Madm/NRBP1 mediates synaptic maintenance and neurodegeneration-induced presynaptic homeostatic potentiation

Highlights

- Presynaptic Madm controls synaptic growth and stability via 4E-BP phosphorylation
- Postsynaptic Madm is sufficient to induce a presynaptic potentiation of release
- Postsynaptic Madm is required for neurodegeneration-induced PHP
- Induction of canonical PHP in degenerating neurons alleviates synaptic degeneration

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In brief
Mushtaq et al. demonstrate that the pseudo-kinase Madm/NRBP1 controls synaptic maintenance at the Drosophila neuromuscular junction via the regulation of cap-dependent translation in neurons. In the presence of synaptic degeneration, postsynaptic Madm is sufficient to induce presynaptic homeostatic potentiation (PHP) that acts neuroprotective and promotes structural and functional synaptic stability.
**Madm/NRBP1 mediates synaptic maintenance and neurodegeneration-induced presynaptic homeostatic potentiation**

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**SUMMARY**

The precise regulation of synaptic connectivity and function is essential to maintain neuronal circuits. Here, we show that the *Drosophila* pseudo-kinase Madm/NRBP1 (Mlf-1-adapter-molecule/nuclear-receptor-binding protein 1) is required presynaptically to maintain synaptic stability and to coordinate synaptic growth and function. Presynaptic Madm mediates these functions by controlling cap-dependent translation via the target of rapamycin (TOR) effector 4E-BP/Thor (eukaryotic initiation factor 4E binding protein/Thor). Strikingly, at degenerating neuromuscular synapses, postsynaptic Madm induces a compensatory, transsynaptic signal that utilizes the presynaptic homeostatic potentiation (PHP) machinery to offset synaptic release deficits and to delay synaptic degeneration. Madm is not required for canonical PHP but induces a neurodegeneration-specific form of PHP and acts via the regulation of the cap-dependent translation regulators 4E-BP/Thor and S6-kinase. Consistently, postsynaptic induction of canonical PHP or TOR activation can compensate for postsynaptic Madm to alleviate functional and structural synaptic defects. Our results provide insights into the molecular mechanisms underlying neurodegeneration-induced PHP with potential neurotherapeutic applications.

**INTRODUCTION**

The relay and processing of information in the nervous system relies on functional and structural stability of synaptic connectivity. Any impairments of these stability parameters will perturb the communication between partner neurons or peripheral targets such as muscles and may lead to progressive psychiatric or neurodegenerative disease conditions. In contrast with the molecular mechanisms controlling synapse formation,1–12 our understanding of the mechanisms ensuring structural and functional maintenance of synapses is currently much less advanced in both invertebrate and vertebrate systems. At the *Drosophila* neuromuscular junction (NMJ), a model system particularly well suited to correlate morphological and functional aspects of synaptic parameters,3–4 it has been demonstrated that a core cytoskeletal network linking cell adhesion molecules via the ankyrin2/spectrin cytoskeleton to the presynaptic actin and microtubule cytoskeleton is essential to maintain structural synaptic stability.5–16 Posttranslational modifications of protein-protein interactions within this network control the balance between synaptic growth and maintenance and are used to implement synaptic plasticity.5–7,10,15 Similarly, it has been demonstrated that functional parameters are maintained at the NMJ by signaling mechanisms that efficiently compensate for perturbations in synaptic release or neurotransmitter detection. The underlying molecular mechanisms are currently best understood for presynaptic homeostatic potentiation (PHP), in which postsynaptic perturbations of glutamate receptors (GluRs) are efficiently compensated by a transsynaptic mechanism that increases presynaptic release to ensure maintenance of appropriate postsynaptic muscle excitation.17,18 Central to the postsynaptic mechanisms involved in PHP induction is the target of rapamycin (TOR) signaling pathway that induces a retrograde signal via translational control mechanisms.19–21 Although the precise retrograde mechanisms that relay the induction signal to the presynaptic motoneuron still remain largely enigmatic, it has become clear that alterations of active zone components and structures are utilized to achieve compensatory presynaptic release.17,20,22–30 A first link between structural and functional stability has been provided by the demonstration that PHP can be induced to restore presynaptic release at degenerating NMJs.31 In addition, it has recently been demonstrated that PHP-like mechanisms...
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can be activated at degenerating NMJs in Drosophila and mice as an endogenous neuroprotective mechanism to decelerate progressive neurodegeneration.32

Here, we extend on these findings by identifying the pseudo-kinase Mif-1-adapter-molecule/nuclear-receptor-binding protein 1 (Madm/NRBP1) as a novel regulator of structural and functional synaptic stability and by defining a molecular pathway underlying endogenous neurodegeneration-induced PHP. The absence of Madm leads to neurodegeneration and defects in synaptic growth, organization, and function. By combining genetic, pharmacological, and functional assays, we identify unique roles of presynaptic and postsynaptic Madm that are required for the maintenance of synaptic stability and function and that utilize the transsynaptic PHP machinery to offset presynaptic deficits. Mechanistically, we show that Madm regulates cap-dependent translation downstream of TOR. Together, our work advances our understanding of the mechanisms linking neurodegeneration and compensatory synaptic plasticity mechanisms.

RESULTS

Madm is required for synaptic growth and maintenance
To identify novel regulators of synaptic maintenance, we previously performed an in vivo RNA interference (RNAi)-based screen of the Drosophila kinome and phosphatome.5 As a part of this approach, we also screened kinase-associated proteins (not included in the published dataset) and identified the pseudo-kinase Madm as a novel presynaptic regulator of synaptic maintenance and development. Madm lacks the conserved ATP-binding motif of active kinases and has been previously identified as a growth-promoting adaptor protein (Figure 1A).33,34 A role in nervous system development or function has not been described to date. To determine the role of Madm in synapse development and maintenance, we first verified available genetic tools (Figure 1A) using an antibody that we raised against full-length Madm protein. The genetic tools include an RNAi line (MadmV27346) targeting the first exon of Madm, an EP insertion in the 5’ UTR of Madm (MadmEP3137), and the previously published alleles MadmD72 (potential null allele) and MadmD52 (partial loss-of-function allele carrying a point mutation in an essential protein interaction domain)33 (Figure 1A). Both mutant alleles are homozygous lethal, but trans-heterozygous combinations with a deficiency encompassing the entire Madm genomic locus (Df(3R) Exel728) survive to the third instar larval stage (Figure S1A). Western blot analysis of larval brain extracts demonstrated high expression of Madm protein (~70.5 kDa) in the nervous system that could be efficiently knocked down by neuronal expression of MadmRNAi. Similarly, the hypomorphic (MadmD52) and the potential null allele (MadmD72) efficiently reduced protein levels, whereas MadmD52 affected expression levels only partially (Figure 1B) consistent with the molecular nature of this point mutation.33 The Madm antibody also recognizes a non-specific band in close proximity to Madm, which can be observed in all genetic conditions (Figure S1C). We used these validated tools to determine the presynaptic and postsynaptic contributions of Madm for synapse development and maintenance.

