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Membrane and synaptic defects leading to neurodegeneration in *Adar* mutant *Drosophila* are rescued by increased autophagy.

Anzer Khan[#1,4], Simona Paro[#2], Leeanne McGurk[#2], Nagraj Sambrani[1], Marion C. Hogg[2], James Brindle[2], Giuseppa Pennetta[3], Liam P. Keegan[1,2]* and Mary A. O'Connell[1,2]*

[1]CEITEC Masaryk University, Kamenice 735/5, A35, Brno, CZ 62500, Czech Republic. [2]MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine at the University of Edinburgh, Crewe Road, Edinburgh EH4 2XU, UK. [3]Centre for Integrative Physiology, Euan MacDonald Centre for Motor Neurone Disease Research, Hugh Robson Building, University of Edinburgh, George Square, Edinburgh, EH8 9XD UK. [4] National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic

[#] Joint first authors

* Corresponding authors

Telephone number ++ 420 54949 5460

Email addresses: Liam.Keegan@ceitec.muni.cz, mary.oconnell@ceitec.muni.cz

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Background

In fly brains the *Drosophila* Adar (adenosine deaminase acting on RNA) enzyme edits hundreds of transcripts to generate edited isoforms of encoded proteins. Nearly all editing events are absent or less efficient in larvae but increase at metamorphosis; the larger number and higher levels of editing suggest editing is most required when the brain is most complex, which is consistent with the fact that *Adar* mutations affect the adult brain most dramatically. However, it is unknown whether *Drosophila* Adar RNA editing events mediate some coherent physiological effect. To address this question, we performed a genetic screen for suppressors of *Adar* mutant defects. $Adar^{5G1}$ null mutant flies are partially viable, severely locomotion defective, aberrantly accumulate axonal neurotransmitter pre-synaptic vesicles and associated proteins and develop an age-dependent vacuolar brain neurodegeneration.

Results

The genetic screen revealed suppression of all $Adar^{5G1}$ mutant phenotypes tested by reduced dosage of the *Tor* gene, which encodes a pro-growth kinase that increases translation and reduces autophagy in well-fed conditions. Suppression of $Adar^{5G1}$ phenotypes is due to increased autophagy; overexpression of *Atg5,* which increases canonical autophagy initiation, reduces aberrant accumulation of synaptic vesicle proteins and suppresses all *Adar* mutant phenotypes tested. Endosomal microautophagy (eMI) is another Tor-inhibited autophagy pathway involved in synaptic homeostasis in *Drosophila*. Increased expression of the key eMI protein Hsc70-4 also reduces aberrant accumulation of synaptic vesicle proteins and suppresses all $Adar^{5G1}$ mutant phenotypes tested.

Conclusions

1 These findings link *Drosophila Adar* mutant synaptic and neurotransmission defects to
2 more general cellular defects in autophagy; presumably, edited isoforms of CNS
3 proteins are required for optimum synaptic response capabilities in the brain during the
4 behaviourally complex adult life stage..
5
6
7 **Background**

8 *Drosophila melanogaster* has a single *Adar* (adenosine deaminase acting on
9 RNA) gene encoding an orthologue of the vertebrate ADAR2 RNA editing enzyme [1].
10 In both vertebrates and *Drosophila* ADAR RNA editing in CNS transcripts is targeted
11 to pre-mRNA exons that form RNA duplexes with flanking intron sequences. Editing
12 events are frequently located in coding regions, leading to the generation of alternative
13 edited and unedited isoforms of CNS proteins (for review [2]). ADAR2 in mammals is
14 required for editing a glutamine codon to arginine at the *Gria2 Q/R* site in the transcript
15 encoding a key glutamate receptor subunit [3]. This editing event regulates the calcium
16 permeability of AMPA class glutamate receptors and loss of this editing event leads to
17 seizures and neuronal cell death. Thus, mice lacking *Adar2* die within 3 weeks of birth
18 however, *Adar2; Gria2*^{R} transgenic mice with the chromosomal *Gria2* gene mutated
19 to encode arginine are normal indicating that *Gria2 Q/R* is the key editing site in
20 vertebrates [4]. The number of edited transcripts and edited sites is very much greater
21 in *Drosophila* than in vertebrates. Editing sites recognition is conserved; uman ADAR2
22 expressed in *Drosophila* rescues *Adar*^{5G1} null mutant phenotypes [5] and correctly edits
23 hundreds of *Drosophila* transcripts encoding ion channels subunits and other CNS
24 proteins [6-10].

Our hypothesis is that during the evolutionary increase in site-specific RNA editing events in advanced insects, there has been selection for editing events that allow production of alternative edited and unedited isoforms of CNS proteins [11]; edited isoforms are also more abundant in adult brains than in larval brains in *Drosophila*.RNA editing has also been evolutionarily expanded in cephalopod molluscs [12], consistent with the idea that more RNA editing may be able to enhance some brain function(s). Recent results reveal the complexity of RNA editing in *Drosophila* neurons, showing that different neuronal populations have distinct editing signatures [13]. The extreme opposite hypothesis to ours, that editing events are evolutionary accidents, appears less likely since many editing events are well conserved within insects or cephalopods respectively and are under positive selection during evolution [14]. However, it is still possible that the many different editing events serve many different and unconnected purposes. We set out to define the key effects of *Drosophila* Adar RNA editing by identifying genetic suppressors of *Adar* null mutant phenotypes and determining the mechanisms of action of these suppressors.

Adar expression increases strongly at pupation and the number of edited sites and editing efficiencies at most sites are higher after metamorphosis in the brain of the adult fly [6, 15]. In *Drosophila*, transcripts with high and conserved editing include *paralytic (para)* [16], *shaker*, *shaker b* and *cacophony (cac)* [17] transcripts which encode the pore-forming subunits of axonal voltage-gated sodium, potassium or calcium channels respectively. At the axon terminus presynaptic active zones are formed above *cacophony* channels clustered in the presynaptic membrane; in the active zones neurotransmitter synaptic vesicles are tethered for rapid neurotransmitter release followed by rapid endocytosis to recycle and refill the vesicles, [18]. The *cacophony* channel triggers calcium entry into presynaptic boutons when it is activated in response

to an action potential [19]. Other transcripts that are edited, especially in the adult brain, such as *Synapsin* [20], *Synaptotagmin 1*, *Endophilin A*, *Munc* [21], encode key proteins involved in the formation and function of neurotransmitter synaptic vesicles.

The *Drosophila Adar^{5G1}* null mutant fly shows reduced viability, lack of locomotion, ataxia and to age related neurodegeneration [6]. In larval motorneurons targeted *Adar* RNAi knockdown, leads to increased motorneuron excitability; reciprocally, *Adar* overexpression in motorneurons leads to reduced neuronal excitability [22]. *Adar^{5G1}* mutant larval neuromuscular junctions have defects in calcium-regulated synaptic transmission and increased numbers of boutons [23] with increased numbers of synaptic vesicles and increased levels of the pre-synaptic proteins Synapsin [20], Endophilin A, Synaptotagmin 1 and others [24]. A much weaker hypomorphic *Adar^{hyp}* mutant that has a nearly normal capacity for locomotion when stimulated, exhibits an aberrantly increased sleep pressure associated with the inability to achieve a normal sleep-mediated reduction of pre-synaptic vesicles and associated proteins and synaptic signaling [25]. This defective locomotion due to persistent halting in the hypomorphic *Drosophila Adar^{hyp}* mutant is similar to what we observe in the more severely affected *Adar^{5G1}* null mutant. In the *Adar^{hyp}* adult brain the sleep defect is due to neuronal excesses of neurotransmitter synaptic vesicles held in a reserve pool that is not readily releasable and difficult to deplete, and the level of presynaptic proteins is elevated, consistent with defects in axonal active zones in brain neurons similar to those observed at larval neuromuscular junctions [25].

