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MINI-REVIEW

MAP kinases in the mammalian circadian system – key regulators of clock function

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Abstract

Over the past 7 years, there has been spectacular progress in our understanding of the molecular basis of the circadian pacemaker in many species, from yeast to mammals. However, the biochemical signalling mechanisms that underpin synchronization of the clock to environmental cues are still poorly understood. Recently, attention has been focused on the role of mitogen-activated protein (MAP) kinase in biological timekeeping. It has been proposed that signal transduction via the MAP kinase cascades allows environmental information to be assimilated intracellularly within the circadian clock to produce changes in the phasing of clock gene expression, which, in turn, underlies clock-controlled phase-resetting of biological rhythms. This review examines the evidence for MAP kinase, particularly extracellular regulated kinases 1/2, involvement in the circadian clock and looks at the putative upstream regulators and downstream substrates of this signalling system.

Keywords: cAMP response element binding protein, circadian, clock, Elk-1, mitogen-activated protein kinase, suprachiasmatic nuclei.

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The circadian system

All eukaryotes possess endogenous timekeeping mechanisms that allow an organism to predict cyclical changes in its environment and to adjust its physiology accordingly. The most marked and well studied of such biological timekeeping systems is the circadian clock, generating rhythms with periodicities of approximately 24 h. Output from this endogenous oscillator imposes temporal architecture on physiological, endocrine and behavioural parameters, allowing an animal to maximally exploit its environment. However, since the period of the circadian clock is not exactly 24 h, it must be capable of being 'entrained' or synchronized to appropriate environmental cues. For most organisms, the most potent entraining factor (Zeitgeber) is the ambient light/dark cycle, with other important Zeitgebers being social interaction, food availability and temperature cycles (Mrosovsky 1996).

In mammals, the master circadian clock is localized to the suprachiasmatic nuclei (SCN) of the ventral anterior hypothalamus (Ralph *et al*. 1990; Reppert and Weaver 2002). The SCN are paired nuclei bilateral to the third ventricle and immediately dorsal to the optic chiasm. The major neural input pathway to the SCN is from the ganglion cells of the retina. This retinohypothalamic tract (RHT) is a monosynaptic, glutamatergic projection that synapses on neurones in the ventral and caudal SCN. The retinal ganglion cells giving rise to the RHT are directly photosensitive (Berson *et al*. 2002), and transmission via the RHT underlies the entrainment of the SCN clock to the light-dark cycle (Ebling 1996). Inhibition of glutamatergic transmission in the SCN blocks photic induction of gene expression and alteration of clock phase (Abe *et al*. 1991; Colwell *et al*. 1991). *In vitro* studies of clock phase-shifting illustrate that application of glutamate or NMDA reset the SCN clock's rhythm in electrical output

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Abbreviations used: AA-NAT, arylalkylamine-*N*-acetyltransferase; AVP, arginine vasopressin; CAMKII, calcium/calmodulin kinase II; CRE, cAMP response element; CREB, cAMP response element binding protein; Dexras, dexamethasone-induced Ras-protein; ERK, extracellular regulated kinase; JNK, c-jun-N-terminal kinase; MAP, mitogen-activated protein; MKP, map kinase-dependent phosphatase; RSK, ribosomal S6 kinase; RHT, retinohypothalamic tract; SCN, suprachiasmatic nuclei.

in a temporal pattern similar to the action of light on behavioural rhythms (Ding *et al*. 1994).

The underlying molecular mechanism allowing the SCN to generate a stable circadian period consists of interlocking transcriptional/translational feedback/forward loops of clock genes and their protein products (e.g. *Per1*, *Per2*, *Cry1 + 2*, *BMAL*, *Clock*, *Rev-erb* α, *Dec1 + 2*; reviewed in Reppert and Weaver 2002; Hastings *et al*. 2003). At present, one issue that remains to be addressed is identification of the signal transduction mechanisms responsible for the integrating and processing of sensory information received by the SCN. Such mechanisms presumably underpin changes in clock gene expression and, ultimately, alterations in the timing of clock output.

