ADAR-mediated RNA editing suppresses sleep by acting as a brake on glutamatergic synaptic plasticity

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It has been postulated that synaptic potentiation during waking is offset by a homoeostatic reduction in net synaptic strength during sleep. However, molecular mechanisms to support such a process are lacking. Here we demonstrate that deficiencies in the RNA-editing gene Adar increase sleep due to synaptic dysfunction in glutamatergic neurons in Drosophila. Specifically, the vesicular glutamate transporter is upregulated, leading to over-activation of NMDA receptors, and the reserve pool of glutamatergic synaptic vesicles is selectively expanded in Adar mutants. Collectively these changes lead to sustained neurotransmitter release under conditions that would otherwise result in synaptic depression. We propose that a shift in the balance from synaptic depression towards synaptic potentiation in sleep-promoting neurons underlies the increased sleep pressure of Adar-deficient animals. Our findings provide a plausible molecular mechanism linking sleep and synaptic plasticity.

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chronically unfulfilled sleep need contributes to numerous medical problems including depression, pain, hypertension, diabetes and cardiovascular disease. On a shorter timescale, even 1–2 nights of poor sleep result in attention deficits that can prove costly, or even deadly, in situations in which reaction time is critical. Remarkably, despite decades of intense study, the mechanisms that control sleep need and the cellular functions that they fulfill are largely unknown. In recent years much attention has focused on the hypothesis that sleep need arises from an experience-dependent increase in net synaptic strength during waking. According to the same hypothesis, sleep homeostatically reverses this increase to maintain average synaptic strength within an optimal dynamic range for synaptic plasticity. Although much experimental data support this hypothesis, criticisms persist and detailed mechanistic support for the proposed phenomenon is lacking.

We set out to identify novel sleep-regulating genes in the fruit fly, Drosophila melanogaster, due to the genetic tractability of this organism as well as the striking parallels between sleep/wake behaviour in flies and mammals, which suggest that core functions of sleep are evolutionarily conserved. This notion has been reinforced in recent years by identification of sleep-regulating genes and signalling mechanisms in flies and mice that are believed to share similar functions, including cyclic AMP signalling, voltage-gated K⁺ channels, and dopamine among others. Notably lacking, however, have been molecular discoveries relating sleep to defined forms of synaptic plasticity.

We hypothesized that if sleep is indeed functioning to maintain overall synaptic strength within a physiological range, then identifiable genes should exist that reflect the reciprocal relationship between both processes. For example, if net potentiation during waking truly drives the need to sleep, then genetic lesions resulting in increased synaptic strength should cause an increase in sleep, whereas genetic lesions resulting in decreased synaptic strength should cause a decrease in sleep. Finally, we reasoned that the resulting dysregulation should shed light on the mechanistic relation between synaptic plasticity and sleep, for which very little information is currently known.

To test these ideas we performed a genetic screen in Drosophila and found that the RNA-editing gene Adar is required for flies to maintain normal waking. Consistent with a related role in synaptic plasticity, Adar acts as a brake on sleep-promoting glutamatergic signalling through postsynaptic AMPA and NMDA receptors by reducing the reserve pool (RP) of synaptic vesicles available for release during sustained trains of neuronal activity. We therefore conclude that Adar suppresses sleep in Drosophila by negatively regulating short-term potentiation.

Results

ADAR is required for wake maintenance. We systematically screened for neuronal genes that control sleep need in D. melanogaster and tested to what extent their mechanisms of action involve synaptic plasticity. Our approach involved coupling the GAL4/UAS system to RNA interference (RNAi)-dependent knockdown of genetic targets specifically in the nervous system and then assaying for effects on daily sleep. After retesting promising lines, we found that efficient knockdown of the conserved RNA-editing gene Adar (Adenosine deaminase acting on RNA) led to increased sleep in both male and female animals (elav > Adar RNAi; Fig. 1a–c; Supplementary Fig. 1a,b; Supplementary Fig. 2a), an effect that was recapitulated with a hypomorphic Adar allele (Adar hyp) that expresses just 20% of normal Adar protein (Fig. 2a,b). Consistent with a deficit in sleep/wake control rather than in locomotion, Adar-deficient animals were at least as active during waking as controls (Fig. 1d; Supplementary Fig. 1c) and had inactive periods that could be fully overcome by mechanical agitation (Fig. 1e). To determine which component of sleep/wake control is regulated by Adar, we analysed the durations of both sleep and wake bouts and found that depletion of Adar selectively affected the latter, leading to destabilization of the waking state (Fig. 1f,g; Supplementary Fig. 1d,e).

