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Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in *Drosophila*

Krisztina Foltenyi[1,2], Ralph J Greenspan[2] & John W Newport[1,3]

Epidermal growth factor receptor (EGFR) signaling in the mammalian hypothalamus is important in the circadian regulation of activity. We have examined the role of this pathway in the regulation of sleep in *Drosophila melanogaster*. Our results demonstrate that rhomboid (Rho)- and Star-mediated activation of EGFR and ERK signaling increases sleep in a dose-dependent manner, and that blockade of *rhomboid* (*rho*) expression in the nervous system decreases sleep. The requirement of *rho* for sleep localized to the pars intercerebralis, a part of the fly brain that is developmentally and functionally analogous to the hypothalamus in vertebrates. These results suggest that sleep and its regulation by EGFR signaling may be ancestral to insects and mammals.

The function of sleep remains elusive and there is scant information about the signaling transduction pathways that are involved in it. In recent years, the development of a promising model system in *Drosophila* has yielded evidence that the fruit fly shows the canonical hallmarks of sleep, as previously defined in mammals. The sleep state includes consolidated periods of inactivity, an increased arousal threshold, and a homeostatic drive to recover sleep after deprivation[1,2]. Furthermore, *Drosophila* responds to the same pharmacological agents that are known to modulate arousal in mammals[2,3], and sleep levels in *Drosophila* decline and become fragmented with age[4]. Here we have shown the role of a classical signaling pathway, common to both flies and mammals, that further supports the power of the fly model system for discovering the molecular modulators of sleep in their anatomical context.

Rhythmic transcription and secretion in the suprachiasmatic nucleus (SCN) of transforming growth factor–α (TGF-α), a ligand for the EGFR (or ErbB-1 in mammals), produces rhythmic behaviors, including sleep. These become irregular in response to the elimination of the rhythmicity of SCN TGF-α secretion, or with the use of a hypomorphic *Egfr* allele[5]. Although the ErbB family of receptor tyrosine kinases may have a role in the endogenous regulation of sleep[6], no direct evidence for this exists. We have addressed this question in *Drosophila*, where the ErbB family is conserved, but with far less complexity than in mammals.

The ErbB family in mammals consists of four members that can form either homodimers or heterodimers. In contrast, the *Drosophila* ErbB family has only one member, EGFR, and four known activating ligands. The activation of three of these ligands, the TGF-α homologs Spitz, Gurken and Keren, requires the processing proteins Star, a transmembrane cargo receptor, and members of the rhomboid family[7], which are integral membrane proteases that cleave the membrane-bound ligand into its soluble form (reviewed in refs. 8,9). Overproduction of Rho proteins produces ectopic secretion of activated ligand, leading to a potent stimulation of EGFR signaling, which is further intensified when coexpressing Star[7,10].

We found that triggering the EGFR pathway by gain-of-function induced excessive sleep in *Drosophila*, and that this behavioral change correlated with the activation of extracellular signal–regulated kinase (ERK), a well-established downstream effector of EGFR signaling. Conversely, loss-of-function in this pathway by cell-specific blockade of *rho* expression had the reciprocal effect of decreasing sleep levels and disrupting sleep consolidation. Finally, we have shown that this decrease in sleep is dependent on the inhibition of *rho* in the pars intercerebralis, a part of the fly brain that may be functionally analogous to the hypothalamus in mammals.

RESULTS

Sleep control by EGFR: gain-of-function

To test the role of neural EGFR in regulating sleep, we asked whether the activation of the EGFR pathway could modulate sleep levels in *Drosophila*. To do so, we conditionally expressed upstream EGFR pathway components that are known to activate the receptor in adults under heat-shock control (*hs*-Gal4)[11] to exert precise temporal control of expression. We found that heat shock induction of rhomboid-1 and Star resulted in an increase in sleep levels throughout the circadian cycle compared with baseline (**Fig. 1a**,**b**), with continued higher sleep levels for the following 2 d. The effect on sleep was most pronounced during the light period immediately following Rho and Star expression, a time when flies are normally highly active. By the third day after heat shock, sleep levels had dropped to below normal, and recovery from

[1]Department of Biology, University of California San Diego, 9500 Gilman Dr., La Jolla, California 92093, USA. [2]The Neurosciences Institute, 10640 John Jay Hopkins Dr., San Diego, California 92121, USA. [3]Deceased. Correspondence should be addressed to R.J.G. (greenspan@nsi.edu).

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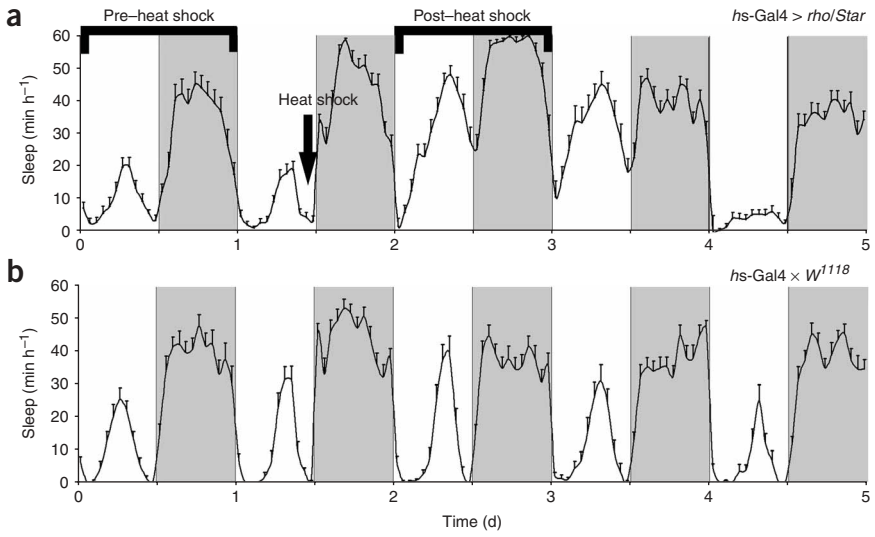

Figure 1 Increased sleep during day and night after activation of EGFR. (**a**,**b**) Time courses of sleep levels of *hs*-Gal4 > *rho*/*Star* (**a**) and *hs*-Gal4 × w^{1118} (**b**) flies for 5 d, beginning from the second full day after loading 2-d-old flies onto monitors. Shading represents lights off. The first bracket above day 1 depicts baseline sleep, and the second bracket above day 3 represents post–heat shock sleep. The second day was considered a time of manipulation and recovery, and thus was excluded from any further analysis in these experiments. Arrow denotes a 60-min, 37 °C heat shock. Behavior was assayed at 23 °C. Each data point is the group average for that hour; error bars represent ± s.e.m.

this compensatory decrease in sleep required another 3–4 d. Thus, an ectopic or elevated level of EGFR ligand increases sleep.