Stable synapses are characterized by the precise apposition of the presynaptic active zone marker Bruchpilot (Brp) to postsynaptic clusters of GluRs. In contrast with control animals, all allelic combinations of Madm mutations showed synaptic degeneration phenotypes (Figures 1D, 1E, 1I, S1D–S1F, S7A, and S7C). At these sites, postsynaptic GluR clusters were no longer opposed by Brp, and the presynaptic motoneuron membrane was fragmented despite the presence of the subsynaptic reticulum marker Discs large (Figures 1D, 1E, S1F, S2E, S2F, S7A, and S7C). These phenotypes were evident at NMJs of all muscle groups in third-instar larvae (Figures S2A–S2C). The synaptic degeneration phenotypes were analogous to phenotypes previously identified for mutations in signaling32 or cytoskeletal genes that are essential for synaptic maintenance.7,9–11,13–16

Presynaptic expression of a wild-type copy of Madm in Madm mutants using either a neuronal Gal4 (nG1) or a motoneuron-specific Gal4-driver (nG2) line rescued synaptic stability (Figures 1F, 1I, and S1D). Interestingly, muscle-specific expression (mG1) of Madm also provided a partial rescue of the synaptic degeneration phenotype (Figures 1G, 1I, and S1D). Simultaneous presynaptic and postsynaptic expression completely restored synaptic stability similar to neuronal expression alone (Figures 1H, 1I, and S1D).

In addition to the synaptic degeneration phenotype, we observed severe synaptic growth defects in Madm mutants. In control animals, type Ib NMJs on muscle 1 are organized into two stereotypic branches (Figures 1C and S2D). In Madm mutants, these NMJs failed to extend on the muscle surface, and synaptic boutons clustered at the site of muscle innervation (Figures 1D, 1E, S2A–S2C, S2E, and S2F). Quantification of
Figure 2. Presynaptic Madm controls synaptic growth and maintenance via 4E-BP regulation

(A and B) In contrast with controls (A), a neuronal knockdown of TOR resulted in synaptic degenerations and NMJ morphology defects (B). Scale bars in (A) apply to (A) and (B) and represent 5 μm.

(C) Quantification of the synaptic retraction frequency on muscles 1/9 and 2/10. Mean ± SEM, two-tailed unpaired t test; n = 10 and 11 animals.

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NMJ growth revealed a significant reduction in the number of boutons (Figure 1J; Table S1) that could be rescued by presynaptic, but not postsynaptic, expression of Madm (Figures 1J, S2G, and S2H; Table S1). Interestingly, postsynaptic Madm partially restored synaptic bouton organization (Figure S2H). Combined presynaptic and postsynaptic expression fully restored NMJ growth and organization to control levels (Figures 1J and S2; Table S1). Because our newly generated Madm antibody did not efficiently work for in situ immunohistochemical analysis, we monitored the distribution of GFP-tagged Madm that we used in all our rescue assays (Figures 1, 2, 3, S1, and S2G–S2I). In motoneurons, GFP-Madm localized to the presynaptic nerve terminal (Figure S3A). In muscles, GFP-Madm localized to the postsynaptic compartment and was enriched within the sub-synaptic reticulum (SSR), a distribution that was not observed for cytoplasmic GFP (Figures S3B–S3D). Consistent with prior analysis of wild-type Madm,43 we did not observe any nuclear localization of Madm in the ventral nerve cord (Figures S3E and S3F). Based on these results, we conclude that presynaptic Madm regulates synaptic growth and maintenance, whereas postsynaptic Madm contributes partially to NMJ organization and stability.

Presynaptic control of synaptic growth and stability
We next asked how presynaptic Madm controls synapse stability and growth. To identify relevant signaling pathways, we performed a trans-heterozygous genetic interaction assay with components of the two major growth promoting pathways at the larval NMJ, the transforming growth factor β (TGF-β)/BMP (Bone Morphogenic Protein), and the TOR signaling pathways (Figure S4A).46–48 Although we did not observe any significant interactions between Madm and components of the TGF-β/BMP pathway, trans-heterozygous combinations of Madm and TOR pathways members (TCS2/gigas; rheb) significantly impaired NMJ growth compared with control genotypes (Figure S4B). Indeed, Madm has been previously implicated in TOR signaling in intestinal stem cells in Drosophila.49 To test the relevance of TOR signaling for synaptic maintenance, we knocked down the serine-threonine kinase TOR in larval neurons using a previously characterized RNAi line.44,45 The TOR knockdown resulted in a significant increase in synaptic degeneration and an impairment of NMJ morphology that resembled the Madm mutant phenotype (Figures 2A–2C and S4C). We could further verify this requirement of TOR for synaptic stability by a pharmacological inhibition of TOR by rapamycin feeding (Figure 2D). TOR controls cellular growth by promoting cap-dependent translation through phosphorylation-dependent inhibition of the negative regulator of translation 4E-BP (eukaryotic initiation factor 4E binding protein/Thor) and phosphorylation-dependent activation of the positive regulator S6-kinase (S6K).46–48 We therefore monitored the phosphorylation status of these regulators of cap-dependent regulation using larval brain extracts. Consistent with prior reports, the knockdown of TOR resulted in an almost complete loss of 4E-BP phosphorylation without altering levels of 4E-BP protein (Figure 2E). In the absence of Madm, we also observed a highly significant reduction in the levels of phosphorylated 4E-BP (Figures 2F and 2G). Importantly, pan-neuronal or motoneuron-specific, but not muscle, expression of wild-type Madm efficiently restored 4E-BP phosphorylation in Madm mutants without affecting the levels of non-phosphorylated 4E-BP (Figures 2F, 2G, S4D, and S5). Consistent with the severe reduction of 4E-BP phosphorylation in the absence of Madm, application of rapamycin to Madm mutants did not further increase the synaptic degeneration phenotype (Figure 2D). In contrast with the effects on 4E-BP phosphorylation, we did not observe a significant reduction in the phosphorylation status of S6K in these brain extracts (Figures 2H and S5D). These data indicate that Madm promotes synaptic growth and stability by inhibiting the activity of the negative regulator of cap-dependent translation 4E-BP that is encoded by the gene Thor in Drosophila. We therefore hypothesized that genetic removal of the negative regulator 4E-BP in Madm mutants may alleviate the Madm-associated phenotypes.

The homozygous viable 4E-BP mutation, Thor15, reduced the levels of phosphorylated and non-phosphorylated 4E-BP below the detection level (Figures 2F and 2G). Consistent with prior studies,50 we did not observe any significant alterations of synaptic growth in Thor mutant animals (Figures 2J and 2L). Importantly, in homozygous double-mutant Madm*Thor animals, we observed a partial rescue of the synaptic growth defect (Figures 2I–2L). Analysis of synaptic stability in these animals revealed that removal of 4E-BP also provided a partial but...
significant rescue of the synaptic degeneration phenotype (Figure 2M). We could attribute this rescue of synaptic growth and stability to presynaptic 4E-BP because only the presynaptic, but not the postsynaptic, RNAi-mediated knockdown of 4E-BP in Madm mutant animals rescued the phenotypes (Figures 2N and S4E). To further investigate this interaction between Madm and 4E-BP, we overexpressed 4E-BP in presynaptic motoneurons alone and in combination with Madm. Overexpression of 4E-BP alone caused a significant decrease in synaptic bouton number that was efficiently restored by co-expression of Madm (Figures S4F–S4I) with no effects on synaptic maintenance (Figure S4J). These data are consistent with Madm counteracting ectopic levels of 4E-BP by increasing the fraction of inactive 4E-BP.

