Disorders of the Nervous System

The Proline/Arginine Dipeptide from Hexanucleotide Repeat Expanded C9ORF72 Inhibits the Proteasome

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Abstract

An intronic hexanucleotide repeat expansion (HRE) mutation in the C9ORF72 gene is the most common cause of familial ALS and frontotemporal dementia (FTD) and is found in ~7% of individuals with apparently sporadic disease. Several different diamino acid peptides can be generated from the HRE by noncanonical translation (repeat-associated non-ATG translation, or RAN translation), and some of these peptides can be toxic. Here, we studied the effects of two arginine containing RAN translation products [proline/arginine repeated 20 times (PR20) and glycine/arginine repeated 20 times (GR20)] in primary rat spinal cord neuron cultures grown on an astrocyte feeder layer. We find that PR20 kills motor neurons with an LD50 of 2 μM, but in contrast to the effects of other ALS-causing mutant proteins (i.e., SOD or TDP43), PR20 does not evoke the biochemical signature of mitochondrial dysfunction, ER stress, or mTORC down-regulation. PR20 does result in a time-dependent build-up of ubiquitylated substrates, and this is associated with a reduction of flux through both autophagic and proteasomal degradation pathways. GR20, however, does not have these effects. The effects of PR20 on the proteasome are likely to be direct because (1) PR20 physically associates with proteasomes in biochemical assays, and (2) PR20 inhibits the degradation of a ubiquitylated test substrate when presented to purified proteasomes. Application of a proteasomal activator (IU1) blocks the toxic effects of PR20 on motor neuron survival. This work suggests that proteasomal activators have therapeutic potential in individuals with C9ORF72 HRE.

Key words: ALS; frontotemporal dementia; lysosome-autophagy; motor neuron; proteasome

Significance Statement

Peptides made up of two alternating amino acids, proline/arginine (PR) or glycine/arginine (GR), are thought to contribute to the pathophysiology of familial ALS and frontotemporal dementia (FTD) caused by expansion of the intronic microsatellite repeat sequence GGGGCC in the C9ORF72 gene. Here, we show that proline/arginine repeated 20 times (PR20) is toxic to motor neurons and inhibits substrate flux through the proteasome and the lysosomal-autophagy pathway. Stimulation of the proteasome alleviates this toxicity, suggesting that targeting the PR20-proteasome interaction may have therapeutic potential.
Introduction

ALS is an adult-onset, fatal neurodegenerative disease that manifests with progressive weakness, muscle wasting, spasticity, and respiratory failure (Wijesekera and Leigh, 2009), signs and symptoms that result from the death of upper and lower motor neurons (Rowland and Shneider, 2001). Approximately 10% of ALS cases are caused by single gene mutations; the remaining ~90% of cases are sporadic (Kiernan et al., 2011).

To date, the most common genetic abnormality underlying familial ALS is an expansion of the hexanucleotide sequence GGGGCC in the intron located between exons 1a and 1b of the C9ORF72 gene (Renton et al., 2011; DeJesus-Hernandez et al., 2011). The majority of normal individuals have fewer than 8 GGGGCC repeats, whereas patients can have hexanucleotide repeat expansions (HREs) consisting of several hundreds or even thousands of repeats (Rutherford et al., 2012). Pre-mRNA transcripts containing the HRE adopt a G-quadruplex structure that can lead to sequestration of RNA-binding proteins and reduced abundance of the mature C9ORF72 mRNA (Conlon et al., 2016; Lee et al., 2016; Lin et al., 2016). In some experimental platforms, the pathophysiology of C9ORF72 HRE can at least partially be linked to these mRNA structures (Haeseler et al., 2014).

Additionally, the pre-mRNA produced from the HRE undergoes translation despite the lack of the ATG start codon. This repeat-associated non-ATG (RAN) translation results in the production of 5 different dipeptide repeat (DPR) proteins, depending on the reading frame and on the translation of the sense or antisense strand. The arginine-rich RAN translation products PR\textsubscript{n} and GR\textsubscript{n}, as well as GA\textsubscript{n} (where the n represents the number of dipeptide repeats) have been shown to result in neurodegeneration (Mizielińska et al., 2014; Zhang et al., 2014). Recent work establishes that DPRs can undergo cell-to-cell transfer (Westergard et al., 2016).

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Briefly, an astrocyte feeder layer was prepared from the cortex of newborn Sprague Dawley rat pups [postnatal day 2 (P2)] and grown to ~80% confluency. Subsequently, dissociated embryonic day 15 (E15) spinal cord neurons were added. One to two days later, AraC (5 mM) (catalog #C6645; Sigma) was added for 24 h to arrest astrocyte proliferation. Cultures were maintained in glia-conditioned medium supplemented with the following trophic factors (1.0 ng/ml each): human neurotrophin-3, human neurotrophin-4, human brain-derived neurotrophic factor, and rat ciliary neurotrophic factor (Alomone Labs). Half of the culture medium was replaced on a biweekly basis.

**Toxicity assays and LD$_{50}$ determination of PR$_{20}$ or MG132**

Days in vitro (DIV)14 mixed spinal cord cultures were exposed to PR$_{20}$ or GR$_{20}$ by addition of synthetic peptide to the media one time. Cultures were fixed 5 d later with freshly prepared 4% paraformaldehyde for 20 min, washed extensively with 0.1 M PBS, pH 7.4, and immunostained with SMI32 to identify motor neurons. Immunopositive cells >25 µm in diameter are motor neurons based on their costaining for peripherin and ChAT, and islet cell-stained concentrations for a more precise definition. Approximately 300 µl of media from each dish was removed and pooled with other dishes, PR$_{20}$ stock solution was added, and the mixture was then added back to the corresponding culture dish, so that each dish contained a final concentration of 2 µM. All plates were incubated with PR$_{20}$ for 48 h.

**Ubiquitylation assay**

The ubiquitylation assay involved acute time point treatments with PR$_{20}$. 16 h prior to scheduled lysis, two dishes were treated to achieve a final concentration of 2 µM PR$_{20}$ using the methods described in the PR$_{20}$ treatment for biochemical assays subsection. This was repeated 8 h prior to lysis, 4 h prior to lysis, and 2 h prior to lysis. Samples were then lysed in RIPA buffer as described in Cell lysis for ubiquitin blots and processed for Western blot analysis.

