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ERK signaling pathway regulates sleep duration through activity-induced gene expression during wakefulness

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Abstract

Wakefulness is accompanied by experience-dependent synaptic plasticity and an increase in activity-regulated gene transcription. Wake-induced genes are certainly markers of neuronal activity and may also directly regulate the duration of and need for sleep. We stimulated murine cortical cultures with the neuromodulatory signals that are known to control wakefulness in the brain and found that norepinephrine alone or a mixture of these neuromodulators induced activity-regulated gene transcription. Pharmacological inhibition of the various signaling pathways involved in the regulation of gene expression indicated that the extracellular signal–regulated kinase (ERK) pathway is the principal one mediating the effects of waking neuromodulators on gene expression. In mice, ERK phosphorylation in the cortex increased and decreased with wakefulness and sleep. Whole-body or cortical neuron–specific deletion of Erk1 or Erk2 significantly increased the duration of wakefulness in mice, and pharmacological inhibition of ERK phosphorylation decreased sleep duration and increased the duration of wakefulness bouts. Thus, this signaling pathway, which is highly conserved from Drosophila to mammals, is a key pathway that links waking experience–induced neuronal gene expression to sleep duration and quality.

Introduction

Sleep is a behavior defined as a period of immobility and reduced ability to respond to external stimuli, which is homeostatically controlled (such that longer wakefulness periods lead to compensatory increase in sleep duration and intensity). Sleep is conserved across evolution (*1*). *C. elegans*, *Drosophila*, fish, reptiles, birds and mammals satisfy all the behavioral criteria of sleep (immobility, reversibility, decreased response to stimuli, homeostatically regulated) (*2-7*).The presence of sleep across evolution suggests that it must be regulated at a more basic cellular and molecular level than at the global complex brain structure. Evidence indicates that wakefulness is associated with increased transcription of activity-regulated (plasticity) genes. As demonstrated by several microarray experiments in mice and rats, the expression of one group of genes is consistently increased by extended wakefulness (typically synaptic activity-regulated genes such as *Arc, Bdnf, Egr, or Homer1a*), whereas another group (including *Cirbp* and *Dbp*) is decreased in abundance (*8-11*). Because the large majority of genes induced by prolonged wakefulness are activity-regulated genes [mostly immediate-early (IEG) and stress-related genes], it is not clear whether their changes in expression merely represents neuronal activity or might directly have an effect on sleep. Nevertheless, a prominent hypothesis about sleep functions posits that sleep is “the price to pay” for waking experience-dependent plasticity (*12*).

Non-rapid eye movement (NREM) sleep is characterized by wide-spread synchronous firing of cortical neurons and such synchronous activities occur

also in isolated cortical islands, cortical slices, under anesthesia, and even in dissociated cortical cultures (*13-20*). Moreover, we and others have shown that primary cortical cultures can be stimulated by waking neuromodulators (including monoaminergic, glutamatergic, cholinergic, and hypocretinergic neurotransmitters or agonists) to induce tonic firing that invariably returns to the default synchronous burst firing 24 hours later (*18-21*). Stimulated cultures show remarkably similar transcriptional changes as do cortical tissues of mice subjected to 6 hours of sleep deprivation (*18*). Here, we used this simple in vitro model to dissect the molecular and cellular pathways leading to activity-regulated gene expression. We found that major activity-regulated genes were primarily induced through the ERK pathway. We also found that whole-body or cortical neuron-specific deletion of *Erk1* and *Erk2* genes or inhibition of ERK phosphorylation in wild-type animals strongly decreased sleep duration. Thus, the ERK pathway, one of the most evolutionary conserved cellular pathways, critically controls the mammalian sleep duration, as previously evidenced in *Drosophila*.

Results:

Norepinephrine is the major neuromodulator for activity-regulated gene expression

The cell culture model provides a powerful tool to assess the transcriptional correlates of sleep- and wakefulness-like states. To assess the contribution of each neurotransmitter in our original waking cocktail separately, we stimulated cortical cultures with each (Fig. 1A) and assessed the expression of 3 major

candidate genes: *Homer1a*, *Dbp,* and *Arc*. AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NE (norepinephrine), histamine, DA (dopamine), and 5HT (serotonin) alone had an inducing effect similar to (but less so than) that of the cocktail on *Homer1a* expression (Fig. 1B). A similar pattern of expression was also obtained for *Arc* (Fig. 1A). The expression of *Dbp* was assessed because it has been consistently found in several studies to decrease during wakefulness, as opposed to the above IEGs. The only neuromodulator that significantly decreased *Dbp* expression was NE (Fig. 1C).

Noradreneric neurons of the locus coeruleus are the major source of wakefulness neurotransmitter NE with diffuse projections throughout the brain and affect alertness, gene expression and higher brain functions (*22-24*). To understand how NE affects our candidate gene expression each NE receptor type was blocked prior to NE addition to cortical cultures (Fig. 1, D to F). The α_1-adrenergic antagonist prazosin did not prevent the effect of NE on *Dbp* expression, while either the α_2-adrenergic antagonist yohimbine or the β-adrenergic antagonist propranolol consistently inhibited the effect of NE on gene expression (Fig. 1, E and F). α_2-adrenergic and β-adrenergic receptors have opposite effects on adenylyl cyclase, with α_2-adrenergic stimulation inhibiting and β-adrenergic stimulation activating adenylyl cyclase-mediated cAMP signaling. Therefore, we postulated that the observed effects of NE, as well as our cocktail, on gene expression might be mediated through another signaling pathway.

ERK signaling critically controls activity-regulated gene expression

A large number of signaling pathways converge to the final transcription of activity-regulated genes, amongst them our candidate genes (*Arc*, *Homer1a* and *Dbp as well as Bdnf*). To find the candidate pathway (or pathways) responsible for the expression changes observed in these genes, we pretreated the cortical cultures for 30-60 min with pharmacological inhibitors to systematically block each of the major pathways: ERK1/2 (using U0126), protein kinase A (PKA; using H89), mitogen-activated protein kinase p38 (using SB203580), PI3K (using LY294002), cJun N-terminal kinase (JNK; using JNK inhibitor II), Ca^{2+}-calmodulin-dependent kinase 2 (CAMK2, using KN-93), Rho kinase (ROCK; using "Rho kinase inhibitor") and actin polymerization (using latrunculin B). Two pathways emerged as major candidates: that mediated by PKA and the other by ERK. Blocking the PKA pathway with H89 reduced the expression of *Bdnf*, *Homer1a,* and *Arc* (Fig. 2, A to D). Blocking the ERK pathway with U01236 decreased the expression of *Arc* and *Homer1a*, increased that of *Dbp*, and also decreased that of *Bdnf,* although not significantly (Fig. 2C). Thus, the only pathway inhibitor that consistently affected gene expression, and the only one to alter that of *Dbp,* was the ERK pathway inhibitor. NE also activates the ERK pathway (*25*), independently of adenylyl cyclase but most probably by transactivation of epidermal growth factor (EGF) receptors. To assess the activation of the ERK pathway by our cocktail, the phosphorylation of ERK was assessed in the cultured neurons. Cultures were either sham-treated or stimulated with the waking cocktail, and proteins were harvested 15 minutes later to quantify the phosphorylation of ERK1 and ERK2 (ERK1/2 or simply ERK hereafter). ERK

phosphorylation was rapidly induced by the cocktail of neurotransmitters as opposed to sham (water) or no stimulation (control) (Fig. 2E). The ratio of phosphorylated to total ERK (p-ERK/ERK) increased almost 4 fold after cocktail stimulation as compared to sham-stimulated dishes, whereas dishes that were not stimulated showed a 3 fold decrease in the p-ERK/ERK ratio as compared to sham-stimulated dishes (Fig. 2F). These data suggest that wakefulness, similar to our cocktail of neurotransmitter, might activate ERK in vivo.