To elucidate whether *Adar* null mutant phenotypes have a coherent underlying basis, we performed a pilot genetic screen on Chromosome II for suppressors of the *Adar^{5G1}* null mutant reduced viability. We find that reduced dosage of *Tor* (*Target of rapamycin*) is a potent suppressor of *Adar* mutant phenotypes. Tor is a kinase is

essential for several cellular processes including increased translation and reduced autophagy under well-fed conditions (for review [26, 27]). Electron microscopic analysis reveals that neurodegeneration in *Adar*^{5G1} mutant fly retina is associated with abnormal, large, intracellular membrane-bounded vacuoles. These vacuoles appear to contain cellular components and are likely to result from aberrant activity of the endosome/autophagy/lysosome system. Tor protein levels are increased in the *Adar*^{5G1} mutant and reducing *Tor* gene dosage suppresses these defects by increasing autophagy and clearing excess pre-synaptic proteins. There is no extensive cell death in the *Adar*-mutant CNS. The findings are consistent with the hypothesis that *Drosophila Adar* function has an evolutionarily selected biological role related to synaptic plasticity and CNS protection.

Results

Reduced *Tor* gene dosage suppresses *Adar* mutant reduced viability, open field locomotion defects and reduced longevity

To elucidate which mechanisms mediate *Adar* mutant phenotypes we performed a pilot screen for heterozygous deletions that increase the number of adult male *Adar*^{5G1} flies eclosing from pupae in crosses , (*Adar* is on Chr. X and males have one gene copy). When virgin female *y,Adar*^{5G1}*,w /FM7, Bar* flies are crossed with male *w*^{1118} and male progeny that eclose from pupae are counted, the ratio of male *y,Adar*^{5G1}*,w* to male *FM7 Bar* progeny obtained is only about twenty percent (see *w*^{1118} control cross at the bottom of Figure S1). This reduced viability at eclosion from the pupa reflects the death of *Adar*^{5G1} mutants during embryonic, larval and pupal stages. Therefore, virgin female *y,Adar*^{5G1}*,w /FM7, Bar* flies are crossed with male *w, Df(2)/SM5 Cy*, suppression of this

Adar^{5G1} reduced viability measured by the proportion of live *Adar*^{5G1}*; Df(2)/+* mutant flies eclosing from pupae can be used for a genome-wide screen of deficiencies.

We performed a trial screen of thirty five *DrosDel* deficiencies [28] covering seventy percent of the left arm of chromosome II for deficiencies that when heterozygous act as suppressors of the reduced viability of male *Adar*^{5G1}mutant flies (Figure S1). *DrosDel* deficiencies are a series of genetically engineered deficiencies covering most of the *Drosophila* euchromatin that each delete about thirty genes on average [28]. The most robustly rescuing deficiency identified by the screen, *Df(2L)ED778*, substantially increases (to 80%), and the partially overlapping *Df(2L)ED784* deficiency somewhat increases, *Adar*^{5G1} mutant viability. The viability of *Adar*^{5G1} is increased by eight deficiencies and decreased by others. The level of suppression differ greatly between deficiencies, with many giving slight suppression that makes the results noisy and not ideal for a larger genome-wide screen. As we obtained a robust result from two deficiencies in this pilot screen, we decided to study these further.

We tested mutations in individual genes within the rescuing *Df(2L)ED778* deficiency and the partially overlapping *Df(2L)ED784* deficiency, and within some other partially rescuing deficiencies, for rescue of *Adar* mutant viability. *DrosDel* deletions are excellent for rapid genome coverage in genetic screens but, for unknown reasons, inability to map effects of deletions down to reduced copy numbers of single genes within the deletions is very common. In this case, single gene mutations in the *Tor* gene, but not mutations in other genes within the deleted regions were found to increase viability (Figure. 1A) and open field locomotion (Figure 1B) [29, 30] in in *Adar*^{5G1}*;Tor*^{k17004} / + and *Adar*^{5G1}*;Tor*^{MB07988} / + flies; lifespanalso appears to be increased (Figure 1C), (we are unable to perform the appropriate Kolmogorov–Smirnov

1 test for statistical significance with our small sample size in 3 replicates). These *Tor*
2 mutants are homozygous lethal P-element insertions at different positions in *Tor* that
3 are presumed null mutants.

4 Open field locomotion was measured by recording crossing of individual flies
5 over lines in a gridded Petri dish (three 2 min. measurements on each of 10 or more
6 flies for each line) as previously described [17]. In this assay, even wildtype flies may
7 stop moving for part of the 2-minute measurement period. However, the *Adar* mutant
8 flies tend to stop within a few tens of seconds and to not move again thereafter. The
9 $Adar^{5G1}$ mutant flies, also show leg tremors and difficulty in walking straight without
10 stumbling, (Supplementary Video V1 show $Adar^{5G1}$ mutant walking defects and
11 Supplementary Video V2 shows rescue in $Adar^{5G1}$; $Tor^{MB07988}$ / +).

12 Reduced *Tor* gene dosage may directly correct an aberrantly increased activity
13 of Tor in $Adar^{5G1}$. Immunoblot analysis of $Adar^{5G1}$ mutant total head protein extracts
14 show that Tor protein is present at a significantly increased level in $Adar^{5G1}$ (Figure 1
15 D). Increased Tor protein is likely to lead to increased levels of activated Tor but
16 unfortunately, there is no available antibody to detect specifically the active,
17 phosphorylated form of *Drosophila* Tor.

18

19 **Reduced *Tor* gene dosage also suppresses *Adar* mutant age-dependent**
20 **neurodegeneration**

21 The $Adar^{5G1}$ null mutant neurodegeneration has been described previously [5,
22 6, 8, 31]. The *Drosophila* ADAR protein is normally present in nuclei of all brain
23 neurons in wildtype and is entirely absent in the $Adar^{5G1}$ null mutant, that deletes the
24 entire *Adar* transcribed region [6]. Neurodegeneration develops more quickly in certain
25 brain regions. In brains of 23 day and 30 day $Adar^{5G1}$ mutant flies the calyces of the

1 mushroom bodies (MB) and the retina (Figure 2C, D, S2), show filled vacuoles not
2 observed in 23 day *w*^{1118} flies (Figure 2A, B). Within the retina neurodegeneration is
3 evident at 23 days as a narrowing of photoreceptors with separations appearing between
4 ommatidia (Figure 2D, S2). Heterozygous *Tor* mutations suppress the *Adar* mutant
5 neurodegeneration in retina and mushroom body neuropil in *Adar*^{5G1}*;Tor*^{k17004} / +
6 (Figure 2E, F) and *Adar*^{5G1}*;Tor* ^{MB07988} / + (Figure 2G, H). Neurodegeneration in the
7 *Adar*^{5G1} null mutant is 100% penetrant and is never observed in the brain of wildtype
8 flies. We do not quantitate the number of the vacuoles as their size variation is too large,
9 instead we state whether it occurs or not.
10 Prominent vacuoles in the brain appear particularly in the mushroom body (MB)
11 calyces. The mushroom body calyces are neuropil regions composed of olfactory
12 projection neuron axons and dendrites of mushroom body Kenyon cells; the cell bodies
13 of the Kenyon cells are located above the calyces and their nuclei stain darkly with
14 haematoxylin. Vacuoles may develop within the large boutons at the pre-synaptic
15 termini of olfactory projection neurons which extend axons from the olfactory lobes
16 beneath the brain reach to the mushroom body calyces [32]. Large round boutons at
17 the ends of projection neuron axons are surrounded by many fine Kenyon cell dendrites.
18 Both olfactory projection neurons and Kenyon cells have now been shown to be
19 cholinergic [33], consistent with our earlier observations that *Adar*^{5G1}*; ChAT>Adar 3/4*
20 flies expressing active ADAR under *choline acetyltransferase ChAT-GAL4* driver
21 control in cholinergic neurons [34] show rescue of vacuolization in MB calyces and
22 retinas of 30 day *Adar*^{5G1} brains [1, 17, 35].
23
24 **The *Adar* mutant neurodegeneration involves aberrant membrane processes and**
25 **formation of large brain vacuoles**