**Loss of Madm affects synaptic transmission**

It has been previously observed that severe synaptic degeneration was accompanied by defects in neuronal transmission. Therefore, we next performed electrophysiological analyses and observed a significant decrease in evoked excitatory junction currents (EJCs) in Madm mutants compared with controls (Figures 3A and 3C). Because we detected no alteration of quantal size (mEJC) (Figures 3A and 3B), the quantal content (QC) at these NMJs was severely reduced (Figures 3D and 3E). Surprisingly, expression of Madm in either the presynaptic or postsynaptic compartment in Madm mutants was sufficient to restore EJC amplitudes and QC to control values (Figures 3A and 3C–3E; Table S1). We included all parental Gal4 and UAS genotypes as controls in this dataset and could thereby exclude any contributions of the genetic background (Figures S6A–S6C). Interestingly, simultaneous presynaptic and postsynaptic expression of Madm in Madm mutants significantly increased QC above control values (Figures 3D and 3E). This indicates that Madm may have the potential to potentiate neurotransmitter release.

**Transsynaptic control of synaptic function**

To disentangle the tissue-specific requirements of Madm, we knocked down Madm either presynaptically or postsynaptically using Madm-specific RNAi (Figures 1B and S1B). Knockdown
of Madm in the presynaptic motoneuron induced synaptic degeneration (Figures S7A and S7C–S7E), whereas postsynaptic knockdown of Madm did not impair synaptic maintenance (Figures S7B, S7D, and S7E). Surprisingly, neither postsynaptic nor postsynaptic knockdown of Madm altered synaptic transmission. However, a simultaneous presynaptic and postsynaptic knockdown led to a significant reduction of EJCs without affecting spontaneous (mEJC) neurotransmitter release (Figures 4A–4C) resulting in a QC reduction comparable with Madm mutants (compare Figures 4D and 4E with 3D and 3E).

Interestingly, a recent study demonstrated that a compensatory ENaC (epithelial sodium channel)-dependent PHP is expressed at degenerating NMJs in flies and mice to ensure the maintenance of neurotransmitter release (Figures S8A–S8C). Consistent with prior observations, benzamil treatment did not alter synaptic transmission in animals lacking presynaptic Madm, which did not have synaptic degeneration phenotypes, in contrast, acute application of benzamil to NMJs lacking presynaptic Madm resulted in a significant decrease in QC that could be completely reversed by washing out the ENaC channel blocker (Figures S8E and S8F). In animals lacking postsynaptic Madm, which did not have synaptic degeneration phenotypes, the application of benzamil did not alter synaptic release properties (Figures 4H and 4K). Similarly, NMJs lacking both presynaptic and postsynaptic Madm and that already displayed reduced QC level compared with controls before benzamil application (Figures 4E and 4H) did not show any further reduction of QC on benzamil application (Figures 4H and S8L). These data are consistent with an ENaC-dependent presynaptic potentiation of neurotransmitter release in animals lacking postsynaptic Madm that ensures normal excitation of postsynaptic muscles despite the presence of functional and neurodegenerative defects. Because this potentiation of release was not present in animals lacking both presynaptic and postsynaptic Madm, it indicates that postsynaptic Madm is required for this process.

To further demonstrate that the presynaptic PHP machinery is essential for the maintenance of neurotransmitter release in the presence of neurodegeneration, we used two independent genetic manipulations that have been previously demonstrated to block PHP expression in the presynaptic nerve terminal. First, we performed knockdown of presynaptic Madm in the background of a homozygous mutation of the schizophrenia-susceptibility gene dysbindin (dysb). It has been demonstrated that Dysb is necessary for the expression and maintenance of PHP.23,25,29 In agreement with prior reports, homozygous dysb mutations alone did not impair spontaneous or evoked neurotransmission (Figures S9A–S9D).23 In contrast, and consistent with our pharmacological (benzamil) inhibition of PHP, absence of dysb (dysb<sup>000128</sup>) in animals lacking postsynaptic Madm (Madm RNAi) resulted in a significant decrease of evoked release (EJC) and QC, but no alteration of mEJCs (Figures S9A–S9D). These data indicate that the rescue of presynaptic release, which we previously observed after postsynaptic expression of Madm in Madm mutant animals, was likely due to a Madm-dependent activation of the presynaptic PHP machinery (Figures 3C–3E).

To directly test this hypothesis, we repeated the Madm rescue assays in a homozygous dysb mutant background. In dysb/Madm double mutants, the expression of Madm in the postsynaptic muscle was no longer sufficient to rescue EJC amplitude or QC (Figures S9E–S9I). To further validate these results, we performed analogous experiments in the presence of a heterozygous brp mutation. Prior work showed that removal of a single copy of brp is sufficient to interfere with the expression of PHP without affecting normal release properties.26 Consistently, a heterozygous brp mutation did not interfere with synaptic transmission. However, presence of this mutation prevented the rescue of QC in Madm mutants by postsynaptic Madm (Figures S8A–S8C), thereby validating our results obtained with the homozygous dysb mutation. Together, these tissue-specific knockdown and rescue experiments demonstrate that, in the absence of presynaptic Madm and therefore in the presence of synaptic degeneration, postsynaptic Madm induces the maintenance of presynaptic neurotransmitter release via molecular components of the PHP machinery. Based on these results, we next asked whether the observed partial rescue of the structural neurodegenerative phenotype observed in the postsynaptic rescue (Figures 1G and 1I) also depends on a
PHP-machinery-dependent restoration of synaptic release properties. Indeed, the presence of either the homozygous dysb mutation (Figures 5J–5M) or the presence of the heterozygous brp mutation (Figure S9D) in the rescue genotype significantly increased the frequency of synaptic retraction events compared with the postsynaptic Madm rescue (Figures 5M and S8D). Thus, postsynaptic Madm is necessary for the expression of a PHP-like response to counteract impairments of synaptic function and maintenance caused by the absence of presynaptic Madm.

**Postsynaptic Madm is sufficient to induce retrograde synaptic potentiation**

Based on these observations, we next asked whether Madm can directly induce synaptic potentiation in control animals. Overexpression of Madm in motoneurons did not lead to any changes in release properties (Figures 6A, 6B, and S9A–S9C). In contrast, muscle overexpression of Madm resulted in a significant enhancement of evoked synaptic release (EJC and QC) without altering NMJ morphology (Figures 6C, 6D, and S8D–S8F). Interestingly, similar effects have been previously observed after postsynaptic overexpression of TOR.20 TOR acts as a postsynaptic mediator of PHP by promoting cap-dependent translation through phosphorylation-dependent activation of S6K and phosphorylation-dependent inhibition of the negative regulator of translation 4E-BP/Thor.19-21,47,49 This postsynaptic initiation of translation induces a retrograde signal that promotes presynaptic potentiation.20,23