**MG-132 flux assay**

MG-132 (Sigma) was used to inhibit proteasomal function. Cells were initially treated with PR$_{20}$ for a total of 48 h using the method outlined in PR$_{20}$ treatment for biochemical assays subsection. Four hours prior to lysis, these cells were treated with MG-132 or vehicle. The MG-132 was kept in a stock solution in DMSO. This was added directly to culture to achieve a final concentration of 5 µM. Cells were then lysed in RIPA buffer and immunoblotted for ubiquitin.

**Cell lysis**

Lysis buffer was prepared containing 1% (v/v) protease inhibitor cocktail, 1% (v/v) PMSF, and 1% (v/v) leucine in 1% RIPA buffer solution on ice. Culture dishes were washed once with ice-cold PBS to remove any remaining media. A total of 125 µl (35-mm dish), 150 µl (6-well plate), or 250 µL (60-mm dish) of lysis buffer was added to each plate, and cells were scraped into a cold 1.5-ml tube. Samples were sonicated for 30 s at 20% intensity, placed on a rotator at 4°C for 20 min, and then centrifuged for 15 min at 13,200 rpm. Supernatant was then removed from each tube for further analysis.

**Cell lysis for ubiquitin blots**

Ubiquitin analysis requires the use of deubiquitinase (DUB) inhibitors. Lysis without these inhibitors runs the risk of degradation of the substrates to be targeted. As such, N-ethylmaleimide (Sigma), a potent DUB inhibitor, was used in the lysis buffer. The lysis buffer was prepared containing 1% (v/v) protease inhibitor cocktail, 1% (v/v) PMSF, 1% (v/v) leucine, and 50 µM NEM in 1% RIPA buffer solution. The remainder of the lysis procedure follows Cell lysis.

**Membrane stripping**

Once scanning of the nitrocellulose membranes probed for 4EBP1/P-4EBP1 and S6K/P-S6K had been completed, Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) was used to strip the blot of antibodies. The membranes were washed twice in PBS-T for 10 min each and then placed in the stripping buffer for 22 min. The membranes were then washed twice once again in PBS-T for 10 min. They were then placed in blocking
solution (5% w/v milk in PBS-T) for 45 min. This resulted in membranes with sample attached and no antibodies, ready to be probed for actin.

**In vitro Ub-Sic1 degradation assays**

Polyubiquitylated Sic1 proteins with PY degron motifs and T7 tag (Ub-Sic1) was prepared as previously described by Choi et al. (2016). Different concentrations of PR$_{20}$ peptides (0, 50, or 500 nM) and purified affinity-purified human proteasomes (5 nM) were preincubated 20 min in proteasome assay buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 2 mM ATP, 10 mM MgCl$_2$, and 1 mM DTT] on ice, and 20 nM reconstituted Ub-Sic1 was subsequently added. Degradation of Ub-Sic1 and PR$_{20}$ peptides was monitored by immunoblotting using anti-T7 and anti-HA antibodies, respectively. Band intensities were quantified using ImageJ software (version 1.48k, NIH) from three independent immunoblotting assays ($n = 3$).

**Direct interaction between PR$_{20}$ and human proteasomes**

Whole-cell extracts from a stable HEK293T cell line harboring HTBH-tagged β4 subunits were prepared as in Han et al. (2014) and incubated with HA-tagged PR$_{20}$ or GR$_{20}$ peptides (2 μM) 2 h at 4°C. The resulting proteasome–dipeptide complexes were pull-downed with streptavidin agarose beads (Millipore) for 3 h at 4°C. Unbound proteasomes in the supernatants were discarded, and the pellets were mixed with 2× SDS sample buffer. Different amounts of samples were resolved by SDS-PAGE/immunoblotting against HA and a proteasome core particle α3 subunit.

**Autophagic flux assay**

Primary spinal neurons were dissected from E15 rat pups and cultured at a density of 2.5 × 10$^5$ cells/ml on a laminin/poly-lysine-coated 96-well plate in motor neuron media. Four days after plating, neurons were transfected with Lipofectamine 2000 (Invitrogen). DNA-Lipofectamine mixtures were incubated with neurons for 20 min followed by rinsing. Neurons were then placed in 100 μL of conditioned media that was collected immediately prior to transfection and half fresh motor neuron media containing 4 μM PR$_{20}$, resulting in a final concentration of 2 μM PR$_{20}$.

Optical pulse labeling experiments were performed using an automated microscopy platform previously described by Arrasate et al. (2004) and Barmada et al. (2014). A Nikon Eclipse Ti inverted microscope equipped with a PerfectFocus system, 20× objective lens and an Andor iXon3 897 EMCCD camera were used to acquire images. Samples were illuminated with a Sutter Instruments Lamba XL lamp. The microscope and related components were encased in custom-built plexiglass enclosure set to maintain the temperature at 37°C and CO$_2$ at 5%. Semrock GFP and TRITC filters were used for excitation and detection. A Semrock 405-nm long-pass filter was used for photoactivation. Image acquisition and stage movements were accomplished in an automated fashion using μManager and original code written in BeanShell. For all experiments, photoconversion occurred 24 h following transfection. GFP and TRITC images were acquired immediately following activation and subsequently every 2 h for 12 h. Images were acquired in a similar 12-h window starting 48 h after PR$_{20}$ treatment. Using in-house image analysis scripts, neuronal cell bodies were identified based on their fluorescent intensity and morphology, and a ROI was drawn around the perimeter of each cell at each time. Single-cell TRITC intensity values were recorded and used to fit a first order exponential decay curve for individual neurons and calculate a Dendra2-light chain 3 (LC3) half-life for each cell.

**Statistical analysis**

To determine whether IU1 treatment had an effect on cell survival, a one-tailed, one sample Student’s t test was used to test the hypothesis that IU1 would improve cell survival when used with PR$_{20}$.

