nature neuroscience

Modulation of $GABA_A$ receptor desensitization uncouples sleep onset and maintenance in *Drosophila*

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Many lines of evidence indicate that GABA and $GABA_A$ receptors make important contributions to human sleep regulation. Pharmacological manipulation of these receptors has differential effects on sleep onset and sleep maintenance insomnia. Here we show that sleep is regulated by GABA in *Drosophila* and that a mutant $GABA_A$ receptor, Rdl^{A302S}, specifically decreases sleep latency. The drug carbamazepine (CBZ) has the opposite effect on sleep; it increases sleep latency as well as decreasing sleep. Behavioral and physiological experiments indicated that Rdl^{A302S} mutant flies are resistant to the effects of CBZ on sleep latency and that mutant RDL^{A302S} channels are resistant to the effects of CBZ on desensitization, respectively. These results suggest that this biophysical property of the channel, specifically channel desensitization, underlies the regulation of sleep latency in flies. These experiments uncouple the regulation of sleep latency from that of sleep duration and suggest that the kinetics of $GABA_A$ receptor signaling dictate sleep latency.

Insomnia is the most common sleep problem and affects approximately one third of the adult American population[1]. Patients with insomnia are generally subdivided into three categories: sleep onset insomnia, sleep maintenance insomnia and terminal insomnia (early-morning awakening coupled with an inability to return to sleep)[2]. The biological basis for these insomnia classifications remains unknown. Nonetheless, a single class of drugs, agonistic modulators of $GABA_A$ receptors, effectively ameliorates these diverse symptoms[2,3]. $GABA_A$ receptors are a family of pentameric ligand-gated Cl^- channels[4] and are a major source of inhibitory currents throughout the CNS[5,6]. These receptors are also an important target for pharmacologic treatment of many other neurological disorders in addition to sleep[7].

The fruit fly *Drosophila melanogaster* is an ideal model for dissecting the relationships between molecules and behaviors, as well as between different sleep states[8,9]. As in mammals, it has been shown that the sleep-like state of *Drosophila* is associated with reduced sensory responsiveness and reduced brain activity[10,11], and is subject to both circadian and homeostatic regulation[12,13]. Researchers have also identified a number of genes[14,15], circuits[16,17] and biological processes[18] that affect fly sleep. However, there is no reported role for GABA and no reported manipulation of GABA receptors in fly sleep studies.

The first $GABA_A$ receptor mutant was isolated from pesticide-resistant *Drosophila* and the locus was named *Resistant to dieldrin* (*Rdl*)[19,20]. Similar to mammalian $GABA_A$ receptors, RDL channels mediate fast inhibitory neurotransmission[21] and are expressed in the CNS[22]. Notably, the mutation that causes the insecticide resistance phenotype (A302S)[20] specifically decreases the rate of RDL desensitization with little or no effect on other channel properties[23]. As a consequence, the mutant receptor has a longer single channel open duration and, therefore, increased channel current, at least under certain conditions (see below). Because of these characteristics, and because this mutation does not have obvious effects on health or viability, we decided to establish the importance of GABAergic transmission to sleep in flies and to examine the effects of the *Rdl* mutation.

Interestingly, flies with this mutant $GABA_A$ receptor subunit slept more, primarily because of decreased sleep latency. CBZ, which accelerates RDL desensitization, had the opposite effect and increased sleep latency. Behavioral and electrophysiological experiments indicated that the *Rdl* mutant was largely resistant to the effects of CBZ, both its ability to alter desensitization and to decrease sleep latency. These results suggest that these two phenomena are mechanistically linked. To account for these observations, we propose that sleep is initiated by fast-firing GABAergic neurons.

RESULTS

To determine whether GABAergic transmission is important for sleep in *Drosophila*, we first used the GAL4/UAS system[24] to decrease GABA release by expressing the hyperpolarizing potassium channel Shaw[25] in GABAergic neurons. Total sleep was reduced, due in turn to an increase in mean wake-episode duration and to a decrease in sleep-bout duration. There was also an increase in the night-time sleep latency, consistent with a role for GABAergic tone in regulating both the initiation and maintenance of sleep (**Fig. 1**).