Since we know that the primary step processing photic information in the SCN involves glutamate, clues as to the signal transduction mechanisms involved in regulating the effect of light on the SCN clock may be gleaned from examining the signalling mechanisms involved in other CNS, glutamate-dependent neuronal processes. One candidate signalling system is the extracellular regulated kinase1/2 (ERK) cascade. ERKs are members of the mitogen-activated protein (MAP) kinase superfamily of threonine/tyrosine kinases, and have classically been investigated for roles in cell proliferation and differentiation. The ERKs are also strongly expressed in post-mitotic neurones, suggesting roles for these factors in neurophysiological and neurochemical processes such as synaptic plasticity (English and Sweatt 1997; Coogan *et al*. 1999). Indeed, activation of the ERK pathway appears necessary for long-term changes in synaptic efficacy and ensuing physiological processes such as associative memory and visual system development (Di Cristo *et al*. 2001; Adams and Sweatt 2002). However, it is only comparatively recently that the possible role of ERK in timekeeping processes has been examined.

Circadian and photic regulation of ERK phosphorylation

Obrietan *et al*. (1998) published the first report examining circadian and photic regulation of ERK phosphorylation in the mouse SCN. They found that in mice maintained in constant darkness, levels of the active, phosphorylated form of ERK (p-ERK) in the SCN showed significant variation across the circadian cycle, with levels being high during the subjective day and low during the subjective night. However, total levels of ERK did not vary across the circadian cycle, indicating that the changes in p-ERK are due to post-translational phosphorylation/dephosphorylation events. When mice are exposed to light pulses during the subjective night, phases at which light causes phase-shifts of behavioural rhythms, p-ERK is rapidly and significantly up-regulated in the SCN. This effect of light on p-ERK expression is phase-gated; light pulses presented during the subjective day, which do not alter behavioural rhythms, fail to induce any changes in p-ERK expression. These results on p-ERK regulation in the mouse SCN have been replicated in the Syrian hamster, the best characterized animal model from a chronobiological perspective. p-ERK levels oscillate under both diurnal and constant conditions, with peak expression during the day and the nadir during the night (Coogan and Piggins 2003). Photic stimulation up-regulates p-ERK expression in a phase-dependent manner similar to that described above for the mouse.

The kinetics of light-induced phosphorylation of ERK show that light pulses rapidly cause a large increase in p-ERK expression globally in the SCN (Obrietan *et al*. 1998; Butcher *et al*. 2003; Coogan and Piggins 2003; Nakaya *et al*. 2003). These levels of p-ERK quickly decline on termination of the pulse, with levels back at baseline values 1 h after initiation of the pulse. If the photic stimulation is not terminated and lights are left on, elevated levels of p-ERK are sustained and only decline towards baseline 90–120 min into the pulse (Butcher *et al*. 2003; Coogan and Piggins 2003). This finding suggests that the ERK cascade in the SCN is under autoregulatory feedback. Such a mechanism is implicated in hippocampal cellular activity, where tetanic stimulation initiates a rise in p-ERK that subsequently subsides after 30 min (Davis *et al*. 2000). This decline in p-ERK is accompanied by an increase in the expression of the MAP kinase-dependent phosphatase-1 (MKP-1), which is then capable of dephosphorylating ERK back to its inactive form. Interestingly, both MKP-1 and MKP-3 are expressed in the SCN (Butcher *et al*. 2003). Experiments with multiple light pulses suggest that any phosphatase-mediated desensitization may only be transient, as light pulses given at intervals of 30 min are capable of repeatedly inducing p-ERK, albeit with a declining magnitude (Butcher *et al*. 2003). These data tie in with what is known about the rapidity at which the circadian clock can be reset using closely spaced photic stimuli (Best *et al*. 1999).