ERK phosphorylation increases and decreases with wakefulness and sleep

As we have previously reported (*18*), our in vitro model reliably mimics the transcriptional signature of in vivo sleep and wakefulness in the mouse cortex. To confirm our in vitro results, we assessed the in vivo phosphorylation of ERK after consolidated NREM sleep or wakefulness. We sacrificed mice after 12 minutes of NREM sleep at light onset (between Zeitgeber time “ZT” 0 and ZT1, first hour of the light period), and the corresponding controls that were maintained awake by gentle handling during the same interval. The amount of phosphorylated ERK (p-ERK) was quantified in the cortex. ERK phosphorylation was strongly induced by 12 minutes of wakefulness as compared to the amount detected in those undergoing NREM sleep (Fig. 2G). The p-ERK/ERK ratio during NREM sleep was nearly 3 fold lower than it was during wakefulness (Fig. 2H), very similar to our cell culture results (Fig. 2F).

ERK1/2 loss-of-function mice have decreased bouts of sleep

ERK phosphorylation may be simply a correlate of wakefulness without any direct regulation of sleep and wakefulness by the ERK pathway. To understand the role of the ERK pathway in sleep, we first investigated the effects of genetic deletion of *Erk1* or *Erk2*. *Erk1* deletion does not result in any observable phenotype, whereas *Erk2* deletion results in embryonic lethality (*26*). Therefore, conditional *Erk2* knockout (KO) mice ($MAPK^{flox/flox}$) were bred into *Emx1-Cre* mice resulting in deletion of *Erk2* in cortical neurons. *Emx1* is a marker of cortical neurons (*27*) (expressed in 88% of cortical neurons). These mice are viable despite important changes in brain structure (reduced cortical thickness) and behavioral deficits (*28*). Total wakefulness duration during the 24-hour baseline recordings was significantly increased in both *Erk1* KO and *Erk2* KO mice as compared to their wild-type littermates, whereas the amount of NREM sleep was significantly decreased in *Erk2* KO mice (Fig. 3). Also, during the dark period after 6 hours of sleep deprivation, wakefulness was increased in both genotypes and NREM sleep was decreased (Fig. 3). These data suggest that the ERK pathway directly regulates NREM sleep duration.

Inhibition of ERK phosphorylation decreases the amount of sleep in mice

Because of the morphological and behavioral changes (in *Erk2* KO mice) as well as the fact that germline inactivation might lead to compensation (between ERK1 and ERK2, as well as between ERK and other signaling pathways), we next continuously perfused a selective inhibitor of ERK phosphorylation (U0126) in the lateral ventricle of C57BL/6J mice and quantified vigilance states and the spontaneous locomotor activity. The

inhibition of ERK phosphorylation significantly decreased NREM sleep and wakefulness was increased by nearly 100 min (Fig. 4). The large increase in wakefulness was mainly distributed during the dark periods (Fig. 4). Indeed, wakefulness during the dark period was significantly increased in treated mice by almost 80 min ($p<0.01$), and NREM sleep was decreased by the same amount ($p<0.01$). REM sleep also showed a tendency to decrease in treated mice and overall nearly half of the total sleep during the dark period was lost. After a period of 6 hours sleep deprivation, treated mice again showed significantly less NREM sleep ($p<0.001$). The decrease in NREM sleep was even more marked during the dark period after sleep deprivation. More specifically, NREM sleep was decreased ($p<0.001$) by one hour during the dark period following sleep deprivation (Fig. 4). ERK phosphorylation changes rapidly with vigilance states and spontaneous phosphorylation and its inhibition can only be assessed in relation with the previous duration of sustained wakefulness or sleep. As an index of the inhibition of ERK phosphorylation, 4 mice under U0126 and 4 mice under DMSO perfusion were sacrificed after 6 hours of sleep deprivation and the amount of phosphorylated ERK was quantified by western blot (fig. S1). U0126 reduced total ERK phosphorylation by 29.30% ($p=0.06$) and ERK2 phosphorylation by 37.85% ($p<0.05$), indicating that even after 6h of imposed wakefulness, U0126 is able to reduce ERK phosphorylation (fig. S1).

To assess whether in addition to sleep-wake distribution, the quality of sleep was also modified we quantified the frequency and the contribution of different uninterrupted bouts of vigilance states in *Erk1*, *Erk2* KO, and in U0126-treated

mice. Not only was the amount of sleep observed in the 24 hour time frame decreased in *Erk2* KO and U0126-treated mice, but wakefulness was also less fragmented (Fig. 5). Sustained bouts of wakefulness were significantly increased in duration in *Erk2* KO and U0126-treated mice (Fig. 5, A and B), while short (<16 seconds) wakefulness bouts were significantly less frequent in U0126-treated mice during baseline and also during recovery (Fig. 5, C and D). These differences again occurred mostly during the dark periods (active period). As a consequence of long sustained wakefulness bouts, the number of long bouts (>900 sec) were significantly decreased in *Erk2* KO and U0126-treated mice (Fig. 5, E and F). Together, these results indicate that the ERK pathway decreases the fragmentation of sleep and consolidates wakefulness.

Inhibition of ERK phosphorylation increases spontaneous locomotor activity

Wakefulness and locomotor activity are often confounded. To assess whether the inhibition of ERK phosphorylation increases wakefulness or activity or both, spontaneous locomotor activity of mice under U0126 perfusion was recorded by infrared sensors. U0126-treated mice were not only awake but also more active (Fig. 6A). Although the increase in activity is more evident during the dark (active) phase, even the short wakefulness bouts during the light period showed a significant increase in locomotor activity (Fig. 6, B and C). To understand whether this increase in activity is due to more locomotion or just to the increased time spent in wakefulness, we normalized the activity with the time spent in wakefulness, recorded by electroencephalogram (EEG).

Treated mice were not only more awake, but when awake they displayed more than 2 fold increase in locomotor activity (Fig. 6D).

ERK signaling controls vigilance states distribution but not sleep homeostasis

Changes in the quantity and quality of vigilance states affect the homeostatic regulation of sleep. Accordingly, longer wakefulness bouts and consolidated NREM sleep can be interpreted as an increase in sleep need and intensity. To evaluate the homeostatic regulation of sleep, we assessed the quantity and the time course of NREM sleep EEG slow wave activity [or delta (1-4 Hz) power density from spectral analysis]. Delta power was increased in U0126-treated mice during both baseline and recovery ($p<0.03$ and $p<0.0004$, respectively). We also performed a simulation of the time course of delta activity (see Material and Methods) to explain the observed changes. The analysis revealed that the observed differences in delta power could be explained by changes in time spent in different vigilance states (fig. S2).

