Introduction

Increased human life expectancy has brought with it an increased burden of age-related loss of function and pathology, including sleep disorders, which affect ~50% of the population over 65 years old. Older people often have difficulty in initiating and maintaining sleep, associated with reduced quality of life, poor health, and increased mortality [1]. Sleep in *Drosophila* and humans has marked similarities, making the fruit fly a powerful genetic model in which to study sleep syndromes [2]. Several drugs and signalling pathways affect sleep in flies and mammals in a similar manner, including arousal-promoting dopaminergic signalling [3,4]. Like humans, flies are more active by day and sleep mostly during the night, and this pattern deteriorates with age: duration of day sleep increases and of night sleep decreases, and sleep bouts become shorter and more often interrupted by waking periods, known collectively as sleep fragmentation [5].

We hypothesised that evolutionarily conserved mechanisms that ameliorate ageing itself could also ameliorate the deterioration in sleep quality in older individuals. Mutants that reduce insulin/insulin-like growth factor (IGF) signalling (IIS) can extend healthy lifespan in the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila*, and the mouse, and may also ameliorate human ageing [6–8]. In *C. elegans* and *Drosophila*, extension of lifespan by reduced IIS requires the single forkhead Box O (FOXO) transcription factor [9–12]. Effects of IIS on ageing are also mediated through its interaction with the Target of Rapamycin (TOR) signalling pathway. The TOR pathway interacts with IIS in part through phosphorylation of the AKT kinase [13] and TOR also regulates translation, through 4E-BP and S6K activity [14], and autophagy, through atg genes [15]. Down-regulation of TOR signalling by the TOR-specific inhibitor rapamycin extends lifespan in flies and mammals [16,17]. It is not clear if reduced IIS and/or TOR activity can delay neural and behavioural senescence, because increased activity in the nervous system itself can be neuroprotective in specific disease states [18], and extended lifespan is not invariably accompanied by amelioration of age-related loss of behavioural function [19]. However, IIS regulates processes...
Author Summary

Sleep is essential for human health, but the quality of this fundamental physiological process declines with age and reduces quality of life. We therefore investigated the mechanisms by which ageing impairs sleep. We used the fruit fly Drosophila, whose sleep has many features in common with that of humans, including the age-related decline in quality. We examined the role of the insulin/IGF (IIS) and TOR signaling network, which has an evolutionarily conserved role in ageing. We found that flies with reduced IIS activity had improved sleep quality at night and higher activity levels by day. Importantly, day activity and night sleep were regulated through distinct mechanisms—day activity by the key IIS transcription factor dFOXO, adipokine hormone, and octopaminergic signalling—whereas night sleep was mediated through TOR and dopaminergic signalling. Surprisingly, acute inhibition of TOR, by rapamycin, even in old flies, improved sleep quality, suggesting that age-related sleep decline is reversible even after it has commenced. Given the high evolutionarily conservation of IIS and TOR function, our results implicate potential therapeutic targets to improve sleep quality in humans.

Sleep in flies is defined as a resting period of no activity that lasts for 5 min or longer. While asleep, flies have a characteristic posture and increased arousal threshold, and longer sleep bouts include a deep sleep state characterised by electrophysiological changes and regulated by molecules involved in synaptic plasticity and pruning [23, 25, 26]. At all ages tested, dilp2-3,5 mutants slept more at night and less by day than did controls (Figure 1E–F). In addition, they had fewer waking periods, and hence sleep bouts, during both day and night, and longer night sleep bouts (Figure 1G–H). Longer sleep periods occurred mainly in the dilp2-3,5 mutants (Figure 1I). Thus, reduced IIS induced more day activity periods but increased both night sleep duration and sleep consolidation, and these phenotypes were already evident in young flies.

To determine if the unaltered circadian rhythm and the activity and sleep phenotypes of dilp2-3,5 mutants are a general feature of reduced IIS in Drosophila, we measured these traits in flies in which IIS was down-regulated by ubiquitous expression (driven by da-Gal4 driver) of a dominant-negative form of the single fly IIS receptor (da-Gal4/UAS-INRDN) [22]; all of the phenotypes seen in the dilp2-3,5 mutant were present (Figure S1A–G).

Consistent with previous studies [5], sleep fragmentation increased with age in w^{ubab} control flies. Day sleep increased while night sleep declined (Figure 1E), and the number of day and night sleep bouts increased and night sleep bout duration decreased with age (Figure 1G–H). In contrast, sleep fragmentation showed little or no increase with age in dilp2-3,5 mutants. Night sleep duration did not change (Figure 1E), day sleep duration did not change either (Figure 1E), while day and night sleep bouts did not increase with age (Figure 1G). Generalized linear modelling (GLM) indicated that all aspects of sleep fragmentation increased significantly less with age in the dilp2-3,5 mutants than in controls: total day and night sleep, p=0.0019 and p<0.0001, respectively, and day and night sleep bouts, p=0.0017 and p=0.0029, respectively. Reduced IIS thus rescued the age-related sleep fragmentation of old flies (Figure 1J).

