Regulation of Daily Locomotor Activity and Sleep by Hypothalamic EGF Receptor Signaling
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15. The pSG5-CARM1 wild type and CARM1^{189}VLD^{191} → AAA mutant were a kind gift from M. R. Stallcup. CARM1 cDNA was amplified with primers: 5′ GGAATTCATATGGCAGCGGCGGCAGCGACGG 3′ and GAGCAGATCTCTAACTCCCATAGTGCATGGTG-TTGG 3′ and subcloned into pFlag-AcSG2 (PharMingen). Recombinant CARM1 proteins were expressed in SF9 cells via the baculovirus system (PharMingen) and purified through Flag-M2 affinity resin. CARM1 cDNA was also subcloned into pCMX-L2 and pCMX-VP16 vectors to generate CMX-CARM1 and CMX-VP16-CARM1. Flag-tagged p300, ACTR, and PCAF were expressed and purified as described (*8*).
16. Chromatin was preassembled in Sf9 *Drosophila* extract for 4 hours at 27°C, then ~50 μM of each coactivator was added and the incubation extended for another 30 min. Transcription was initiated by addition of Hela nuclear extract and dNTPs. Transcript amounts were analyzed by primer extension to determine the relative levels.
17. Acetylation and methylation assays were performed essentially as described (*8, 9*).
18. Supplemental material is available on *Science* Online at www.sciencemag.org/cgi/content/full/1065961/DC1
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20. Approximately 5×10^6 293T cells were transfected with CMX-CARM1 alone or with CMX-Flag CBP 1 day before the labeling. We added 50 μl of 486 μCi/ml S-AdoMet to 5 ml of methionine-depleted DMEM with dialyzed serum, and then applied to cells and incubated at 37°C for 4 hours. Immunoprecipitation with anti-CBP (A22, Santa Cruz) was performed as described (*38*).
21. Purified CREB proteins were in vitro phosphorylated by PKA (*33*). EMSA was performed as described (*38*).
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28. CARM1 was subcloned into pLNCX (Clontech) between Hind III and Cla I sites. Phenix cells were transfected with pLNCX-CARM1 to produce retrovirus, which was then used for infection of PC12 cells. G418 (400 μg/ml) was used to select stably transfected cells. Expression of CARM1 was confirmed by Western blotting.
29. Portions of p300 (amino acids 1 to 300, 301 to 800, 1060 to 2000, and 1500 to 2414) were expressed as GST fusion proteins in *Escherichia coli* as described in D. Chakravarti *et al.* [*Nature* **383**, 99 (1996)].
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39. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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Regulation of Daily Locomotor Activity and Sleep by Hypothalamic EGF Receptor Signaling

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The circadian clock in the suprachiasmatic nucleus (SCN) is thought to drive daily rhythms of behavior by secreting factors that act locally within the hypothalamus. In a systematic screen, we identified transforming growth factor-α (TGF-α) as a likely SCN inhibitor of locomotion. TGF-α is expressed rhythmically in the SCN, and when infused into the third ventricle it reversibly inhibited locomotor activity and disrupted circadian sleep-wake cycles. These actions are mediated by epidermal growth factor (EGF) receptors on neurons in the hypothalamic subparaventricular zone. Mice with a hypomorphic EGF receptor mutation exhibited excessive daytime locomotor activity and failed to suppress activity when exposed to light. These results implicate EGF receptor signaling in the daily control of locomotor activity, and identify a neural circuit in the hypothalamus that likely mediates the regulation of behavior both by the SCN and the retina.

Circadian rhythms of behavior in mammals are robust and precise. For example, in constant darkness and temperature, the circadian rhythm of locomotor activity in laboratory rodents persists indefinitely (*1*) and is accurate to within a few minutes per day (*2, 3*). The circadian clock driving locomotor activity and other circadian behaviors, such as the sleep-wake cycle, is located within the suprachiasmatic nucleus (SCN) of the hypothalamus (*4*).

The molecular mechanisms by which the SCN drives circadian rhythms of locomotor activity and other behaviors are unknown. Intriguing clues, however, have come from SCN transplant studies. In animals made arrhythmic by SCN lesions, SCN grafts drive circadian rhythms of locomotor activity (*5*), even if the grafts are encapsulated (to prevent extension of axons but allow diffusion of secreted factors) (*6*). A study of "temporal chimeras" (*7*), hamsters with functional SCN tissue of both wild-type and short-period mutant genotypes, indicated that the SCN inhibits locomotor activity at one phase and promotes it at another, with inhibition dominating when the two influences coincided. These and related studies (*8–10*) suggest that the SCN drives circadian rhythms of locomotor activity by secreting at least one "locomotor inhibitory factor" at one phase and at least one "locomotor activating factor" at another. Although the effects of SCN grafts are mediated by factors secreted into the third ventricle of the hypothalamus in a paracrine fashion, it is possible in the intact animal that the secreted SCN factors act synaptically (*6*).

