Homeostatic plasticity can be induced and expressed to restore synaptic strength at neuromuscular junctions undergoing ALS-related degeneration

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Abstract
Amyotrophic lateral sclerosis (ALS) is a debilitating neurodegenerative disease characterized by motor neuron dysfunction and progressive weakening of the neuromuscular junction (NMJ). Hereditary ALS is strongly associated with variants in the human C9orf72 gene. We have characterized C9orf72 pathology at the Drosophila NMJ and utilized several approaches to restore synaptic strength in this model. First, we demonstrate a dramatic reduction in synaptic arborization and active zone number at NMJs following C9orf72 transgenic expression in motor neurons. Further, neurotransmission is similarly reduced at these synapses, consistent with severe degradation. However, despite these defects, C9orf72 synapses still retain the ability to express presynaptic homeostatic plasticity, a fundamental and adaptive form of NMJ plasticity in which perturbation to postsynaptic neurotransmitter receptors leads to a retrograde enhancement in presynaptic release. Next, we show that these endogenous but dormant homeostatic mechanisms can be harnessed to restore synaptic strength despite C9orf72 pathogenesis. Finally, activation of regenerative signaling is not neuroprotective in motor neurons undergoing C9orf72 toxicity. Together, these experiments define synaptic dysfunction at NMJs experiencing ALS-related degradation and demonstrate the potential to activate latent plasticity as a novel therapeutic strategy to restore synaptic strength.

Introduction
Amyotrophic lateral sclerosis (ALS) is a debilitating disease characterized by the progressive loss of motor neurons and synaptic dysfunction at the neuromuscular junction (NMJ), ultimately leading to muscle atrophy. This neurodegenerative disease arises from complex genetic and environmental causes, but abnormalities in two key genes, C9orf72 and superoxide dismutase 1 (SOD1), are most commonly associated with hereditary forms of ALS (1–3). Expression of disease-associated versions of these two genes are sufficient to induce neuronal degeneration in both mammalian and Drosophila models (4–10). However, a detailed analysis of NMJ structure, function, and plasticity has yet to be performed in these model systems, and effective strategies for delaying or restoring synaptic strength remain lacking.

The most common genetic contribution to ALS is associated with a hexanucleotide repeat expansion in the first intron of C9orf72 (GGGGCCn), associated with 20–40% of familial cases (1,2), and 5–20% of sporadic forms of ALS (11). Typical healthy individuals carry less than 30 copies of this repeated region, while ALS patients can harbor hundreds or even thousands of copies (12). This intronic hexanucleotide repeat is thought to give rise to repetitive, structured RNA that can undergo Repeat-Associated Non-ATG Translation (RANT), producing dipeptide repeat (DPR) proteins (2,13–18). Both repetitive RNAs and peptides have been suggested to interfere with diverse cellular...
processes including RNA processing (19), nucleolar function (20–23), and nucleocytoplasmic trafficking (8,13) to ultimately contribute to motor neuron degeneration (4,5,15). Several C9orf72 repeat expansion models have been developed for *Drosophila* (5,8,15), where fundamental insights, conserved in mammalian models, have been revealed (4,21,24).

The *Drosophila* NMJ has been established as a powerful model synapse to study synaptic biology as well as a process called presynaptic homeostatic plasticity (PHP). Here, pharmacological or genetic perturbations that disrupt postsynaptic receptor function lead to decreased responsiveness to neurotransmitter. However, synaptic strength is maintained at baseline levels because of a homeostatic increase in presynaptic release that precisely compensates for reduced receptor functionality (25,26). Importantly, PHP is a fundamental and evolutionarily conserved form of adaptive synaptic plasticity, observed at the NMJs of rodents (27,28) and humans (25,29). However, it is not known whether NMJs undergoing ALS toxicity are capable of expressing PHP, and whether these endogenous mechanisms can be activated for therapeutic potential.

We have systematically evaluated *Drosophila* ALS models to define NMJ dysfunction and test to what extent, if any, these synapses can express PHP. In particular, we determined the manipulation that causes the most severe NMJ degradation, finding that transgenic overexpression of a 100× dipeptide repeat (GR) in *Drosophila* motor neurons led to the most extreme reduction in the number of synaptic boutons. Next, we characterized pre-and postsynaptic defects in this model, revealing reductions in bouton and active zone number as well as neurotransmission. Intriguingly, PHP can be both acutely induced and chronically expressed in C9orf72 NMJs, and we show that synaptic strength can be restored to wild-type levels through genetic manipulations which either activate PHP signaling or increase postsynaptic neurotransmitter trafficking. Finally, activation of the dual leucine zipper kinase (DLK) regenerative machinery lead to decreased responsiveness to neurotransmitter.

**Results**

**Assessing the severity of *Drosophila* ALS models at the larval NMJ**

Over the past several years, a variety of manipulations have been designed to model ALS-related pathogenesis in *Drosophila* (5,6,9,10,15). In general, these approaches were focused on exploiting the speed and power of this genetic system to induce pathology, test for modifiers, and reveal fundamental mechanisms involved in ALS toxicity. While these efforts have succeeded in discovering insights that are conserved in mammalian systems (4), a detailed characterization of synaptic structure and function at the neuromuscular junction has yet to be performed. We therefore utilized a combination of genetics, quantitative imaging, and electrophysiology approaches to define the impact of ALS-related transgenic expression in motor neurons on synaptic structure, function, and plasticity at the larval NMJ.

