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REVIEW

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Neurotransmitters of the retino-hypothalamic tract

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Abstract The brain's biological clock, which, in mammals, is located in the suprachiasmatic nucleus (SCN), generates circadian rhythms in behaviour and physiology. These biological rhythms are adjusted daily (entrained) to the environmental light/dark cycle via a monosynaptic retinofugal pathway, the retinohypothalamic tract (RHT). In this review, the anatomical and physiological evidence for glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) as principal transmitters of the RHT will be considered. A combination of immunohistochemistry at both the light- and electron-microscopic levels and tract-tracing studies have revealed that these two transmitters are co-stored in a subpopulation of retinal ganglion cells projecting to the retino-recipient zone of the ventral SCN. The PACAP/glutamate-containing cells, which constitute the RHT, also contain a recently identified photoreceptor protein, melanopsin, which may function as a "circadian photopigment". In vivo and in vitro studies have shown that glutamate and glutamate agonists such as *N*-methyl-D-aspartate mimic light-induced phase shifts and that application of glutamate antagonists blocks light-induced phase shifts at subjective night indicating that glutamate mediates light signalling to the clock. PACAP in nanomolar concentrations has similar phase-shifting capacity as light and glutamate, whereas PACAP in micromolar concentrations modulates glutamate-induced phase shifts. Possible targets for PACAP and glutamate are the recently identified clock genes *Per1* and *Per2*, which are induced in the SCN by light, glutamate and PACAP at night.

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Introduction

The mammalian biological clock is located in the hypothalamic suprachiasmatic nuclei (SCN), which in the rat consist of a heterogeneous group of approximately 16,000 neurons (van den Pol 1980). The SCN drives diurnal changes in physiology and behaviour, such as hormone secretion, temperature and the sleep-waking cycles, in a predictable manner thereby preparing the body for oncoming events and demands (Klein et al. 1991). When examined under constant conditions (constant darkness or constant light), the endogenous rhythms driven by the clock oscillate with a period length close to 24 h (Latin *circa* + *dies* = circadian). Consequently, the clock needs daily adjustment (entrainment) to be synchronized with the astronomical day length. Without entrainment, the endogenous rhythms will be "free running" resulting in a daily shift in rhythmicity depending on the length of the endogenous period. Two types of "zeitgebers" (photic and non-photic cues) act on the clock and are important for its daily entrainment. The most powerful zeitgeber known is the environmental light/dark cycle that arises because of planetary rotation. Photic information is processed by the retina and reaches the brain via the optic nerves. The signalling pathway to the circadian timing system mediating the light entrainment of the clock is, however, anatomically and functionally different from the neural pathway used for vision. The retinal projection innervating the circadian pacemaker originates from a subset of retinal ganglion cells and is known as the retinohypothalamic tract (RHT; Moore and Lenn 1972; Moore et al. 1995). Lesion of the optic nerves results in free running of the circadian rhythm and blindness (Morin and Cummings 1981), whereas selective lesions of the RHT fibres to the SCN result in "circadian blindness" but not in loss of vision

(Johnson et al. 1988a). Retinal projections also reach other parts of the circadian timing system, such as the intergeniculate leaflet (IGL) of the lateral geniculate complex (Pickard 1985). Cells in the IGL integrate photic and non-photic information and project to the SCN providing feedback regulation of the pacemaker via the geniculo-hypothalamic tract (Moore 1995). Photic information from the RHT is also modulated within the SCN by non-photic input via neural projections originating mainly from the median raphe nucleus of the midbrain (Rea et al. 1994; Pickard et al. 1996, 1999; Meyer-Bernstein and Morin 1996; Meyer-Bernstein et al. 1997). At least one of the photoreceptors mediating photic information to the circadian timing system is functionally different from the classical photoreceptors used for vision (von Schantz et al. 2000). These observations are based on findings in mice lacking the classical photoreceptors, i.e. rods (*rd/rd* mice) or both rods and cones (*rd/rd/cl* mice). These mice strains are visually blind as a result of severe degeneration of the retina but retain the ability to entrain to the light/dark cycle (Foster et al. 1991; Freedman et al. 1999) most likely due to an intact RHT (Provencio et al. 1998). The photopigment mediating light information to the clock is not known (see Bellingham and Foster 2002) but a good candidate for a "circadian photopigment" is a recently identified opsin, melanopsin (Provencio et al. 2000), which is exclusively expressed in the ganglion cells of the RHT (Hannibal et al. 2002; Hattar et al. 2002; Gooley et al. 2001). Until recently, the primary neurotransmitter of the RHT was considered to be the excitatory amino acid glutamate (for reviews, see Ebling 1996; Rea 1998). A few years ago, the widespread neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP; Vaudry et al. 2000) was found to be co-stored with glutamate in the rat RHT (Hannibal et al. 2000). Functional studies have provided evidence that PACAP alone or in concert with glutamate is involved in light signalling to the clock (Harrington et al. 1999; Chen et al. 1999; Nielsen et al. 2001).

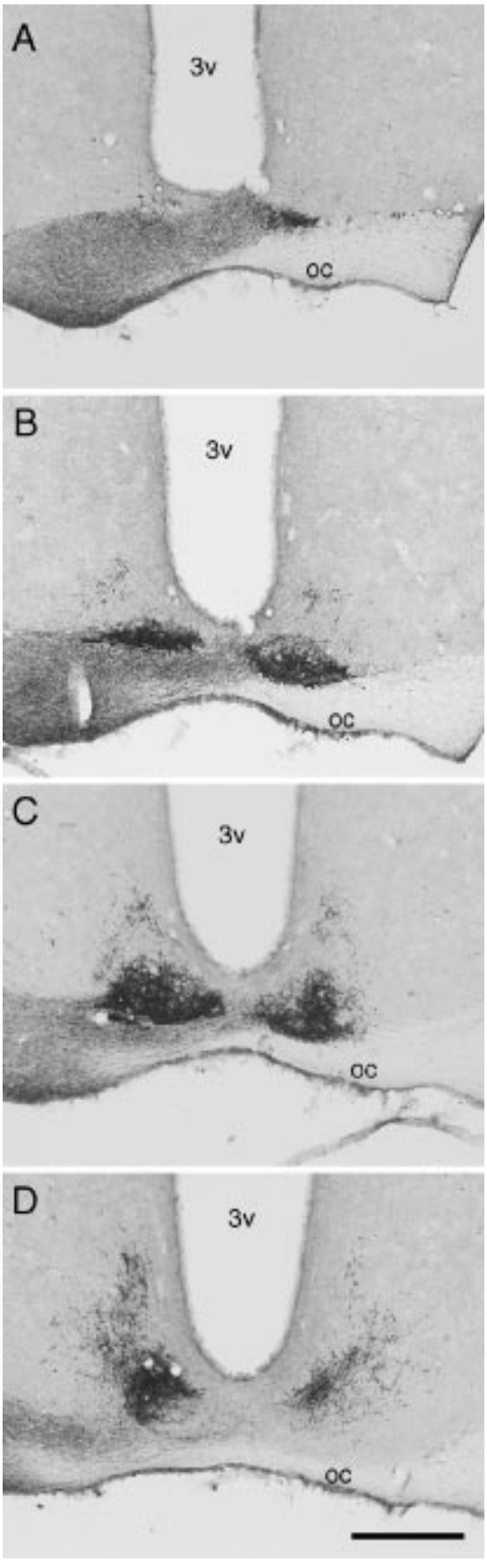
To be established as a neurotransmitter of the RHT mediating light signalling to the circadian timing system, a substance should fulfil the following criteria: (1) it should be located in the RHT, (2) it should be released by light stimulation, (3) it should affect the cells of the SCN similar to light (i.e. it should phase-shift the endogenous rhythm, change the electrical activity of SCN neurons and stimulate signalling pathways mediating light-induced phase shift) and (4) its effects should be blocked by specific antagonists. This review focuses on potential neurotransmitters of the RHT in the light of the above-mentioned criteria. A description of the molecular core clock, which is the target for the light-induced phase shift is described in detail elsewhere (Reppert and Weaver 2001; King and Takahashi 2000; Okamura et al. 2002; Stanewsky 2002).