Transplant experiments indicate that the receptors for the secreted SCN factors are located

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near the third ventricle of the hypothalamus (*10*). The major projection of the SCN is to the subparaventricular zone (SPZ) (*11*), a little understood hypothalamic region flanking the third ventricle. Lesions of the SPZ disrupt circadian regulation of locomotor activity (*12*), making the SPZ a likely location for receptors of secreted SCN locomotor factors, whether synaptic or paracrine.

Under a 24-hour light-dark (LD) cycle, the daily timing of locomotor activity depends on both light and the circadian clock. Light influences locomotor behavior in two ways. First, it resets the circadian clock (*13*) via the retinohypothalamic tract (RHT), a direct projection from the retina to the SCN (*4*) and other hypothalamic sites (*14, 15*). Second, it acts acutely in an effect termed "masking." In nocturnal animals like hamsters and mice, light suppresses locomotor activity (*16*) independently of the circadian clock, requiring neither a genetically functional clock (*17*) nor an intact SCN (*18, 19*). Nevertheless, both masking (*20*) and circadian clock resetting (*21*) involve similar or identical photoreceptors in the inner retina, raising the possibility that masking, like clock resetting, is mediated by the RHT, which directly projects to the SPZ (*15*). The molecular basis of masking is unknown.

Screen for secreted SCN "locomotor factors." We performed a systematic molecular and behavioral screen to identify locomotor factors secreted by the SCN. To find secreted factors not previously documented in the SCN, a hamster SCN cDNA library was screened in a yeast secretion-trap system (*22, 23*). A behavioral screen followed in which newly identifed and previously documented (*9, 24–26*) SCN factors were tested for an effect on circadian locomotor activity by constant infusion into the third ventricle of hamsters for 2 to 3 weeks (*27*). In general, constant infusion of an SCN locomotor factor should alter locomotor activity reversibly without affecting the underlying SCN circadian clock. A locomotor inhibitory factor, for example, should block locomotor activity for the duration of the infusion. Because the SCN clock should not be affected, the circadian rhythm of locomotor activity should reappear with its expected phase and period upon cessation of the infusion. In contrast, constant infusion of SCN factors involved only in outputs other than locomotor activity should have no effect on locomotor behavior.

Chronic infusions of artificial cerebrospinal fluid (aCSF) into the third ventricle had little effect on the circadian rhythm of running-wheel behavior, causing at most a modest reduction in overall activity without affecting the period, phase, or precision of the rhythm (Fig. 1, upper left). Altogether, 32 secreted peptide or protein factors were tested at least twice each (singly or in pools), of which 11 were among the newly identified SCN factors and the rest were previously documented. Most had little or no effect on locomotor behavior (*27*). For example, coinfusion of neuropeptides thought to be coreleased from SCN neurons (vasoactive intestinal polypeptide, peptide histidine-isoleucine, gastrin-releasing peptide, and neuromedin-C) (*28*) had no effect on the amount or precision of circadian running-wheel activity (Fig. 1, upper right).

One peptide, transforming growth factor-α (TGF-α), behaved exactly as expected for a SCN locomotor inhibitory factor. TGF-α, localized in the SCN (*26*), completely blocked running-wheel activity during the ~3-week infusion. Upon cessation of the infusion, the running-wheel activity rhythm quickly reappeared with the expected phase and period ($n = 6$) (Fig. 1, lower left). The only known receptor for TGF-α is the epidermal growth factor receptor (EGFR), which is also activated by EGF (*29*). To determine whether TGF-α was acting through the EGFR, we tested EGF, which is not detectably expressed in adult hamster hypothalamus (*19*). EGF also reversibly blocked running-wheel activity ($n = 4$) (Fig. 1, lower right), implicating the EGFR as the relevant receptor for TGF-α.

TGF-α and EGFR in the hypothalamus. TGF-α mRNA was highly expressed in the SCN and piriform cortex, with less expression in the caudate-putamen and the supraoptic nuclei (Fig. 2A). *TGF*-α mRNA expression in the SCN showed a circadian rhythm (Fig. 2B) (*30, 31*) that was comparable in amplitude to the rhythms of *Cry1* and *Cry2* (*32*), known clock-controlled SCN transcripts. The phase of the *TGF*-α rhythm agrees with that expected for a locomotor inhibitory factor—its peak (CT6) corresponded to the time of locomotor quiescence and its trough (CT18) to a time of locomotor activity. As expected, TGF-α protein was also detected (*33, 34*) in SCN cells (Fig. 2C).