1 What is the defect underlying the *Adar*^{5G1} mutant neurodegeneration that is
2 strongly suppressed by reduced *Tor* dosage? To examine the *Adar*^{5G1} mutant
3 neurodegeneration at higher resolution, we performed an electron microscopic analysis
4 of retinas and optic laminae of aged *Adar*^{5G1} mutant flies. Transmission electron
5 microscope (TEM) sections parallel to the surface of the eye are particularly suitable
6 for study because these sections show a highly regular pattern of photoreceptors and
7 support cells within the repeating ommatidia (Figure 3A, B). TEM images of sections
8 through the retina of 25 day old *Adar*^{5G1} show large membrane-bounded vacuoles
9 between or within support cells that surround the photoreceptors (R1-R7/8) (Figure 3C,
10 arrows). Other defects in *Adar*^{5G1} resemble those seen with autophagy mutants, such as
11 autophagic-like vesicles (Figure 3D, E, F), multilamellar vesicles (Figure 3G, H), and
12 membrane-bounded vesicles budding from the rhabdomeres of photoreceptors in more
13 advanced stages of degeneration (Figure 3I-L).
14 This data suggests that the *Adar* mutant neurodegeneration does not involve
15 death of neurons in the first instance, but it does reflect development and enlargement
16 of aberrant intracellular vacuoles like those observed in lysosomal storages diseases
17 that cause defects in autophagy. It is likely that the aberrant vacuoles between
18 ommatidia develop within the retinal pigment cells that import red and brown pigment
19 precursors from the hemolymph and process and store them in membrane-bounded
20 pigment granules that are a type of lysosome-related organelle. We did not obtain TEM
21 sections through mushroom body calyces but sections through the optic lamina where
22 the cellular arrangements are more difficult to interpret in EM also show aberrant
23 multilamellar vesicles and membrane overgrowth
24 Aberrant intracellular membrane processes typify the *Adar* mutant
25 neurodegeneration, which does not appear to involve extensive neuronal death. TUNEL

1 assays did not detect neuronal death in the *Adar*^{5G1} mutant brain (Figure S3 A- D), and
2 few Lysotracker-positive nuclei are seen in brain (Figure S3 B), although cell death
3 does occur outside the brain in head fat cells (Figure S3 A-D). *Adar*^{5G1}*;ChAT>p35* flies
4 expressing the viral anti-apoptotic protein p35, which inhibits most *Drosophila*
5 caspases [36, 37] still show vacuolization in the MB calyces and retina at 30 days
6 (Figure S2 E, F), indicating that vacuolization is not prevented by blocking apoptosis.
7
8 **Suppression of *Adar* mutant phenotypes by reduced *Tor* or by increased**
9 **expression of Atg5**
10 We next focused on understanding the mechanism of suppression of *Adar*
11 mutant phenotypes by reduced *Tor* gene dosage. *Tor* is a key gene controlling growth
12 and autophagy [27]; suppression of *Adar* mutant phenotypes by reduced *Tor* gene
13 dosage may be due to decreased translation or to increased autophagy in the *Adar*^{5G1}*;*
14 *Tor* / + flies.
15 Tor is a protein kinase that, when active, increases translation by
16 phosphorylation of the ribosomal protein S6 kinase (S6K) protein that increases its
17 activity and by phosphorylation of the eIF 4E BP translation inhibitor that reduces its
18 inhibitory activity [38, 39]. Reduced *Tor* gene dosage should reduce translation in the
19 *Adar*^{5G1}*; Tor/*+ double mutants. However, mimicking translation-decreasing effects of
20 reduced *Tor* gene dosage by decreasing S6 kinase activity in cholinergic neurons in
21 *Adar*^{5G1}*; ChAT>S6K*^{KQ} flies expressing a dominant negative S6K [40], or *Adar*^{5G1}*;*
22 *ChAT>Thor* flies with increased expression of translation-inhibiting eIF 4E-BP (*Thor*),
23 did not show suppression of *Adar*^{5G1} mutant open-field locomotion (Figure 4A). This
24 indicates that reduced translation is not the primary mechanism by which reduced *Tor*
25 suppresses the *Adar* mutant phenotypes.

Since suppression of the *Adar* mutant phenotypes by reduced *Tor* does not appear to be due to reduced translation, the suppression may instead be due to increases in some type of autophagy. Increased autophagy could be consistent with the clearing of the large vacuoles in aged *Adar* mutant brains and retinas by reduced *Tor* dosage. Activated Tor suppresses autophagy by phosphorylating Atg1, the key protein for activation of canonical autophagy. Increased expression of key autophagy proteins is able to increase canonical autophagy [27]; *Adar*^{5G1}*; ChAT>Atg5* flies [41], show increased viability and rescue of *Adar*^{5G1} mutant locomotion defects (Figure 4A), and neurodegeneration (Figure 4B, C). Therefore, suppression of *Adar*^{5G1} mutant phenotypes appears to be due to increased autophagy caused by the reduced *Tor* gene dosage.

Tor is activated by growth-promoting extracellular signals such as insulin as well as by intracellular signals; Tor locates to the surface of the lysosome and is activated there by amino acids being returned from the lysosome to the cytoplasm. The insulin receptor acts through PI3 kinase (PI3K) and the serine-threonine protein kinase AKT to phosphorylate the Tuberous Sclerosis Complex (TSC), releasing it from the Rheb (Ras homolog enriched in brain) protein in the lysosomal Tor protein complex and activating Tor [42]. If reduced *Tor* gene dosage suppresses *Adar* mutant phenotypes because it reduces effects of growth-promoting signals such as insulin then the effect of reduced *Tor* gene dosage should be mimicked by increasing TSC protein dosage. Surprisingly, *Adar*^{5G1}*; ChAT>TSC1*, *TSC2* (Figure 4A), with reduced signaling to Tor through the insulin pathway do not show strong rescue of *Adar*^{5G1}mutant locomotion defects. This suggests that any aberrant axonal growth signal in the *Adar*^{5G1}mutant is not due to alteration in an upstream signal through the insulin receptor, nor through the anaplastic lymphoma kinase that may substitute for insulin receptor in the brain, that

1 also signals through PI3K [43] to the Tor complex 1 (TORC1). If suppression of the
2 *Adar* mutant phenotype by reduced Tor is not due to changed responsiveness to external
3 signals such as insulin, then it may be due to an intracellular effect. Since Tor is
4 activated on lysosomes there may be an aberrant intracellular feedback from autophagy
5 that leads to increased Tor.