Neuroanatomical studies

Identification of the RHT

The RHT is an anatomically and functionally distinct retinofugal pathway mediating the photic entrainment of circadian rhythms. During the first part of the twentieth century, several investigators described a retinohypothalamic projection but it was Moore and Lenn (1972) and Hendrickson et al. (1972) that conclusively verified a direct projection to the SCN by using injection of tritiated leucine or proline into the posterior chamber of the eye followed by autoradiographic visualization. These pioneering studies were subsequently confirmed by investigations using the subunit B of cholera toxin (ChB) as an anterograde tracer. By injecting a conjugate of ChB and horseradish peroxidase (CT-HRP) into the vitreous body of the eye, the RHT projections have now been demonstrated in several mammalian species (Pickard and Silverman 1981; Johnson et al. 1988b; Levine et al. 1991; Murakami et al. 1989; Murakami and Fuller 1990; Mikkelsen 1992; Cooper et al. 1993; Tessonneaud et al. 1994). Recently, a RHT projection has also been shown in humans by means of post mortem in vitro tracing with neurobiotin (Dai et al. 1998). These studies have demonstrated that the major part of the RHT projection terminates in the SCN. In the rat, this projection forms a dense aggregation of nerve fibre terminals at the chiasmal border and a dense plexus in the ventro-lateral part of the SCN. Only a few terminals are present in the medial portion of the SCN (Fig. 1). In species such as the rat, the RHT projects mainly to the contralateral SCN, whereas in the hamster, mouse and blind mole rat, the contralateral and ipsilateral projections are approximately equal (Johnson et al. 1988b; Levine et al. 1991; Mikkelsen 1992; Cooper et al. 1993; Abrahamson and Moore 2001). The functional significance of this species difference is unclear. In addition to the SCN, the RHT projects to the anterior hypothalamic area, the retrochiasmatic area and the lateral hypothalamus. Projections are also found in the perifornical area, dorsal hypothalamus and zona incerta (Johnson et al. 1988b; Levine et al. 1991; Mikkelsen 1992). Retinal projections considered as part of the RHT also reach several thalamic nuclei and the amygdaloid complex. Of these projections, which seem to be axonal collaterals from the RHT (Pickard 1985), the projections to the IGL and the pretectum seem to be important for the circadian timing system (Johnson et al. 1989; Mikkelsen and Vrang 1994).

Neonatal rats and hamsters treated with mono-sodium glutamate show severe retinal degeneration and visual blindness but retain their ability to entrain to light (Pickard et al. 1982; Chambille and Serviere 1993). These observations suggest that a distinct subset of retinal ganglion cells gives rise to the RHT and have been confirmed by retrograde tracing experiments with horseradish peroxidase injections into the SCN. This approach (Pickard 1980, 1982; Pickard and Silverman 1981; Murakami et al. 1989) has revealed that the ganglion



cells projecting to the SCN of hamster and cat resemble ganglion cells classified as type III or W according to Perry (1979). These findings have been corroborated by transneural infection with a mutant strain of the swine alpha herpes virus, the Bartha strain of pseudorabies virus (PRV-Bartha; Moore et al. 1995). When injected intraocularly, PRV-Bartha seems to have a special preference for the ganglion cells giving rise to the RHT. After replication, the virus is transported anterogradely to the SCN and infects neurons in the retino-recipient zone. By trans-synaptic spread, the virus is then taken up by axon terminals within the SCN and transported retrogradely to the contralateral retina. Here, the virus spreads in a time-dependent manner (Card et al. 1991). At the optimal time point of the infection (in rat, 85–90 h after injection of the virus into the eye), a homogeneous subset of retinal ganglion cells is labelled (Moore et al. 1995). The infected ganglion cells are widely distributed and display sparsely branching processes. These neurons, whose number is even smaller than that of neurons visualized by the injection of retrograde tracers such as HRP and FluoroGold into the SCN, establish the RHT (Moore et al. 1995). The same approach has enabled the RHT to be visualized in normal and in visually blind (*rd/rd* mutant) mice (Provencio et al. 1998). These blind mice are able to entrain to light despite the lack of rods and cones (Foster et al. 1991).

Neurotransmitters of the RHT

Glutamate

There is accumulating evidence that glutamate is a neurotransmitter of the RHT (for a review, see Ebling 1996). Several studies have demonstrated glutamate immunoreactivity in nerve terminals of the SCN. Using post-embedding techniques and electron microscopy, van den Pol was the first to show glutamate immunoreactivity within presynaptic nerve terminals in the rat SCN, although their origin was not identified (van den Pol and Tsujimoto 1985; van den Pol 1991). This issue was addressed a few years later by De Vries et al. (1993) who used intraocular injection of CT-HRP and post-embedding immunohistochemistry with colloidal gold particles to show that, for the rat, the retinal nerve terminals displayed a significantly higher content of glutamate immunoreactivity than the postsynaptic dendrites and non-retinal terminals. Similar results were obtained in mice (Castel et al. 1993). Using pre-embedding techniques and double immunohistochemistry at both the light- and

Fig. 1A–D Photomicrographs of four sections through the rat SCN in the rostro-caudal direction showing the retinohypothalamic projection following immunohistochemical staining of the anterograde tracer cholera toxin subunit B with biotinylated secondary antibodies and biotinylated tyramide/avidine-biotin-peroxidase and diaminobenzidine as amplification (*3v* third ventricle, *oc* optic chiasma). *Bar* 400 µm