The EGFR was detected in multiple areas of the adult hamster brain, as reported for rat (*35*), but an additional high concentration was detected by immunohistochemistry in the SPZ, the major target field of the SCN (Fig. 2D). The majority of labeled cells had neuronal morphology when viewed at high magnification (Fig. 2E). The EGFR is thus localized as predicted for a receptor for an SCN locomotor factor. TGF-α and its receptor, the EGFR, therefore satisfy pharmacological, temporal, and anatomical predictions for playing a role in circadian inhibition of locomotor activity by the SCN.

Effect of TGF-α on the sleep-wake cycle. To evaluate the physiological effects of TGF-α in greater detail, we monitored the electroencephalogram (EEG), the electromyogram

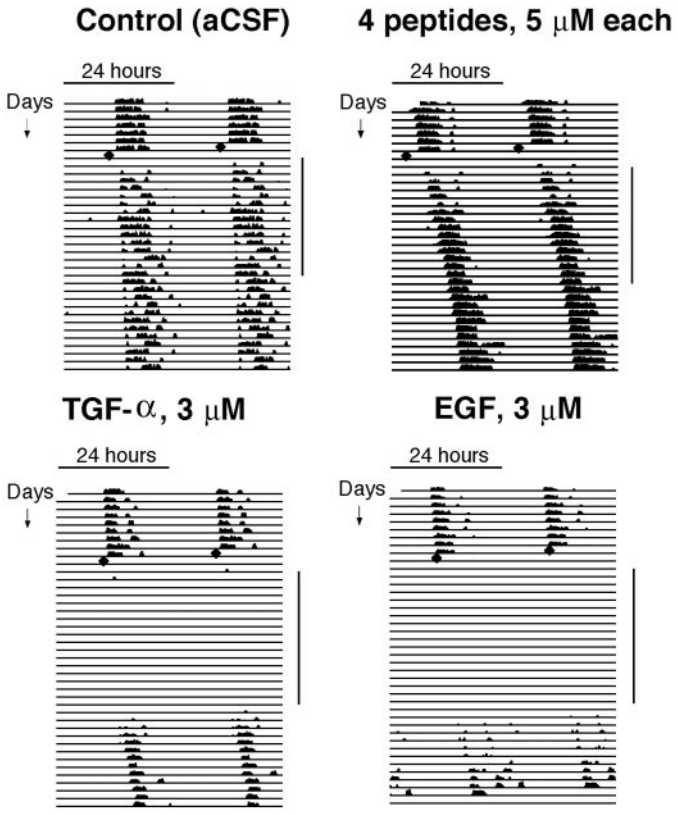

Fig. 1. Reversible inhibition of locomotor activity by TGF-α. Double-plotted running-wheel activity records of hamsters in constant dim light are examples from a behavioral screen for secreted SCN locomotor factors (*27*). Factors were infused into the third ventricle (0.5 μl/hour) for 2 to 3 weeks via an implanted cannula with an osmotic minipump, and locomotor activity was monitored. Two days are represented horizontally, and lines on the vertical axis represent successive days. Tick marks, running-wheel revolutions (>0 per 10-min bin); height of each mark, number of revolutions; solid diamond, time of cannulation; and bar at the right of the record, period of infusion. Control infusions of artificial CSF (aCSF) caused a modest reduction in activity, and the pool of four SCN neuropeptides produced no substantial effect. TGF-α produced a reversible inhibition of locomotor activity without affecting the phase or period of the circadian clock ($n = 6$). EGF ($n = 4$) had an identical effect.

(EMG), bodily movement, and body temperature of hamsters kept in constant darkness during control or TGF-α infusions into the third ventricle (*36*). During control infusions, episodes of waking, non-REM sleep, and REM sleep were normal for hamsters (*37*), and the circadian component of sleep-wake regulation was evident (Fig. 3, top left). As expected, there was a circadian rhythm of bodily movement (Fig. 3, middle left), an assay that reports positional changes and exploratory behavior (*36*), rather than the strong locomotor drive that the running-wheel activity assay reports. Also evident was a circadian rhythm of body temperature (Fig. 3, bottom left), which in rodents is mainly a consequence of physical activity rather than an independent rhythm (*38*).

