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REVIEW

Synaptic homeostats: latent plasticity revealed at the *Drosophila* neuromuscular junction

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Abstract

Homeostatic signaling systems are fundamental forms of biological regulation that maintain stable functionality in a changing environment. In the nervous system, synapses are crucial substrates for homeostatic modulation, serving to establish, maintain, and modify the balance of excitation and inhibition. Synapses must be sufficiently flexible to enable the plasticity required for learning and memory but also endowed with the stability to last a lifetime. In response to the processes of development, growth, remodeling, aging, and disease that challenge synapses, latent forms of adaptive plasticity become activated to maintain synaptic stability. In recent years, new insights into the homeostatic control of synaptic function have been achieved using the powerful *Drosophila* neuromuscular junction (NMJ). This review will focus on work over the past 10 years that has illuminated the cellular and molecular mechanisms of five homeostats that operate at the fly NMJ. These homeostats adapt to loss of postsynaptic neurotransmitter receptor functionality, glutamate imbalance, axonal injury, as well as aberrant synaptic growth and target innervation. These diverse homeostats work independently yet can be simultaneously expressed to balance neurotransmission. Growing evidence from this model glutamatergic synapse suggests these ancient homeostatic signaling systems emerged early in evolution and are fundamental forms of plasticity that also function to stabilize mammalian cholinergic NMJs and glutamatergic central synapses.

Keywords Homeostasis · Neuromuscular junction · Synaptic plasticity · Neurotransmission

Introduction

Homeostatic plasticity is a fundamental form of physiological regulation that strives to maintain neural activity within optimal ranges by altering diverse aspects of neuronal structure and function. Synapses are fundamental units of nervous system function and as such are major substrates for homeostatic modulation. Underscoring the importance of these processes, improper homeostatic regulation of synapses is associated with a variety of neurological, neuropsychiatric, and neurodegenerative diseases [1–4]. Evidence for the homeostatic control of synaptic function has been demonstrated in the central and peripheral nervous systems of diverse organisms, from invertebrate flies and crustaceans to rodents and humans. In these systems, homeostatic synaptic plasticity operates across temporal scales from seconds to days and can target distinct processes in pre- and postsynaptic compartments to enable stable intrinsic, synaptic, and circuit activity. Although several molecules and pathways have been identified to function in specific forms of adaptive synaptic plasticity, fundamental questions about synaptic homeostats remain enigmatic, including how they sense the state of the system to detect deviations from set point levels of activity and how intrinsic, anterograde, and retrograde homeostatic signaling systems are embedded within a broader synaptic dialogue.

The *Drosophila* larval neuromuscular junction (NMJ) has been established as a premiere model system to illuminate the genes and mechanisms that orchestrate homeostatic synaptic plasticity (Fig. 1). Beyond the genetic tractability, advanced electrophysiological methods, and quantitative imaging approaches that are well known in this system, there are features of this synapse that render it particularly attractive for studies of synaptic homeostasis. First, the fly NMJ is a model glutamatergic synapse, with a high degree of conservation in the fundamental machinery and challenges that constitute and confront glutamatergic synapses in the

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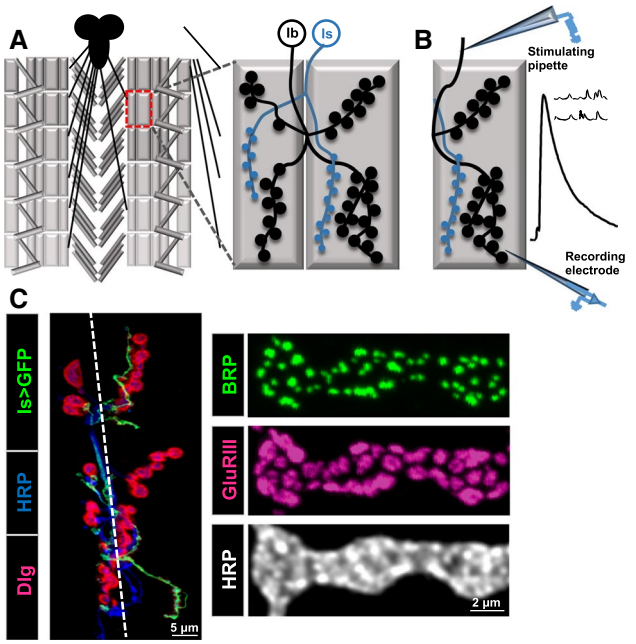

Fig. 1 Synaptic structure and function at the *Drosophila* NMJ. **a** Schematic illustrating the third instar *Drosophila* larvae dissected to expose the repeated segmented musculature and the brain (top, black) with motor nerves innervating each muscle hemi-segment. Inset: schematic of the NMJ at muscles 6 and 7. Note that two different motor neurons, the tonic Ib and phasic Is, each bifurcate to innervate both muscles. **b** Schematic of the fly NMJ preparation used to record electrophysiological signals. Excitatory postsynaptic potentials (EPSPs) are evoked using a stimulating electrode connected to the motor nerve (above), while a recording electrode connected to an amplifier detects spontaneous miniature excitatory postsynaptic potentials (mEPSPs) and EPSPs. Example traces of EPSP and mEPSP events are shown to the right. **c** Confocal image of the muscle 6/7 NMJ immunostained with a neuronal membrane marker (HRP, blue) and a postsynaptic density marker (DLG, magenta) that labels synapses made at Ib NMJs. A transgene expressing the green fluorescent protein (GFP, green) is driven specifically in the Is motor neuron. Right: high-resolution confocal image of NMJs immunostained with the presynaptic active zone scaffold BRP (green), the postsynaptic glutamate receptor subunit GluRIII (magenta), and the neuronal membrane marker HRP (white)

mammalian central nervous system [5]. Second, because reliable muscle contraction is central to behavior and survival, the NMJ is built for stability. Thus, the NMJ has been engineered by evolution to adapt to a variety of diverse challenges to ensure robust muscle contraction. Importantly, these responses can be clearly defined as "homeostatic" and distinguished from other forms of Hebbian and short-term plasticity, which can be difficult to cleanly separate at central synapses. Third, the NMJ is a powerful model for studying neuronal injury and neurodegeneration, where forward genetic screens have discovered fundamental pathways that mediate signaling during injury and degeneration. Further, models for NMJ diseases such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) have been developed at the *Drosophila* NMJ [6–8]. Finally, the stereotypical size and structure of presynaptic active zones and postsynaptic glutamate receptor fields, immense growth, and diversity of motor inputs make the *Drosophila* NMJ uniquely powerful for investigating homeostatic adaptations to synaptic structure and function. One well-established form of homeostatic plasticity, referred to as presynaptic homeostatic potentiation, has been studied for over 20 years at the fly NMJ [9, 10]. However, new homeostats have recently been revealed that can work in isolation or in combination to robustly stabilize synaptic strength at the fly NMJ.

This review will focus on recent advances in our understanding of the phenomenology, design, and cellular, molecular, and physiological mechanisms of synaptic homeostats that operate at the *Drosophila* NMJ. We will focus on highlighting key advances, enduring controversies, and outstanding questions in the best understood homeostat at the fly NMJ—presynaptic homeostatic potentiation. We will then introduce and discuss advances made in our understanding of four additional homeostats that have emerged from work using the *Drosophila* NMJ over the past five years. Lastly, we will briefly discuss how these homeostats operate independently and in conjunction to stabilize synaptic function over both acute and chronic time scales, and end with major open questions and future targets of investigation that will continue to drive new insights in this system to illuminate fundamental principles of relevance to many systems.

Presynaptic homeostatic potentiation (PHP)

A robust model of homeostatic synaptic plasticity has been established at the *Drosophila* NMJ over the past 20 years. Here, genetic and pharmacological manipulations that reduce postsynaptic glutamate receptor (GluR) functionality trigger a trans-synaptic, retrograde signal to the presynaptic neuron. This signal in turn instructs the neuron to precisely increase presynaptic neurotransmitter release, offsetting diminished postsynaptic sensitivity and maintaining stable muscle excitability and synaptic strength (Fig. 2). This process, referred to as presynaptic homeostatic potentiation (PHP), parallels similar processes observed at the NMJs of rodents [11–13] as well as humans [14, 15]. Importantly, this same phenomenon has recently been demonstrated in the mammalian central nervous system [16]. PHP was initially discovered in the course of characterizing the postsynaptic GluRs that drive muscle contraction at the fly NMJ. These GluRs show homology to kainate-type excitatory ionotropic GluRs and consist of two receptor subtypes composed of the essential GluRIIC, GluRIID, and GluRIIE subunits and either GluRIIA ("A type") or GluRIIB ("B type") subunits [17, 18]. "A type" GluRs drive most of the current during synaptic transmission at the NMJ, while "B type" GluRs