A fascinating finding reported both in the mouse and the hamster is that, in addition to a circadian rhythm in the total expression of p-ERK, there is a time-dependent regulation of intra-SCN p-ERK distribution (Obrietan *et al*. 1998; Lee *et al*. 2002; Coogan and Piggins 2003; Nakaya *et al*. 2003). During the subjective day, p-ERK is spontaneously expressed in the ‘shell’ area of the SCN (Fig. 1), with a pattern resembling that of, but not co-existing with, cells synthesizing the neuropeptide arginine-vasopressin (AVP; Lee *et al*. 2002). During the subjective night, p-ERK is expressed solely in a core of SCN neurones at the mid-caudal level of the SCN (Fig. 1). Expression of p-ERK by these SCN neurones is dependent on the presence of a retinal innervation, as optic enucleation (blinding) ablates this ‘core’ p-ERK staining (Lee *et al*. 2002). The functional significance of the differences in p-ERK distribution with circadian time is not known, although these findings support the idea of

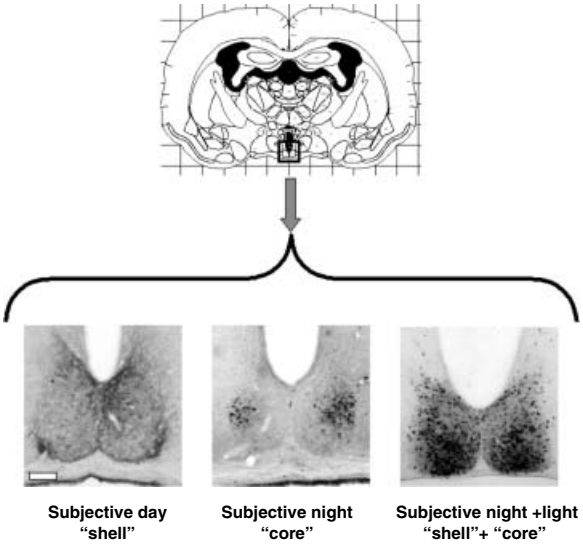

Fig. 1 Circadian and photic regulation of p-ERK in the hamster SCN. Diagram depicting the anatomical location of the SCN (after Morin and Wood 2001), with photomicrographs below showing the regulation of p-ERK immunostaining in the hamster SCN with time and light treatment. Scale bar = 100 μm.

functional compartmentalization within the SCN clock (Hamada *et al*. 2001). It is proposed that the 'core' area of the SCN is primarily responsible for processing incoming photic information, and that the 'shell' region of the SCN represents a subpopulation of spontaneously rhythmic SCN neurones that, in turn, receive photic information from 'core' neurones. Both shell and core areas of the SCN synthesize neuropeptides that are hypothesized to be humoral outputs of the SCN. Neurones of the shell SCN express AVP, and the expression of AVP is directly under clock control (Jin *et al*. 1999). Interestingly, expression of AVP by SCN explants has been shown to be sensitive to inhibitors of the ERK pathway (Arima *et al*. 2002). Therefore, ERK cascade activation may be a key event in the generation of at least one output from the SCN. In support of this idea, in the *Drosophila* circadian system, ERK has been shown to be a key component in the mechanism synthesizing pigment-dispersing factor, a vital output of the clock (Williams *et al*. 2001).

Physiological role for ERK cascade in the circadian clock

The results described in the above section may be criticized for being phenomenological in nature; p-ERK expression changes in the SCN with respect to circadian time and photic conditions, but the key question is whether ERK phosphorylation is a key event in mediating the running and resetting of the clock. In order to address this question, a number of studies have utilized specific pharmacological inhibitors of the ERK cascade. *In vitro* results show that glutamate-induced phase-shifting of the electrical discharge rhythm of SCN neurones, a process that closely mimics the effects of light *in vivo*, is attenuated by pre-treatment with the MEK (MAP kinase kinase) inhibitor PD98059 (Tischkau *et al*. 2000).