Age-related night sleep fragmentation was also ameliorated in da-Gal4/UAS-INRDN flies. GLM indicated that, while day behaviours did not differ, age-related night sleep fragmentation of da-Gal4/UAS-INRDN flies increased less with age than in both genetic controls: total night sleep, p<0.0001 and p=0.0003 da-Gal4/+ UAS-INRDN/+; respectively; night sleep bouts p=0.011 and p=0.016 da-Gal4/+ UAS-INRDN/+, respectively (Figure S1G). Amelioration of age-related sleep fragmentation is thus a general feature of reduced IIS in Drosophila.

Results

Reduced IIS Affected Activity and Sleep Patterns But Not Circadian Rhythm

To investigate the effects of reduced IIS on activity and sleep, we measured activity patterns of long-lived, dilp2-3,5 mutant flies and controls [21]. Activity and circadian rhythm can be correlated [23], and we therefore first measured both of them under 12:12 h light:dark (LD) and constant dark (DD) conditions over a 3-d period. Control, w^{ubab} flies showed typical circadian rhythmicity, which was unaltered in dilp2-3,5 mutants (Figure 1A). However, in the mutants day activity was significantly increased, whereas night activity was significantly reduced, a pattern that was maintained as the flies aged (Figure 1B–C). Although day activity was higher in the dilp2-3,5 mutants, wakefulness (average activity per awake minute [24]) was not significantly altered (Figure 1D), suggesting that dilp2-3,5 mutants had a greater number of active periods during the day.
Figure 1. Reduced IIS affected activity and sleep and ameliorated age-related sleep fragmentation. (A) Locomotor activity over 9 d of wDah control and dilp2-3,5 mutant flies under 12:12 h LD and constant darkness 12:12 h DD (n = 12, age 20 d). Mean free running period (τ) in DD ± s.e.m. (B) Average activity count data (30 min bins) under 12:12 h LD conditions (25 d wDah n = 48, dilp2-3,5 n = 31). (C) dilp2-3,5 mutants were more active during the day and less active during the night compared to controls. (D) There was no significant difference in wakefulness (average activity per waking minute). (E) dilp2-3,5 mutants slept more at night and less during the day than controls. (F) Minutes of sleep per 30 min (25 d wDah n = 48, dilp2-3,5 n = 31). (G) Day and night sleep of dilp2-3,5 mutants were interrupted by fewer waking periods compared to controls. (H) dilp2-3,5 flies had longer sleep bouts during the night. (I) Longer sleep bouts were more prevalent in dilp2-3,5 mutants (age 25 d). (J) wDah control flies, but not dilp2-3,5 mutants, show a significant age-related increase in night sleep bouts (age 10 d, 25 d, 45 d, 55 d, and 65 d). (B–F) wDah, n = 31, 43, 46, 26, and 31 for ages 10 d, 25 d, 45 d, 55 d, and 65 d, respectively; dilp2-3,5, n = 31, 31, 32, 29, and 43 for ages 10 d, 25 d, 45 d, 55 d, and 65 d, respectively. Kruskal Wallis test with Dunn’s multiple comparison (selected pairs). ***p < 0.001, **p < 0.01, and *p < 0.05. Error bars represent s.e.m. doi:10.1371/journal.pbio.1001824.g001
A normal LD cycle was therefore required for the daytime, but not the nighttime, activity and sleep phenotypes of dilp2-3,5 mutants.

Loss of FOXO Affected Day Behaviours of INRDN Flies

To identify molecular mechanisms that mediated the activity and sleep phenotypes of IIS mutants, we investigated the role of the transcription factor dFOXO, which is essential for lifespan extension by reduced IIS in Drosophila [12]. dfxo/dilp2-3,5 double null mutants are lethal, but dfxo/INRDN double mutants are viable. In a wild-type background, loss of dfxo function did not induce any activity or sleep phenotypes (Figure 2 and Figure S3). However, loss of dfxo in INRDN flies specifically affected day, but not night, phenotypes (Figure 2 and Figure S3). INRDN;dfxo double mutants showed strongly reduced day activity and increased day sleep duration, without changes in wakefulness, and the number of day sleep bouts did not differ significantly between INRDN;dfxo double mutants and controls (Figure 2 and Figure S3). Reduced IIS thus affects day sleep and activity through dFOXO, but not sleep through a different route.