The increase in sleep was dose-dependent, as seen by expressing Rho and Star at higher levels with two copies of the heat shock driver (**Fig. 2a–f**). As the effect on sleep was most apparent during the daytime because of a ceiling effect at night, a comparison of sleep parameters between experimental and control groups during the lights-on periods showed that the increase in total sleep was due to an increase in both sleep bout number and bout duration, indicating that flies with upregulated EGFR activity initiated sleep more often and maintained the state longer than did normal flies (**Fig. 2f** and **Supplementary Table 1** online). Rho alone was sufficient to cause an increase in sleep (**Fig. 2d**,**e**), but to a lesser extent than when coexpressed with Star. Star, in contrast, is not rate-limiting in this pathway[7,10,12]. In sum, activation of EGFR signaling increased sleep in a dose-dependent manner, ranging from the smallest effect by Rho alone, to the strongest with Rho and Star being driven by two copies of *hs*-Gal4.

To further confirm that the Rho-induced increase in sleep was indeed due to EGFR activation, we also tested the effect of overexpressing a soluble form of the EGFR ligand Spitz (s-Spitz) that does not require processing by Rho[13]. Driving this highly potent and specific ligand also caused an increase in sleep levels (**Fig. 2b**,**d**,**f**), confirming the role of EGFR in these gain-of-function experiments.

To determine that the Rho and Star overexpressing flies (*hs*-Gal4(×2) > *rho*/*Star*) were not just sick or paralyzed, we measured the percentage of flies that responded to the change in lighting conditions following the first day after heat shock. We found that 91% of the flies that had spent most of that afternoon asleep were startled into activity by crossing the infrared monitor beam at least once when the lights were shut off (**Fig. 2g**,**h**). Normal flies also showed a consistent, immediate response to lights-off or lights-on with a burst of locomotor activity, even if the lights went on in the middle of the night when they were sleeping (data not shown). To further verify that all of the experimental groups were also normal during periods of activity, we calculated the change in counts per waking minute, which was our measure of normal locomotor activity. This determined that even though all genotypes had a transient decrease in their counts after heat shock, no experimental group differed statistically from all of the controls ($P > 0.05$, Tukey-Kramer Honestly Significant Difference (HSD) Test, **Supplementary Table 1**). These latter controls confirmed that the activation of Rho and EGFR signaling in the adult fly causes a dose-dependent increase in sleep levels and sleep consolidation without any detectable adverse effects on locomotion or responsiveness to environmental changes.

Additional controls also showed that the increase in sleep as a result of Rho and Star could be completely suppressed by a dominant negative form of EGFR, $EGFR^{DN}$ (ref. 14, **Fig. 2d–f**) indicating that the effect is mediated by EGFR. Because $EGFR^{DN}$ acts downstream of Rho and Star, it does not influence the total level of initial overexpression of these two processing proteins. Thus, the successful suppression of Rho- and Star-induced sleep with $EGFR^{DN}$ also signified that this must be due to the functional activities of Rho and Star, and not simply a by-product of their high levels.

To further ensure that the increase in sleep was due to the catalytic activity of Rho and not to a toxic side-effect resulting from its high copy number, we tested a point mutant of the protease in which a histidine residue was exchanged for tyrosine in the catalytic domain of the protein, rendering it unable to cleave ligand (Rho^{H281Y})[15]. Overexpression of Rho^{H281Y} using heat shock failed to increase sleep levels (**Fig. 2c–f**), which was most clearly demonstrated by the lack of activity suppression during evening hours (with a slight increase in daytime sleep that was most likely a residual effect of the heat shock itself, as also seen in the *hs*-Gal4 × w^{1118} control, **Fig. 2d**). These results demonstrated that both Rho and EGFR have to be functional to achieve the increased sleep levels that are observed with the ectopic activation of EGFR signaling.

Sleep control by EGFR: loss-of-function

To determine whether the endogenous pathway is required for normal sleep regulation, we produced flies with reduced Rho activity (**Fig. 3a–h** and **Supplementary Tables 2** and **3** online). Because loss-of-function mutations of the pathway are either lethal or have widespread developmental effects, we made use of a previously described RNA interference (RNAi) construct against *rho*, under the control of the Gal4-binding upstream activating sequence (UAS-rho^{DN})[16], and targeted its expression to the nervous system. As a preliminary control, we confirmed that this RNAi construct was truly functional in neurons (as this is not always the case) and targeted *rho* by showing that it could reduce endogenous Rho protein levels in whole fly heads when expressed with the pan-neural driver *elav*-Gal4 (**Fig. 3h**).

When we expressed rho^{DN} in all neurons using *elav*-Gal4, sleep levels decreased markedly from 8.3 ± 0.11 h to 2.9 ± 0.23 h during the night (**Fig. 3a**,**e**). However, *elav*-Gal4 > rho^{DN} showed developmental defects that included lethality when flies were reared above 25 °C, missing facets in the anterior portion of the eye at lower temperatures, and abnormally