To test for group differences in the flux assay, a permutation test was used. This is a nonparametric statistical test that was implemented in MATLAB. The goal of the test was to determine whether the +MG132/−MG132 ratio changed significantly in the presence of PR$_{20}$. The test first quantifies this by taking the average of each experimental group and then finding the difference of ratios. It then permutes two samples located in two groups randomly, and determines that difference between averages again. This is repeated 50,000 times, and a histogram is plotted. This histogram represents the distribution of random noise in the sample. Since a higher difference in the +MG132/−MG132 ratio is a more “significant” result, the percentage of the random noise produced from 50,000 permutations that is higher in magnitude than the true difference (calculated from unpermuted data) is the $p$ value.

Permutations of a data point in a +MG132 into a group labeled −MG132 (or vice versa) were not allowed. This is because these permutations would produce an obviously significant result, as MG132 is a known proteasomal inhibitor that significantly increases ubiquitin levels in the cell. Thus, this group difference was not interesting to us.

To assess group differences in the autophagic flux assay, the two-sided Kolmogorov–Smirnov test was used. This nonparametric test determines the probability that two continuous, one-dimensional probability distributions are drawn from the same distribution or not.

**Results**

**PR$_{20}$ added to media is taken up by neurons and results in death**

The difficulty of distinguishing between toxic HRE mRNA and DPR proteins has impeded our understanding of the mechanism underlying C9ORF72 HRE pathophysiology. As reported in Science (Kwon et al., 2014), the McKnight lab devised a strategy to overcome this problem: they studied U2OS cells and human astrocytes directly exposed to synthetic DPR proteins. They found that HA-tagged DPR proteins are taken up by these cells and evoke a variety of biochemical changes. PR$_{20}$ and GR$_{20}$ were shown to be toxic in a concentration range of 10-30 μM depending on the specific assay. We built upon this
approach by applying synthetic, HA-tagged, PR20 or GR20 dipeptides to cultures of rat spinal cord neurons grown on an astrocyte feeder layer, referred to as mixed spinal cord cultures hereafter. We find that PR20 is toxic to cells: 5 d after a single application of PR20, we observe the death of motor neurons with a LD50 of 2 \( \mu \text{M} \) (Fig. 1A). No significant motor neuron death was seen with 2 \( \mu \text{M} \) PR20 at the 48-h time point. In pure astrocyte cultures, we found no cell death when PR20 was applied up to 10 \( \mu \text{M} \) (Fig. 1B).

Kwon et al. (2014) show that 30 min after application to U2OS cells or human astrocytes, PR20 accumulates in the nucleus, colocalizing with the nucleolar protein fibrillin. This demonstrates that cells can take up extracellular DPR proteins, which is consistent with the recent demonstration of cell-to-cell transfer of DPRs in vitro (Westergard et al., 2016). We investigated the fate of PR20 in mixed spinal cord cultures through immunoblotting and

Figure 1. PR20, but not GR20, is toxic to motor neurons: kinetics of cellular accumulation and disposal. Mixed spinal cord neuron cultures or pure astrocyte cultures were exposed to PR20- or GR20-containing media; survival assays and biochemical interrogations followed. A, The survival of motor neurons was determined 5 d after exposure to various concentrations of PR20. The LD50 of PR20 is \(-2 \mu \text{M}\). B, The survival of astrocytes was determined using the colorimetric XTT assay 5 d after exposure to various concentrations of PR20 or GR20. No death was seen up to 10 \( \mu \text{M} \) DPR. C, The survival of motor neurons was determined 5 d after exposure to various concentrations of GR20. No death was seen up to 35 \( \mu \text{M} \) GR20. D, Immunoblotting for HA-tagged PR20 at various time points after addition of 2 \( \mu \text{M} \) PR20 to mixed spinal cord cultures. PR20 is first detectable in cell lysates approximately 0.5 h after exposure and rises to a maximum at 1.0-2.0 h. The Western blot signal declines thereafter and is undetectable at the 48-h time point. E, In a 2-h pulse-chase paradigm after 2 \( \mu \text{M} \) PR20 application, Western blot signal is detectable in the soluble and insoluble fractions at the 2-h time point, and the signal diminishes thereafter to a barely detectable level by the 6-h time point. F, In a 2-h pulse-chase paradigm after 10 \( \mu \text{M} \) GR20 application, Western blot signal is detectable in the soluble fraction only at the 2-h time point, and the signal diminishes thereafter to a barely detectable level by the 4-h time point. G, Mixed spinal cord cultures were pulsed for 2 h with 2 \( \mu \text{M} \) PR20 and the chase media contained MG132 (5 \( \mu \text{M} \) or vehicle. Cell lysates were prepared at time intervals thereafter, and quantitative image analysis of the resultant Western blots showed that the decrement in PR20 abundance was the same in the +MG132 condition in comparison with the –MG132 condition. H, Mixed spinal cord cultures were pulsed for 2 h with 2 \( \mu \text{M} \) PR20, and the chase media contained bafilomycin A1 (Baf A1) (400 nM). Cell lysates were prepared at time intervals thereafter, and quantitative image analysis of the resultant Western blots showed that the decrement in PR20 abundance was the same in the +Baf A1 condition in comparison with the –Baf A1 condition.
immunocytochemistry. After application of $2 \mu M$ PR20, the immunoblot signal is first detected after 30 min and is maximal at the 1- to 2-h time points (Fig. 1D). Despite the continual presence of PR20 in the media, the immunoblot signal diminishes over time and is undetectable at the 48-h time point. To gain further insight into the kinetics of the observed decrease in PR20 levels in cell lysates over time, we pulsed cells with $2 \mu M$ PR20 for 2 h, washed, replaced with fresh media (lacking PR20), and made cell lysates at regular intervals thereafter. Upon probing for PR20 in the soluble and insoluble fractions of cell lysates, we saw maximal immunoreactivity at the 2-h time point and a rapid decline in soluble and insoluble PR20 over the subsequent 4 h (Fig. 1E). After a single 2-h application of $2 \mu M$ GR20, the peptide is weakly detectable by immunoblotting in mixed spinal cord lysates. To more easily follow GR20 by immunoblotting, we applied $10 \mu M$ GR20 to cultures and performed the same kinetic analysis as described in Figure 1D. In this assay system, GR20 is detectable in cell lysates as early as 15 min and maximally accumulates in cells over the first hour of exposure. GR20 immunoblot signal falls off rapidly thereafter and is undetectable at the 24-h time point. In the pulse-chase assay, GR20 is maximal at the 2-h time point (immediately after the removal of GR20 containing media) and is rapidly cleared from the soluble fraction of cells over the next 2-4 h (Fig. 1F). GR20 is not detectable in the insoluble fraction under these assay conditions. To determine whether the proteasome was involved in the decrease in PR20 abundance, we compared the kinetics of the PR20 signal when MG132 or vehicle was included in the chase media. The loss of PR20 was indistinguishable under these two conditions (Fig. 1G). Parallel studies using bafilomycin A1 monitored by the abundance of KDEL-tagged chaperones BiP and PDI). Biochemical interrogations at earlier time points (25% of LD50) revealed no change in cytosolic GR20 immunoreactivity in motor neurons and other cells (Fig. 2G–I), but not astrocytes (Fig. 2J–L).