Reduced IIS Induced Day Hyperactivity through AKH and Octopaminergic Signalling

Like glucagon in mammals, AKH is a peptide that acts antagonistically to insulin to increase lipolysis and glycogenolysis in the fly fat body [29], the equivalent of mammalian liver and white adipose tissue. AKH expression is increased in larvae and adults lacking brain insulin-producing cells [29], and AKH has been shown to increase activity in flies [30]. We therefore hypothesised that reduced IIS could act through increased AKH release and AkhR function to regulate day activity.

To test the role of AKH in the day activity and sleep phenotypes of IIS mutants, we generated dilp2-3,5 mutants lacking the AkhR receptor (CG11325). Loss of AkhR had no effect on day activity or sleep of wild-type flies, but it abrogated the increased day activity of IIS mutants, without affecting night activity, night sleep duration, or number of night sleep bouts (Figure 3A and Figure S4A). Tolbutamide increases AKH release through targeting the sulphonylurea receptor on AKH-producing cells [20]. In control flies, treatment with tolbutamide increased day activity, whilst reducing day sleep behaviours. In contrast, tolbutamide treatment did not increase day activity in dilp2-3,5, dfxo, or AkhR mutants (Figure 3B and Figure S4B). Reduced IIS thus acts through increased AKH activity to induce its day activity and sleep phenotypes.

AKH increases activity in cockroaches through activation of the AkhR in octopaminergic cells [31], and we therefore hypothesised that reduced IIS might affect the level of the arousal-promoting, biogenic amine octopamine [32]. To determine if changes in the level of octopamine underlay the day activity and sleep phenotypes of IIS mutants, we used mass spectrometry to measure octopamine and found that levels were significantly increased in head extracts from IIS mutants (Figure 3C). Loss of AkhR abrogated the increased octopamine levels of dilp2-3,5 mutants, suggesting that AkhR mediated the effect of reduced IIS on octopaminergic-signalling-mediated day activity (Figure 3C). To address the mechanism underlying this increase in octopamine levels, we examined the dopamine/octopamine biosynthetic pathway, but found expression of enzymes within this pathway to be unaltered (Figure S3A). However, tyramine, the precursor of octopamine, was significantly reduced (Figure S3B), suggesting increased enzymatic activity of tyramine β-hydroxylase in IIS mutants.

To determine if increased day activity of IIS mutants was caused by increased octopaminergic signalling, we fed flies with mianserin hydrochloride, an inhibitor of octopaminergic signalling, which acts by inhibiting octopamine-induced cAMP increase [33]. Brief (2 d) feeding with mianserin hydrochloride did not affect activity or sleep of wild-type flies, but abrogated the increased day activity, sleep, and bout number but not the night sleep phenotypes of IIS mutants, indicating that reduced IIS induces activity through increased octopaminergic signalling (Figure 4A–B). However, enhanced octopaminergic signalling...
Reduced IIS thus acts through reduced AKH and octopaminergic signalling to induce day activity and sleep phenotypes in Drosophila.

Reduced TOR Activity Mediated the Effects of Reduced IIS on Night Activity and Sleep

IIS increases activity of the TOR kinase, which regulates translation through 4E-BP and S6 Kinase (S6K) and autophagy through atg genes [34]. TOR is inhibited by rapamycin, which also extends yeast, worm, fly, and mouse lifespan [16,17,35,36]. Feeding wild-type flies with rapamycin increased night sleep duration and bout length and reduced number of sleep bouts, but did not affect day sleep, wakefulness, or activity (Figure 5A–B), suggesting that TOR might mediate the effects of reduced IIS on night sleep. In support of this hypothesis, rapamycin did not affect the night activity and sleep phenotypes of IIS mutants (Figure S6A). Night sleep fragmentation occurs during ageing, and to determine if acute treatment of old flies with rapamycin could ameliorate their sleep fragmentation, we treated 42-d-old flies over a brief, 3-d, period, which resulted in increased night sleep duration, fewer night sleep bouts, and increased bout length, without affecting day behaviours (Figure 5C). Reduced TOR signalling thus mediated the effects of reduced IIS on night activity and sleep, and acute inhibition with rapamycin could rescue the sleep fragmentation of ageing flies.
Reduced Insulin Signalling and Sleep Decline

S6K Mediated Effects of Reduced IIS on Night Sleep

Rapamycin-mediated lifespan extension in Drosophila is dependent on decreased S6K activity and increased autophagy, but not on 4E-BP [16,17]. Neither loss of 4E-BP nor blocking autophagy counteracted the rescue of night sleep fragmentation by rapamycin (Figure 5D–E and Figure S6B–C). However, ubiquitous expression of constitutively active S6K did suppress the effect of rapamycin (Figure 5F and Figure S6D); these findings demonstrate that rapamycin acted through reduced S6K activity to rescue night sleep fragmentation.

