conditions result in a multi-fold increase in protein 3-nitrotyrosine (6). One such relevant example is in sporadic ALS, where HPLC analysis revealed an approximately seven-fold increase in both the concentration of cerebrospinal fluid 3-nitrotyrosine and 3-nitrotyrosine/free tyrosine ratio between ALS and control patients (7). As the free tyrosine levels remained consistent between the ALS and control patients, an increase in protein nitrotyrosine--and not free tyrosine nitration--is likely (6). More importantly, protein nitration does occur in the initiation of motor neuron disease (6). Furthermore, it is the structure of a protein and not its abundance or the number of tyrosine residues a protein has that dictates nitration

selectivity (8). Specifically, the presence of the aromatic ring on the surface of the protein, the location of tyrosine in the loop structure, proximity to a negative charge, and the absence of neighboring cysteines dictates preferential nitration (9).

Through the nitration of tyrosine residues, the biological oxidant peroxynitrite ($ONOO^-$) induces motor neuron death (10,11). Additionally, peroxynitrite is produced as a by-product of trophic factor deprivation (12). This is because motor neurons cultured without brain-derived neurotrophic factor (BDNF) express mRNA transcripts for nitric oxide synthase (NOS), whereas BDNF-treated motor neurons do not. Furthermore, trophic factor-deprived motor neurons display immunofluorescence for neuronal NOS, whereas trophic-factor treated motor neurons do not. When NOS inhibitors were added to trophic factor-deprived motor neurons or when BDNF was added, apoptosis was reduced. Moreover, superoxide scavengers added to trophic factor-deprived motor neurons displayed a decrease in apoptosis and nitrotyrosine residues. Taken together, these results indicate that superoxide and nitric oxide combine endogenously following trophic factor deprivation to produce peroxynitrite and induce apoptosis (12).

As for evidence of tyrosine residue nitration, an experiment showed that when tyrosine-containing peptides were added to a culture of motor neurons, apoptosis was reduced. The peptides acted as competitive targets for the nitrating free radical intermediates generated from peroxynitrite (10). These results suggest that tyrosine nitration activates specific pathways that lead to cellular death. In a separate study, the specific target for peroxynitrite-induced apoptosis was found to be the 90kDa heat

shock protein (Hsp90), as it was the only putative nitrated protein to have significantly poorer motor neuron outcomes after being delivered intracellularly (11).

The members of the Hsp90 family are comprised of highly conserved and ubiquitous molecules that act as protein chaperones (13). Hsp90 molecules are atypical in the sense that their main function is not to fold proteins but to stabilize and activate over 200 signaling proteins known as clients (14). Some of these clients include the purinergic receptor P2X7 and important kinases such as p38, which mediates apoptosis (15,16). The N-terminal domain of Hsp90 binds and regulates clients and co-chaperones. The N-terminal domain also contains the ATPase activity (13,17). Additionally, the middle domain interacts with clients and co-chaperones (13). Lastly, the C-terminus interacts with the largest group co-chaperones, with each co-chaperone in the group containing a tetratricopeptide repeat (TPR) (18).

It is not just the intrinsic anatomy of Hsp90 that determines how it interacts with its ligands, but also post-translational modifications and client-induced co-chaperone specificity. In terms of post-translational modifications, phosphorylation occurs the most, and an experiment in endothelial cells demonstrated that--following Hsp90 phosphorylation--an increased association between endothelial nitric oxide synthase (eNOS) and Hsp90 occurs (19). Furthermore, another method of post-translational modification, S-nitrosylation, produces a similar effect in the association between Hsp90 and eNOS (19). As for co-chaperone recruitment, phosphorylation of the middle domain of Hsp90 enhances the association of the co-chaperone Aha1 (20). Additionally, the

recruitment of co-chaperones is client-specific, as the remodeling co-chaperones (associate after the client) PPIases, Fkbp51, Fkbp52, and Cyp40 are selective for the steroid receptor in steroid-Hsp90 complexes (21)

Ultimately, discovering the consequences of Hsp90 modifications is crucial for accurately describing a myriad of cellular processes such as cell survival, hormone signaling, and apoptosis as well as certain disease states (e.g. ALS) (13). The specific modification and consequence relevant to this study is that the nitration of specific residues in Hsp90 confers neurotoxicity in a gain-of-function manner (11).

Hsp90's toxic gain-of-function is triggered specifically by nitration to its tyrosine residues. When five susceptible tyrosine residues were replaced by phenylalanine, which is resistant to nitration, the intracellularly-delivered, peroxynitrite-treated Hsp90 remained non-toxic to motor neurons (11). Through recombinant protein synthesis via site-specific unnatural amino acid replacement, five different Hsp90 proteins were created, each containing a single nitrotyrosine residue at a specific residue unique to each other (11). Only the Hsp90 proteins with nitrotyrosine at positions 33 and 56 were shown to induce motor neuron death, mimicking the aforementioned results regarding peroxynitrite-treated Hsp90. In fact, only the nitration of one tyrosine residue is necessary and sufficient to precipitate a toxic gain-of-function (11).

Although it is widely known that nitrated Hsp90 and the activation of the FADD Fas pathway leads to motor neuron death, little is known about how the structure, and

therefore function, of the intermediate step between them--the P2X7 receptor (P2X7R)--facilitates the activation of the Fas pathway and subsequent apoptosis. However, it is known that P2X7R is a complex consisting of at least 11 proteins, including Hsp90, and that it is a ligand-gated channel (22,23). Moreover, P2X7R precipitates an influx of calcium once activated (22). This calcium is imperative in the apoptotic pathway for motor neurons. An experiment illustrated that the mobilization of FasL was reduced after a calcium chelator was introduced intracellularly following Hsp90 peroxynitrite treatment (11). Because FasL interacts with the Fas death receptor and because FasL mobilization to this receptor was reduced, motor neuron death was not observed (11).

Furthermore, following Hsp90 inhibition and nitration, P2X7R is capable of becoming activated independent of a ligand and then triggering apoptosis, indicating that the interplay between Hsp90 and P2X7R is paramount in the survival of motor neurons (11,24). Additionally, blocking P2X7R with an antagonist and preventing calcium influx decreases motor neuron death in rats afflicted with spinal cord injuries, but this method doesn’t prevent the ligand independent activation bypass (25). The composition of P2X7R complex in ALS individuals may be more conducive to triggering motor neuron death via Hsp90 nitration than healthy individuals. In order to study this phenomenon, we focused on motor neurons derived from the inducible neuroprogenitor cells (iNPCs) of human participants. This is because P2X7R is both species specific and cell specific (26,27).