We draw a number of conclusions from these experiments. First, PR20 is toxic to motor neurons with an LD50 of 2.0 $\mu M$, a value that is 25% of the LD50 of 8.4 $\mu M$ reported by Kwon et al. (2014) that kills human astrocytes. We do not see the astrocytic DPR toxicity which we attribute to procedural differences: (1) Kwon et al. used human astrocytes, whereas we used freshly prepared rat astrocytes; and (2) we applied DPRs once, whereas Kwon et al. did so repeatedly.

Second, when cells contain the maximal amount of PR20 (approximately 2 h after treatment), we observe that it accumulates in the nuclei of neurons and astrocytes. As such, PR20 acting directly on neurons may be noxious and/or a sublethal insult to astrocytes might be contributing to motor neuron death. Although punctate accumulations of PR20, as reported previously (Wen et al., 2014), are also visible, the extent to which nuclear or punctate PR20 confers toxicity is not known.

Third, even at a ~20 times higher concentration, GR20 is not toxic to motor neurons. When cells contain the maximal amount of GR20 (approximately 2 h after treatment), we find that it selectively accumulates in the cytosol of neurons only. Possible explanations for the benignity of GR20 in our system are that the nucleus is the site of DPR protein toxicity and/or that astrocytes contribute to motor neuron injury. The absence of GR20 in either or both of these locales apparently renders motor neurons largely insensitive to GR20.

Fourth, PR20 accumulates to a higher degree and for a longer time than GR20 in cells, although the half-life of these dipeptides is similar (on the orders of hours). Neither the ubiquitin-proteasome nor the lysosome-autophagy pathway is likely to be responsible for the time-dependent loss of PR20. Owing to how we crafted the sensitivity of Western blotting for DPR proteins, we are probably underestimating the relative abundance of these proteins in our cell lysates. Thus, we suspect that DPR proteins are not completely eliminated from cells within 48 h. It is noteworthy that HA-tagged PR20 and GR20 have similar molecular weights (6.1 vs 5.3 kDa, respectively) and identical calculated isoelectric point (e.g., pI = 12.37). This suggests that the biological effects of small basic peptides are sequence dependent, implying that they interact with distinct intracellular molecules.

**PR20 does not significantly impact mTORC, ER stress, or mitochondrial dysfunction**

Cell and animal models of ALS based on the expression of mutant proteins that cause familial ALS (e.g., mTDP43, mSOD1) have implicated reduced mTORC activity, mitochondrial dysfunction, and ER stress (Kong and Xu, 1998; Lim et al., 2012; Betz and Hall, 2013; Saxena et al., 2013; Perera et al., 2014) in the pathophysiology. To understand the mechanism underlying DPR protein toxicity, we asked whether PR20 evoked similar biochemical changes in cells.

Upon treating cultures with $2 \mu M$ PR20 for 48 h, we used immunoblotting techniques to probe for these biochemical changes (Fig. 3A,B). We see no statistically significant differences in the activation level of the mTOR pathway (monitored by the abundance of phosphorylated S6K and 4EBP1), mitochondrial dysfunction (monitored by the abundance of phosphorylated AMPK), or ER stress (monitored by the abundance of KDEL-tagged chaperones BiP and PDI). Biochemical interrogations at earlier time points similarly show no effect of PR20 on these pathways (data not shown). These observations suggest that in this experimental paradigm, PR20 does not influence pathways previously implicated in mTDP43 and mSOD toxicity. One caveat here is that our culture system contains astrocytes, motor neurons, and other neurons and biochemical changes within a subpopulation of cells might be ob-
Figure 2. PR20 and GR20 accumulate in distinct subcellular locations and distinct cell populations. Mixed spinal cord cultures were exposed to 2 μM PR20 or GR20 for 48 h, fixed, and processed for immunocytochemistry. **A**, Staining with SMI32 reveals large multipolar motor neurons. The inset shows a single 0.5-μm slice from the confocal data at the level of the nucleus. **B**, The same field as in **A**, stained for HA-PR20 reveals nuclear staining as well as scattered puncta. The inset shows a single 0.5-μm slice at the same level as in (**A**), suggesting nuclear PR20 in the motor neuron. **C**, A merge image of **A** and **B** reveals nuclear PR20 in multiple cells, including the labeled motor neuron. The inset shows the same single 0.5-μm slice from **A** and **B** unambiguously demonstrating PR20 immunoreactivity in motor neurons. Calibration bar = 35 μm. **D**, Staining for GFAP reveals abundant astrocytes in these cultures. **E**, The same field as in **D**, stained for HA-PR20 reveals nuclear staining as well as scattered puncta. **F**, A merge image of **D** and **E** reveals nuclear PR20 in astrocytes. **G**, Mixed spinal cord cultures were exposed to 2 μM GR20 for 48 h, fixed, and processed for immunocytochemistry. Staining with SMI32 reveals large multipolar motor neurons. Inset shows a single 0.5-μm slice from the confocal data at the level of the nucleus. **H**, The same field as in **G**, stained for HA-GR20 reveals cytoplasmic staining. Inset show a
scurred when we generate cell lysates from this heterogeneous cell population. The cost of using maximally healthy astrocyte/neuronal cocultures that approximate the \textit{in vivo} complexity of cell-cell interactions is the difficulty of studying the biochemistry of a distinct subtype of neuron.