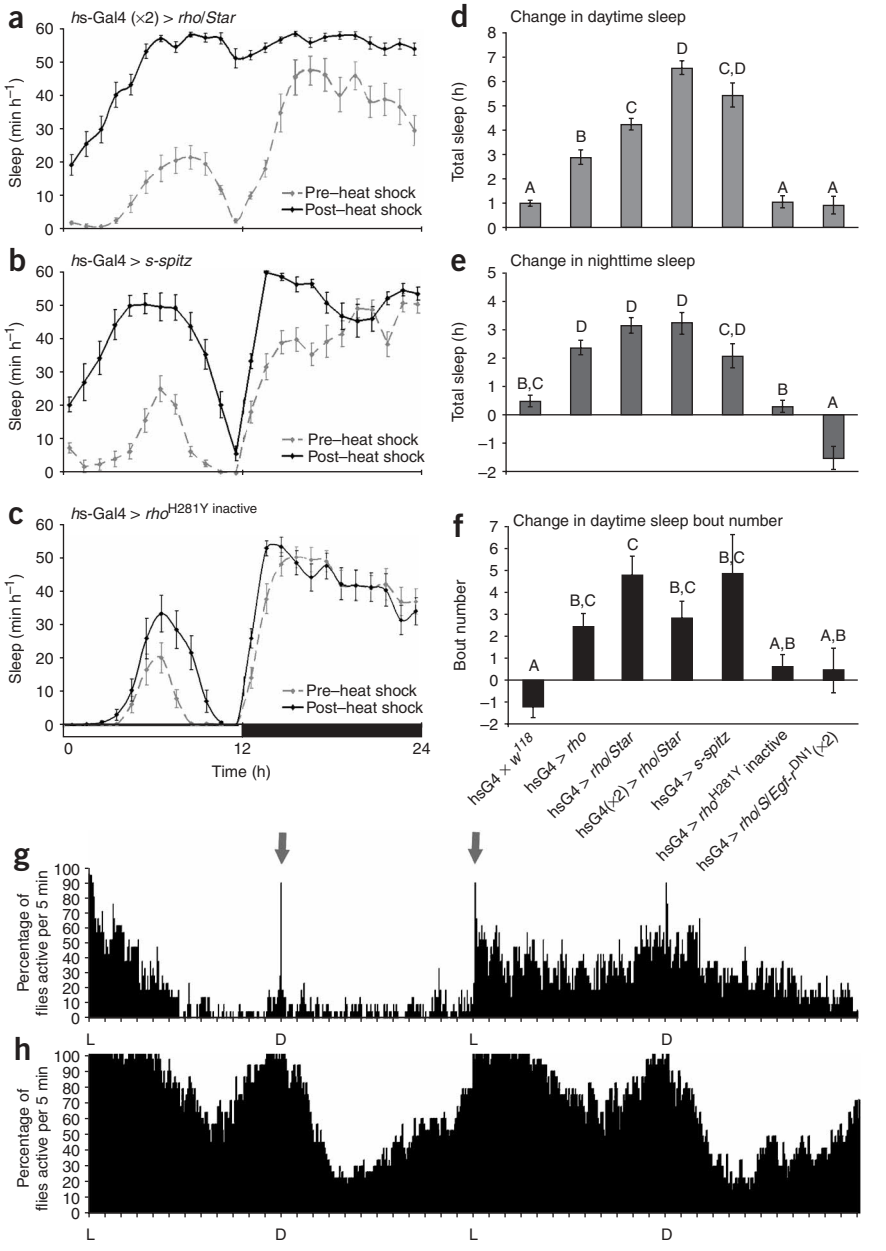

Figure 2 Dose-dependent increase in sleep after overexpression of Rho or Rho and Star. (**a**–**c**) Average activity traces of the effect of stimulating EGFR signaling (**a**,**b**) and a control trace (**c**) are shown. *hs*-Gal4(×2) > *rho*/*Star* ($n = 22$) (**a**), *hs*-Gal4(×2) > *s-spitz* ($n = 22$) (**b**), *hs*-Gal4 > rho^{H281Y} ($n = 28$) (**c**). (**d**,**e**) Histograms showing the change in the total average daytime (**d**) and nighttime (**e**) sleep for each genotype (labeled below **f**). (**f**) Change in daytime sleep bout number. Error bars represent ± s.e.m. To normalize for strain differences in baseline sleep, pre–heat shock sleep levels were subtracted from post–heat shock levels for each genotype. Letters above bars represent statistically significant groups as determined by Tukey-Kramer HSD test for normally distributed data ($P < 0.05$). All groups labeled with letter(s) different from any other group are statistically distinguishable from each other; that is, a group labeled 'B' is different from a group labeled 'A' or 'C', but is not different from a group labeled 'B,C'. (**g**) The same *hs*-Gal4(×2) > *rho*/*Star* flies are shown as in **a** for the first two whole days after heat shock. Shown are the percent of flies active in a single 5-min collection bin. Ninety-one percent of the flies that had spent most of that afternoon asleep were startled into activity (beam crossing) at least once when the lights were shut off (arrow). D, dark; L, light. (**h**) Untreated control for comparison. The immediate effect of lights-off was obscured for the control group due to high activity before and after such transitions.

low locomotor activity (measured as counts per waking minute) in adults (**Supplementary Table 3**). Nonetheless, this result demonstrated a possible requirement for Rho in sleep. To identify *rho* RNAi–mediated sleep-impaired flies without any visible developmental defects, we screened 48 neural drivers with more restricted expression patterns (**Supplementary Table 2**), and found four lines with a significant effect on sleep: c767, 50Y, c687 and 386Y (**Fig. 3b–e**, **Supplementary Tables 2** and **3**). None of the other tested drivers produced clear sleep defects, including drivers with expression patterns in the eye, optic lobes, mushroom bodies or glial cells.

Comparing nighttime sleep levels in *elav*-Gal4 and three of the most restricted lines driving UAS-rho^{DN} (c767, 50Y and c687) revealed that the duration of sleep bouts was markedly shortened, but accompanied by an increase in the number of times flies attempted sleep as compared with their controls (**Fig. 3f**,**g** and **Supplementary Table 3**). These changes indicate that these flies did have a sleep need, but were unable to maintain the sleep state. All of the examined sleep parameters (total nighttime sleep, bout number and bout duration, **Fig. 3e–g**) were statistically indistinguishable between the three restricted drivers ($P > 0.05$, Kruskal-Wallis Test), suggesting that the same sleep mechanism was being impaired in all three cases, thus further strengthening the validity of the phenotype. Moreover, these flies expressing rho^{DN} did not show any lethality or observable developmental abnormalities, and locomotor activity (counts per waking minute) was normal relative to one or both of their respective controls (**Supplementary Table 3**).