Discussion

Our cell culture model provides a powerful tool to assess molecular and cellular correlates of sleep-wake regulation. One of our major discoveries (*18*) is that transcriptional changes induced by sleep deprivation can be reliably reproduced in vitro by chemical stimulation. We therefore followed the transcription of key candidate genes to investigate the molecular pathways

activated by wakefulness and chemical stimulation. Surprisingly, NE alone could reproduce the effect of the whole cocktail on the expression of our candidate genes, confirming the essential role of NE in wakefulness and associated neuronal plasticity (*29*). To understand through which pathway NE stimulates gene expression its receptors were pharmacologically blocked by antagonists. Blocking either α-2 or β-adrenoreceptors inhibited the effects of NE on gene expression. Our results are in agreement with several studies reporting the major role of NE in state-dependent gene expression (*23, 30, 31*). Rats depleted of NE (through locus coeruleus lesions) in the cortex show a reduction of known transcripts that increase during wakefulness, most of which are reported to be implicated in synaptic plasticity and response to cellular stress (*23*). Cirelli *et al*. (*32*) showed also that NE depletion reduced the homeostatic response to sleep deprivation. Taken together these data confirm the importance of synaptic plasticity-related genes induced by NE during wakefulness in sleep-wake regulation.

Because α_2- and β-adrenoreceptors have opposite effects on adenylyl cyclase, wherein α_2 stimulation inhibits and β stimulation activates cAMP signaling, our findings indicate that another intracellular signaling pathway might be involved. Neurotransmitter-mediated stimulation of cortical cultures pre-treated with relatively specific inhibitors of various signaling pathways revealed a key role for ERK phosphorylation by MEK (mitogen-activated protein kinase kinase). We also found similar changes in ERK phosphorylation in vivo as a function of sleep and wakefulness. Our results strongly suggest that transcriptional changes induced by NE and sleep deprivation occur through the ERK pathway, as already suggested (*25*). Our

work is limited to NREM sleep and no significant changes in REM sleep was observed. Interestingly REM sleep also activates the ERK pathway while the noradrenergic system is silenced during this state. REM sleep-induced activation of the ERK pathway depends mainly on cholinergic mechanisms, and is therefore different from the effects of wakefulness (*33*). Obviously, the ERK pathway is not the only one activated by wakefulness or chemical stimulation. Accordingly, for instance blocking the PKA pathway also effects the expression of several plasticity-related genes (such as *Bdnf* and *Homer1a*). It has also been shown that both ERK and PKA are required to induce changes in synaptic plasticity (*34*). Nevertheless, the ERK pathway was further investigated here in vivo because, as opposed to other pathways, the inhibition of its phosphorylation by U0126 affected the transcription of *Dbp*.

Our finding that the ERK pathway is activated both in vivo and in vitro by wakefulness and chemical stimulation confirms also the observations in *Drosophila* (*35*). Foltneyi *et al*. (*35*) found that ERK phosphorylation through EGFRs dramatically increased the amount of sleep in *Drosophila*. Accordingly, we hypothesize that ERK phosphorylation by NE stimulation might result from trans-activation of EGFR. Another study also implicated ERK phosphorylation both in the regulation of sleep and plasticity in *Drosophila* (*36*). We showed here that ERK loss-of-function (mainly loss of ERK2 via gene deletion) or inhibition of ERK phosphorylation in vivo reduced sleep duration and consolidated both sleep and wakefulness. More specifically, when *Erk2* was deleted or when ERK phosphorylation was inhibited, longer uninterrupted wakefulness bouts were observed that might

result in consolidated subsequent sleep. Changes in the homeostatic marker of sleep (EEG delta activity) are consistent with the distribution of sleep and wakefulness after inhibition of ERK phosphorylation. Given that phosphorylation is a fast process, the long lasting changes in vigilance states observed here should be due to the downstream changes in gene transcription. Indeed, ERK phosphorylation occurs quickly and reaches a maximum within 15 min (*37*). Therefore, sustained wakefulness is required to maintain ERK phosphorylation. This explains our finding that the effect of ERK phosphorylation inhibition was most evident during the dark periods when mice stay awake for long bouts. The phosphorylation occurs through a kinase cascade involving RAS and RAF activation, which, in turn, phosphorylate MEK (target of U0126) that phosphorylates ERK. Double-phosphorylated ERK activates MAPK-interacting kinases MNK1 and MNK2, ELK1, and ribosomal S6 kinase (RSK) leading to the phosphorylation of c-MYC and CREB and the activation of target gene transcription. The role of phosphorylated CREB in sustained wakefulness, subsequent sleep, and memory are well documented (*38, 39*). Note that phosphorylated ERK also activates target protein translation by phosphorylating MNK, thus causing activation of eIF4E. This downstream effector of ERK pathway has also been implicated in sleep and plasticity (*40*).

The fact that ERK inhibition, as opposed to other signaling pathways (including PKA), affects *Dbp* expression is intriguing. Detailed analysis of signaling pathways involved in synchronizing peripheral circadian rhythms found no evidence that the ERK pathway plays any role while SRF-dependent

actin dynamics (tested in our screening by inhibiting Rho kinase and actin polymerization without any effect) was the key pathway (*41*). The MAPK p38 (also inhibited in our experiments without any effect) was found to be a potential kinase of the circadian rhythm-associated protein Period (PER) that also plays a major role in stress-response signaling (*42*). ERK phosphorylation is activated in the suprachiasmatic nucleus following a light pulse, and inhibition of this process by U0126 strongly reduces ERK phosphorylation and attenuates light entrainment of circadian rhythms without affecting clock-timing properties (*43*). These observations suggest that changes we report here for sleep duration are unlikely the result of the role of the ERK pathway in circadian rhythms. DBP is part of the positive feedback loop of the circadian molecular clock as opposed to PER and CRY, which are part of the negative feedback loop. Although the exact mechanism remains to be investigated, our results suggest that either activated ERK leads to the inhibition of *Dbp* transcription or that this inhibition occurs following activation of *Per2*.

Inhibiting ERK phosphorylation not only increased wakefulness but also the locomotor activity mainly during the active (dark) phase, independent of the time spent awake. Given that ERK phosphorylation can be induced by cellular stress, one could argue that the increase in ERK phosphorylation during wakefulness could be a correlate of stress response. However, several studies suggest that this hypothesis is unlikely. Mongrain *et al*. (*44*) showed that adrenalectomy that abolished increased corticosterone concentrations in 3 different inbred mouse strains dramatically reduced the expression of many stress-related genes after sleep deprivation but not that of major activity-

regulated genes such as *Homer1a*. They also found that sleep deprivation in adrenalectomized mice still activated the ERK pathway, suggesting that under these conditions, extended wakefulness *per se* more than the associated stress is responsible for changes in activity-regulated genes. In flies, oxidative stress or mechanical disturbances as well as waking induced by starvation did not result in ERK activation, as opposed to sleep deprivation (*36*).

Although U0126 used at 10 μM in vivo cannot completely inhibit ERK phosphorylation (*45*), the partial inhibition was still sufficient to increase wakefulness and to consolidate NREM sleep and wakefulness. Given the changes in candidate gene transcription and in vigilance states, we conclude that the accumulation of activity-regulated and sleep-related genes are principally mediated by ERK activation. Accordingly, inhibiting ERK phosphorylation reduces the accumulations of these transcripts and therefore longer consolidated wakefulness bouts are required to translate waking experience into sleep.

We have suggested that one of the sleep functions could be to protect the neuronal membrane homeostasis against overstimulation, as suggested by a large release of phospholysolipids following sleep deprivation and in vitro stimulation (*18*). Inhibition of the ERK pathway was shown to reduce the release of arachidonic acid (*46*). ERK has also been shown to phosphorylate and activate phospholipase A2 (*47, 48*). These findings suggest that ERK activation during extended wakefulness mediates the release of lysophospholipids, which if not counterbalanced by sleep will cause damage to cell membrane integrity.