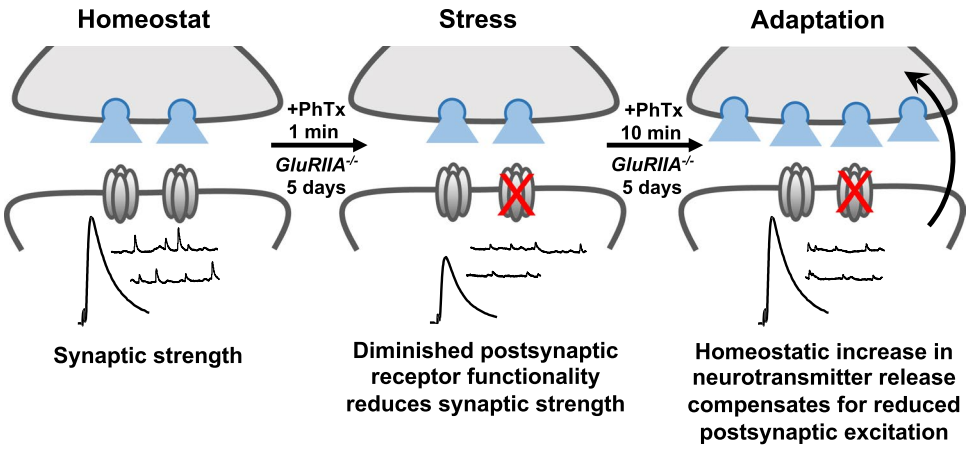

Fig. 2 Presynaptic homeostatic potentiation at the *Drosophila* NMJ. Schematic illustrating presynaptic homeostatic potentiation (PHP) as modeled at the *Drosophila* NMJ. Homeostat: baseline synaptic transmission is shown with representative electrophysiological traces of miniature excitatory postsynaptic potentials (mEPSPs) and an evoked excitatory postsynaptic potential (EPSP), a measure of synaptic strength. Stress: when postsynaptic glutamate receptor (GluR) functionality is diminished following acute pharmacological blockade with philanthotoxin-433 (PhTx) or chronic genetic loss of the *GluRIIA* subunit, mEPSP amplitude (quantal size) is reduced, with a corresponding decrease in synaptic strength. Adaptation: however, over both acute and chronic time scales, EPSP amplitudes are restored to baseline values despite continued diminishment of GluR functionality due to a homeostatic increase in presynaptic neurotransmitter release (quantal content). Hence, a reduction in postsynaptic excitability is sensed in the muscle and transduced into a retrograde signal that ultimately leads to a precise enhancement in presynaptic release that maintains stable synaptic strength

rapidly desensitize [19, 20]. In *Drosophila* mutations of the *GluRIIA* receptor subunit, spontaneous neurotransmission was reduced, but evoked synaptic strength was maintained due to what is now referred to as PHP [10]. Over the following two decades, significant insights have been achieved in identifying key genes and mechanisms involved in PHP expression in *Drosophila* [9, 21, 22], where forward genetic screening approaches have identified ~ 25 genes, have been found that function in the presynaptic neuron to be necessary for PHP expression [23, 24]. These genes function to homeostatically modulate at least two key presynaptic processes that enhance neurotransmitter secretion: increases in (1) presynaptic Ca^{2+} influx and (2) the size of the readily releasable vesicle pool [25, 26]. This work has established a strong foundation to understand how presynaptic neurotransmitter release is adaptively increased following the reception of retrograde signaling from the postsynaptic compartment. Candidate retrograde signals have also been proposed [27, 28], although much remains to be learned before the nature of retrograde signaling can be confirmed and integrated into what is known about PHP. Several recent reviews have more fully covered these topics [9, 22] and will not be discussed further here. However, important advances have been made in understanding four fundamental questions about PHP signaling: (1) How is PHP signaling itself initiated in the postsynaptic muscle? (2) Is active zone structure a target for PHP modulation? (3) Is PHP input, target, and/or synapse specific? (4) How do acute vs chronic PHP signaling differ? Here, we will focus on new insights made into these four areas of PHP signaling at the NMJ.

Postsynaptic PHP induction requires signal transduction systems utilizing phosphorylation and ubiquitination

Our understanding of the expression mechanisms that ultimately enhance glutamate release in the presynaptic terminal has been significantly enhanced in recent years. In contrast, far less is known about how loss or pharmacological blockade of postsynaptic GluRs is sensed and transduced into retrograde signaling. It is clear that loss or pharmacological blockade of GluRIIA-containing receptors, one of two GluR subtypes at the fly NMJ, is necessary to initiate retrograde PHP signaling. In addition, PHP can be induced and expressed over rapid timescales ("acute PHP") by 10 min incubation in a toxin, philanthotoxin-433 (PhTx) that targets GluRIIA-containing receptors, or over days of larval development by genetic loss of *GluRIIA* ("chronic PHP"). When PHP was first described in mutants that have lost the postsynaptic *GluRIIA* receptor subunit, it was immediately speculated that reduced Ca^{2+} influx due to loss of GluRs might be the initiating factor that drives retrograde PHP signaling. This model was supported by the first insights into PHP induction reported a few years later, where constitutively active Ca^{2+}/calmodulin kinase II (CaMKII) in the postsynaptic muscle of *GluRIIA* mutants blocked the expression of PHP [29], a finding recently revisited and confirmed

[30]. More recently, immunostaining of active (phosphorylated) CaMKII revealed a reduction in pCaMKII signals at postsynaptic compartments in *GluRIIA* mutants [30, 31] and even after acute pharmacological challenge [32]. Together, these results may appear to indicate that reduced Ca^{2+} influx through loss or blockade of GluRIIA-containing receptors is necessary for PHP induction. However, incubation in saline lacking Ca^{2+} has no impact on acute PHP induction or expression [32]. This suggests that either Ca^{2+} influx is not required for acute PHP induction, or that PHP induction works differently in pharmacological perturbation vs genetic loss of postsynaptic GluRIIA-containing receptor function.

Over the past few years, forward genetic approaches have revealed the first new players involved in postsynaptic inductive PHP signaling. First, a role for postsynaptic translation in chronic PHP expression through *target of rapamycin* (*Tor*) and associated translational control factors was found [33–35]. Importantly, postsynaptic overexpression of *Tor* was demonstrated to be capable of artificially triggering instructive PHP retrograde signaling [32, 34], which remains the only known manipulation capable of inducing PHP expression in the absence of GluR perturbation. While this work suggested that translation may play a role in chronic PHP expression, rapid PHP, triggered by PhTx application, is translation-independent and can be robustly expressed in the presence of inhibitors of protein synthesis [32, 36–38]. This finding predicted that posttranslational signaling processes may be involved in PHP induction. Indeed, a genetic screen of all kinases and phosphatases encoded in the *Drosophila* genome identified a role for postsynaptic phosphoinositide-3-kinase (PI3K) signaling in both acute and chronic PHP expressions [39]. In particular, genetic evidence suggested that PI3K signaling interfaced with Rab11-dependent membrane trafficking in the postsynaptic muscle during PHP signaling [39]. While a postsynaptic role for another kinase in addition to CaMKII in PHP signaling is intriguing, it remains unclear how PI3K-mediated signal transduction is related to GluR loss, CaMKII activity, or retrograde PHP signaling.

Finally, a forward genetic screen specifically designed to identify postsynaptic factors required for retrograde PHP signaling identified the sleep gene *insomniac*. Specifically, the ubiquitin ligase *Cullin-3* (*Cul3*) and its putative adaptor *insomniac* (*inc*) were found to be necessary in the postsynaptic muscle to enable both chronic and acute retrograde PHP signaling [40]. Interestingly, both Inc and Cul3 rapidly accumulate at postsynaptic compartments following pharmacological blockade of GluRs, where they function to mono-ubiquitinate substrates at the postsynaptic density [40]. Further, both *inc* and *Cul3* genetically interact with the extracellular matrix component *multiplexin*, a candidate retrograde signal. A secondary candidate screen of Inc/Cul3 interacting genes led to the discovery of *peflin*, which encodes a Ca^{2+} binding protein with five EF hand domains, to be required postsynaptically for PHP expression. Intriguingly, mammalian studies revealed peflin to function as a Ca^{2+}-sensitive co-adaptor for Cul3 to mono-ubiquitinate Sec31 in neurons, which ultimately controls membrane trafficking to secrete collagen [41]. One attractive possibility is that at the *Drosophila* NMJ, postsynaptic peflin responds to Ca^{2+} signaling in the postsynaptic compartment to recruit Inc/Cul3-dependent mono-ubiquitination and target membrane trafficking during PHP. This works provides a foundation from which to understand how rapid changes in Ca^{2+} signaling through diminished GluR functionality are sensed to enable ubiquitination of postsynaptic substrates, which together with CaMKII might drive retrograde PHP signaling. In addition, this work identifies an intriguing molecular link between sleep and synaptic homeostasis. In summary, while emerging evidence suggests a vital role for phosphorylation, ubiquitination, and perhaps Ca^{2+} signaling in the postsynaptic signal transduction cascade elicited by PHP induction, a major future challenge for this field will be to generate a coherent framework connecting these processes with the elusive retrograde signaling system that instructs a precise increase in presynaptic neurotransmitter release in response to diminished GluR function.

PHP expression targets active zone structure for homeostatic modulation

Several lines of evidence indicate that the presynaptic active zone cytomatrix is targeted for homeostatic modulation in the context of PHP signaling. First, several genes encoding active zone components have been found to be necessary for PHP expression, including the CaV2 Ca^{2+} channel *Cacophony* (*Cac*; [38]) and its auxiliary subunit $\alpha_2\delta$ [42], the piccolo homolog *fife* [43], the scaffolds *RIM* (Rab3-interacting molecule; [44]), *Rbp* (*Rim binding protein*; [45]), *Unc13A* [36], and the kainate receptor *DKaiR1D* [46, 47]. Second, presynaptic Ca^{2+} levels are enhanced during PHP [25] and the abundance of Cac is rapidly increased at active zones following PHP induction [48, 49]. Third, experiments in which Ca^{2+} is buffered at presynaptic terminals suggest that synaptic vesicle-Ca^{2+} coupling is modulated following PHP induction [50]. Finally, and most provocatively, active zone structure is rapidly remodeled during PHP, leading to apparent increases in the abundance and nano-scale organization of active zone components [26, 32, 36, 48, 49, 51, 52]. The first evidence for PHP-dependent active zone remodeling was found in confocal imaging studies in which the fluorescence intensity of antibodies labeling the active zone scaffold Bruchpilot (Brp), the *Drosophila* homolog of ELKS/Cast, was enhanced after PhTx application or in *GluRIIA* mutants [26]. Subsequent studies confirmed

and extended this work, in which the active zone components Brp, Cac, Unc13, and Rbp were rapidly enhanced at active zones by confocal imaging [32, 36, 48, 49]. Super resolution imaging using STimulated Emmision Depletion (STED) microscopy revealed a "nano-modular" increase in the number of active zone modules at individual active zones [36, 48]. These structures are thought to correlate with enhanced presynaptic release [48, 49]. Together, these insights establish active zone components, and their nanoscopic arrangement and levels, to be targets for homeostatic modulation that may ultimately contribute to the potentiation of neurotransmitter release.