During TGF-α infusions, episodes of waking, non-REM sleep, and REM sleep were normal in appearance, amount, and duration (*19*). Thus, the blockade of running-wheel behavior by TGF-α was not due to hypersomnolence or other gross disturbances of cortical physiology but rather to a more discrete action. However, TGF-α infusion altered the timing of the sleep-wake cycle; normal circadian regulation was replaced by a highly regular and reproducible ultradian rhythm of 5 to 6 cycles per day (Fig. 3, top right). Like controls, animals infused with TGF-α were physically active (Fig. 3, middle right), indicating that the blockade of running-wheel activity was not due to a general blockade of motor function. Nonetheless, the circadian rhythm of bodily movement was disrupted or diminished (Fig. 3, middle right), suggesting that TGF-α plays a role in the circadian regulation of activities less vigorous than wheel-running without acting as an inhibitor. Body temperature showed an ultradian rhythm that precisely followed the sleep-wake rhythm (Fig. 3, bottom right) with a lag of ~30 min, providing an independent measure of the ultradian physiological oscillation produced by TGF-α.

The ultradian sleep-wake and temperature rhythm produced by third ventricle infusion of TGF-α closely resembled the effect of a focal excitotoxic lesion of SPZ neurons (*12*). This ultradian rhythm is normally inhibited by circadian control and is disinhibited when SPZ neurons fail to relay SCN circadian information to sleep-wake circuits (*12*). Our results indicate that chronic TGF-α administration uncouples SPZ neurons from sleep-regulatory circuits and that SPZ neurons expressing the EGFR transmit circadian information from the SCN to sleep-wake centers, in addition to likely regulating circadian locomotor activity.

Genetic analysis of the role of the EGFR in locomotor activity. If the EGFR mediates circadian inhibition of locomotor activity in a nonredundant manner, then mice with a loss-of-function mutation in the EGFR should exhibit excessive activity during the light period (day) in LD cycles and during subjective day in constant darkness (DD) (times when mice are normally quiescent). We monitored the running-wheel behavior of mice with *waved-2*, a point mutation in the EGFR that causes an 80 to 95% decrease in ligand-stimulated receptor tyrosine kinase activity (*39*). Unlike EGFR-null mutants that die in the early postnatal period or earlier (*40*), *waved-2* mice develop into viable and essentially normal adults.

The running-wheel activity of *waved-2* mutant mice (*41*) showed entrainment to a 12-hour light, 12-hour dark (12:12 LD) cycle and had an appropriate circadian period in DD (Fig. 4A) (*19*), indicating that the fundamental properties of the SCN circadian clock were normal. How-

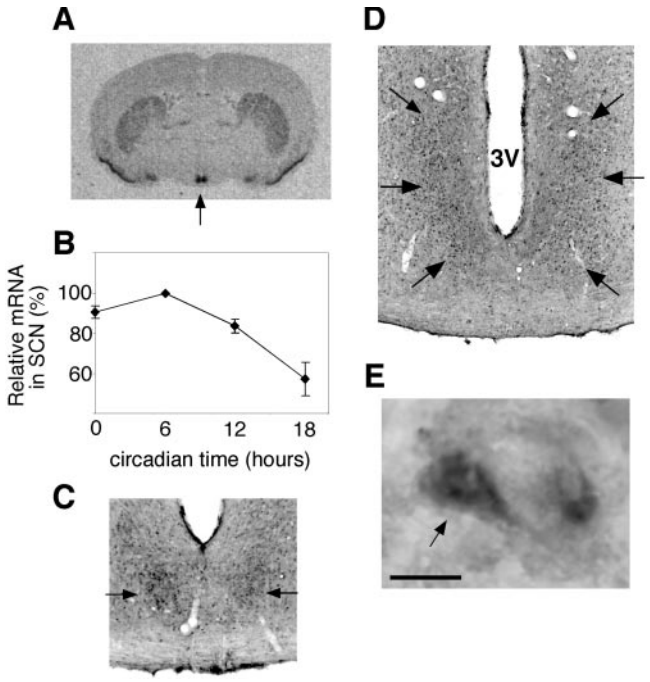

Fig. 2. TGF-α and the EGFR in the hypothalamus. (**A**) In situ hybridization (*31*) showing *TGF-α* mRNA in a coronal section of hamster brain obtained at CT6. Arrow, SCN. (**B**) Densitometric analysis of *TGF-α* mRNA levels in the SCN at different circadian times, plotted relative to the maximum signal ($n = 5$ animals for each time-point; error bars, SEM). A rhythm with the same phase and amplitude was found in animals under LD cycles (*45*). (**C**) Immunolabeled hamster brain section showing TGF-α protein in SCN neurons (*34*). Arrows, bilateral SCN. (**D**) Low-magnification view (10×) of immunolabeled coronal hamster brain section showing the EGFR in neurons (dark cell bodies) in the SPZ (roughly delimited by arrows) (*34*). Far fewer immunolabeled cells were seen in the lateral hypothalamus, and almost none were seen in the thalamus (*45*). 3V, third ventricle. (**E**) Typical neuronal morphology of EGFR-expressing cells in the SPZ (magnification: 100×). Note large triangular cell body with prominent proximal dendrite (arrow). Bar, 10 μm.