Thus our findings indicate that the ERK pathway, a highly evolutionary conserved signal transduction pathway from *Drosophila* to mammals, plays a major role in controlling plasticity and sleep-related gene transcription and translation and that this, together with other functions of ERK (metabolic, oxidative stress, and proliferation or apoptosis) regulates vigilance states.

Material and Methods

Animal handling

C57BL/6J, MAPK$^{flox/flox}$, and Emx1-Cre mice were purchased from The Jackson Laboratory. *Erk1* KO mice were provided by Dr Binnaz Yalcin (University of Lausanne) and Dr. Gilles Pages (University of Nice Sophia Antipolis). All animals were on C57BL/6J genetic background and maintained under standard animal housing conditions with free access to food and water and a 12 hour light-12 hour dark cycle (lights on at 7 a.m.). All experiments were approved by the Vaud Cantonal Veterinary Office (Switzerland).

Primary Cortical Cultures

Cultures were prepared as detailed in (*18*). Briefly, E17-E18 mouse embryos were collected to prepare cortical cultures. Timed-pregnant mice were killed by cervical dislocation and embryos were removed and decapitated in PBS at 4°C. Cortices from embryos were dissected and cut into small pieces in a dissection solution. After enzymatic digestion for 30 min with papain (10 U/ml; Roche 108014), cells were dissociated by 12 to 15 triturations using a glass pipette in Neurobasal medium completed with B27 (Invitrogen), 0.5 mM Glutamax-I (Invitrogen) and penicillin streptomycin antibiotics (1%) (complete

NBM). After every 4 or 5 triturations, the cells in suspension were transferred to a separate tube. Isolated cells were centrifuged during 4 min at 150*g* and resuspended in 2 ml of complete NBM. One to two million cells were harvested on Petri dishes (35 mm diameter) previously coated with poly-L-lysine (0.1mg/ml). Cultures were maintained in an incubator at 37°C with 5% CO_2. Half of the medium was replaced every 7 days.

Stimulation protocol

A cocktail was prepared freshly with AMPA (1 µM, Sigma-Aldrich), noradrenaline (1 µM, Sigma-Aldrich), kainic acid (kainate), ibotenic acid, serotonin, histamine dihydrochloride, dopamine hydrochloride, and N-methyl-D-aspartate (NMDA) (each 1 µM, from Tocris Bioscience), carbachol (10 µM, Sigma-Aldrich), and orexin (0.01 µM, Sigma-Aldrich). Distilled H_2O was added in sister cultures as control in all stimulation protocols. To avoid differences in gene expression caused circadian rhythms, stimulations and RNA and protein extractions from different conditions were performed at the same circadian time. RNA was extracted three hours after stimulation and again 24 hours later (recovery).

Pharmacology

For the norepinephrine study, we selectively blocked each receptor subtype with yohimbine, phentolamine, prazosin (each 10 µM, from Sigma-Aldrich) or propranolol (10 µM, from Merck Millipore) 30 min before stimulation with norepinephrine (1 µM, from Sigma-Aldrich). For the pathway study, LY294002 (PI3K inhibitor, Cell Signaling Technology, 50 µM) or SB203580 (p38 inhibitor, Merck Millipore, 10 µM) was added 1 hour before simulation with the cocktail,

and H89 (PKA inhibitor, Sigma-Aldrich, 10 µM), KN-93 (CAMKII inhibitor, Sigma-Aldrich, 0.5 µM), JNK inhibitor II (Merck Millipore, 10 µM), U0126 (ERK1/2 inhibitor, Cell Signaling Technology, 10 µM), Latrunculin B (actin polymerization inhibitor, Merck Millipore, 0.3µM), or Rho kinase Inhibitor (Merck Millipore, 100nM) was added 30 min before stimulation.

RNA extraction and analysis

RNA from cells was extracted with the RNA easy mini kit from Qiagen and RNA from cortex was extracted with the RNA easy lipid midi kit (Qiagen). All RNA samples were treated with DNase and conserved at -80 °C. RNA quantities were assessed with a NanoDrop ND-1000 spectrophotometer and the quality of RNA was controlled on Agilent 2100 bioanalyzer chips. Three to 6 µg of RNA were obtained from cortical cultures and 100 to 150 µg from cortices.

Gene expression analyses were performed as described in Hinard *et al*. (*18*). Briefly, to quantify relative RNA expression from cortical cultures or from mouse cortex, RNA was first reverse transcribed: RNA (500 ng) was mixed with random hexamer (1 µg), dNTP (1 µL of 10 mM), RNAsin (1 µL of 40 U/µL), superscript II (1 µL of 200 U/µL) (each from Invitrogen) completed to 20 µl volume with buffer. Denaturation was performed at 65 °C for 5 min followed by reverse transcription by warming up the samples to 28 °C for 10 min, and then to 42°C for 60 min. The resulting cDNAs were diluted 1:10 in water and mixed with primers and probes of target genes by a robot (Tecan, Switzerland). Target genes were then amplified by real time qPCR in an ABI prism HT7900 detection system (in triplicate). A qBase v1.3.5 software was used to quantify the relative level of the RNA by the delta Ct method (DDCt).

Three housekeeping genes (*EEF1a1, RPS9* and *TBP*) were measured to normalize relative expression levels by calculating their geometric means. Controls were made with a sample without superscript II (Non Amplified Control). In the cultures, the gene expression after stimulations was normalized using the mean of the control samples for each culture separately. Primers and TaqMan probes for candidate genes are reported in table S1.

Protein extraction, Western blot and quantification

To quantify the ratio of phosphorylated/total ERK, the mice were sacrificed after the experiment by cervical dislocation. Cortices were extracted and frozen in liquid nitrogen. Proteins were extracted using RIPA buffer with a cocktail of protease inhibitor (Roche). Trituration in RIPA was done to homogenize the samples on ice. Samples were left on ice during 15 min, centrifuged at 4°C and 2000g for 10 min. Supernatants were collected and proteins were quantified using BCA protein assay kit (Thermo Scientific) and the absorbance was measured with a Saphire 2 Tecan. 20-40 μg of protein were heated at 95 °C for 5 min in the Leammli buffer and loaded on a 12% SDS-Page gel, and transferred onto a PVDF membrane. Membranes were incubated with either ERK1/2 antibody or phosphorylated ERK1/2 primary antibody (each 1:2000, Cell Signaling Technology) for 2 hours followed by a secondary polyclonal Goat anti-rabbit (1:2000, Dako) for 2 hours. Membranes were revealed with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare), and developed with a SRX-101A (Konica Minolta).

EEG Recording and Analysis

EEG and EMG implantations were performed under deep anesthesia. Briefly, two EEG electrodes (Frontal 1.7mm right from the midline and at 1.5mm anterior to the bregma; Parietal at 1.7mm right of midline and at 1.0mm anterior to lambda) and 3 anchor screws were placed on the skull. Two semi-rigid gold wires were used as EMG electrodes and were inserted into the neck muscle along the back of the skull. EEG and EMG wires were connected to a micro-connector, cemented to the skull and connected to recorders through a swivel. EEG and EMG signals were amplified, filtered, analog-to-digital converted (2 kHz), and down-sampled and stored at 200 Hz. Spontaneous locomotor activity was measured by infra-red sensors (with ClockLab). Movements were quantified as count per minutes once every 5 minutes. For drug perfusion, micro-osmotic pumps from Alzet (model 1002) were prepared one day before the surgery following the instruction of the manufacturer and filled with U0126 (1.52 μg/μl) suspended in 40% DMSO in artificial cerebrospinal fluid (ACSF), or 40% DMSO in ACSF as control. The pumps were placed in the back of the mice and connected to a cannula (Brain infusion kit 1; 3-5mm; Alzet) placed in the right lateral ventricle (1mm lateral to midline; 0.3mm anterior to lambda; 2.2mm deep) and perfusion was made at a rate of 0.25 μl/hour.