Having shown that decreased signaling in the cells driven by 50Y, c687 and c767 resulted in decreased sleep, we wanted to show that the same cells were capable of producing the gain-of-function phenotype that was observed earlier after systemic overexpression of EGFR pathway signaling components. For this purpose, we sought a set of conditions that were capable of producing a transient and local increase in EGFR signaling, while avoiding constitutive expression during development or prolonged use of high temperatures, as either of these would result in the masking of the phenotype as a result of desensitization or stress, respectively. To this end, we used the highly potent secreted ligand s-Spitz (**Fig. 2b**), in combination with the temperature sensitive Gal4 suppressor $Gal80^{ts}$ [17]. Given the potency of s-Spitz, our rationale was that a minimal temperature shift to relieve $Gal80^{ts}$ repression ought to produce a level of expression sufficient to induce at least a partial gain-of-function phenotype. We reared the Gal4; *tubulin*-$Gal80^{ts}$; UAS-*s-spitz* flies at 18 °C to ensure tight

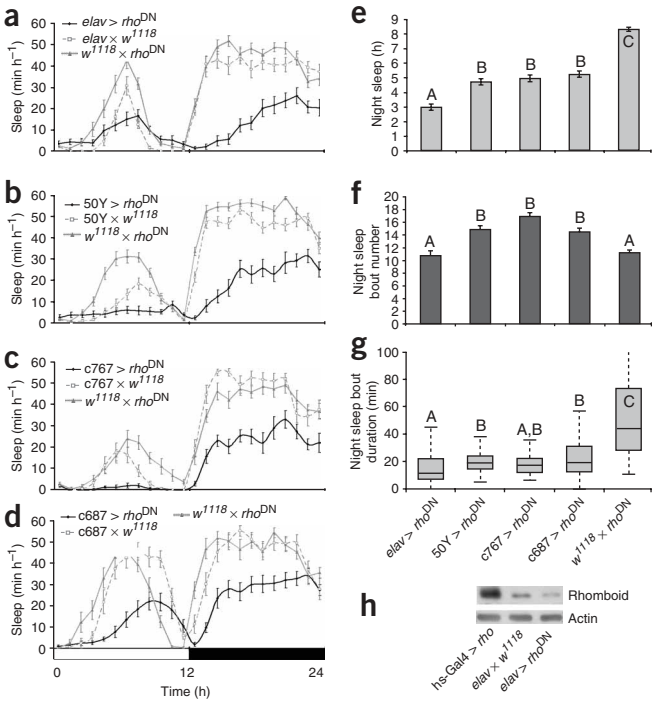

Figure 3 Reduced sleep after directed *rho*-RNAi expression. (**a**–**d**) Representative traces of sleep levels for one 24-h light:dark cycle for flies with UAS-*rho*^{DN} (*rho* RNAi) driven by specified Gal4 drivers (black), and their controls. All time intervals shown are the fourth or fifth day after the start of monitoring of 1–2-day-old female flies. Points represent group average ± s.e.m. *elav*–Gal4 > *rho*^{DN} ($n = 29$) (**a**), 50Y > *rho*^{DN} ($n = 19$) (**b**), c767 > *rho*^{DN} ($n = 15$) (**c**), c687 > *rho*^{DN} ($n = 25$) (**d**). (**e**,**f**) Histograms for the total nighttime sleep levels (**e**) and the number of nighttime sleep bouts (**f**) for *elav* > *rho*^{DN}, 50Y > *rho*^{DN}, c767 > *rho*^{DN} and c687 > *rho*^{DN} (error bars represent ± s.e.m.) and their common control (labeled at the bottom of panel **g**). Letters above bars represent statistically significant groups as determined by Tukey-Kramer HSD test for normally distributed data ($P < 0.05$). (**g**) Box plots for nighttime sleep bout duration. The nonparametrically distributed data are shown as a median depicted by horizontal lines in boxes; the upper and lower box limits the 75% and 25% quantiles, and vertical dashed lines above and below the boxes represent the 95% and 5% quantiles. Letters represent statistically significant groups as determined by ten independent group-wise or pair-wise comparisons using the Wilcoxon/Kruskal-Wallis Test, ($\alpha' = 0.005$). (**h**) Immunoblot against Rho in *elav* > *rho*^{DN} and controls. *hs*-Gal4 > *rho* was included as a positive control to show the location of the Rho band. Actin loading control is included.

suppression of expression and then tested them at 21 °C to minimize any shock caused by a sudden rise in temperature. By the third night after this gentle induction, flies expressing s-Spitz both conditionally and locally slept an average of 1 h more per night than either of their respective controls (which were also sleeping more than usual as a result of the low temperature, **Fig. 4**). We also included c774, which showed no effect with *rho*^{DN} (**Supplementary Table 2**) as a negative control, and found that s-Spitz also had no effect on sleep levels with this driver. These experiments showing the reciprocal effects of inhibition and activation confirmed the role of cells driven by 50Y, c687 and c767 in the regulation of sleep through the EGFR pathway.

Sleep deprivation carried out on flies expressing *rho*^{DN}, to see whether they were able to mount a sleep rebound in a state of increased sleep need, revealed that they did show a rebound, but recovered less sleep than controls (for example, ~17% in 50Y > *rho*^{DN} flies as compared with ~40% in controls, **Supplementary Fig. 1** online). These sleep deprivation experiments are consistent with our findings that impairing Rho function in flies made them short sleepers that could not maintain a prolonged sleep state, and had difficulty settling down after being stimulated.

To determine that the changes observed in sleep patterns were not simply masking a shift in circadian timing, we tested flies for period under constant dark conditions and for phase under light:dark conditions. Our results show that the regulation of sleep by Rho did not substantially influence either the period or the phase of the circadian rhythm in flies that were inhibited for Rho (**Fig. 5**) or in flies overexpressing Rho and Star (data not shown). Period values were all within the normal range, although the variance was so low that they were statistically different, and phase was not statistically different between c767 > *rho*^{DN} and one of its two controls. Only the total level and consolidation of sleep were being influenced, not the circadian timing of sleep. In the mammalian studies, the EGFR signals originated from the SCN, but the analogous situation was not the case in this study, as inhibiting *rho* expression in the circadian regulatory cells with drivers *period*-, *Pdf*-, *cry*- or *timeless*-Gal4 did not change sleep patterns (**Supplementary Table 2**). Therefore, the effects that we observed on sleep regulation by aberrant EGFR signaling were most likely a signal coming from a region of the brain that lies downstream of circadian control and is involved in the sleep process itself.