At present, it remains unclear how exactly active zones are adaptively remodeled during PHP signaling and how these changes translate into a precise tuning of neurotransmitter release. Clearly, a posttranslational mechanism must mediate the homeostatic remodeling of active zone structure, since PHP can be rapidly expressed in the presence of translational blockers [32, 38] and active zones are remodeled in these conditions [36]. In principle, an increase in the abundance of Ca^{2+} channels at individual active zones provides an attractive mechanism to enhance presynaptic Ca^{2+} influx [25, 49], while an expansion in the area and nano-modular arrangement of active zone scaffolds such as Brp and Unc13A enables an increase in the number of synaptic vesicles available for release during PHP [53, 54]. Indeed, both processes are known to contribute to increased release probability during PHP [25, 26]. Further support for increased abundance of active zone material comes from the requirement of axonal motors that transport synaptic cargo. In particular, the axonal motors *aplip-1* (App-like interacting protein), *srpk79D* (serine-arginine protein at 79D), and the lysosome adaptor *arl-8* (arf-like GTPase-8) were necessary for the rapid remodeling of active zones following PHP induction [36, 48]. Indeed, Arl-8 transports both active zone and synaptic vesicle components [55], and synaptic vesicle markers were also observed to be rapidly enhanced at presynaptic terminals during PHP [48]. While this evidence is convincing, a recent study leveraging localization microscopy (dSTORM) proposed that an increase in the density of active zone components leads to "compaction" during PHP, with no net change in protein abundance [52]. It remains to be determined how a compaction of the active zone may promote neurotransmitter release, and how the nano-modular changes observed using STED microscopy relate to the reported enhancement in density. Finally, while the homeostatic remodeling of active zone structure is necessary to sustain potentiated neurotransmission induced by the chronic loss of *GluRIIA*, PHP can still be acutely induced and expressed even when active zones are not remodeled [36, 48]. An exciting question for the field in the future will be to illuminate how active zones are instructed to remodel following PHP induction and the importance of this process over both acute and chronic time scales for the expression of homeostatic plasticity at synapses.

Input, target, and synapse specificity of PHP expression

The development of quantal Ca^{2+} imaging approaches and the discovery of input-specific driver lines have enabled the dissection of how distinct motor inputs contribute to PHP at the *Drosophila* NMJ. Most muscles at the fly larval NMJ are innervated by two motor inputs, a tonic (type Ib) and phasic (type Is), which differ in morphological and physiological properties [56, 57]. A binary transcriptional control system has been well established in *Drosophila* which consists of the yeast transcriptional activator Gal4 and the Gal4-responsive enhancer UAS (upstream activation sequence) [58]. Importantly, Gal4-driver lines have been recently discovered that express in a subset of motor neurons, including ones specific for Is or Ib motor inputs [59–61]. In vivo quantal Ca^{2+} imaging in postsynaptic muscles revealed that PHP is exclusively expressed at tonic type Ib inputs in *GluRIIA* mutants [31]. Additional support for input-specific PHP expression was found through imaging of active zones, where Brp was demonstrated to be enhanced at Ib terminals in *GluRIIA* mutants, but no change was observed at Is inputs [51]. These results were confirmed and extended in a subsequent study, in which input-specific Gal4 driver lines [60] were used with optogenetic stimulation to selectively evoke release at Ib vs Is inputs [62]. This study made two important findings. First, while loss of *GluRIIA* did indeed drive PHP primarily at Ib boutons, rapid PHP induced by acute application of PhTx targeted Is inputs for homeostatic potentiation. Second, under conditions of very high extracellular Ca^{2+} saline conditions, Ib and Is inputs lose their distinctions and both contribute to enhanced release in response to PhTx application or loss of *GluRIIA*. While these findings are intriguing, however, it is not clear how physiologically relevant the observed changes are at highly elevated Ca^{2+} conditions. In addition, large differences in the strength of spontaneous neurotransmission are known to exist at Ib vs Is terminals [63], and because the muscle is isopotential, this optogenetic approach is unable to accurately distinguish input-specific baseline miniature transmission nor quantify the differences in *GluRIIA* mutants and PhTx application. New approaches will need to be developed to silence all evoked and spontaneous transmission at specific inputs to accurately assess input-specific synaptic function and homeostatic plasticity.

In addition to input-specific contributions to PHP, there is now evidence for target and synapse specificity of PHP expression at the fly NMJ. Specifically, a single Is and Ib motor neuron bifurcates at the muscle 6/7 NMJ to innervate two adjacent targets (see Fig. 6). Recently, a genetic

manipulation was developed that enabled the loss of *GluRIIA* selectively at one muscle without impacting GluR expression at the adjacent target [30]. Remarkably, PHP was specifically expressed at terminals innervating the target muscle with reduced GluR expression, while no changes in presynaptic release or synaptic strength were observed at terminals shared by the same neuron innervating the adjacent target muscle. This provided the first evidence that PHP can be induced and expressed locally at a subset of synapses from a single motor neuron without impacting function at terminals innervating an adjacent target. Not only did this study demonstrate that PHP can be expressed with target specificity, but it strongly suggests that PHP can be induced and expressed with synapse specificity, where individual active zone-GluR dyads may be the fundamental units that PHP targets for modulation.

Intriguing differences are apparent between acute and chronic PHP induction and expression

Although the field has largely described acute and chronic PHP to be essentially the same phenomenon, recent work has established these processes exhibit major differences in both the induction and expression mechanisms, suggesting they may in fact be distinct processes. First, while some genes are necessary for both acute and chronic PHP expressions, several genes are needed selectively for chronic PHP expression and are dispensable for acute PHP [32, 36, 48, 64–66]. Second, although acute PHP is robustly expressed in the absence of new protein synthesis [32, 38], chronic PHP appears to require genes and pathways that modulate and promote new protein synthesis [34, 35]. Third, while active zone remodeling is necessary to sustain the chronic expression of PHP, acute PHP can be induced and expressed in the absence of active zone remodeling or even the scaffold Brp itself [36, 48]. Finally, chronic PHP targets Ib inputs for homeostatic potentiation [30, 31], while acute PHP primarily targets Is motor inputs [62]. These differences may result from the specific perturbation to postsynaptic GluRs—a chronic, genetic absence throughout development of GluRIIA-containing receptors compared to an acute, pharmacological disruption of existing GluRIIA-containing receptors. Distinctions in the postsynaptic structure opposite Is and Ib terminals may also contribute to the differences between chronic and acute PHP induction and expression. Indeed, the postsynaptic compartment opposite Ib inputs is composed of an elaborate subsynaptic reticulum that is not apparent at Is terminals. Interestingly, mutations that perturb this elaborate SSR structure also disrupt chronic PHP expression [67]. While it remains unclear how or why acute and chronic PHP appears to target distinct motor inputs for homeostatic potentiation, this question is certain to be an exciting topic for future studies.

Presynaptic homeostatic depression (PHD)

In contrast to the advances in our mechanistic understanding of PHP, relatively little is known about an inverse process referred to as presynaptic homeostatic depression (PHD). The first evidence for this form of presynaptic homeostatic plasticity, while not appreciated as such, was found while characterizing mutations in genes involved in synaptic vesicle endocytosis. In these mutants, defects in vesicle re-formation resulted in synaptic vesicles with increased size, leading to a concomitant increase in the amplitude of spontaneous neurotransmission [68–70]. However, instead of exhibiting a concomitant increase in evoked amplitude, as would be expected, no change in EPSP amplitude was observed in these mutants. This phenomenon, now referred to as PHD (Fig. 3), was clearly articulated in a seminal study in which overexpression of the *vesicular glutamate transporter* in motor neurons (vGlut-OE) enhanced synaptic vesicle size and miniature amplitude, but stable synaptic strength was maintained due to a homeostatic reduction in presynaptic neurotransmitter release [71]. As a result, increased quantal size is observed, but stable EPSP values are maintained due to a homeostatic reduction in presynaptic glutamate release (quantal content). In this initial study, it was considered that PHD could be an adaptive response to excess glutamate release and may even involve a presynaptic glutamate autoreceptor. Alternatively, PHD may stabilize synaptic strength as a response from a retrograde signal emitted by the muscle, akin to PHP. Although a handful of subsequent studies have shed some mechanistic insight into PHD, fundamental questions about this process remain a mystery, and not a single gene required for PHD expression has yet been identified. Here, we will discuss what is known about the induction and expression mechanisms of PHD and consider the nature of this homeostat.

PHD induction

On the surface, PHP and PHD appear to be similar in principle as synaptic homeostats but simply inverse in direction. Both forms of adaptive plasticity involve an increase or decrease in miniature amplitude and a compensatory change in presynaptic glutamate release that is inverse in direction and that ultimately results in stable synaptic strength. However, a deeper examination reveals fundamental differences in these presynaptic forms of homeostatic modulation. It is clear that PHP induction occurs in the postsynaptic muscle and is initiated by genetic loss or pharmacological blockade of GluRIIA-containing receptors [10, 38]. Indeed, spontaneous activity alone