The P2X7R complex is cell-specific: in a study via Western Blotting with constructed monoclonal and polyclonal P2X7R antibodies, P2X7R was monomeric in brain glia-astrocyte cells and multimeric in peritoneal macrophages and peripheral bone marrow (26). Furthermore, the P2X7R complex is species-specific: human P2X7R was 10-100 times more sensitive to ligand activation via the ATP agonist BzATP than the rat model, despite the fact that human and murine P2X7R share 80% homology in their sequences (27). This sensitivity stems from the C-terminal domain of P2X7, which is longer than any other member of the P2X family and is responsible for pore formation, transduction, and signaling (28). Most importantly, the C-terminus is the site where Hsp90 binds (29).

The purpose of this study, then, was to investigate whether or not there are post-translational differences in the P2X7R composition between healthy and ALS patients. We hypothesized that mass spectrometry would reveal that P2X7R is particularly vulnerable to ligand-independent activation by Hsp90 nitration in ALS patients. If so, new targets for ALS therapeutics may materialize.

Experimental Procedures

Differentiation of motor neurons

iPSC-derived NPCs of CRTL-155 and SOD-210 provided by our collaborator Kathrin Meyer from Nationwide Children's Hospital were differentiated to motor neurons. The differentiation process took 28 days to complete. The cells were plated on poly-L-ornithine and laminin coated plates with 10mL of media consisting of DMEM/F12

(Thermo Fisher Scientific, Waltham, Massachusettes), B27 (1:50 final) (Thermo Fisher Scientific), the γ-secretase inhibitor DAPT (1:1,000 final) (Sigma Aldrich, St. Louis, Missouri), retinoic acid (1:10,000 final) (Sigma Aldrich) , dorsomorphin (1:1000 final) (Tocris Bioscience, Bristol, United Kingdom) and antibiotic-antimycotic (100X) (1:100 final, Thermo Fisher Scientific) for 3 days at 37ºC. After that, smoothened agonist (SAG) (1:1,000 final) (Sigma Aldrich) was added, and the cells were incubated at 37ºC for another 3 days. Then, muscle extract (Sigma Aldrich) was added (1 μM final) along with a mixture of the following trophic factors: Brain-Derived Neurotrophic factor (BDNF) (Invitrogen, Carlsbad, California, 10 ng/ml final), Glia-Derived Neurotrophic Factor (GDNF) (Invitrogen, 0.1 ng/mL final), and Ciliary Neurotrophic Factor (CNTF) (Invitrogen, 10 ng/mL final). Media were changed every 2 days for 6 additional days and incubated at 37ºC. At the last media change, the cells were incubated at 37ºC with the Hb9:GFP lentivectors in culture media at a multiplicity of infection (MOI) of approximately 20. Twenty four hours later, the media was replaced by 10 mL of new media consisting of neurobasal media (Thermo Fisher Scientific), B27 (1:50 final), antibiotic-antimycotic (100X) (1:100 final), and the trophic factors BDNF (10 ng/mL final), GDNF (0.1 ng/mL final) and CNTF (10 ng/mL final). Media were changed every two days until the cells expressed green fluorescent protein under fluorescent microscopy.

Fluorescent cells were harvested by mild digestion with Accutase (Thermo Fisher Scientific, Waltham, Massachusetts). Cell suspensions were subjected to cell sorting at the Flow Cytometry and Cell Sorting Facility of the Department of Environmental and

Molecular Toxicology. All cells expressing GFP were separated. Motor neurons were centrifuged, the supernatant removed and the pellet frozen at -80°C until used.

Lentivirus Production

Dulbecco's Modified Eagle Medium (DMEM) complete with 10% FBS and 4 mM L-alanyl-L-glutamine was prepared. Then, 25 mM of chloroquine phosphate was prepared. Lastly, 1 mg/mL Polyethylenimine (PEI) was prepared and adjusted to have a pH of 7.

3.8*10^6 HEK 293T cells (TakaraBio, Kusatsu, Shiga, Japan) were seeded onto 10 cm tissue culture plates in DMEM complete with 10% FBS and 4 mM L-alanyl-L-glutamine and incubated at 37°C, 5% CO2 for ~20 hours. Then, the media was replaced with 10 mL of fresh DMEM with an addition of 10 µL of 25 mM chloroquine diphosphate/10 ml of complete media. After that, a mixture with the following 3 transfection plasmids (Table 1) was created: 1.3 pmol psPAX2, 0.72 pmol pMD2.G, 1.64 pmol of Hb9:GFP and OptiPro SFM (Thermo Fisher Scientific) was added until there was 500 µL of media. Next, the transfection mixture was diluted by the addition of 56.7 µL of 1 mg/mL PEI. This was done by adding PEI dropwise while gently flicking the diluted DNA tube (i.e. tube with OptiPro SFM).

The mixture was incubated for 20 minutes at room temperature before being added dropwise to the 293T cells. The cells were incubated overnight at 37°C, and the media was replaced with 15 mL of DMEM complete. Then, the cells were incubated again at

37°C, and the viruses were harvested at 48, 72, and 96 hours after transduction. Next, the viral supernatant was centrifuged at approximately 500g for 5 minutes to pellet any of the 293T cells that were collected during harvesting. Lastly, the viral supernatant was filtered through a 0.45 µm PES filter, snap frozen in liquid nitrogen, and stored at -80°C until further use.

Table 1 Description of the plasmids in the transfection mix

Plasmid	Final concentration	Purpose	Source
psPAX2	2600 pM	Packaging Plasmid	Addgene, Watertown, Massachusetts
pMD2.G	1440 pM	Envelope Plasmid	Addgene, Watertown, Massachusetts
Hb9:GFP	3280 pM	Transfer Plasmid	Addgene, Watertown, Massachusetts

Concentration of Lentivirus

Lentivirus-containing supernatants were harvested and centrifuged at 500g for 10 minutes. Next, the supernatant was transferred to a sterile container and Lenti-X Concentrator (TakaraBio) was added at a ratio of 1 Lenti-X Concentrator:3 supernatant. Then, the mixture was incubated at 4°C overnight. The mixture was centrifuged at 1500g for 45 minutes, leading to the formation of a white pellet. The supernatant was carefully removed, and the high-titer, virus-containing pellet was resuspended in 1/10 to 1/100th of the original volume with complete DMEM. Lastly, the sample was stored at -70°C in single-use aliquots.

Colony Formation Titering Assay

DMEM complete with 10% FBS and 4 mM L-alanyl-L-glutamine was prepared. Additionally, some of the DMEM complete was used to make a DMEM mixture with 10 µg/mL polybrene.