Scoring of vigilance states was performed visually in 4 second epochs as described previously (*49*). Recordings included two baseline days, followed by 6 hours sleep deprivation and 18 hours recovery. A discrete Fourrier transform analysis was performed on the EEG signal to calculate the EEG power densities. For delta power (1-4Hz) calculation, baseline light and dark

periods were divided respectively into 12 and 6 intervals with an equal number of NREM epochs. Light period of the recovery was divided in 8 intervals. For sleep fragmentation and analysis, the frequency and the amount of the 3 different vigilance states were calculated according to Franken *et al*., (*49*). Simulation of the time-course of the EEG delta power was performed based on the distribution of sleep-wake epochs, as described in Franken *et al*., (*50*). Basically, the process S (EEG delta power) was considered to increase during wakefulness or REM sleep following an exponential function: $S_{t+1} = UA - (UA - S_t)*e^{-dt/\tau_i}$, and to decrease exponentially during NREM sleep ($S_{t+1} = LA + (S_t - LA)*e^{-dt/\tau_d}$) (time resolution of iteration dt = 4s), with UA as an upper (UA = 282%) and UL as an lower (LA = 55%) asymptote with time constant of increase τ_i = 7.9 h and decrease τ_d = 1.9 h.

C57BL/6J mice aged 9-10 weeks were sleep deprived for 6 hours at the beginning of the light period by gentle handling. Gentle handling consisted in cage-tapping, introduction of paper towels in the cage, or approaching a plastic pipette next to the mouse as soon as sleeping behavior was observed. Sleep-deprived mice and undisturbed controls were euthanized by cervical dislocation. The brain and liver were collected and rapidly frozen on dry ice, and stored at -80 °C.

Supplementary Material

Fig. S1. In vivo inhibition of ERK phosphorylation after 6 hours of sleep deprivation.

Fig. S2. Time course of delta power during NREM sleep.

Table S1. Primers and probes for gene expression analyses

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Figure Legends

Fig. 1. Candidate gene expression in cultured neurons after stimulation with each constituant of the waking cocktail. (**A to C**) 3 dishes from 3 independent cultures were stimulated for 3 hours with neuromodulator: NMDA, AMPA, kainic acid (KA), ibotenic acid (Ibot), serotonin (5HT), histamine (Hist), dopamine (DA) or norepinephrine (NE) (each 1 μM); carbachol (CCh; 10 μM); or orexin A (OrexA, 0.01 μM). Data are mean normalized mRNA expression + SD relative to sham-stimulated cultures (Control). (**D to F**) Gene expression after stimulation with sham (control) or NE (1 μM, 3 hours) in cultures pre-treated with NE antagonist (yohimbine, prazosin or propranolol, 10 μM for 30 min). Data are mean + SD from 6-9 independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus control; t-tests with Holm correction.

Fig. 2. Signaling pathway analysis. (**A to D**) 3 dishes from 3-4 cultures were pretreated with a pharmacological inhibitor then stimulated with cocktail. Data are mean + SD from 3 dishes of 3-4 independent cultures. Expression of each mRNA was normalized to that of XXX and each calculated relative to that in cocktail-treated cultures. (**E**) Western blot of total ERK (upper) and phosphorylated ERK1/2 (lower), 15 min after stimulation with the cocktail, water or nothing (control). (**F**) Quantification of the p-ERK/ERK ratio from (E) using ImageJ Data are mean + SD, n=4, 3 independent westerns. Blue horizontal lines connect conditions with significant differences. (**G**) Western blot of total ERK (upper) and p-ERK1/2 (lower) in the cortex of mice after 12 min of consolidated NREM sleep or wakefulness. (**H**) Quantification of the p-ERK/ERK ratio from (G) using ImageJ. Data are mean + SD, n=4, 3

independent westerns. *p<0.05, **p<0.01, ***p<0.001, t-test with Holm correction when required (A to D).

Fig. 3. Effects of genetic deletion of *Erk1* and *Erk2* on vigilance states amount. Left: Hourly amounts of the various vigilance states during a baseline period (first 24 hours; grey areas indicate the dark periods), a period of sleep deprivation (hours 24-30), and a period of recovery (hours 30-48) in *Erk1* wild-type (WT, blue line, n=6), *Erk1* knockout (KO, orange line, n=7), *Erk2* WT (black line, n=7) and *Erk2* KO (green line, n=8) mice that were awake (top) or undergoing NREM or REM sleep (middle and bottom, respectively. Right: Quantification of the corresponding amount of vigilance states during the 24-hour baseline and 12-hour recovery dark periods. Data are mean + SD; *p<0.05, two-way ANOVA with Holm correction for multiple tests.

Fig. 4. Effects of ERK phosphorylation inhibition on vigilance states amount. Left line graphs: Average hourly amounts (mean ± SEM) of the various vigilance states during a baseline period (first 24 hours; grey areas indicate the dark periods), a period of sleep deprivation (hours 24-30), and a recovery period (hours 30-48) in mice that were awake (top) or undergoing NREM or REM sleep (middle and bottom, respectively) and intraventricularly perfused with either U0126 (red, n=9 mice) or the solvent DMSO (blue, n=7 mice). Right bar graphs: Quantification of the corresponding amount of vigilance states during the 24-hour baseline (total), 12-hour baseline dark, 24-hour recovery (total), and 12-hour recovery periods. Data are mean + SD;

*p<0.05, **p<0.01, ***p<0.001; two-way ANOVA with Holm correction for multiple tests.

Fig. 5. Effects of *Erk1* and *Erk2* deletion and ERK phosphorylation inhibition on vigilance states quality. (**A and B**) The duration of sustained wake bouts (>900 sec) in *Erk1* KO (n=7) and *Erk2* KO (n=8) mice and their wild-type (WT, n=6 for *Erk1*, n=7 for *Erk2*) controls, and in U0126-treated (n=9) or control (DMSO, n=7) mice during the 24-hour baseline (A) and 18-hour recovery (B). (**C and D**) The frequency of short wake bouts (<16 sec) during the 24-hour baseline (C) and 18-hour recovery (D). (**E and F**) The frequency of long wake bouts (>900 sec) during the 24-hour baseline (E) and 18-hour recovery (F). Data are mean + SD from 6-9 mice per condition; *p<0.05, t-test.

Fig. 6. Spontaneous locomotor activity after inhibition of ERK phosphorylation. (**A**) Mean locomotor activity over 2 days of baseline treatment with U0126 (red line, n=9 mice) or DMSO (blue line, n=7 mice). Dashed area around lines indicates ± 1 SEM. (**B and C**) Quantification of total locomotor activity during the 12-hour light or dark period. (**D**) The ratio between locomotor activity counts per minute of EEG-recorded wakefulness. Data are mean + SD from 7 or 9 mice, as indicated in (A); ***p<0.001, t-test.

