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**A neuroprotective role for microRNA *miR-1000*
mediated by limiting glutamate excitotoxicity**

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Summary

Evidence has begun to emerge for roles of microRNAs as regulators of synaptic signaling, specifically acting to control postsynaptic responsiveness during synaptic transmission. In this report, we provide evidence that *Drosophila miR-1000* acts presynaptically to regulate glutamate release at the synapse by controlling expression of the vesicular glutamate transporter (VGlut). Genetic deletion of *miR-1000* leads to elevated apoptosis in the brain as a result of glutamatergic excitotoxicity. The seed-similar *miR-137* regulates VGlut2 expression in mouse neurons. These conserved miRNAs share a neuroprotective function in the brains of flies and mice. *Drosophila miR-1000* shows activity-dependent expression, which might serve as a mechanism to allow neuronal activity to fine-tune the strength of excitatory synaptic transmission.

Introduction

microRNAs (miRNAs) have emerged in recent years as important regulators of homeostatic mechanisms. Changes in miRNA expression and activity have been linked to neurodegenerative disorders¹⁻⁴. A growing body of evidence suggests neuroprotective roles for miRNAs in the aging brain^{5,6 7-9}, as well as in the control of synaptic function and plasticity^{10,11}. Mouse *miR-134* acts postsynaptically to regulate synapse strength¹² and *miR-181* and *miR-223* regulate glutamate receptors, thereby affecting postsynaptic responsiveness to glutamate^{13,14}. *miR-1*, a muscle-specific microRNA, acts in a retrograde fashion at the neuromuscular junction to regulate the kinetics of synaptic vesicle exocytosis¹⁵. However, there are few examples of miRNAs acting directly in the presynaptic terminal to control synaptic strength. *miR-485*, which is found presynaptically, has been shown to control the expression of synaptic vesicle protein, SV2A, thereby affecting synapse density and GluR2 receptor levels¹⁶.

Here, we report that *Drosophila miR-1000* regulates neurotransmitter release from presynaptic terminals. *miR-1000* regulates expression of the vesicular glutamate transporter (VGlut) that loads glutamate into synaptic vesicles. Genetic ablation of *miR-1000* leads to glutamate excitotoxicity, resulting in early-onset neuronal death. Presynaptic regulation of *miR-1000* is activity dependent and could serve as a mechanism for tuning synaptic transmission. We present evidence that this regulatory relationship is conserved in the mammalian CNS, with the seed-similar miRNA, *miR-137*, conferring neuroprotection through regulation of VGlut2. The consequences of misregulation of glutamatergic signaling can be severe: excitotoxicity due to excessive glutamate release has been implicated in ischemia and traumatic brain injury, as well as in neurodegenerative conditions such as Parkinson's disease, Alzheimer's disease and ALS¹⁷⁻²¹.

Results

miR-1000 mutants show age progressive neurodegeneration

To examine the roles of miRNAs in neurodegenerative processes, we generated mutants in *Drosophila* miRNA genes by targeted homologous recombination. *miR-1000* was of interest because its expression is CNS-specific throughout development (**Fig. 1a**). *miR-1000* levels were high in newly eclosed adult flies and decreased by half during the first 2 weeks of adult life (**Fig. 1b**). Production of a targeted knockout allele of the *miR-1000* gene is described in **Supplementary Fig. 1** (referred to as KO1). We made a second independent mutant using a vector that allows retargeting of the locus by Recombinase Mediated Cassette Exchange²² (RMCE), and used RMCE to insert GFP and Gal4 reporters into the *miR-1000* locus, replacing the miRNA (**Fig. 1c**; KO2 refers to the RMCE allele with the mini-white cassette excised). We also used RMCE to replace the *miR-1000* hairpin in its endogenous context to create a rescued mutant allele (**Fig. 1c**, referred to as RMCE rescue). The RMCE rescue allele restored *miR-1000* expression to ~half of normal levels (**Fig. 1d**) and was used in subsequent experiments to test for genetic rescue of the mutant phenotypes. Three combinations of *miR-1000* mutant alleles were examined (*miR-1000*^{KO1}/*miR-1000*^{KO2}, *miR-1000*^{KO1}/Df(3R)Exel6203 and *miR-1000*^{KO2}/Df(3R)Exel6203). Mutants showed a modest reduction in survival during embryonic stages, but developed normally thereafter, when reared under conditions of low crowding to minimize competition-induced stress (**Supplementary Fig. 2**). The survival deficit was rescued by restoring miRNA expression using the RMCE rescue allele.

miR-1000 mutant flies showed sharply reduced adult lifespan, with survival declining rapidly by 2 weeks of age (**Fig. 2a**; $p < 0.0001$ comparing *miR-1000*^{KO1}/*miR-1000*^{KO2}, *miR-1000*^{KO1}/Df and *miR-1000*^{KO2}/Df with their respective controls; Kaplan-Meier analysis using the log rank test). Lifespan was significantly rescued by restoring *miR-1000* expression in the mutant background using the RMCE rescue allele ($p < 0.0001$ comparing RMCE rescue with the other mutant combinations). *miR-1000* mutants also showed an early-onset movement disorder. At 2 days of age, 50-60% of flies were impaired in their ability to perform in a climbing assay (**Fig. 2b**, ANOVA: $p < 0.0001$). Performance worsened by day 10, with ~80% of flies impaired. The climbing defect was rescued by restoring *miR-1000* expression using the RMCE rescue allele (**Fig. 2b**, $p < 0.0001$). Shortened adult lifespan and movement disorder are features characteristic of *Drosophila* models of neurodegeneration²³.

To ask if the defects in the *miR-1000* mutant resulted from neurodegeneration, we used an antibody to the activated form of Caspase 3 to examine *miR-1000* mutant brains for evidence of apoptotic cell death. Caspase 3-positive cells were observed in 2 day-old *miR-1000* mutant brains, but not in control brains (**Fig. 2c**,

d). By 10 days, the number of Caspase positive cells increased >10-fold in the mutant, but remained low in the control (**Fig. 2c, e**). The increase in number of apoptotic cells was significant compared to the controls at both ages. These increases were attributable to loss of *miR-1000*, because restoring *miR-1000* expression using the rescue allele reduced apoptosis. The increase in the number of apoptotic cells between 2 and 10-day mutant brains was significant (ANOVA: $p < 0.0001$), suggesting age-progressive degeneration.

Suppression of neuronal cell death by expression of the *Drosophila* inhibitor of apoptosis protein (DIAP1) reduced the number of Caspase 3-positive cells in the mutant brain (**Fig. 2f**) and improved survival of *miR-1000* mutants (**Fig. 2g**, $p < 0.0001$ comparing mutant with and without DIAP1). These data establish a link between apoptosis and the reduced lifespan observed in the mutant. Vacuole formation, a characteristic of age-related neurodegeneration²⁴, was observed in mutant brains at 10 days of age (**Supplementary Fig. 3**). Vacuolization is not observed in brains of normal animals until much later in life. The mutants also showed elevated sensitivity to oxidative stress (**Supplementary Fig. 4**), another characteristic feature of *Drosophila* neurodegeneration models. Taken together, these findings indicate that *miR-1000* mutants suffer from early-onset neurodegeneration.

Misregulation of the vesicular glutamate transporter

miRNAs typically repress their targets. Among 374 computationally predicted *miR-1000* targets, we selected several for analysis based on their CNS functions (**Supplementary Fig. 5**). The level of the Vesicular glutamate transporter mRNA (*VGlut*; CG9887) increased ~4 fold in RNA isolated from heads of *miR-1000* mutants (**Fig. 3a**). This was offset by ~half in the rescued mutant, consistent with the partial restoration of miRNA expression. The other predicted targets did not show this pattern of upregulation in the mutant and downregulation in the rescue, and were not considered further (**Supplementary Fig. 5**). The 3' UTR of *VGlut* mRNA contained two predicted *miR-1000* sites (**Fig. 3b**). Luciferase assays showed that *miR-1000* was able to downregulate a reporter containing the *VGlut* 3' UTR and that this effect was abrogated by mutating the predicted target sites to disrupt seed pairing to the miRNA (**Fig. 3c**). Thus, *VGlut* mRNA is a direct target of *miR-1000*.