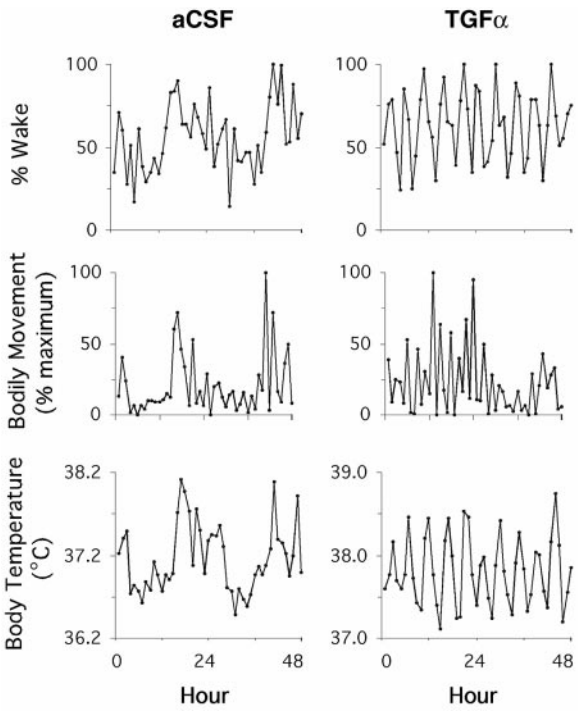

Fig. 3. Disruption of the circadian rhythm of sleep-wake behavior by infusion of TGF-α into the third ventricle (controls, $n = 3$; TGF-α, $n = 5$). EEG, EMG, bodily movement, and body temperature were monitored simultaneously in individual cannulated hamsters (*36*). Data are displayed in 1-hour bins. The control was infusion of aCSF.

ever, in LD cycles, *waved-2* mice were abnormally active during the day compared to wild-type mice of identical genetic background, and this abnormal activity substantially degraded the precision of activity onsets at night (Fig. 4A). As expected, wild-type mice had very little daytime running-wheel activity, only 1.4 ± 0.57 (SEM)% of the total, whereas mutants had 11.5 ± 4.75%. Heterozygotes were similar to wild types (3.9 ± 0.72%), and overall there was a significant effect of genotype [analysis of variance (ANOVA), $P < 0.02$] (*41*). These results demonstrate that the EGFR mediates inhibition of locomotor activity under LD cycles.

When the mice were in DD, we could detect no statistically significant difference among the genotypes in the amount of running-wheel activity during subjective day or in the distribution of activity during subjective night (although abnormalities observed in the *waved-2* mutant mice in LD cycles often appeared to persist). Thus, it is possible that the EGFR does not play a role in the circadian inhibition of locomotor activity, but is somehow restricted to acting only under LD cycles. Alternatively, this partial loss-of-function mutation might not produce a strong locomotor phenotype in DD because of redundancy in the circadian control of locomotor inhibition. The latter seems more likely given the broad evidence for the involvement of TGF-α and the EGFR in the circadian inhibition of locomotor activity and sleep (Figs. 1 through 3).

Why is the locomotor phenotype of *waved-2* mutants different in a LD cycle and in DD? To address this question, we monitored masking of running-wheel behavior in response to 3- and 6-hour light pulses during subjective night (*41*). Wild-type mice showed complete inhibition of running-wheel activity during the light pulses, whereas *waved-2* mutants showed little inhibition (Fig. 4B) [inhibition, 95% ± 1.1 (SEM), 90% ± 1.9, and 53% ± 20.9 for wild-type, heterozygous, and homozygous mice, respectively; $P < 0.01$, ANOVA] (*41*). These results demonstrate that EGFR activity is required for normal masking responses and consequently for the proper organization of daily locomotor activity in a 24-hour LD cycle, in addition to any role in the circadian regulation of locomotor activity.