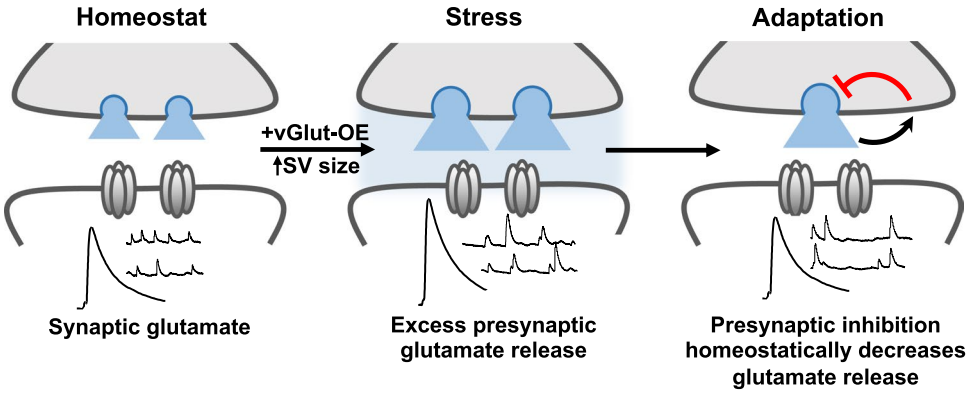

Fig. 3 Presynaptic homeostatic depression at the *Drosophila* NMJ. Schematic of presynaptic homeostatic depression (PHD) as modeled at the *Drosophila* NMJ. Baseline synaptic transmission is shown. Stress: a homeostatic challenge of excess glutamate release is induced by overexpression of the *vesicular glutamate transporter* in motor neurons (vGlut-OE), which leads to an enlargement in the size of synaptic vesicles. Quantal size (mEPSP amplitude) is increased due to enhanced glutamate release from individual synaptic vesicles, with an expected concomitant increase in synaptic strength. Adaptation: excess presynaptic glutamate release is sensed to induce presynaptic inhibition and reduce glutamate release (quantal content) to maintain glutamate balance at the NMJ

is sufficient to communicate the information necessary to induce PHP expression, at least in the case of acute induction [38]. Ultimately, a retrograde signal from the postsynaptic compartment, received by the presynaptic neuron, instructs a precise increase in neurotransmitter release. In contrast, early studies on GluRs at the fly NMJ demonstrated that the opposite of *GluRIIA* loss, increased expression of GluRIIA-containing receptors, results in the opposite effect, enhanced miniature amplitude [10, 19]. However, no change in presynaptic function was observed, resulting in a non-homeostatic increase in EPSP amplitude; this finding was subsequently confirmed and extended [51]. Together, this body of work provided evidence that PHP is a unidirectional phenomenon, increasing neurotransmitter release when postsynaptic GluRs are reduced, while no inverse plasticity process is apparently induced when postsynaptic GluRs are enhanced.

These observations lead to two possibilities regarding PHD induction. First, PHD may involve a retrograde signaling system akin to PHP, a possibility that has been entertained [72]. If this model were correct, it would need to be induced through mechanisms that do not depend on enhanced GluR functionality in the postsynaptic cell. However, to date, there is no known postsynaptic manipulation capable of inducing retrograde PHD expression. Indeed, the only process known to be capable of inducing PHD originates in the presynaptic compartment—an increase in synaptic vesicle size—caused by vGlut-OE or defective synaptic vesicle endocytosis, as recently demonstrated in *endophilin* and *minibrain* mutants [48, 73]. Thus, an alternative model has been postulated in which PHD is induced specifically as a response to excess glutamate release from the presynaptic neuron due to enhanced synaptic vesicle size [51]. In this study, PHD was shown by quantal Ca^{2+} imaging to be expressed at both Is and Ib inputs, not to be induced by increased postsynaptic *GluRIIA* overexpression, and to operate with apparent obliviousness to the excitability state of the postsynaptic cell [51]. Therefore, rather than being a homeostat that stabilizes synaptic strength, PHD may actually be a glutamate homeostat focused on maintaining glutamate balance in response to excess glutamate release itself.

This is an attractive hypothesis when glutamate clearance and the biology of the peripheral nervous system of *Drosophila* larvae are considered. Ambient glutamate is toxic in the nervous system and can lead to excitotoxicity and neurodegeneration [74], and degeneration is actually observed in the fly brain following vGlut-OE [75]. Nonvesicular release of glutamate by peripheral glia functions at the *Drosophila* larval NMJ to modulate glutamate receptor clustering [76]. However, the major glutamate clearance mechanism in the brain involves transporters on neurons and glia that sequester excess glutamate, yet in the fly larval periphery, these transporters are not expressed [77]. Therefore, PHD may have evolved as a mechanism of synaptic control to maintain glutamate homeostasis, reducing neurotransmitter release in response to excess emission. Such a model implies a glutamate autoreceptor that must detect excess glutamate to drive presynaptic inhibition. The sole metabotropic glutamate receptor encoded in the *Drosophila* genome, *mGluRA*, is an attractive candidate, as it is present at presynaptic motor terminals and can inhibit release in response to excess glutamate released [78]. However, at present, the nature of PHD induction and of this homeostat remains unclear, and future studies will be needed to clarify these fundamental questions about this form of homeostatic plasticity.

Expression mechanisms of PHD

Although a major outstanding question centers on the nature of the induction mechanism that drives PHD, some advances have been made in our understanding of PHD expression mechanisms. In the original study that defined PHD, it was shown that vGlut-OE induces a precise reduction in presynaptic release probability without any obvious changes to synaptic growth or structure [66]. Failure analysis further supported a functional decrease in release probability as the primary adaptation that led to PHD. More recently, several studies have clearly demonstrated that PHP and PHD require distinct genetic mechanisms, as mutations in genes that block PHP expression have no impact on PHD [51, 65, 72]. Like PHP, PHD appears to involve a reduction in presynaptic Ca^{2+} influx without any apparent changes in the action potential waveform as assessed through voltage imaging [72]. However, in contrast to PHP, no obvious changes in active zone components, including Brp and endogenously tagged Ca^{2+} channels, are apparent after PHD induction [48, 49]. Nor is a reduction in the readily releasable pool of synaptic vesicles observed in PHD [51, 72] as an increase is found in PHP. Thus, if the increased Ca^{2+} influx during PHP results from the apparent increase in Ca^{2+} channel abundance, then the reduced Ca^{2+} influx that diminishes release in PHD likely involves functional changes to Ca^{2+} channels [49]. Thus, while a reduction in presynaptic Ca^{2+} influx may be what ultimately tunes down neurotransmitter release to achieve PHD, major questions remain about how Ca^{2+} influx is targeted by the still unknown upstream mechanisms controlling PHD induction and signal transduction.

Potentially novel forms of PHD

A phenomenon was recently reported that resembles the PHD described above but does not rely on enhanced synaptic vesicle size. Here, *Drosophila* larvae were terminally arrested at third instar larval stages (ATI) by disrupting hormonal signaling required for pupariation [79]. As a result, ATI larvae continue to grow for up to 35 days, in contrast to the typical 5-day-larval period, before dying. Remarkably, both pre- and postsynaptic structures expand, with increased numbers of presynaptic boutons, active zones, and postsynaptic muscles and receptor fields observed [79]. Consistent with increased GluR abundance, an increase in mEPSP amplitude is observed, although no change in synaptic vesicle size was found. However, EPSP amplitudes are maintained at levels similar to wild type throughout the ATI lifespan due to an apparent reduction in presynaptic neurotransmitter release that parallels PHD [79]. Indeed, a reduction in presynaptic release probability was observed without any reductions in the apparent abundance of presynaptic active zone components, consistent with a functional reduction. Although ATI NMJs appear to mimic PHD electrophysiologically, there is a major distinction: ATI NMJs do not have any apparent increase in synaptic vesicle size [79]. Thus, if the apparent PHD observed at ATI NMJs is the same process detailed in vGlut-OE and endocytosis mutants, then this would indicate that excess global release of glutamate, rather than from individual synaptic vesicles, must be the key inductive event that drives presynaptic homeostatic depression. This idea would also be consistent with a variety of *Drosophila* mutants that, like ATI NMJs, exhibit synaptic overgrowth but normal synaptic strength [34, 80, 81]. However, it is also possible that the apparent presynaptic homeostatic plasticity process observed in ATI NMJs may actually be a novel form that is mechanistically distinct from conventional PHD, perhaps coupled to developmental processes coordinating synaptic growth and muscle size.

Adaptive changes at NMJs in the context of injury and disease

A relatively unexplored question is how synapses adapt as neurons experience injury and disease processes, and to what extent homeostatic plasticity mechanisms are engaged. The past 15 years has witnessed a wealth of understanding about the intrinsic changes and signaling systems in neurons that respond to axonal injury, with key factors such as DLK (dual leucine zipper kinase), SARM (sterile armadillo/toll-interleukin receptor homology domain molecule), and the biosynthetic enzyme NMNAT (nicotinamide mononucleotide adenyltransferase; NMNAT) playing crucial roles in coordinating and executing regenerative and degenerative programs in neurons [82, 83]. The fly NMJ has proved to be an important system in these seminal discoveries and investigations [84–86]. In addition, the *Drosophila* NMJ has served as a model system to interrogate various neurodegenerative and neurological diseases, including Huntington's [87, 88], Alzheimer's [89–91], and Parkinson's diseases [92, 93], as well as Fragile X Syndrome [94], ALS (Amyotrophic Lateral Sclerosis; [7, 95, 96]), and SMA (Spinal Muscular Atrophy; [6, 97, 98]. However, the adjustments and adaptations that occur at synapses in these conditions are just beginning to be understood. Of particular importance, synapses are tripartite nodes of inter-cellular communication, and it is therefore necessary to understand not only how the intrinsic properties of neurons change during injury and disease, but also how uninjured but synaptically connected neurons, muscles, and glia sense and respond to neuronal injury and disease states. Here, we will cover recent advances on these questions using the *Drosophila* NMJ as a model system.

Highwire targets presynaptic release sites to reduce neurotransmitter release

Cellular damage and injury trigger a coordinated and programmed response in neurons, with one major control point mediated by the evolutionarily conserved Phr1/Highwire/Rpm-1 (PHR) protein. In *Drosophila*, the E3 ubiquitin ligase Highwire (Hiw) coordinates both regenerative and degenerative responses to axonal injury. Following injury such as a nerve crush, Hiw protein levels are reduced [99]. This initiates at least two distinct responses. First, one function of Hiw is to constitutively degrade the mitogen-activated protein kinase kinase kinase (MAPKKK) Wallenda (Wnd), the fly homolog of dual leucine zipper kinase (DLK) [100]. Wnd activates a downstream pathway controlled by the Jun N-terminal kinase (JNK) and the transcription factor Activator Protein-1 (AP-1) to enable both regenerative and degenerative signaling programs [99, 100]. Reduced Hiw enables Wnd/DLK to execute its downstream signal transduction program through AP-1 to change a large number of cellular processes; the impacts of this signaling on synaptic homeostasis are discussed in the following section. Second, loss of Hiw also has direct, Wnd-independent consequences on presynaptic function, which therefore enables rapid and local modulation of neurotransmitter release in response to early stages of neuronal adaptation to injury.

hiw was identified in a forward genetic screen for mutants that alter synaptic structure and named for the uncoordinated movement observed in adult flies lacking this gene [101]. The initial characterization of synaptic function in *hiw* mutants revealed two primary defects: a reduction in quantal size and an additional reduction in presynaptic neurotransmitter release (quantal content; [101]). Subsequent work revealed that active Wnd signaling is responsible for the reduced quantal content, while a Wnd-independent function of Hiw mediates the reduced presynaptic neurotransmitter release [102]. Furthermore, Hiw was shown to regulate levels of the NAD + biosynthetic enzyme NMNAT, a potent axonal maintenance factor [103]. Separately, NMNAT was also shown to be required for maintaining active zone structural integrity by interacting with the active zone scaffold Brp [104]. Interestingly, loss of NMNAT results in ubiquitination, mislocalization, and aggregation of Brp and subsequent active zone degeneration [104]. Despite these advances in our understanding of the roles of Hiw on synaptic function, it remained elusive how Hiw modulated presynaptic function through a Wnd-independent mechanism.