TOR up-regulates translation [13], and inhibition of protein synthesis through cycloheximide administration enhances sleep in mammals [37]. To probe a possible role of reduced protein synthesis in consolidation of sleep in flies, we fed them cycloheximide. This resulted in increased day and night sleep duration and bout length and reduced number of sleep bouts, and in reduced day and night activity (Figure S7A), suggesting that inhibition of protein synthesis increases sleep in flies, and could account for the effects of TOR and S6K on night sleep. Prolonged cycloheximide feeding (5 d) did not increase mortality (unpublished data), and nor did it further affect activity or sleep patterns, but we cannot exclude the possibility that reduced activity of the flies was a toxic side-effect of the drug (Figure S7B). These findings suggest a role of reduced protein synthesis in the night sleep phenotypes from reduced IIS and TOR activity.

Reduced IIS Altered Dopamine Receptor Expression

Dopaminergic signalling regulates sleep in flies [3,4], and disruption of the dopamine receptor 1 gene (DopR1) decreases night activity, increases night sleep, and decreases night sleep fragmentation [38], phenotypes that we also observed in the present study (Figure 6A). We therefore investigated the role of dopaminergic signalling in mediating the effects of rapamycin on night sleep phenotypes, by administration of rapamycin to DopR1 flies (Figure 6A). In contrast to the effects in wild-type controls, rapamycin did not affect night activity, night sleep, night sleep bout number, or bout duration of DopR1 mutants (Figure 6A). GLM indicated that response of DopR1 mutants to rapamycin was significantly different from that of wild-type flies for night activity (p < 0.0001), night bout number (p = 0.025), and night bout length (p = 0.015), but not for night sleep (p = 0.57). Furthermore, dilp2-3,5;DopR1 double mutants did not differ in their activity and sleep phenotypes from dilp2-3,5 mutants (Figure 6B). These results indicate that reduced dopaminergic signalling mediates the effects of reduced IIS on night sleep activity and sleep phenotypes. We therefore investigated the mechanisms mediating the effects of reduced IIS and TOR activity on dopaminergic signalling.

We first tested whether the night phenotypes of the IIS mutants were a consequence of decreased DopR levels, by quantifying DopR1 transcript levels in dilp2-3,5 and INRAS flies. Surprisingly, transcript levels of DopR1 were increased in both dilp2-3,5 mutants and INRAS flies, and also in INRAS flies lacking dfoxo (Figure 6C), suggesting a compensatory response of dopamine receptor expression to a different lesion in dopaminergic signalling.

We next determined whether overall dopamine levels in the flies were affected by reduced IIS. We quantified dopamine synthesis in dilp2-3,5 mutants but found no difference in either the expression levels of dopamine biosynthetic enzymes (Figure S3A) or in total dopamine (Figure 6D). However, after treatment with rapamycin or reduced IIS, expression levels of dopamine transporter (DAT) were increased (Figure 6E). Under normal physiological conditions, dopamine signalling is regulated by the level of extracellular dopamine and the rate of DAT-mediated clearance of dopamine from the synaptic cleft. Increased DAT expression in Drosophila IIS mutants therefore blunt dopaminergic signalling and induce the compensatory changes seen elsewhere in the pathway.
If increased DAT activity in IIS mutants does indeed regulate their night sleep phenotypes, then these should be disrupted by pharmacological inhibition or overactivation of dopaminergic signalling. 3-Iodo-L-tyrosine (3IY) inhibits dopaminergic signalling by inhibiting tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of dopamine, and hence reducing dopamine levels. If the nighttime behaviours of IIS mutants are mediated through increased DAT activity, and therefore reduced dopaminergic signalling, short-term treatment with 3IY should not affect IIS mutant behaviours, whereas controls should show behaviours associated with reduced dopaminergic signalling. Although 3IY treatment had no effect on day behaviours of wild-type flies, it decreased their night sleep duration, reduced night sleep fragmentation, and increased the length of night sleep periods (Figure 6F). In contrast, IIS mutants showed no response to 3IY at night, with fewer, longer sleep bouts (Figure 6F).

Methamphetamine (METH) increases dopaminergic signalling, by preventing dopamine clearance from the synaptic cleft by DAT and increasing dopamine efflux through DAT from the presynaptic cells. METH would therefore be predicted to revert the night activity and sleep phenotypes of IIS mutants towards those of controls. In accordance with the results of previous studies [3], short-term exposure (12 h) to METH increased both day and night activity in wild-type flies, decreased their day and night sleep, and reduced their day and night sleep bout number and bout length (Figure 6G). In contrast, METH treatment had no effect on the day phenotypes of dilp2-3,5 mutants, whereas it significantly increased their night activity and reduced their night sleep and sleep bout length to levels similar to those of treated controls (Figure 6G). Night sleep phenotype of the IIS mutants was thus more responsive to METH treatment, as predicted if reduced IIS increases DAT activity. Taken together, these results indicate that reduced IIS induced night activity and sleep phenotypes by modulating DAT activity.