6 To determine whether increased autophagy may be rescuing *Adar* mutant
7 defects by clearing aberrant accumulations of synaptic vesicles, we measured levels of
8 the presynaptic protein Synaptotagmin1 that is associated with the synaptic vesicles in
9 heads of *Adar*^{5G1} mutant and rescued flies by immunoblotting. Immunoblotting of head
10 protein extracts with anti-Synaptotagmin 1 antibodies demonstrate that there is an
11 aberrant accumulation of Synaptotagmin 1 in *Adar*^{5G1}mutant heads [25] (Figure 4D)
12 that is lowered by reduced *Tor* or by increased *Atg5* expression.

13

14 **Increased autophagic vesicles but incomplete clearance of ref(2)p in the *Adar*^{5G1}**
15 **mutant**

16 To assess canonical autophagy in the *Adar*^{5G1} mutant and rescues, we examined
17 levels of ref(2)p protein. ref(2)p is the *Drosophila* orthologue of the mammalian p62
18 canonical autophagy adapter protein (also called Sequestosome1), that brings
19 ubiquitinated cargo to canonical autophagosomes; p62 is degraded in the process and
20 p62 accumulates when canonical autophagy is defective [44]. If canonical autophagy is
21 operating normally in *Adar*^{5G1} and increased in heads of *Adar*^{5G1}*; Tor* ^{k17004} /+ double
22 mutant or *Adar*^{5G1}*; ChAT>Atg5* flies then levels of p62 protein should be normal in
23 *Adar*^{5G1} and reduced in the double mutants [45]. However, p62 protein levels are
24 twofold higher than normal in *Adar*^{5G1} head protein extracts and increase further in the
25 double mutants, (Figure 4E), in particular with increased Atg5. This suggests that

1 canonical autophagy mighr not benot functioning perfectly in the *Adar*^{5G1} mutant
2 background, even though it partially clears excess synaptic vesicles proteins (see
3 below).

4 Larval fat cells are used to study autophagy in *Drosophila*, as these cells are
5 much larger than brain neurons and form a single sheet of cells in which autophagy is
6 readily induced by starvation of the larvae and detected by staining the lysosomes in
7 live cells with acidic Lysotracker dye. Staining larval fat cells from well-fed larvae of
8 the *Adar*^{5G1} mutant with Lysotracker dye shows the presence of increased numbers of
9 lysosomes in the *Adar*^{5G1} mutant, even in the absence of starvation (Figure 5E,F)
10 relative to equivalent wildtype *w*^{1118} cells (Figure 5B,C). Starvation increases the
11 number of lysosomes further in the *Adar*^{5G1} mutant cells (data not shown). Expression
12 of *ADAR 3/4* (Figure 5H, I), protein in *Adar*^{5G1} mutant fat cells under the control of the
13 CollagenIV-GAL4 (*CgIV-GAL4*) driver is sufficient to eliminate the elevated basal
14 autophagy in the *Adar*^{5G1} mutant, as indicated by the loss of Lysotracker vesicle
15 staining.
16
17 **Rescue of *Adar* mutant phenotypes by increased expression of the endosomal**
18 **microautophagy (eMI) protein Hsc70-4**
19 Recent studies have shown that a different type of starvation-inducible, Tor-
20 inhibited autophagy called endosomal microautophagy (eMI), occurs in *Drosophila*
21 neurons and is especially important in presynaptic active zones [46-49]. To test whether
22 increased eMI rescues *Adar*^{5G1} mutant phenotypes we used the *ChAT-GAL4* and *Act*
23 *5C-GAL4* drivers to increase expression of the Hsc70-4 protein by directing expression
24 of *UAS-Hsc70-4*. Increasing Hsc70-4 in cholinergic neurons increases locomotion
25 (Figure 6A); on the other hand, knocking down of *Hsc70-4* in cholinergic neurons does

1 not improve the *Adar*^{5G1} mutant phenotype (Figure 6A). When acting as a chaperone
2 for neurotransmitter synaptic vesicles Hsc70-4 acts together with an interacting partner
3 protein called Small glutamine-rich tetratricopeptide repeat protein (Sgt), as an ATP-
4 driven molecular chaperone protein. In eMI Hsp70-4 acts without Sgt to recruit
5 KFERQ-motif proteins to endosomes [46]. The Sgt protein favors the more general
6 chaperone role of Hsc70-4 in synaptic vesicle cycling and suppresses its function in
7 eMI. Therefore, we also increased eMI with an *UAS-Sgt RNAi* construct to decrease
8 expression of Sgt specifically in cholinergic neurons and this also dramatically
9 suppressed the *Adar*^{5G1} mutant locomotion defect (Figure. 5A); knockdown of Sgt with
10 the ubiquitous *Act 5C-GAL4* driver is lethal. Increased eMI in the *Adar*^{5G1} mutant
11 background also suppresses neurodegeneration. Overexpression of Hsc70-4 (Figure 6,
12 B, C) or knocking down *Sgt* (Figure 6, D, E) in *Adar*^{5G1} with *ChAT-GAL4* suppresses
13 the *Adar*^{5G1} mutant neurodegeneration in retina and mushroom body
14 Immunoblotting of head protein extracts with anti-Synaptotagmin 1 antibodies
15 demonstrates that the aberrant accumulation of Synaptotagmin 1 in *Adar*^{5G1}mutant
16 heads (Figure 6F) is dramatically reduced by increased Hsc70-4 expression. We
17 conclude that increased eMI suppresses the *Adar*^{5G1} mutant phenotypes. The reduction
18 of Synaptotagmin 1 to below wildtype levels is surprising but synaptic vesicle-
19 associated proteins are normally present at levels that probably reflect retention of a
20 reserve of older protein molecules in association with the no-longer readily releasable
21 reserve pool of synaptic vesicles [50-52]. We also see a less dramatic decrease in the
22 level of Synaptotagmin 1 when reducing the level of Tor or overexpressing Atg5 in the
23 *Adar*^{5G1} mutant background (Figure 4D)ncreased Atg5 is likely to be lowering
24 synaptotagmin 1 through increased canonical autophagy and is unlikely to be acting
25 within the eMI pathway as Atg5 has been reported to not be required for eMI [47].

1 We also examined the level of ref(2)p when overexpressing Hsc70-4 or
2 knocking down Sgt in *Adar*^{5G1} (Figure 6G). We did not observe any significant
3 difference in ref(2)p levels between head extracts of *Adar*^{5G1} mutant, *Adar*^{5G1}*; ChAT*
4 *> Hsc70-4* or *Adar*^{5G1}*; ChAT > Sgt RNAi* flies. This suggests that, as expected,
5 increased Hsc70-4 does not increase canonical autophagy or significantly change levels
6 of ref(2)p.
7 Since increasing eMI suppresses the *Adar*^{5G1} mutant phenotypes, it is possible
8 that eMI might be insufficient in *Adar*^{5G1}. To investigate this, we determined the level
9 of Hsc70-4 protein by immunoblotting head protein extracts (Figure 6H) and by
10 measuring its expression by qPCR (Figure 6I). By both methods, we observe a small
11 but significant decrease in Hsc70-4 level in *Adar*^{5G1}.
12
13 **Discussion**
14 RNA editing by Adar is required to maintain the integrity of the CNS in adult
15 *Drosophila* [6]. To find suppressors of the *Adar*^{5G1} null mutant phenotype, we
16 performed an initial screen for genetic suppressors that increase the viability of *Adar*^{5G1}
17 and discovered a key role for Tor-regulated autophagy in all *Adar* mutant phenotypes
18 (Fig. 1 A-C, Fig. 2, E-H). Tor protein is abnormally increased in *Adar*^{5G1} mutant heads
19 (Fig. 1 D); therefore, suppression of *Adar* mutant defects by reduced *Tor* gene dosage
20 is, at least in part, a true rescue i.e. reducing Tor directly corrects a defect in the *Adar*
21 mutant rather than simply activating some entirely unrelated bypass pathway.
22 Consistent with an autophagy defect, the *Adar*^{5G1} mutant neurodegeneration
23 shows resemblances to neurodegenerations in *Drosophila* models of lysosomal storage
24 diseases, a class of neurodegenerations in which lysosomes accumulate different
25 intracellular components [53]. The most distinctive abnormal intracellular components