Two classes of ALS models have thus far been developed in *Drosophila*: those based on disease-relevant mutations in the human SOD1 gene (6,10), and those centered on pathological hexanucleotide repeat expansions in the human C9orf72 gene (5,8,15) (Fig. 1A). Overexpression of these aberrant isoforms of human gene regions in either the adult eye or entire nervous system of *Drosophila* causes neurodegeneration and/or premature lethality (5,6,8,9,15). To model SOD1-related pathology, we obtained a transgenic line that expresses the A4V variation that expresses the A4V variation, as well as DPR-only transgenes engineered to produce guanine-arginine (GR) repeats (15).

To determine which ALS model led to the most consistent and severe pathogenesis at the NMJ, we utilized the Gal4/UAS system to express each transgene in motor neurons and examined synaptic structure at third-instar stages. We reasoned that if these transgenes led to NMJ degeneration, bouton numbers should be reduced. Of all the transgenes tested, only three led to significant reductions in bouton numbers compared to wild-type controls. Overexpression of 58 copies of the (G4C2) repeat (OK371-Gal4; UAS-(G4C2)58X) or the 36× and 100× GR dipeptide repeats (GR.36 or GR.100; OK3-Gal4/UAS-GR) caused significant reductions in bouton number (Fig. 1B). However, NMJs expressing the 58× (G4C2) repeats also exhibited a significant reduction in muscle size (Fig. 1C) and did not survive past the early third instar stage, suggesting some level of non-specific toxicity. In contrast, expression of either GR transgene led to a specific reduction in synaptic bouton number without a change in muscle size (Fig. 1C), surviving through late pupal stages. Finally, we utilized electrophysiology as an additional filter for NMJ pathology in these models. Surprisingly, baseline synaptic transmission appeared unperturbed following expression of the 58× (G4C2) transgene (data not shown). In contrast, EPSP amplitude was greatly reduced following expression of both 36× and 100× GR transgenes (Fig. 4A). Given these results, we focused on characterizing motor neuron expression of GR.36 and GR.100 transgenes in further detail at the larval NMJ (abbreviated GR.36-OE and GR.100-OE), using a GA.100 DPR transgene (GA.100-OE) as an additional control (15).

Expression of GR repeats in motor neurons reduces synapse number and function

Having established an optimal manipulation that leads to NMJ degeneration, we went on to perform a detailed analysis of presynaptic NMJ structure in GR.36-OE and GR.100-OE. We performed immunohistochemistry at the NMJ with antibodies that label synaptic vesicles (vGlut), neuronal membrane (HRP) and active zones (BRP), quantifying synaptic structure in wild type relative to GA.100-OE, GR.36-OE and GR.100-OE. This analysis revealed a ~40% reduction in neuronal membrane surface area as well as bouton size (Fig. 2A, C and D) following GR expression in motor neurons, consistent with retraction of synapses. No changes were observed in GA.100-OE NMJs relative to wild type (Fig. 2A, C and D), confirming no apparent toxicity with equal overexpression of the 100× GA dipeptide repeat (15). Further, we found reductions in the total number of active zones per NMJ, the size of individual active zone puncta, and the staining intensity per active zone puncta (Fig. 2B, E, G and H). However, we observed no difference in the density of active zone puncta (Fig. 2F), nor in the size of the postsynaptic muscle (data not shown). To further characterize the reduction in presynaptic

components, we quantified levels of three synaptic vesicle markers: vGlut (vesicular glutamate transporter), Synapsin, and Synaptotagmin. While vGlut staining intensity was unchanged, both Synapsin and Synaptotagmin were reduced at GR.100-OE synapses, consistent with a general reduction in presynaptic components (Supplementary Material, Fig. S1A and B). Although synaptic bouton number was also reduced at the muscle 6/7 NMJ in GR.36-OE NMJs (Fig. 1B), these animals did not show a significant reduction in neuronal membrane surface area or active zone number at muscle 4 NMJs, suggesting that longer GR repeats induce more severe pathology across distinct synapses. Thus, expression of GR repeats, but not GA repeats, in motor neurons induces presynaptic degradation, including a reduction in the size and number of active zones.

One possibility is that overexpression of GR.100 in motor neurons could inhibit developmental growth of presynaptic terminals. Alternatively, GR.100 NMJs could expand during development, but subsequently undergo degeneration and retractions of synapses. In this case, previous studies have demonstrated synaptic ‘footprints’ are left, in which postsynaptic densities remain unaposed by presynaptic components (30). To assay synaptic footprints, we co-stained NMJs with the postsynaptic marker DLG and the presynaptic marker BRP and quantified the percentage of NMJs with footprints, defined as multiple DLG puncta unaposed by presynaptic BRP strutures. While these footprints were very rare in control NMJ, nearly half of all GR.100-OE NMJs exhibited footprints (Fig. 2l and J). This is consistent with GR.100-OE causing retraction and degeneration of established synapses, although we cannot rule out that some aspects of synaptic development are impacted in this condition.