Fig. 1

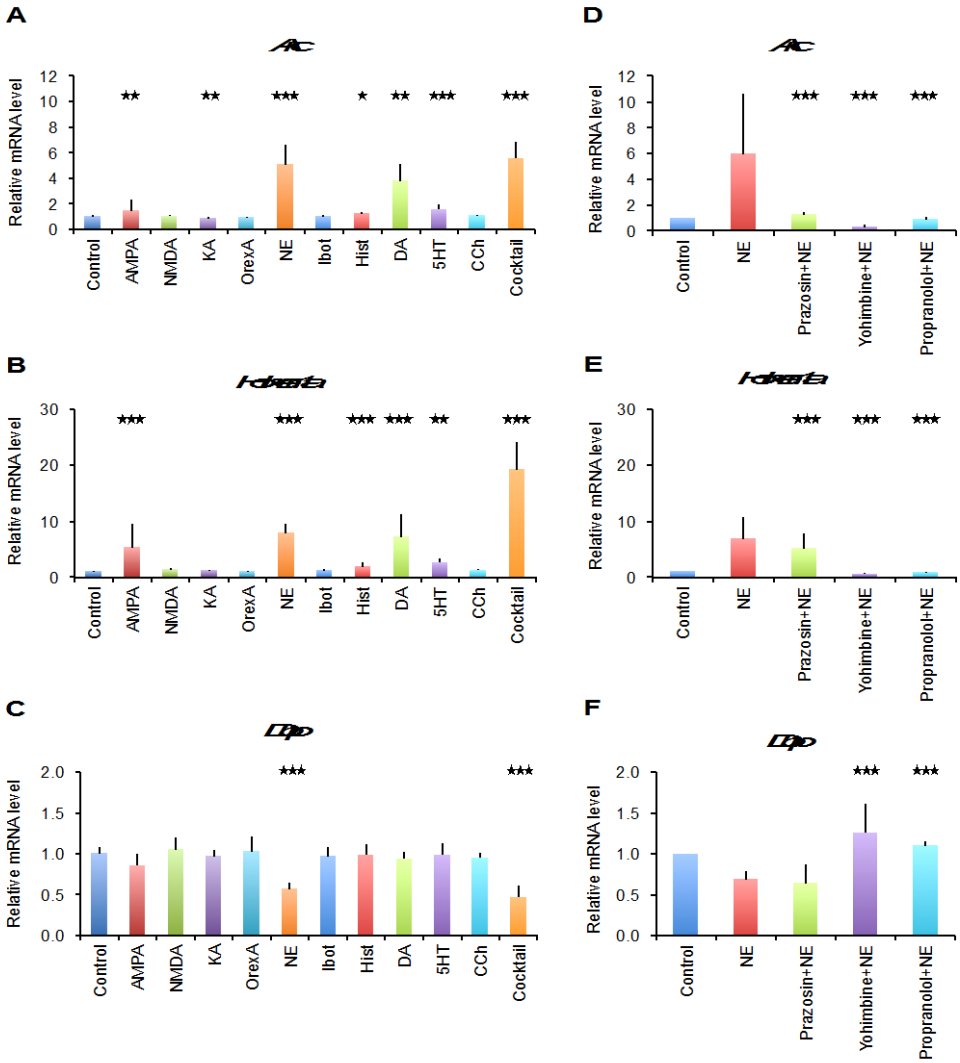
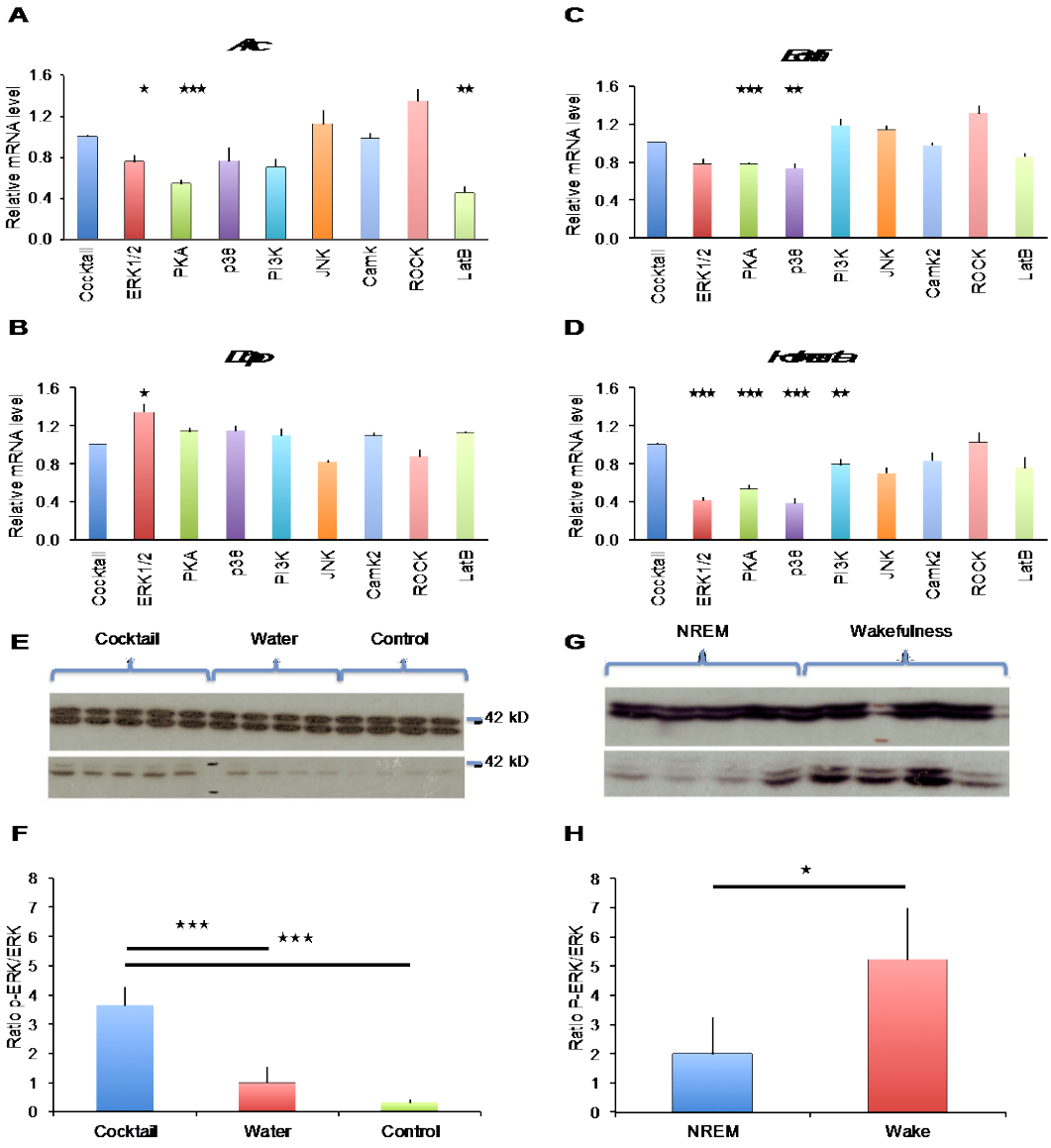

A
D
B
E
C
F
Relative mRNA level
Control
AMPA
NMDA
KA
OrexA
NE
Ibot
Hist
DA
5HT
CCh
Cocktail
Prazosin+NE
Yohimbine+NE
Propranolol+NE

Fig. 2


A
C
B
D
Relative mRNA level
1.6
1.2
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0.4
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ERK1/2
PKA
p38
PI3K
JNK
Camk
Camk2
ROCK
LatB
E
Cocktail
Water
Control
42 kD
42 kD
G
NREM
Wakefulness
F
Ratio p-ERK/ERK
8
7
6
5
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3
2
1
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Cocktail
Water
Control
H
Ratio P-ERK/ERK
NREM
Wake