1 in the vacuoles in the *Adar*^{5G1} mutant eyes and brains (Fig. 2, A-F), apart from double
2 membrane autophagosomes (Fig. 3 F) are the multilamellar membrane whorls (Fig.
3 3H). These have been identified in cell bodies in other *Drosophila* mutants such as
4 *eggroll* [54], *swiss cheese* [55-57], and *benchwarmer/spinster* [58] and are
5 characteristic of the human neurodegenerative Tay-Sachs disease [53, 59]. The
6 formation of large vacuoles in *Adar* mutant mushroom body calyces might be directly
7 related to accumulation of large numbers of neurotransmitter-containing presynaptic
8 vesicles and associated presynaptic proteins such as Synaptotagmin 1 in the brain [25],
9 which is prevented by reduced *Tor* gene dosage or by increased *Atg5* (Fig. 4D) or
10 increased *Hsc70-4* (Fig. 6E) expression to increase autophagy.

11 Which type of Tor-regulated autophagy is involved in the suppression of *Adar*
12 mutant phenotypes? Canonical autophagy (CA) is be still sufficiently functional to
13 mediate rescue of *Adar*^{5G1} mutant phenotypes (Fig. 4A-D), even though it may also be
14 somewhat impaired in *Adar*^{5G1}. Immunoblots show that ref(2)p protein, the *Drosophila*
15 homolog of the vertebrate p62 adapter for canonical autophagy of ubiquitinated
16 proteins, is increased in *Adar*^{5G1} and increased much more with reduced Tor or
17 increased Atg5 (Fig. 4E). *Adar*^{5G1} larval fat cells also show increased Lysotracker-
18 positive acidic autophagosomal and lysosomal vesicles (Fig. 5 E,F). This impeded CA
19 in *Adar*^{5G1} might arise because some proteins that have edited isoforms, play important
20 roles in CA. [60]Transcripts of *cacophony (cac)* and *straightjacket (stj)* encode subunits
21 of the pre-synaptic voltage-gated calcium channel that is also required for fusion of
22 lysosomes with autophagosomes and endosomes. Loss of function mutations of *cac* or
23 *stj* impair neurotransmission and lysosome function in neurons, leading to some
24 accumulation of p62 protein [61], although it is not known whether loss of only the
25 edited isoforms of these proteins is sufficient to cause any similar defect. Other edited

transcripts encoding CA-associated proteins include *Atg14*, *Atg17, AMPKalpha*, and *Foxo* (Table S1); all of these, in addition to probable involvement of edited synaptic-vesicles associated proteins in membrane fusion events in CA [61], suggest that both CA and synaptic vesicle are among processes affected by proteins encoded by edited transcripts in CNS. An additional possible explanation for why ref(2)p clearance is impeded in *Adar*^{5G1} is that CA is affected by Dicer-2-mediated aberrant innate antiviral immune induction that occurs in *Adar*^{5G1}-mutant heads (Deng et al., 2020, Nat. Comms, in press), which is likely to result from accumulated unedited intracellular dsRNA in *Adar*^{5G1} , paralleling the mouse *Adar1* mutant interferon induction through antiviral dsRNA sensors [63-65] [64]. In mammalian cells innate immune induction impedes CA in by diverting p62 from its role as the receptor for ubiquitinated proteins in CA to instead form a cytoplasmic innate immune signaling platform; p62 and other CA substrates now accumulate because they are less efficiently turned over by CA [60]. This cross-regulation of p62 by innate immune signaling helps to redirect CA to innate immune defense and it is likely that a similar effect also acts on ref(2)p in *Drosophila*; this could in part account for the *Adar*^{5G1} mutant ref(2p protein accumulation.

The increased ref(2)p in the *Adar* mutant may also lead to the increased Tor activation. In vertebrates the p62 protein associates with TORC1 on the cytosolic surface of the lysosome; increased p62 contributes to increased Tor activation by intracellular amino acids returning from the lysosome [66]. Aberrant Tor activation through this cell-autonomous pathway in *Drosophila* [67] might explain why we could not mimic the *Tor/+* rescue of *Adar* mutant phenotypes by genetic manipulations that interfere with extracellular hormone and growth-related signaling to TORC1 e.g. by increased expression of the TSC1 and TSC2 proteins that repress Tor in the growth signaling pathways (Fig. 4A).

Endosomal microautophagy (eMI) has recently been described as an important new autophagy pathway involved in proteostasis at presynaptic active zones in *Drosophila* [46, 47]. *Drosophila* eMI targets proteins containing KFERQ motifs to endosomes using the homologous KFERQ-recognition protein (Hsc70-4 in *Drosophila*, HSPA8 in humans) that is also used in lysosomal chaperone-mediated autophagy (CMA) in vertebrates. *Drosophila* is believed to lack CMA as it does not have a homolog of the alternatively spliced isoform of lysosomal LAMP2A protein required to recruit HSPA8 to lysosomes [46, 47]. Increased expression of the key Hsc70-4 protein or decreased Sgt increase eMI and rescue *Adar* mutant locomotion defects (Fig. 6A), neurodegeneration (Fig. 6B-D) and elevated Synaptotagmin 1 levels in *Adar* mutant heads (Fig. 6F), without affecting ref(2)p levels (Fig. 6G). Immunoblots for Hsc70-4 indicate that this protein is at a lower level in *Adar* mutant heads (Fig. 6H,I); this suggests that eMI may be insufficient or suppressed by increased Tor in the *Adar* mutant. Similar to the p62 adapter during CA, the Hsc70-4 cargo selector is believed to also be turned over as KFERQ target proteins are recruited and destroyed during eMI It is not known how activated Tor suppresses eMI; it has been proposed that Atg1 is also involved [47]; possibly the reduced Hsc70-4 observed in *Adar*^{5G1} is part of the mechanism of eMI suppression by increased Tor..

Since rescue of *Adar* mutant locomotion defects by expression of Adar requires expression of the catalytically active Adar protein, we expected that RNA editing of some target transcript might be essential to rescue locomotion [1]. For instance, editing of the transcript encoding *Synaptotagmin 1* might be required because this leads to production of an edited isoform with a different residue close to those that determine the calcium responsiveness of synaptic vesicle exocytosis, potentially affecting the calcium-dependence of the synaptic vesicle cycle [24]. Or editing of the transcript