To ask whether upregulation of *VGlut* contributes to the defects in the *miR-1000* mutant, we used two genetic approaches to selectively reduce *VGlut* levels in the *miR-1000* mutant background. First, one copy of the *VGlut* gene was removed using a chromosomal deletion (referred to as *Df(VGlut)*). Second, we introduced a UAS-RNAi transgene to selectively deplete *VGlut* transcript in *miR-1000*-expressing cells in the mutant, using the *miR-1000*^{K02}-*Gal4* knock-in allele. Both of these strategies restored climbing ability in *miR-1000* mutants (**Fig. 3d**). Both

strategies also reduced apoptosis in the mutant brain (**Fig. 3e**) and extended the lifespan of the miR-1000 mutant (**Fig. 3f**). Taken together, these findings indicate that the defects in the miR-1000 mutant result from *VGlut* overexpression in cells expressing this miRNA.

Excess synaptic glutamate release

Glutamate is the predominant excitatory neurotransmitter in most regions of the *Drosophila* CNS ²⁵. We investigated the synaptic actions of *miR-1000* by examining the glutamatergic neuromuscular junctions (NMJ). *miR-1000* is expressed in presynaptic motor neurons, visualized by the *miR-1000^{KO2}-Gal4* knock-in allele driving UAS-CD8-GFP (**Fig. 4a**). The number and size of synaptic boutons was increased in the NMJ of the miR-1000 mutant (**Fig. 4a**). Electrophysiological recording of spontaneous synaptic activity at the NMJ was used to investigate the role of elevated *VGlut* mRNA levels on synaptic transmission. Spontaneous miniature excitatory junction potentials (mEJPs) were larger in miR-1000 mutants (**Fig. 4b**), with a significant increase in amplitude (**Fig. 4c, d**). This increase in excitatory synaptic transmission was suppressed by restoring miRNA expression using the rescue allele (**Fig. 4c, d**). The frequency of mEJPs also increased in the mutant, and was rescued by restoring miRNA expression (**Fig. 4e**). Because *VGlut* and *miR-1000* are not expressed in muscle, it is likely that the increased mEJP amplitude and frequency were due to excess release of glutamate from presynaptic motor neurons in the miR-1000 mutants. Increased frequency is likely due to the increase in bouton number.

Next, we measured mEJPs in miR-1000 mutants in which the increase in *VGlut* was counteracted by expression of *VGlut-RNAi* in miR-1000 expressing cells (**Fig. 4f, g**). mEJP amplitude and frequency were significantly reduced by lowering *VGlut* levels in the mutant background (**Fig. 4h, i**). These observations suggest that the observed increase in *VGlut* expression was responsible for the increased excitatory synaptic signaling in the miR-1000 mutant.

Mutant defects due to glutamate excitotoxicity

To ask whether elevated glutamate release was responsible for the miR-1000 mutant phenotype, we made use of memantine, a glutamate receptor antagonist used to treat Alzheimer's disease ²⁶. Flies were fed on food containing memantine and climbing performance was measured after one day. Drug-treated mutants showed a significant improvement compared to the untreated mutants (**Fig. 5a**). The drug had little or no effect on control or rescued mutant flies.

Next, we tested the effects of reducing expression of NMDA, AMPA and metabotropic glutamate receptors in the mutant background. NMDAR are important in excitotoxic cell death in the mammalian brain ²⁷ and this seems to

be the case in *Drosophila* as well: reducing NMDAR levels by mutating one copy of the *NMDAR1* gene improved survival of *miR-1000* mutant animals (**Fig. 5b**, $p < 0.0001$); improved their performance in the climbing assay (**Fig. 5c**); and suppressed apoptotic cell death in the mutant (**Fig. 5d**). Similar results were obtained by reducing levels of the *Drosophila* AMPA type receptor GluR1 or the metabotropic glutamate receptor encoded by the *mGluRA* locus (**Supplementary Figs. 6 and 7**). Reducing activity of the muscle specific glutamate receptors, GluRIIA and GluRIIB receptors was ineffective (**Supplementary Fig. 7**). Climbing performance of the *miR-1000* mutant was improved by blocking apoptosis by expression of DIAP1 in cells expressing mGluRA (**Fig. 5e**), indicating that loss of these cells contributes to the mutant phenotype.

Next, we measured mEJPs in *miR-1000* mutants with reduced levels of the NMDAR, AMPA or mGluR receptors (**Supplementary Fig. 8**). Lowering mGluR significantly counteracted the increased mEJP amplitude in the *miR-1000* mutant ($p = 0.004$) (**Supplementary Fig. 8**). Reducing levels of NMDAR or AMPA receptors did not have a significant effect in this assay. The increased frequency of mEJPs in the *miR-1000* mutant was not significantly restored by lowering the levels of any of the three receptors (**Supplementary Fig. 8**), consistent with this effect being presynaptic in origin.

Taken together these findings provide evidence that the neurodegeneration observed in the *miR-1000* mutants is due to glutamate excitotoxicity, mediated through NMDA, AMPA and metabotropic glutamate receptors.

Conservation of VGlut regulation

In light of the importance of VGlut regulation by *miR-1000*, we asked whether this regulation is conserved in mammals. *miR-1000* is not found in mammals, but the seed-similar miRNA *miR-137* is conserved (**Fig. 6a**; ref 28). Among the three mammalian vesicular glutamate transporters, VGlut1, VGlut2 and VGlut3²⁹⁻³¹, we found a predicted target site for *miR-137* in the 3' UTR of the human VGlut2 transcript (**Fig. 6b**). Luciferase reporter assays showed that the human and mouse VGlut2 3'UTRs were regulated by *miR-137*, as well as by *Drosophila miR-1000* (**Fig. 6c** and **Supplementary Fig. 9**). This repression was relieved after mutating the predicted seed match in the VGlut2 3'UTR.

To ask whether *miR-137* regulates VGlut2 in mammalian neurons, we depleted *miR-137* in cultures of primary cortical neurons derived from E16 mouse embryos. We observed an increase in VGlut2 mRNA and an increase in the level of VGlut2 protein following transduction of cells with a lentiviral vector to deplete *miR-137* (**Fig. 6d, e**). We found more Caspase-3-positive cells in *miR-137* depleted cultures, indicating an increase in apoptosis (**Fig. 6f**). To test the function of *miR-137* *in vivo*, we injected lentivirus to deplete *miR-137* or control

lentivirus into the dentate gyrus region of the hippocampus. *miR-137* is expressed in the dentate gyrus region of the hippocampus³². Experimental and control viruses were injected into opposite brain hemispheres of the same mice. Elevated VGlut2 protein levels were observed in the hippocampus on the *miR-137* depleted side of the brain, but not on the control side (**Fig. 6g** and **Supplementary Fig. 10**). Thus, the regulatory relationship between *miR-1000/miR-137* and VGlut2 appears to be conserved in mouse.

Activity dependent regulation of *miR-1000*

To ask whether neuronal activity regulates *miR-1000* activity, we turned to the *Drosophila* visual system. The adult visual system is a major site of *miR-1000* expression: *miR-1000* and *VGlut* are coexpressed in photoreceptors as well as in neurons of the retina, lamina and medulla (**Supplementary Fig. 11**).