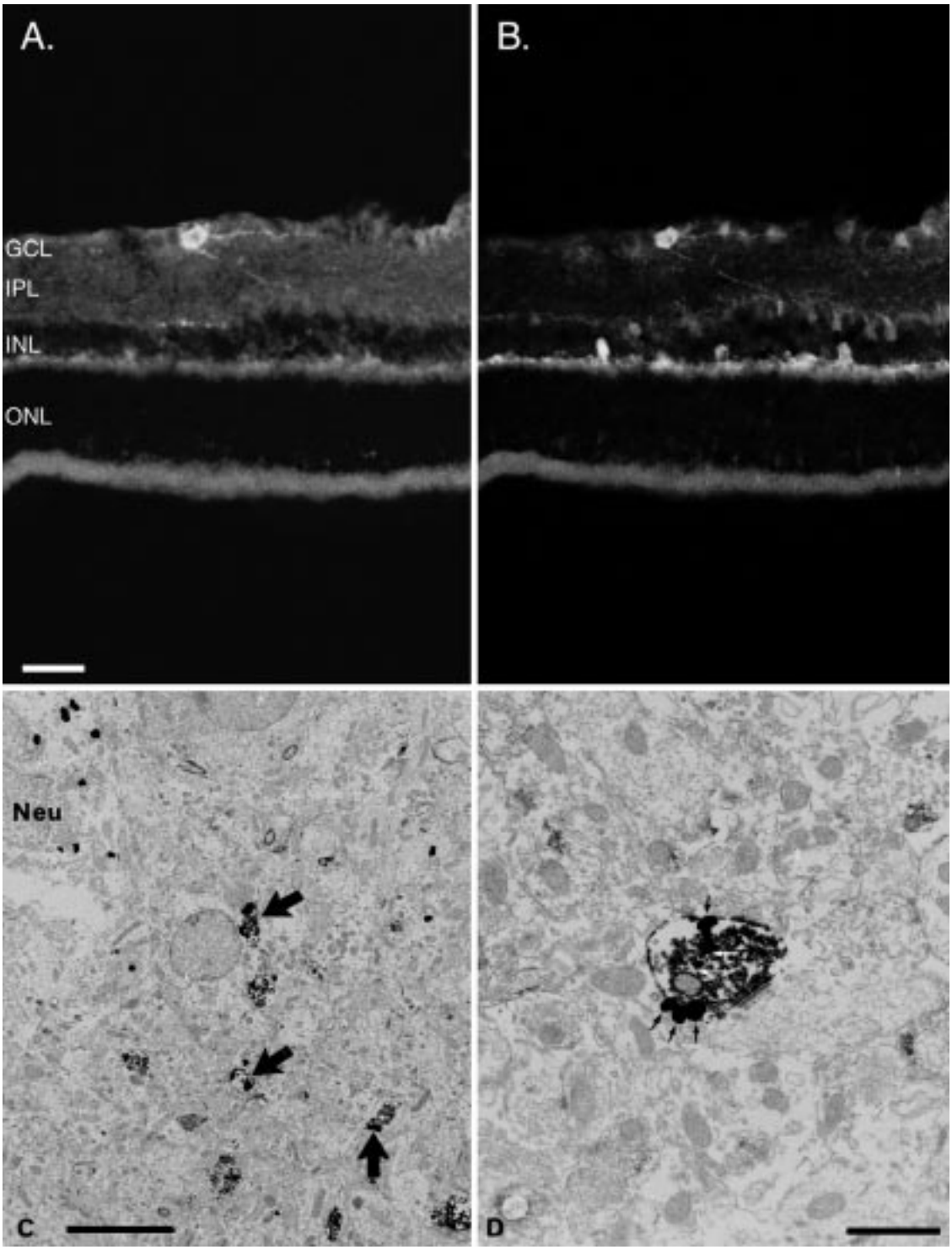


Fig. 2A–D Double immunostaining of PACAP and glutamate in the retina and suprachiasmatic nucleus of rat (*IPL* inner plexiform layer; *ONL* outer nuclear layer; *INL* inner nuclear layer; *GCL* ganglion cell layer; *Neu* neuron). Fluorescence photomicrographs showing double immunostaining of PACAP (**A**) and glutamate (**B**) in a sagittal section of rat retina. PACAP immunoreactivity was co-localized with glutamate in a subpopulation of glutamate-immunoreactive ganglion cells. Glutamate-positive cells were also located in the inner nuclear layer (*INL*) from which processes were observed to project towards the outer plexiform layer (**B**). **C, D** Electron micrographs showing double immunostaining for PACAP and glutamate in the suprachiasmatic nucleus of the rat. PACAP was visualized by the use of horseradish-peroxidase-labelled antibodies with tyramide amplification. Glutamate was demonstrated by the use of 1-nm gold-labelled antibodies. The gold particles were later silver-intensified. **C** Low-power electron micrograph of PACAP-immunoreactive nerve terminals. Several of these nerve terminals are also immunoreactive for glutamate (*arrows*). **D** High-power electron micrograph of a double-labelled nerve terminal making an axodendritic synapse. Several silver-intensified gold particles are present (*arrows*). *Bars* 50 µm (**A, B**), 6 µm (**C**), 1 µm (**D**). Modified from Hannibal et al. (2000)