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Received 27 November 2007; accepted 3 January 2008; published online 27 January 2008; doi:10.1038/nn2046

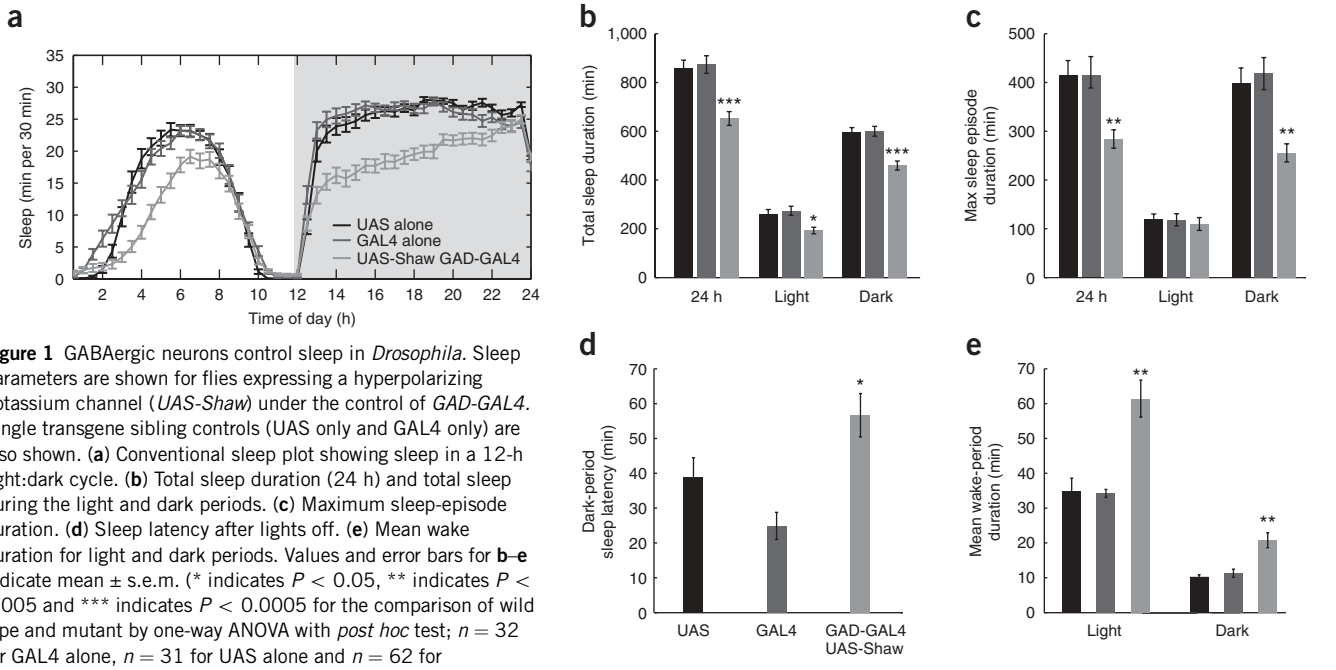

Figure 1 GABAergic neurons control sleep in *Drosophila*. Sleep parameters are shown for flies expressing a hyperpolarizing potassium channel (*UAS-Shaw*) under the control of *GAD-GAL4*. Single transgene sibling controls (UAS only and GAL4 only) are also shown. (**a**) Conventional sleep plot showing sleep in a 12-h light:dark cycle. (**b**) Total sleep duration (24 h) and total sleep during the light and dark periods. (**c**) Maximum sleep-episode duration. (**d**) Sleep latency after lights off. (**e**) Mean wake duration for light and dark periods. Values and error bars for **b**–**e** indicate mean ± s.e.m. (* indicates $P < 0.05$, ** indicates $P < 0.005$ and *** indicates $P < 0.0005$ for the comparison of wild type and mutant by one-way ANOVA with *post hoc* test; $n = 32$ for GAL4 alone, $n = 31$ for UAS alone and $n = 62$ for *GAD-GAL4;UAS-Shaw*).

To investigate the potential role of *Rdl*, the sleep pattern of an extensively outcrossed homozygous Rdl^{A302S} strain, Rdl^{MDRR} (see Methods), was assayed under standard light-dark (12-h:12-h light-dark cycles) conditions (**Fig. 2**). As $GABA_A$ currents in Rdl^{A302S} fly neurons have longer single channel open durations[23], we surmised that these flies might have increased sleep, as a result of either decreased sleep latency and/or increased sleep duration. Indeed, Rdl^{MDRR} flies had decreased sleep latency (compare the beginning of dark period in the control and Rdl^{MDRR} genotypes in **Fig. 2a**; see **Fig. 2c**, left for quantification). They also had a small, but significant, increase in total daily (24 h) sleep ($P < 0.005$; **Fig. 2b**, left).