The frozen virus was thawed at 37ºC in a warm water bath and 10-fold serial dilutions of lentivirus into DMEM complete containing 10 µg/mL polybrene were prepared. Next, 150 µL of a viral dilution, along with 1,000 cells, were added to each well in a 6-well plate (each well received a unique dilution). After that, the plate was incubated for 48-72 hours at 37ºC and the media was replaced with 1.5 mL of DMEM complete containing the appropriate amount of antibiotic-antimycotic (100X) determined through minimum toxic experimentation. Then, the cells were incubated at 37ºC for approximately 2 weeks, and every 3-4 days, the media was replaced with DMEM complete containing the appropriate amount of antibiotic-antimycotic (100X).

When the negative control cells (untransduced) died and colonies were visible, the media was aspirated from the wells and the cells were washed with 1 mL of PBS. Next, 0.1% crystal violet solution was filtered through a 0.22 µm filter to remove any precipitates. Each well was stained for 10 minutes at room temperature with 1 mL of 0.1% crystal violet; after which, the stain was removed, the cells were washed with PBS, and the colonies were counted. As such, the transduction units per mL (TU/mL) were calculated.

Table 2. Summary of serial dilution and virus addition

Dilution	Volume of Lentivirus or Previous Dilution (µL)	Volume of DMEM Complete Containing 10 µg/mL Polybrene (µL)	Volume of Virus Added to Plate (µL)	Volume of Cells Added to Plate (µL)	Final Viral Dilution
1:10	100 of Stock Virus	900	150	1,350	1:100
1:100	100 of 1:10	900	150	1,350	1:1,000
1:1,000	100 of 1:100	900	150	1,350	1:10,000
1:10,000	100 of 1:1,000	900	150	1,350	1:100,000
1:100,000	100 of 1:10,000	900	150	1,350	1:1,000,000

Immunoprecipitation

Purified motor neurons were resuspended in immunoprecipitation (IP) lysis buffer (Thermo Fisher Scientific).The motor neurons were then homogenized by 3 cycles of freeze and thaw followed by passage through a needle. One-half mg of motor neuron proteins derived from the previous step were brought to a volume of 300 µL in lysis buffer. Then, 4 µg of polyclonal anti-P2X7 receptor antibody (Alomone, Jerusalem, Israel) was added and the mixture was incubated for 2 hours on ice with agitation. 15 minutes before the end of the incubation, the protein A/G agarose beads (Pierce

Biotechnology, Rockford, Illinois) were washed. After that, 30 µL of protein A/G agarose beads were added, and the samples were incubated for an additional hour. The samples were washed 3 times with an equal amount of lysis buffer (30 µl) and centrifuged at 14,000 RPM for 2 minutes. The supernatant was discarded carefully such that the protein A/G agarose beads were not carried away with the supernatant.

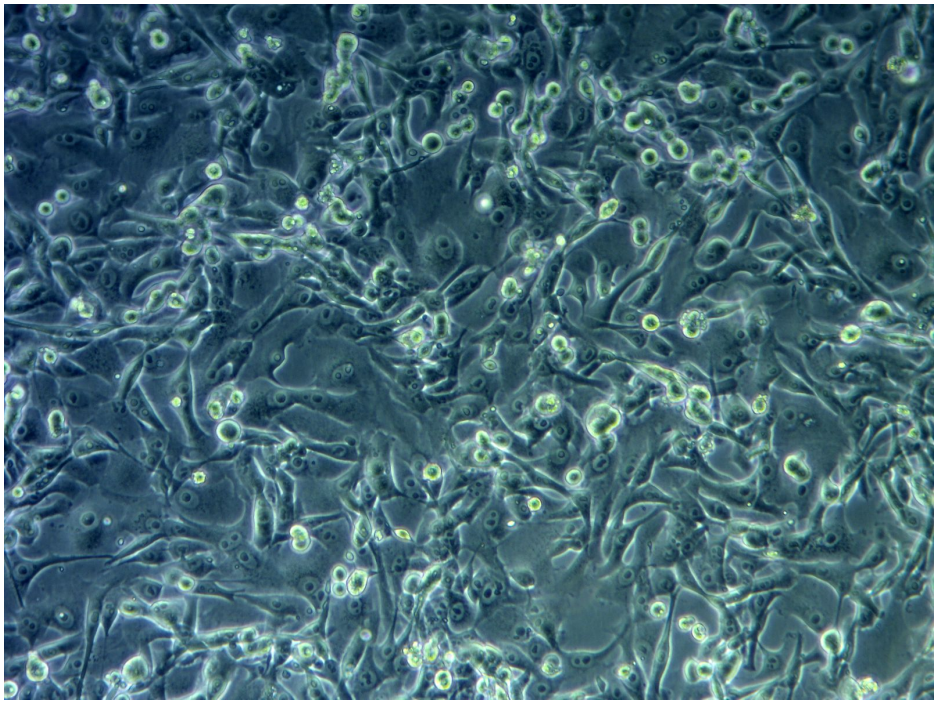
Following supernatant disposal, 10 µl of 3X sample buffer was added to the sample. This was then heated up to 100°C so that the protein of interest could detach from the protein A/G agarose beads. After being heated up, this mixture was centrifuged at 14,000 RPM at 4°C for 10 minutes and the supernatant was saved. The proteins were separated by PAGE on 10% gel, and the bands in the gel were cut and sent for mass spectrometric analysis.