Given the reduction in synaptic bouton number and presynaptic active zone structures at GR.100-OE NMJs, we next considered whether any changes were induced in the postsynaptic muscle. The Drosophila NMJ is a model glutamatergic synapse, with two classes of postsynaptic receptor complexes composed of the common subunits GluRIIA, GluRIID, GluRIla, and one of two additional subunits, GluRIIB and GluRIIB (30–32). GluRIIB-containing receptors drive most of the current in response to glutamate binding, with less current being contributed through GluRIIB-containing receptors (31,33). Some studies in other neurodegenerative models have suggested changes in the postsynaptic compartment following NMJ pathogenesis (34,35), and we therefore characterized the state of the postsynaptic muscle following GR.100-OE. In particular, a shift in the GluRIIB:GluRIIA ratio could alter the response of the muscle to presynaptic glutamate release, and may therefore contribute to NMJ dysfunction.

We co-stained NMJs with antibodies against GluRIIA, GluRIIB, and the common subunit GluRIID. We observed a reduction in the total number of glutamate receptor puncta per NMJ, with no changes in puncta size in GR.100-OE (Fig. 3A–Q), consistent with a postsynaptic reflection of degraded presynaptic architecture.
Interestingly, we also found a significant increase in both GluRIIA and GluRIIB intensity at GR.100-OE NMJs, with the most notable change being in GluRIIB staining intensity levels (Fig. 3D). In contrast, GR.36-OE and GA.100-OE expression did not lead to any significant change in postsynaptic receptor levels. The changes in postsynaptic receptor levels observed at GR.100-OE synapses may suggest a compensatory response in muscle to reduced presynaptic bouton number and function.

Finally, we assessed the physiological impact of GR expression in motor neurons. We performed electrophysiology at the NMJ and observed a significant reduction in mEPSP frequency in both GR.36-OE and GR.100-OE NMJs (Fig. 4A and C), consistent
with reduced active zone number. However, we observed normal quantal size (mEPSP amplitude) in all genotypes (Fig. 4A and B), indicating a failure to functionally increase postsynaptic responsiveness to glutamate despite the observed changes in receptor expression. Lastly, evoked EPSP amplitudes were reduced by ~50% in both GR.36-OE and GR.100-OE NMJs (Fig. 4A and D), consistent with the reductions in active zone number at these NMJs. This reduction in quantal content (Fig. 4B) leads to an overall reduction in synaptic strength following GR expression in motor neurons. Notably, overexpression of the putatively non-toxic GA.100 DPR transgene had no noticeable effects on spontaneous or evoked neurotransmission. Thus, motor neuron overexpression of GR, but not GA, dipeptide repeats, leads to NMJ degradation, reducing synaptic bouton and active zone number as well as baseline neurotransmission.

**Figure 3.** Postsynaptic glutamate receptor levels are increased following presynaptic expression of GR repeats. (A) Representative images of muscle 4 NMJs in the indicated genotypes immunostained with antibodies against three postsynaptic glutamate receptor subunits: GluRIIA, GluRIIB, and GluRIID. Quantification of total glutamate receptor puncta number (B), glutamate receptor size (C), and normalized mean intensity (D) in the indicated genotypes. Error bars indicate + SEM. Statistical comparisons to wild type were made using a 2-tailed Student’s t-test. *P ≤ 0.05; **P 0.01; ***P > 0.001; detailed statistical information for represented data (mean values, SEM, n, p) is shown in Supplementary Material, Table S1.

**GR.100-OE NMJs retain the capacity to express presynaptic homeostatic potentiation**

Homeostatic control of synaptic strength has been demonstrated at NMJs in Drosophila, rodents, and humans (25,26,29,36). We next set out to test to what extent NMJs undergoing ALS-related degeneration are capable of expressing this adaptive form of synaptic plasticity, referred to as presynaptic homeostatic potentiation (PHP). PHP can be induced by acute pharmacological blockade of glutamate receptors using Phlanthotoxin-433 (PhTx) (37). This causes a reduction in mEPSP amplitude, as expected, but EPSP amplitude remains similar to baseline levels because of a rapid, homeostatic increase in presynaptic release (quantal content) that compensates for reduced muscle sensitivity and restores synaptic strength. We confirmed that in both wild-type and GA.100-OE NMJs, application of PhTx led to the expected ~50% reduction in mEPSP amplitude and stable EPSP amplitudes due to a ~200% increase in presynaptic release (quantal content) (Fig. 5A–C). Application of PhTx to NMJs expressing GR repeats also led to the expected reduction in mEPSP amplitude, while the homeostatic increase in quantal content was also observed, which maintained baseline levels of synaptic strength (Fig. 5A–C). This demonstrates that despite reduced synapse number and baseline transmission at GR.100-OE NMJs, an adaptive increase in presynaptic efficacy can be triggered following acute pharmacological disruption of postsynaptic receptors.

We went on to assess whether the expression of PHP can be maintained over chronic time scales in the face of GR.100-OE induced NMJ degradation. PHP can be induced and expressed over several days of larval development by genetic loss or reduction of postsynaptic glutamate receptors (32,36,38). Although GR.100-OE NMJs can acutely express PHP, maintaining this potentiated state of presynaptic release is more energetically demanding and may not be sustained during NMJ degradation. Postsynaptic glutamate receptors can be reduced over chronic times scales through muscle expression of an RNA-interference transgene targeted against the common glutamate receptor subunit GluRIII (GluRIII-RNAi (38)). As with PhTx-induced PHP, control NMJs exhibit reduced mEPSP amplitudes and a homeostatic increase in quantal content following GluRIII-RNAi expression (Fig. 5A, B and D). Similarly, GluRIII-RNAi; GR.100-OE synapses also exhibited reduced mEPSP amplitudes and...
retained the capability to homeostatically increase release in response over chronic time scales (Fig. 5A, B and D). Because this manipulation requires both pre- and post-synaptic Gal4 expression, we controlled for muscle expression of GR.100. Muscle expression of GR.100 does lead to early pupal lethality (data not shown), consistent with some level of cellular toxicity. We therefore examined synapse morphology and BRP puncta number in these animals compared to controls. Although bouton and BRP numbers are reduced in muscle GR.100-OE (Supplementary Material, Fig. S2A–C), muscle size is also reduced, and the ratio of BRP number to muscle size remains similar to that of wild type (Supplementary Material, Fig. S2D–E).