Postsynaptic overexpression of TOR and Madm resulted in quantitatively similar increases of EJCs and QC. This increase in presynaptic release was completely abolished when Madm was overexpressed in muscles of homozygous dysb mutant animals or heterozygous brp mutant animals (Figures 6E–6I). This demonstrates that postsynaptic Madm, like TOR, utilizes the presynaptic PHP machinery to potentiate presynaptic release.20,23 We next addressed whether postsynaptic Madm also utilizes cap-dependent translation mechanisms to induce these changes in synaptic transmission. Analysis of the phosphorylation status of S6K and 4E-BP in animals overexpressing Madm in muscles revealed an almost 2-fold increase in phosphorylated (activated) S6K. This increase of pS6K is comparable with the changes observed in GlurIIA mutant animals that induce TOR-dependent PHP (Figures 6J, 6K, and S10A).20 In addition, we observed a significant increase in the fraction of phosphorylated, and thus inactivated, 4E-BP (Figures 6J, 6L, 6M, S9H, and S10B). Consistent with prior reports, we did not observe any increase in phosphorylated 4E-BP in GlurIIA mutant animals. This is likely due to the fact that alterations in phosphorylation levels in response to PHP induction mechanisms occur locally at the synapse and may not significantly alter overall p4E-BP levels in muscles.19

**Madm and PHP**

Because postsynaptic Madm is required for a PHP-like mechanisms in animals lacking presynaptic Madm and displaying synaptic degeneration, we next asked whether Madm is also required for canonical, GlurIIA-mutation-induced PHP. The requirement of TOR for PHP has been demonstrated by removal of a single copy of TOR in the background of the PHP-inducing GlurIIA mutation.20 Consistent with prior studies, we observed a significant decrease in the amplitude of miniature release events (mEJCs) in the absence of GlurIIA (GlurIIA<sup>SP16/SP16</sup>, but no reduction in the amplitude of evoked neurotransmitter release (EJCs). This reflects a compensatory increase in QC and represents PHP (Figures 7A–7D, S11A, and S11B).17,18 Removing one copy of Madm in this background did not alter release properties (Figures S11A and S11B). We validated this result by analyzing GlurIIA-Madm double-mutant animals (GlurIIA<sup>SP16/SP16, Madm<sup>S323/D</sup></sup>). However, despite a reduction in quantal response (mEJCs) compared with Madm single mutants, the EJC amplitudes of double-mutant animals remained at the level of Madm mutants, and QC was significantly increased to control levels (Figures 7A–7D). Thus, despite the impairment of synaptic release, PHP can still be efficiently induced in Madm mutant animals (Figure 7E). Because Madm impinges on the same translational control machinery as TOR, we next tested whether TOR-dependent induction of PHP depends on the presence of Madm. Similar to our observations for GlurIIA-induced PHP, overexpression of TOR in muscles of Madm mutants significantly increased QC, almost back to control levels (Figures 7A–7D). Thus, two independent PHP-inducing manipulations are able to restore QC in Madm mutants back to control levels (Figure 7E), and these effects are comparable with the compensatory effects we observed for postsynaptic Madm in rescue assays (Figure 3).

We therefore tested whether these manipulations may also restore the structural synaptic degeneration phenotypes. Analysis
of the frequency of synaptic retractions in Madm mutants that carry either homozygous GluRIIA mutations or are overexpressing TOR postsynaptically revealed a significant rescue of the synaptic degeneration phenotype comparable with the postsynaptic Madm rescue (Figures 7F–7I). However, these postsynaptic manipulations failed to restore the synaptic growth deficits observed in Madm mutants (Figure S11C). Thus, postsynaptic induction of homeostatic plasticity does not only restore functional but also structural synaptic stability defects in Madm mutants.

Finally, we asked whether postsynaptic Madm is not only required in the absence of presynaptic Madm but may be part of a general compensatory mechanisms evoked by presynaptic neurodegenerative processes. Thus far, an endogenous expression of PHP had been reported only for mutations in the neuronal specific cytoskeletal adaptor protein Ankyrin2 in Drosophila. We could confirm these findings and observed that ank2 mutants (ank22001/200d) expressed benzamil-sensitive PHP (Figures 7J–7L and S11D–S11F). We then knocked down Madm specifically in the postsynaptic muscles of ank2 mutants. In these animals, we no longer observed a significant reduction of the QC on benzamil application (Figures 7J, 7K, 7M, and S11D–S11F). These data indicate that the expression of a neurodegeneration-induced, ENaC-dependent compensatory release depends, at least in part, on the presence of postsynaptic Madm.

**DISCUSSION**

Here, we identify the adaptor protein and pseudo-kinase Madm as a novel regulator of cap-dependent translation and define molecular mechanisms underlying neurodegeneration-induced PHP. Whereas presynaptic Madm is required for synaptic growth and maintenance, postsynaptic Madm is essential to offset the functional synaptic impairments caused by the absence of presynaptic Madm. In response to ongoing presynaptic neurodegeneration, postsynaptic Madm evokes synaptic release enhancements via the molecular machinery necessary for the expression of PHP, even though Madm itself is not required for canonical PHP expression. Mechanistically, presynaptic and postsynaptic Madm activate the cap-dependent translation machinery downstream of the TOR complex to initiate a cap-dependent translation response. Consistently, postsynaptic activation of the TOR pathway or induction of GluRIIA-mediated PHP is sufficient to functionally substitute for the absence of postsynaptic Madm. Importantly, these postsynaptic alterations do not only restore functional parameters but also alleviate structural synaptic degeneration phenotypes. Madm-dependent fine-tuning of cap-dependent translation on both sides of the synaptic terminal may thus represent a novel module to appropriately maintain functional and structural synaptic stability.

**Madm and neurodegeneration**

Absence of Madm in motoneurons affects three key NMJ parameters: synaptic growth, maintenance, and function. The synaptic degeneration always starts at the distal-most bouton, and this phenotypic trait is shared with previously identified regulators of synaptic stability that link synaptic cell adhesion molecules to the actin and microtubule cytoskeleton, or promote transsynaptic growth like the TGF-β pathway, or represent regulatory signaling molecules such as kinases and phosphatases. Madm and its vertebrate ortholog NRBP1 have been previously identified as adaptor proteins for a number of signaling pathways, contributing to the control of cell growth and proliferation during normal development and in cancer. The molecular level, we now demonstrate that Madm, like TOR, is required for the phosphorylation of 4E-BP in neurons. Phosphorylation of 4E-BP relieves its inhibitory effect on the translation regulator eIF4E that in turn initiates cap-dependent translation. Our data are in agreement with the prior observation that Madm can influence 4E-BP phosphorylation induced by an ectopic TOR-pathway activation in Drosophila midgut cells. Consistent with Madm acting as a negative regulator of 4E-BP, the absence of 4E-BP in Thor+Madm double mutants partially alleviated the synaptic growth and degeneration phenotypes, and a presynaptic knockdown of TOR partially phenocopied the Madm growth and stability phenotypes. We therefore propose that Madm and TOR promote cap-dependent translation necessary for synaptic growth and maintenance. This function of translational regulation is likely evolutionary conserved because a block of TOR signaling in a mouse model of amyotrophic lateral sclerosis (ALS) accelerated NMJ degeneration. However, despite the regulation of downstream targets of the TOR signaling pathway that we and others observed, Madm affects only a specific part of the TOR signaling pathway because Madm mutants do not share morphological phenotypes with mutations in positive or negative regulators of the TOR pathway.