Despite the extensive outcrossing of the Rdl^{MDRR} A302S mutant chromosome, we made two additional chromosome combinations to validate these results. We assayed an Rdl^{MDRR}/Rdl^{1} combination, which creates a hemizygous Rdl^{A302S} allele because of the Rdl^{1} null mutation[26]. We also assayed an independent homozygous Rdl^{A302S} combination, made by crossing our outcrossed Rdl^{MDRR} chromosome with an independent Rdl^{A302S} mutant chromosome, Rdl^{CB1}. The latter was previously identified in another ethyl methanesulfonate screen on a different background chromosome. Both of the new combinations had a very similar sleep latency phenotype (**Fig. 2c**, middle and right), and the Rdl^{A302S}/Rdl^{1} combination had little effect on total sleep duration (**Fig. 2b**, right). None of these genotypes had substantial increases in locomotor activity during active periods, indicating that these sleep phenotypes are not the result of simple hyperactivity (data not shown). We conclude that the most consistent effect of the Rdl^{A302S} mutation is to decrease sleep latency.

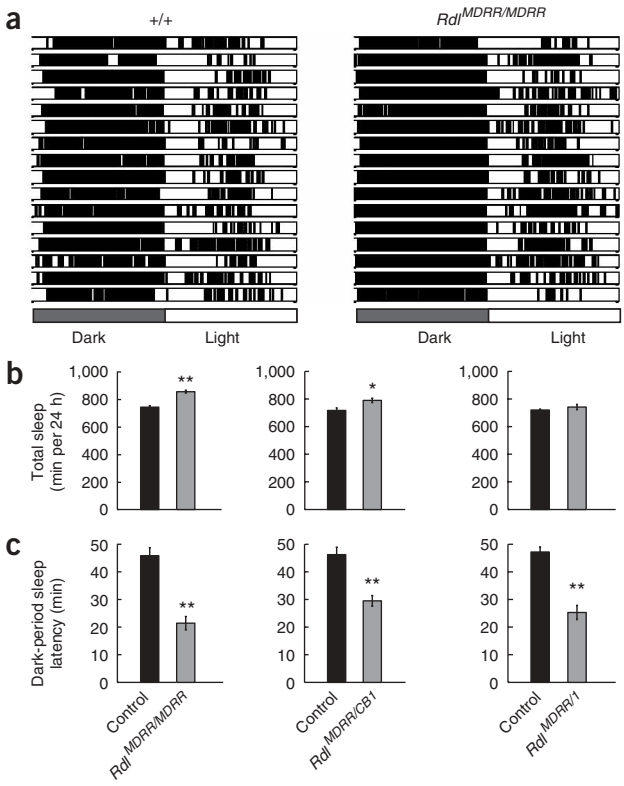

Figure 2 Rdl^{A302S} flies show decreased sleep latency and increased sleep-episode duration. Rdl^{MDRR} and Rdl^{CB1} are independently isolated A302S point mutations in the *Rdl* gene. Wild-type sibling controls for each experimental genotype are shown. (**a**) Raster plots showing the sleep/wake pattern of 16 individual wild-type (+/+, left) and Rdl^{MDRR} (right) flies during a 24-h (12 h:12 h dark:light) period. White areas represent periods of wakefulness (periods of movement, or inactivity <5 min), whereas the black areas show sleep periods (inactive periods >5 min). (**b**) Quantification and comparison of total 24-h sleep between wild-type ($n = 135$) and Rdl^{MDRR} ($n = 155$, left) flies, wild-type ($n = 51$) and Rdl^{MDRR}/Rdl^{CB1} ($n = 49$, middle) flies, and wild-type ($n = 277$) and Rdl^{MDRR}/Rdl^{1} ($n = 50$, right) flies. (**c**) Sleep latency after lights off for same genotypes. Values and error bars for **b** and **c** indicate mean ± s.e.m. (* indicates $P < 0.005$, ** $P < 0.0005$ for the comparison of wild type and mutant by two-tailed Student's *t*-test).