In behavioural studies, Butcher *et al*. (2002) found that micro-injection of the MEK1/2 inhibitors U0126 (into the lateral ventricles) or SL327 (systemically) in the mouse attenuated light-induced phase delays *in vivo*. Phosphorylation of ERK therefore appears to be a key event in photically-elicited phase delays during the early subjective night. Butcher *et al*. (2002) also reported that neither U0126 nor SL327 alone altered circadian clock phase. However, the half-life of these compounds may be too short to cause profound enough changes to alter the spontaneous expression of clock genes. Also, the inhibitors were given during the early subjective night when endogenous levels of p-ERK in the SCN are very low and therefore may not be significantly altered by the blockade of positive drive to p-ERK from MEK1/2.

Phosphorylation of ERK also appears to be a key event in mediating the phase-shifting effects of light in the Syrian hamster during the late subjective night. Micro-injection of U0126 into the third ventricle during the late subjective night prior to a light pulse attenuated the subsequent phase advance (Coogan and Piggins 2003). However, Yokota *et al*. (2001) reported that neither U0126 nor PD98059 was effective at blocking light-induced phase delays in the Syrian hamster. There are two possible explanations for this apparent discrepancy: (i) the use of two different phases in the studies of Coogan and Piggins (2003) and Yokota *et al*. (2001) in the hamster, and species difference between mice and hamsters in their dependence on p-ERK for photically-induced phase delays; or (ii) the shorter time (15 min vs. 30 and 45 min) used for pre-treatment with the MEK inhibitors by Yokota *et al*. (2001) may not have allowed sufficient inhibition of MEK prior to the time the light pulse was presented.

The attenuation of photic resetting of rodent behavioural rhythms by MEK inhibitors suggests that the ERK cascade is integrally involved in photic entrainment in adult mammals. In neonates, the SCN clock is entrained to the light/dark cycle via entrainment to maternal rhythms (Reppert and Schwartz 1986). Synchronization of the neonatal clock to maternal Zeitgebers is mimicked by agonists to the dopamine D1 receptor. Recently, it has been reported that intracellular signalling events elicited by a synchronizing D1 dopamine receptor agonist are dependent on activation of the ERK cascade (Schurov *et al*. 2002). These results raise the possibility that ERK phosphorylation also plays a key role in entrainment of the fetal SCN clock to the maternal rhythm.

Regulators and substrates for the ERK cascade in the SCN

It is well established that phase resetting of the SCN clock requires changes in gene expression (Zhang *et al*. 1996), and

it follows that if ERK is involved in the molecular mechanisms that underlie phase resetting, then activation of the ERK cascade must alter gene expression. Indeed, in NIH-3T3 fibroblast cells in culture, blockade of the ERK cascade leads to attenuation of *Per1* and *Per2* oscillations (Akashi and Nishida 2000; Oh-Hashi *et al*. 2002). Putative targets of the ERK cascade are transcription factors that have binding elements on promoters of known clock genes. The best studied of these in the SCN is the cAMP response element binding protein (CREB), which binds to the cAMP response element (CRE) consensus sequence present on photically-inducible clock genes and drives transcription following its phosphorylation on two serine residues (SER 133, SER142; Ginty *et al*. 1993; Gau *et al*. 2002).