1 encoding *Synapsin* might be required because this changes an important residue that is
2 phosphorylated by cAMP-dependent protein kinase A (PKA); edited synapsin may
3 limit aberrant synaptic vesicle accumulation and clustering [20, 25]. Therefore, rescue
4 of locomotion defects by reduced Tor or increased autophagy without restoring editing
5 of any target transcript is surprising.
6
7 **Conclusion**Altering flows of membranes and proteins through Tor-regulated
8 autophagy processes is surprisingly sufficient to overcome *Drosophila Adar* mutant
9 synaptic synaptic defects, locomotion defects and age-dependent neurodegeneration,
10 presumably by rejuvenating synaptic vesicle pools (hese *Adar* mutant defects are
11 summarized in Figure 7). This suggests that that controlling such flows is also a major
12 biological role of Adar RNA editing in *Drosophila*.Can we therefore propose an overall
13 coherent role of ADAR2-type RNA editing in CNS of vertebrates and invertebrates?
14 The independent evolutionary expansions of ADAR2-type RNA editing events in
15 transcripts encoding CNS proteins in advanced insect groups and in cephalopods
16 suggests involvement in brain function and more complex cognition, behavior and life-
17 cycles. In vertebrates, the homologous ADAR2 is a cycling protein that mediates
18 circadian effects [68]; ADAR2 editing also mediates a type of homeostatic postsynaptic
19 plasticity through regulated editing of transcripts encoding glutamate receptor subunits
20 [69, 70] and the seizures that develop in *Adar2* mutant mouse pups also involve
21 widespread effects of aberrant synaptic plasticity [71]. It is likely that *Drosophila* Adar
22 is also involved in circadian rhythms [72], and *Drosophila* Adar is also involved in
23 synaptic plasticity during sleep [25]. Aberrantly increased sleep arises because the
24 increased reserve pools of presynaptic neurotransmitter synaptic vesicles cannot be
25 reduced normally during sleep. The role of Adar we outline here acts to protect the brain

through effects on synaptic plasticity. Adar RNA editing may be involved in circadian changes in synaptic plasticity and may even mediate beneficial effects of sleep on the brain.

Methods

Fly maintenance and fly strains

All fly stocks were raised on standard corn meal-agar medium. Fly stocks were maintained at 18°C and crosses were performed at 25°C. Flies used in aging experiments were maintained in tubes not supplemented with additional yeast, to prevent flies from becoming stuck to the yeast. A single fly was maintained in a vial and each vial was tipped-on daily. The wild-type control strains were either w^{1118}. The GAL4 driver lines and balancer lines were obtained from the Bloomington Stock Centre. Detailed genotypes of individual strains used are as follows;

Tor^{k17004}: *y[1] w[67c23]; P{w[+mC]=lacW}Tor[k17004]/CyO,*

$Tor^{MB07988}$: *w[1118]; Mi{ET1}Tor[MB07988]*

$S6K^{KQ}$ (dominant negative): *w[1118]; P{w[+mC]=UAS-S6k.KQ}2*

Thor: w[*]; P{w[+mC]=UAS-Thor.wt}2

Atg6: y; UAS-Atg6-2D; Sb/Tm6b (from U. Pandey)

Atg5: y[1] w[1118]; wg[Sp-1]/CyO; P{w[+mC]=UAS-eGFP-drAtg5}16

Atg1[6A]: y,w,hsflp;; UAS-Atg1[6A], (from T. Neufeld)

Atg1[GS10797](EP line): y,w,hsflp; Atg1[GS10797], (from T. Neufeld)

TSC1, TSC2: y,w,hsFlp; UAS-TSC1, UAS-TSC2, (from T. Neufeld)

UAS-Hsc70-4: w[126]; P{w[+mC]=UAS-Hsc70-4.K71S}G

BDSC #28709 - y^1 v^1*; P{TRiP.JF03136}attP2 (Hsc70-4 RNAi)*

1 *BDSC #61267 - y^1 v^1; P{TRiP.HMJ23046}attP40 (sgt RNAi)*

2

3 The GAL4 binary system was used to express transgenes in the *Adar* mutant
4 background. The *$Adar^{5G1}$* mutant strain was combined with *ChAT>-GAL4* and virgin
5 females of these strains were crossed to males of the transgenic lines bearing the
6 *Drosophila UAS-cDNA* constructs. Female genotype is *y, $Adar^{5G1}$, w / w, FM7, Bar;*
7 *(ChAT-GAL4.7.4)19B,(UASGFP.S65T)T2 / (ChATGAL4.7.4)19B,(UASGFP.S65T)T2*.

8

9 ***DrosDel* screen for suppressors of reduced viability in the *$Adar^{5G1}$* mutant.**
10 To screen for suppressors of *$Adar^{5G1}$* mutant reduced viability we crossed virgin female
11 *y, $Adar^{5G1}$, w /FM7, Bar* in groups of five with males from the *DrosDel / SM5 Cy* lines.
12 Taking male non-*Curly* progeny, we counted the *$Adar^{5G1}$; DrosDel* / + and *FM7 Bar;*
13 *DrosDel /* + flies that eclosed from pupae and determined the ratio of male *y, $Adar^{5G1}$,*
14 *w; Df /* + to sibling male *FM7; Df* / +progeny for each deficiency. *DrosDel* deficiencies
15 are marked with mini-w^+. Tests of *Tor* mutants were performed in the same way.

16

17 **Open field locomotion assays**.
18 Open field locomotion was measured by recording crossing of individual flies over lines
19 in a gridded Petri dish (three 2 min. measurements on each of 10 or more flies for each
20 line) as previously described [17]. The data are presented as the average number of lines
21 crossed by a fly in the 2 min. period. The flies are collected on the day of eclosion from
22 the pupae. Next morning, when effects of CO_2 anesthesia have worn off, they are
23 individually introduced to the measuring dish and the measuring period begins after
24 tapping the dish once on the bench. The test measures the flies maximized movement
25 response to an initial stimulation and to a new environment.

1

Histology techniques

For standard haematoxylin-eosin stained sections *Drosophila* heads were fixed at room temperature in Carnoy's fixative for 4 hours. For detecting cell death, the terminal deoxynucleotidyl transferase Biotin-dUTP nick end-labelling (TUNEL) kit from Roche was used. *Drosophila* heads were fixed for 4 hours at room temperature in 4% paraformaldehyde. The heads were embedded into paraffin wax with standard histology procedures. Sections were cut at 6μm and either stained with haematoxylin and eosin for pathological analysis or stained for cell death according to the TUNEL kit instructions. Images were captured using a compound microscope, which comprised of a Coolsnap HQ CCD camera (Photometrics Ltd, Tucson, AZ) with Plan-neofluar objectives (Carl Zeiss, Welwyn Garden City, UK). Images were captured with neofluar objectives at 40X (with a numerical aperture of 1.3) for eyes and at 63X and 40X (with a numerical aperture of 1.25) for mushroom bodies. Color additive filters (Andover Corporation, Salem, NH) installed in a motorized filter wheel (Ludl Electronic Products, Hawthorne, NY) were used sequentially to collect red, green and blue images, which were then superimposed to form a color image. Image capture and analysis were performed with in-house scripts written for IPLab Spectrum (Scanalytics Corp, Fairfax, VA). The brightness and contrast were altered with the advanced histogram section in either IP Lab Spectrum or Adobe Photoshop. This was done by manually setting the minimum and maximum pixel intensities on the histogram. If necessary, the gamma was altered on the histogram. The images shown are representative examples from samples of 10-20 heads sectioned for each age and genotype.

24

Electron microscopy

The *Adar*^{5G1} mutants and w^{1118} controls were aged to 25 days or longer from parallel collections. The proboscis was removed in Schneider's insect media and the heads were fixed for at least one hour in 2.5% glutaraldehyde and subsequently fixed in 1% osmium tetroxide in Sorenson's buffer. The heads were dehydrated and embedded into resin. Survey sections of 0.5μm were cut through the frontal brain and ultra-thin sections were cut at the regions of interest. The sections were stained with 2% aqueous uranyl acetate for 15 minutes and lead citrate (supplied by Leica) for five minutes. The tissue sections were viewed with a Philips CM 100 Compustage (FEI) transmission electron microscope and digital images are collected with an AMT CCD camera (Deben). The brightness and contrast were altered manually with the advanced histogram section in either IP Lab Spectrum or Adobe Photoshop by setting the minimum and maximum pixel intensities on the histogram. If necessary, the gamma was altered on the histogram.