Two possible explanations for the increase in sleep caused by reduction in Adar are altered sleep homeostasis and increased sleep pressure. To discriminate between these possibilities we sleep-deprived Adar hypomorphs and control flies during the

Figure 1 | Adar stabilizes the waking state to suppress sleep. (a) Representative sleep profiles of male elav > Adar RNAi and controls. (b) Quantification of sleep in a. Pan-neuronal knockdown of Adar increases sleep in elav > RNAi animals relative to controls. (c) Representative western blot of fly brains indicates efficient knockdown of ADAR expression in elav > Adar RNAi flies. (d) Waking activity is not reduced in elav > Adar RNAi animals. (e) Sleep in elav > Adar RNAi animals is acutely reversible by mechanical perturbation. (f) Sleep maintenance is unaffected in elav > Adar RNAi animals. (g) The wake state is destabilized in elav > Adar RNAi animals relative to controls. For each panel: elav > + (n = 39); elav > Adar RNAi (n = 54); + > Adar RNAi (n = 39). For all figures *, **, *** and **** indicate P < 0.05, 0.01, 0.001 and 0.0001, respectively, and error bars represent s.e.m.
ADAR suppresses glutamatergic signalling. Previous studies in Drosophila have suggested that ADAR protein is expressed broadly throughout the brain\(^{12}\). To map where Adar functions to modulate sleep behaviour, we initially performed an anatomical screen in which we coupled UAS-Adar RNAi to a variety of well-characterized GAL4 drivers that express in populations of circadian clock neurons, established sleep-regulating regions of the brain, and in neurons distinguishable from one another by their distinct neurotransmitter systems (Supplementary Table 1). We also screened an additional collection of \(~500\) randomly selected GAL4 lines derived from cloned putative enhancer fragments\(^{13}\). Out of both collections, the GAL4 driver 40B03 was most effective at recapitulating the increase in sleep observed with pan-neuronal knockdown of Adar (Supplementary Fig. 4).

As controls to confirm that knockdown of Adar by 40B03-GAL4 led to increased sleep, rather than a physical disability or generalized defect in CNS function, we performed a series of additional experiments. In the first, we measured the responsiveness of knockdown animals to an arousal stimulus of fixed intensity. Consistent with the rapidly reversible nature of sleep, we found that the tendency of 40B03 \(>\) Adar RNAi flies to remain immobile could be fully overcome by mechanical stimulation (Supplementary Fig. 5a). In a second experiment, we fed flies with the caffeine analogue, IBMX, and found that it was able to efficiently maintain waking in 40B03 \(>\) Adar RNAi animals (Supplementary Fig. 5b). In a third experiment, we tested climbing ability and found that 40B03 \(>\) Adar RNAi flies showed no performance deficits relative to controls (Supplementary Fig. 5c). In a fourth experiment, we tested whether increased sleep in 40B03 \(>\) Adar RNAi animals occurs through effects on selected neuronal circuits or through a general depression of neuronal function. We reasoned that widespread effects should sensitize animals to other general CNS depressants. To test this possibility we measured the amount of time it took for flies to stop responding to a repeated mechanical
Elevated DVGLUT and NMDAR activity increase sleep. Since Adar is known to alter synaptic transmission by unresolved mechanisms at the neuromuscular junction (NMJ)\textsuperscript{19}, which is glutamatergic in flies, we hypothesized that Adar's effects on sleep might be mediated by alterations in glutamatergic signalling in the central brain. To test this hypothesis, we compared sleep in Adar null mutants (Adar\textsuperscript{-/-})\textsuperscript{20} alone and in the presence of transgenic Adar expressed exclusively in glutamatergic neurons (Adar\textsuperscript{P},OK371 > Adar). As expected, Adar\textsuperscript{-/-} mutants exhibited an increase in sleep, and this phenotype was significantly attenuated in Adar\textsuperscript{P},OK371 > Adar animals (Fig. 3d). Thus, Adar expression in glutamatergic neurons is sufficient to restore nearly normal sleep to Adar-deficient animals.