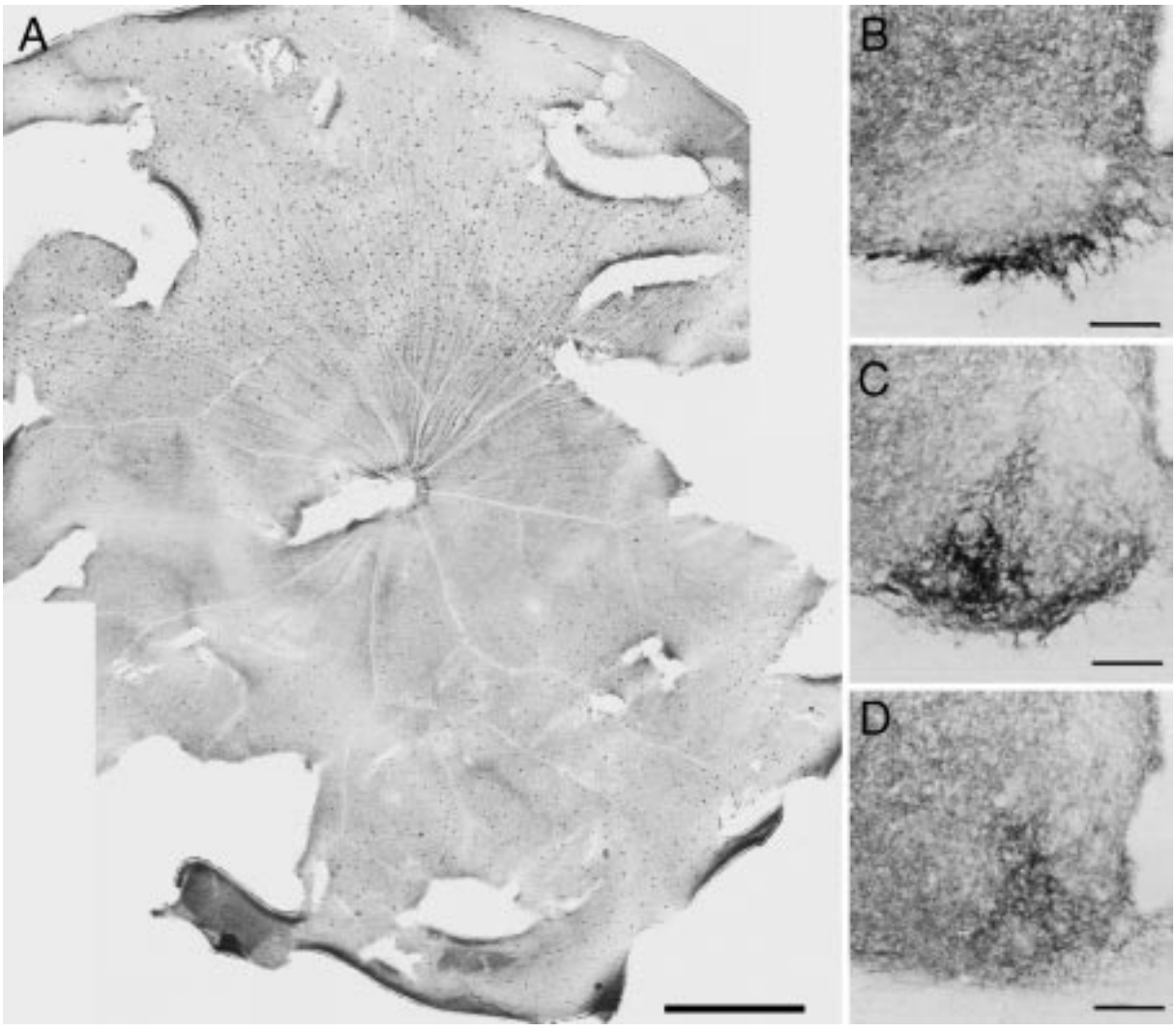


Fig. 3A–D PACAP staining in the retina and SCN in rat. **A** Photomicrograph of whole-mount rat retina stained for PACAP. Note the higher accumulation of PACAP-immunoreactive retinal ganglion cells in the superior part of the retina and the axon projections towards the retinal papilla. **B–D** Three rostro-caudal sections through the rat SCN stained for PACAP. PACAP-immunoreactive fibres in the SCN correspond to the RHT projection. *Scale bars* 1 mm (**A**), 100 µm (**B–D**). Modified from Hannibal et al. (1997, 2002) with permission from Journal of Neuroscience

electron-microscopical levels, we recently demonstrated that, in the rat, glutamate is co-stored with PACAP in RHT-projecting ganglion cells and their terminals (Fig. 2; see also below and Hannibal et al. 2000).

PACAP

PACAP is a neuropeptide of the vasoactive intestinal polypeptide (VIP)/secretin family of regulatory peptides and is widely distributed in the central and peripheral nervous system (for reviews, see Vaudry et al. 2000; Arimura 1998). PACAP is found in two biologically active forms: PACAP-38 consisting of 38 amino acids and PACAP-27, the C-terminally truncated form. PACAP-38 is the dominant form in tissues (Hannibal et al. 1995, 1998; Fahrenkrug and Hannibal 1996; Hannibal and Fahrenkrug 2000). By use of a specific mouse monoclonal antibody, PACAP was identified in nerve terminals within the SCN and in a population of retinal ganglion cells (Fig. 3). Tract-tracing studies with ChB and bilateral enucleation have indicated that PACAP immunoreactivity is located in the RHT (Hannibal et al. 1997, 2001a). This has been confirmed in whole-mount preparations of rat retina by using PRV-Bartha virus tracing and double immunohistochemistry for PACAP: virus-containing retinal ganglion cells have been shown to be PACAP immunoreactive (Fig. 4; see also Hannibal et al. 2001a) indicating that PACAP-containing retinal ganglion cells constitute the RHT projection to the SCN. Interestingly, the PACAP-containing RHT persists in rats that have been neonatally blinded by mono-sodium glutamate treatment, although the amount of PACAP is reduced by the treatment (Hannibal et al. 2001a).

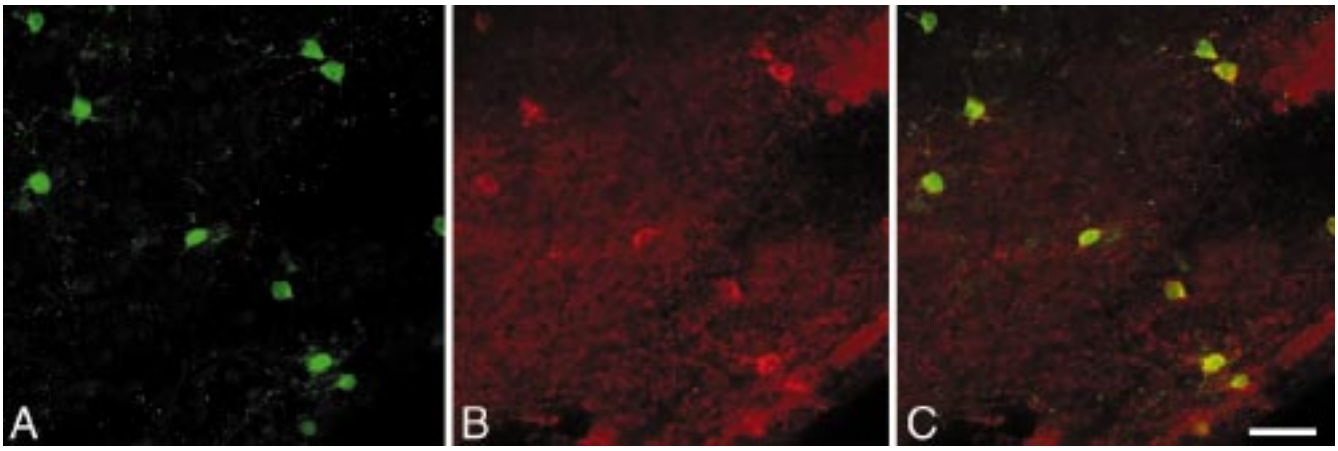


Fig. 4 Confocal laser scanning images showing co-localization of PRV-Bartha virus (**A**) and PACAP (**B**) and PRV-Bartha/PACAP (**C**) in a whole-mount preparation of rat retina. Animals received an intravitreal injection of virus 85–90 h before fixation. *Bar* 50 μm (see also Hannibal et al. 2001b)

Co-existence of PACAP and glutamate

By using double immunofluorescence and polyclonal antibodies against L-glutamate in combination with a mouse monoclonal PACAP antibody, PACAP was found to be co-stored with glutamate in a subpopulation of glutamatergic retinal ganglion cells and in nerve terminals in the ventro-lateral SCN (Fig. 2; see also Hannibal et al. 2000). The co-localization of PACAP and glutamate in the same axon terminals was confirmed by ultrastructural studies using pre-embedding techniques and double immunostaining with a combination of biotinylated tyramide-diaminobenzidine (DAB) and silver-intensified gold-labelled antibodies. These results demonstrated that PACAP and glutamate are co-stored in retinal terminals in the retino-recipient zone of the SCN (Fig. 2C, D; see also Hannibal et al. 2000).

Other neurotransmitters of the RHT

Excitatory amino acids

Apart from glutamate, a number of closely related molecules and derivates may also function as neurotransmitters of the RHT. Of these molecules, L-aspartate and N-acetylaspartylglutamate (NAAG) are the best studied. Optic nerve stimulation increases the concentration of L-aspartate in the SCN (Liou et al. 1986), and the injection of L-aspartate into the SCN results in minor phase advance during subjective day (De Vries and Meijer 1991). The presence of L-aspartate has been shown within cells of the SCN but, so far, L-aspartate has not been demonstrated in the RHT (Csaki et al. 2000). On the other hand, NAAG, which can act as an endogenous ligand for glutamate receptors, has been found in the RHT (Moffett et al. 1990) but its physiological significance remains to be clarified.