**Neurodegeneration-induced PHP**

Surprisingly, postsynaptic Madm is both required and sufficient to offset the synaptic release impairments associated with the

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**Figure 6. Postsynaptic Madm is sufficient to induce synaptic potentiation**

(A–D) Analysis of presynaptic (A and B) and postsynaptic (C and D) Madm overexpression. (A and C) Representative traces of mEJCs and EJCs. (B and D) Quantification of mEJC amplitude, EJC amplitude, and QC demonstrated that only postsynaptic overexpression (D) is sufficient to potentiate presynaptic release. Mean ± SEM. Student’s t test for pairwise comparison; n = 8, 12 (A and B), 14, or 16 (C and D) cells, ≥ 8 animals per genotype. (E–I) Representative traces (E) and quantifications of mEJCs (F), EJCs (G), and QC (H and I) showed Madm overexpression induced synaptic potentiation comparable with postsynaptic TOR. Presence of homozygous dysb or heterozygous brp mutations suppressed the induced increases in EJC amplitude and QC; (I) QC normalized to controls. Mean ± SEM. ANOVA and Sidak’s multiple comparison tests; n = 17, 16, 13, 15, and 14 cells, ≥ 8 animals per genotype. (J–M) Western blot analysis of muscle extracts for phosphorylated S6K, phosphorylated 4E-BP, and non-phosphorylated 4E-BP. (J) Images of representative western blots, (K) quantification of phosphorylated S6K, (L) quantification of phosphorylated 4E-BP, and (M) quantification of non-phosphorylated 4E-BP; all values were normalized to Tubulin levels. GluRIIA(S94D/S95D) led to an enhancement of phosphorylated S6K, but not of phosphorylated 4E-BP. In contrast, postsynaptic overexpression of Madm promotes phosphorylation of both proteins. Mean ± SEM. ANOVA and Dunnett’s multiple comparison tests; n = 4, 3, and 3 biological replicates, with each replicate representing 5 animals. ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05 and ns (non-significant) p ≥ 0.05. See also Figures S9A–S9H and S10.
synaptic degeneration induced by the loss of presynaptic Madm. Using complementary genetic approaches, we demonstrate that postsynaptic Madm induces a compensatory increase in presynaptic release by utilizing the presynaptic machinery associated with the expression of PHP. It has been demonstrated that perturbations of postsynaptic GluRs that decrease the perception of quantal release events (mEJCs) are efficiently offset by a compensatory increase in presynaptic release.17,18 By pharmacological and genetic inhibition of three components of the PHP expression system, we demonstrate that postsynaptic Madm utilizes the same molecular machinery. First, acute inhibition of the presynaptic epithelial sodium channel ENaC, which is necessary for the expression of PHP,30,32,52 revealed that an ENaC-dependent mechanism ensures maintenance of synaptic transmission in animals lacking only presynaptic Madm. Because such a compensatory increase was not present in animals lacking both presynaptic and postsynaptic Madm, it demonstrates an essential role of postsynaptic Madm in this process. Second, removal of the schizophrenia-susceptibility gene dysb that is essential for PHP expression23,25,29 in animals either expressing presynaptic Madm RNAi or in Madm mutants that were rescued by postsynaptic expression of Madm completely blocked the compensatory increase in QC. Finally, removal of a single copy of brp, a major structural component of the active zone that is necessary for the expression of PHP,20,22 also blocked the rescue of the neurotransmission phenotype. Together, these experiments demonstrated that absence of presynaptic Madm causes the synaptic transmission defects observed in Madm mutants, and that postsynaptic Madm is essential to induce the compensatory PHP-machinery-dependent increase in presynaptic release.

How does postsynaptic Madm potentiate synaptic release? Consistent with postsynaptic Madm being sufficient to compensate for the decrease in synaptic release in the absence of presynaptic Madm, ectopic expression of Madm in the muscle is sufficient to enhance presynaptic release above control levels. To our knowledge, such a feature has thus far been observed only for TOR and LRRK2.20,21 Both TOR and LRRK2 mediate postsynaptic potentiation via a positive regulation of cap-dependent translation.20,21 Our data show that Madm activates the same cellular machinery as TOR and LRRK2, and overexpression of Madm and TOR resulted in qualitatively similar increases in QC. Similar to TOR-dependent synaptic potentiation,20,22 the Madm-dependent increase in presynaptic release required the PHP expression factors Brp and Dysb. Furthermore, our analysis of downstream translational regulators of TOR signaling revealed that Madm is sufficient to significantly increase the phosphorylation status of S6K and 4E-BP and thereby activate cap-dependent translation. However, in contrast with TOR and LRRK2 that depend on each other for postsynaptic potentiation and are required for the GluRIIA-dependent PHP,21 Madm is not necessary for GluRIIA- or TOR-dependent PHP. Nevertheless, Madm, TOR, and GluRIIA mutations likely induce identical trans-synaptic compensatory mechanisms in line with prior findings that global increases in translation may be read out as a specific PHP induction signal.25,62 Because a pseudo-kinase Madm does not have the capacity to directly phosphorylate the translational regulators, however, there is accumulating evidence that pseudo-kinases can serve as essential adaptors to contribute to target-specific post-translational modifications as demonstrated both for Madm and its vertebrate ortholog NRBP1.43,63,64 Uncovering the precise mechanisms of how Madm contributes to the efficient phosphorylation of S6K and 4E-BP will be of great importance to our understanding of these signaling pathways.

**Neurodegeneration and PHP**

Two studies previously addressed the relationship between neurodegeneration and PHP. In a Drosophila model of ALS that is based on presynaptic overexpression of an extended GR-dipeptide repeat, it was observed that induction of PHP via a postsynaptic knockdown of the GluRIIA subunit or via postsynaptic overexpression of TOR is sufficient to restore the synaptic strength deficits associated with presynaptic degeneration.21 In addition, Orr and colleagues20 recently demonstrated that functional defects associated with synaptic degeneration in ankyrin2 mutant

**Figure 7. Induction of PHP alleviates synaptic neurodegeneration**

(A) Representative traces of mEJCs and EJCs.
(B) Quantification of mEJC amplitudes show a significant decrease in GluRIIA and GluRIIA:Madm double-mutant animals. Postsynaptic overexpression of TOR in Madm mutants did not alter mEJC amplitudes.
(C) Quantification of EJC amplitudes demonstrated a significant decrease to identical levels in Madm and GluRIIA; Madm double mutants despite the observed decrease in mEJCs in the double mutants. Postsynaptic overexpression of TOR in Madm mutants enhanced EJC amplitudes compared with Madm mutants.
(D) Quantification of QC shows a significant increase in GluRIIA mutants in GluRIIA:Madm double-mutant animals and a decrease in Madm mutants. Interaction between GluRIIA and Madm double mutants compared with controls. This decrease in QC can be significantly rescued in GluRIIA; Madm double mutants or by postsynaptic overexpression of TOR.
(E) mEJC, EJC amplitudes, and QC normalized to controls to highlight the rescue of QC (see also Figures 4E–4M, S5, and S7). Mean ± SEM. ANOVA and Dunn’s multiple comparison tests; n = 17, 13, 12, 14, and 16 cells, ≥ 8 animals per genotype.
(F–I) Analysis of synaptic degeneration. A partial rescue of synapse degeneration was observed on induction of PHP by loss of Dysb in muscles of ankyr2 mutants. The same data are plotted along a unitary line; each data point represents the QC of a single animal before (x axis) and after (y axis) benzamil application (K). The data are plotted along a unitary line; each data point represents the QC of a single animal before (x axis) and after benzamil application (K).