Immunoblotting

Male flies (minimum 15 flies) of the desired genotype were collected and aged for 2 days and then homogenized in NB Buffer (150mM NaCl, 50mM Tris-HCl pH 7.5, 2mM EDTA, 0.1% NP-40). Protein concentration was determined with Pierce BCA Protein Assay Kit. An equal amount of protein was loaded in each lane of a Tris-Glycine Gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA, incubated with primary antibody overnight. The next day it the membrane was incubated with secondary antibody and developed with Pierce ECL Western Blotting Substrate. Anti-Ref2P(Antibody Registry ID: AB_2570151 (1:1000) was a gift from Tor Erik Rusten (University of Oslo), anti-synaptotagmin (1:500) (Developmental Studies Hybridoma Bank, DSHB Hybridoma Product 3H2 2D7, Antibody Registry ID:

AB_528483), anti-Hsc70-4 (1;1000) was a gift from Konrad Zinsmaier (Bronk et.al, Neuron 2001) , anti-Tor antibody (Antibody Registry ID: AB_2568971) (1:1000) was a gift from Gábor Juhász, anti-Tublin (Developmental Studies Hybridoma Bank, DSHB Hybridoma Product 12G10 , Antibody Registry ID: AB_1157911) (1:5000). Imaging was performed with ChemiDoc™ XRS+ System and signal intensity was quantified with Image J software and statistical analyses were done with the t-test.

qPCR

RNA from approximately 20 fly heads was isolated with Tripure and cDNA generated with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). qPCR reactions were performed with The LightCycler® 480 SYBR Green I Master mix and the primers are listed in Supplementary Table 2 were used to measure expression levels. Expression levels were normalized to those of RP49 and t-tests were used for statistical analysis.

Lysotracker staining of larval fat cells.

Drosophila larvae were collected and brains and fat body dissected in cold PBS. The tissue of interest was incubated with LysoTracker® Red DND-99, Molecular Probes, Invitrogen (1 µl of dye in 10ml of cold PBS) for 5 minutes in ice. After five 2-minute washes in PBS, the tissue was mounted in Vectashield DAPI and viewed with a fluorescent microscope.

Statistical Analyses

Two sample data were analyzed by Student's *t*-test. A *p*-value of <0.05 was considered statistically significant. More than three groups, *p*-values were calculated by a one-way ANOVA followed by Tukey's test. The significance of differences between variables

was described based on *p*-values *: p-value < 0.05. **: p-value < 0.005. ***: p-value < 0.001 and n.s (not significant). Error bars: SEM (Standard Error of Mean for biological replicates).

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in the supplementary Data and Materials files.

Author contributions

All authors read and approved the final manuscript.

Anzer Khan performed immunoblots, qPCR and other assays.

Simona Paro performed *Drosophila* screen.

Leeanne McGurk performed TEM and microscopy.

Nagraj Sambrani generated some *Drosophila* strains.

Marion C. Hogg performed microcopy.

James Brindle assisted with experiments.

Giuseppa Pennetta designed experiments.

Liam P. Keegan designed experiments and wrote the manuscript.

Mary A. O'Connell designed experiments and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Conflict of interest

This work does not involve any conflict of interest

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25

26 **Figure legends**

27 **Figure 1: Reduced *Tor* gene dosage rescues *Adar*^{5G1} mutant phenotypes.** *Tor*
28 mutations increase (A) viability at eclosion from the pupae, n = 3 (B) open field
29 locomotion, n > 8 and (C) lifespan in *Adar*^{5G1}mutant flies. *FM7* is a first chromosome
30 balancer strain.n = 3 (D) Immunoblot with antibody to *Drosophila* Tor protein of
31 *Adar*^{5G1}mutant and wildtype (*w*^{1118}) fly head protein extracts. n = 3 Quantitation of
32 immunoblot data shows increased level of Tor in *Adar*^{5G1}. *p*-values in (A and B) were
33 calculated by a one-way ANOVA followed by Tukey's test. The significance of
34 differences between variables was described based on *p*-values *: p-value < 0.05. **:

1 p-value < 0.005. ***: p-value < 0.001 and n.s (not significant). Error bars: SEM
2 (Standard Error of Mean for biological replicates). p-value in D were calculated by
3 Student ‘s t-test. Source data values are included in the additional file 6.
4
5 **Figure 2. Rescue of *Adar*^{5G1} mutant neurodegeneration by reduced *Tor* gene
6 dosage.** Images show representative 6 microns thick haematoxylin and eosin stained
7 sections through mushroom body calyces (left panels, (63X)) and retinas (right panels,
8 40X) of (A, B) 23-day *w*^{1118}, (C, D) 23-day *Adar*^{5G1}; *ChAT-GAL4 / +,* (E, F), 25 day
9 *Adar*^{5G1}; *Tor*^{K170048} / + and (G, H) 23 day *Adar*^{5G1}; *Tor*^{MB07988} / +. Scale bars: 20µm.
10
11 **Figure 3: EM analysis of retinal degeneration in the *Adar*^{5G1} mutant.** (A) The
12 ommatidia of *w*^{1118} at 25 days. Each ommatidium comprises seven photoreceptor cells
13 surrounded by and separated from neighboring ommatidia by thin pigment cells
14 containing red pigment granules. (B) An ommatidium of 25 days old *w*^{1118} at higher
15 resolution. The photoreceptor cells with light-detecting rhabdomeres (Rb) appear
16 normal. The R7/R8 photoreceptor is indicated. Organelles such as mitochondria are
17 identifiable (arrow). (C) Retina of the *Adar*^{5G1} mutant at 25 days showing pigment cells
18 with large vacuoles between ommatidia (arrows). (D) Higher resolution image of a
19 single ommatidium in 25 days old *Adar*^{5G1} with vacuole (V) between photoreceptor
20 cells of two ommatidia. (E) Magnification of area within the circle in (D). Interrupted
21 membrane (arrow) was observed inside the vacuole. (F) Magnification of area within
22 the square in (D). Membrane-bounded vesicles (arrows) in the photoreceptors contain
23 cellular components in an autophagosome-like structure surrounded by two or more
24 membrane layers. (G, H) Multilamellar membrane structures (arrows) in a
25 photoreceptor cell and within a glial cell close to the basement membrane between the

retina and the lamina in *Adar*^{5G1}. (I) Single membrane-bounded vesicles pinching off from the photoreceptor (arrows) in early stages of photoreceptor degeneration in *Adar*^{5G1}. (J) Larger multilamellar membrane structures budding off from the extracellular membrane of photoreceptor cells into the ommatidial cavity (arrows) at more advanced stages of degeneration in *Adar*^{5G1}. (K) Extensive loss of pigment cells separating ommatidia in advanced stages of neurodegeneration in *Adar*^{5G1}. Photoreceptor cell cytoplasm and extracellular membrane are abnormal and vesicles bud from the rhabdomeres (arrows). (L) Abnormal exocytosis from the rhabdomere in late stages. The extracellular membrane of the photoreceptor is not well defined.