**PR\textsubscript{20} induces cytoplasmic degradation dysfunction through diminished proteasomal flux**

A variety of disease-causing proteins can cause UPS inhibition (Bence et al., 2001). Thus, we asked whether PR\textsubscript{20} influences the ubiquitylation status of the proteome over time. Upon immunoblotting for total cellular ubiquitin at various time points after the application of PR\textsubscript{20} (vs vehicle) to cultures, we find that PR\textsubscript{20} treatment is associated with a progressive increase in total ubiquitylated protein levels. This is statistically significant at the 2-, 8-, and 16-h time points (Fig. 4A). We considered the possibility that the smear of ubiquitin immunoreactivity might contain PR\textsubscript{20} immunoreactivity. When we performed immunoblots for HA in these samples, we saw all the PR\textsubscript{20} immunoreactivity ran at \textasciitilde10 kDa, the molecular weight we see of pure peptide (Fig. 4A). Thus, this assay provides no evidence that PR\textsubscript{20} is ubiquitylated.

The increase in the steady-state amount of ubiquitin over time could be attributed to an increase in the rate of ubiquitylation or a decrease in the rate of degradation of ubiquitylated substrates (Fig. 4B). This is analogous to the autophagy protein LC3-II whose increased abundance in

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**Figure 3.** PR\textsubscript{20} neither influences the activation of TORC or AMPK nor ER stress. Mixed spinal cord cultures were exposed to PR\textsubscript{20} for 48 h and then processed for immunoblotting. \textbf{A}, Representative immunoblot images of biochemical markers of TORC activation (e.g., phospho-4EBP1 and phospho-S6K), AMPK activation, or activation of the ER stress response (KDEL) shows no difference between PR\textsubscript{20} versus vehicle-treated cells. \textbf{B}, Quantification of the bands with intensity values from samples were averaged and normalized to actin loading controls. Dark grey bars correspond to the presence of PR\textsubscript{20}, and light grey bars correspond to levels without PR\textsubscript{20}. Error bars represent SE.
Figure 4. Total ubiquitin levels rise over time in the presence of PR20; PR20, but not GR20, inhibits substrate flux through the ubiquitin-proteasomal system. Mixed spinal cord cultures were exposed to PR20 or GR20 and subjected to biochemical interrogations. A, Duplicate or triplicate cell lysate samples probed for ubiquitin in the absence of PR20 (-PR20) or at 2, 4, 8, or 16 h after PR20 application. A progressive build-up in total ubiquitin levels occurs over time. The panel below shows quantification of these data with total ubiquitin levels normalized to actin loading controls. Error bars represent SE. Blotting for HA-tagged PR20 shows that DPR is present in the lysates but does not migrate as a high molecular weight species as would be expected if PR20 was ubiquitylated. Equal amounts of total protein are present in each lane, as reported by the actin blot. B, Cartoon describing two different mechanisms for increased steady state ubiquitin levels: enhanced ubiquitylation versus decreased proteasomal degradation. C, Total ubiquitin levels from cells treated with PR20 or vehicle for 48 h and then MG132 or vehicle for 4 h. The difference between the +/− MG132 lanes represents the flux of ubiquitylated substrates over 4 h. Representative ubiquitin blots demonstrate a smaller difference between the +/− MG132 lanes in PR20-treated cultures versus vehicle-treated cultures. The permutation test was applied to determine whether statistically significant group differences exist, and the results are shown in the lower panel. There is a reduced flux of ubiquitylated substrates in the PR20-treated cells versus vehicle-treated cells. Six independent biological replicates were tested for each condition. *p < 0.05 by the permutation test. D, Representative ubiquitin blots demonstrate no difference between the +/− MG132 lanes in GR20-treated cultures versus vehicle-treated cultures. The permutation test was applied to determine whether statistically significant group differences exist, and the results are shown in the lower panel. No group differences were found, and at least 10 independent biological replicates were tested for each condition. n.s., not significant.
cells under certain conditions can be attributed to an increase in the rate of production or a decrease in the rate of degradation (which reflects increased or decreased flux through the lysosomal-autophagy pathway, respectively). Although several approaches can be taken to resolve the mechanism, in the autophagy field, the turnover assay is most widely used: the difference in LC3-II levels between samples with or without an autophagy inhibitor (i.e., chloroquine or bafilomycin A1) is used to report flux (Mizushima et al., 2010; Klionsky et al., 2012). We have taken a similar approach here using MG-132, a potent proteasomal degradation inhibitor (Goldberg, 2012). The difference in the quantity of ubiquitylated proteins from cells treated with MG-132 for 4 h versus vehicle reflects the size of the ubiquitylated protein pool normally degraded by the UPS. The larger the difference, the more proteins are degraded. Thus, this is a measure of flux through the UPS.

Parallel sets of mixed cultures were treated with PR20 or vehicle for 48 h, and subsequent experimental groups were treated with MG-132 or vehicle for 4 h. Upon lysis, whole-cell ubiquitin levels were determined through immunoblotting. The average of these was taken for the whole-cell ubiquitin levels were determined through im-

This observation suggests that PR20 accumulation in cells specifically acts as a proteasomal inhibitor.

We wondered how soon after application to cultures did PR20 inhibit proteasomal flux. Specifically, at the time point when PR20 appears maximally within cells (i.e., 2 h; see Figure 1D), was proteasomal flux impaired? We repeated the ±MG132 flux assay after 2 h of PR20 exposure and found no statistically significant (p < 0.05) group differences were found; proteasomal flux was reduced in the presence of PR20 (Fig. 4C). In parallel, we examined the effect of GR20 on total ubiquitin levels with or without MG-132. GR20 neither influenced total ubiquitin levels nor proteasomal flux (Fig. 4D). Together with the finding of elevated total ubiquitylated protein levels in PR20-treated (but not GR20-treated) cultures over time, these observations suggest that PR20 specifically acts as a proteasomal inhibitor.