Together with evidence that synaptic vesicles accumulate at the NMJ in Adar mutants\textsuperscript{19}, these data prompted us to measure protein levels of the Drosophila vesicular glutamate transporter (DVGLUT) in fly heads. Consistent with a defect in synaptic signalling in central glutamatergic neurons, we found a striking increase in DVGLUT protein in flies depleted of Adar (Fig. 4a; Supplementary Fig. 2b). To determine whether this increase was responsible for the increase in sleep we observed in Adar-deficient animals, we paired Adar hypomorphs with a single copy of either a strong hypomorph or a null dvglut allele\textsuperscript{12}. On their own, neither of the heterozygous dvglut alleles affected sleep, but we found that each one was able to suppress the excess sleep of Adar hypomorphs (Fig. 4b). Thus, compensating for elevated DVGLUT restores normal sleep to Adar-deficient animals. Interestingly, even more severe reductions in dvglut expression led to significantly less sleep than in controls (Supplementary Fig. 6), suggesting that levels of glutamatergic signalling must be tightly maintained within a narrow range to avoid excesses and shortfalls in daily sleep.

To determine the identities of postsynaptic mediators of increased glutamatergic signalling in Adar-deficient animals, we knocked down various glutamate receptor transcripts in the fly genome while simultaneously reducing Adar expression (Supplementary Fig. 7). Interestingly, knockdown of either of two NMDA-type glutamate receptors, NR1 or NR2, was sufficient...
to partially or completely restore normal sleep to animals depleted of Adar. Similar results were obtained with two independent NR2 RNAi lines (Fig. 4c). Since activation of NMDA receptors is known to require simultaneous synaptic release of glutamate and depolarizing current through AMPA-type glutamate receptors, AMPA receptor signalling would also be expected to be involved in Adar-dependent increases in sleep. Consistent with this expectation, we observed restoration of normal sleep to Adar-deficient animals on knockdown of the AMPA receptor transcript GluRI (Supplementary Fig. 7). Thus, Adar is required to reduce signalling through excitatory glutamate receptors in the brain.

**An expanded RP increases sleep in Adar mutants.** Enhanced excitatory glutamate signalling through AMPA/NMDA receptors could be achieved through several distinct mechanisms related to elevated DVGGLUT expression. First, each synaptic vesicle could contain more DVGGLUT, thus increasing the amount of glutamate packaged into each vesicle, which would result in an increase in quantal size. In the absence of accessible and relevant neurons in the adult central nervous system from which to characterize synaptic properties, we tested this possibility by measuring spontaneous miniature excitatory postsynaptic potentials (mEPSPs) at the larval glutamatergic NMJ, which is a model for central glutamatergic synaptic transmission. In this preparation, a null allele of Adar has been reported to exhibit increased mEPSP amplitude. However, these changes were not detectable with more moderate reductions in Adar that are still able to increase sleep (Figs 5a–c; 2a,b). Second, more vesicles could be released per action potential that arrives at presynaptic terminals. However, we did not measure a difference in quantal content in Adar hypomorphs relative to controls, as reflected in the amplitude of evoked EPSPs at the NMJs of both groups of animals (Fig. 5d–f). These data strongly suggest that reductions in Adar that are sufficient to increase sleep do not alter the amount of glutamate in each synaptic vesicle or the number of vesicles released per action potential during baseline synaptic transmission.

These measurements also permitted us to clearly distinguish changes in synaptic vesicle size and number from potential expansions or contractions of different vesicle pools that could alternatively underlie constitutive potentiation of glutamatergic synapses. Specifically, we hypothesized that the increased sleep observed in Adar-depleted flies was mediated by an increase in availability of glutamatergic vesicles during sustained neuronal activity. In support of this hypothesis, a previous study reported that Adar mutants accumulate synaptic vesicles and vesicle-related proteins at the NMJ. We also tested our hypothesis by stimulating axons at the NMJ for 10 min at 15 Hz to deplete synaptic vesicle pools while measuring the quantal content per stimulus (Fig. 6a). As described by others, we found that change in quantal content followed two temporally distinct phases: an initial period involving rapid decay followed by a subsequent period of slower, more sustained decay. Such changes have been attributed to fast and slow depletion of what are often referred to as the readily releasable pool (RRP) and RP of synaptic vesicles, respectively. Intriguingly, in Adar hypomorphs with increased sleep we found that the fast phase decayed more quickly (Fig. 6b) and the slow phase decayed more slowly than in controls (Fig. 6c). These data are consistent with both an expanded RP in Adar mutants and chronically potentiated glutamatergic signalling, which could be a signal to sleep.