**Mean ± SEM. Two-way ANOVA; n = 9 and 9 animals. Larvae were reared at 29°C (J–M). **p ≤ 0.001, *p ≤ 0.01, *p ≤ 0.05 and ns (non-significant) p ≥ 0.05. See also Figures S11A–S11F.**
animals are partially offset by an endogenous induction of homeostatic compensation that is sensitive to the ENaC inhibitor benzamil. This compensatory mechanism explains the maintenance of normal postsynaptic excitation that we previously observed at NMJs with mild synaptic degeneration phenotypes in the absence of presynaptic Ankyrin2 or α-Spectrin.1,13

Our analysis of Madm complements these studies and provides novel insights into neurodegeneration-specific PHP. First, we show that synaptic strength deficits of Madm mutants can be efficiently restored by ectopic induction of PHP as observed in an ALS model of degeneration.31 We now demonstrate that this neuroprotective role of PHP is not limited to electrophysiological parameters but is also sufficient to alleviate structural synaptic degeneration phenotypes. This observation is consistent with the observed neuroprotective role of PHP in a mouse model of ALS.32 Furthermore, analogous to Orr and colleagues,12 we now demonstrate that presynaptic degeneration caused by the knockdown of a synapse stability-promoting factor (Madm) induces an endogenous PHP mechanism that compensates deficits in synaptic strength. With postsynaptic Madm, we now identify a first molecular factor that is specifically required for neurodegeneration-induced PHP, but not essential for other forms of PHP. Importantly, also in ank2 mutant animals, postsynaptic Madm is, at least partially, required for the induction of a compensatory presynaptic increase in synaptic release. Because these data thus far rely on a pharmacological block of the presynaptic ENaC channel, a treatment that induces significant variability in synaptic release, further studies will be necessary to determine whether Madm participates in all cases of neurodegeneration-induced potentiation.

The unique requirements of Madm for this process raise the question how the NMJ might sense ongoing synaptic degeneration and disassembly that requires a compensatory increase in presynaptic release. Recent studies ruled out that alterations in calcium or ion influx through GluRs can represent the sensor for PHP.25,65,66 Instead, it has been proposed that conformational changes of receptor complexes may induce different forms of PHP.65 Interestingly, at degenerating NMJs, the density of GluR clusters significantly increases at sites lacking the presynaptic active zone marker Brp (see, e.g., Figures 1D, 1E, 5J, 7F, S1F, S7A, and S7C). We previously observed identical increases in GluR cluster density at synaptic degenerations in ank2, neuroglian, casein kinase2, and hts/adducin mutant animals.7,14,16 It is thus conceivable that conformational changes in GluR cluster density or structure that are caused by synaptic degeneration represent a unique signal to induce a compensatory mechanism to offset potential decreases in neurotransmitter detection.

Limitations of the study
In our study, we provide evidence that Madm controls synapse maintenance and neurodegeneration-induced PHP by regulating the ratio between inactive and active Thor/4E-BP. As a pseudo-kinase, Madm is unable to directly phosphorylate target proteins, and it therefore remains unclear how Madm controls these processes. Our genetic data indicate that Madm may recruit TOR for these processes, but the molecular and biochemical mechanisms remain unresolved. In addition, although we clearly implicate Madm as a specific factor for neurodegeneration-induced PHP, the general relevance of Madm for this form of PHP and the cellular mechanisms underlying neurodegeneration-specific PHP induction remain unknown.

STAR Methods
Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111710.