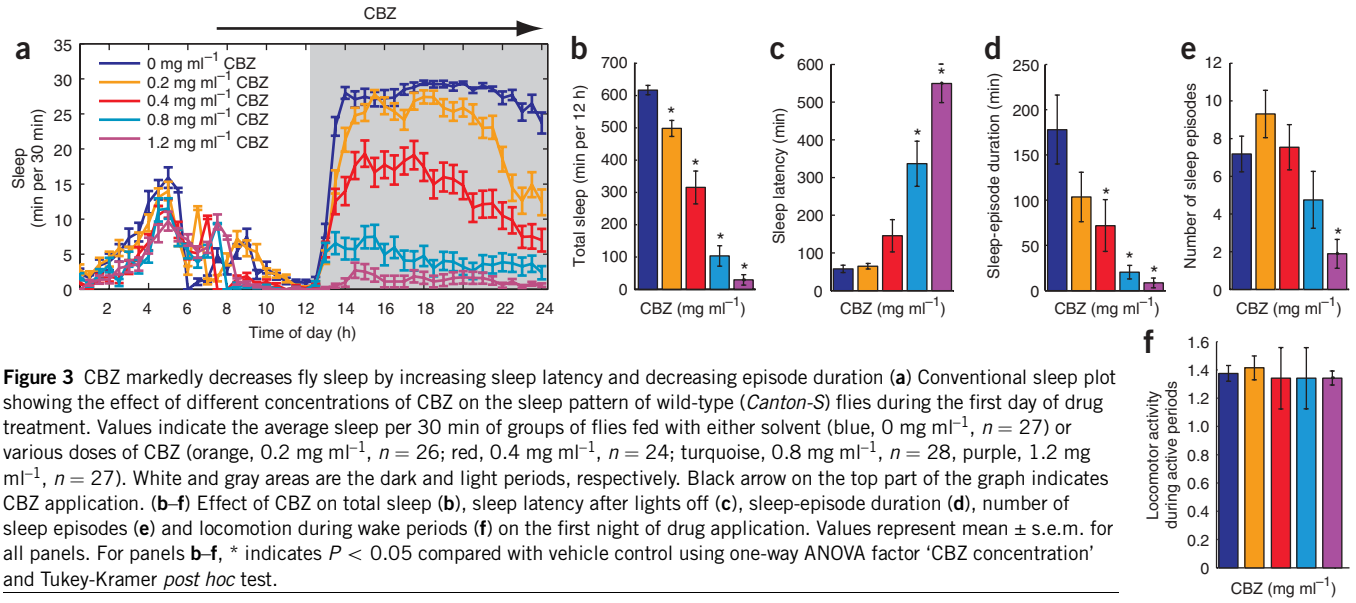

Figure 3 CBZ markedly decreases fly sleep by increasing sleep latency and decreasing episode duration (**a**) Conventional sleep plot showing the effect of different concentrations of CBZ on the sleep pattern of wild-type (*Canton-S*) flies during the first day of drug treatment. Values indicate the average sleep per 30 min of groups of flies fed with either solvent (blue, 0 mg ml^{-1}, $n = 27$) or various doses of CBZ (orange, 0.2 mg ml^{-1}, $n = 26$; red, 0.4 mg ml^{-1}, $n = 24$; turquoise, 0.8 mg ml^{-1}, $n = 28$, purple, 1.2 mg ml^{-1}, $n = 27$). White and gray areas are the dark and light periods, respectively. Black arrow on the top part of the graph indicates CBZ application. (**b**–**f**) Effect of CBZ on total sleep (**b**), sleep latency after lights off (**c**), sleep-episode duration (**d**), number of sleep episodes (**e**) and locomotion during wake periods (**f**) on the first night of drug application. Values represent mean ± s.e.m. for all panels. For panels **b**–**f**, * indicates $P < 0.05$ compared with vehicle control using one-way ANOVA factor 'CBZ concentration' and Tukey-Kramer *post hoc* test.

In a search for a complementary approach to better understand the effect of the Rdl^{A302S} mutation, we first assayed the sleep effects of a number of drugs on wild-type flies. Only the human anticonvulsant CBZ had a marked dose-dependent effect, with the highest dose (1.2 mg ml^{-1}) almost completely inhibiting night-time sleep (**Fig. 3a**,**b**). This was the result of both an increase in sleep latency (**Fig. 3c**) and a decrease in sleep-episode duration (**Fig. 3d**). The number of sleep episodes appeared to show a biphasic response, with an increase at a low dose and a prominent, significant decrease at a high dose ($P < 0.05$; **Fig. 3e**). Locomotor activity during active periods was unchanged (**Fig. 3f**), indicating that the sleep effects were not the result of hyperactivity.

To relate these CBZ effects to the Rdl^{A302S} phenotypes described above, we assayed the sleep of the homozygous Rdl^{A302S} strain after CBZ feeding (**Fig. 4**). The drug effects were less potent than in wild-type flies (**Fig. 4a**,**b**; also compare **Fig. 4a** with **Fig. 3a**). This was because the sleep latency of Rdl^{A302S} flies was almost completely resistant to CBZ; wild-type flies showed a large dose-dependent increase in sleep latency, whereas Rdl^{A302S} latency remained unaffected, even at high levels of the drug (**Fig. 4c**). This suggests that the latency effects of CBZ and the Rdl^{A302S} mutation share a common mechanism. In contrast, the mutant flies were equally sensitive to the sleep-duration effects of the drug (**Fig. 4d**). These data pharmacologically uncouple sleep latency from sleep duration.