Substance P

Substance P (SP) has also been suggested as a neurotransmitter of the RHT. This undecapetide is widely distributed (Halliday et al. 1995) and is considered to be a neurotransmitter in both the central and the peripheral nervous systems (Harrison and Geppetti 2001). SP immunoreactivity within the SCN varies among species. In mouse and hamster, only a few SP-immunoreactive nerve fibres and cell bodies are located in the SCN (Piggins et al. 2001a; Abrahamson and Moore 2001). In the rat, SP-immunoreactive cell bodies and nerve fibres are confined to the ventral part of the SCN. The nerve fibres have been suggested to originate from the eye because their number decreases after enucleation (Takatsuji et al. 1991; Mikkelsen and Larsen 1993). However, these observations have not been confirmed by other studies (Otori et al. 1993; Hartwich et al. 1994; Hannibal and Fahrenkrug 2002). This discrepancy has raised the question as to whether the SP fibres belong to the RHT or represent intrinsic fibres originating from SP perikarya in the ventral SCN (Mikkelsen and Larsen 1993; Piggins et al. 2001a). This issue has been recently addressed by the injection of ChB into the eye and the simultaneous immunocytochemical demonstration of this anterograde tracer, PACAP and SP. The results have demonstrated that SP-immunoreactive fibres in the rat SCN do not originate from the eye. In this context, it is also noteworthy that the SP immunoreaction is located in amacrine and displaced amacrine cells but not in retinal ganglion cells containing PACAP immunoreactivity (Hannibal and Fahrenkrug 2002). Functionally, SP has been shown to phase-shift the endogenous rhythm in vitro (Shibata et al. 1992; Kim et al. 2001), whereas the results from in vivo injection of SP and/or antagonists are conflicting (Piggins and Rusak 1997; Challet et al. 1998). Nevertheless, a modulatory role of SP in light-induced phase shifting is likely, irrespective of whether SP is an intrinsic or an afferent neurotransmitter in the SCN (Kim et al. 2001).

Neurotransmitter receptors in the SCN

Glutamate receptors

Glutamate is the endogenous ligand for a large family of receptors belonging to two families: the ionotropic glutamate receptors, viz. *N*-methyl-D-aspartate (NMDA) and non-NMDA and the AMPA and kainate-preferring receptors, and the metabotropic glutamate receptors, i.e. G-protein-coupled receptors. NMDA, non-NMDA and metabotropic receptors have all been identified within the SCN by radioligand binding, in situ hybridization histochemistry and immunohistochemistry (for a review, see Ebling 1996). Subtypes of NMDAR1C are expressed in the entire SCN, whereas NMDAR2C is restricted to the dorso-medial SCN. The localization raises questions regarding the role of NMDAR2C in photic entrainment (Ebling 1996). mRNAs encoding the AMPA (GluR1, the GluR2 and the GluR4), the kainate (GluR6 and GluR7) and the metabotropic (mGluR1 and mGluR5) receptors have also been demonstrated within the SCN (Ebling 1996; for functional data, see Kopp et al. 2001).

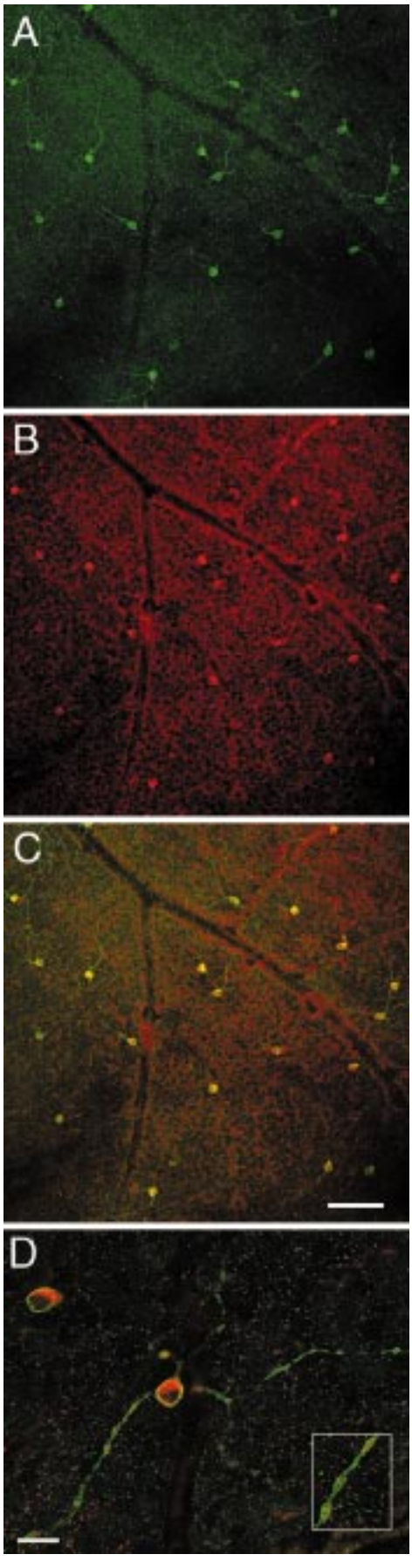
PACAP receptors

PACAP exerts its function via two classes of G-protein-coupled receptors. (1) The type I receptor corresponding to the PAC1 receptor is coupled to adenylate cyclase and phospholipase C depending on the splice variant (Spengler et al. 1993) and binds PACAP with a 1000-fold higher affinity than VIP. (2) The type II receptor corresponding to the VPAC1 and VPAC2 receptors binds PACAP and VIP with equal affinity and is coupled mainly to adenylate cyclase (Harmar et al. 1998). The type II receptors have a relatively restricted distribution within the CNS (Usdin et al. 1994; Vaudry et al. 2000; Sheward et al. 1995), whereas the PAC1 receptor is widely distributed in the brain and spinal cord (Vaudry et al. 2000; Shioda et al. 1997; Hashimoto et al. 1996). In situ hybridization studies have demonstrated the PAC1 (Hannibal et al. 1997; Cagampang et al. 1998a) and the VPAC2 (Lutz et al. 1993; Sheward et al. 1995; Cagampang et al. 1998b) but not the VPAC1 receptor in the SCN (Sheward et al. 1995; Usdin et al. 1994). The expression of both PAC1 and VPAC2 receptor mRNAs in the SCN show a circadian rhythm with a low amplitude with peaks during subjective day and mid subjective night (Cagampang et al. 1998a) and mid to late subjective day and late subjective night (Cagampang et al. 1998b), respectively. So far, little is known about the phenotype of SCN cells expressing the two receptors.