Figure 4. Presynaptic overexpression of GR repeats degrades synaptic strength at the NMJ. (A) Representative electrophysiological traces of evoked (EPSP) and spontaneous (mEPSP) responses from wild type, GA.100-OE, GR.36-OE, and GR.100-OE NMJs. Quantification of mEPSP amplitude (B), mEPSP frequency (C), EPSP amplitude (D), and quantal content (E) for the indicated genotypes. Error bars indicate ±SEM. Statistical comparisons to wild type were made using a 2-tailed Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001; detailed statistical information for represented data (mean values, SEM, n, p) is shown in Supplementary Material, Table S1.

Figure 5. Homeostatic control of presynaptic function persists despite GR induced NMJ degradation. Representative EPSP and mEPSP traces of wild type (A) and GR.100-OE (B) under baseline conditions and following acute application of the postsynaptic glutamate receptor antagonist philanthotoxin (PhTx). Following application, mEPSP amplitude is reduced, but EPSP amplitudes are maintained at baseline levels due to a homeostatic increase in presynaptic release (quantal content). Similarly, a chronic reduction in mEPSP amplitude can be induced through genetic reduction of glutamate receptor levels by postsynaptic knock down of the glutamate receptor subunit GluRIII (GluRIII-RNAi: w; OK6-Gal4/+; BG57-Gal4, GluRIII-RNAi). Note that both wild type and GR.100-OE NMJs exhibit reduced mEPSP amplitude following PhTx application or GluRIII-RNAi, but maintain baseline EPSP levels, demonstrating robust homeostatic compensation. (C) Quantification of mEPSP and quantal content values for the indicated genotypes following PhTx application, normalized to baseline values (absence of PhTx application). (D) Quantification of mEPSP and quantal content values for the indicated genotypes in combination with GluRIII-RNAi, normalized to control genotypes (absence of GluRIII-RNAi). Note that GR.100 (Muscle) indicates a control for muscle-only expression of GR.100 (UAS-Poly-GR.100/+; BG57-Gal4/+). Error bars indicate ±SEM. Detailed statistical information for additional controls, represented data, and absolute values (mean values, SEM, n, p) are shown in Supplementary Material, Table S1.
Further, synaptic function is not perturbed in muscle GR.100-OE, and PHP can be expressed similarly to controls (Fig. 5D and Supplementary Material, Table S1). Thus, despite the toxicity due to GR expression in motor neurons, GluRIII-RNAi; GR.100-OE synapses retain the capacity to robustly express PHP in response to both acute and chronic perturbation of postsynaptic neurotransmitter receptors.

Activating retrograde homeostatic signaling restores synaptic strength in GR.100-OE synapses

Although NMJs experiencing C9orf72 toxicity are capable of robustly expressing PHP following glutamate receptor perturbation, this latent plasticity is dormant during pathogenesis. Thus, to adaptively increase presynaptic release and restore synaptic strength, we set out to test whether these latent PHP mechanisms can be activated at GR.100-OE NMJs without the counterproductive receptor perturbation. One way to activate PHP signaling without disrupting postsynaptic receptors is through postsynaptic overexpression of the translational regulator Target of Rapamycin (Tor). Chronic overexpression of Tor in muscle (Tor-OE) has been shown to trigger retrograde PHP signaling, leading to increased presynaptic release and potentiated levels of synaptic strength (39) (Fig. 6A). We first confirmed that Tor-OE increased EPSP amplitude without affecting mEPSP amplitudes, leading to increased presynaptic release and enhanced levels of synaptic strength in otherwise wild-type NMJs (Fig. 6A). Importantly, Tor-OE combined with GR.100-OE had a similar effect, potentiating presynaptic release without altering mEPSP amplitude, in effect activating latent PHP expression during presynaptic degradation and restoring synaptic strength to wild-type levels (Fig. 6A–C). We confirmed that pre- and postsynaptic expression of Tor in motor neurons did not impact synaptic physiology (OK6-Gal4/+; MHC-Gal4/UAS-TOR), and controlled for postsynaptic expression of GR.100-OE (OK6-Gal4/GR.100; MHC-Gal4/+−) (Supplementary Material, Table S1). Thus, activation of PHP retrograde signaling at an NMJ experiencing GR-related degeneration restores synaptic strength to wild-type levels, providing a foundation for an intriguing therapeutic approach to counteract ALS-related NMJ toxicity.