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AUTHOR CONTRIBUTIONS
Z.M., K.A., and J.P. conceptualized the study and designed the experiments. Z.M., K.A., D.A.L., and I.D.K. performed the biochemistry experiments. Z.M., K.A., and J.P. conceptualized the study and designed the experiments. Z.M., and L.M.L. performed and analyzed the electrophysiology experiments. Z.M., K.A., and J.P. wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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### STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse anti-BRp      | DSHB   | nc82 RRID:AB_2314866 |
| Mouse anti-Synapsin | DSHB   | 3C11 RRID:AB_528479  |
| Rabbit anti-dGluRIIC| (Pielage et al.) | N/A |
| Rabbit anti-Dlg     | (Pielage et al.) | N/A |
| Guinea pig anti-Dlg | (Pielage et al.) | N/A |
| Rabbit anti-GFP     | Invitrogen | Cat# A-6455 |
| Mouse anti-GFP      | Invitrogen | Cat# A-11120 |
| Rabbit anti-Madm    | This study | N/A |
| Mouse anti-tubulin  | DSHB   | E7 RRID:AB_2315513  |
| Alexa Fluor® 647 AffiniPure Goat Anti-HRP | Jackson ImmunoResearch Laboratories, Inc | Cat# 123-605-021 |
| CyTM3 AffiniPure Goat Anti-HRP | Jackson ImmunoResearch Laboratories, Inc | Cat# 123-165-021 |
| Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 | Invitrogen | Cat# A32723TR |
| Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 | Invitrogen | Cat# A-11011 |
| Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 | Invitrogen | Cat# A-11008 |
| Rabbit anti-pS6K (T398) | Cell Signaling | Cat# 9209S |
| Rabbit anti-p4EBP 1 (T37/46) | Cell Signaling | Cat# 2855S |
| Rabbit anti-np4EBP (T46) | Cell Signaling | Cat# 4923S |
| Goat anti-Rabbit HRP-linked IgG | Cell Signaling | Cat# 7074S |
| Goat anti-Mouse HRP-linked IgG | Cell Signaling | Cat# 7076S |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Benzamid            | Sigma-Aldrich | Cat# B2417 |
| Bouin' s fixative   | Sigma-Aldrich | Cat# HT10132 |
| Rapamycin           | LC Laboratories | Cat# R-5000 |
| ProLong® Gold Antifade Mountant | Thermofisher Scientific | Cat# P36934 |
| SuperSignal® West Pico PLUS Chemiluminescent Substrate | Thermo Scientific | Cat# 34577 |
| Protease inhibitor cocktail (PIC), MinicompLete | Roche | Cat# 11836153001 |
| Phosphatase inhibitor cocktail (PIC) | APExBiO | Cat# K1012 |
| WesternBright® Sirius® chemiluminescent sunstrate | Advansta | Cat# K12043C20 |
| pENTR™/D-TOPO™ Cloning Kit, with One Shot™ TOP10 Chemically Competent E. coli | Invitrogen | Cat# K240020 |
| His Spin-Trap       | GE Healthcare | Cat# GE28-4013-53 |
| NuPAGE™ 4 to 12%, Bis-Tris, 1.0 mm, Mini Protein Gel | Invitrogen | Cat# NP0322BOX |
| X-ray film          | Fujifilm | N/A |
| **Experimental models: Organisms/strains** |        |            |
| w1118               | Bloomington Drosophila Stock Center | BDSC# 5905 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| elavC155-Gal4 (nG1); P[GawB]elav[C155] | Bloomington Drosophila Stock Center | BDSC# 458 FlyBase: FBti0002575 |
| Oki371-Gal4 (nG2); w[1118]; P[w/+;w hs] = GawB| Bloomington Drosophila Stock Center | BDSC# 26160 FlyBase: FBti00079687 |
| da-Gal4; w; P[w/+;w hs] = GAL4-daG32UH1, Sb[1]/TM6B, Tb[1] | Bloomington Drosophila Stock Center | BDSC# 55851 FlyBase: FBti0013991 |
| BG57-Gal4 (mG2); w; P[w/+;w hs] = UAS-wah, HA)7; P[w/+;w hs] = GawB| Bloomington Drosophila Stock Center | BDSC# 32556 FlyBase: FBti0010275 |
| UAS-dicer 2; w[1118]; P[w/+;w hs] = UAS-Dcr-2.2)D2 | Bloomington Drosophila Stock Center | BDSC# 54650 FlyBase: FBti0011335 |
| UAS-Madm | This study | N/A |
| Madm^{TOPReEx2783}; w[1118]; Df(3R)Exel7283/TM6B, Tb[1] | Bloomington Drosophila Stock Center | BDSC# 7952 FlyBase: FBti00038297 |
| Madm^{EP3137}; w[1118]; P[w/+mC] = EP|Madm[EP3137]/TM6B, Tb[1] | Bloomington Drosophila Stock Center | BDSC# 17093 FlyBase: FBti0011335 |
| Madm^{542}; (Gluderer et al.) | N/A |
| Madm^{543}; UAS-GFP Madm | (Gluderer et al.) | N/A |
| Thor^2; y[1] w течение; Thor^2 | Bloomington Drosophila Stock Center | BDSC# 9559 FlyBase: FBti0117673 |
| GluRIIB-Gal4 (mG1); y[1] w течение; Mi[Trojan-GAL4.2]GluRIIB[M03631-TG4.2]; Dr[1]/TM63, Sb[1] | Bloomington Drosophila Stock Center | BDSC# 60333 FlyBase: FBti0168143 |
| UAS Thor; w течение; P[w/+mC] = UAS-Thor.wt| Bloomington Drosophila Stock Center | BDSC# 9147 FlyBase: FBti0064612 |
| GluRIIA[SP16]; w течение; GluRIIA[SP16]/In(2LR)Gla, wg[GLA-1] PP O 1[Tb] | Bloomington Drosophila Stock Center | BDSC# 64202 FlyBase: FBti0085982 |
| UAS TOR-myc; w течение; P[w/+mC] = UAS-Tor.myc2 | Bloomington Drosophila Stock Center | BDSC# 53727 FlyBase: FBti0156877 |
| dysbe00128; w[1118]; PBac[w/+mC] = RB/Dysb[e01028]/TM6B, Tb[1] | Bloomington Drosophila Stock Center | BDSC# 17918 FlyBase: FBti00041464 |
| brp^{59}; (Kittel et al.) | FlyBase: FBti0194770 |
| Madm RNAi; w[1118]; P[(GD7155) v27347] | Vienna Drosophila RNAi Center | VD# v27347 FlyBase: FBti0121864 |
| Thor RNAi; y течение sc^v течение sev^v течение \text{[P]}(y+7.7) \text{v+[t1]}.8 = TRIP,HMS01555/attP40 | (Kim et al.)^{70}, (Toshniwal et al.)^{71} Bloomington Drosophila Stock Center | BDSC# 36667 FlyBase: FBti0146678 |
| Tor RNAi; y течение sc^v течение sev^v течение \text{[P]}(TRIP,HMS01114)attP2 | Bloomington Drosophila Stock Center | BDSC# 34639 FlyBase: FBti0140808 |
| ankyrin^{2001}; w[1118]; PBac[w/+mC] = WHJ/Ank2[Y02001]CG32373[R02001]/TM6B, Tb[1] | (Pielage et al.)^{13} | BDSC# 18502 FlyBase: FBti0181704 |

**Oligonucleotides**

| Madm CDS forward primer | 5'-CACCATGTCAAATAGCCAAGCG AATG-3' | N/A |
| Madm CDS Reverse primer | 5'-TCAATGCTGCTGTCGTGCCC-3' | N/A |

**Recombinant DNA**

| pUASSatB-10xUAS destination vector | Invitrogen | Cat# 11803012 |
| Gateway pDEST17 vector | Invitrogen | |

**Software and algorithms**

| Fiji/ImageJ | https://imagej.nih.gov/ij/ | RRID: SCR_002285 |
| Imaris (Bitplane 7.0) | http://www.bitplane.com/imaris/imaris | RRID: SCR_007370 |
| Clc genome work bench 7.0 | Qiagen | https://digitalinsights.qiagen.com/ |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for reagents should be directed to and will be fulfilled by the lead contact, Jan Pielage (pielage@bio.uni-kl.de).

Materials availability
The reagents generated in this study are available upon request and will be shared by lead contact.

Data and code availability
- All datasets reported in this work are available with lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly stocks
Flies were raised on standard fly food at room temperature. All genetic crosses were performed at 25°C unless otherwise mentioned in figure legends. w^{1118} was always crossed to parental lines and served as a control. The details of the fly strains used can be found in the resources table.

METHOD DETAILS

Generation of Madm transgenes and antibody
The full length Madm ORF was amplified from cDNA LD28657 obtained from the Drosophila Genomic Research Center (Indiana, USA) using the following primers: 5'-CACCATGCTAAATAGCAGCAGATG-3' and 5'-TCAATTGCTCCTAGGTGCCC-3'. The ORF was cloned into pENTR using TOPO cloning (Invitrogen) and exchanged into the pUASattB-10xUAS destination vectors with and without N-terminal EGFP tag. Constructs were verified via sequencing and injected into the attP40 genomic landing site (BestGene Inc, California, USA).

The N-terminal 6xHis-tagged full-length Madm construct was generated using Gateway cloning (pDEST17 vector) (Invitrogen). The protein was expressed, and further purification was performed under denaturing conditions using Spin-Trap Columns following standard procedures (GE Healthcare). Polyclonal rabbit anti-Madm antibodies were generated and purified by Davids Biotechnologie (Regensburg, Germany). Pre-sera of rabbits were checked for reactivity before immunization.

Western blot
Larval brains were dissected in pre-chilled HL3 buffer containing protease inhibitor (mini-complete, Roche), lysed in NP40-based lysis buffer, transferred into 2X sample buffer (Invitrogen) and boiled for 10 min at 95°C. Proteins were separated on 8–12% NuPage gels (Invitrogen). Subsequently, protein was transferred to Invitrolon™ PVDF membranes (Invitrogen) following standard procedures. Membranes were blocked in 5% skim milk solution in TBST containing 0.2% of Triton X-100 at room temperature for 1 h followed by incubation with primary antibodies rabbit anti-Madm (1:500) or mouse anti-Tubulin (loading control; 1:1,000; Developmental Studies
Hybridoma Bank) at 4°C overnight. Antibodies were diluted in blocking solution. Secondary HRP-conjugated anti-rabbit or anti-mouse antibodies (Jackson Immunoresearch) were applied at 1:10,000 for 2 h at room temperature. Protein was detected by chemiluminescence using ECL substrate (SuperSignal West Pico Kit, Thermo Scientific) and the fluorescence signal was developed on an X-ray film (Fujifilm).