Figure 4: Decreased Tor, or increased Atg5 to increase autophagy, suppress *Adar*^{5G1} mutant phenotypes. A. Rescue of *Adar*^{5G1} mutant open field locomotion defects in *Adar*^{5G1}; *Tor*^{K170048} / +, *Adar*^{5G1}; *Tor*^{MB07988} / +, *Adar*^{5G1}; *ChAT>Atg5* and *Adar*^{5G1}; *ChAT>Atg1* flies but not in *Adar*^{5G1}; *ChAT>Thor* or *Adar*^{5G1}; *ChAT>S6K*^{KD} or *Adar*^{5G1}; *ChAT>TSC1,TSC2* flies. n > 8. B. Representative images of MB calyx (63X) and (C) retina (40X) in 30-day *Adar*^{5G1}; *ChAT>Atg5*. Scale bars: 20µm. D Immunoblot with antibody to Synaptotagmin 1 of head protein extracts of *Adar*^{5G1}, *w*^{1118}, *Adar*^{5G1}; Tor^{K17004} / + and *Adar*^{5G1}; *ChAT > Atg5* flies. Quantitation of immunoblot data shows increased Synaptotagmin 1 in *Adar*^{5G1} is reduced by decreased *Tor* or by increased *Atg5*. n ≤ 3. E. Immunoblot with antibody to ref(2)p, the *Drosophila* p62 canonical autophagy protein, of head protein extracts of *w*^{1118}, *Adar*^{5G1}mutant, *Adar*^{5G1}; Tor^{K17004} / + and *Adar*^{5G1}; *ChAT > Atg5* flies. Quantitation of immunoblot data shows that increased ref(2)p, *Drosophila* p62 protein, in *Adar*^{5G1} is not reduced but increased by decreasing *Tor* or by increasing *Atg5*. n ≥ 3. *p*-values were calculated by a one-way ANOVA followed by Tukey's test.. The significance of differences between variables was described based on *p*-values *: p-value < 0.05. **:

p-value < 0.005. ***: p-value < 0.001 and n.s (not significant). Error bars: SEM (Standard Error of Mean for biological replicates). Source data values are included in the additional file 6.

Fig. 5. ADAR protein expression rescues the autophagy-related phenotype in *Adar*^{5G1} larval fat cells. The fat bodies of A-C: wild-type strain w^{1118}, D-F: *Adar*^{5G1}*;CgIV>*, G-I: *Adar*^{5G1}*;CgIV*>UAS-dAdar3/4, have been dissected and live-stained with DAPI (A,D,G) and Lysotracker (B,E,H) dyes (merges in C,F,I). Wild-type fat body does not show any Lysotracker staining (B and C). *Adar*^{5G1} mutant fat cells have an increased activation of autophagy as indicated by increased Lysotracker staining in lysosomes (E and F). Expression of the *UAS-dAdar3/4* transgene in the *Adar*^{5G1} mutant fat cells is sufficient to rescue the elevated basal autophagy (H and I). Scale bars: 50 µm.

Figure 6. Increased Hsc70-4 suppresses *Adar*^{5G1} mutant phenotypes. A. Rescue of *Adar*^{5G1} mutant open field locomotion defects in *Adar*^{5G1}*; ChAT>Hsc70-4* and *Adar*^{5G1}*; ChAT>Sgt RNAi* flies with increased endosomal microautophagy. $n \geq 10$. B. Representative images of MB calyx (40X) and (C) retina in 30-day *Adar*^{5G1}*; ChAT>Hsc70-4* (40X). (D) Representative images of MB calyx (40X) and (E) retina in 30-day *Adar*^{5G1}*; ChAT>SgtRNAi* (40X). F. Immunoblot detection of the presynaptic protein Synaptotagmin1 in *w*^{1118}, *Adar*^{5G1} mutant, *Adar*^{5G1}*; ChAT>Hsc70-4, Adar*^{5G1}*; ChAT>Sgt RNAi* and *Adar*^{5G1}*; Act5c>Hsc70-4* head protein extracts. Quantitation of the immunoblot data is shown below; levels of Synaptotagmin 1 compared to tubulin in each of the different head protein extracts. $n \leq 3$. G. Immunoblot to detect ref(2)p, the *Drosophila* p62 autophagy protein, in total head proteins of *Adar*^{5G1} mutant, *w*^{1118}

1 wildtype, *Adar*^{5G1}*; ChAT>Hsc70-4* and *Adar*^{5G1}*; ChAT>Sgt RNAi* flies. n ≤ 3. H.
2 Immunoblot to detect Hsc70-4 protein in total head protein extracts of w^{1118} wildtype,
3 *Adar*^{5G1} mutant, *Adar*^{5G1}*; ChAT>Hsc70-4* and *Adar*^{5G1}*; ChAT>Sgt RNAi* flies and
4 *Adar*^{5G1}*; Act5c>Hsc70-4*. n = 3. I. qPCR of *Hsc70-4* from w^{1118} wildtype and *Adar*^{5G1}
5 heads showing that *Hsc70-4* is significantly decreased in *Adar*^{5G1} heads. n = 6, *p*-values
6 in (A,E,G and H) were calculated by a one-way ANOVA followed by Tukey's test..
7 The significance of differences between variables was described based on *p*-values *:
8 p-value < 0.05. **: p-value < 0.005. ***: p-value < 0.001 and n.s (not significant). Error
9 bars: SEM (Standard Error of Mean for biological replicates). *p*-values in H were
10 calculated by Student 's *t*-test. Source data values are included in the additional file 6.
11
12 **Fig. 7 Summary of *Adar* mutant phenotypes.** In the *Adar* mutant, aberrantly
13 increased Tor leads to inadequate autophagy, reduced synaptic vesicle clearance and
14 neurodegeneration.
15
16
17 **Additional files**
18
19 **Additional file 1**
20 **Figure S1- Screen of *DrosDel* deletions on Chromosome 2L for rescue of *Adar*^{5G1}**
21 **viability.** Ratio of *Adar*^{5G1} to *FM7 Bar* genotypes among male progeny in the presence
22 of *DrosDel* deficiencies, or in their absence (w^{1118} cross at the bottom) (expressed as a
23 percentage). Progeny are obtained by crossing *Adar*^{5G1} */ FM7, Bar* virgin females to
24 males to w^{1118} males or males of *DrosDel/SM5, Cy* deficiency stocks.
25

Additional file 2

Figure S2- ***Adar*^{5G1} neurodegeneration at 30 days.**

Images of 6 micron thick haematoxylin and eosin stained sections through mushroom body calyces (left panels, (63X)) and retinas (right panels, 40X) of 30-day *Adar*^{5G1}.

Additional file 3

Figure S3-Neuronal cell death is not prominent in heads of 25-day-old *Adar*^{5G1} mutant flies. (A) TUNEL staining to detect apoptotic cells in head sections from 25-day-old *Adar*^{5G1} mutant flies stained with DAPI to detect nuclei. TUNEL-positive nuclei are not detected in neurons However TUNEL-positive nuclei are conspicuous in head fat bodies of 25-day-old *Adar*^{5G1} mutant flies (boxed area in A). (B) Magnification of area boxed in A (C) Haematoxylin and eosin stained section serial to A, white box indicates fat body tissue. (D) Magnification of area boxed in C. (E, F) Images show representative 6 micron thick haematoxylin and eosin stained sections through mushroom body calyces (left panels, (63X)) and retinas (right panels, 40X) of 30-day *Adar*^{5G1}; *ChAT>UAS-p35.* Scale bars: 20μ**m**

Additional file 4

Supplementary Table 1: List of Adar edited transcripts encoding proteins required for autophagy.

Additional file 5

Supplementary Table 2: Primers used for qPCR

Additional file 6

Excel sheet containing source data file for Figure 1A ,1B ,1C.1D ,4A,4D,4E,6A,6F,6G,6H and 6I.

1

2 **Additional file 7-** Video of *Adar*^{5G1} null mutant showing locomotion defect.

3 **Additional file 8** – Video of Adar^{5G1}; *Tor*^{K170048} Double mutant, which shows

4 locomotion defect is recued when Tor dosage is reduced in the *Adar* null mutant

5 background.