Sleep correlates with activated ERK

To further confirm that the modulation in sleep levels via Rho perturbation was a result of EGFR pathway activity, we analyzed the time course of the activation of the downstream EGFR target ERK in fly heads, and compared it with the duration of the sleep behavioral phenotype. We used the gain-of-function *hs*-Gal4 > *rho*/*Star* flies in this experiment because endogenous levels of phosphorylated ERK (ppERK) are very low to begin with, and further decreases in loss-of-function flies were therefore irresolvable. Our results show that the increase in sleep in flies overexpressing Rho and Star followed the same

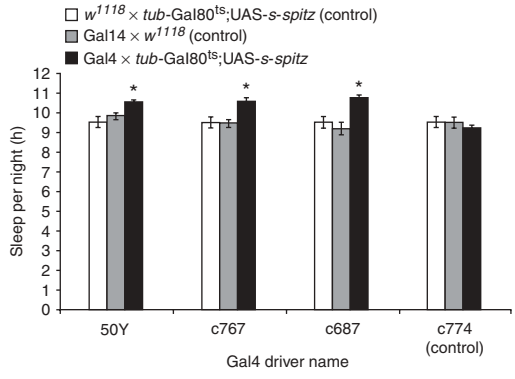

Figure 4 Increased sleep in c767, 50Y and c687 flies overexpressing EGFR ligand. C767, 50Y and c687 Gal4 drivers were used in combination with the conditional Gal4 inhibitor Gal80^{ts} to drive the expression of the secreted EGFR ligand, s-Spitz. Flies were reared at 18 °C and assayed at 21 °C. Data shows sleep levels on the third night at 21 °C for each driver group that includes the experimental genotype (black) and both of its relevant controls. The c774 group was included as a negative control to demonstrate the necessity of the spatial specificity of s-Spitz expression. Stars represent statistically significant groups as determined by the Tukey-Kramer HSD test for normally distributed data ($P < 0.05$), error bars represent ± s.e.m.

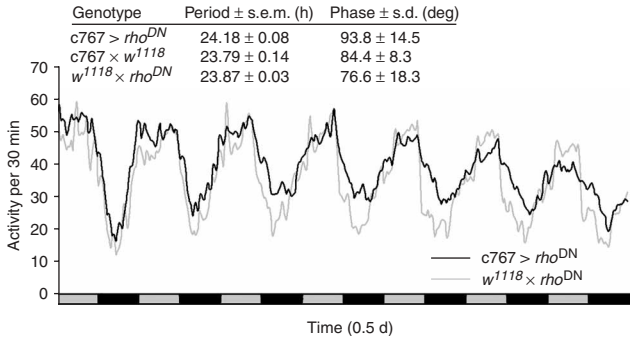

Figure 5 Lack of circadian shift after inhibition of sleep with rho^{DN}. Mean activity for c767 > rho^{DN} flies and the control cross are shown for 7 consecutive days monitored under constant dark conditions after a 3-d entrainment ($n = 32$ for all groups). Black bars under graph represent night, gray bars subjective day. Table insert shows quantitative analysis of phase and period for experimental and control genotypes.

time course as ERK activation in the head (**Fig. 6a**,**b**). In contrast to the strong correlation of sleep increase and ppERK, neither of these correlated closely with the time course of Rho protein levels, which persisted far longer than ERK activation (**Fig. 6c**). The basis for different time courses of ppERK and Rho protein levels is unexplained, but may indicate a molecular or behavioral feed-back mechanism, such as the documented downregulation of the activated EGFR through degradation[18–20]. Under such circumstances, the presence of Rho would no longer have an effect on ERK activation.

The effects on ppERK correlated well with the effects on sleep in other genotypes that we tested. Overexpression of Rho alone also increased ppERK, as it did sleep, but with a slower time course for both phenotypes than when it was coexpressed with Star (**Fig. 6d**), reflecting the known synergism between Rho and Star in activating their ligand substrate.

Control flies (*hs*-Gal4 × w^{1118}) only showed an initial ppERK spike (**Fig. 6b**,**c**), seen in both the experimental and control groups and attributable to the stress of the heat shock itself[21], and otherwise showed only those mild fluctuations associated with the general diurnal effects of the circadian rhythm on ERK activation[22]. Additionally, overexpression of the mutant Rho^{H281Y} or the dominant negative $EGFR^{DN}$ coexpressed with Rho and Star did not change ppERK levels (**Fig. 6d**). Thus, these experiments demonstrated that active EGFR signaling is required for the hyperphosphorylation of ERK kinase, and that there is a marked parallel between the time course of activated ERK and increased sleep levels.

To determine the location in the fly brain where this elevation in ppERK was occurring, we carried out immunohistochemical staining for ppERK in flies overexpressing Rho and Star. We compared experimental and control fly brains at the time point showing the greatest difference in sleep behavior (~23 h after heat shock, see **Fig. 1**), and found the most notable difference to be in an axonal tract that projects though the dorsal protocerebrum and median bundle into the tritocerebrum (**Fig. 6e**,**f**), a part of the brain that receives inputs from the pars intercerebralis.

Rho in the pars intercerebralis affects sleep

As 50Y, c767 and c687 driving *rho* RNAi exerted similar effects on nighttime sleep patterns, we examined whether their expression patterns also included a common group of cells by the use of a membrane-bound (green fluorescent protein) GFP transgene. A comparison of their expression patterns revealed that all three inserts drove expression prominently in a set of neurons in the pars intercerebralis, which project into the tritocerebrum (**Fig. 7a–c**), the same region that was