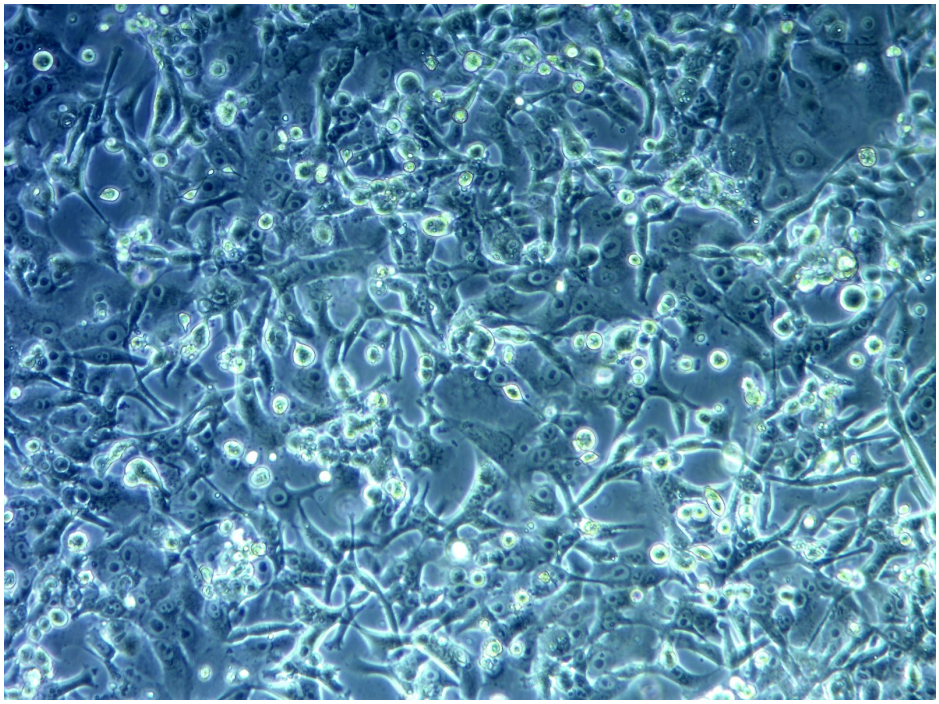
Results

Motor neuron differentiation progression

Figure 1 shows the partial differentiation process of the inducible neuroprogenitor cells (iNPCs) to motor neurons. Even on day 5, there is a visible increase in the proportion of cells expressing a jagged motor neuron phenotype (aka dendrites and axons) as opposed to the round appearance typical of iNPCs. The iNPCs were differentiated up to the point of green fluorescence expression in