The experiments also shed light on the mechanisms underlying sleep homeostasis. A decrease in sleep-episode duration with CBZ should normally increase sleep pressure, resulting in a compensatory increase in sleep-episode number. The CBZ-dependent block

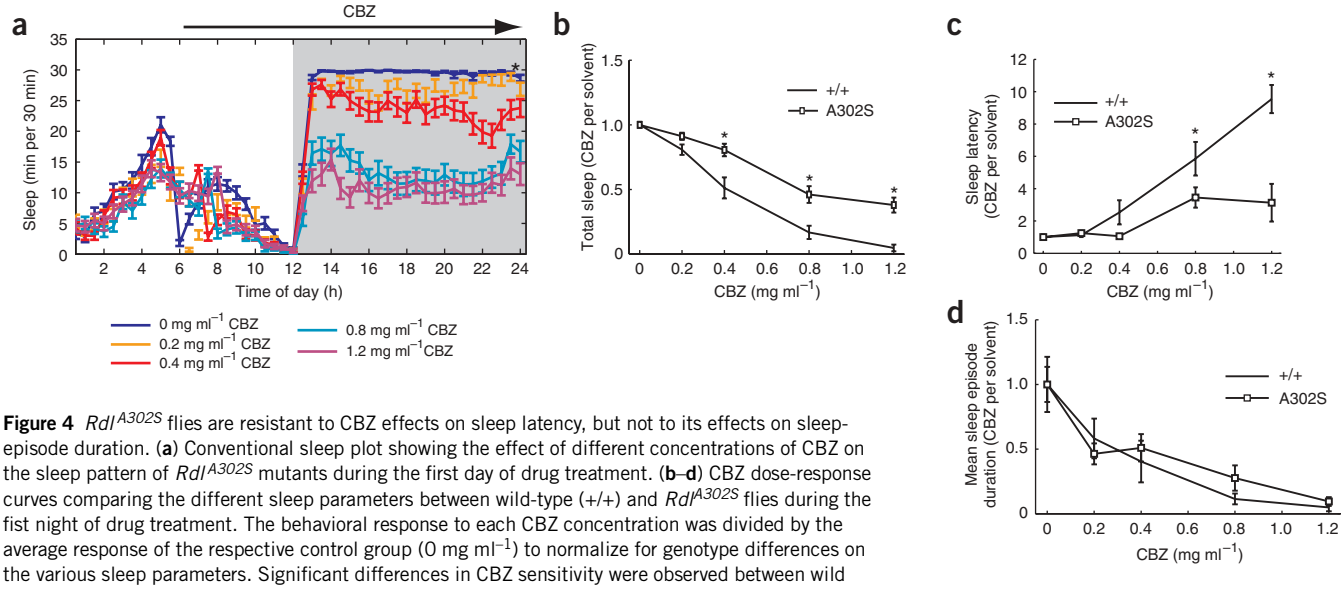

Figure 4 Rdl^{A302S} flies are resistant to CBZ effects on sleep latency, but not to its effects on sleep-episode duration. (**a**) Conventional sleep plot showing the effect of different concentrations of CBZ on the sleep pattern of Rdl^{A302S} mutants during the first day of drug treatment. (**b**–**d**) CBZ dose-response curves comparing the different sleep parameters between wild-type (+/+) and Rdl^{A302S} flies during the fist night of drug treatment. The behavioral response to each CBZ concentration was divided by the average response of the respective control group (0 mg ml^{-1}) to normalize for genotype differences on the various sleep parameters. Significant differences in CBZ sensitivity were observed between wild type and Rdl^{A302S} in total sleep (0.4, 0.8 and 1.2 mg ml^{-1}; **b**) and sleep latency (0.8 and 1.2 mg ml^{-1}; **c**), but not mean sleep-episode duration (**d**). * indicates $P < 0.05$ by two-way ANOVA with 'genotype' and 'CBZ concentration' as factors. In order of increasing dose, $n = 27$, 26, 24, 28 and 27 for the A302S mutant Rdl^{MDRR}.