For detection of phosphorylated proteins, larvae were dissected in cold HL3 containing protease inhibitor cocktail (mini-complete, Roche) and phosphatase inhibitor cocktail (APEXBio) on ice. Samples were lysed in NP40 buffer containing protease inhibitor and phosphatase inhibitor cocktail, transferred to 2x sample buffer and processed at 75°C for 10 min. After electrophoresis proteins were transferred onto Invitronotr™ PVDF membranes (Invitrogen). Membranes were incubated with primary antibodies (rabbit p4E-BP (T37/46), 1:1.000, np4E-BP (T46), 1:1.000, pS6K (T398), 1:1.000; Cell Signaling) or mouse anti-tubulin (1:1000; Developmental Studies Hybridoma Bank) at 4°C overnight. HRP-conjugated secondary anti-rabbit or anti-mouse antibodies (Cell Signaling) were applied at 1:3.000 dilutions for 2 h at room temperature. Protein was detected by chemiluminescence using ECL substrate (Western bright Sirius, Advansta) and Odyssey® XF Imager (Li-cor).

Immunohistochemistry

Wandering third-instar larvae were dissected in pre-chilled standard HL3 saline. Preparations were fixed for 5 min with Bouin’s fixative (Sigma-Aldrich) and washed in PBST containing 0.1% of Triton X-100. Larval preparations were incubated with primary antibodies overnight at 4°C and with secondary antibodies for 2 h at room temperature. The following antibodies were used: anti-Brp (nc82) 1:250, anti-Synapsin (3c11) 1:50 (Developmental Studies Hybridoma Bank, Iowa), rabbit anti-dGluRIC 1:3,000,14 rabbit anti-GFP 1:2,000 (#A-6455, Invitrogen), rabbit anti-DLG 1:10,000,14 Guinea pig anti DLG 1:700,14 and mouse anti-GFP 1:500 (#A-11120, Invitrogen). Alexa Fluor 488/568 conjugated secondary antibodies were used at 1:1,000 dilution (Invitrogen). Alexa Fluor 647 or Cy3 conjugated anti-HRP antibodies were used at 1:500 and 1:1,000 dilutions respectively (Jackson Immunoresearch laboratories). Images were acquired using a LSM700 (Zeiss) confocal microscope with 40x/1.3 NA and 63x/1.4 NA oil immersion objectives or a Leica Stellaris 8 confocal microscope using a 20x/0.75 NA air objective.

Images for quantifications were acquired under identical settings. Imaris (Bitplane), Fiji (ImageJ) and Photoshop (Adobe) were used for linear image processing and analysis.

Rapamycin treatment

Third-instar larvae were treated with rapamycin as described previously. Briefly, rapamycin was prepared as a stock solution in absolute ethanol. The final concentration in food was 200 μM for rapamycin and 1% for ethanol. After thoroughly mixing the rapamycin with freshly prepared fly food, the vials were left at room temperature for 24 h to allow evaporation of the ethanol. Blue food coloring dye was added to the rapamycin food to ensure that only fed larvae were used for experiments. Fly food containing 1% ethanol was used as control. Larvae were allowed to feed for 24 h at 25°C before dissection and immunohistochemical analysis.

Quantification of phenotypes

For the quantification of synaptic degeneration, postsynaptic footprints (representing synaptic retraction events) of NMJs were analyzed in animals stained for the presynaptic active zone marker Brp and the postsynaptic receptor marker GluRIC. NMJs with one or more boutons retracted were considered as an affected NMJs. The frequency of synaptic relocations represents the percentage of NMJs with relocations per animal. The severity of synaptic degeneration was classified based on the number of destabilized boutons as follows: 1–2 synaptic boutons, 3–6 synaptic boutons, ≥7 synaptic boutons or total elimination, scored 1, 2 and 3 respectively. Quantifications were performed on muscles 1/9 and 2/10 of segments A3-A6.

Morphology quantifications were performed on larval NMJs stained with presynaptic Synapsin, HRP and postsynaptic Discs-large. Quantifications were performed on muscle 1 of segments A3 and A4. The number of boutons and retractions were quantified using a TCS SPE DM5500Q (Leica) microscope with 40X/0.75 NA air objective.

Quantification of Western blots was done on equally treated independent biological replicates using the “Gel Analysis method” of Fiji (ImageJ). Briefly, the density of proteins was measured and normalized to the genetic control running on the same blot. These data were normalized to the loading control (tubulin).

Electrophysiological recordings

Two electrode voltage-clamp recordings were performed on third-instar larvae on muscle 6 in abdominal segments A3 as described previously. Briefly, larvae were dissected in pre-chilled Ca2+-free HL3 (70mM NaCl, 5mM KCl, 10mM MgCl2, 10mM NaHCO3, 115mM sucrose, 5mM trehalose, 5mM HEPES) which was replaced by HL3 containing 0.45 mM Ca2+ for voltage-clamp recordings. Sharp intracellular glass electrodes with 15–20 MΩ resistance filled with 3 M KCl were used for recordings. Muscles were clamped at –70 mV by injecting current for both mEJC and EJC recordings. mEJCs were collected for 60 s and for EJCs, segmental nerves of respective muscles were stimulated with suprathreshold pulses delivered by isolated pulse stimulator (A-M systems) for at least 15 APs at 1 Hz. Only the recordings with input muscle resistance of ≥3 MΩ and holding current of ≤5 nA were considered for quantifications. All recordings were amplified and acquired with the help of an AxoClamp 900A (Molecular Devices) and digitized by Digidata 1550b (Molecular Devices).
Recordings were analyzed for mEJCs and EJCs with the help of Clampfit 10.7 (Molecular devices). mEJCs recorded for 60 s were averaged for each muscle cell. A minimum of 15 APs were recorded and averaged for each muscle cell. QC was quantified by dividing average EJC amplitude with average mEJC amplitude for each individual cell.

Single electrode current clamps were performed for benzamil (Sigma) experiments as described previously. Briefly, benzamil was prepared as a stock solution in DMSO. The final working concentration was 50 μmol for benzamil and 0.01% for DMSO. Both pre- and post-benzamil recording buffers were supplemented with equal concentration of DMSO. Preparations were washed at least 12 times after benzamil treatment. Only recordings with a muscle resistance ≥3 MΩ and a resting membrane potential ≤−60 mV were considered for analysis. Muscle health was tested by measuring muscle resistance at each step before recording. In all cases mEJCs were recorded for 60 s and for EJCs 30 APs at the rate of 1 Hz were recorded.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analyses were performed using GraphPad 9 (Prism). All data were analyzed for normal distribution by D’Agostino-Pearson omnibus and Shapiro–Wilk normality tests. One-way, two-way ANOVA and Student t-test were performed accordingly, to compare the phenotypes between different genotypes with relevant posthoc corrections as indicated in the figure legends and Table S1. Significance levels were defined as following: ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05 and ns (non-significant) p ≥ 0.05.