the motor neurons. Unfortunately, the motor neuron culture containing the fluorescent motor neurons died; therefore, immunoprecipitation and mass spectrometry of the P2X7R complex were not

performed. Additional differentiations to progress further than the fluorescence step and to reach immunoprecipitation and mass spectrometry were not successful.





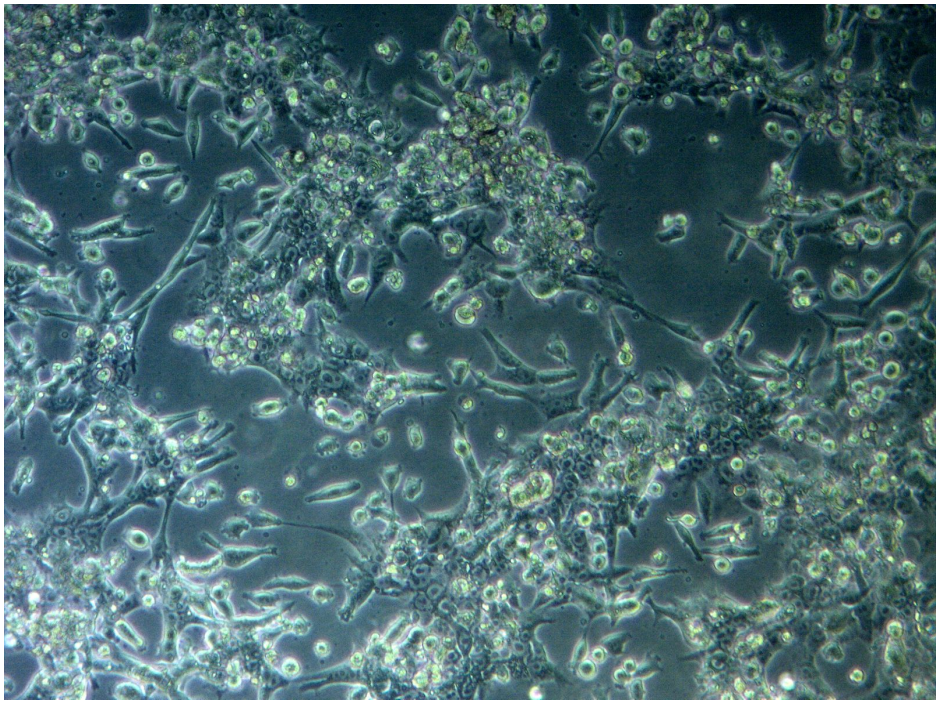
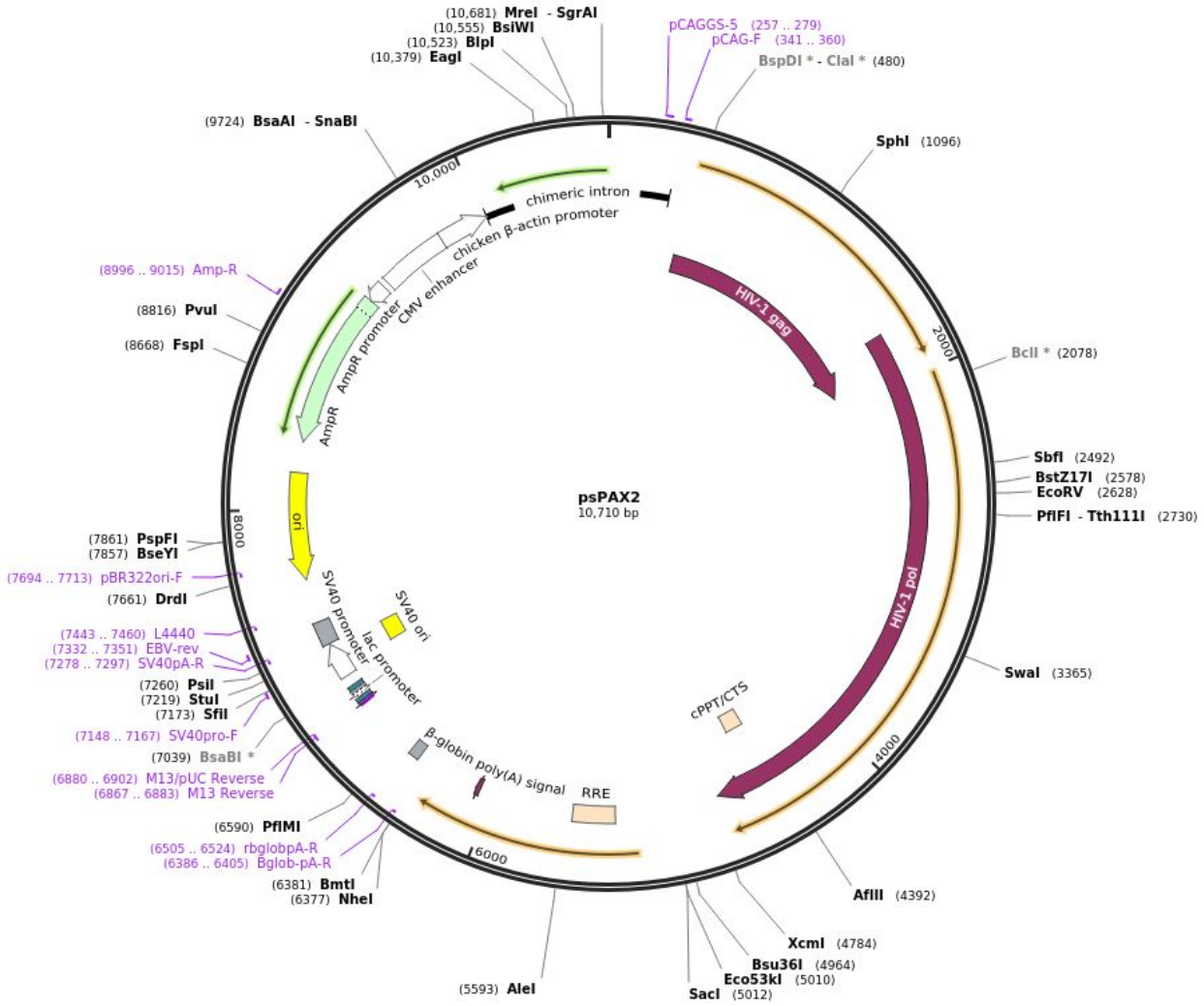
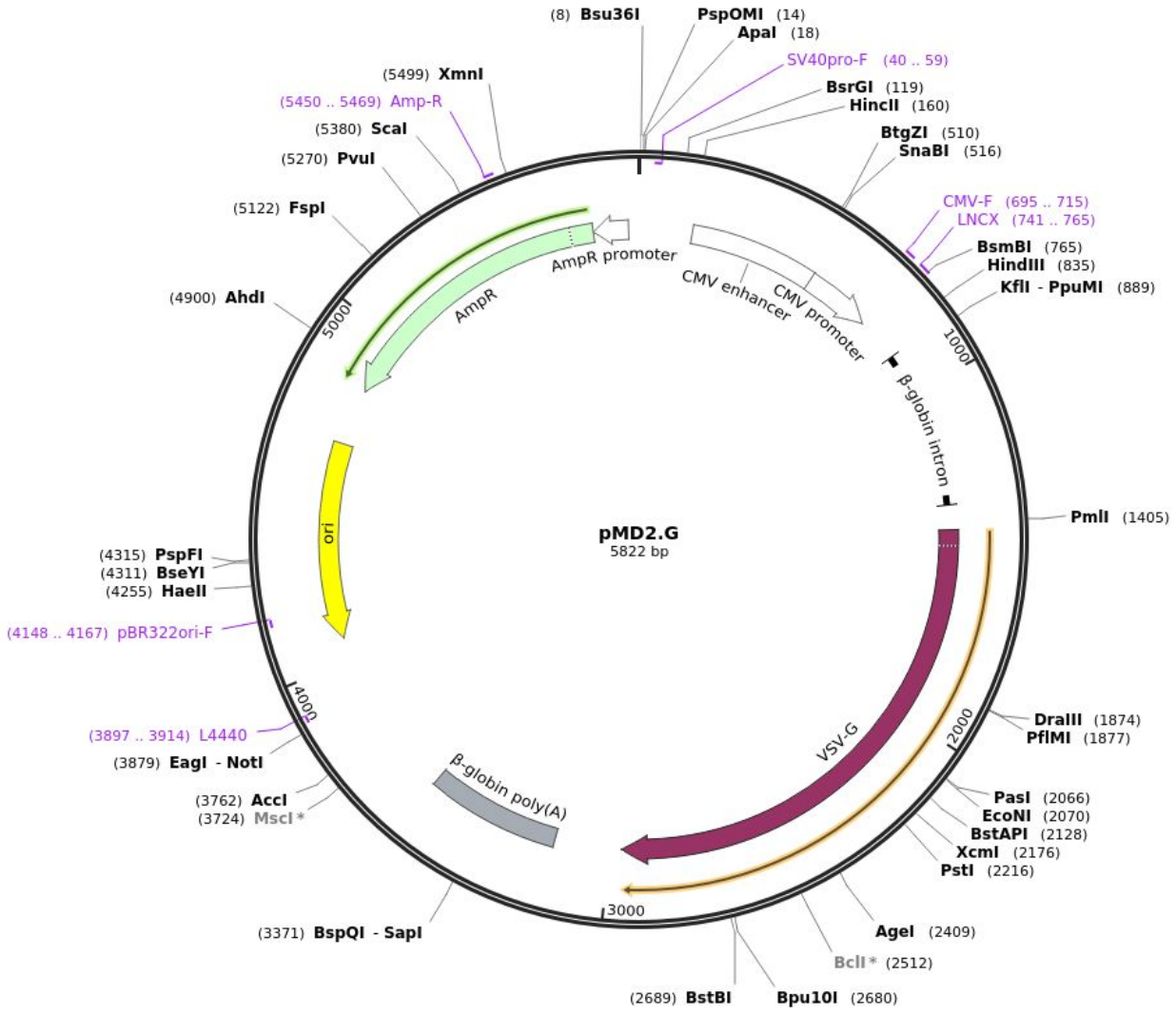