Increased postsynaptic receptor expression restores synaptic strength at GR.100-OE NMJs

Our analysis of the postsynaptic muscle at GR.100-OE NMJs suggested that although expression levels of glutamate receptors appear to increase, perhaps as an attempted compensatory adaption to reduced presynaptic release (Fig. 2D), this does lead to any observable functional impact (Fig. 4D). We therefore considered whether increased expression of postsynaptic receptors to a sufficiently elevated level could restore synaptic strength in GR.100-OE NMJs. Postsynaptic overexpression of the GluRIIA subunit (GluRIIA-OE) increases mEPSP amplitude without changing presynaptic neurotransmitter release, leading to enhanced synaptic strength without any induction or expression of PHP (33,40). We first confirmed that postsynaptic overexpression of GluRIIA does indeed lead to increased levels of GluRIIA-containing receptors, at the expense of the lower conducting GluRIIB-containing receptors, in both a wild type and GR.100-OE NMJs (Fig. 7A and B). This results in the expected increase in mEPSP amplitude in both manipulations (Fig. 7C and D). GluRIIA-OE NMJs exhibited no change in presynaptic release, as expected, leading to enhanced synaptic strength (Fig. 7C, E and F). Importantly, while presynaptic release remained depressed in GR.100-OE NMJs, synaptic strength was restored to wild-type levels due to the enhanced postsynaptic sensitivity to neurotransmitter (Fig. 7C, E and F). Thus, adaptive postsynaptic receptor scaling can compensate for degraded presynaptic efficacy in GR.100-OE NMJs, restoring proper levels of synaptic strength.

Given that synaptic strength is restored to wild-type levels in GluRIIA-OE; GR.100-OE NMJs, we next assayed whether or not this adaptive strategy could also improve the motor deficits associated with C9orf72 repeat overexpression (9). We first confirmed that overexpression of GR.36 and GR.100 (Fig. 7H), but not GA.100 (Supplementary Material, Table S1) in motor neurons does indeed cause marked reductions in larval crawling behavior using an established gridline crossing mobility assay (41) (Fig. 7G; see Methods and Supplementary Material, Table S1). Remarkably, overexpression of GluRIIA completely rescues these motor deficits, restoring locomotor behavior to wild-type levels (Fig. 7G). Together, this demonstrates that both enhanced presynaptic release, triggered by activation of PHP signaling, or increased postsynaptic sensitivity to neurotransmitter, induced by increased receptor expression, can restore GR.100-OE NMJs to wild-type levels of synaptic strength.

Activation of the Wallenda/Dlk injury response pathway is not neuroprotective against C9orf72 toxicity

Neuronal injury initiates regenerative signaling pathways, enabling adaptive changes that include inhibited anterograde axonal transport, increased synaptic growth, and reduced neurotransmission (42,43). One of the best understood mediators of this regenerative response is the dual leucine zipper kinase (DLK) pathway, which is activated following injury (44). In healthy Drosophila motor neurons, the E3 ubiquitin ligase Highwire (Hiw) constitutively degrades the DLK homolog Wallenda (Wnd) (45). However, following injury, Hiw ceases degradation of Wnd, enabling activation of a MAP kinase pathway that ultimately changes genes expression in the nucleus and activates regenerative adaptations in the neuron (46–48). Thus, genetic loss of hiw leads to constitutive activation of the DLK signaling pathway, converting the motor neuron into a persistent regenerative state. As a final test of therapeutic potential, we assessed whether engaging this regenerative signaling pathway might be neuroprotective against the degenerative toxicity induced by DPR overexpression in the GR.100-OE model. First, we confirmed the exuberant synaptic growth and reduced neurotransmission induced by the absence of hiw (Fig. 8A and F). Indeed, hiw mutants show extensive synaptic overgrowth, with increased neuronal membrane surface area and bouton number, without a change in overall active zone number (Fig. 8A–E). We reasoned that if regenerative signaling was neuroprotective against further degeneration due GR expression, then expression of GR.100 in a hiw mutant background might not produce degeneration, in effect appearing similar to hiw mutants alone. However, while hiw; GR.100-OE NMJs also exhibit the same distinctive synaptic morphology characteristic of hiw mutants, a reduction in neuronal membrane surface area, bouton size and active zone number was observed compared with hiw mutants alone, similar to the effect of GR.100-OE in wild-type synapses (Fig. 8A–E). Functionally, GR.100-OE in a hiw mutant background further weakened synaptic transmission beyond the reduction observed in hiw mutants alone, also similar to the degenerative impact of GR.100-OE in wild-type NMJs (Fig. 8F–I). Thus, the DLK injury response pathway, while
neuroprotective in response to neuronal injury such as axotomy (44), is not neuroprotective against the pathological toxicity inflicted by GR.100 expression in motor neurons.

**Discussion**

Several models have been developed using transgenic expression of disease-relevant ALS sequences to induce neuronal degeneration in Drosophila. Thus far these studies have focused primarily on the gross effects of degeneration and lethality caused by overexpression of aberrant forms of hSOD1 or C9orf72-derived repeats (5,6,8,9,15). To understand the specific impacts of these manipulations on the neuromuscular junction, the synapse relevant to ALS, we have performed a detailed analysis of synaptic structure, function and plasticity. We went on to demonstrate that one form of adaptive synaptic plasticity, presynaptic homeostatic potentiation (PHP), could be triggered to restore synaptic strength. Similarly, increased postsynaptic expression of neurotransmitter receptors could compensate for reduced presynaptic release and restore synaptic strength and even larval mobility. However, regenerative signaling does not protect or slow motor neurons from GR-related degradation. Together, these experiments define synaptic dysfunction in an established ALS model while both highlighting and constraining possible therapeutic processes to target.