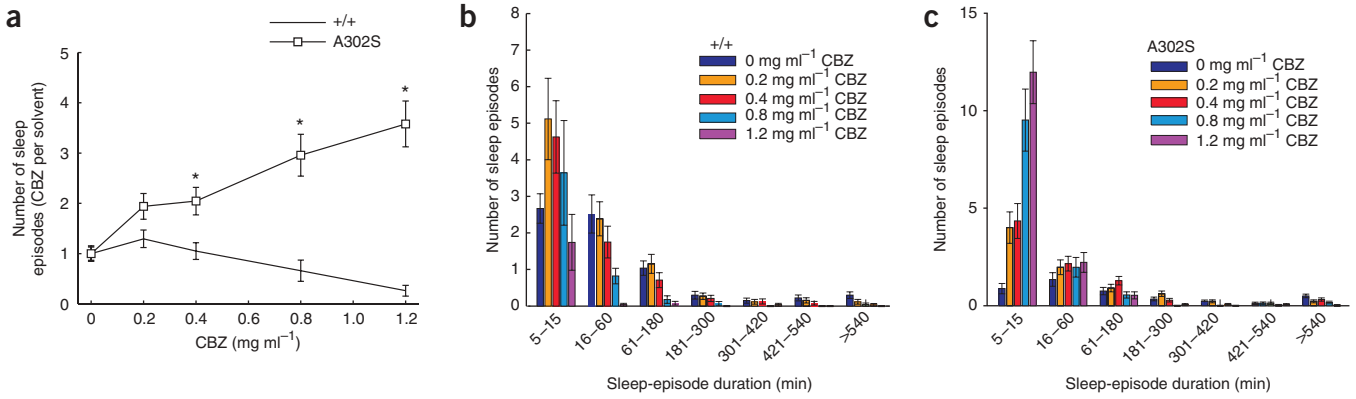

Figure 5 The *Rdl*^{A302S} mutation rescues sleep homeostasis. (**a**) Significant differences in CBZ sensitivity were observed between wild type and *Rdl*^{A302S} in the number of sleep episodes at 0.4, 0.8 and 1.2 mg ml^{-1}, showing that the *Rdl*^{A302S} mutant can respond homeostatically to sleep deprivation. * indicates $P < 0.05$ by two-way ANOVA with 'genotype' and 'CBZ concentration' as factors. (**b**,**c**) Sleep-episode bout length distribution in wild-type (**b**) and *Rdl*^{A302S} (**c**) flies with increasing amounts of CBZ.

of sleep initiation, as well as sleep duration, in wild-type flies apparently affected this compensation by preventing an increase in the number of sleep episodes (**Fig. 5a**). In contrast, *Rdl*^{A302S} flies were able to increase the number of sleep episodes and, therefore, compensate for the decreased sleep duration, presumably because their ability to fall asleep was less affected by the drug (**Fig. 5a**). This was also apparent in the marked difference in short 5–15 min sleep episodes between genotypes; only *Rdl*^{A302S} flies were able to initiate more short episodes despite increasing drug concentrations (**Fig. 5b**,**c**). These data further support the idea that sleep latency and duration are separable processes in flies and suggest that the *Rdl* $GABA_A$ receptor is the target responsible for the sleep latency effects of CBZ.

To assay whether CBZ might directly affect the RDL channel, we expressed RDL in *Xenopus* oocytes and measured current in the presence of increasing concentrations of CBZ. CBZ specifically increased RDL desensitization without affecting peak amplitude after a single GABA pulse (**Fig. 6a**,**b**). However, an increase in desensitization might alter the peak current amplitude of GABA pulse trains if they were sufficiently frequent to prevent channel recovery before the next pulse. Indeed, CBZ had a peak amplitude effect with this kind of GABA pulse-train stimulation protocol (**Fig. 6c**). We concluded that the GABA application frequency determines the effect of CBZ on current amplitude. Importantly, the CBZ effect on RDL desensitization was completely blocked by the A302S mutation, which has a potent effect on desensitization without drug (ref. 23 and **Fig. 6d**).

These data imply that the ability of the *Rdl* channel to desensitize is critical for sleep onset and provide a coherent, biophysical explanation for the drug's, as well as the mutant's, effects on sleep latency. Because desensitization is expected to have more significant effects on total current at synapses receiving high frequency input (**Fig. 6**), we propose a model (**Supplementary Fig. 1** online) in which fast-firing GABAergic (sleep initiation) neurons induce sleep onset by inhibiting wake-promoting neurons. The desensitization-induced synaptic current reduction at sleep initiation–wake promoting synapses is enhanced by CBZ, which leads to a faster current decrease and increased sleep latency. The A302S mutant channel shows reduced desensitization, as well as reduced CBZ-sensitivity, consistent with the observed *in vivo* effects in these flies. The lack of a differential (A302S versus wild type) CBZ effect on sleep duration suggests that other, slower-firing GABAergic inputs maintain sleep (sleep maintenance neurons) and that the CBZ effects on sleep duration may also involve effects on other sleep-relevant molecules.