To determine whether our observations at the larval NMJ were relevant to altered sleep in Adar-deficient adults and whether a larger RP size was responsible for the increase in sleep we observed in Adar mutants, we focused on the possible genetic interaction between Adar and Synapsin (Syn). Syn encodes a synaptic vesicle protein that is thought to act as a barrier to
transitions from the RP to the RRP\textsuperscript{24}. A reduction in the amount of Syn should thus lower the barrier to this transition. By this logic, if increased sleep in Adar mutants results from an expanded glutamatergic RP, then reducing the levels of Syn should compensate for this effect. To test this hypothesis we coupled a hypomorphic mutation in Adar that increased sleep with a heterozygous null mutation in Syn (Syn\textsuperscript{97}; ref. 25) and measured rates of depletion of the RP and RRP during high-frequency presynaptic stimulation of the NMJ. Consistent with our hypothesis, the rate of depletion of the RP was restored nearly to control levels in Adar\textsuperscript{hyp} mutants. Importantly, sleep was not reduced by heterozygous Syn\textsuperscript{97} mutants. Thus, it is likely that glutamatergic signalling is tightly regulated to maintain proper levels and timing of sleep—a process in which we have now implicated Adar.

In elucidating the pathway through which Adar acts to regulate sleep, we found two points at which synaptic plasticity might be implicated. The first is based on our observation that the sleep-promoting effects of Adar mutants require AMPA and NMDA receptors. NMDA receptors are frequently involved in synaptic plasticity\textsuperscript{33–35}, making them prime candidates for potentiated synaptic responses that have been proposed to accumulate during waking and in turn drive compensatory sleep need. In fact, a recent report has described NR1 as a novel sleep-promoting gene in Drosophila\textsuperscript{36}. Here we have confirmed that NMDA receptors promote sleep, and we have demonstrated that they are required for the sleep phenotype of Adar mutants. Thus, it is likely that a major role of wild-type Adar is to act as a presynaptic brake on NMDA-dependent postsynaptic potentiation.

We were able to more thoroughly investigate the second mechanism by which Adar appears to regulate glutamatergic synaptic plasticity to influence sleep. In this case, we found that basal synaptic transmission in glutamatergic neurons was not altered by depletion of Adar. That is, Adar hypomorphs had
normal glutamate loading into synaptic vesicles, postsynaptic responses to spontaneously released glutamate, and number of glutamatergic vesicles released per evoked excitatory postsynaptic potential. However, upon sustained stimulation, Adar mutants exhibited decreased synaptic depression that could be compensated by reducing Syn, a gene that limits depletion of the RP\textsuperscript{31}. Notably, reducing Syn was also sufficient to reduce sleep in Adar mutants to control levels. Our data thus support the hypothesis that Adar mutants have an expanded synaptic RP, which reduces depression of sleep-promoting glutamatergic neurons to increase intrusions of sleep into the waking state.

Expansion of the RP of synaptic vesicles, like those we have observed in Adar mutants, has been shown to substantially increase the amount of information transmitted per burst of action potentials due to decreased short-term depression\textsuperscript{32}. In essence, this phenomenon shifts the balance of depression and potentiation towards the latter. We suggest that in glutamatergic sleep-promoting neurons, this shift translates to an increased probability of sleep onset from spike trains that would normally be subthreshold for the behaviour.