Figure 1. Differentiation of inducible neuroprogenitor cells (iNPCs) to motor neurons. Day 1 (top), day 2 (middle), and day 5 (bottom).

Lentivector production and fluorescence

The Hb9:GFP lentivectors were produced successfully in the HEK 293T cells. Figure 2 illustrates that the packaging plasmid psPAX2, the envelope plasmid pMD2.G, and the transfer plasmid Hb9:GFP were combined in order to manufacture a lentivector that would fluoresce green under the presence of the motor neuron-specific Hb9 promoter (30,31). The motor neurons were transduced with the harvested lentivectors at day 12 of differentiation, and the motor neurons expressed fluorescence several days later. Figure 3 exemplifies this. Lastly, images of the green-fluorescing motor neurons were taken but were not able to be recovered.





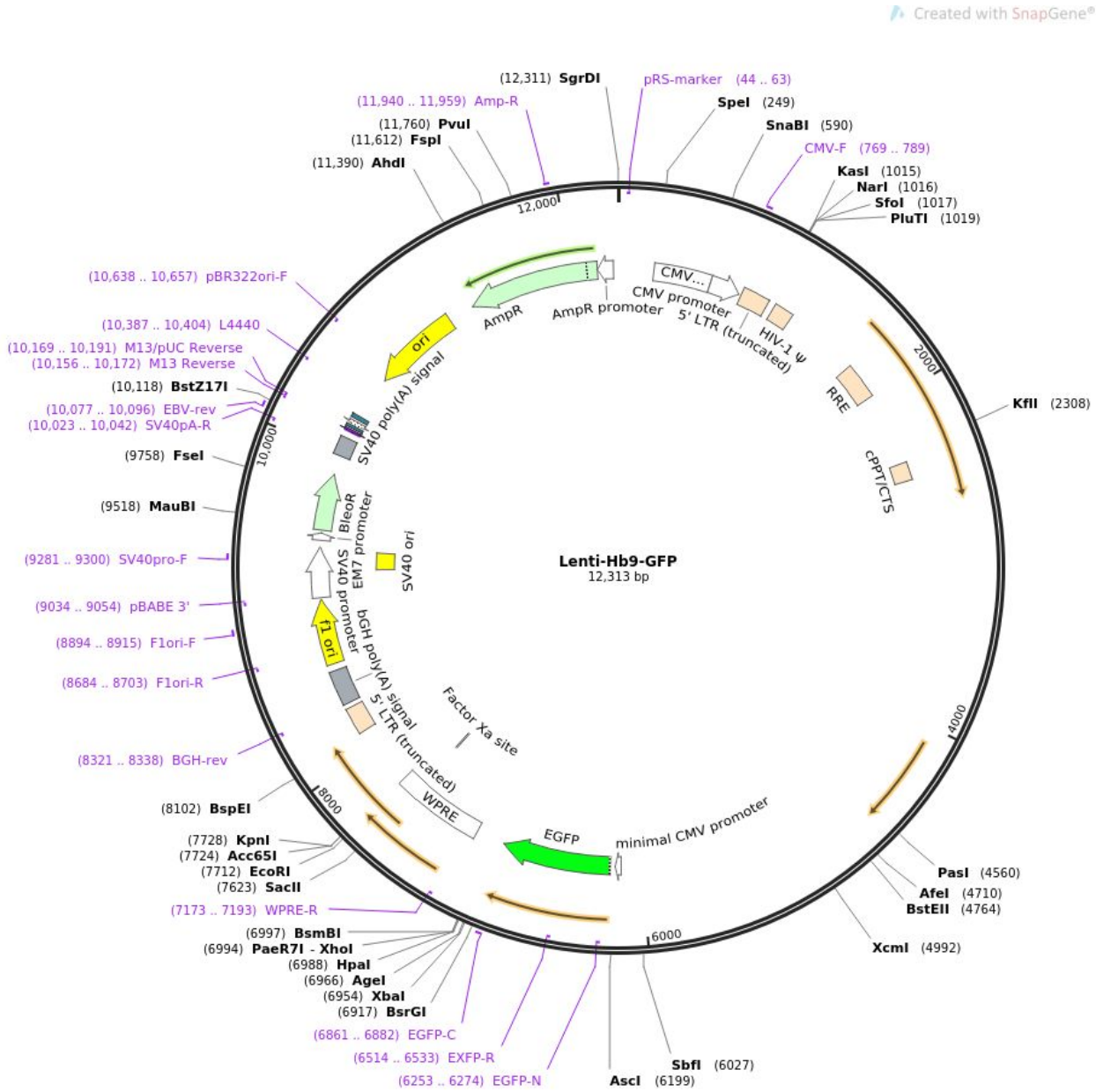


Figure 2. **Plasmid maps of the plasmids that were combined in HEK 293T cells to create the Hb9:GFP lentivectors. PsPAX2 is the packaging plasmid, pMD2.G is the envelope plasmid, and Hb9:GFP is the transfer plasmid.**

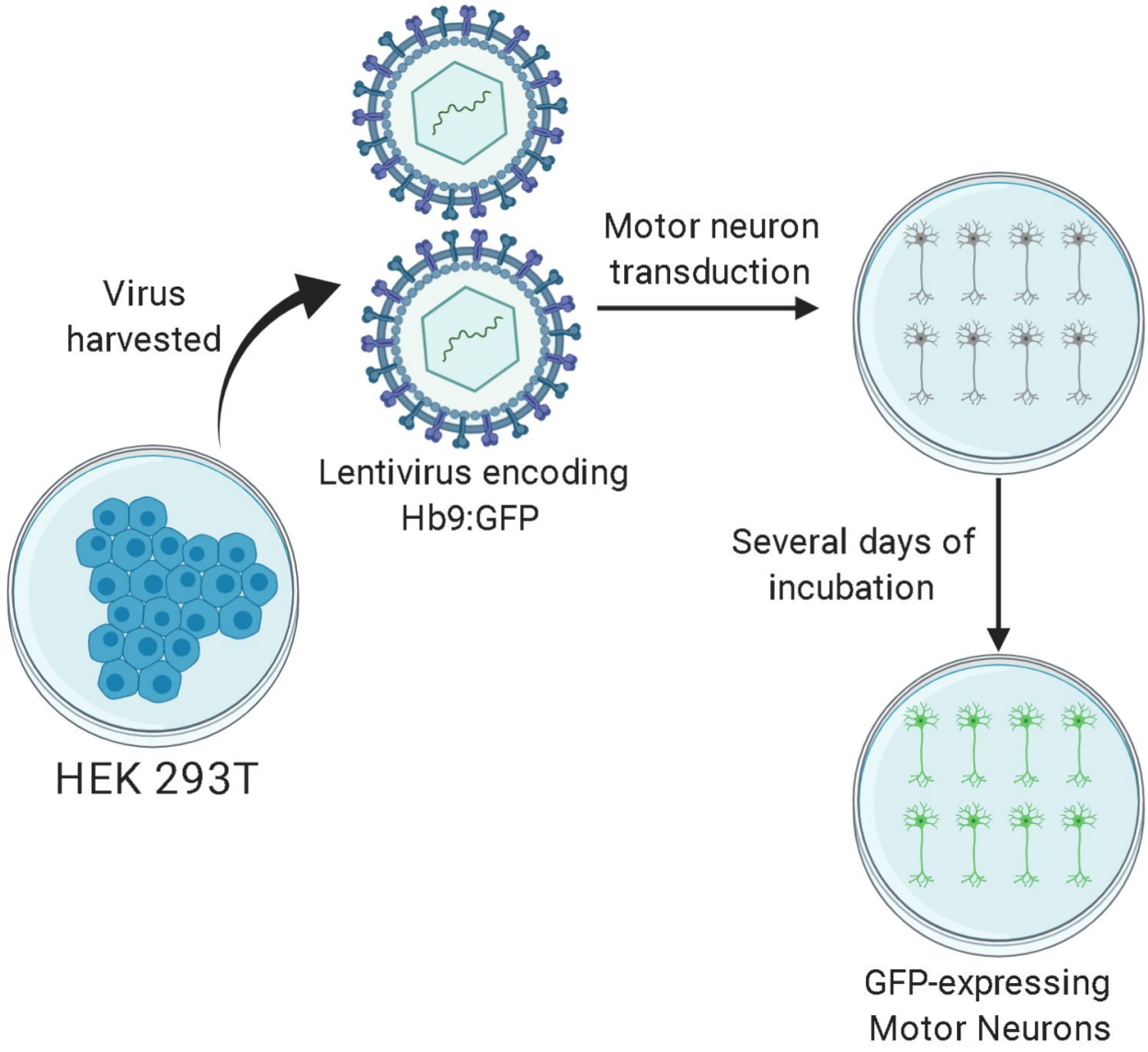


Figure 3. **Diagram detailing how lentivector-induced GFP expression was achieved in the motor neurons**

Discussion

The P2X7 receptor (P2X7R) complex is implicated in the death of motor neurons following the nitration of Hsp90. The P2X7R complex is both cell and species specific

and has been found to be present in humans via *in situ* hybridization (26,27,32). However, it is unclear to what extent the composition of the complex has on facilitating the Fas-FADD cell death pathway. The purpose of this study was to investigate whether there are post-translational compositional differences in the P2X7R complex. If differences exist, new therapies may be developed to target the ALS-specific P2X7R complex.