We next asked whether PR20 influences flux through the lysosomal-macroautophagy pathway (referred to as autophagy hereafter) as well. As mentioned above, one commonly used biochemical approach is to monitor changes in the amount of 1A/1B-LC3, a microtubule-associated protein that acts as both an autophagy marker and substrate, in the presence or absence of an autophagy inhibitor (Mizushima et al., 2010; Klionsky et al., 2016). Unfortunately, this approach is not well suited to investigations using mixed cultures because of the difficulty of resolving the active, lipidated form of LC3, termed LC3-II, from the unmodified LC3-I using immunoblot techniques (Zhai et al., 2015). Moreover, traditional immunoblotting and immunocytochemical techniques can be insensitive and are often difficult to accurately quantify. To overcome these problems, we adopted an optical pulse labeling approach (Tsvetkov et al., 2013; Barmada et al., 2014) to monitor autophagic flux. In this technique, a Dendra2-LC3 fusion protein is introduced into cells. Upon exposure to 405-nm light, the excitation and emission spectra of Dendra2 are irreversibly red shifted. Because LC3 is an autophagy substrate, the rate of Dendra2-LC3 degradation (estimated by the time-dependent loss of red fluorescence) presents a measure of autophagic flux (Koga et al., 2011; Tsvetkov et al., 2013; Barmada et al., 2014; Loos et al., 2014). An example of this phenomenon is shown in Figure 5A.

Primary spinal neurons were transfected with a plasmid encoding Dendra2-LC3, and subject to optical pulse labeling using automated longitudinal fluorescence microscopy (Barmada et al., 2014). The half-life of Dendra2-LC3 was measured in thousands of primary spinal neurons on single-cell basis. Four experiment groups were investigated: (1) Dendra2-LC3 + vehicle; (2) Dendra2-LC3 + GR20; (3) Dendra2-LC3 + PR20; and (4) Dendra2-LC3 + MG132. We observed a wide distribution of neuronal Dendra2-LC3 half-lives in all groups (Fig. 5B), consistent with previous studies (Barmada et al., 2014). Treatment with 2 μM PR20 peptide led to a significant shift in the population distribution, with a reduction in the peak around ~20 h and a longer tail of half-lives Dendra2-LC3 (Fig. 5B). Compared with the control, treatment with PR20 extended the half-life of Dendra2-LC3 by ~5.6 h (p = 2 × 10^-16). Compared with the control, treatment with GR20 increased the half-life of Dendra2-LC3 by 1.5 h (p = 0.0038). Finally, compared with the control, MG132 led to a more marked rightward shift of the curve, and this treatment extended the half-life by 11.9 h (p = 2.2 × 10^-16).

Next, we looked at the distribution of Dendra2 half-lives operating under the presumption that this soluble cytosolic protein is likely to be degraded by the UPS (Fig. 5C). Four experiment groups were investigated: (1) Dendra2 + vehicle, (2) Dendra2 + GR20, (3) Dendra2 + PR20, and (4) Dendra2 + MG132. Compared with the vehicle, MG132 treatment led to a marked rightward shift of the curve and type-specific reporters of UPS flux could reveal differences otherwise obscured by our Western blots.
the difference between the Dendra2 half-life in MG132 versus vehicle-treated cells (32.3 vs 29.6 h) was statistically significant ($p < 10^{-5}$). Dendra2 half-life in PR$_{20}$- or GR$_{20}$-treated cultures (30.5 and 31.0 h, respectively) did not differ in a statistically significant manner from vehicle-treated cells.

We draw a number of conclusions from these results. First, PR$_{20}$ (and to a far lesser extent GR$_{20}$) retards flux through the autophagy pathway. Second, to the degree that Dendra2 is degraded by the UPS, PR$_{20}$ and GR$_{20}$ had no statistically significant effect. Together, these observations suggest that PR$_{20}$ acts in a relatively selective manner to slow flux through the autophagy pathway. Third, inhibition of the proteasome impairs autophagic flux. This was an unexpected observation, since several prior studies using cell lines have reported autophagy induction upon UPS inhibition (Ding et al., 2007; Lan et al., 2015; Bao et al., 2016). Our findings in neurons may indicate a cell-type-specific relationship between degradative pathways. Alternatively, a nonspecific toxicity to the autophagy pathway may occur when cell function is impaired by UPS blockage. Future work will be required to understand this effect of MG132 on autophagy in neurons.

**PR$_{20}$ imparts direct inhibitory effects on the proteasome in vitro**

Considering the major role of the proteasome in the degradation of ubiquitylated substrates, we focused on the effects of PR$_{20}$ on the UPS. PR$_{20}$ could impede UPS
flux in a variety of ways, such as: (1) reducing access of ubiquitylated substrates to the proteasome and their proper presentation by shuttle factors, (2) increasing de-ubiquitylation, (3) impeding transiting of substrates into the catalytic core of the proteasome, or (4) inhibiting proteolysis. To help distinguish among these possibilities, we began by asking whether PR20 directly associates with proteasomes. Cell lysates from HEK293T cells engineered to express biotinylated proteasomes were incubated with PR20 or GR20. Avidin-coated beads were then used to purify proteasomes, and the pulldown material was subjected to immunoblotting. We find that PR20, but not GR20, associates with proteasomes in this assay and increasing the amounts of proteasomes leads to more pulldown of PR20 (Fig. 6A). In light of this observation, we hypothesized that PR20 directly inhibits proteasomal substrate degradation.

To investigate the mechanism of action of PR20 on the proteasome, we turned to an *in vitro* system with predefined components. Incubation of purified proteasomes with a test substrate (Ub-Sic1) leads to time- and ATP-dependent degradation of Sic1 (Verma et al., 2001). Here, we preincubated proteasomes with PR20 at various molar and subsequently followed the time-dependent degradation of Ub-Sic1 or PR20.

Ub-Sic1 or PR20 levels were determined at 0, 5, and 15 min after exposure to purified 26S proteasomes (Fig. 6B–D). In these experiments, we see a time-dependent loss of Ub-Sic1 and when PR20 is added to these reactions. There is no change in the abundance of PR20 or the alpha 3 subunit of the proteasome over time. Thus, with or without PR20, we see specific degradation of Ub-Sic1 in this assay, but PR20 is not itself degraded by the proteasome (Fig. 6B).