Although our studies flesh out mechanistic details linking modulation of short-term plasticity to sleep, hints of such a connection exist from other studies as well. For example, loss-of-function mutations in the Fragile X mental retardation 1 (Fmr1) gene cause an expansion of total synaptic vesicles in mice\textsuperscript{33} and an increase in sleep in Drosophila\textsuperscript{34}. Conversely, loss of calcineurin impairs synaptic vesicle recycling in mice, resulting in enhanced synaptic depression\textsuperscript{40}, and causes reduced sleep in Drosophila\textsuperscript{31,42}. Although these effects and our own findings are consistent with a positive correlation between short-term synaptic potentiation and sleep, other findings suggest a more complex relationship. For example, loss of Rab3a in mice reduces the probability of synaptic vesicle release following a train of action potentials\textsuperscript{43}, thereby increasing synaptic depression, and increases non-REM sleep\textsuperscript{44}. In addition, loss of the Rab3-interacting molecule RIM\textsubscript{1\alpha} reduces presynaptic calcium influx in mice, thereby increasing short-term facilitation\textsuperscript{45}, and reduces REM sleep\textsuperscript{46}. Rab3a and RIM\textsubscript{1\alpha} mutant mice have other phenotypes as well, thus complicating the establishment of a clear relationship between short-term plasticity and sleep. Nonetheless, although the directionality of change for each phenomenon may depend on which neural circuits are impacted, there is ample evidence linking short-term plasticity and sleep in various genetic perturbations. Our own findings underscore this idea.

It will be interesting to determine if similar mechanisms link sleep to potentiating responses involved in other forms of behavioural plasticity. The link between RNA editing and glutamatergic plasticity that we have described may be particularly fruitful to explore in other contexts as well. Hints of such a relationship have been described previously. For example, early observations of the role of RNA editing in the brain showed that ADAR functions postsynaptically to limit Ca\textsuperscript{2+} permeability and channel conductance of GluR2-containing receptors\textsuperscript{47,48}. Furthermore, editing of GluR2, GluR3 and GluR4 at the R/G position has been shown to alter receptor gating kinetics, resulting in more rapid desensitization and recovery from desensitization\textsuperscript{49}. Together with evidence that RNA editing is reduced in glutamate excitotoxic diseases such as ALS\textsuperscript{50–52}, we suggest that a major function of RNA editing in the nervous system is to limit glutamatergic signalling. Since we have shown that ADAR acts as a presynaptic glutamatergic brake in flies, it will be interesting to determine whether this function is conserved in mammalian nervous systems and whether ADAR is also able to achieve the same effect through postsynaptic regulatory mechanisms.

Methods

Fly strains. D. melanogaster were grown at room temperature (20–22 °C) on standard cornmeal media with yeast. Unless otherwise indicated, all animals were outcrossed a minimum of 5 times into a w\textsuperscript{118} is031 genetic background. Adar\textsuperscript{HSA} and Adar\textsuperscript{BP} were obtained from Dr. Robert Reenan. Adar\textsuperscript{9} and UAS-Adar were obtained from Dr. Gabriel Haddad. dygut\textsuperscript{1} and Djf2/dygut\textsuperscript{2} were obtained from Dr. Aaron DeAntonio. The Adar RNAi line, 776a, was obtained from the Vienna Drosophila Resource Center (http://stockcenter.vdrc.at/control/main) and was used in the presence of UAS-Dicer in all experiments. UAS-CDB-RFP, UAS-Dicer (second and third chromosome insertions), elav-Gal4, dygut\textsuperscript{1}UAS::RFP, OK371-Gal4, Syn\textsuperscript{2+}, NRI RNAi (HMS02200), NR1 #1 RNAi (HMS02192), NR2 #2 RNAi (HMS02176), GluR8 RNAi (HMS02153), GluR8 RNAi (JF02752), GluR8 RNAi (JF03145), GluR1 RNAi (JF03145), GluR1 RNAi (JF03154), GluR1 RNAi (JF08154), GluR1d RNAi (JF02035), GluRIIE RNAi (JF01962), Clamy1 RNAi (JF02987), CG3822 RNAi (JF01873), CG5621 RNAi (JF01840) and CG1155 RNAi (JF03425), mGluR4 RNAi (JF01958): refs 53, 54, 40B03-Gal4 and all other Gal4 lines\textsuperscript{26} were obtained from the Bloomington Stock Center (http://flystocks.bio.indiana.edu/). Male flies were used for all behavioural assays unless otherwise indicated.