Because *waved-2* mutants entrain to LD cycles (Fig. 4A) and show appropriate phase-shifts to light-pulses (*19*), the retinal photoreceptors thought to underlie both circadian phase-shifting and masking (*20*) and the transmission of luminance information by the RHT to the hypothalamus must be intact. Thus, the defect is very likely manifested within the hypothalamus or in downstream circuits. Taken together, our results implicate EGFRs expressed by hypothalamic SPZ neurons in the inhibitory regulation of locomotor activity, likely in response both to light and to the circadian secretion of TGF-α from the SCN.

TGF-α and EGF in the retina. Because EGFR signaling is required for masking (Fig. 4B), and masking does not require an intact SCN (*18, 19*), the ligands for the EGFR that mediate masking must come from a source outside the SCN. If the documented projection from retinal ganglion cells to the SPZ (*15*) mediates masking, at least a subset of retinal ganglion cells should express one or more ligands for the EGFR. TGF-α and EGF are found in adult human retina, with TGF-α immunoreactivity observed in Muller glia and ganglion cells and weak EGF immunoreactivity reported throughout the retina (*42*). To extend these observations, we performed immunohistochemistry (*43, 44*) for TGF-α and EGF on sections from adult mouse retinas. TGF-α was expressed in Muller glia and throughout the ganglion cell layer (Fig. 5, left). In contrast, EGF expression was confined to a few cells in the inner nuclear layer (*19*) and to a small, but widely distributed subset of cells in the ganglion cell layer (Fig. 5, right). In number and distribution, this subset closely resembles the retinal ganglion cells that give rise to the RHT (*45*). These results are consistent with direct regulation of the EGFR on SPZ neurons by retinal

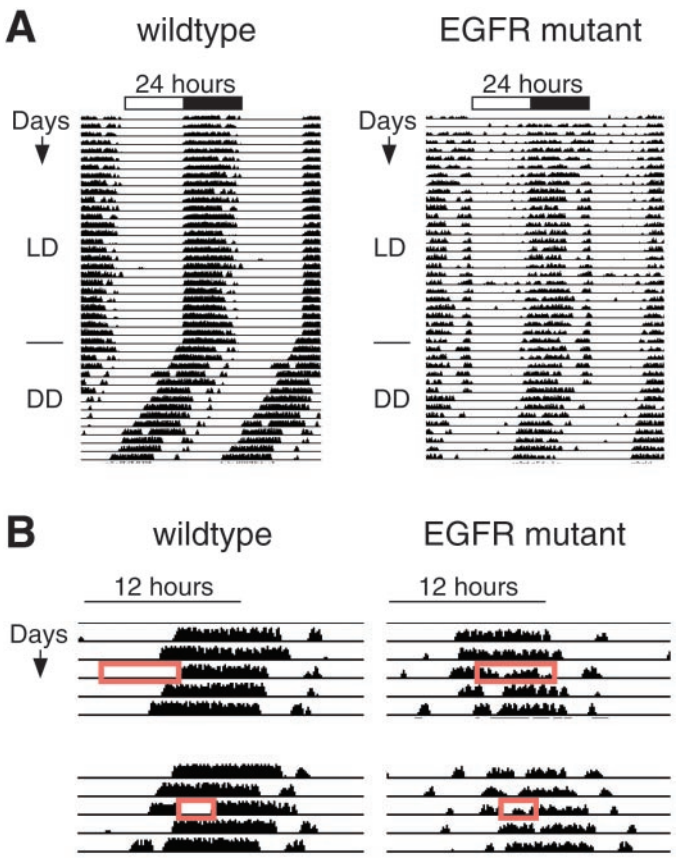

Fig. 4. Abnormal locomotor behavior of EGFR mutant mice. (**A**) Double-plotted running-wheel records of littermate wild-type and homozygous *waved-2* mutant mice housed under the same conditions. LD, 12:12 light-dark cycle, represented by open/solid bars, respectively, at top. Horizontal line at the left of each record marks the transition from LD into constant darkness, DD. Note excessive daytime activity in mutant (*41*). (**B**) Defect in the masking response to light in EGFR mutant mice. Single-plotted running-wheel records magnified to show acute responses to 3- and 6-hour light pulses (represented by red boxes). Records are from the same two mice as in (A), exposed together to the same light-pulses. In this example, the 6-hour light pulse fell at different circadian phases for the two mice because of a small difference in their circadian periods. The phase of light-pulse administration was not a factor in the masking responses of wild-type or mutant mice (*19*).