The substrate of Hiw that functions to inhibit neurotransmitter release at presynaptic terminals was recently identified to be NMNAT itself [105]. A series of genetic experiments demonstrated that Hiw serves to degrade local NMNAT levels, and that enhanced NMNAT levels following loss of Hiw alter Brp and active zone structure. In particular, NMNAT overexpression in wild type and in *hiw* mutants was shown to reduce presynaptic release probability, likely due to the alteration in Brp and active zone structure. Further, active zone ultrastructure was irregular in *hiw* mutants, suggesting that local increases in NAD +, due to loss of Hiw, may depress neurotransmitter release through alteration in release site structure. How excess NAD + perturbs Brp and active zone structure is unclear, but one possibility is that NAD + influences Brp regulatory mechanisms including acetylation [106–108]. Alternatively, the mechanism could be less direct and result from metabolic changes in the presynaptic terminal. Together, this work raised the intriguing possibility that one consequence of loss of Hiw function, induced by axonal injury, is to reduce presynaptic neurotransmission, perhaps as an adaption that promotes neuronal repair or degeneration.

Neuronal injury homeostatically modulates the set point of synaptic strength

Although Hiw has Wnd-independent functions as discussed above, one major role of Hiw is to directly regulate Wnd/DLK signaling. Normally, Hiw constitutively degrades Wnd/DLK. However, following axonal injury, this degradation no longer occurs, leading to increased Wnd protein levels and activation of an intrinsic signaling system that transforms the neuron into a state of a persistent programmed response to injury [86, 99]. A key question is whether uninjured but synaptically connected cells can detect injury signaling in neurons and, if so, how they respond.

One crucial clue to this question came from the initial characterization of Hiw and Wnd mutants. These studies revealed that the postsynaptic responsiveness to glutamate (quantal size) was reduced when neuronal Wnd signaling was activated, suggesting presynaptic Wnd signaling might provoke reduced postsynaptic sensitivity. A recent study indeed demonstrated that postsynaptic glutamate receptor levels were diminished, along with the postsynaptic scaffold Discs Large, at the fly NMJ in response to acute (hours) or chronic (days) active Wnd signaling in motor neurons [102]. Because the postsynaptic muscle does not directly experience Wnd signaling, this suggests it receives an anterograde signal from the neuron to remodel and reduce the postsynaptic apparatus. The identity of this signal remains unknown, although it appears to be activity independent, since reduced postsynaptic GluR levels persist in *hiw* mutant motor neurons in which evoked activity is blocked [102].

This result raised a conundrum. Typically, a reduction in postsynaptic GluRs is the key initiating event that induces retrograde PHP signaling at the fly NMJ, which in turn increases neurotransmitter release. However, in neurons with active Wnd signaling, no change in quantal content was observed despite the reduction in postsynaptic GluR

levels [102]. Two possibilities could explain this apparent failure to express PHP. First, the neuron might be incapable of increasing presynaptic glutamate release despite receiving retrograde signaling from the postsynaptic cell, perhaps due to the metabolic changes induced by Wnd signaling. Alternatively, the postsynaptic muscle may fail to communicate the retrograde signal to the neuron. A variety of experiments showed that a concerted change in the muscle led to the blockade of retrograde PHP signaling in response to active neuronal Wnd signaling. When the motor neuron with active Wnd signaling receives the retrograde PHP signal through a genetic manipulation in the muscle, it now becomes capable of robustly increasing neurotransmitter release (Fig. 4). A final set of experiments found that in response to active neuronal injury-related signaling, GluR levels become diminished in the postsynaptic muscle to reduce synaptic strength, and this new set point is stabilized through a targeted inhibition of retrograde PHP signaling. One attractive possibility is that the set point of synaptic strength becomes homeostatically reduced following injury to allow time for the decision of neuronal repair or degeneration to be adjudicated.

Homeostatic plasticity, neurodegeneration, and disease at the fly NMJ

The *Drosophila* NMJ has served as a powerful model to illuminate fundamental insights into a variety of neurodegenerative diseases. For the NMJ diseases ALS and SMA in particular, models have been developed that parallel important features of disease pathology [6, 7, 95, 97]. However, to what extent homeostatic plasticity processes are engaged in the context of disease pathology, if at all, was not considered in these initial studies. In principal, adaptive plasticity such as PHP could be exploited as therapeutic targets to maintain synaptic function and delay NMJ degeneration and dysfunction.

Evidence for homeostatic control of the NMJ circuit has recently emerged from a fly model of SMA. The human disease SMA is caused by loss of function alleles of the gene *smn1* (Survival motor neuron1; [109]). Mutants in the *Drosophila* homolog of *smn1* exhibit many similar features of the human disease, with muscle atrophy, defective locomotion, and altered motor circuit patterns [6, 97, 98]. However, electrophysiological recordings from fly *smn* mutant NMJs revealed an unexpected increase in synaptic strength [98] due to increased quantal content, as no changes were observed in quantal size. Importantly, *smn* expression in either the motor neuron of the muscle failed to rescue mutant phenotypes; rather, *smn* expression was required in pre-motor inputs [98]. Additional work demonstrated the loss of pre-motor input onto motor neurons themselves, induced by pre-motor expression of *Kir2.1*, phenocopied the enhanced synaptic strength observed in *smn* mutants. This phenomenon may be an adaptive form of plasticity similar to PHP, in which neurotransmitter release is enhanced. However, in this case, no apparent defects in postsynaptic GluRs are observed, and increased neurotransmitter release may in fact be induced through reduced synaptic drive onto motor neurons. This might indicate that adaptive presynaptic plasticity, akin to PHP, can be induced through manipulations of

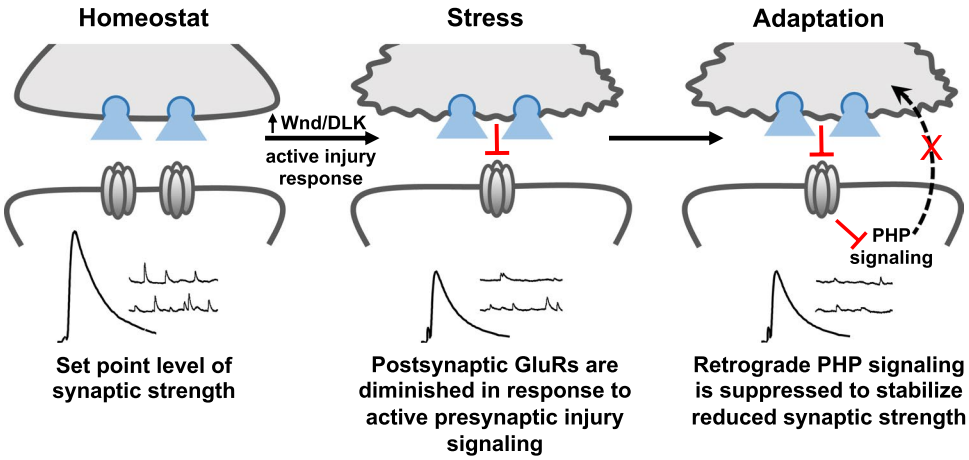

Fig. 4 Neuronal injury homeostatically reduces the set point of synaptic strength. Schematic illustrating how the postsynaptic muscle responds to active injury-related signaling in the presynaptic motor neuron. Baseline neurotransmission is shown. Stress: following activation of injury-related signaling in the motor neuron through Wallenda (Wnd), the fly homolog of the dual leucine zipper kinase (DLK), the postsynaptic muscle senses this signaling and reduces GluR abundance at the postsynaptic compartment, which in turn decreases synaptic strength. Adaptation: although normally this reduction in GluR induces retrograde PHP signaling in the muscle, this postsynaptic signal transduction pathway is suppressed as the muscle responds to active presynaptic Wnd signaling. Therefore, the set point level of synaptic strength is deliberately reduced and stabilized through a suppression of retrograde PHP signaling as the NMJ acclimates to injury

activity of inputs onto motor neurons, without any perturbations to the NMJ itself.

In a fly model of another motor neuron disease, ALS, PHP was shown to be capable of being expressed to restore NMJ function [96]. In particular, an expansion of a pathological hexanucleotide repeat (Gly-Arg) in the human *C9orf72* gene has been linked to ALS [110]. A fly model was developed in which a 100xGly-Arg repeat was overexpressed in motor neurons, resulting in degeneration of the NMJ [8]. Presynaptic terminals and active zone numbers were severely reduced, and synaptic "footprints"—postsynaptic specializations lacking a presynaptic input—were observed at NMJs expressing the 100xGR repeat [96]. Electrophysiological recordings correspondingly found that EPSP amplitudes were reduced by over 50% compared to controls, consistent with reduced active zone number. Next, the extent to which NMJs undergoing ALS-related degeneration retain the capacity to express PHP was examined. Intriguingly, PHP could be robustly expressed at 100xGR NMJs to restore synaptic strength despite the severe degeneration [96]. It is important to note that while these ALS NMJs have the potential to express PHP, a "trigger" is necessary to activate this dormant plasticity, as PHP does not appear to be expressed in this model of ALS, despite NMJ degradation, and is only elicited following GluR perturbation. Interestingly, however, in separate mouse models of ALS that do not target C9orf72 pathology, PHP was proposed to be expressed at early stages of degeneration and termed "homeostatic neuroprotection," which served to delay disease progression [11].