We observed that direct overexpression of arginine-containing dipeptide repeats in motor neurons caused more extensive and consistent loss of synaptic structure than other ALS models. Indeed, arginine-containing DPR-only constructs were more effective than constructs designed to produce both repetitive RNA and a variety of DPRs. This suggests that the toxicity caused by 30× and 58× (G4C2) RNA repeats either induce a novel pathology unrelated to GR translation, or do not lead to the same levels of GR translation compared to the GR.36 and GR.100 transgenes. Non-canonical RAN translation is likely to be a less efficient process in Drosophila compared with standard ATG translation, and toxic DPRs may therefore not accumulate as rapidly compared with direct translation. In addition, while both the 30× and 58× repeats have been found to have neurodegenerative effects in adult Drosophila photoreceptors (5,9), the relatively short larval development may not allow sufficient time to cause degeneration in larval motor neurons. Also, while it is not clear whether C9orf72-related degeneration in mammals is caused by loss of endogenous C9orf72 function, production of repetitive RNAs, build-up of DPRs, or a combination of all of these (12), it is clear from fly models that expression of arginine-containing DPRs alone, and not RNA, is sufficient to produce toxicity (15). It has been proposed that the degradation of presynaptic structures due to GR expression results from defective gene expression due to aberrant nuclear pore trafficking (8,9), while RNA export and processing may also be defective (8,13,19–23). In contrast to GR expression, overexpression of a disease form of hSOD1 (A4V) appeared to have little effect on synaptic structure, although there is recent indication that engineered mutations in the endogenous sod1 gene in Drosophila produces more penetrant larval phenotypes (10). Nonetheless, we have found that protein coding GR repeats produce NMJ degradation at Drosophila larval NMJs that most closely parallels disease pathogenesis observed in mouse models and humans (49–53).

We have used the GR.100 DPR model to establish a more complete understanding of the structural and functional consequences of ALS toxicity at neuromuscular junctions. First, we find that expression of GR.100 causes a dramatic retraction of synaptic membrane and active zone structures. This is consistent with the ‘dying back’ phenomenon observed in ALS cases, where retraction and dysfunction at the NMJ occurs prior to motor neuron cell death (54,55), suggesting that this Drosophila model parallels early stages of this disease. We observe a clear reduction in all presynaptic components, similar to what has been observed in human ALS tissue and mammal models (49–52).

Next, our electrophysiological studies demonstrate loss of presynaptic efficacy in GR.100-OE that matches the observed degradation of synapses. Reductions in presynaptic release...
have also been observed in human ALS patients, consistent with NMJ dysfunction observed in early stages of ALS (53). Interestingly, while decreases in presynaptic neurotransmitter release (quantal content) are observed in both this Drosophila model and human ALS patients, human patients also present with reduced quantal size (53), which we do not observe. This may reflect pathology that occurs in later stages of ALS, not re-capitated in this fly model, or, alternatively, that compensatory upregulation of neurotransmitter receptors in early stages maintains quantal size, as observed in Figure 3. Furthermore, while muscle-autonomous pathology is observed in SOD1 mutant mice (56), we find that C9orf72 DPR expression in muscle alone, while certainly toxic, does not cause synaptic defects. Nonetheless, clear loss of presynaptic release and synaptic strength at the NMJ is observed in both fly and mammalian models (54, 57, 58).

Most therapeutic strategies to combat C9orf72 pathogenesis thus far have focused on either interfering with nuclear transport machinery to alleviate the build-up of toxic DPR proteins (8, 9) or targeting repetitive RNAs themselves (13, 18, 59, 60). In contrast, we have focused on restoring functionality to an already compromised synapse. Indeed, we demonstrate that homeostatic plasticity can be induced and expressed at GR.100-OE degrading NMJs. Further, the therapeutic potential of this approach was demonstrated by activating PHP signaling through postsynaptic expression of Tor, which effectively restores synaptic strength to wild-type levels. Although Tor signaling has yet to be tested in mammals, PHP is conserved at rodent and human NMJs (25, 27–29), and there is evidence in the rodent central nervous system that postsynaptic Tor-mediated signaling drives a retrograde enhancement in presynaptic efficacy (61–63), hinting at the therapeutic potential of this approach. Together, this highlights the potential of the latent plasticity that exists in motor neurons to be expressed despite disease pathogenesis, and presents an attractive therapeutic target.