Discussion

IIS Signalling and Sleep

Sleep syndromes are highly prevalent in elderly humans and, with a continuing increase in life expectancy and a greater...
Figure 6. Dopamine receptor mutants do not respond to rapamycin treatment. (A) DopR1 mutants had similar activity and sleep features as rapamycin-fed flies. Behaviour of DopR1 mutants was not affected by rapamycin feeding (age 10 d, n = 64 for all genotypes). Flies were fed with rapamycin for 9 d. (B) dilp2-3;5, DopR1 mutants had similar activity and sleep features as dilp2-3;5 mutants (n = 64 for all genotypes). (C) QRT-PCR analysis of dopamine receptor (DopR1) expression normalized to Rpl32 expression and controls in head extracts of dilp2-3;5 mutants (age 10 d, n = 9) and da-Gal4/UAS-INRDN flies and da-Gal4/UAS-INRDN;dfxox mutants (age 10 d, n = 3). (D) Mass spectrometry measurement of dopamine levels in head extracts of female flies (age 10 d, n = 3). (E) QRT-PCR analysis of DAT expression, normalized to Rpl32 expression (n = 9). (F) Behaviour of IIS mutants after short-term exposure (2 d) to the tyrosine hydroxylase inhibitor 3IY (5 mg/ml) (age 35 d, n = 32 for all genotypes). IIS mutants differed from controls in the nighttime activity and sleep response to 3IY treatment, but not in bout or bout length (activity p = 0.044, sleep p = 0.014, bout number p = 0.276, night bout length p = 0.463, GLM). (G) Behaviour of IIS mutants after short-term (12 h) exposure to METH (1 mg/ml) (age 25 d, n = 48 for all genotypes). IIS mutants differed from controls in daytime behaviours after METH treatment (activity p = 0.005, sleep p < 0.0001, bout number p = 0.0002, bout length p = 0.031, GLM) along with nighttime bouts (p < 0.0001) and bout length (p < 0.0001), whereas nighttime activity and sleep did not differ (activity p = 0.796, sleep p = 0.352, GLM). (A, B, G) Kruskal Wallis test with Dunn’s multiple comparison of selected pairs. (F) Individual comparisons by Mann–Whitney U test. (C–E) Two-tailed t test. ***p < 0.001, **p < 0.01, and *p < 0.05. Error bars represent s.e.m. (C, E, F) QRT-PCR analysis normalized to RNApolII expression shown in Figure S5D.

doi:10.1371/journal.pbio.1001824.g006
Reduced Insulin Signalling and Sleep Decline

The consumption factor FoxO is an important downstream mediator of IIS. In C. elegans all aspects of IIS are dependent on daf-16, the worm ortholog of foxO [9]. In contrast, in Drosophila IIS-mediated lifespan extension is dependent on dfoxo, whereas several phenotypes of reduced IIS are dfxo-independent [12]. Activity and sleep were unaffected by the loss of dfxo in wild-type flies. In contrast, under low IIS conditions loss of dfxo specifically affected daytime behaviour, with night time behaviours unaffected. Reduced IIS therefore affects day and night sleep and activity through distinct mechanisms. It also uncouples the effects of IIS on lifespan and on night sleep consolidation, since dfxo is essential for extended longevity of flies with reduced IIS. dFOXO has been previously shown to increase neuronal excitability, possibly via transcription of ion channel subunits or other regulators [45].

We suggest that a possible such regulator could be octopaminergic signalling, known to promote arousal in Drosophila [32,46]. Octopamine, the arthropod equivalent of noradrenaline, regulates several behavioural/physiological processes, including glycogenolysis and fat metabolism, as well as synaptic and behavioural plasticity [47]. Moreover, octopamine can affect sleep by acting on insulin-producing cells in the fly brain, thus linking IIS and sleep/activity [46]. Indeed, we found that IIS mutants have increased octopamine levels and, importantly, pharmacological inhibition of octopaminergic signalling reverted the increased day activity of IIS mutants. Noteworthy, mRNA expression of octopamine biosynthetic enzymes was not changed, but tyramine levels were significantly reduced, suggesting that increased translation, reduced degradation, or increased activity of the tyramine-β-hydroxylase regulates octopamine levels in IIS mutants. In contrast to day activity, increased lifespan of IIS mutants was not affected by pharmacological inhibition of octopaminergic signalling, thus separating longevity from the day activity phenotype.