One major limitation of using the *Drosophila* NMJ to study neurodegeneration is the short 4-day stage of the third instar. This brief temporal window limits its use for interrogating dynamic processes and disease progression over chronic timescales. This is particularly important in the context of neurodegeneration studies, since the phenotype often only appears in later disease states. Using a genetic manipulation to delay the onset of pupariation from third instar stages, it is possible to extend the larval period to ~ 9 days [111] or to even completely block pupariation to arrest larvae at third instar (ATI) stages for 35 days [79]. Using mutations in the gene *stathmin*, which encodes a surveillance factor for axonal damage and degenerative signaling [112, 113], the authors examined degeneration in the ATI background [79]. Although *Drosophila stathmin* mutants exhibit some degree of NMJ degeneration at standard third instar stages [112], the ATI extension enabled the study of severe NMJ degeneration with resolution and detail that was not apparent when limited to the conventional short larval stage. Interestingly, a recent study demonstrated that *stathmin2* is reduced in mammalian ALS models and that upregulation of *stathmin2* can be neuroprotective against some ALS-related neuronal degeneration [114]. Thus, the ATI system has the potential to serve as a useful tool for assessing and discovering disease phenotypes not possible to examine over conventional larval growth periods.

Homeostatic adaptations to synaptic overgrowth

Drastic changes in synapse number, morphology, and structure occur throughout development, maturation, and aging in the nervous system, enabling the flexibility necessary to wire the brain and to adjust neurotransmission following experience and in disease. However, these persistent and dynamic changes to synapses pose a major challenge to the stability of neural function. Homeostatic mechanisms ensure physiologically stable levels of functionality in the face of ongoing alterations to synapse numbers and structure. These homeostatic adaptations have been observed during developmental pruning, sleep/wake behavior, and experience-dependent plasticity [115–117], and the molecular mechanisms involved remain an active and exciting area of investigation.

The *Drosophila* NMJ has been used to characterize the integration and coordination of synaptic growth and function. This NMJ expands over 100-fold during 5 days of larval development and uses an elaborate program to coordinate the growth and structure of pre- and postsynaptic structures. In particular, bouton numbers, containing active zones, are steadily added while in the muscle, the subsynaptic reticulum (SSR) expands through new membrane addition as postsynaptic glutamate receptor fields mature [118–120]. Some of the key molecular mechanisms and signaling pathways that regulate NMJ growth have been well studied, including Wnt signaling [121], Bone Morphogenetic Protein (BMP) signaling [122, 123], and autophagy-related signaling [124, 125]. Further, progress has been made in specifically identifying retrograde and trans-synaptic pathways that coordinate pre- and postsynaptic scaling during NMJ growth such as BMP signaling [126], where insulin receptor signaling in the muscle through the guanine nucleotide exchange factor dPix was recently demonstrated to coordinate a developmental coupling of neuronal terminals with their targets [127]. Finally, it has been observed that the NMJ maintains robust neurotransmission despite major perturbations to synaptic growth, morphology, and structure. This is apparent throughout development where stable muscle excitation is maintained despite immense differences in the volume, architecture, and passive electrical properties of the muscle [56, 128–130]. At terminal larval stages, synaptic strength remains constrained within narrow physiological ranges despite a broad variation in synaptic growth [23, 24, 80]. Even in manipulations that extend larval stages to permit continuous growth of NMJs and muscles beyond the typical

4-day larval period [111], synaptic strength remains stable throughout 35 days of larval growth [79]. Thus, throughout normal larval development and even in a novel life stage not selected for in evolution where NMJs expand beyond typical growth programs, robust mechanisms exist that maintain stable functionality. In subsequent sections, we highlight recent work that has advanced our understanding of how synaptic function and growth are coordinated.

Active zones are substrates for homeostatic adaptations to synaptic overgrowth

As synapses that innervate a single target grow and elaborate, synaptic strength should increase if there are no compensatory changes. However, while a large range in the number of synapses is observed across wild-type NMJs, NMJ strength, as measured by electrophysiology, is constrained within far narrower physiological ranges [80]. This implies that adaptive countermeasures exist that stabilize synaptic strength while enabling flexibility in NMJ growth. This question was addressed in a recent study in which extreme synaptic overgrowth was observed in mutations of *endophilin* (*endo*), a key gene involved in synaptic vesicle endocytosis at the *Drosophila* NMJ [68, 70]. However, despite a doubling in the number of NMJ boutons and active zones, synaptic strength in *endo* mutants remains similar to wild type [48]. Although *endo* NMJs exhibited this increase in the number of active zones, the area of individual active zones, as measured by immunofluorescence labeling of active zone components, was reduced along with their immunofluorescence intensity [48]. Further experiments demonstrated that while the apparent abundance of active zone components was reduced at individual active zones in *endo* mutants, the overall level of active zone material across the entire NMJ was conserved between *endo* and wild-type NMJs. Super resolution imaging with STED microscopy demonstrated a decrease in the diameter of individual active zones, with a reduction in the "nano-modular" arrangement of active zone sub-structures. This suggests that neurons may be endowed with mechanisms that regulate total neurotransmitter release per NMJ independent of the total number of release sites, with pliability of their numbers and size that can inversely scale to maintain global neurotransmitter output (Fig. 5). Together, this work suggested that active zone nano-structure is targeted for homeostatic modulation to maintain stable neurotransmitter output independently of the number of synapses established.

There is evidence that this homeostatic scaling of active zone structure described in *endo* mutants may be a general mechanism utilized at NMJs confronting synaptic overgrowth. A morphology-based forward genetic screen isolated *Drosophila* mutants that displayed extreme synaptic overgrowth and undergrowth [80]. Interestingly, of the five overgrowth mutants isolated, all exhibited increased active zone number that scaled with bouton numbers but normal synaptic strength [80]. However, in each mutant active zone area was reduced, phenocopying the scaling phenomenon observed in *endo* mutants. This indicates that scaling of active zone area and structure may be a one mechanism employed by NMJs to adaptively adjust neurotransmitter output in response to increased synapse growth.

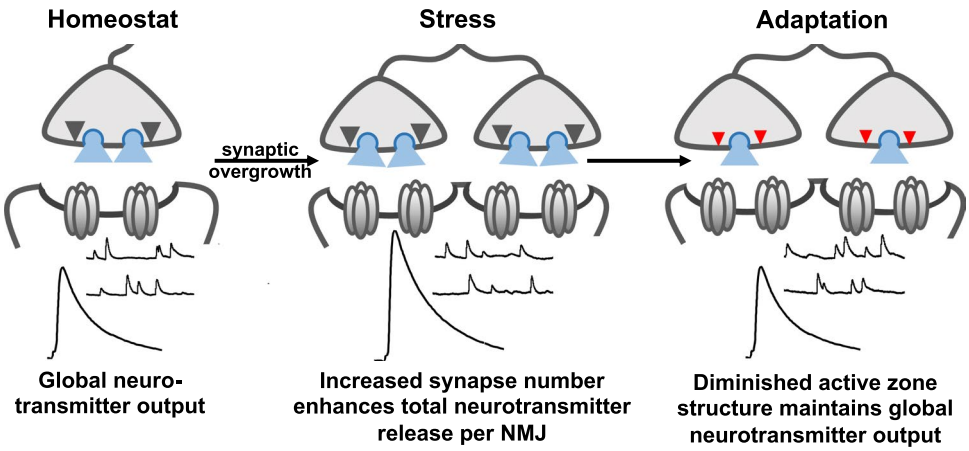

Fig. 5 Active zone structure is homeostatically scaled to compensate for increased synaptic growth. Schematic illustrating how the *Drosophila* NMJ stabilizes synaptic strength when confronted with synaptic overgrowth. Homeostat: global neurotransmitter release onto a particular target is shown in the baseline state. Stress: synaptic overgrowth results in increased bouton and synapse numbers, which should elevate global neurotransmitter output onto the particular target and, in turn, enhance synaptic strength. Adaptation: however, presynaptic active zone structure is remodeled such that an apparent reduction in the abundance of active zone material (represented by red triangles) serves to inhibit presynaptic neurotransmitter release probability. This homeostatic adaptation maintains global neurotransmitter output onto specific targets, stabilizing synaptic strength in response to presynaptic overgrowth

Homeostatic adaptations to synaptic undergrowth

It is clear that in many cases, mutants that exhibit synaptic undergrowth, defined as NMJs with reduced bouton and synapse number, NMJ function is similarly reduced [80, 131]. However, mutations in the synaptic vesicle-associated GTPase *rab3* show normal synaptic strength despite having only about one third the number of active zones compared to wild type [132]. In these mutants, each individual release site was increased ~ 300% in size, as measured by immunofluorescence intensity of the active zone scaffold Brp [132]. This finding immediately suggested an inverse phenomenon to the *endo* mutant NMJs discussed above, in which increased protein levels at individual active zones served to increase release probability and compensate for the reduction in overall number. Indeed, *rab3* mutants showed a similar homeostatic scaling of active zone size and nano-structure with number [48], similar to *endo* mutants but inverse in direction. However, in addition to active zone scaling, there is evidence that other mechanisms can be utilized to compensate for synaptic undergrowth and maintain stable synaptic strength. For example, of the undergrowth mutants isolated in the screen discussed above [80], each mutant also showed normal synaptic strength. However, two novel mechanisms were found that served to maintain stable neurotransmission: (1) an increase in postsynaptic GluR abundance offset reduced neurotransmitter output onto the muscle or (2) a concomitant increase in bouton size accommodated more active zones per bouton, in effect maintaining stable synapse number despite reduced bouton number. Although the induction and signaling mechanisms involved in these forms of adaptive plasticity remain unclear, these studies highlight synaptic structure as a target for homeostatic modulation to offset defects in synaptic growth and maintain stable synaptic strength.