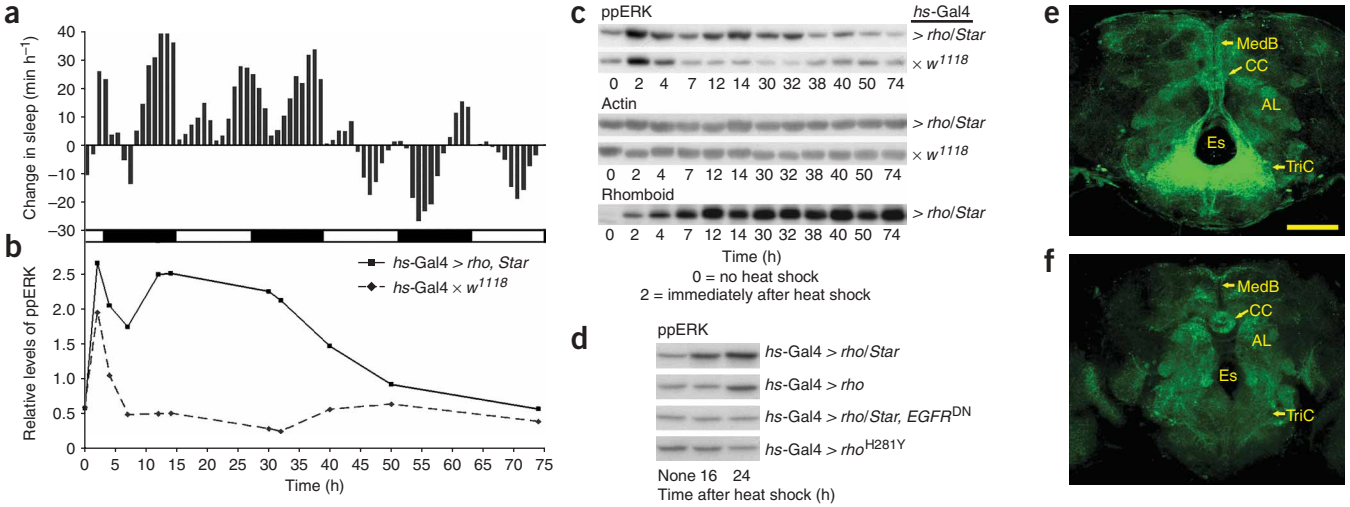

Figure 6 Increased sleep correlated with increased levels of ppERK. (**a**) Histogram of mean population ($n = 19$) change in sleep levels over 3 d in light:dark conditions starting 1 h before heat shock for *hs*-Gal4 > *rho*/*Star* flies and non–heat shocked controls, showing the number of minutes per hour that the flies slept more (positive) or less (negative) than did the controls. (**b**) Levels of ppERK, normalized to actin loading control on immunoblots, prepared from fly heads collected at marked times. (**c**) Immunoblots for the data quantified in **b**, using antibodies against ppERK, actin and Rho. Flies in monitors for **a** and flies in vials for **b** and **c** were heat shocked together and then separated for further monitoring and collection. This was done twice with similar results. (**d**) Immunoblots stained against ppERK at 0, 16 and 24 h after heat shock. *hs*-Gal4 > *rho*/*Star* (line 1) served as a positive control for the others. (**e**) Cumulative *Z*-series stack of a whole-mount *hs*-Gal4 > *rho*/*Star* brain 23 h after heat shock, stained for ppERK (green). (**f**) *hs*-Gal4 × w^{1118} control, also heat shocked and collected at the same time. A confocal slice through the central complex showing equal staining in both conditions served as the standardizing control (shown embedded in the projections). Secondary antibody failed to stain in the brain (data not shown). AL, antennal lobe; CC, central complex; Es, esophagus; MedB, median bundle; OL, optic lobe; TriC, tritocerebrum. Scale bar equals 100 μm.

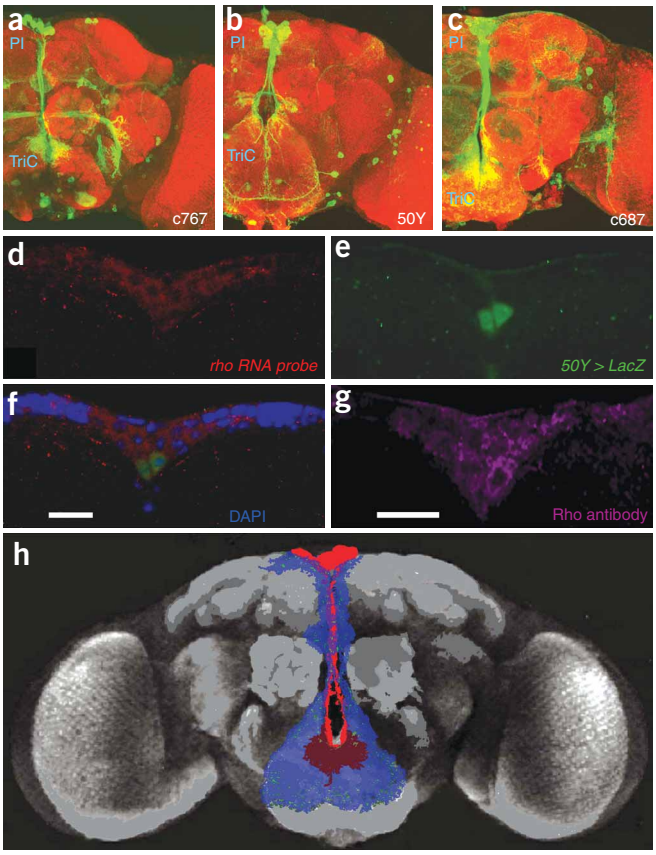

Figure 7 Rho is expressed in pars intercerebralis (PI) cells. (**a**–**c**) Whole-mount brains showing the expression pattern of drivers c767, 50Y and c687, as revealed by a membrane-bound form of GFP that was expressed under the control of a UAS promoter (UAS-mCD8::GFP.L) (green). The cells at the top are part of the PI, whose axons innervate the tritocerebrum. The neuropil-staining antibody nc82 was used to visualize overall brain structure (red). (**d**–**f**) A 2-μm frontal section from the PI region of a 50Y > *LacZ* brain costained for *rho* with antisense RNA (**d**, red) and antibody to β-Gal (**e**, green) is shown, and **d** and **e** were merged (**f**), along with DAPI staining (blue), to show the overlap between *rho*, 50Y and cell nuclei. (**g**) The PI region in an independently stained w^{1118} brain with antibody to Rho showing the same pattern as the *rho in situ* hybridization (**d**). Scale bars represent 20 μm. (**h**) Brain regions involved in the effects of Rho, EGFR and ERK on sleep. Composite false-colored assembled image of the c767 driver pattern in the PI, median bundle and tritocerebrum (bright red), and activated ERK (ppERK) in response to Rho and Star overexpression (blue), overlaid onto an image of a *Drosophila* brain. The expression patterns were 'grabbed' from the appropriate stained brains by the 'magic wand' tool in Photoshop, and adjusted to fit onto the scale of the portrayed brain.

heavily stained for ppERK after heat shock induction of Rho and Star (**Fig. 6e**). To further confirm that there is overlap between the drivers in the pars intercerebralis region, we used those with the narrowest expression patterns, 50Y and c767, simultaneously to express GFP, and then counted cell bodies in the pars intercerebralis. 50Y had 14–15 Gal4-expressing cells in the pars intercerebralis and c767 had 11–12, and in brains expressing both drivers there were 18–21 cells labeled with GFP. Therefore there is a likely overlap in 6–7 cells (there was also a much brighter GFP signal in 6 cells when using both drivers, data not shown). Although these 6–7 cells are not necessarily the only ones mediating the abnormal sleep effect of UAS-rho^{DN}, there is nonetheless a direct overlap between 50Y and c767.