Figure 7. Postsynaptic overexpression of GluRIIA increases quantal size and restores synaptic strength and locomotor behavior in GR.100-OE. (A) Representative images of glutamate receptor immunostaining at NMJs of wild type, postsynaptic overexpression of GluRIIA (GluRIIA-OE), and postsynaptic overexpression of GluRIIA with GR.100-OE (GluRIIA-OE Æ GR.100-OE). Note that GluRIIA-OE increases GluRIIA expression and reduces GluRIIB expression. (B) Quantification of mean intensity levels of glutamate receptor staining from the indicated genotypes. (C) Representative EPSP and mEPSP traces from indicated genotypes. Note that GluRIIA-OE causes an increase in postsynaptic sensitivity to glutamate, resulting in enhanced quantal size, no change in quantal content, and a concomitant increase in EPSP amplitude. Quantification of mEPSP amplitude (D), EPSP amplitude (E), and quantal content (F) in the indicated genotypes. (G) Schematic showing larval mobility assay. Single larvae are allowed to crawl freely on an agarose plate placed on top of 5 mm grid paper. The number of gridlines crossed in 2 minutes is manually scored. (H) Locomotor behavior is improved following elevated postsynaptic receptor expression in GR.100-OE NMJs. Quantification for the mobility assay for wild type, GR.36-OE, GR.100-OE, and GluRIIA-OE third-instar larvae without and following GluRIIA overexpression. Error bars indicate SEM. Statistical comparisons were made using a 2-tailed Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001; ns = not significant, P > 0.05. Detailed statistical information for represented data (mean values, SEM, n, p) is shown in Supplementary Material, Table S1.
Figure 8. Constitutive regenerative signaling is not neuroprotective against GR.100-OE induced degeneration. (A) Representative images of wild type, highwire (hiw) mutant, and hiw; GR.100-OE (hiw; OK6-Gal4/UAS-GR.100) NMJs. Presynaptic neuronal membrane is immunostained with anti-HRP (magenta) and anti-vGlut (green). (B) Representative images of BRP puncta in these genotypes. Quantification of neuronal membrane surface area (C), bouton size (D), and BRP puncta number per NMJ (E) in the indicated genotypes. (F) Representative EPSP and mEPSP traces of the indicated genotypes. Quantification of mEPSP amplitude (G), EPSP amplitude (H), and quantal content (I) in the indicated genotypes. Error bars indicate ±SEM. Statistical comparisons were made using a 2-tailed Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001; ns = not significant, P > 0.05. Detailed statistical information for represented data (mean values, SEM, n, p) is shown in Supplementary Material, Table S1.
While work with SOD1 mutant mice has revealed defects in the muscle compartment during ALS disease progression (56), less is known about the role or response of the muscle cell to C9orf72-related neurodegeneration. We show that muscle-only expression of GR.100, while toxic, does not impact synaptic strength. We further demonstrate that postsynaptic receptor clusters are reduced in GR.100-OE NMJs, reflecting the reduction in presynaptic structures. However, we also observed an apparent increase in receptor levels, suggesting a possible compensatory adaptation in the muscle to reduced presynaptic release. Interestingly, alterations to postsynaptic receptor subunit composition is observed upon denervation at human and rodent NMJs (54,35), and homeostatic scaling of postsynaptic glutamate receptors has been widely observed in the mammalian central nervous system (64) and even at the Drosophila NMJ (65). However, activation of regenerative signaling was not neuroprotective against degeneration by C9orf72 DPs. Interestingly, the degeneration observed in ALS is similar in many ways to axotomy-induced Wallerian degeneration, and a genome-wide association study of ALS patients found links with genes associated with Wallerian degeneration (54,66). This suggested that activation of Wallenda signaling, due to the absence of Highwire, could be protective against ALS degeneration. However, GR.100-CE caused an even further reduction in synaptic area and strength in highwire mutants, indicating that at least this GR toxicity cannot be ameliorated by regenerative signaling. Although regenerative signaling is not neuroprotective, there may be an unappreciated level of adaptive plasticity of receptor trafficking that exists at NMJs, parallel to the more famous processes in central neurons, that could be utilized to delay and restore NMJ function during disease progression.

The establishment of ALS models in Drosophila now enables the powerful and extensive genetic toolkit of this system to be exploited to further investigate disease pathogenesis, progression, and therapeutic opportunities. Indeed, modifier screens have already been utilized using degeneration in the Drosophila eye to reveal nuclear pore components as genetic interactors of C9orf72-related pathology (8,9). Additional modifier screening approaches, perhaps modeled after successful screens assaying larval NMJ growth, structure, and even function (67–70), will almost certainly reveal unanticipated insights into ALS pathogenesis with therapeutic potential. In addition, new RNA-seq and translational profiling techniques in Drosophila (71–73) can now be leveraged to generate cell-type specific RNA libraries following C9orf72 pathogenesis. This powerful approach has the potential to discover the key genes, likely mis-expressed due to defective nucleocytoplasmic trafficking following disruption of the nuclear pore (6,9), that ultimately leads to presynaptic degeneration at the NMJ. Finally, the Drosophila model system offers a nearly complete collection of mutant and RNAi lines targeting every gene in the genome (74) to further investigate genes involved in ALS disease pathogenesis. Together, these unbiased approaches in Drosophila and other systems, combined with human genetic studies of ALS patients and emerging induced pluripotent stem cell technologies, provide a strong foundation from which to define pathogenic processes and identify therapeutic interventions in ALS and other neurodegenerative diseases.

Materials and Methods

Fly Stocks: Drosophila stocks were raised at 25 °C on standard molasses food. The w1118 strain is used as the wild type control unless otherwise noted, as this is the genetic background of the transgenic lines and other genotypes used in this study. Detailed information on the transgenic and mutant fly lines used is this study can be found in Supplementary Material, Table S1. Standard X, second (CyO; CyO, Weep; CyO, GFP), and third chromosome balancers (TM3, Tb; Sb; TM6B, MKRS) and genetic strategies were used for all crosses and for maintaining mutant lines.