IIS Regulates Octopaminergic Signalling through AKH

The effect of reduced IIS on day sleep/activity was mediated through AKH, the equivalent of human glucagon, an antagonist of insulin [28,29,48]. In flies, AKH coordinates the response to hunger through mobilizing energy stores and increasing food intake [49], as well as inducing a starvation-like hyperactivity [30,48]. Loss of AKH receptor (AkhR) abrogated the increased activity of IIS mutants without affecting night sleep. These results demonstrate that day and night phenotypes of IIS mutants can be uncoupled, suggesting that the increased night sleep of IIS mutants is not just a compensatory consequence of increased day activity.

dilp2-3,5 mutants have increased octopamine levels, and loss of AkhR in the dilp2-3,5 mutant background reduced their octopamine level back to wild-type levels, suggesting that AkhR-mediated regulation of octopamine controls day hyperactivity in IIS mutants. In support of our findings, octopaminergic cells mediate the increased activity effect of AKH in other insects [31]. Flies lacking dFOXO did not respond to chemically induced AKH release, suggesting that AKH affects activity through dFOXO. Therefore, we suggest that dFOXO and AkhR act through overlapping mechanisms to enhance octopaminergic signalling and induce activity.

In flies, AkhR is highly expressed in fat body and its loss alters lipid and carbohydrate store levels [50]. Therefore, AkhR might indirectly enhance octopaminergic signalling through alterations in lipid and carbohydrate metabolism. In support of this idea, lipid metabolism affects sleep homeostasis in flies [51]. Additionally, AkhR expression in octopaminergic cells could regulate octopamine synthesis and release in flies [31]. Interestingly, expression of AkhR is altered in dfxo mutants [52], thus implicating dFOXO in AkhR regulation. Both are highly expressed in fat body, an important organ for metabolism in flies, and fat-body-specific insulin receptor may regulate AkhR function through dFOXO activation.

In larval motor neurons, dFOXO increases neuronal excitability [45] and octopamine increases glutamate release, suggesting there is at least a spatial functional link between the two [47,53]. Thus, together with a possible role in AkhR synthesis, dFOXO could act downstream of octopamine to increase activity.

IIS Regulates Night Sleep through TOR

To determine the mechanism underlying the IIS-dependent amelioration of age-related sleep decline, we investigated downstream components and genetic interactors of IIS. One such interactors that affects health and ageing is TORC1. TORC1 is a major regulator of translation, through S6K, 4E-BP, and of autophagy, through ATG1 [14,15]. Inhibiting TOR signalling, and thus translation, by rapamycin treatment in wild-type flies recapitulated the sleep features of IIS mutants, even in old flies. This rescue of sleep quality was blocked by ubiquitous expression of activated S6K, suggesting that reduced S6K activity is required for the rescue. Our findings, together with previous results showing...
S6K to regulate hunger-driven behaviours, highlight the importance of S6K as a regulator of behaviour in flies [54]. Thus, manipulating TOR signalling can improve sleep quality through S6K.

In mammals, rapamycin treatment has beneficial effects on behaviour throughout lifespan. Although complete block of TOR activity is detrimental for long-term memory [55], a moderate decrease through rapamycin treatment can improve cognitive function, abrogate age-related cognitive deterioration, and reduce anxiety and depression [56]. Moreover, increased TOR activity throughout development is detrimental for neuronal plasticity and memory [36]. In flies, rapamycin prevents dopaminergic neuron loss in mutants with parkinsonism [57]. Although the role of TOR in brain function has not been well studied in flies, the advantageous effect of rapamycin in both mammalian brain function and sleep in flies may be mediated through common neurophysiological mechanisms.

Gene expression studies have suggested that protein synthesis is up-regulated during sleep [2,58], which may be an essential stage in macromolecular biosynthesis [59-61]. Consistent with this, inhibiting protein synthesis in specific brain domains prolongs sleep duration in mammals, suggesting that sleep is maintained until specific levels of biosynthesis occur and aids in explaining the ubiquitously conserved need for sleep [37,62]. Here, brief cycloheximide treatment prolonged night sleep and increased consolidation in flies, indicating an evolutionarily conserved role for protein synthesis inhibition on sleep regulation. Contrary to reduced IIS, cycloheximide reduced day activity, possibly due to the global effect of cycloheximide on protein synthesis or due to toxic defects in flies’ physiology. Decreased protein synthesis rates may enhance the necessity for increased sleep duration, to allow sufficient synthesis of proteins and other macromolecules during sleep, allowing organisms to be healthy and functional during the day.