Sleep deprivation and rebound. Flies in DAM2 Drosophila activity monitors (Trikinetikas). Sleep deprivation was performed as described ( NIH, National Institutes of Health). For all experiments. For all behavioural assays unless otherwise indicated.

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Complete Protease Inhibitors). Lysates were cleared of particulate debris by centrifugation at 5,000g for 5 min at 4 °C before protein quantification. Lysates were resolved on 10-well 4–12% NuPAGE SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were probed using 1:500 mouse anti-HA (Covance), 1:10,000 rabbit anti-DVGLUT2 and 1:10,000 mouse anti-actin primary antibodies (EMD Millipore) followed by 1:5,000 anti-mouse, 1:5,000 anti-rabbit and 1:10,000 anti-mouse HRP antibodies, respectively (VWR). Visualisation of secondary antibodies was achieved using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Images in the main figures have been cropped for presentation. Full size images are presented in Supplementary Fig. 2.

Electrophysiology. All dissections and recordings were performed in modified HL3 saline containing (in mM): 70 NaCl, 5 KCl, 10 MgCl2, 10 NaHCO3, 115 Sucrose, 5 Trehalose, 5 HEPES and 0.4 CaCl2 (unless otherwise specified), pH 7.2. Neuromuscular junction sharp electrode (electrode resistance between 10 and 35 MΩ) recordings were performed on muscles 6 and 7 of abdominal segments A2 and A3 in wandering third instar larvae. Larvae were cultured in standard molasses medium, raised at 25 °C and dissected; the guts, trachea and ventral nerve cord were removed from the larval body walls with the motor nerve carefully cut, and the preparation was rinsed several times with HL3 saline. Recordings were performed on an Olympus BX51WI microscope using a ×40/0.80 water-dipping objective. Recordings were acquired using Axoclamp 900A amplifiers, Digidata 1440A acquisition system and pClamp 10.5 software (Molecular Devices). Electrophysiological sweeps were digitized at 10 kHz, and filtered at 1 kHz. Data were analysed using Clampfit (Molecular devices), MiniAnalysis (Synapsoft) and Excel (Microsoft). mEPSPs were recorded in the absence of any stimulation, and cut motor axons were stimulated at ~5 mA for 3 ms to elicit mEPSPs. To fine tune stimulus intensity, an ISO-Flex stimulus isolator was used (A.M.P.L.). Intensity was adjusted for each cell, set high enough to consistently elicit full responses in both axes innervating the muscle segment. Average mEPSP amplitude, EPSP amplitude, and quantal content were calculated for each genotype with corrections for nonlinear summation58. Muscle input resistance (RM) and resting membrane potential (Vrest) were monitored during each experiment. Recordings were rejected if the Vrest was more depolarized than ~60 mV, if the RM was less than 5 MΩ, or if either measurement deviated by more than 10% during the experiment. For synaptic vesicle rundown experiments, postsynaptic responses for each preparation were fit to a single exponential curve RPR or averaged into 30 s bins and fit from 60 s to 9 min by linear regression (RP).

Data analysis and statistics. Replicates (n values) represent the number of biological replicates for each experimental condition. Bar graphs depict the median and 25th to 75th percentiles (box) and minimum/maximum values (whiskers). Unless otherwise indicated, all data are expressed as a fold change of control. All dissections and recordings were analysed with unpaired Student’s t-test followed by Welch’s correction for comparisons between two groups. For experiments of single factor design, we analysed data using one-way analysis of variance followed by the Tukey–Kramer test for multiple comparisons or Sidak’s multiple comparisons test for select comparisons. Two-way analysis of variance followed by Sidak’s multiple comparisons test was used to analyse experiments of two-factor design. For datasets from non-Gaussian distributions, comparisons were performed using Kruskal–Wallis test followed by Dunn’s multiple comparisons test. All statistical tests were two-sided and performed using Prism 6.0 for Mac OS X (GraphPad Software).

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Author contributions
J.E.R., D.K.D. and W.J.J. conceived and designed the experiments; J.E.R. and J.P. performed the experiments. J.E.R. and W.J.J. analysed the data and wrote the manuscript with contributions from D.K.D.

Additional information

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