In this study, we were only able to observe motor neuron fluorescence. Nevertheless, this fluorescence let us detect the cells in culture that were actually motor neurons (i.e. purification). We used inducible neuroprogenitor stem cells (iNPCs) derived from both healthy and ALS patients, matured them into motor neurons, and transduced them with Hb9:GFP-containing lentivectors. Through this method, we identified what cells in our culture were motor neurons, because they were the ones that fluoresced green. More importantly, the de facto presence of fluorescence indicated that the lentivectors were successfully produced in the HEK 293T cells.

Our fluorescence results are consistent with a previous study which also transduced motor neurons with an Hb9:GFP-containing lentivector. The motor neurons in that study fluoresced green following transduction (31). In addition, a different study utilizing the same vector architecture as our study--but with an RFP transfer plasmid instead of a GFP transfer plasmid--demonstrated fluorescence (33). Ultimately, the motor neurons in our study fluoresced because the motor neurons recognize the Hb9 promoter in the lentivector as one of their own, subsequently transcribing and translating the fluorescent

protein of choice. The Hb9 promoter has been inductively established to be in all motor neurons via staining for the Hb9 protein (34).

Unfortunately, even though fluorescence was observed in one culture, that culture died. Furthermore, the following cultures died before motor neuron maturation. There are several reasons why the fluorescent cell culture and subsequent differentiations could have stopped progressing. Mainly, the culture may have been exposed to non-sterile environments for too long, such as when we observed the cells for fluorescence. Another possibility is that the culture became contaminated during media changes. This could have occurred via cross-contamination with a pipette that became unknowingly unsterilized following non-air contact in the fume hood. Lastly, the culture might have been incubated improperly. For example, the water in the pan may have been contaminated or cross-contamination may have occurred between a researcher's lab coat/skin and the incubator. Furthermore, the chamber doors may have been open for a long enough period of time to let in pathogens.

Not only was there an issue with maintaining a viable culture but there was also an issue with recovering images for the motor neuron fluorescence. These images might have been taken improperly on the fluorescence microscope or the files containing the images might have been mismanaged. Additionally, separate images were captured on a cellular device and were backed up into the cloud; however, the cellular device was destroyed and the cloud backup was not viewable/corrupted/inadequately maintained.

As there was no cell culture that could be immunoprecipitated nor images documenting fluorescence, a future experiment will have to achieve motor neuron fluorescence again but with proper documentation before proceeding to immunoprecipitation and subsequent mass spectrometry. If the mass spectrometry illustrates a compositional difference in the P2X7R complex between healthy and ALS patients, future studies will be needed to test whether different ALS-specific P2X7R complex ligands induce a safe and neuroprotective response. These studies will start *in vitro*--and given an efficacy signal--they will then proceed to clinical trials.

Although the original purpose of this study was to analyse the composition of the P2X7R complex, which was not realized, we were still able to corroborate the lentivector method of motor neuron purification employed in previous studies and that utilizing the Hb9 promoter is a sufficient way to achieve this purification.

References

1. ALS, Amyotrophic Lateral Sclerosis, Lou Gehrig's disease [online] https://www.hopkinsmedicine.org/neurology_neurosurgery/centers_clinics/als/conditions/als_amyotrophic_lateral_sclerosis.html
2. Greenacre, S. A., and Ischiropoulos, H. (2001) Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. Free Radic. Res. 34, 541–581
3. Nagase, M., Yamamoto, Y., Miyazaki, Y., and Yoshino, H. (2016) Increased oxidative stress in patients with amyotrophic lateral sclerosis and the effect of edaravone administration. Redox Rep. 21, 104–112
4. Adams, L., Franco, M. C., and Estevez, A. G. (2015) Reactive nitrogen species in cellular signaling. Exp. Biol. Med. (Maywood). 240, 711–717
5. Yang, H., Zhang, Y., and Pöschl, U. (2010) Quantification of nitrotyrosine in nitrated proteins. Anal Bioanal Chem. 397, 879–886
6. Bruijn, L. I., Beal, M. F., Becher, M. W., Schulz, J. B., Wong, P. C., Price, D. L., and Cleveland, D. W. (1997) Elevated free nitrotyrosine levels, but not protein-bound nitrotyrosine or hydroxyl radicals, throughout amyotrophic lateral sclerosis (ALS)-like disease implicate tyrosine nitration as an aberrant in vivo property of one familial ALS-linked superoxide dismutase 1 mutant. Proc Natl Acad Sci U S A. 94, 7606–7611
7. Tohgi, H., Abe, T., Yamazaki, K., Murata, T., Ishizaki, E., and Isobe, C. (1999) Remarkable increase in cerebrospinal fluid 3-nitrotyrosine in patients with sporadic amyotrophic lateral sclerosis. Ann. Neurol. 46, 129–131
8. PACHER, P., BECKMAN, J. S., and LIAUDET, L. (2007) Nitric Oxide and Peroxynitrite in Health and Disease. Physiol Rev. 87, 315–424
9. Souza, J. M., Daikhin, E., Yudkoff, M., Raman, C. S., and Ischiropoulos, H. (1999) Factors determining the selectivity of protein tyrosine nitration. Arch. Biochem. Biophys. 371, 169–178
10. Ye, Y., Quijano, C., Robinson, K. M., Ricart, K. C., Strayer, A. L., Sahawneh, M. A., Shacka, J. J., Kirk, M., Barnes, S., Accavitti-Loper, M. A., Radi, R., Beckman, J. S., and Estévez, A. G. (2007) Prevention of Peroxynitrite-induced Apoptosis of Motor Neurons and PC12 Cells by Tyrosine-containing Peptides. J. Biol. Chem. 282, 6324–6337
11. Franco, M. C., Ye, Y., Refakis, C. A., Feldman, J. L., Stokes, A. L., Basso, M., Mera, R. M. M. F. de, Sparrow, N. A., Calingasan, N. Y., Kiaei, M., Rhoads, T. W., Ma, T. C., Grumet, M., Barnes, S., Beal, M. F., Beckman, J. S., Mehl, R., and Estévez, A. G. (2013) Nitration of Hsp90 induces cell death. PNAS. 110, 4449–4450
12. Estévez, A. G., Spear, N., Manuel, S. M., Radi, R., Henderson, C. E., Barbeito, L., and Beckman, J. S. (1998) Nitric Oxide and Superoxide Contribute to Motor Neuron Apoptosis Induced by Trophic Factor Deprivation. J Neurosci. 18, 923–931
13. Hoter, A., El-Sabban, M. E., and Naim, H. Y. (2018) The HSP90 Family: Structure, Regulation, Function, and Implications in Health and Disease. Int J Mol Sci. 10.3390/ijms19092560