Consequently, varying ambient light levels provided a simple means to control photoreceptor activity. Compared to animals reared under a standard 12/12 light-dark cycle, dark-reared animals should have low photoreceptor activity, whereas animals reared in constant light should have higher activity. *miR-1000* transcript levels were significantly reduced in dark-reared animals (**Fig. 7**, $p = 0.018$); animals reared in constant light showed increased *miR-1000* expression compared to animals reared under a normal 12/12 light dark cycle ($p = 0.049$). Animals carrying the *VGlut* 3'UTR GFP reporter transgene, showed higher GFP expression in the dark-reared animals (where *miR-1000* was lower than the controls, $p = 0.009$) and lower GFP expression in the light-reared animals (where *miR-1000* was higher than the controls, $p = 0.029$). These findings provide evidence that *miR-1000* regulates *VGlut* levels in an activity dependent manner.

Discussion

Although postsynaptic regulation of glutamate receptor activity has been well studied, much less is known about presynaptic regulation of glutamatergic signaling. Our findings suggest that *miR-1000* acts presynaptically to regulate *VGlut* expression and thereby control synaptic glutamate release. It is tempting to speculate that this could provide a mechanism for tuning synaptic output and locally modulate synaptic strength. Such a mechanism would be most useful if the miRNA itself could be regulated in an activity-dependent manner. We provide evidence that *miR-1000* expression is regulated by light *in vivo*, presumably reflecting photoreceptor activity in the eye. This in turn leads to light-regulated regulation of *VGlut* reporter levels. These findings lend support to the notion of activity-dependent regulation of *miR-1000* activity. An in depth exploration of these issues awaits the development of new methods to monitor changes in presynaptic miRNA levels in real time.

Failure of *miR-1000* mediated regulation of VGlut leads to excess glutamate release and results in excitotoxicity. Consistent with these findings, Gal4-directed overexpression of *VGlut* has been reported to cause neurodegeneration³³. Interestingly, elevated levels of VGlut have been associated with excitotoxicity in vertebrate animal models of epilepsy and traumatic brain injury. The GAERS rat epilepsy model shows elevated levels of VGlut2 but not of VGlut1¹⁸. Similarly, in a model of stroke, ischemic injury was found to result in elevated expression of VGlut1, but not of VGlut2¹⁷. VGlut1 levels are regulated by methamphetamine treatment³⁴, likely contributing to excitotoxic consequences of methamphetamine abuse. VGlut1 levels have also been reported to increase in rat brains following antidepressant treatment³⁵.

In the mouse, *miR-223* acts on postsynaptic glutamate receptors and has a neuroprotective role *in vivo*¹⁴. Our findings provide evidence that *miR-1000* has a neuroprotective role mediated through regulation of presynaptic glutamate release, and that this regulatory mechanism is conserved for *miR-137* and VGlut2 in the mouse. Together, these studies show miRNA-mediated regulation of glutamatergic activity acts pre- and post-synaptically to modulate synaptic transmission and to protect against excitotoxicity. In this context, it is noteworthy that *miR-137* is reported to be enriched at synapses³⁶. Interestingly, *miR-137* levels were found to be low in a subset of Alzheimer's patients with elevated serine palmitoyltransferase 1 expression leading to increased ceramide production³⁷. Single nucleotide polymorphisms affecting *miR-137* target sites could lead to low-level constitutive overexpression of its targets, even when the SNP is present in one copy. A single nucleotide polymorphism affecting *miR-137* has also been identified as a risk factor for schizophrenia^{38,39}. It will be of interest to learn whether misregulation of VGlut2 expression contributes to these neurological conditions. Our findings raise the possibility that miRNA mediated regulation makes VGlut and other miRNA target genes possible risk factors in neurodegenerative disease.

Experimental Procedures

Fly strains

Df(3R)Exel6203 (removes *miR-1000*), Df(2L)BSC37 (removes *VGlut*), *P(EP)Nmdar1^{EP331}* (*NMDAR* allele) *Mi(MIC)mGluRA^{MI02169}* (*mGluRA* allele), *PBac(WH)Glu-R1^{f05411}* (*Glu-R1* allele), Df(2L)Exel8016 (removes *GluRIIA*, *GluRIIB*), *P(GawB)VGlut^{OK371}* were obtained from Bloomington stock center. *P(GawB)mGluRA^{NP6701}* (*mGluR*-GAL4) was obtained from DGRC, Kyoto and *P(GD834)v2574* (*VGlut*-RNAi), *P(GD707)v1793* (*mGluR*-RNAi-1), *P(GD707)v1794* (*mGluR*-RNAi-2) were obtained from the Vienna Drosophila Research Center. Control flies were *yw* unless otherwise indicated. All data reported were from male flies. Females produced comparable results (not shown).

Mutant generation

miR-1000^{KO1} and *miR-1000^{KO2}* deletion mutants were generated by targeted homologous recombination⁴⁰ and Recombinase-Mediated Cassette Exchange²², as described⁴¹. *miR-1000* is located in the first intron of the *msi* gene, but represents an independent transcription unit, transcribed from the opposite strand of the DNA. Left and right homology arms were amplified by PCR from genomic DNA. The 4436 bp left homology arm was amplified with primers: LF-TTAACATTGTTGAGCGTCATTAGC, LR-ACGAAGGTAACCATATCTCAGACC; the 4199 bp right arm was amplified with: RF-AATACTGTGGCGACTTTAGGTAGG and RR-CAGCACTTTATTCAATTCACTTGG.

Following recombination, 141 bp spanning the miRNA hairpin was deleted and replaced with a *mini-white* cassette to create the RMCE allele *miR-1000^{KO2}*. The Gal4 and GFP knock-in alleles were made by replacing the *mini-white* cassette with Gal4 (863 bp) or GFP (720 bp) coding sequences using PhiC31 mediated recombination. The RMCE rescue allele introduced 202 bp spanning the miRNA hairpin (amplified with primers F- 5' CTGCGAAATAAATCACTCCAATAA 3' and R- 5' CGTTTTTCTCAGGTTTTCCTGTCC 3').

The presence of the intron-containing *mini-white* marker is expected to disrupt *msi* function. The *mini-white* marker in the *miR-1000* allele is flanked by loxP sites and was excised by crossing the mutant to heat shock-CRE flies. After CRE mediated excision, a single LoxP site is left in the *msi* intron. Genetic complementation tests showed that *msi* function was not compromised in the *mini-white*-excised *miR-1000* mutant strains (*miR-1000^{KO1}*), or in the *mini-white*-excised *miR-1000* RMCE alleles (*miR-1000^{KO2}*, depicted in Fig. 1c). Deletion of the miRNA in the various mutants was confirmed by genomic DNA PCR and by miRNA quantitative-PCR. All genetic tests were done using flies carrying two independently generated alleles or an allele in trans to a chromosomal deletion, Df(3R)Exel6203, removing the *miR-1000* locus.

Quantitative RT-PCR

Total RNA was extracted from 15-20 fly heads with TRIzol reagent (Invitrogen). For miRNA PCR, RNA from each sample was subjected to cDNA synthesis by TaqMan RT kit (Applied Biosystems). Mature miR-1000 transcript level was measured by using TaqMan miRNA assays and normalized to U14 or U27 control primers. For *Drosophila* VGlut mRNA qRT-PCR, total RNA was treated with RNase-free DNase. First strand cDNA was synthesized using oligo-dT primers and SuperScript RT-III (Invitrogen). qRT-PCR was performed using SYBR green (Applied Biosystems). Measurements were normalized to Ribosomal Protein 49 (rp49 5'- F-5'GCTAAGCTGTCGCACAAA 3' and R-5' TCCGGTGGGCAGCATGTG 3'). Primers used to assay *Drosophila* VGlut mRNA levels were F- 5' TACTAGCTGAGCAGCAAGATGAAG 3' and R- 5' TGCGAT ATCTCAAACCTTCTCCATA 3'. For mouse VGlut2 qPCR, F- 5' AGG TGC AAT GGA AAA ATT TAA GAC 3' and R- 5' GGC TGA CTG ATG AGC AGT AAA TAA 3' were used and b-act, F- 5' ATG CTC CCC GGG CTG TAT 3' and R- 5' CAT AGG AGT CCT TCT GAC CCA TTC 3' were used for normalization. For mouse miRNA qPCR, Taqman microRNA assay for miR-137 (Applied Biosystems) were used and normalized to U6 control primers. Primers used to measure GFP levels were F- 5' TGG CAA TAT GAA AGT CTG CAT C 3' and R- 5' TGC CAT CCA CAT ACG TAC AGT T 3'.

