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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.

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

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15. The pSG5-CARM1 wild type and CARM1LFV127→AAA mutant were a kind gift from M. R. Stallcup. CARM1 cDNA was amplified with primers: 5′-GGAGTACCATGACGAGGCGGCGGCGGCGG-3′ and GAGCAGACCTCTACACTCCCTACATGAGATGTC-3′ and subcloned into pFlag-ACG2 (Phar-Mingen). 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 pcDNA3-L2 and pcDNA3-V16 vectors to generate CMX-CARM1 and CMX-VFP16-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. Activation 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
20. Approximately 5 × 10⁵ 293T cells were transfected with CMX-CARM1 alone or with CMX-Flag-GBP 1 day before labeling. We added 50 μL of 485 μM 5-iodo-5-deoxyuridine (Ci/ml) and subcloned into pFlag-ACG2 (Phar-Mingen). Recombinant CARM1 proteins were expressed in SF9 cells via the baculovirus system (PharMingen) and purified through Flag-M2 affinity resin, and then applied to cells and incubated at 37°C for 4 hours. Immunoprecipitation with anti-CBP (A22, Santa Cruz) was performed as described in (38).
21. Purified CREB proteins were in vitro phosphorylated by P38 (EMSA was performed as described (38)).
24. CARM1 was subcloned into plNCK (Clontech) between Hind III and Cla I sites. Phenix cells were transfected with pNCK-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.
25. Portions of p300 (amino acids 1 to 301, 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)].
27. W. X. Xu et al., data not shown.
35. 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.
36. We are grateful to C. Park and F. Wolfgang for the peptide analysis. We acknowledge M. Stallcup and H. R. Hershman for providing reagents. We thank Y. Ouyang, A. Lee, H. Jugulon, and J. Havstad for technical help, and L. Ong and E. Stevens for administrative assistance. We are grateful to R. Yu, R. Lin, W. Xie, T. Steronsdorff, C. Tsai for critical reading of the manuscript. R.M.E. is an investigator of the Howard Hughes Medical Institute at the Salk Institute and March of Dimes Chair in Molecular and Developmental Biology. Supported by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases (RO1DK57978).
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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.
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 identified 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, confusion of neuropeptides thought to be coreleased from SCN neurons (vasoactive intestinal polypeptide, peptide histidine-isoleucine, gastrin-releasing peptide, and neuropeptide-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 agreed 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...
that TGF-β diminished (Fig. 3, middle right), suggesting a 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-β 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-β was not due to hypoactivity or other gross disturbances of cortical physiology but rather to a more discrete action. However, TGF-β 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, right), indicating that the blockade of running-wheel behavior 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-β 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-β.

The ultradian sleep-wake and temperature rhythm produced by third ventricle infusion of TGF-β 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-β 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-
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 ± 7.2%), 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 EGFR 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 EGFR are found in adult human retina, with TGF-α immunoreactivity observed in Muller glia and ganglion cells and weak EGFR immunoreactivity reported throughout the retina (42). To extend these observations, we performed immunohistochemistry (43, 44) for TGF-α and EGFR on sections from adult mouse retinas. TGF-α was expressed in Muller glia and throughout the ganglion cell layer (Fig. 5, left). In contrast, EGFR 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...
TGF-α, or perhaps more likely, EGF.

Hypothalamic EGF signaling and the daily regulation of behavioral activity. In the nervous system, TGF-α and EGF 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 EGF signaling in SPZ neurons likely mediates this regulation. Genetic analysis demonstrated that EGF 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 EGF signaling in SPZ neurons. According to this view, luminecence 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 EGF receptor on SPZ neurons, inhibiting locomotor activity. TGF-α, secreted in a circadian fashion from the SCN, activates the EGF receptor 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.

References and Notes


23. Briefly, the secretion trap was designed as follows: The host yeast strain lacks invertase, an enzyme that must be secreted to grow on sucrose. A cDNA library prepared from microdissected hamster SCNIs was then inserted into a yeast expression vec- tor, escodine, which lacks invertase, the promoter that allows the yeast to grow in the absence of sucrose. Transformation of the yeast with this vector resulted in a yeast line that can grow in the absence of sucrose from the cDNA library has both an invertase homo- nine and a functional signal sequence in-frame with invertase. Positives thus include both transmembrane and secreted proteins.
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 a mixture of ketamine (150/60 mg/kg body weight, administered i.p.) and a benzodiazepine hepatic steel cannula (Plastics One, Wallingford, CT) was placed stereotaxically into the anterior thalamus and visualized by reaction with 3,3′-diaminobenzidine (Sigma).
31. Brießy, the secretion trap was designed as follows: The anterior segment and lens were re- moved from a hamster (Homo sapiens, 12-month-old) and frozen to −80 °C. The anterior and posterior segments were then homogenized in a RIPA buffer. A 1:50 dilution of the homogenate was plated on 96-well plates, and a temperature and activity trans- mitting unit (Mini Mitter) was fixed on the back of the animal, allowing for the continuous monitoring of the implanted transmitter. After implantation, the animals were placed in DD and monitored for 7 days. Brießy, male hamsters were entrained to a 12/12 LD cycle, and an osmotic minipump was placed sub- cutaneously and connected to the cannula by tubing filled with 24 μl of aCSF [144 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1.2 mM CaCl2, 2 mM NaH2PO4 (pH 7.4)].
32. 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 sat- urate any receptors near the third ventricle so as to mask an effect. Hashed to constant light, and running-wheel activity was monitored for >30 days, aCSF and after cannula placements were histologically documented. Same SCN-secreted factors were revealed after anal- ysis of running-wheel records. Known SCN-secreted fac- tors had no apparent effect on the amount of locomotor activity included brain-derived neurotrophic factor, vasoactive intestinal polypeptide, peptide histidine-isoleucine, glial growth factor, secreted in a circadian fashion from the SCN, activates the EGF receptor 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.


Sulfuryl-35-labeled ribonucleotides were made from mouse TGF-α and EGF polymerase chain reaction (PCR) prod- ucts into which the T7 and T3 sites were incor- porated, respectively, at each end. PCR primers were: TGF-3: 5′-aattaaccctcactaaaggg-gttagctgtgtgccagg-3′; TGF-3: 5′-ttcttcacaagcagcactcgccccgtggtcagtgcagcc-3′). Primers were hybridized to the cDNA, and mRNA quan- tification was performed as above. Genomic DNA from mice was purified and a temperature and activity trans- mitting unit (Mini Mitter) was fixed on the back of the animal, allowing for the continuous monitoring of the implanted transmitter. After implantation, the animals were placed in DD and monitored for 7 days. Brießy, male hamsters were entrained to a 12/12 LD cycle, and an osmotic minipump was placed sub- cutaneously and connected to the cannula by tubing filled with 24 μl of aCSF [144 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1.2 mM CaCl2, 2 mM NaH2PO4 (pH 7.4)].

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