Next, we asked whether endogenous *rho* was expressed in these pars intercerebralis cells. Using fly brains of genotype 50Y > *LacZ*, we found that *rho* was present in many of the large pars intercerebralis cells (**Fig. 7d**, red), and there was some overlap with 50Y cells marked by a *LacZ* transgene (**Fig. 7e**,**f**, green). DAPI DNA staining revealed that *rho* was not present in all cells, and that in those cells that did express *rho*, the RNA transcript was restricted to the cytoplasm, as expected (**Fig. 7f**). To strengthen confidence in the specificity of the *rho* RNA expression pattern, we stained w^{1118} brains for Rho protein with antibody to Rho (**Fig. 7g**), and observed similar results in the pars intercerebralis (note that Rho is also expressed in other parts of the brain).

DISCUSSION

The findings reported here show a previously unknown role for EGFR and ERK signaling in sleep regulation and consolidation in *Drosophila* (**Supplementary Fig. 1**). In the adult fruit fly, EGFR is expressed ubiquitously throughout the nervous system[23], where its only known role is in the maintenance and survival of neurons[24]. Our results demonstrate that the overexpression of EGFR pathway signaling components Rho and Star in *Drosophila* causes an acute, reversible and dose-dependent increase in sleep that tightly parallels an increase in phosphorylated ERK in the head. The ability of a dominant-negative EGFR to block the activation of ERK, as well as the known selectivity of Rho for these ligands[8,9], argues that the manipulation is specific to the EGFR pathway. In contrast to the increase in sleep amount after Rho overexpression, inhibiting its expression led to a significant decrease in sleep. Notably, this decrease in sleep was due to a marked shortening of the duration of sleep episodes accompanied by an elevation of sleep bout number. This observation suggests that flies have an increased need for sleep, but are unable to stay asleep, which is perhaps analogous to insomnia in humans. Therefore, we propose that the EGFR pathway is essential for sleep maintenance.

The brain regions that appear to be involved in the influence of signaling by Rho, EGFR and ERK on sleep are the pars intercerebralis, median bundle and tritocerebrum. The cells of the pars intercerebralis contain Rho and generate EGFR ligand that activates ERK in the receiving cells in the tritocerebrum (**Fig. 7h**). We identified the pars intercerebralis as the region that is responsible for EGFR ligand secretion by demonstrating that inhibiting Rho in this region resulted in decreased sleep, and that the cells in that region expressed endogenous Rho. We identified the tritocerebrum though the system-wide overexpression of the EGFR ligand–processing components Rho and Star, which resulted in a localized hyperactivation of ERK. This is presumably because an ectopic presence of Rho and Star will only result in heightened EGFR signaling if the cells contain endogenous ligand precursor.

Although the mushroom body is the only region of the *Drosophila* brain that has been reported to have an effect on sleep[25,26], we did not observe Rho expression in the mushroom body, nor did inhibiting Rho with UAS-rho^{DN} in this structure have any effect on sleep levels (**Supplementary Table 2** and data not shown). However, it is reasonable to expect that the regulation of sleep would involve multiple brain regions and pathways, and that the regulation, versus the function, of sleep could be two distinct, but linked, processes.

Cells of the pars intercerebralis send out axonal projections though the median bundle and then bifurcate, innervating the tritocerebrum or running alongside the esophageal canal to innervate the endocrine gland corpora cardiaca[27]. Our results indicate that the pars intercerebralis cells innervating the corpora cardiaca are not the ones responsible for the observed decrease in sleep, as Gal-4 drivers that are active in these cells[28] did not produce a significant drop in sleep

levels when expressing *rho* RNAi (**Supplementary Table 2** and data not shown). Developmental studies have led to the postulate that the pars intercerebralis and the corpora cardiaca are the developmental equivalent of the mammalian hypothalamic-pituitary axis[29–31]. The hypothalamus is a major center in the mammalian brain for the regulation of arousal[32–35], and the SCN, which is a part of the hypothalamus, has already been shown to regulate circadian activity through EGFR signaling.

Vertebrate studies have only investigated EGFR signaling in the subparaventricular zone, a region located immediately adjacent to the SCN, and this region did not affect total sleep levels, but did alter its timing[5]. In addition, evidence in mammals for a role of EGF in sleep *per se* is equivocal[6]. Our results directly demonstrate that the disruption of EGFR ligand production affects sleep though the pars intercerebralis and not though the circadian control center of the *Drosophila* brain. It also suggests that the pars intercerebralis shares some functional, as well as developmental, homology with the mammalian hypothalamus through its crucial and conserved involvement in regulating sleep and its maintenance with neural hormones such as the EGFR ligands.

In the fly, a single member of the EGFR family binds both the TGF-α–like family of ligands (Spitz, Gurken and Keren) and the neuregulin-like ligand Vein[9]. In vertebrates, these ligands bind to specific ErbB family members, with ErbB-1 (EGFR) binding EGF and TGF-α, whereas ErbB-3 and ErbB-4 bind the neuregulins[36]. In mammalian systems, ErbB-2 and ErbB-4 cofractionate, coimmunoprecipitate and colocalize in cultured rat hippocampal neurons with the postsynaptic density protein PSD-95 (also known as SAP90), and show exclusion from presynaptic terminals *in vivo*[37,38]. Similarly, ERK colocalizes with, and directly phosphorylates, PSD-95, as is the case with the ErbB receptor–family members[39,40]. In the fly, EGFR interacts with the postsynaptic density protein Discs Large (Dlg), the *Drosophila* homolog of PSD-95 (ref. 41).

ERK has a role in synaptic plasticity that is conserved among *Aplysia, Drosophila* and mammals[42,43]. A recent study shows that ERK directly phosphorylates the pore-forming α subunit of the A-type potassium channel Kv4.2, a member of the Shal-type (Shaker-like) family[44]. This broadens the role of ERK beyond the realm of cell proliferation, differentiation, and even long-term memory consolidation, and suggests that it may also contribute to the more immediate alterations of the electrical properties of the neuronal membrane.