3'UTR Reporter assays

The *Drosophila* VGlut 3'UTR luciferase reporter was made by cloning the 3 Kb 3'UTR of VGlut into a tubulin-promoter-firefly luciferase vector⁴² using primers F-5' TCATAGCTTTTAGTTGTAGTCGGAAA 3' and R-5' TTCGTTGGGACTTGTACAAATAAATA 3'. Mutations were introduced into the predicted miR-1000 sites by PCR using primers 1F- 5'TCATAGCTTTTAGTTGTAGTCGGAAA 3', 1R- 5'ACTAGGCTTACTTCACTAGGTTTAGACGCTTACACAAGCAGTATTGTTGT 3', 2F- 5'ACAACAATACTGCTTGTGTAAGCGTCTAAACCTAGTGAAGTAAGCCTAGT 3', 2R- 5'TGGTTAACATATTTATGTTATTTTAGACCACACAGCCCCGAGAGCTTAAG 3', 3F-5' TGGTCTAAAATAACATAAATATGTTAACCAGAAAGCCACGGTTAACTTT 3', 3R- 5'TTCGTTGGGACTTGTACAAATAAATA 3'. *miR-1000* was expressed under tubulin promoter control from a plasmid containing 200 bp spanning the miR-1000 hairpin (primers F-5' TGCGAAATAAATCACTCCAATAA 3' and R- 5'GTTTTTCTCAGGTTTTCCTGTCC 3'). Hsa-VGlut2 3'UTR was amplified by primers F-5' AAAGTTGCAAGCATATCAACCA 3' and R-F' TGTATGATTAATGCACTGCTTT 3'. Mutations were induced by PCR fragments, amplified from following primers: 1F- 5' AAAGTTGCAAGCATATCAACCA 3', 1R-5' GGAATCCTCATCTCGATGGGTACACAAATGTTGCAATTCTTG 3', 2F- 5' CCATCGAGATGAGGATTCATATAAATTTCTGTCTGTATATTACC 3', 2R- 5' TGTATGATTAATGCACTGCTTTTT 3'. Hsa-miR-137 was amplified by F-5' CAGCTTGGTCTCTGACTCTCT 3' and R-5'AAGAAAGTGCTACCTTGGCAAC 3'. Mouse VGlut2 3'UTR was amplified by F- 5' TGCTAGTTGCTGGATTCATTTG 3'

and R- 5' CAAACCACACCGGAAATAATGT 3'. Mutations were introduced into 3'UTR by PCR using following primers- 1F' TGCTAGTTGCTGGATTCATTTG 3', 1R- 5' CACACAGGAGGTTTTATGCTATCCTCATCTCGATGGGTAC 3', 2F- 5' CCCATCGAG ATGAGGATAGCATAAAACCTCCTGTGTGT 3', 2R- 5' CAAACCACACCGGAAATAATGT 3'. Serum free S2 cells were transfected in 24-well plates with 25 ng of the firefly luciferase reporter, tubulin-promoter *Renilla* luciferase control plasmids and 250 ng of the miRNA-expression plasmid or empty vector. Transfections were performed in triplicate. Dual-luciferase assays (Promega) were performed 2.5 days after transfection according to the manufacturer's protocol. Experiments were performed four times.

Drosophila VGlut 3'UTR GFP reporters were made by cloning the 3'UTR of the *VGlut* gene downstream of EGFP in pCasper-tubulin-EGFP vector, using the PCR primers mentioned above for amplifying the wild type *Drosophila* VGlut 3'UTR.

Immunocytochemistry

Adult brains were dissected in ice cold Phosphate Buffered Saline (PBS) and fixed in 4% Paraformaldehyde for 30 min. Brains were washed several times with PBS+ 0.1% Triton-X (PBT) before blocking with 5% NGS for 16hrs at 4°C. Brains were incubated in primary antibody for 36-48hrs at 4°C. Antibodies: Mouse anti-Dlg (1:10, from Developmental Studies Hybridoma Bank), Rabbit anti-caspase3 (1:100, Cell Signaling), Chicken anti-GFP (1:2000; Abcam), Rabbit DVGlut (1:10000, DiAntonio lab), mouse VGlut2 (1:200, Abcam). Fluorescent secondary antibodies (Invitrogen) were used at 1:500 overnight at 4°C. Brains were washed several times in PBT and stained with DAPI (1:1000) for 20 minutes before mounting. Confocal microscope images were processed using ImageJ and Photoshop. Caspase3 positive cells were counted from stacks of confocal sections using Imaris (Bitplane).

Embryo in-situ hybridization

miR-1000 probe was generated by 1145 bp long PCR fragment, amplified with F- 5' AAATCAGGGAGTGGTCGTTG 3' and R-5' ACCACCCTCGAATGTGTAGC 3'. Probe was detected with AP-coupled anti-DIG Fab fragments (Roche #1093274; 1:2000; 2 hr RT) and visualized with NBT/BCIP (Roche #1682326; 30–120 min).

Lifespan assay

Flies were reared under conditions of controlled crowding (50 larvae/vial), temperature and humidity. Groups of 20 male or female flies were collected at eclosion and aged separately. Flies were transferred to fresh vials every 2nd day and the number of dead flies was recorded.

Climbing assay

Groups of 20 male or female flies, reared under conditions of controlled crowding, were collected when freshly eclosed. Flies were aged for 2 or 10 days

without further exposure to CO₂. Before testing, flies were transferred to a graduated cylinder made from a 25ml plastic pipette, and allowed to rest for half an hour before the assay. Flies were tapped down to the bottom of the tube and the number of flies climbing above 5cm in the cylinder was counted. Three trials were done for each sample and the results averaged. The experiment was repeated with 3 independently reared replicates and measured at the same time of day (from 2 to 5pm) to avoid differences due to circadian cycles in activity. Data from these experiments were pooled analyzed by ANOVA.

Drug feeding

Memantine hydrochloride (Sigma) was dissolved in water and added to a final concentration of 0.01 mM of drug in standard fly food. Food was stored overnight to ensure even distribution of the drug before use. Climbing assays were performed after 24hrs on drug-treated and mock-treated flies.

Electrophysiology

Spontaneous miniature events were recorded from muscle 6 of segment A3 and A4 from third instar larvae. Larvae were dissected in ice-cold calcium free HL3.1 medium (70 mM NaCl, 5 mM KCl, 4 mM MgCl₂, 10 mM NaHCO₃, 5 mM Trehalose, 115 mM Sucrose, 5 mM HEPES, pH-7.2). Segmental nerves and brain were removed before recordings. Recordings were done with HL3.1 medium with 0.8 mM CaCl₂, pH-7.2 at room temperature. Sharp glass electrode filled with 3 M KCl was used for recordings. Spontaneous events were recorded by Clampex 6 and Clampex 10. Data were analyzed using minianalysis (www.synaptosoft.com).

Oxidative stress assay

5 day old mutant (*miR-1000*^{KO1}/*miR-1000*^{KO2}), *miR-1000*^{KO2}/+, Rescue and control flies were grown on 2.5% H₂O₂ with 10% sucrose and 1% agar for giving oxidative stress. Number of dead flies was counted every 12hrs.

Light Dark assay

Aged matched white pupae were kept under constant dark or constant light until they eclosed. Control samples were raised under 12hrs light-dark cycle. RNA was extracted from heads of freshly eclosed flies and Quantitative RT-PCR was done.