In the absence of PR20, there is progressive reduction in Ub-Sic1 abundance, and quantification of signal shows experimental group differences by ANOVA ($F_{(2,9)} = 773.0$, $p < 0.001$). Post hoc analysis reveals statistically significantly less Ub-Sic1 at the 5- and 15-min time points in comparison with the starting level ($p < 0.001$ and $p < 0.001$). At a molar ratio of 1:10 or 1:100 (proteasomes: PR20), there is also a progressive reduction in Ub-Sic1 abundance and quantification of signal shows experimental group differences by ANOVA ($F_{(2,9)} = 679.5$, $p < 0.001$) and $F_{(2,9)} = 762.6$, $p < 0.001$, respectively. Post hoc analysis reveals statistically significantly less Ub-Sic1 at the 5-min ($p < 0.01$ and $p < 0.01$) and 15-min ($p < 0.01$ and $p < 0.01$) time points in comparison with the starting level.

Qualitatively it appears, however, that the progressive reduction of Ub-Sic1 is less pronounced in the presence of PR20. To quantify this, we monitored the difference between the Ub-Sic1 signal at the starting point versus the 5- or 15-min time point (e.g., $Δ_{0-5}$ and $Δ_{0-15}$). We then compared the $Δ_{0-5}$ of the three experimental groups (e.g., in the absence of PR20: 1:10 proteasomes:PR20 and 1:100 proteasomes:PR20) and found group differences by ANOVA ($F_{(2,9)} = 32.42$, $p < 0.001$) (Fig. 6D). Post hoc analysis reveals statistically significant less $Δ_{0-5}$ in the 1:10 and 1:100 experimental groups compared with the...
no PR20 group (p < 0.05 and p < 0.05). Similarly, we compared the Δ0–15 of the three experimental groups and found group differences by ANOVA (F(2,9) = 8.667, p = 0.008) (Fig. 6D). Post hoc analysis reveals statistically significantly less Δ0–15 in the 1:10 and 1:100 experimental group compared with the no PR20 group (p < 0.05 and p < 0.05). Together, these observations suggest that PR20 operates at the level of the proteasome to impede the function of the UPS. This observation is consistent with the hypothesis that PR20 causes ubiquitylated substrate build-up due to impaired proteasome function.

Pharmacological inhibition of the proteasome is toxic to motor neurons, reduces flux of ubiquitylated substrates, and can be rescued by DUB inhibition

If PR20 kills motor neurons by partial inhibition of the proteasome, then partial inhibition of the proteasome by pharmacological means might: (1) be toxic to motor neurons and, critically, (2) impair proteasomal flux to a similar degree as seen with PR20. These predictions presuppose that proteasomal inhibition by PR20 and by MG132 (for example) are equivalent in terms of pharmacodynamics, pharmacokinetics, and selectivity versus nonselectivity in ubiquitylated substrate build-up. We added varying concentrations of MG132 one time to our mixed spinal cord cultures and found an LD50 of ~100-150 nM when motor neuron number was determined 5 d later (Fig. 7A). We then studied proteasomal flux by incubating sets of DIV14 cultures with 150 nM MG132 or vehicle for 48 h and then treating half of each set with 5 μM MG132 or vehicle for 4 h before creating cell lysates and probing for ubiquitin (as described above in Figure 4). With n = 6 replicates in each experimental group, the permutation test found a statistically significant reduction in proteasomal flux in the 150 nM pretreatment group compared with the control (p = 0.027) (Fig. 7B,C). Finally, we asked whether proteasomal activation with the USP14 DUB inhibitor IU1 (Lee et al., 2010) could protect motor neurons from MG132 toxicity. Parallel sets of dishes were treated with 150 nM MG132 (or vehicle) and 5 μM IU1 (or vehicle). Counts of motor neurons 5 d later revealed statistically significant group differences by ANOVA (F(8,119) = 5.924, p < 0.001). The post hoc analysis revealed statistically significantly fewer motor neurons in the MG132-treated group in comparison with all other experimental groups (p = 0.001). No other group differences were found (Fig. 7D). Thus, pharmacological inhibition of the proteasome that achieves a reduc-
cytochemical quantification of motor neurons. A, The dose-response curve shows that MG132 kills motor neurons with an LD50 of ~ 100-150 nM. B, Mixed spinal cord cultures were exposed to 150 nM MG132 or vehicle for 48 h, and the parallel groups of cultures were exposed to 5 μM MG132 or vehicle for 4 h. Cell lysates were prepared and blotted for total ubiquitin levels. The difference of the ubiquitin immunoreactivity in the cultures treated with 5 μM or vehicle reports the flux of substrates through the UPS over 4 h. Representative blots are shown. C, Quantification of the data from the flux assay and statistical analysis by the permutation tests reveals a statistically significant inhibition of flux through the UPS in cultures treated with 150 nM for 48 h (p = 0.027). D, Number of motor neuron in mixed spinal cord cultures after exposure of 150 nM MG132 or vehicle and 5 μM IU1 or vehicle. MG132 leads to a statistically significant reduction in motor neuron number, and this is reversed by IU1 treatment. IU1 alone is not toxic.

Induction of the proteasome rescues neuronal survival in the presence of PR20

Finally, we asked whether IU1 protects against PR20-induced motor neuron death. We applied PR20 (or vehicle) and IU1 (or vehicle) to cultures and quantified the number of motor neurons alive after 5 d of treatment with each condition (Fig. 8). Statistically significant group differences were found by ANOVA (F_{(8,132)} = 7.408, p < 0.001). The post hoc analysis indicated that a statistically significant decrease in motor neuron survival occurs in the presence of PR20 and this is rescued to control levels upon treatment with IU1 (p < 0.05). IU1 did not influence the survival of vehicle-treated cells. This suggests that the PR20-mediated proteasomal degradation dysfunction plays a role in the lethality that the dipeptide imparts on cells.