Fig. 3

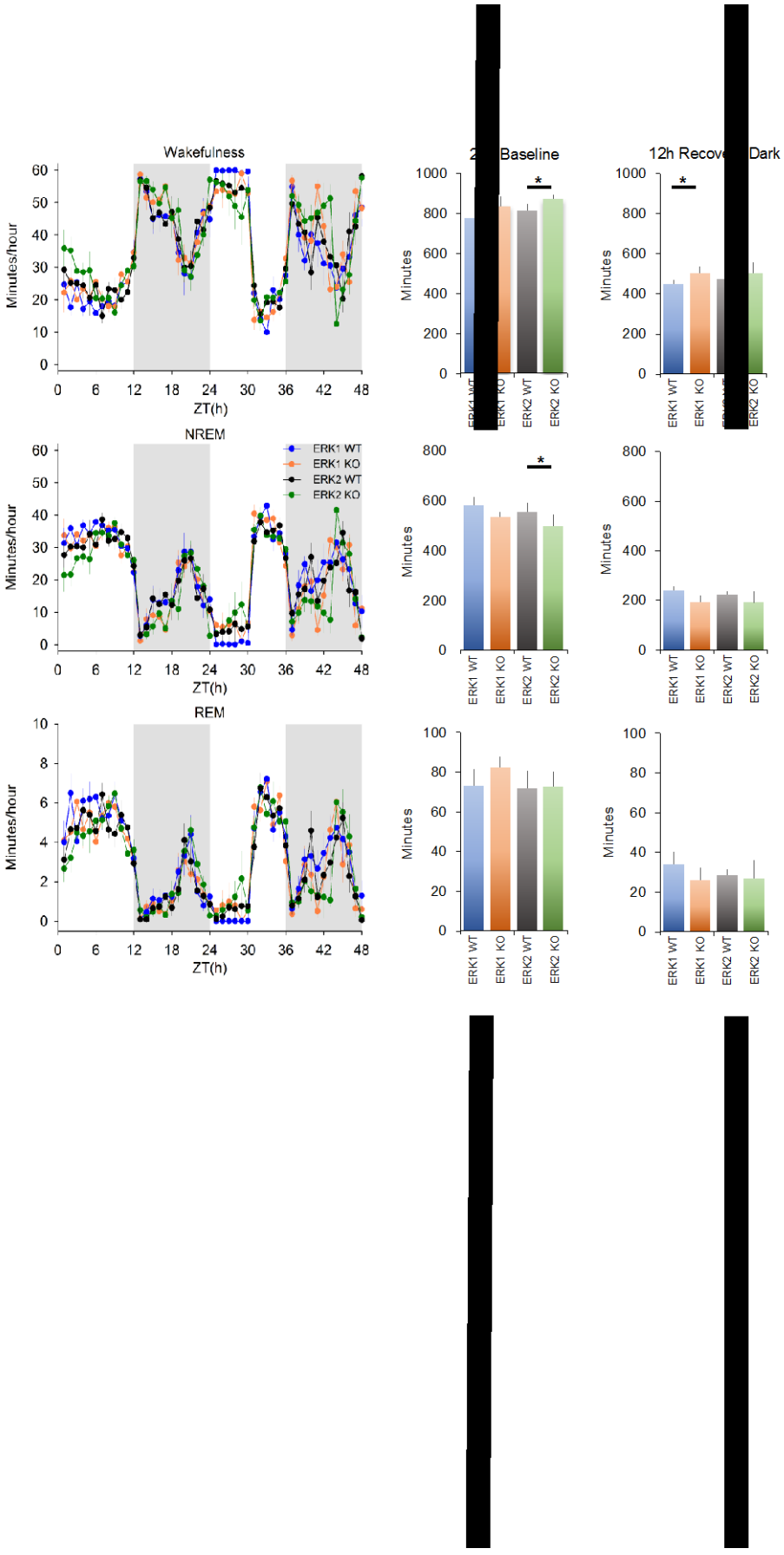

Wakefulness
NREM
REM
Minutes/hour
ZT(h)
ERK1 WT
ERK1 KO
ERK2 WT
ERK2 KO
Baseline
12h Recov
Dark
Minutes
*

Fig. 4

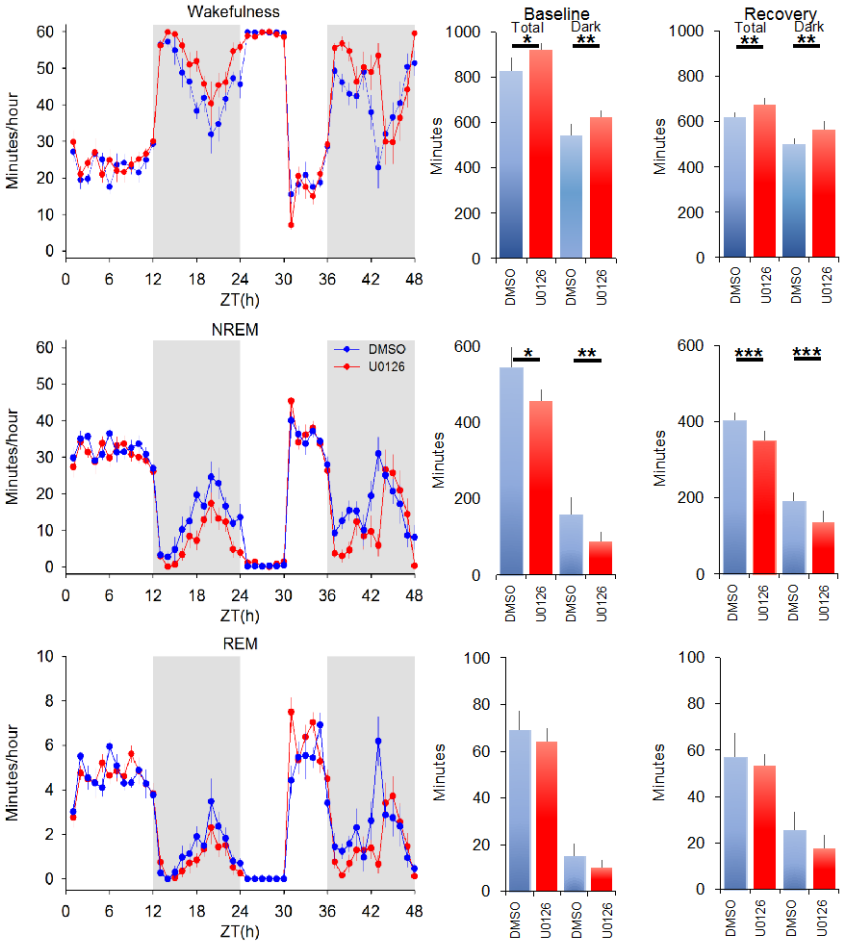

Wakefulness
Baseline
Recovery
Total
Dark
NREM
REM
DMSO
U0126
Minutes/hour
Minutes
ZT(h)