Cortical cell culture and lentiviral transduction

For culture of mouse cortical neurons, tissue was excised from brains of E16 mouse embryos and digested with trypsin for 15 minutes at RT with gentle shaking. Cells were pelleted and washed before resuspending in serum neurobasal medium (NB medium, Glutamax, FBS (Gibco #21103049, #35050-061, #10437028), B27 and Penicillin/streptomycin (Invitrogen, #15140122, #17504-044). 5 X 10⁵ cells/ml were plated in each well in serum neurobasal medium and incubated at 37°C with 5% CO₂ for one day. Next day, serum

neurobasal medium was replaced with plain neurobasal medium (NB medium, B27, Glutamax). Medium was changed every 3rd day. Cells were transduced with lentivirus at DIV7 for RNA and protein analysis and at DIV3 for immunostaining. Scrambled hairpin control and miRZip-137 lentivirus plasmids were from Systems Biosciences (#MZIP000-PA-1, # MZIP137-PA-1) and packaged using pPACKH1 HIV Lentivector Packaging Kit (#LV500A-1) according to the manufacturer's instructions. Lentiviruses titers ranged from 10^5 - 10^6 transducing units/ μ L. Cells were harvested on day 14 after transduction for RNA and protein extraction and on day 10 after transduction for immunostaining.

Immunoblots

Cells were lysed in 62.5 mM Tris-HCl pH 6.8, 25% Glycerol, 2% SDS, 0.01% Bromophenol Blue by heating at 100°C for 5 min. Proteins were fractionated on an 8% SDS-PAGE gel and transferred to nitrocellulose. Membranes were blocked in 3% milk and incubated with primary antibodies to mouse VGluT2 (Abcam ab79157, 1:1000 dilution) and Rabbit beta-actin (Sigma A-2066, 1:1000 dilution) overnight at 4°C. Membrane was incubated with VGluT2 and beta-actin antibody consecutively to avoid non-specific bands. Membranes were washed with PBST (PBS, 0.1% Tween-20) and incubated with HRP-conjugated secondary antibodies (Jackson immunoresearch #115035062 for mouse, #111035003 for rabbit) for 2hrs RT. Membranes were exposed using ECL according to the manufacturer's instructions (GE Healthcare).

Stereotactic injection and immunolabeling of mouse brain sections

C57BL male mice were handled and caged according to IACUC protocols (3 animals/cage). Stereotaxic injection of 1.5 μ L of lentiviral vectors into the dentate gyrus of 10 week-old mice was done under anesthesia with isoflurane gas (3% induction and 1% maintenance). Stereotaxic coordinates used for the dentate gyrus injection were 2 mm posterior, 1.6 mm lateral and 2.3 mm deep, relative to Bregma. Virus injections were conducted at 150 nl/min using a Hamilton, Microliter # 95 syringe with 33 gauge needle (Hamilton, # 7762-06) and a microsyringe pump (UltraMicroPump4, SYS-Micro4 Controller, World precision instruments, USA). Virus titer for miR-Zip 137 (#MZIP137VA-1) was 7.48×10^9 ifu/ml and for scrambled control (#MZIP000VA-1) was 3.53×10^9 ifu/ml. After injection, animals were treated with antiseptic cream, sutured and placed on a warm plate for recovery. Mice were maintained under 12hr light/dark cycle and sacrificed 14 days after injection. They were perfused trans-cardinally with 0.9% saline followed by 4% paraformaldehyde for fixation. Brains were post-fixed in 4% paraformaldehyde overnight, then washed with PBS 3-4 times for 30 min each, immersed in 15% sucrose followed by 30% sucrose and kept at 4°C for 2 days. Coronal sections of 40 μ m were prepared using a sliding microtome. Sections were stored at -20°C in 0.1 M phosphate buffer containing 25% glycerin and 30% ethylene glycol. Labeling of free-floating sections was carried out as

follows: every 6th section in the series was selected for immunostaining and washed in Tris buffered Saline (TBS). Sections were permeabilized and blocked for 1 hour at RT in TBS containing 0.25% triton and 3% normal donkey serum (TBST). Sections were incubated with primary antibodies at 4°C for 2 days. After 3 washes for 10 min with TBS, sections were incubated with TBST for 10 min at RT, followed by secondary antibodies in TBST for 4 hours at RT. DAPI was added to sections after 2 washes with TBS for 10 min. Sections were mounted in 2.5% 1,4 diazabicyclo [2.2.2] octane/polyvinyl alcohol on Poly-lysine coated slides (Menzel-Glaser, Thermo Scientific) and imaged using Zeiss LSM700 confocal. Mouse brain overview images were taken using tilescan function and auto stitched using Zeiss LSM700 confocal. Virus GFP and VGlut2 staining were quantified using imageJ. Pixel by pixel intensity quantification was done using Matlab.

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Author contributions

PV performed all experiments. MRA and AT contributed to the mouse lentiviral injection experiments. PV, GA and SMC designed the experiments and wrote the manuscript.

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Figure legends

Figure 1: *miR-1000* expression and mutant alleles.

(a) *miR-1000* expression at different developmental stages. Upper panel: in situ hybridization to detect *miR-1000* in a stage 14 embryo (lateral view). Expression in brain and ventral nerve cord were prominent. Middle panel: *miR-1000* GFP knock-in allele showing expression in the larval brain and ventral nerve cord. Lower panel: *miR-1000* was widely expressed in the adult brain, prominently in the mushroom bodies and visual system. Scale bars: 50 μ M.

(b) Quantitative miRNA PCR. RNA was isolated from wild-type control adult heads at 2, 15 and 30 days of age. Data were normalized to U14 and U27 RNAs. Data show mean $\pm$ s.d. for 3 biological replicates ($p < 0.001$ comparing 2 and 15 d, t-test, 2 tailed unequal variance).

(c) Diagram illustrating the targeting strategy for the *miR-1000* RMCE knockout alleles (KO2). An AttP-flanked *mini-white* reporter replaced *miR-1000*. AttB-flanked cassettes were used to replace the *mini-white* cassette with GFP, Gal4 or the *miR-1000* hairpin.

(d) Quantitative PCR showing *miR-1000* levels in RNA isolated from heads of flies of the indicated genotypes. Data were normalized to U14 and U27 and show mean $\pm$ s.d. for 3 biological replicates. $p < 0.001$ comparing *miR-1000*^{KO1}/*miR-1000*^{KO2} with control and rescue (t-test, 2 tailed unequal variance). In all Figures, KO1 indicates the *miR-1000*^{KO1} allele described in Supplementary Fig. S1 with the *mini-white* cassette removed; KO2 indicates the *miR-1000*^{KO2} RMCE allele with the *mini-white* cassette removed. "Rescue" indicates flies homozygous for the RMCE rescued *miR-1000*^{KO2} allele.

Figure 2: Phenotypic characterization of miR-1000 mutants.

(a) Adult lifespan. KO1: *miR-1000*^{KO1}; KO2: *miR-1000*^{KO2} RMCE allele with mini-white removed; Rescue: flies homozygous for the RMCE allele with the miRNA reintroduced. Df: Df(3R)Exel6203, a deletion removing *miR-1000*. Control was *yw*. Analyzed using Kaplan-Meier statistics and the Mantel-Cox log rank test: $p < 0.0001$ comparing *miR-1000*^{KO1}/*miR-1000*^{KO2}, *miR-1000*^{KO1}/Df and *miR-1000*^{KO2}/Df with *miR-1000*^{KO1}/+, *miR-1000*^{KO2}/+ Df/+ and *yw* controls. The controls were not significantly different from each other. $p < 0.0001$ comparing rescue with *miR-1000*^{KO1}/*miR-1000*^{KO2}, *miR-1000*^{KO1}/Df and *miR-1000*^{KO2}/Df. $N > 60$ flies/genotype; rescue $n = 104$.