Photopigments in the retina responsible for light signalling to the SCN

The photopigment responsible for light activation of the RHT and its cellular localization in the retina is unknown. Previous studies in retinally degenerated mutant mice lacking rods (*rd/rd*; Foster et al. 1991) and both rods and cones (*rdta/cl*; Freedman et al. 1999) have demonstrated that the classical photoreceptor cells, the rods and cones, are dispensable for the circadian light response (von Schantz et al. 2000). Similarly, many blind people lacking conscious perception of light exhibit normal photic entrainment of the circadian rhythm (Czeisler et al. 1995). Two photopigments have been considered as "circadian photopigments", the cryptochromes and melanopsin. The vitamin-B_2-based cryptochromes CRY1 and CRY2 are found in the mammalian retina (Miyamoto and Sancar 1999). Studies with knock-out mice lacking one or both *Cry* genes have shown that the CRY molecules are important as central clock core components but not necessary for photic signalling to the brain (Vitaterna et al. 1999; van der Horst et al. 1999). Action spectrum analyses for light entrainment of locomotor activity suggest that an opsin-based photopigment with an absorption peak around 500 nm is responsible for circadian photoentrainment in mammals (Provencio and Foster 1995; Takahashi et al. 1984; Bellingham and Foster 2002). Melanopsin is a newly discoverd photopigment recently found in the inner retina (Provencio et al. 2000). It belongs to one of four opsins expressed outside the retinal photoreceptor layer. Our recent finding that melanopsin is exclusively expressed in the PACAP-containing retinal ganglion cells that constitute the RHT suggests that melanopsin is a circadian photopigment (Fig. 5, see also Hannibal et al. 2002). Melanopsin expression has been demonstrated by in situ hybridization with melanopsin cRNA probes and by immunohistochemistry using specific antibodies raised against a fusion protein containing the C-terminal part of mouse melanopsin (Hannibal et al. 2002). In co-localization studies the density of ganglion cells containing melanopsin and PACAP has been found in a range of 31 to 39 cells/mm^2 in the superior half of the retina and 5 to 9 cells/mm^2 in the lower half of the retina. The functional significance of this distribution pattern remains to be determined (Hannibal et al. 2002). Melanopsin immunoreactivity has been located at the surface of the perikarya and the dendritic processes of retinal ganglion cells, thereby increasing the light perceiving area of the cell (Fig. 5).

Although the functional role of melanopsin as a circadian photopigment remains to be established, we recently found that white light induces c-*fos* immunoreactivity in PACAP-containing retinal ganglion cells. This immunoreactivity is sustained only in the PACAP-containing retinal ganglion cells as long as light is turned on (Fig. 6, see also Hannibal et al. 2001a) suggesting that these cells are directly photosensitive possibly via activation of the melanopsin photopigment. This notion is supported by electrophysiological studies of the flat-mount in vitro preparation of rat retina (Berson et al. 2002; Hattar et al. 2002). In these experiments, SCN-projecting ganglion cells identified by retrograde tracing respond to light, despite a chemical blockade of synaptic transmis-



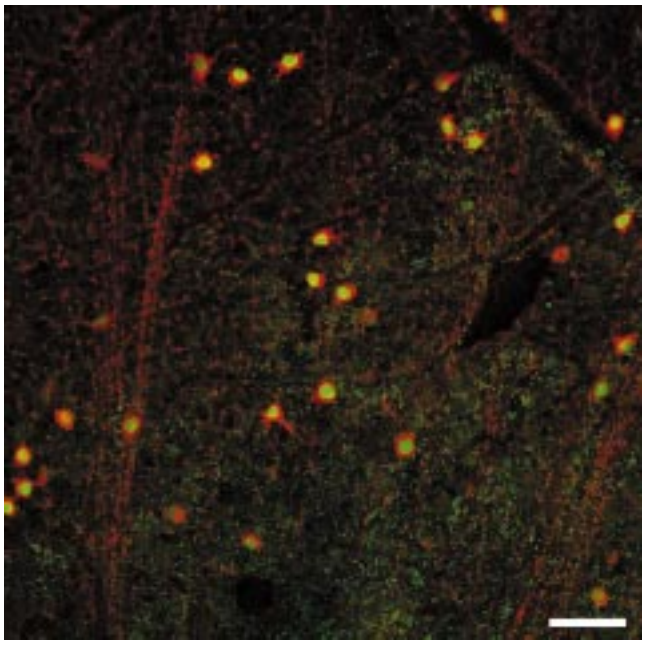


Fig. 6 Confocal laser scanning image showing co-localization of PACAP-IR (*red*) and c-*fos*-IR (*green*) in retinal ganglion cells in a retinal whole-mount from a rat kept in constant light for 19 h. PACAP and c-*fos* immunoreactivities are completely co-localized in the ganglion cells. *Bar* 50 µm. See also Hannibal et al. 2001a

sion (Berson et al. 2002). Furthermore, the light response does not reflect electric coupling to rods and cones because the SCN-projecting cells are sustained in a depolarizing state and not in a hyperpolarizing state as are the ganglion cells coupled to rods and cones. This indicates that such retinal ganglion cells are directly photosensitive. Together with the demonstration of melanopsin immunoreactivity in SCN-projecting ganglion cells (Hattar et al. 2002), the results point towards melanopsin as being a good candidate for a photopigment involved in light signalling to the circadian timing system.

Behavioural and physiological studies

Before considering the functional data concerning potential neurotransmitters of the RHT, the effects of light stimulation on the circadian timing system will be briefly discussed. The physiological properties of a "photo-

◀ **Fig. 5A–D** Melanopsin is exclusively located in PACAP-immunoreactive retinal ganglion cells. **A–C** Low-power confocal laser scanning photomicrographs showing a randomly selected part of the retina double-immunostained for melanopsin (**A**), PACAP (**B**) and melanopsin/PACAP (**C**). **D** High-power photomicrograph showing double-immunostaining of melanopsin (*green*) and PACAP (*red*) in the same ganglion cells of whole-mount retina. Note the punctate melanopsin immunoreactivity on the surface of the cell body and processes. *Insert* in **D** shows high magnification of a melanopsin/PACAP-containing dendrite. *Bars* 100 µm (**A–C**), 20 µm (**D**). From Hannibal et al. (2002) with permission from Journal of Neuroscience