Fig. 5

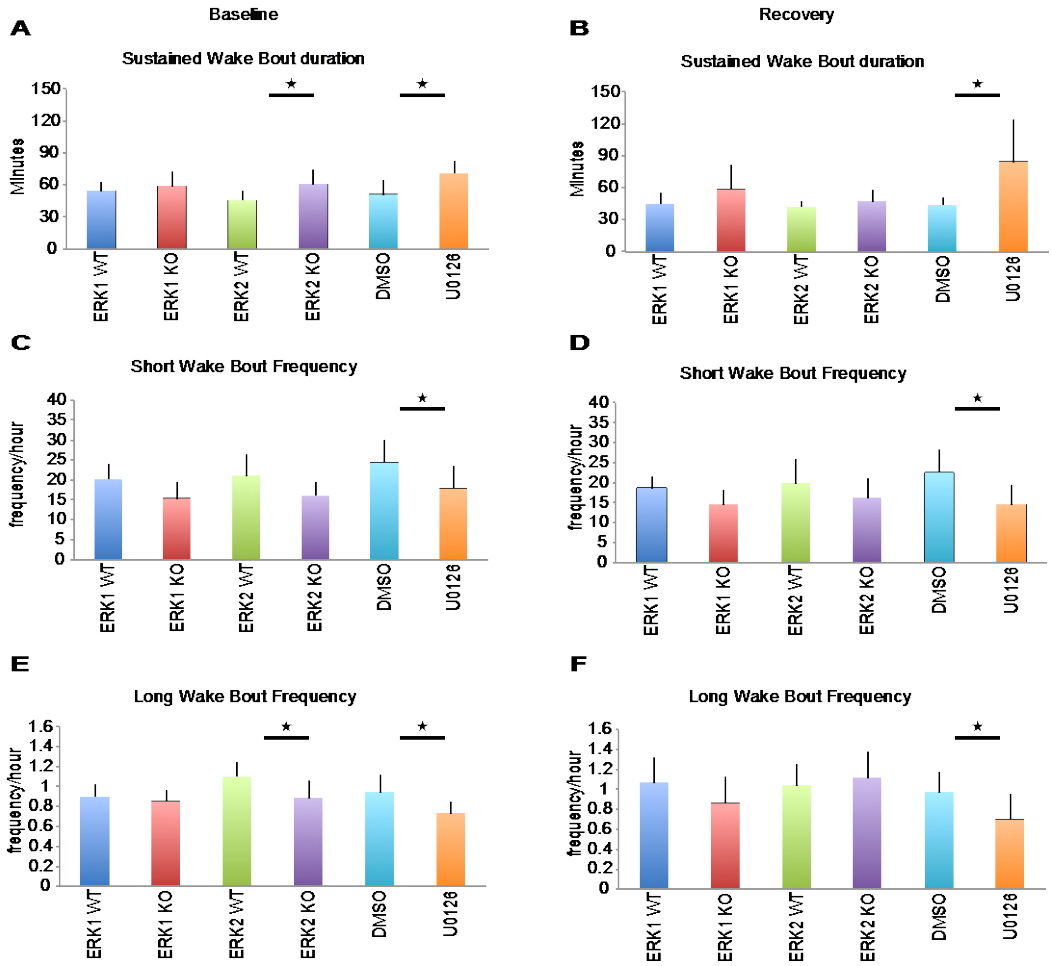
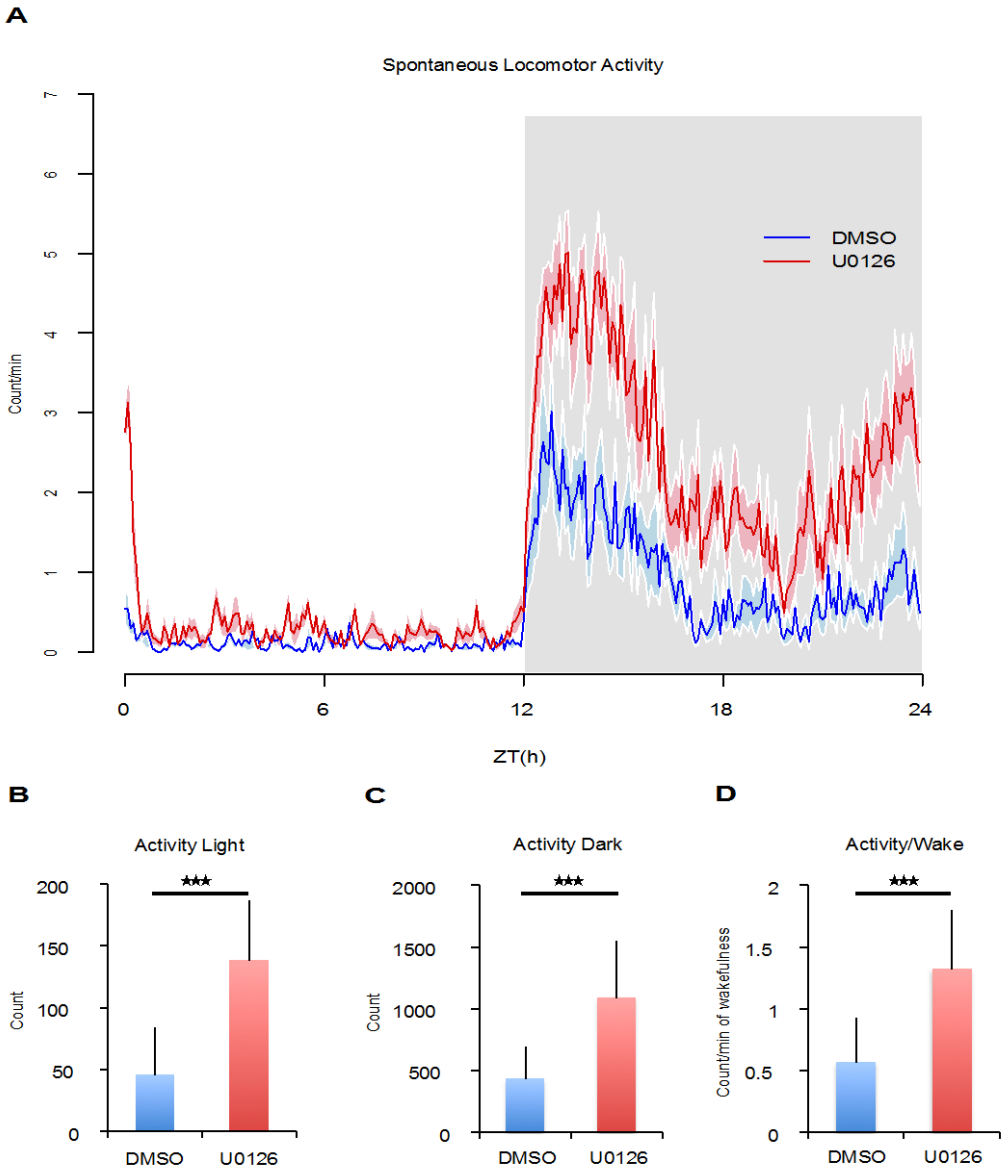

A
Baseline
Sustained Wake Bout duration
Minutes
150
120
90
60
30
0
ERK1 WT
ERK1 KO
ERK2 WT
ERK2 KO
DMSO
U0126
B
Recovery
Sustained Wake Bout duration
C
Short Wake Bout Frequency
frequency/hour
40
35
30
25
20
15
10
5
0
D
Short Wake Bout Frequency
E
Long Wake Bout Frequency
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
F
Long Wake Bout Frequency

Fig. 6


A
Spontaneous Locomotor Activity
Count/min
ZT(h)
DMSO
U0126
B
Activity Light
Count
***
C
Activity Dark
Count
***
D
Activity/Wake
Count/min of wakefulness
***