Target-specific homeostatic plasticity at the *Drosophila* NMJ

A subset of motor neurons innervates multiple postsynaptic muscles at the fly NMJ and must maintain sufficient excitation of their targets to enable stable circuit and locomotor functionality. A unique combination of 35 motor neurons innervates 30 distinct muscle segments at the larval NMJ [133], and this stereotypic anatomical arrangement provides a platform to investigate target-specific mechanisms that homeostatically maintain synaptic strength. A seminal study reported over 20 years ago used a manipulation to bias innervation on one target at the expense of the adjacent target [134]. In particular, the cell adhesion factor *Fasciclin II* (*FasII*) was overexpressed in a single muscle to increase innervation on one target, while also causing reduced innervation on the adjacent target (schematized in Fig. 6). Remarkably, synaptic strength was maintained at levels similar to wild type on both targets. This phenomenon parallels plasticity observed in the mammalian brain where synaptic strength is adjusted in a target and synapse-specific way [135–137]. Since this initial study, new insights have been achieved regarding target- and input-specific plasticity at the *Drosophila* NMJ, particularly over the last few years. Here we will discuss these important studies.

A homeostatic increase in postsynaptic GluR abundance maintains synaptic strength at hypo-innervated targets

The first clue as to how NMJ transmission is stabilized at hypo-innervated targets came from electrophysiological recordings of spontaneous neurotransmission in the original Davis and Goodman 1998 study, where an increase in miniature amplitude was observed. One possibility discussed was that multivesicular release, detected as an apparent increase in quantal size, could potentially explain the increased quantal size (and reduced quantal content) observed at hypo-innervated NMJs. Alternatively, a postsynaptic mechanism may be responsible for the increased quantal size, most likely due to a change in the abundance, subtype, and/or functionality of postsynaptic GluRs. More recent studies have demonstrated that indeed, the abundance of GluRs was homeostatically enhanced at hypo-innervated targets [102, 138]. This increase in GluR abundance was similar in magnitude to the reduction in bouton numbers and targeted both GluRIIA- and GluRIIB-containing receptor subtypes, while no change in presynaptic function was observed [138]. Taken together, these studies show that in the case of biased innervation between two target muscles, a reduction in innervation is offset by a homeostatic increase in GluR abundance per postsynaptic receptor field which serves to compensate for the reduced global neurotransmitter output to maintain stable muscle excitation (Fig. 6). Like many of the homeostats described at the NMJ, little is known about how reduced innervation is sensed as well as the nature of the downstream inductive signals that lead to the adaptive increase in GluR abundance.

A target-specific reduction in the number and structure of presynaptic active zones maintains synaptic strength at hyper-innervated targets

In contrast to hypo-innervation, an entirely distinct adaptation functions to maintain stable synaptic strength on hyper-innervated NMJs (Fig. 6). In this case, miniature amplitudes on hyper-innervated muscles were found to be unchanged [134] and no differences in postsynaptic GluR levels or subtypes were observed [138]. Recordings from single boutons

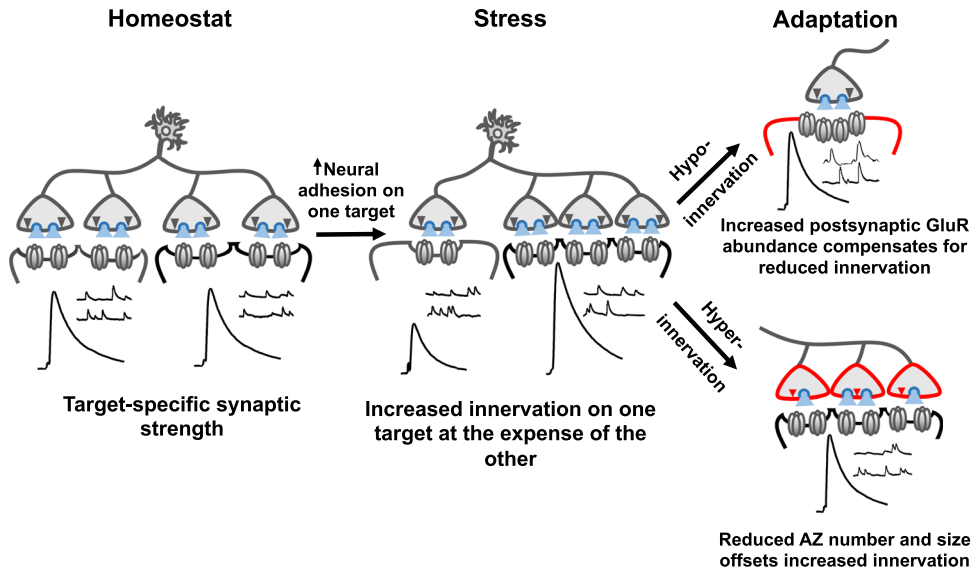

Fig. 6 Biased innervation elicits distinct pre- and postsynaptic adaptations at terminals of a single neuron. Schematic illustrating biased innervation across two targets shared by a single motor neuron at the *Drosophila* NMJ. Homeostat: baseline synapse number and neurotransmission are shown. Stress: overexpression of the neuronal cell adhesion factor *fasII* selectively on one of the two muscles (labeled with a black outline) increases innervation from a single motor neuron on that muscle at the expense of the adjacent muscle (labeled with a gray outline). This would be expected to result in reduced synaptic strength (EPSP amplitude) on the hypo-innervated target and potentiated EPSP amplitude on the hyper-innervated target. Adaptation: however, EPSP amplitudes remain the same on both targets. On the hypo-innervated target, reduced neurotransmitter output (quantal content) is observed due to reduced innervation, as expected. However, a homeostatic increase in postsynaptic GluR abundance increases quantal size, which compensates for reduced quantal content, to maintain EPSP amplitude. In contrast, a completely different adaptation is observed on the hyper-innervated target. Here, no change in postsynaptic GluRs is observed. Rather, a target-specific, homeostatic reduction in both the number and size of active zones (represented by triangles) diminishes neurotransmitter release to offset increased innervation

at hyper-innervated targets further revealed lowered release probability per bouton, indicating a homeostatic reduction in presynaptic release probability per bouton compensates to maintain stable total neurotransmitter output [134]. Recent work investigating this phenomenon found that overall release probability on hyper-innervated targets was not different from wild type, consistent with reduced release probability per bouton [138]. Interestingly, imaging of release sites on hyper-innervated targets provided a clue as to how this target-specific, homeostatic reduction in release probability may be achieved. In particular, a reduction in both the density and intensity of the Ca^{2+} channel Cac and the scaffold Brp at active zones was observed at hyper-innervated NMJs, while the total fluorescence intensity of these proteins on hyper-innervated targets was unchanged compared to wild type [138]. These findings parallel the studies discussed above in which the total abundance of active zone proteins remains constant while the number and structure of individual release sites can vary at presynaptic terminals. However, in this case, the elasticity of active zone number and size is target specific, as no changes in active zone number or structure per bouton were found at terminals from the same axon that hypo-innervated the adjacent muscle [138]. Thus, a target-specific reduction in the number and apparent abundance of material at release sites per bouton may stabilize synaptic strength at hyper-innervated muscles (Fig. 6).

Input-specific homeostatic plasticity

As mentioned in the PHP section above, most muscles in *Drosophila* are innervated by two different motor inputs, phasic type Is (small) and tonic type Ib (big). These motor inputs differ in both morphological and functional characteristics [56, 57, 139]. Type Is motor inputs fire with phasic patterns, have high release probabilities, depress during trains of stimulation, and exhibit small terminal boutons with low elaboration of the subsynaptic reticulum (SSR) [63, 140]. In contrast, type Ib inputs fire with tonic patterns, have lower release probabilities, facilitate during trains of stimulation, and exhibit large NMJ boutons with elaborate SSR structure [141]. The recent development of quantal in vivo calcium imaging approaches [31] and the identification of Gal4 drivers specific for Is and Ib [59, 61] have enabled the dissection of input-specific functions and plasticity at the fly NMJ. Quantal imaging revealed that chronic PHP in *GluRIIA* mutants primarily targets Ib inputs for homeostatic potentiation [31] and specifically remodels active zones at Ib terminals without impacting release sites at Is [51]. In

contrast, rapid pharmacological PHP primarily targets Is terminals for homeostatic potentiation [62]. These findings suggest that distinct inputs respond differently to acute vs chronic homeostatic PHP signaling at the NMJ, although the mechanism for this specificity is unclear. In contrast, other forms of homeostatic plasticity at the fly NMJ, such as PHD and biased innervation, similarly impact both Is and Ib inputs for homeostatic modulation [51, 134]. These findings raise a fundamental question: do type Is and/or Ib motor neurons innervating the same muscle target adaptively respond when transmission is perturbed from the other input?

Three recent studies have begun to shed light on this question of inter-input synaptic plasticity. First, the tetanus neurotoxin light chain blocks all evoked neurotransmitter release when expressed in *Drosophila* motor neurons, while spontaneous miniature transmission persists [142]. When tetanus toxin is expressed in phasic Is motor inputs at the fly NMJ, quantal Ca^{2+} imaging reveals no apparent functional changes in transmission from the tonic Ib motor input innervating the same target [31], indicating at least tonic Ib inputs do not functionally compensate when evoked transmission from phasic Is inputs is blocked. However, structural synaptic plasticity was observed at tonic Ib terminals when tetanus toxin was expressed in Is inputs [59], with an apparent increase in bouton number found but with little functional change, consistent with what was observed with quantal imaging. Interestingly, however, genetic ablation of phasic Is motor inputs did induce some compensatory increase in functional neurotransmission in tonic Ib neurons, without apparent changes in synaptic structure at the Ib inputs [59, 61]. In this case, the increase in neurotransmission at Ib inputs was variable depending on the specific NMJ, with some Ib inputs having no apparent change in function [61]. Regardless, in no case was the increase in synaptic strength at Ib inputs sufficient to be "homeostatic" and restore NMJ transmission to baseline values. In contrast, little structural or functional changes were observed at Is terminals when Ib motor inputs were ablated or blocked in evoked transmission [59, 61].