(b) Climbing assays show the percentage of flies that climbed >5 cm in a 30 cm column. Data represent at least 3 independent biological replicates (20 flies/batch). Analyzed by ANOVA, Holm-Sidak multiple comparison test: $p < 0.0001$ mutants vs controls and rescue. Error bars: mean $\pm$ s.d.

(c) Caspase3 positive cells in 2d and 10d brains. Anti-Caspase3 (red). Anti-Dlg (green) shows brain morphology.

(d-e) Counts of Caspase3 positive cells in brains at 2d and 10d. Note the different Y-axis scale for the 10d sample. $n=8$ brains each. p values: ANOVA using the Holm-Sidak multiple comparison test.

(f) Counts of caspase3 positive cells from *miR-1000* mutant brains $\pm$ UAS-DIAP1 expressed under mGluRA-GAL4 control. $n = 14$ for *miR-1000*^{KO1}/*miR-1000*^{KO2}; *mGluRA-GAL4*, UAS-DIAP1, $n=7$ for *miR-1000*^{KO1}/*miR-1000*^{KO2}; *mGluRA-GAL4*, $n = 14$ for control. p values: ANOVA using the Holm-Sidak multiple comparison test.

(g) Adult lifespan. Data were analyzed using Kaplan-Meier statistics and the Mantel-Cox log rank test: $p < 0.0001$ comparing *miR-1000*^{KO1}/*miR-1000*^{KO2}, *mGluRA-GAL4* with UAS-DIAP1; *miR-1000*^{KO1}/*miR-1000*^{KO2}, *mGluRA-GAL4*. $n = 106$ flies for control; 119 for *miR-1000*^{KO1}/*miR-1000*^{KO2}, *mGluRA-GAL4*; 131 for UAS-DIAP1; *miR-1000*^{KO1}/*miR-1000*^{KO2}, *mGluRA-GAL4*.

Figure 3: *miR-1000* acts by regulating VGlut

(a) Quantitative RT-PCR showing VGlut transcript levels in RNA isolated from heads of animals of the indicated genotypes. Data was normalized to rp49 and represent the average of at least 3 independent experiments $\pm$ s.d. $p < 0.01$ comparing mutant vs control and mutant vs rescued mutant (unpaired t-test).

(b) Predicted target sites for miR-1000 in the VGlut 3'UTR. Red: mutations introduced in the 3'UTR reporters for luciferase assays.

(c) Luciferase reporter assay. Data: average of 4 independent experiments $\pm$ s.d. $p < 0.0001$ ANOVA, Holm-Sidak multiple comparison test.

(d) Climbing performance at day 2. Color-coding of genotypes for panels d-f shown below. KO1 indicates *miR-1000*^{KO1}; KO2 indicates the RMCE allele *miR-1000*^{KO2}, both with *mini-white* removed. KO2-GAL4 indicates GAL4 knock-in allele, *miR-1000*^{KO2}-GAL4. Df(VGlut) is Df(2L)BSC37. Average of at least four independent experiments $\pm$ s.d. $p < 0.0001$ ANOVA, Holm-Sidak multiple comparison test.

(e) Counts of Caspase3 positive cells in 10d brains. Left: $n = 20$ for *miR-1000*^{KO2}/*miR-1000*^{KO2}-Gal4, $n = 20$ for *VGlut-RNAi*, *miR-1000*^{KO2}/*miR-1000*^{KO2}-Gal4, $n = 11$ for control. Right: $n = 12$ for *miR-1000*^{KO1}/*miR-1000*^{KO2}, $n = 11$ for *Df(VGlut)/+*; *miR-1000*^{KO1}/*miR-1000*^{KO2} and $n = 9$ for control. A small number of brains of each genotype, including control, showed abnormally high levels of cell death and were excluded. ANOVA, Holm-Sidak multiple comparison test.

(f) Adult lifespan analyzed using Kaplan-Meier statistics and the Mantel-Cox log rank test: $p < 0.0001$ comparing *VGlut-RNAi*, *miR-1000*^{KO2}/*miR-1000*^{KO2}-Gal4 with *miR-1000*^{KO2}/*miR-1000*^{KO2}-Gal4; $p < 0.0001$ comparing *Df(VGlut)/+*; *miR-1000*^{KO1}/*miR-1000*^{KO2} with *miR-1000*^{KO1}/*miR-1000*^{KO2}. $n = 87$ flies for control; 84 each for *miR-1000*^{KO1}/*miR-1000*^{KO2} and *miR-1000*^{KO2}/*miR-1000*^{KO2}-Gal4; 190 for *Df(VGlut)/+*; *miR-1000*^{KO1}/*miR-1000*^{KO2}; 197 for *VGlut-RNAi*, *miR-1000*^{KO2}/*miR-1000*^{KO2}-Gal4.

Figure 4: Excess glutamate release in miR-1000 mutants

(a) Confocal micrographs showing neuromuscular junctions on larval muscles 6 and 7. Top: *miR-1000^{KO2}-Gal4* knock-in allele/+. Bottom: *miR-1000^{KO2}-Gal4* allele/*miR-1000^{KO1}* mutant. Muscle was labeled with antibody to Myosin heavy chain (MHC). Nuclei labeled with DAPI. Green: *miR-1000^{KO2}-Gal4 >UAS-CD8-GFP* was used to visualize *miR-1000* expression in the NMJ. Anti-VGluT (red). Images are maximum projections from a stack of optical sections. Scale bar = 20μM.

(b) Representative traces of spontaneous mEJPs recorded from larval muscle 6. Neuronal connections to the brain were cut to eliminate evoked responses. For panels a-d KO indicates flies homozygous for the *miR-1000^{KO1}* allele with *mini-white* excised. Rescue indicates flies homozygous for the RMCE rescue allele.

(c) Cumulative probability distribution of mEJP amplitudes.

(d) Median amplitude of mEJPs. n = 8 for KO, n = 11 for control and rescue. One electrophysiology trace from the mutant gave aberrantly large amplitude, and was omitted. Data analyzed by ANOVA, Tukey's multiple comparison test.

(e) mEJP frequency. n = 8 for KO, n = 11 for control and rescue. Data analyzed by ANOVA, Tukey's multiple comparison test.

(f) Representative traces of spontaneous mEJPs at the NMJ of the *miR-1000^{KO2}/miR-1000^{KO2}-GAL4* knock-in *miR-1000* mutant ± *VGluT-RNAi*. *KO2-Gal4* indicates the *Gal4* knock-in allele.

(g) Cumulative probability distribution of mEJP amplitudes.

(h) Median amplitude of mEJPs. Data were analyzed using the Mann-Whitney test. *miR-1000^{KO2}/miR-1000^{KO2}-GAL4* (n = 10) and *VGluT-RNAi, miR-1000^{KO2}/miR-1000^{KO2}-GAL4* (n = 13).

(i) mEJP frequency analyzed using the Mann-Whitney test. *miR-1000^{KO2}/miR-1000^{KO2}-GAL4* (n = 10) and *VGluT-RNAi, miR-1000^{KO2}/miR-1000^{KO2}-GAL4* (n = 13).

Figure 5: Rescue by manipulating glutamate receptor levels

(a) Climbing assay performed on control and *miR-1000^{KO1} / miR-1000^{KO2}* mutant flies fed for one day on food containing 0.01mM Memantine or with food lacking drug ($p = 0.01$ treated vs untreated). Data represent the average of at least three independent experiments $\pm$ s.d. Data were analyzed by ANOVA using the Holm-Sidak multiple comparison test.