Alternatively, the effect of protein synthesis inhibition on night sleep could be the result of reduced expression of specific sleep regulators. We found that DopR1 and dIlp2-3,5 mutants share night phenotypes and that rapamycin did not affect sleep of DopR1 mutants, suggesting that TOR acts on dopaminergic signalling to affect night sleep. Reduced IIS elevated expression of DopR1, independently of dFOXO, in accordance with data from mammals [63]. This effect may be feedback caused by down-regulation of dopaminergic signalling in IIS mutants, although not through direct regulation of DopR. Under normal physiological conditions, dopamine signalling is determined by the level of extracellular dopamine and the rate of DAT-mediated dopamine clearance from the synaptic cleft [64]. The rate of dopamine clearance is dependent on the turnover rate of DAT and the number of functional transporters at the plasma membrane [65-67]. We found that reduced IIS and rapamycin treatment induced increased expression of DAT, suggesting an increased rate of dopamine clearance from the synaptic cleft, and thus a reduction in the amplitude of dopamine signalling, without changes in total dopamine levels. DAT function and IIS have recently been linked in mammals. DAT function increases upon insulin stimulation and is diminished on insulin depletion, through alterations in DAT membrane localization [68]. However, IIS-dependent regulation of DAT subcellular localization in Drosophila has not yet been demonstrated. Our data suggest down-regulating dopaminergic signalling, either by loss of DopR1 or increasing DAT levels, is beneficial for sleep quality. In agreement with this we show that artificially increasing dopaminergic signalling, through short-term METH treatment, increases both day and night activity and reduces night sleep, and reverts the beneficial effect of reduced IIS on night behaviours. In mammals, cocaine administration, which enhances dopaminergic signalling, increases TOR activity. Also, rapamycin blocks cocaine-induced locomotor sensitization [69]. Interestingly, cocaine stimulates S6K phosphorylation in rat brains, and this effect is blocked by rapamycin. Taken together, these results show that in flies and mammals dopaminergic and IIS/TOR signalling may interact in similar ways.

Conclusions

In conclusion, reduced IIS extends lifespan in diverse organisms. Here we have shown that it can also ameliorate age-related sleep fragmentation, but that the mechanisms by which it does so are distinct from those by which it extends lifespan. Reduced IIS affected day activity and sleep phenotypes through increased octopaminergic signalling, but enhanced octopaminergic signalling did not increase lifespan. Similarly, in Drosophila increased lifespan from reduced IIS requires dfoxo, but the night sleep phenotypes of IIS mutants were independent of this transcription factor. Reduced IIS thus acts through multiple pathways to ameliorate different aspects of loss of function during ageing. IIS links metabolism and behaviour through its components, such as S6K and dFOXO, which act through different neuronal circuits and neurons to affect sleep (Figure S8, including interactions shown in [45,53]). The strong evolutionary conservation of these circuits and their functions suggests that pharmacological manipulation of IIS effectors could be beneficial in treatments of sleep syndromes in humans.

Materials and Methods

Fly Stocks and Fly Husbandry

All mutant chromosomes and transgenes were backcrossed into a white Dahomey (Dh-D) wild-type strain for at least eight generations. Fly stocks were kept at 25°C on a 12 h light and 12 h dark cycle (12:12h LD) and were fed a standard sugar/yeast/agar diet (SYA) [70]. In all experiments we used virgin females that were reared at controlled larval densities. Adult flies were kept in SYA food vials (10 to 30 flies per vial) prior to behavioural analysis. During activity recording, flies were fed with SYA food. The daughterless-Gal4 (da-Gal4) driver and the lines UAS-dInRA1409K and UAS-S6KSTDETE were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). The dfoxo944 mutant was kindly provided by Cathy Slack [12]. The atg5 RNAi was kindly provided by Thomas P. Neufeld [71]. 4E-BP and AkbR null mutants [72] were kindly provided by Paul F. Lasco and Ronald P. Kuhnlein, respectively. DopR1 mutants were kindly provided by David Anderson [38].

Circadian, Sleep, and Activity Analysis

For circadian rhythm analysis, 20-d-old virgin females were placed in an activity monitoring system (DAM2, Trikinetics) and were entrained in 12:12 h LD conditions for 5 d prior to a 5-d period of activity recordings under 12:12 h DD (free-running condition). During the 10 d of recording, flies were kept continuously in the activity monitoring system. Activity recordings were collected in 30 min intervals and rhythmicity of individual flies was analysed using the MAZ package. Periodicity was calculated using the autocorrelation method [73]. As a measure of significance for the rhythmic components, we used a Monte Carlo approach [73].

For sleep analysis, virgin females were kept under 12:12 h LD conditions for 3 d in the activity monitoring system. Activity was measured in 5 min intervals, and data from the third day were analysed in Excel. Sleep was defined as an interval of 5 min or
more of nonactivity. Wakefulness, the average activity per waking minute, was calculated as previously described [24]. For analysis of aged flies (Figure 1 and Figure S2), we used flies from the same collection, kept with SYA food vials throughout their life. Sleep and activity of randomly selected individuals, at different ages, were recorded for 5 d in the activity monitoring system. All behavioural data (activity, sleep duration, wakefulness, sleep bout number, and sleep bout length) are represented by mean values.