14. Mollapour, M., and Neckers, L. (2012) Post-translational modifications of Hsp90 and their contributions to chaperone regulation. Biochim Biophys Acta. 1823, 648–655
15. Hsp90interactors.pdf [online] https://www.picard.ch/downloads/Hsp90interactors.pdf
16. Porras, A., and Guerrero, C. (2011) Role of p38α in apoptosis: implication in cancer development and therapy. Atlas of Genetics and Cytogenetics in Oncology and Haematology. 10.4267/2042/44993
17. Zuehlke, A. D., and Neckers, L. (2016) Clients Place Unique Functional Constraints on Hsp90. Trends Biochem. Sci. 41, 562–564
18. Muller, P., Ruckova, E., Halada, P., Coates, P. J., Hrstka, R., Lane, D. P., and Vojtesek, B. (2013) C-terminal phosphorylation of Hsp70 and Hsp90 regulates alternate binding to co-chaperones CHIP and HOP to determine cellular protein folding/degradation balances. Oncogene. 32, 3101–3110
19. Li, J., Soroka, J., and Buchner, J. (2012) The Hsp90 chaperone machinery: conformational dynamics and regulation by co-chaperones. Biochim. Biophys. Acta. 1823, 624–635
20. Xu, W., Beebe, K., Chavez, J. D., Boysen, M., Lu, Y., Zuehlke, A. D., Keramisanou, D., Trepel, J. B., Prodromou, C., Mayer, M. P., Bruce, J. E., Gelis, I., and Neckers, L. (2019) Hsp90 middle domain phosphorylation initiates a complex conformational program to recruit the ATPase-stimulating cochaperone Aha1. Nature Communications. 10, 2574
21. Röhl, A., Rohrberg, J., and Buchner, J. (2013) The chaperone Hsp90: changing partners for demanding clients. Trends Biochem. Sci. 38, 253–262
22. Raoul, C., Henderson, C. E., and Pettmann, B. (1999) Programmed Cell Death of Embryonic Motoneurons Triggered through the FAS Death Receptor. J Cell Biol. 147, 1049–1062
23. Kim, M., Jiang, L.-H., Wilson, H. L., North, R. A., and Surprenant, A. (2001) Proteomic and functional evidence for a P2X7 receptor signalling complex. EMBO J. 20, 6347–6358
24. Strayer, A. L., Dennys-Rivers, C. N., Ricart, K. C., Bae, N., Beckman, J. S., Franco, M. C., and Estevez, A. G. (2019) Ligand-independent activation of the P2X7 receptor by Hsp90 inhibition stimulates motor neuron apoptosis. Exp. Biol. Med. (Maywood). 244, 901–914
25. Wang, X., Arcuino, G., Takano, T., Lin, J., Peng, W. G., Wan, P., Li, P., Xu, Q., Liu, Q. S., Goldman, S. A., and Nedergaard, M. (2004) P2X7 receptor inhibition improves recovery after spinal cord injury. Nature Medicine. 10, 821–827
26. Kim, M., Spelta, V., Sim, J., North, R. A., and Surprenant, A. (2001) Differential Assembly of Rat Purinergic P2X7 Receptor in Immune Cells of the Brain and Periphery. J. Biol. Chem. 276, 23262–23267
27. Metzger, M. W., Walser, S. M., Aprile-Garcia, F., Dedic, N., Chen, A., Holsboer, F., Arzt, E., Wurst, W., and Deussing, J. M. (2017) Genetically dissecting P2rx7 expression within the central nervous system using conditional humanized mice. Purinergic Signal. 13, 153–170
28. Adinolfi, E., Pizzirani, C., Idzko, M., Panther, E., Norgauer, J., Di Virgilio, F., and Ferrari, D. (2005) P2X7 receptor: Death or life? Purinergic Signal. 1, 219–227

29. Migita, K., Ozaki, T., Shimoyama, S., Yamada, J., Nikaido, Y., Furukawa, T., Shiba, Y., Egan, T. M., and Ueno, S. (2016) HSP90 Regulation of P2X7 Receptor Function Requires an Intact Cytoplasmic C-Terminus. Mol Pharmacol. 90, 116–126
30. Lukashchuk, V., Lewis, K. E., Coldicott, I., Grierson, A. J., and Azzouz, M. (2016) AAV9-mediated central nervous system-targeted gene delivery via cisterna magna route in mice. Mol Ther Methods Clin Dev. 3, 15055
31.. Marchetto, M. C. N., Muotri, A. R., Mu, Y., Smith, A. M., Cezar, G. G., and Gage, F. H. (2008) Non-Cell-Autonomous Effect of Human SOD1G37R Astrocytes on Motor Neurons Derived from Human Embryonic Stem Cells. Cell Stem Cell. 3, 649–657
32. Yu, Y., Ugawa, S., Ueda, T., Ishida, Y., Inoue, K., Kyaw Nyunt, A., Umemura, A., Mase, M., Yamada, K., and Shimada, S. (2008) Cellular localization of P2X7 receptor mRNA in the rat brain. Brain Res. 1194, 45–55
33. Hester, M. E., Murtha, M. J., Song, S., Rao, M., Miranda, C. J., Meyer, K., Tian, J., Boulting, G., Schaffer, D. V., Zhu, M. X., Pfaff, S. L., Gage, F. H., and Kaspar, B. K. (2011) Rapid and efficient generation of functional motor neurons from human pluripotent stem cells using gene delivered transcription factor codes. Mol. Ther. 19, 1905–1912
34. Faye, P.-A., Vedrenne, N., Miressi, F., Rassat, M., Romanenko, S., Richard, L., Bourthoumieu, S., Funalot, B., Sturtz, F., Favreau, F., and Lia, A.-S. (2020) Optimized Protocol to Generate Spinal Motor Neuron Cells from Induced Pluripotent Stem Cells from Charcot Marie Tooth Patients. Brain Sciences. 10, 407