These studies revealed several important points about plasticity between phasic Is and tonic Ib motor inputs. First, Ib motor neurons can exhibit both structural and functional plasticity when Is neurons are ablated or silenced, while Is neurons are insensitive to Ib activity. However, it was noted that Is motor inputs require the physical presence of Ib motor inputs, at least in some cases, to properly innervate the appropriate target muscle during development [59]. Second, there is a high degree of variability in the specific plasticity elicited, depending on which NMJ and Is/Ib combination are being manipulated. The reasons for this heterogeneity are unclear, although it might be related to the fact that Ib neurons typically innervate only a single target muscle, while Is neurons innervate a group of several distinct muscle targets. Finally, Ib inputs respond differently to the physical presence of Is motor neurons compared with silencing of evoked activity from Is. This indicates that miniature transmission from Is may be sufficient to communicate some information to induce structural plasticity at Ib, while loss of Is appears to induce only functional changes at Ib. Resolving the signaling mechanisms for these various forms of inter-input synaptic plasticity at the fly NMJ will be an intriguing line of research for the future.

Integration and balancing of multiple homeostatic signaling systems

The homeostatic signaling systems at synapses described above are typically studied in isolation to determine specific mechanistic insights. However, synapses in vivo often need to adapt to multiple homeostatic challenges simultaneously, frequently in opposing directions. The advances in our understanding of the synaptic homeostats discussed above at the *Drosophila* NMJ have provided a new appreciation for how such forms of plasticity work in relation to each other, with two important principles emerging from these studies. First, each homeostatic signaling system appears to be independent, utilizing distinct genes and expression mechanisms. Second, with the exception of injury signaling and PHP, multiple homeostats can be simultaneously induced and expressed to balance synaptic strength without one form disrupting the other, even when the stresses and adaptations are in opposing directions. Here, we discuss recent findings interrogating multiple homeostats operating at the fly NMJ.

PHP can be induced and expressed with PHD or biased innervation to balance synaptic strength

Recent work has demonstrated that PHP can be triggered to be expressed in combination with a second homeostat at the same NMJ. As discussed above, it is clear that PHP and PHD are separate and mechanistically distinct homeostats. Indeed, the core genes required for PHP expression are dispensable for the expression of PHD. These genes include *dysbindin*, *dKaiR1D*, *gooseberry*, *rim*, and *cac,* each of which have been shown to be necessary for PHP expression while being completely dispensable for PHD [46, 51, 65, 72]. However, in the case of injury-related signaling, postsynaptic retrograde PHP signaling is deliberately shunted to stabilize synaptic strength at a reduced set point [102]. This highlights the possibility that one form of homeostatic plasticity may supercede another when more than one homeostat is induced and expressed. However, two recent studies have shown that the opposing homeostats of PHP and PHD can be expressed together to balance synapse strength [51, 72]. Importantly, PHD and PHP can be chronically

expressed together throughout larval development through genetic overexpression of *vGlut* presynaptically and loss of *GluRIIA* postsynaptically [51]. In addition, PHP can be acutely induced and expressed by pharmacological perturbation of GluRs at NMJs chronically expressing PHD, demonstrating a temporal independence of these processes. Finally, mutations that block PHP have no apparent impact on PHD expression, yet still fail to express acute or chronic PHP when combined with vGlut-OE [51], underscoring the independence and specificity of these distinct homeostatic signaling systems.

In addition to simultaneous induction with PHD, PHP can also be expressed at NMJs adapted to hypo- or hyper-innervation. Using the *FasII* manipulation described above to induce the distinct homeostatic adjustments to balance synaptic strength, PHP can be acutely induced by GluR blockade using PhTx application at both hypo- and hyper-innervated targets [138]. Miniature activity is reduced at both targets, as expected, but synaptic strength is maintained due to a homeostatic increase in neurotransmitter release at terminals innervating both postsynaptic compartments. It should be highlighted that PHP is robustly and precisely expressed despite the postsynaptic enhancement in GluR abundance at hypo-innervated targets or the reduction in active zone density and structure at hyper-innervated muscles. Finally, and perhaps most remarkably, chronic PHP could be induced by GluR reduction at just the hyper-innervated muscle to balance synaptic strength without impacting neurotransmission at terminals shared by the same motor neuron hypo-innervating the adjacent target [138]. Thus, PHP can be induced simultaneously with one of three distinct homeostatic adaptions—PHD, hypo-innervation, and hyper-innervation—to balance synaptic strength at the fly NMJ.

PHP, PHD, and active zone scaling can be simultaneously expressed at the same NMJ to maintain synaptic strength

PHP can be expressed with one additional homeostatic challenge—either PHD, hypo- or hyper-innervation—and maintain stable synaptic function. Interestingly, it was shown that PHP can also be induced simultaneously with two additional and bidirectional homeostatic challenges (Fig. 7). In this case, three distinct manipulations were used together to homeostatically challenge NMJs [48]. First, acute or chronic PHP was induced by PhTx application or genetic loss of *GluRIIA*. Second, PHD was induced by overexpression of *vGlut* or using *endophilin* mutations, either of which leads to enlarged synaptic vesicles and excess glutamate release. Third, active zone scaling was induced by *endo* mutation, which increases active zone number but reduces size, or the reciprocal *rab3* mutation, which reduces active zone number but increases their size [48, 132]. These three challenges were combined in various combinations at individual NMJs, and each homeostatic adjustment in neurotransmission was induced and expressed to enable stable synaptic strength [48]. Further, individual active zones were revealed to be capable of simultaneous modification by active zone scaling (*endo* or *rab3* mutants) and PHP, where active zone remodeling due to PHP can occur on top of the remodeling that already occurred in *endo* or *rab3* mutations. These results reveal the extent to which the NMJ is built for stability, where at least three independent homeostats can be induced,

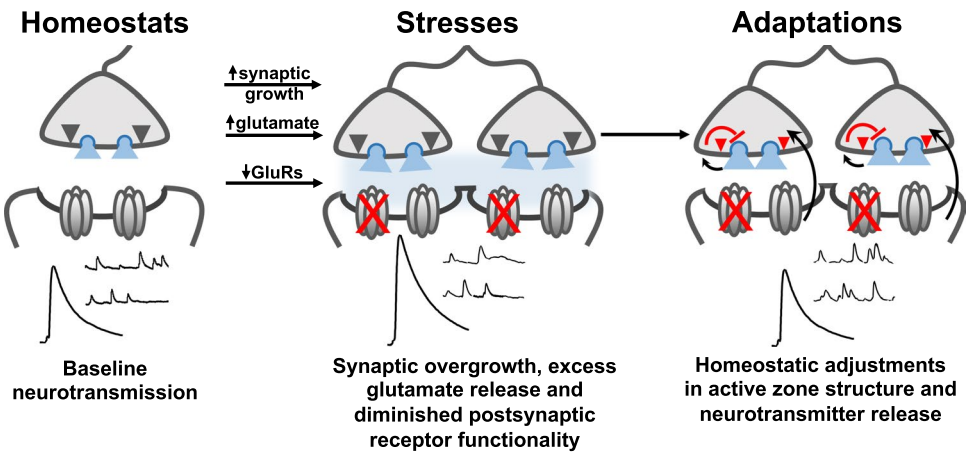

Fig. 7 Three independent homeostats can work simultaneously to balance synaptic strength. Schematic illustrating a single *Drosophila* NMJ confronting three homeostatic challenges at the same time: synaptic overgrowth, excess glutamate release, and pharmacological blockade of postsynaptic GluRs. Remarkably, all three homeostatic adaptations are induced and expressed simultaneously and precisely calibrated to maintain stable synaptic strength. Thus, active zone scaling, PHD, and PHP can be balanced to maintain stable NMJ function

expressed, and balanced simultaneously to establish and maintain synaptic strength.

Conclusions and future directions

The *Drosophila* NMJ has illuminated fundamental principles of synaptic development, growth, transmission, and plasticity for over 40 years and is well positioned to continue to be a premiere model synapse in the future. Going forward, the fly NMJ is well suited to address several impactful questions centering around the homeostatic control of synaptic structure and function. Much has been learned recently about the molecular dialogue between pre- and postsynaptic compartments at this NMJ and the modulation of presynaptic neurotransmitter release from the motor neuron and postsynaptic responsiveness in the muscle. One major challenge for the future will be to describe and manipulate the activity of pre-motor inputs onto motor neurons themselves. This will unlock approaches to determine how (or if) motor neurons adapt to reduced or enhanced excitability, as well as how these responses impact development, function, and plasticity at the NMJ. Intriguing insights into these questions have started to emerge [98, 143–146]. Ultimately, the characterization of pre-motor activity and adaptations to motor units will enable a true appreciation of the full input/output relationships and circuit plasticity of the *Drosophila* NMJ.

Beyond the fly NMJ, powerful systems and approaches have been established to study homeostatic synaptic plasticity in the *Drosophila* brain and in mammalian systems. In the fly central nervous system, for example, homeostatic scaling of synaptic structure and function has been demonstrated in the visual system, where an adaptive remodeling of dendritic arborization occurs in response to chronically elevated or reduced visual activity [147]. In addition, the rodent NMJ is becoming a compelling system to study PHP at a cholinergic synapse [12, 13] and provocative links have been found between PHP and NMJ diseases like ALS [11]. New homeostats beyond PHP are likely to emerge in the coming years at the rodent NMJ. Finally, it has long been clear that a variety of homeostatic mechanisms operate at synapses in the mammalian central nervous system, where new targets for adaptive plasticity, including the structure and location of the axon initial segment [148, 149], have been characterized in addition to GluR scaling and presynaptic homeostatic modulation [150–152]. Of particular note, exciting links and mechanistic relationships between sleep, synapses, and homeostatic plasticity have been revealed in the *Drosophila* and rodent brain [153–157]. The homeostatic control of synaptic activity will continue to be an exciting area of research with fundamental principles discovered at the *Drosophila* NMJ.

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Complaince with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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