Immunocytochemistry: Third-instar larvae were dissected in ice cold 0 CaCl2 HL-3 and fixed in Bouin’s fixative for 5 min as described (75). Larvae were washed with PBS containing 0.1% Triton X-100 (PBST) for 30 min, blocked for 1 h in 5% Normal Donkey Serum (NDS), overnight incubation in primary antibodies at 4 °C, then washed in PBST, incubated in secondary antibodies for 2 h, a final wash in PBST, and equilibration in 70% glycerol. Samples were mounted in VectaShield (Vector Laboratories). The following antibodies were used: mouse anti-Synapsin, 3C11 (1:10; Developmental Studies Hybridoma Bank; DSHB); guinea pig anti-vGlut (75) (1:2000); mouse anti-Bruchpilot (BRP: nc82; 1:100; DSHB); mouse anti-GluRIIA (BB4D2; 1:100; DSHB); rabbit anti-GluRIIB (1:1000; generated by Cocalico Biologicals using the peptide described in (32)); guinea pig anti-GluRIID (1:1000; generated by Cocalico Biologicals using the peptide described in (30)); Rabbit anti-Synaptotagmin Dsyt-CL1 (76); Cyanine 3 (Cy3) or Alexa Fluor 647 conjugated goat anti-HRP (1:200; Jackson ImmunoResearch). Donkey anti-mouse, anti-guinea pig, and anti-rabbit Alexa Fluor 488-, Cy3, and DyLight 405-conjugated secondary antibodies (Jackson Immuno Research) were used at 1:400.

Imaging and analysis: Samples were imaged using a Nikon A1R Resonant Scanning Confocal microscope equipped with NIS Elements software and a 100× APO 1.4 NA oil immersion objective using separate channels with four laser lines (405, 488, 561 and 637 nm). For fluorescence quantifications, z-stacks (step size of 0.175 µm) were obtained using identical gain and laser power settings for all genotypes within an experiment. Representative images were optimized for detection without saturation of the signal. Boutons were counted using vGlut and HRP-stained NMJ terminals on muscle 6/7 and muscle 4 of segment A3, considering each vGlut puncta to be a bouton. The general analysis toolkit in the NIS Elements software was used to quantify BRP puncta number, size, mean staining intensity, and density by applying intensity thresholds and filters to binary layers on this channel. Quantification for the GluR subunits was performed in a similar way by using the binary for GluRIIA to measure values in the GluRIIB and GluRIID channels. Measurements based on confocal images were taken from at least ten synapses acquired from at least four different animals. The footprint retraction assay was performed as previously described (77). M6/7 synapses from segments A2–A5 were examined for DLG boutons lacking BRP puncta. NMJs with at least one such bouton were scored as a retraction.

Electrophysiology: All dissections and recordings were performed in modified HL-3 saline (78–80) containing (in mM): 70 NaCl, 5 KCl, 10 MgCl2, 10 NaHCO3, 115 Sucrose, 5 Treheolose, 5 HEPES, and 0.4 CaCl2 (unless otherwise specified, pH 7.2. Neuromuscular junction sharp electrode (electrode resistance between 10 and 35 MΩ) recordings were performed on muscles 6 and 7 of abdominal segments A3 and A4 in wandering third-instar larvae. Larvae were dissected and loosely pinned; the guts, trachea, and ventral nerve cord were removed from the larval body walls with the motor nerve cut, and the preparation was perfused several times with HL-3 saline. Recordings were performed on an Olympus BX61 WI microscope using a 40×/0.80 water-dipping objective. Recordings were acquired using

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an Axoclamp 900A amplifier, Digidata 1440A acquisition system and pClamp 10.5 software (Molecular Devices). Electrophysiological sweeps were digitized at 10 kHz, and filtered at 1 kHz. Data were analyzed using Clampfit (Molecular devices), MiniAnalysis (Synaptosoft), Excel (Microsoft), and SigmaPlot (Systat) software.

Miniature excitatory postsynaptic potentials (mEPSPs) were recorded in the absence of any stimulation, and cut motor axons were stimulated to elicit excitatory postsynaptic potentials (EPSPs). An ISO-Flex stimulus isolator (A.M.P.I.) was used to modulate the amplitude of stimulatory currents. Intensity was adjusted for each cell, set to consistently elicit responses from both neurons innervating the muscle segment, but avoiding overstimulation. For each recording, at least 100 mEPSPs were analyzed to obtain a mean mEPSP amplitude value. The average single AP-evoked EPSP amplitude of each recording is based on at least 20 EPSPs. Quantal content was estimated for each recording by calculating the ratio of mean EPSP amplitude to mean mEPSP amplitude and then averaging recordings across all NMJs for a given genotype. Muscle input resistance (Rm) and resting membrane potential (Vrest) were monitored during each experiment. Recordings were rejected if the Vrest was above -60 mV, if the Rm was less than 5 MΩ, or if either measurement deviated by more than 10% during the course of the experiment. Larvae were incubated with or without phanilothoxin-433 (Sigma; 20 μM) resuspended in HL-3 for 10 mins, as described (37,67).

**Crawling Assay:** The larval mobility assay was performed as described (41). Single wandering third-instar larvae were placed on a plain 1% agarose plate (100 mm) placed on top of 5 mm grid paper. The number of gridlines crossed in two minutes was scored for at least 10 animals per genotype. The animal was allowed to acclimate on the plate briefly before the assay and the timer was started upon the first peristaltic contraction (considered the first attempt at forward motion).

**Statistical Analysis:** All data are presented as mean ± SEM. Data were compared using either a one-way ANOVA and tested for significance using a 2-tailed Bonferroni post-hoc test, or using a Student’s t-test (where specified), analyzed using Graphpad Prism or Microsoft Excel software, and with varying levels of significance assessed as P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) or not significant. Additional statistical details can be found in Supplementary Material, Table S1.

**Supplementary Material**

Supplementary Material is available at HMG online.

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