Circadian and photic stimuli regulate the phosphorylation of CREB in the SCN (Ginty *et al*. 1993; Obrietan *et al*. 1999; Gau *et al*. 2002). CRE-mediated transcription is also under both circadian and photic control (Obrietan *et al*. 1999) and is required for photic resetting in the SCN and induction of the clock gene *Per1* (Tischkau *et al*. 2002; Travnickova-Bendova *et al*. 2002). Disruption of the ERK cascade using a dominant negative construct of MEK prevents calcium-mediated CRE-driven transcription (Obrietan *et al*. 1999). PD98059 and U0126 also attenuate phosphorylation of CREB- and CRE-driven transcription by excitatory stimuli in rodent SCN neurones and JEG3 cells (Obrietan *et al*. 1999; Travnickova-Bendova *et al*. 2002; Dziema *et al*. 2003). CREB is not directly phosphorylated by ERK; instead, ERK phosphorylates p90 ribosomal S6 kinases (p90RSK), which in turn phosphorylates CREB (Roberson *et al*. 1999). Recently, p90 RSK phosphorylation has been found to mirror that of ERK in the rat pineal gland, and application of U0126 blocks norepinephrine-induced up-regulation of both p-p90RSK and arylalkylamine-*N*-acetyl transferase (AA-NAT, the rate-limiting enzyme in the melatonin synthesis pathway; Ho *et al*. 2003). Given the known importance of CREs in regulating AA-NAT expression (Baler *et al*. 1997), it appears that p90 RSK acts as a pivotal link in the ERK-CREB-gene expression pathway in the vertebrate pineal gland. A very recent report has also indicated that photic stimulation in the early subjective night leads to phosphorylation of p90 RSK in the mouse SCN, and that phosphorylated p90 RSK co-localizes with p-ERK (Butcher *et al*. 2004). Phosphorylation of p90 RSK induced by light is blocked by pre-treatment with U0126, indicating that p90 RSK phosphorylation is indeed a downstream consequence of ERK activation in the SCN (Butcher *et al*. 2004).

Another putative substrate for ERK is Elk-1, a ternary complex transcription factor which, following its phosphorylation, binds to the serum response element (SRE) consensus sequence. Similar to CREs, SREs are present on the promoter sequences of photically-inducible clock genes (Wilsbacher *et al*. 2002). Recently, Elk-1 has been shown to be phosphorylated in the Syrian hamster SCN in response to photic stimulation (Coogan and Piggins 2003). This phosphorylation of Elk-1 is attenuated by U0126. Therefore, Elk-1 and CREB appear to be key transducers of the ERK cascade in the circadian clock.

An additional potential substrate for ERK is a clock gene, the basic helix-loop-helix transcriptional regulator BMAL1, which contains the protein–protein association PAS domains characteristic of a number of clock gene products. In the chick pineal gland, ERK can directly phosphorylate BMAL1, impairing its ability to complex with other PAS factors and activate transcription (Sanada *et al*. 2000). Collectively, the available data strongly suggest that ERK activation may directly feed into the core mechanisms of circadian clocks in a number of species, although in chick pineal cells a recent study has suggested that alteration of ERK activity is not sufficient, or necessary, for entrainment of the chick pineal cells (Yadav *et al*. 2003).

Many photically-inducible immediate early genes (*c-fos*, *JunB*, *EGR-1*) contain CREs and SREs on their promoters. A recent study has shown that U0126 attenuates the photic induction of these factors in the mouse SCN during the early subjective night (Dziema *et al*. 2003). However, in hamster SCN, U0126 does not block c-Fos induction by light pulses during the late subjective night, although p-ERK and p-Elk-1 induction and behavioural phase-advance to photic stimulation are attenuated (Coogan and Piggins 2003). These differences may arise from the species and phase differences used in these studies.

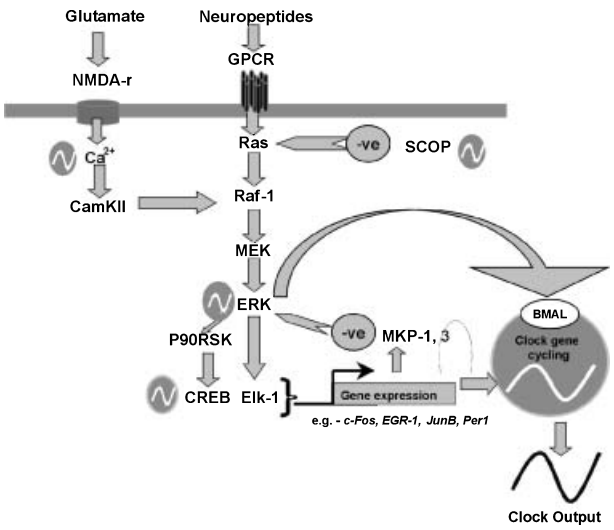

Fig. 2 Schematic of putative signalling involving ERK in the SCN clock. Factors displaying the waveform beside them have shown circadian rhythmicity in their expression/activity. The circadian rhythm in p-ERK in constant conditions may be due to a fluctuating, calcium-dependent positive drive and an oscillating, SCOP-mediated negative drive.