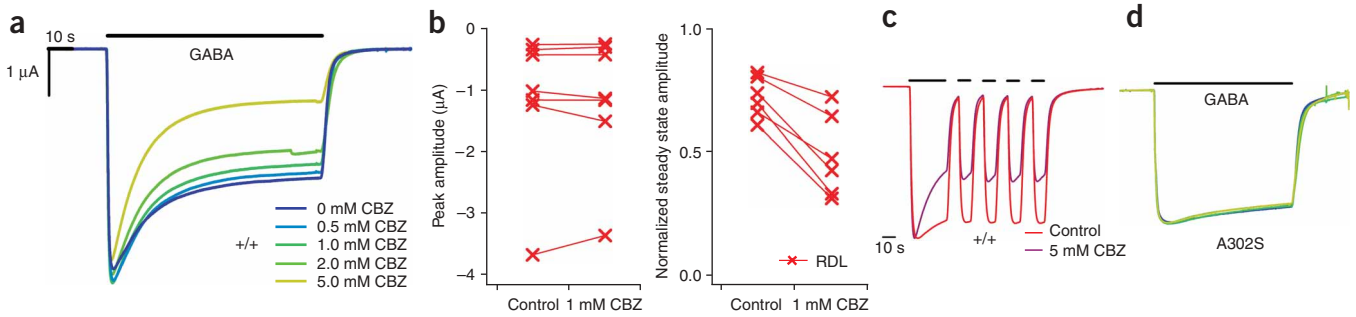

Figure 6 CBZ specifically increases RDL desensitization and the A302S mutation prevents CBZ effects (**a**) Response to 90-s application of 100 µM GABA with variable doses of CBZ, recorded from oocytes expressing RDL held at –60 mV under voltage clamp. (**b**) Comparison of current amplitudes with (1 mM CBZ) and without (control) drug perfusion. Change in peak amplitude was not statistically significant (left, $P > 0.9$, paired *t*-test), whereas steady state amplitude was significantly decreased ($P < 0.005$, paired *t*-test). Steady state amplitude was calculated by normalizing the peak current amplitude to 1 and fitting to a single exponential equation. (**c**) Current evoked by successive pulses of 50 µM GABA with and without CBZ. (**d**) Response of oocytes expressing A302S mutant channel to 100 µM GABA under the same conditions as in **a**. Traces in **c** and **d** were normalized to the first peak amplitude.

DISCUSSION

Here we show that GABA and $GABA_A$ receptors are important for sleep in *Drosophila*. To the best of our knowledge, this is the first demonstration in an animal model that sleep onset and maintenance are differentially regulated by $GABA_A$ receptors, as well as by a pharmacological agent. The ability of *Rdl* mutant flies to mount a homeostatic response to the sleep-depriving drug CBZ further suggests that the sleep-regulating neurons that express this receptor are part of the core sleep circuit. These findings further support the use of *Drosophila melanogaster* as a model for understanding the mechanisms and function of mammalian sleep.

Our results suggest that GABAergic inputs control both the onset and maintenance of sleep, as both latency and duration of sleep episodes were affected by suppressing GABAergic transmission. Our finding that fast-desensitizing $GABA_A$ receptors preferentially controlled sleep initiation suggests that there are fast-spiking GABAergic sleep-initiation neurons that synapse onto RDL-expressing postsynaptic wake-promoting neurons (**Supplementary Fig. 1**). In this model, RDL channel desensitization should decrease wake-promoting hyperpolarization. The virtue of desensitization in this context is that it helps prevent spurious and/or unwanted sleep episodes. Sleep drive must be sufficient for sleep-initiation firing to occur for a sufficiently long time to achieve wake-promoting silencing. Only under conditions that sustain high-frequency sleep-initiation neuron firing does the animal fall asleep. Once wake-promoting neurons are quiescent, putative sleep-maintenance neurons are released from inhibition and act to maintain wake-promoting neurons below their action potential threshold. Other GABA receptor subunits (and/or other transmitter systems) are presumably more important than RDL for sleep-maintenance neurons. At the end of the night, extrinsic inputs, regulated by circadian as well as by homeostatic factors, presumably inhibit the sleep-promoting regions (sleep maintenance and sleep initiation) and the fly wakes up.

The latency assay, the time it takes flies to fall asleep once the lights have turned off at Zeitgeber Time 12, presumably reflects sleep pressure, which influences sleep-initiation neuronal firing. The importance of desensitization to sleep latency (or sleep initiation) is emphasized by the Rdl^{A302S} phenotype, the kinetics of RDL^{A302S} currents, the effect of CBZ on fly sleep and the effect of CBZ on wild-type and mutant receptor physiology. The slow decay kinetics of RDL^{A302S} currents suggest that hyperpolarization of wake-promoting neurons occurs more easily in mutant flies. The stronger and more consistent mutant effect on sleep latency, as compared to sleep duration, indicates that the output of the sleep-initiation neurons is affected more strongly by the mutant receptor than that of sleep-maintenance neurons. Although this could reflect the presence of other GABA receptor subunits in sleep-maintenance neurons, it could also just reflect a slower firing rate. CBZ acts specifically on RDL desensitization and this effect is blocked by the RDL^{A302S} mutation, which explains why the drug effect on sleep latency is substantially reduced in the Rdl^{A302S} genotype.