On the basis of our findings and the published reports on the functions of EGFR, we propose the following cellular mechanism for sleep regulation in *Drosophila* (**Supplementary Fig. 2** online). Star and Rho in the pars intercerebralis produce and secrete ligand to EGFR located at the postsynaptic membrane of neurons in the tritocerebrum, leading to the activation of ERK in these cells. The difference in staining patterns between inactive ERK clustering near synapses (data not shown) and active ERK located out in the axons indicates that the activated ERK, at least in part, translocates from the postsynaptic membrane and spreads out into the axons that fill out the tritocerebrum and other locations to which these cells project. As a result of a lack of ppERK in the cell bodies of these neurons and the reversible nature of the sleep behavior, it is unlikely that these cells are undergoing long-term synaptic structural changes associated with changes in gene expression. Instead, we propose that the action of ppERK occurs at the synapse or in the axon (or both), where it is possibly altering the gating of a neural receptor or channel, and thus changing the membrane properties of the cells. This modification results in an altered brain state that ultimately manifests itself in the sleep behavior of the animal. Such a model is consistent with a previously described mutation in the potassium channel *shaker* (Kv1.4), which has been shown to be incapable of getting much sleep[45].

METHODS

***Drosophila* stocks and conditions.** Flies cultures consisted of yeast, molasses and agar food, and were kept and assayed at 23 °C under 12 h light and 12 h dark conditions. Stocks of *w*;;hs*-Gal4, *w*;*UAS-*rho*;, *w*;;*UAS-*rho*,UAS-*Star*, *w*;;*UAS-*rho*^{DN} and *w**,UAS-*s-spitz* came from A. Guichard and E. Bier (University of California San Diego); 50Y, c687, c767 and c774 were obtained from D. Armstrong. *Elav*-Gal4, *w*^{1118} (#5905), *w*;*UAS-*EGFR*^{DN};UAS-*Egfr*^{DN}, *w*;*UAS-*mCD8::GFP.L* and *tubulin*-Gal80^{ts} stocks came from the Bloomington Stock Center. An *in vivo* ethyl methane sulfonate (EMS) screen identified Rho^{H281Y} using a leaky *hs-rho* construct (A. Guichard, personal communication). After cloning into pUAST and injection (Rainbow Transgenic Fly Service), misexpression of Rho^{H281Y} in wings (MS1096 > *rho*^{H281Y})^{12} confirmed that it was inactive, being indistinguishable from the MS1096 × *w*^{1118} control.

Activity data collection and statistical analysis. Female assays with the TriKinetics *Drosophila* activity-monitoring system on 5% sucrose/1% agar measured sleep in 5-min bins as previously described[2,3]. A vibrating platform enabled sleep deprivation experiments with 10-s vibrations at 3–4-min intervals, processed as previously described[46]. Heat shock experiments involved predrying empty tubes with desiccant overnight at 37 °C to reduce moisture condensation, raising incubator temperature to 37 °C for 1 h, and verifying that flies were not stuck to the tubes.

Statistical analysis was carried out with JMP software. Normality determinations were carried out with the Wilks-Shapiro test, parametrically distributed data was analyzed with one-way ANOVA with the Tukey-Kramer HSD Test as the *post hoc* analysis, and nonparametric was data analyzed with Wilcoxon/Kruskal-Wallis ANOVA tests with the appropriate α level being determined by the Bonferroni correction for multiple independent comparisons ($P < 0.05$)[47]. A combined spectral and autocorrelation analysis[48] enabled calculation of circadian period, and phase calculations used a single 'center of gravity' peak value (CHRONO software)[49].

Western blot. Rho extraction buffer consisted of 2.5% CHAPS, 50 mM KCl, 120 mM NaCl, 2 mM DTT, 20 mM Tris-HCl pH 7.5, protease inhibitor cocktail with EDTA (Roche), and phosphatase inhibitor cocktails I and II when needed (Sigma). We lysed frozen heads (liquid nitrogen) in 15 μl per head (motorized pestle), and then kept them on ice for 45 min, intermittently vortexing them. Total protein concentration measurements were done by the Bradford method, and samples were loaded without heating for Rho detection, but were boiled for 5 min otherwise. Each gel lane consisted of 5 μg protein (~half of a female fly head). We used Rho antibody at a 1:3,000 dilution (from E. Bier), antibody to ppERK (Sigma) at a 1:5,000 dilution, and antibody to actin (JLA20, Developmental Studies Hybridoma Bank) at a 1:10,000 dilution, and quantified band intensities with ImageJ (US National Institutes of Health).

Immunohistochemistry: For ppERK, flies were collected from activity monitors in batches of 7 on ice, the heads and probosci were removed and fixed in 8% paraformaldehyde/PBS (no detergent) for 40 min on a 50 rpm rotating shaker. The brains were then dissected and stained with antibody to ppERK (Sigma) at a 1:200 dilution in 0.2% BSA, 0.3% Triton-X 100, and 0.3% deoxycholate in PBS pH 7.4. All brains were mounted in FluoroGuard Antifade Reagent (Biorad), and acquisition of fluorescently labeled images was carried out on a Leica SP2-AOBS (Leica Microsystems) scanning confocal microscope. The tritocerebral–median-bundle–protocerebral ppERK signal proved to be a single, continuous unit by three-dimensional imaging using the Improvision program Volocity (data not shown). For Rho, after fixing the heads in 6% paraformaldehyde/PBS (1 h), we stained them with antibody to Rho at a 1:500 dilution (blocking buffer, 0.3% Triton-X 100, 0.3% deoxycholate, 5% normal goat serum, 0.2% BSA in PBS for all staining except ppERK). For *Rho in situ* hybridization with β-Gal immunohistochemistry, we removed the heads and probosci of 50Y > *LacZ* flies, placed them on ice, fixed them for 1 h (shaken at 50 rpm) in 6% paraformaldehyde/PBS and 0.1% Triton-X 100, pH 9.5 to

enhance their signal[50], and used antibody to β-galactosidase at a 1:1,000 dilution (Promega). Dinitrophenol (DNP)-labeled *rho* antisense RNA was a gift from D. Kosman and W. McGinnis (University of California San Diego).

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

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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