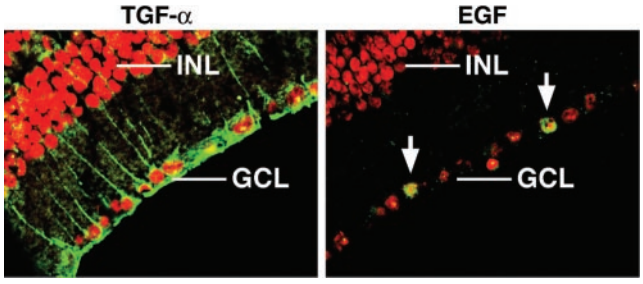

Fig. 5. TGF-α and EGF in the retina. Confocal immunofluorescence images (*44*) with green immunofluorescence representing TGF-α or EGF, as indicated, and red representing fluorescence from ethidium bromide–labeled cell nuclei. INL, inner nuclear layer; GCL; ganglion cell layer. TGF-α is expressed in Muller glia (parallel processes running between the INL and the GCL), and throughout the GCL. EGF is expressed in rare INL cells (not seen in this section) and in a small, widely distributed subset of GCL cells (arrows). The EGF-expressing GCL cells are less abundant than implied in this view, which was selected to show examples of positive cells (magnification: 60×).

TGF-α, or perhaps more likely, EGF.

Hypothalamic EGFR signaling and the daily regulation of behavioral activity. In the nervous system, TGF-α and EGFR have been implicated in diverse developmental processes, such as astrocyte differentiation and neuronal survival, but far less is known about their actions in the adult nervous system (*46*). Our results strongly suggest that TGF-α is a secreted SCN factor involved in the circadian regulation of locomotor activity and sleep and that EGFR signaling in SPZ neurons likely mediates this regulation. Genetic analysis demonstrated that EGFR activity is required for the acute inhibition of locomotor activity in response to light, in addition to a likely role in the circadian inhibition of locomotor activity.

Our results suggest that the independent regulation of behavioral activity by light and by the SCN converge upon EGFR signaling in SPZ neurons. According to this view, luminance information from photoreceptors in the inner retina is transmitted by the RHT to the SCN, where it mediates clock resetting, and to the SPZ, where it mediates masking. EGF (or TGF-α) from retinal ganglion cells mediates masking by activating the EGFR on SPZ neurons, inhibiting locomotor activity. TGF-α, secreted in a circadian fashion from the SCN, activates the EGFR on the same SPZ neurons, contributing to the circadian inhibition of locomotor activity. Thus regulation of behavior by light and by the SCN can be considered as two different inputs to a single hypothalamic circuit that has evolved to regulate behavior precisely in relation to the natural 24-hour LD cycle.