The upstream regulation of the ERK cascade above MEK in the SCN remains to be determined. NMDA receptor activation is required for photic induction of p-ERK (Coogan and Piggins 2003). However, the intracellular events linking NMDA receptor activation and ERK phosphorylation are not clear. Inhibitors of calcium/calmodulin kinase II (CAMKII) block both light-induced behavioural phase shifts (Golombek and Ralph 1994) and *Per1* and *Per2* expression (Yokota *et al*. 2001; Nomura *et al*. 2003), and suppress photic induction of p-ERK (Butcher *et al*. 2002). Therefore, calcium influx via the NMDA receptor channel may activate CAMKII which, in turn, may lead to phosphorylation of ERK via a SynGAP-dependent mechanism (Chen *et al*. 1998).

If such a mechanism contributes to photic induction of p-ERK, it does not explain the spontaneous rhythm in p-ERK. The putative upstream regulators of ERK activation, Raf-1 and Ras, oscillate in phase with p-ERK and p-MEK in the chick pineal gland, a tissue known to contain an endogenous circadian clock (Hayashi *et al*. 2001). A recently described novel protein inhibitor of Ras activation, SCOP, oscillates in the SCN in anti-phase to p-ERK (Shimizu *et al*. 1999). Overexpression of SCOP attenuates up-regulation of p-ERK evoked by depolarization (Shimizu *et al*. 2003). Therefore, spontaneous expression of SCOP may regulate the cyclical expression of p-ERK in the SCN (Fig. 2). Further evidence for the involvement of Ras-type proteins in regulating ERK signalling comes from the finding that dexamethasone-induced Ras-protein 1 (Dexras1) mRNA cycles in the SCN, and this cyclical expression is ablated in the clock gene knockout *Cry1,2*$^{-/-}$ mouse (Takahashi *et al*. 2003). However, *Dexras1* mRNA is high during the early to mid subjective night when p-ERK is low, suggesting that Dexras1 may be involved in photic induction, but not spontaneous expression, of p-ERK.

Future considerations

One of the most important recent sea changes in thinking in chronobiology has been the realization that extra-SCN sites in mammals also express circadian oscillators (Yamazaki *et al*. 2000). It will be of great interest to examine the role of ERK signalling in these peripheral oscillators. There are reports of cyclical expression of p-ERK in the retinas of chicks (Ko *et al*. 2001) and bullfrogs (Harada *et al*. 2000), structures known to express functional circadian clocks. As little is known about the location of circadian clock-expressing cell types in peripheral organs such as the liver and the lungs, it may prove difficult to tease apart the regulation of ERK signalling by circadian clocks and other physiological processes. The use of transgenic models of clock gene knockouts and reporter constructs should facilitate the addressing of such problems.

The ERKs described above are ERK1/2, and while these are the best studied of the MAP kinases in the central nervous system, there are other MAP kinases implicated in neuronal function. For example, ERK5 is thought to belong to a separate pathway to ERK1/2, and is involved in growth factor signalling (Watson *et al*. 2001). P38 and c-jun-N-terminal kinase (JNK), other members of the MAP kinase superfamily, have recently been shown to be under circadian and photic regulation in the SCN (Pizzio *et al*. 2003). P38 has also been implicated in the chick pineal circadian clock (Hayashi *et al*. 2003; Yadav *et al*. 2003). The elucidation of cross-talk and integration between these various MAP kinase pathways in circadian clocks will further our understanding of the roles of MAP kinases in biological timekeeping.

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