Biogenic Amine Measurements

Amine levels in virgin females were measured with mass spectrometry. Fly head extracts were homogenised in 0.1% formic acid and filtered (0.45 μm) at 13,000 g for 10 min (4°C). The filtrate was transferred into total recovery vials (Waters, Milford, MA) and immediately frozen at −20°C. Directly before analysis, samples were thawed.

For absolute quantification of Dopamine, Octopamine, and Tyramine in positive ESI MRM (multireaction monitoring) mode, an Acquity UPLC/Xevo TQ (Waters) with MassLynx and Tyramine in positive ESI MRM mode, samples were thawed.

Supporting Information

Figure S8. An overview of pharmacological sites of action can be found in Supporting Information. For each site, compounds are listed that are used to test the function of these sites. An example is dopamine and its antagonists, which are used to test the dopaminergic system. This figure shows that dopamine and its antagonists are used to test the dopaminergic system.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Prism Software, Inc) and JMP (v.10, SAS institute). For behavioural analysis we used the Kruskal Wallis with Dunn’s multiple comparison test, for multiple comparisons, and Mann–Whitney test, for pair-wise comparisons. GLM was used to determine significance of genotype by treatment or age interactions on normalized activity and sleep behaviour data (where necessary data were transformed to reach normality criteria).
Figure S4  Day hyperactivity, not nighttime behaviours, of IIS mutants is mediated through Akt.R. (A) Nighttime behaviour data of Tolkbamide treated flies in Figure S3B. Kruskal Wallis test with Dunn’s multiple comparison test (selected pairs). ***p<0.001, **p<0.01, and *p<0.05. Error bars represent s.e.m.

Figure S5  Bioamine biosynthetic enzyme expression level is independent of IIS. (A) Bioamine biosynthetic pathways and QRT-PCR analysis of biosynthetic enzyme expression in wild type and mutants (n = 3) expression. (B) Mass spectrometry measurement of tyramine levels in head extracts (age 35 d, n = 6). (C) Survival analysis of wild type and mutants (n = 3) expression.

Figure S6  Effect of rapamycin on daytime behaviour of IIS/ TOR signalling components. (A) Chronic rapamycin treatment (9 d) does not affect activity and sleep of IIS mutants (age 10 d, n = 3) expression. (B) dEBP mutants (n = 19/19), (C) flies with reduced autophagy (da-Gal4/UAS-ATG5-RNAi (a) (n = 20/17)) or genetic controls (da-Gal4/+ (c1) (n = 20/21), and UAS-ATG5-RNAi/+ (c2) (n = 23/19)). (D) flies ubiquitously expressing constitutively active S6K (da-Gal4/UAS-S6K constitutively active) (S6K) (n = 20/17) or genetic controls (da-Gal4/+ (c1), n = 20/21, and UAS-S6K constitutively active) (c2), n = 20/18). Kruskal Wallis test with Dunn’s multiple comparison test (selected pairs). ***p<0.001, **p<0.01, and *p<0.05. Error bars represent s.e.m. (Daytime behaviours from Figure 5D-F).

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Figure S7  Cycloheximide affected both day and night behaviour. (A) Cycloheximide (CHX) treatment (17 mM) reduced day activity and increased day sleep but not wakefulness or day sleep bouts (age 10 d, n = 15/15 control/CHX treated). (B) Extended CHX treatment (5 d) did not alter behavior beyond that of 2 d treatment (n = control/25/25-d-old flies, CHX/25/25-d-old flies, CHX treated) with Dunn’s multiple comparison test (selected pairs). ***p<0.001, **p<0.01, and *p<0.05. Error bars represent s.e.m.

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Figure S8  Model depicting IIS regulation of day activity and night sleep. Reduced IIS increases day activity through AKH, dFOXO, and Octopamine signalling, whereas IIS regulation of night sleep is dependent on TOR and S6K activity, and dopaminergic signaling through DAT activity. Blue ovals indicate pharmacological treatments used in this study. Blue arrows indicate activation, red blocked arrows indicate inhibition, and dashed lines indicate putative interactions.

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Acknowledgments
We are grateful to Matt Piper for his advice on statistical analysis; David Anderson, Thomas P. Neufeld, Cathy Slack, Paul F. Lasco, and Ronenel P. Kilulein for providing flies; Ed Green and Charalambe Kyriacou for their help with circadian rhythm analysis; and Jenny Fröhlich for technical assistance.

Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: AM LST SG LP. Performed the experiments: AM LST SG. Analyzed the data: AM LST SG LP. Wrote the paper: AM LST SG LP. Performed qPCRs: UB OH. Performed mass spectrometry: YH.
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