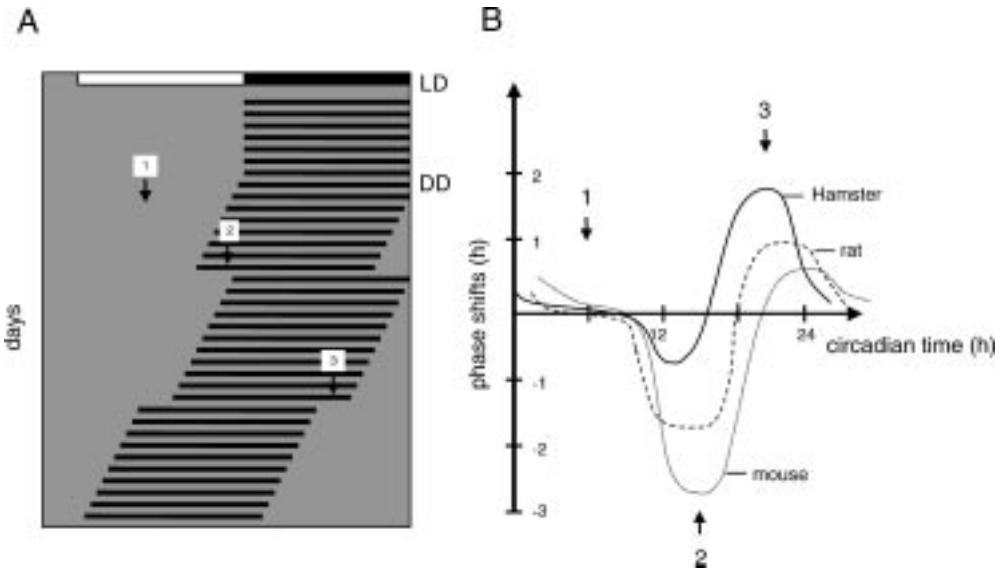


Fig. 7 **A** Schematic presentation of the activity rhythm of a nocturnal animal where each *horizontal line* represents the activity of the animal in 1 day. The animal was entrained to a light/dark photoperiod (*LD*) as represented *above* the record. The animal was then released into constant darkness (*DD*), the activity rhythm now being "free-running". During the free-running period, the animal experienced light pulses during subjective day (*1*), early subjective night (*2*) and late subjective night (*3*). The light pulse given during the day had little or no effect on the phase of the endogenous rhythm, whereas a light pulse given at early subjective night resulted in a phase delay of the overt rhythm (indicated by *2* in **A** and **B**). A light pulse given at late subjective night resulted in a phase advance of the overt rhythm (indicated by *3* in **A** and **B**). **B** Complete phase response curve to light stimulation during a 24-h period. Phase delays are plotted in the negative direction and phase advances in the positive direction. The *horizontal axis* in **B** represents one circadian day. Note the species differences in response to light stimulation. Modified from Meijer and Rietveld (1989) and Daan and Pittendrigh (1976)

entrainment" system is the ability of daily adjustment of the endogenous period length (i.e. free-running period τ) to the astronomical 24-h day length. To fulfil these criteria, the phase shifts induced by light stimulation should have a size [amplitude: $\Delta\phi(\phi)$] that adjusts this deviation [$\Delta\phi(\phi)=\tau-T$, where T always equals 24 h; Daan and Pittendrigh 1976]. τ is a unique property of the circadian pacemaker and varies among individuals and species (Pittendrigh and Daan 1976; Summers et al. 1984; Wright et al. 2001). Similarly, the phase-response curves to light (PRC) obtained by light stimuli applied during a 24-h period to animals kept under constant darkness are species-dependent (Fig. 7B). The general pattern is, however, that light causes a phase delay when applied during late subjective day and early subjective night, whereas light applied between late subjective night and early subjective day phase-advances the endogenous rhythm. Light given during the subjective day has little effect on the phase (Fig. 7). In short, the response to a resetting light stimulus at a given phase of the rhythm is correlated with the individual τ; fast pacemakers with a short τ as is found in the mouse tend to be more delayed and/or less advanced than slow pacemakers with a long τ as is found in rat and hamster (Fig. 7; see also Daan and Pittendrigh 1976).

A candidate neurotransmitter mediating the effects of light in the RHT should be released from the nerve terminals in the SCN and affect the phase of the circadian pacemaker similar to light. Antagonists to the transmitter receptor should decrease or block the light-induced effects, such as phase shifts. These issues have been addressed in detail for glutamate by using in vivo and in vitro models (see Ebling 1996) and to a minor extent for PACAP. The end-point parameter determined in most in vivo experiments is the behavioural phase shift in running-wheel activity (for a description of the method, see Pittendrigh and Daan 1976). Moreover, light-inducible genes, such as the immediate-early-gene *c-fos* (cf. Kornhauser et al. 1996) or clock genes (cf. Chang and Reppert 2001), and phosphorylation of the transcription factor cAMP responsive element binding protein (CREB; Ginty et al. 1993; Obrietan et al. 1999) and the extracellularly regulated kinase p44/42 mitogen-activated protein kinase (ERK/MAPK; Obrietan et al. 1998) have been analysed in the SCN. These molecules seem to be important elements of the light signalling pathways to the clock, because they are rapidly induced/phosphorylated in the SCN by light. In vitro brain-slice preparations containing the SCN (Gillette 1986) have been useful in studying the phase-shifting effects of various neurotransmitters (and agonists). Brain slices in which the optic nerve is left in situ and electrically stimulated have been used to study the effects of various blockers of the RHT transmitters (for a review, see Ebling 1996). Recently, the model has also proved useful for examining changes in clock gene expression after application of RHT transmitters (Nielsen et al. 2001).

Is glutamate released from the RHT?

The extracellular concentration of glutamate and aspartate in hamster SCN was shown, by in vivo microdialysis, to change during the 24-h light/dark cycle, with the highest level being found during the dark phase (Glass et al. 1993; Rea et al. 1993). However, these results had a low resolution because of the sensitivity of the technique and may not necessarily be related to differential amino acid release from RHT nerve terminals. Using an in vitro approach and tritium-labelled amino acids, Liou et al. (1986) have demonstrated that stimulation of the optic nerve causes the release of ^{3}H-glutamate and ^{3}H-aspartate from a brain slice containing the SCN. These findings are supported by the demonstration that the activation of SCN neurons by optic nerve stimulation can be blocked by the application of NMDA antagonists (Shibata et al. 1986; De Vries et al. 1994).

Glutamate phase-shifts the endogenous rhythm

In initial experiments, in vivo injections of L-glutamate into or adjacent to the SCN failed to induce phase shifts in running-wheel activity as seen for light (for discussions of these results, see Meijer et al. 1988; Ebling 1996). In vivo application of NMDA to the SCN was, however, recently shown to mimic light-induced phase shifts in the behavioural rhythm (Mintz and Albers 1997; Mintz et al. 1999). Several in vitro studies with a brain-slice model (Ding et al. 1994; Shibata et al. 1994) also showed that the application of NMDA mimics light-induced phase shift at night. All results support a role for glutamate in light signalling to the clock.

Glutamate antagonist blocks light-induced phase shifts

Further support for glutamate as a mediator of light information to the circadian timing system comes from experiments showing that NMDA and non-NMDA antagonists block light-induced phase shifts at both early and late night (Colwell et al. 1990, 1991; Colwell and Menaker 1992). Moreover, evidence has been provided that glutamatergic signalling pathways involve nitric oxide (Ding et al. 1994, 1997), phosphorylation of CREB (Ding et al. 1997), calcium release via ryanodine receptor activation at early night (Ding et al. 1998) and cGMP-dependent pathways at late subjective night (Prosser et al. 1989; Gillette and Mitchell 2002).

Glutamate induces light-sensitive genes within the SCN similar to light

Light induces the expression of several immediate-early genes including *c-fos* at time points at which light causes a phase shift of the endogenous rhythm. Induction of *c-fos* can be mimicked by the application of glutamate agonists. This induction is seen in the ventro-lateral SCN and can be reduced markedly by adding a glutamate antagonist before the light pulse (reviewed by Kornhauser et al. 1996; Rea 1998). The induction of *c-fos* is, however, not necessarily linked to a light-induced phase shift (Honrado et al. 1996; Hannibal et al. 2001b). At certain time points in the night, light induces *c-fos* but this induction is not followed by a phase shift (Sutin and Kilduff 1992). Recently, light has been shown to stimulate the ERK/MAPK and the CREB signalling pathways in the SCN. These effects are attenuated by a glutamate antagonist (PD 98059) indicating that glutamate receptor activation is followed by the phosphorylation of ERK and CREB (Obrietan et al. 1998). More recently, the clock genes *Per1* and *Per2* have been attributed a role in light-induced phase shifts because they are rapidly induced by light stimulation at those time points at which light phase-shifts the endogenous rhythm (Shigeyoshi et al. 1997; Zylka et al. 1998; Albrecht et al. 1997; Yan et al. 1999; Field et al. 2000; Akiyama et al. 1999). In vitro, the expression of both genes in the SCN is induced at late night by glutamate (Nielsen et al. 2001) and the glutamate-induced phase shift of neuronal firing activity is blocked by *mPer1* antisense-oligonucleotide treatment (Akiyama et al. 1999).