There was an additional, prominent CBZ effect on sleep-episode duration, which was not ameliorated by the Rdl^{A302S} mutation. This suggests that there are additional CBZ targets, which affect wake-promoting activity either directly or indirectly (**Supplementary Fig. 1**). The multiple effects of CBZ on the *Drosophila* sleep circuit are not surprising, as its pharmacology in humans[27] indicates that it interacts with many targets, including sodium channels, $GABA_A$ receptors[28] and adenosine receptors.

Nonetheless, the decrease in fly sleep is the result, in part, of the unexpected CBZ effects on fly $GABA_A$ receptor kinetics. CBZ is widely used in the treatment of multiple neurological disorders, including epilepsy, bipolar disorder and trigeminal neuralgia[29], and therapeutic drugs for these disorders might be expected to increase sleep. Indeed, several studies have reported that patients and healthy volunteers receiving CBZ therapy experience sleep problems, which normally disappear with long-term treatment[30]. It is intriguing that the CBZ effects on fly sleep also decrease with chronic drug treatment (data not shown). Our observations, along with others, further illustrate the potential utility of using *Drosophila* models combined with pharmacological approaches to better understand complex behaviors and disease states.

The similarities between fly and mammalian sleep suggest that altering subtle aspects of $GABA_A$ receptor function in mammals will also lead to discrete effects on sleep structure. Indeed, a human mutation altering desensitization and deactivation of a $GABA_A$ receptor subunit causes a familial type of insomnia[31]. Taken together with our data on the *Drosophila* RDL channel, this suggests that targeting specific aspects of $GABA_A$ receptor function such as desensitization will give rise to more specific and effective sleep therapeutics.

METHODS

Animals. Fly cultures were kept at 25 °C with a 12-h light/dark cycle on cornmeal, yeast, sucrose and agar food. The original *Rdl* A302S allele, Rdl^{MDRR}, which was isolated from the wild[20], and $Rdl^{1}/TM3^{26}$ flies were obtained from the Bloomington Stock Center (Bloomington). Rdl^{CB1} was isolated in a modified dominant F1 screen for insecticide resistance in which *Canton-S* flies were ethyl methanesulfonate mutagenized using previously described methods[32]. For all strains, the third chromosome was crossed into *Canton-S* background; for Rdl^{MDRR} the strain was subsequently outcrossed over six generations and isolated using PCR-ren[33].

Behavioral assays and drug administration. Behavioral assays and analysis were carried out as previously described[12]. For all genotypes, the locomotor activity of 5–7-day-old flies loaded with 5% sucrose in 2% agar was monitored using the Trikinetics system (Waltham) in 24-h light-dark cycles. Data were collected in 1-min bins and a sliding window was applied. Sleep was defined as 5 consecutive min of inactivity[12,34]; sleep latency was measured from the time of lights off to the onset of the first sleep episode. For episodes flanking a given period, quantifications for sleep duration were done with episodes truncated to start and end in the respective periods. Only data from female flies are shown[34]. In all cases where we assayed male flies, we observed qualitatively similar results on sleep latency.

For experiments involving CBZ, a stock solution (20 mg ml^{-1}) was solubilized in 45% (2-hydroxypropyl)-β-cyclodextrin (Sigma) and mixed into the standard agar medium. Experiments were carried out by recording 5–6 baseline d on standard medium, and then switching to CBZ/sucrose/agar 6 h before the onset of the dark period.

Electrophysiological recording. The wild-type *Drosophila Rdl* clone (GH019619) was obtained from the *Drosophila* Genome Collection 1 (DGC1). This clone lacks TM4 and has more stable desensitization kinetics (J.C.C. and J.A., unpublished results). Oocyte collection, injection and electrophysiological recordings were carried out as described[35,36]. Two electrode voltage-clamp experiments were performed 1–5 d post-injection. An oocyte chamber of ~200 µl was perfused with a flow rate of ~10–15 ml min^{-1} and recording were made at a holding potential of –60 mV in Barth's solution (2 mM KCl, 5 mM HEPES, pH 7.6, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 100 mM NaCl). CBZ was dissolved in 45% (2-hydroxypropyl)-β-cyclodextrin (Sigma). Because cyclodextrin forms a complex with these drugs[37,38], the effective concentration of these agents is expected to be much lower that what is indicated in the experiments. We used GABA concentrations that were close to the saturated region of the dose-response curve because we observed that CBZ effects were essentially independent of GABA and that desensitization is more prominent in this range.

Statistical Analysis. Data were analyzed as described in the figure legends using JMP software version 5.0.1.2 for the PC and Macintosh (SAS Institute).

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We are grateful to E. Marder, R. Allada and R. Greenspan for comments on the manuscript. This work was funded by a grant from the US Army (W81XWH-04-1-0158) to M.R. and L.C.G., and MH 067284 to L.C.G.

Published online at http://www.nature.com/natureneuroscience
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