(b) Adult lifespan of *miR-1000* mutant flies with and without reduced expression of NMDAR1. The *Nmdar1^{EP331}* mutant allele was recombined onto the *KO2* RMCE chromosome. Kaplan-Meier survival analysis using the log rank test: $p < 0.0001$. $n = 130$ flies for control; 133 for *miR-1000^{KO1} / miR-1000^{KO2}*; 153 for *NMDAR1, miR-1000^{KO2} / +*; 169 for *NMDAR1, miR-1000^{KO2} / miR-1000^{KO1}*.

(c) Climbing assay performed on 2-day old flies. Genotypes as in panel b. Data represent the average of at least 4 independent experiments $\pm$ s.d. $p = 0.0116$, ANOVA using the Holm-Sidak multiple comparison test.

(d) Counts of Caspase3 positive cells in the brains of 10-day old flies of the indicated genotypes. $n = 15$ brains/genotype. Data were analyzed by ANOVA using the Holm-Sidak multiple comparison test.

(e) Climbing assay performed on 2-day old flies expressing UAS-DIAP1 driven by *mGluR-GAL4* in *miR-1000* mutant allelic combination (*miR-1000^{KO1} / miR-1000^{KO2}*). $p = 0.006$ comparing *miR-1000^{KO1} / miR-1000^{KO2}; mGluR-GAL4* with *miR-1000^{KO1} / miR-1000^{KO2}; mGluR-GAL4 > UAS-DIAP1*. Data represent average of at least four independent experiments $\pm$ s.d. Data were analyzed using the Mann-Whitney test.

Figure 6: VGluT regulation by *miR-137* in mouse neurons

(a) Alignment of *miR-1000* with human and *Drosophila miR-137*. Seed sequences highlighted in blue. Orange: nucleotide differences between human and *Drosophila miR-137* expected to have limited impact on target selection.

(b) Predicted target site of human *miR-137* in the human VGluT2 3' UTR (*Slc17a6*; www.targetscan.org). Pairing shown by vertical lines.

(c) Luciferase activity of a human VGluT2 3'UTR reporter. Regulation was lost when the miR-137 site was mutated (red, residues outside seed sequence were mutated to prevent strong binding at 3' end). Average of 4 independent experiments $\pm$ s.d. $p < 0.001$ comparing intact and mutated UTR in the presence of each miRNA (2-tailed unpaired t-test, unequal variance).

(d) Quantitative RT-PCR showing mouse VGluT2 transcript in primary cultures of E16 mouse cortical neurons transduced to deplete mmu miR-137 or with a scrambled sequence control. Average of > three experiments $\pm$ s.d, analyzed by 2-tailed unpaired t-test (unequal variance).

(e) Immunoblot comparing VGluT2 protein in cortical neurons transduced to deplete miR-137 or with a scrambled sequence control. Note: more protein was loaded in the control lane than in the experimental samples, so the difference in VGluT2 is likely underestimated.

(f) Number of apoptotic cells in cultures of cortical neuron transduced to deplete miR-137 or with a scrambled control. The number of Caspase3 positive cells per image field was normalized to total cells stained with DAPI. At least 3 separate fields were scored from 4 culture dishes for each virus. Data analyzed by 2-tailed unpaired t-test, assuming unequal variance.

(g) Section of a mouse brain labeled to visualize VGluT2 protein and GFP. Left side injected with lentivirus to deplete mmu miR-137. Right side injected with scrambled control virus. Viruses visualized by GFP. Below: measurement of VGluT2 normalized to GFP on experimental and control sides in regions of comparable size. Confocal micrographs for measurement were taken using a 10x objective (images in Fig. S10). $n = 8$ brains. VGluT2 levels were significantly higher in the miR-137 depleted side ($p = 0.02$, paired t-test; error bars $\pm$ s.d.). In addition, VGluT2 values were normalized to GFP on a pixel-by-pixel basis and the average values compared for miR-137 depleted and control hemispheres. The difference was significant ($p = 0.04$, paired t-test).

Figure 7: Activity-dependent regulation of *miR-1000*

Quantitative PCR showing *miR-1000* and VGlut-3'UTR-GFP reporter levels. Data represent the average of at least three biological replicates $\pm$ s.d, analyzed by ANOVA using the Holm-Sidak multiple comparison test.

a **b** **c** **d**

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Supplementary Figure 1

Strategy for targeted knock-out of *miR-1000*

miR-1000 is located in the first intron of the *msi* gene, but represents an independent transcription unit, transcribed from the opposite strand of the DNA. *miR-1000* deletion mutants were generated by targeted homologous recombination, as described {Chen, 2011 #5266}. Left and right homology arms (blue bars) were amplified by PCR from genomic DNA (diagram not to scale). The 4436bp left arm was amplified with: LF-ACGAAGGTAACCATATCTCAGACC, LR-TTAACATTGTTGAGCGTCATTAGC; the 4199bp right arm was amplified with: RF-CAGCACTTTATTCAATTCACTTGG and RR-AATACTGTGGCGACTTTAGGTAGG.

Following recombination, 141 bp spanning the miRNA hairpin was deleted and replaced with a *mini-white* cassette (red). The presence of the intron-containing *mini-white* marker is expected to disrupt *msi* function. The *mini-white* marker in the *miR-1000* allele is flanked by loxP sites (purple triangles) and was excised by crossing the mutant to heat shock-CRE flies. After CRE mediated excision, a single LoxP site is left in the *msi* intron. Genetic complementation tests showed that *msi* function was not compromised in the *mini-white*-excised *miR-1000* mutant strains (*miR-1000*^{KO1}), or in the *mini-white*-excised *miR-1000* RMCE alleles (*miR-1000*^{KO2} depicted in Fig 1c). Deletion of the miRNA in the various mutants was confirmed by genomic DNA PCR and by microRNA quantitative-PCR. All genetic tests were done using flies carrying two independently generated alleles or an allele in trans to a chromosomal deletion (Df(3R)Exel6203) removing the *miR-1000* locus.

supplementary Figure 2

Embryo hatching, pupariation and adult eclosion rate

(a) Embryo hatching rate for the indicated genotypes. KO1 indicates the mini-white excised simple deletion described in Figure S1. KO2 indicates the mini-white excised RMCE deletion allele. Rescue indicates animals homozygous for the miRNA rescue allele in the RMCE background (depicted in Fig 1c). Data represent the average of three independent experiments with $n = 150$ embryos each. Error bars: $\pm$ s.d. p-value of all mutant combination (KO1/Df, KO2/Df and KO1/KO2) is < 0.01 when compared to control and rescue.

(b) Percent pupariation indicates completion of larval development to reach the pupal stage (black bars). Percent eclosion indicates survival of the pupa to adult (grey bars). Data represent the average of three independent experiments with $n = 50$ larvae each. Error bar: $\pm$ s.d.

Supplementary Figure 3

Vacuole formation in the *miR-1000* mutant brain

Confocal microscope images of 10D old adult brains from *miR-1000*^{KO1}/*miR-1000*^{KO2} mutant flies, labeled with antibody to Discs large protein (Dlg). Left panel: overview. Olfactory lobes and mushroom bodies are prominently labeled. Center and right panels show magnified views of olfactory lobes. Arrows indicate vacuoles. Scale bars = 10 μm.

Supplementary Figure 4

Oxidative stress sensitivity in *miR-1000* mutant

Flies were reared on medium supplemented with 2.5% H_2O_2 . KO1/KO2 mutant flies (n = 100), Rescue denotes homozygous RMCE rescue allele (n = 100), KO2/+ heterozygous control (n = 100), yw control (n = 80). $p < 0.0001$ comparing mutant and rescue and comparing mutant with each control (Kaplan Meier analysis using the log rank test).

Supplementary Figure 5

mRNA expression for predicted *miR-1000* targets

miR-1000 targets were predicted using Targetscan (www.targetscan.org). Selected targets were tested by quantitative real-time PCR for changes in RNA level comparing KO1/KO2 mutant, control and RMCE rescue mutant brains. Data were normalized to Ribosomal Protein rp49 and to the *yw* control and represent the average of three independent experiment $\pm$ s.d.