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23. Briefly, the secretion trap was designed as follows: The host yeast strain lacks invertase, an enzyme that must be secreted for the strain to grow on sucrose. A cDNA library prepared from microdissected hamster SCNs was then inserted into a yeast expression vector encoding a mutant invertase, which lacked codons for the initiator methionine and the signal sequence necessary for secretion. After transformation of the host strain with the library, colonies will form on sucrose only in those cases in which a clone from the cDNA library has both an initiator methionine and a functional signal sequence in-frame with invertase. Positives thus include both transmembrane and secreted proteins.
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27. After entrainment to a 14:10 LD cycle for >2 weeks, male Syrian hamsters (Charles River Laboratories) were placed in constant dim light (<1 lux), and running-wheel activity was monitored for >7 days. Animals were anesthetized with ketamine/xylazine (130/26 mg/kg body weight, administered i.p.), and a stainless steel cannula (Plastics One, Wallingford, CT) was placed stereotaxically within the third ventricle and fixed with dental cement. An osmotic minipump (~0.5 μl/hour × 18 to 22 days; Alzet) containing a coded sample was placed subcutaneously and connected to the cannula by tubing filled with 24 μl of aCSF [144 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2 mM $NaPO_4$ (pH 7.4)]. Peptide or protein factors were each at 1 to 5 μM in aCSF in the minipump and cannula; we do not know the concentrations achieved in vivo, but we wanted to saturate any receptors near the third ventricle so as not to miss an effect. Hamsters were returned to constant dim light, and running-wheel activity was monitored for >30 days, after which cannula placements were histologically documented. Sample identities were revealed after analysis of running-wheel records. Known SCN-secreted factors (*9, 24, 25*) that had no apparent effect on the amount of locomotor activity included brain-derived neurotrophic factor, vasoactive intestinal polypeptide, peptide histidine-isoleucine, gastrin releasing peptide, neuromedin-C, substance P, neurokinin A, neuropeptide K, neuropeptide γ, somatostatin, antrin, cholecystokinin, thryotropin-releasing hormone, neurotensin, and neuromedin N. However, because we have not yet documented the stability of these factors under the infusion conditions, negative results are not necessarily meaningful.
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31. Sulfur-35–labeled riboprobes were made from mouse TGF-α and EGF polymerase chain reaction (PCR) products into which the T7 and T3 sites were incorporated, respectively, at each end. PCR primers were: TGF-T3: 5′-aattaaccctcactaaaggggttagctgtgtgccaggctctggag-3′; TGF-T7: taatacgactcactatagggtcagaccactgtctcagagtggcag; EGF-T3: aattaaccctcactaaaggggaatcacggctgtactcttgggtg; EGF-T7: taatacgactcactatagggctgttccatcaaaatgcatgtgtc. No hybridization signals were observed for either sense riboprobe or for the EGF antisense riboprobe. For *TGF-α* mRNA quantification, densitometry was performed by scanning the exposed KodakBioMax MR films (histogram option, Adobe Photoshop). Carbon-14 standards (American Radiolabeled Chemicals) were used to verify that the measured values were within the linear response range of the film.
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34. Hamsters were anesthetized (chloral hydrate, 300 mg/kg body weight, administered i.p.) and perfused with saline followed by 4% paraformaldehyde. Preparation of sections and immunohistochemistry were performed as described (*33*) with rabbit anti-TGFα (1:200; PeproTech) or rabbit anti-EGFR (1:200; Santa Cruz Biotechnology). After overnight incubation with primary antisera, sections were treated for 2 hours with biotinylated donkey anti-rabbit antiserum (1:1000; Jackson ImmunoResearch), followed by a 1-hour incubation with avidin-biotin complex (Vectastain ABC Elite, Vector Laboratories). Immunoreactive cells were visualized by reaction with 3,3′-diaminobenzidine (Sigma).
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36. EEG, EMG, body temperature, and bodily movements were monitored and analyzed as described (*12*). Briefly, male hamsters were entrained to a 12:12 LD cycle for >14 days, and a third ventricular cannula and osmotic minipump were implanted (*27*). At the same time, EEG and EMG electrodes were placed and a temperature and activity transmitter (Mini Mitter) was implanted intraperitoneally, after which the hamsters were placed in DD. Bodily movement signals correspond to movements of the implanted transmitter of ~1 inch with respect to an antenna beneath the cage. Forty-eight–hour continuous sleep EEG and EMG recordings were obtained at least 1 week after cannula implantation and onset of infusions.
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41. Adult male and female *waved-2* mice (The Jackson Laboratory, strain B6EiC3Sn-*a/A-Egfr*^{wa2} *Wnt3a*^{vt}) were individually housed in running-wheel cages in LD (12:12) for 3 to 4 weeks, and wheel-running counts over a 14-day period (second and third weeks) were used for behavioral analysis in LD. One-way ANOVA [by genotype; n = 6 (+/+), 18 (+/–), 5 (–/–)] was performed after arcsine transformation (*47*) of the percentages of total running-wheel activity that occured during the light. There was no significant difference among genotypes in total running-wheel activity. Mice were placed in DD (there were no significant differences in circadian period among genotypes), and after 4 weeks, some mice received 3- or 6-hour light pulses (200 to 300 lux). For quantitation of masking responses, all mice were reentrained to LD 12:12 and again released into DD. On the second day in DD, all mice received a 3-hour pulse at CT 13 to 16. Masking was measured as activity counts during the pulse expressed as a percentage of counts during the same time interval on the preceeding day. ANOVA was perfomed as above. Genomic DNA from ear punches or tails was extracted in 50 mM NaOH at 95°C for 15 min, and genotypes were determined by genomic PCR (EGFR-sense: 5′-ctttacagtaccaagacctatgctc-3′; -antisense: 5′-cccttctcttagatcatccttgctg-3′) and restriction fragment analysis with FokI (the *waved-2* mutation creates a new Fok I site) (*39*).
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44. Retina sections were prepared as described (*43*), except that the anterior segment and lens were removed. Sections (10-μm) were cut on a cryostat, mounted onto gelatinized slides, and stained with rabbit polyclonal anti–TGF-α (*34*) or anti-EGF (1:200; FitzGerald Industries International). The secondary was goat anti-rabbit IgG Alexa Fluor 488 (1:200; Molecular Probes). Sections were counterstained with 1.3 μM ethidium bromide (Sigma) or 4 μM ethidium bromide homodimer-1 (Molecular Probes), and fluorescence was detected with a Bio-Rad MRC-1024 confocal imaging system.
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