Discussion

The mechanism by which C9ORF72 HRE mutations cause ALS/frontotemporal dementia (FTD) (Renton et al., 2011) is an active area of inquiry. Evidence exists for both a toxic RNA gain-of-function and for the production of toxic DPR proteins (Haeusler et al., 2014; Mizielsinska et al., 2014). In an effort to distinguish between the contributions of RNA versus that of DPR proteins, we studied the effects of synthetic dipeptides on mixed cultures. We find that PR20 is taken up by neurons and astrocytes and disrupts both the UPS and the autophagic protein degradation pathways. GR20, however, did not have an effect on the UPS. PR20 has a direct inhibitory effect upon the proteasome when tested in vitro. It is likely that these effects of PR20 are pathophysiological since pharmacological stimulation of the proteasome promotes motor neuron survival in the presence of PR20. The effects of PR20 appear molecularly distinct from mtTDP43- or mSOD-mediated toxicity (Betz and Hall, 2013; Saxena et al., 2013; Perera et al., 2014; Kong and Xu, 1998). Our results suggest a specific mechanism by which proline/arginine DPR proteins are injurious to motor neurons. Since DPR peptides are seen in human autopsy tissues (Ash et al., 2013; Mori et al., 2013), our in vitro observations have implications for human disease.

Among the technical considerations inherent in all disease models, we feel that three merit special discussion. First, many of our experiments are performed after adding PR20 to the culture media, achieving an extracellular concentration of 2 μM. What intracellular concentration of PR20 does this translate into, does it vary over time and/or by cell-type, and most importantly, how do these values compare to the concentration of cellular DPR proteins generated in other experimental platforms? There are no good data on these difficult questions. It is noteworthy that many investigators have deployed the CMV or CAGG promoters to drive high DPR protein expression from an engineered cDNA (Wen et al., 2014; Zhang et al., 2014; Yamakawa et al., 2015). Unfortunately, we lack the quantitative data on the cellular concentration of RAN peptides in these models that could form the basis of comparison with our results. Second, when using purified proteasomes and a ubiquitylated test substrate in vitro (e.g., Ub-Sic1) to determine proteasomal degradation rate, is the PR20-dependent inhibition of the proteasome reporting a physiological event? This is a valid concern, since we only see the PR20 effect when the molar ratio of PR20 to proteasomes is 10:1 or greater. The protein concentration in our in vitro reaction is 20 nM, and it is not possible to substantially increase the concentration because the...
proteins no longer remain soluble. On the other hand, estimates of protein concentrations in cells are about 0.25 g/ml [which for 100-kDa proteins is roughly equivalent to 5 mM (Milo, 2013)], ~5 orders of magnitude greater than that which can be achieved in vitro. The biology of RAN peptides and proteasomes in the crowded environment of cells can, at best, be approximated by in vitro assays, although synthetic PR20 and purified human proteasomes directly interact with each other. Whether the 10:1 ratio is truly reporting on events occurring in vitro will ultimately depend on its predictive value. In light of all of the data we have assembled, we think a reasonable case can be made that the Ub–Sic1/proteasome/PR20 observations are valid and relevant. Third, when DPR proteins are expressed from a cDNA, 36 repeat products (Mizielinska et al., 2014; Tran et al., 2015), 42 repeat products (Wen et al., 2014), 50 repeat products (Zhang et al., 2014), 66 repeat products (Kramer et al., 2016), and 100 repeat products (Yamakawa et al., 2015) are toxic. The extent to which cognate cDNAs are translated to generate the intended full-length product is unclear. In addition, the length of DPRs that are benign and the length beyond which toxicity is detectable remains murky. The selection of 20 repeats of PR or GR in our study was based on the observations of others that this length of DPR can be toxic (Kwon et al., 2014; Kanekura et al., 2016). DPRs as short as 6 repeats (such as GR6 and GA6) can be toxic to neurons; we do not know whether PR6 is toxic and if so, whether this involves the proteasome (Flores et al., 2016). Future studies should investigate these issues as well as determine the prevalence of DPRs of specific lengths in human cells with HRE C9ORF72.

In an attempt to distinguish the effects of toxic mRNA species from those of DPR proteins, many research groups have taken advantage of the degeneracy in the genetic code to create DPR proteins in the absence of GGGGCC repeats. Whether the cognate mRNA by itself is truly nontoxic remains, in our opinion, an open question. G-C-rich RNAs can adopt a variety of secondary structures even without long, uninterrupted stretches of GGGGCC that could attract and sequester RNA-binding proteins. We believe that studies in which cognate RNA is translated to create DPR proteins do not exclude the possibility of RNA toxicity and thus should be interpreted with caution. Only a few groups have studied the effects of synthetic DPR peptides (unambiguously excluding the contribution of mRNA toxicity in models of C9ORF72 HRE toxicity) (Kwon et al., 2014; Chang et al., 2016; Kanekura et al., 2016), and only one studied primary neurons (Flores et al., 2016).

Notwithstanding the above concerns using translation of non-GGGGCC constructs to generate diarnino acid peptide, many groups have investigated the toxicities of various DPR proteins. Wen et al. (2014) showed that PRn is toxic in primary cortical and motor neuron cultures, and Mizielinska et al. (2014) and Wen et al. (2014) found that PRn and GRn, but not PA3, and GA3, are toxic in a fly model. In primary neuron cultures, GA3 and GR3 are toxic (May et al., 2014; Zhang et al., 2014; Flores et al., 2016), and this can be associated with ER stress and cytoplas-
UPS flux, highlights the specificity of the proposed molecular mechanism for PR$_{20}$ toxicity. To understand why the accumulation of ubiquitylated substrates is toxic, it will be important to determine which ubiquitylated substrates accumulate in the presence of PR$_{20}$. Two broad possibilities exist: (1) PR$_{20}$ may nonspecifically inhibit the degradation of ubiquitylated substrates, and this could be toxic owing to the accumulation of damaged/ misfolded proteins; or (2) PR$_{20}$ may inhibit the degradation of a specific subset of ubiquitylated substrates, and this could be toxic owing to the disruption of a select cell biological process. Resolving the contributions of these alternative possibilities may provide mechanistic insight into the pathophysiolog.

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