Only *VGlut* showed the expected pattern for a functional target: upregulation in mutant that is restored toward normal in the rescued mutant.

Nplp1: neuropeptide-like precursor 1

Pdk1: Phosphoinositide-dependent kinase 1

Rodgi: a leucine zipper protein involved in learning and memory.

NMDA1: *N-methyl-D-aspartate receptor-associated protein*, the *Drosophila* NMDA-type Glutamate receptor orthologue.

slgA: a proline catabolic enzyme which has been reported to affect locomotion.

VGlut: *Vesicular Glutamate transporter*

EAAT2: *Excitatory Amino Acid Transporter 2*

Supplementary Figure 6

Rescue by reduced AMPA-type Glutamate receptor activity

S6a: Effect of removing one copy of the *GluR1* gene on lifespan

Longevity of *miR-1000* mutant (KO1/KO2) was improved by removing one copy of the AMPA-type Glutamate receptor gene *GluR1* (*PBac(WH)Glu-R1⁰⁵⁴¹¹*). Kaplan Meier analysis using Log rank test: $p < 0.0001$. The *GluR1* allele was recombined onto the KO2 mutant chromosome.

S6b: Effect of removing one copy of the *GluR1* gene on climbing.

Climbing behavior of *miR-1000* mutant (KO1/KO2) was improved by removing one copy of the AMPA-type Glutamate receptor gene *GluR1*.

S6c: Effect of removing one copy of the *GluR1* gene on apoptosis

Quantification of apoptotic cells in 10 day-old brains of the indicated genotypes. KO2, GluR1 is the homozygous double mutant chromosome. GluR1 is the homozygous *GluR1* mutant. ANOVA: $p < 0.0001$ comparing KO2/KO2 with KO2, GluR1 and the other genotypes. control $n = 10$ brains; $n \geq 14$ other genotypes

Supplementary Figure 7

Rescue by reduced metabotropic Glutamate receptor, mGluRA activity

(a) Effect of removing one copy of the, mGluRA on lifespan

Longevity of *miR-1000* mutant (KO1/KO2) was improved by removing one copy of the metabotropic Glutamate receptor gene *mGluRA*. $p < 0.0001$ comparing KO1/KO2 mutant with KO1/KO2 mutant carrying one copy of the mGluRA mutant (Log rank test).

(b) Effect of removing one copy of the mGluRA on climbing

Climbing behaviour of *miR-1000* mutant was improved by removing one copy of mGluRA gene. $p < 0.001$ comparing KO1/KO2 mutant with KO1/KO2 carrying one copy of mGluRA mutant.

(c) Effects of RNAi mediated depletion of mGluR

Two different UAS-RNAi transgenes were used to deplete the metabotropic Glutamate receptor mGluR. A Gal4 insertion at the mGluRA locus was used to direct transgene expression. mGluR-RNAi-1: *P(GD707)v1793*, mGluR-RNAi-2: *P(GD707)v1794*. RNAi transgenes were recombined onto the KO2 chromosome. Gal4 driven RNAi expression significantly improved lifespan: $p < 0.0001$ comparing KO1/KO2;mGluR-GAL4/+ with and without mGluR-RNAi-1 or mGluR-RNAi-2 (log rank test). Note the partial rescue from the UAS-RNAi, KO2 recombinants as homozygotes without Gal4. This was due to leaky expression of the RNAi transgenes (panel S7d).

(d) RNAi transgene efficiency

Leaky expression of the mGluR^{RNAi} transgenes shown by quantitative PCR.

mGluR mRNA was reduced by both transgenes when present in 2 copies without a Gal4 driver. Gal4

directed expression of the RNAi transgenes further reduced mGluR levels. Data were normalized to rp49 and to the *yw* control flies.

(e) Effect of RNAi-mediated depletion *mGluR* on climbing

Climbing behavior of *miR-1000* (KO1/KO2) was improved by RNAi-mediated depletion of the metabotropic Glutamate receptor *mGluR*. Note the partial rescue from leaky expression of the *mGluR*^{RNAi} transgenes. Addition of Gal4 provided a modest further improvement. Data: average of at least four independent experiments. ANOVA: $p < 0.0001$ comparing KO1/KO2;*mGluR*-GAL4 with KO2, *mGluR*^{RNAi} 1/KO1; *mGluR*-GAL4 and KO2, *mGluR*^{RNAi} 2/KO1;*mGluR*-GAL4.

(f) Effect of reducing *mGluR* on apoptosis

Quantification of apoptotic cells in 10 day-old brains of the indicated genotypes. ANOVA: $p < 0.0001$ comparing KO2/KO2 with all the other genotypes. Control $n = 10$ brains; $n \geq 14$ other genotypes. This data set was collected in the same experiment as the data shown in Fig S6c. The control and KO2/KO2 samples are the same.

(g) Effect of reducing GluRIIA and GluRIIB on climbing

Left: KO1/KO2 mutant carrying one copy of Df(2L)Exel8016, which removes GluRIIA and GluRIIB. Data represent the average of 3 independent experiments. t-test: ns.

Supplementary Figure 8

mEJPs in mutants with reduced Glutamate receptor activity

(a) Representative traces of spontaneous mEJPs at the NMJ from larvae of genotypes miR-1000 mutant (KO1/KO2), with reduced NMDAR1 expression (NMDAR, KO2/KO1), with reduced GluR1 expression (GluR1, KO2/KO1) and with reduced mGluRA expression (KO2/KO1; mGluR).

(b) Cumulative probability distribution of mEJP amplitudes from genotypes indicated in A.

(c) Median amplitude of mEJPs of the mutant (KO1/KO2) compared to mutant with reduced levels of receptors as in A. Significant rescue in mEJP amplitude was observed in miR-1000 mutant with reduced mGluR level (ANOVA: $p = 0.004$ comparing KO1/KO2 with KO1/KO2;mGluR).

(d) mEJP frequency of genotypes as in A. ANOVA: differences not significant.

Supplementary Figure 9

Regulation of the mouse VGlut2 3'UTR by miR-137

Luciferase activity of a reporter containing the mouse VGlut2 3'UTR. Expression of mouse *miR-137* or fly *miR-1000* reduced activity of the intact reporter ($p = 0.0006$ comparing the intact 3' UTR to the control for *miR-137*; $p = 0.0037$ for *miR-1000*). Regulation was lost when the *miR-137* site was mutated ($p = 0.0006$ comparing the intact and mutant 3' UTR for *miR-137*; $p = 0.0064$ for *miR-1000*; The difference between control and mutant 3' UTRs were not significant). The mutations introduced into the *miR-137* sites in the mouse UTR are shown in red. Data represent the average of 5 independent experiments $\pm$ s.d. Data were analyzed by ANOVA using the Holm-Sidak multiple comparison test.

Supplementary Figure 10

Lentiviral depletion of *miR-137* in the mouse brain

Higher magnification images from the brain shown in Figure 6g that were used for measurement of virally expressed GFP and VGlut2. Images from 8 brains were used for the quantification shown in figure 6g.

Supplementary Figure 11

miR-1000 and VGlut expression in the visual system

(a) Optical section showing *miR-1000* expression using the GFP knock-in allele (green) and VGlut protein (red) in the retina of adult eye. Scale bar = 50 μM.

(b) Optical section showing *miR-1000* expression (green) and VGlut-GAL4 driving UAS-CD8-RFP (red) in the lamina and medulla layers of the adult optic lobe. The lamina receives axonal projections from photoreceptors R1-R6 and the medulla receives projections from photoreceptor R7-R8. Scale bar = 50 μM.

(c) Optical section showing *miR-1000* expression (green) and VGlut-GAL4 (red) in medulla and lobula layers of the adult optic lobe. Scale bar = 50 μM.

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