Is PACAP released from the RHT?

A few studies have addressed this issue by an indirect approach. In tissue extracts punched from the SCN, the PACAP concentration is lower during the day than during the night. This variation seems to be diurnal, i.e. to depend on the light/dark cycle, since no concentration difference has been found in specimens from animals kept in constant darkness (Fukuhara et al. 1997). The results may suggest that PACAP is released during the day and stored during the dark phase. As described below, PACAP can phase-shift the electrical rhythm during the subjective day in vitro (Hannibal et al. 1997). Using a horizontal brain-slice preparation and optic nerve stimulation with different stimulation frequencies, Burgoon and Gillette (2000) have shown that, in the SCN, the phase advance of the electrical activity that is induced at mid subjective day is blocked by the application of the specific PACAP antagonist PACAP6–38, indicating that PACAP is released upon optic nerve stimulation. Recently, the light-induced phase shift has been found to differ between homozygous *PAC1* knock-out and wild-type mice, suggesting that PACAP is released from the RHT during light stimulation at night (Hannibal et al. 2001b). This finding conforms to previous observations showing that intracerebroventricular injection of specific neutralizing PACAP antibodies modulates the light-induced phase advance (Chen et al. 1999).

PACAP phase-shifts the endogenous rhythm similar to light

Recent studies have shown that injection of PACAP into the SCN in vivo phase-shifts the endogenous rhythm of running-wheel activity (Harrington et al. 1999; Piggins et al. 2001b) and the electrical firing activity in vitro (Harrington et al. 1999) similar to light. Interestingly, this effect of PACAP is dose-dependent, a maximal response being elicited by nanomolar concentrations. In micromolar concentrations, PACAP has no direct effect on the phase (Hannibal et al. 1997) but it modulates the glutamate-induced phase shift (see below and Chen et al. 1999).

The role of PACAP as a mediator of light signalling to the clock has further been supported by our recent studies with knock-out mice lacking the PAC1 receptor (Hannibal et al. 2001b). Both wild-type and homozygous (*PAC1*$^{-/-}$) mice appear to have an anatomically intact RHT projection to the SCN but show different circadian behaviour during constant darkness and in response to light stimulation (Hannibal et al. 2001b). *PAC1*$^{-/-}$ mice have a significantly shorter τ compared to wild-type mice and responded to light stimulation at early night with a significantly higher sensitivity than wild-type mice. As a result, the homozygous mice respond to a light pulse given at early subjective night with an increased phase delay. In contrast to the wild-type mice, light stimulation of homozygous mice at late subjective night results in a phase delay and not in a phase advance. The findings suggest that PACAP signalling is involved in the regulation of the clock sensitivity to light stimulation and in the mechanism determining the direction of the phase shift at late night (Hannibal et al. 2001b).

As previously mentioned, light entrainment of the clock is believed to involve the induction of c-*fos* (Kornhauser et al. 1996) and the recently identified clock genes *Per1* and *Per2* (Shigeyoshi et al. 1997; Zylka et al. 1998; Albrecht et al. 1997; Yan et al. 1999; Field et al. 2000; Akiyama et al. 1999), because these genes are rapidly induced in the SCN by light stimulation at those time points at which light phase-shifts the clock. Compared with wild-type mice, mice lacking the *PAC1* receptor show larger phase delays in response to light but a marked attenuation of light-induced *mPer1*, *mPer2* and c-*fos* gene expression in the retino-recipient zone of the SCN (Hannibal et al. 2001b). This dissociation between the light-induced phase shift of running-wheel activity and induction of c-*fos*, *Per1* and *Per2* gene expression in the SCN indicates that light-induced behavioural phase shifts are not always dependent of c-*fos*/*clock* gene expression in the SCN. Photic stimulation at late night provokes a phase delay in *PAC1*$^{-/-}$ mice but a phase advance in wild-type mice. However, the light-induced *mPer1* and *c-fos* gene expression of *PAC1*$^{-/-}$ mice is similar to that in wild-type mice suggesting that PACAP and PAC1 receptor signalling play a minor, if any, role in the light-induced gene expression of *mPer1* and c-*fos* at this time point (Hannibal et al. 2001b).

PACAP phase-shifts the endogenous rhythm similar to dark pulses

Whereas light stimulation affects the clock at night, dark pulses presented during the subjective day phase-advance the endogenous rhythm similar to non-photic stimuli (Hastings et al. 1998a). If animals are kept in constant light and then exposed to a dark pulse (2–4 h) in the middle of the subjective day, a phase advance of the endogenous rhythm is observed (Boulos and Rusak 1982; Ellis et al. 1982). In our initial experiments with an in vitro rat brain-slice preparation, application of PACAP in micromolar concentrations to the SCN caused a phase advance in the electrical firing rhythm during the subjective day but not during the subjective night. This effect involved a cAMP/protein kinase-A-dependent pathway (Hannibal et al. 1997). These observations were later confirmed in the hamster (Harrington and Hoque 1997; Harrington et al. 1999). One might speculate that the change from light to darkness may cause a release of PACAP from the RHT, thus explaining the phase advance during subjective day. This assumption is supported by the ability of PACAP to stimulate the phosphorylation of CREB during late subjective day (Kopp et al. 1997; von Gall et al. 1998). It is also possible that PACAP signalling from the RHT interacts with non-photic transmitters released in the SCN, since neuropeptide Y, an important neurotransmitter of the geniculo-hypothalamic tract mediating non-photic information to the SCN (cf. Yannielli and Harrington 2001), blocks the PACAP-induced phase advance during subjective day in vitro (Harrington and Hoque 1997).

PACAP interacts with glutamate signalling during light-induced phase shift

The functional significance of the co-existence of PACAP and glutamate in the RHT is not fully understood. PACAP has been reported to modulate glutamatergic signalling in the SCN (Chen et al. 1999; Kopp et al. 2001). In an in vitro brain-slice model, Chen et al. (1999) showed that PACAP when applied in micromolar concentrations together with glutamate at early night potentiated the glutamate-induced phase delay, whereas the specific antagonist PACAP6–38 blocked the glutamate-induced phase shift at CT14. In contrast, PACAP (in micromolar concentrations) blocked the glutamate-induced phase-advance, when co-administered with glutamate at late subjective night, and co-administration of PACAP6–38 potentiated the glutamate-induced phase shift at this time point. The clock-controlled modulatory effects of PACAP on glutamate signalling were confirmed by in vivo experiments. The intraventricular injection of a specific PACAP antibody resulted in a potentiation of the light-induced phase advance (Chen et al. 1999) suggesting that PACAP is an important modulator of glutamate-induced phase shift. A modulatory role of PACAP on glutamate signalling was further supported

by Kopp et al. (2001) who analysed the effects of PACAP and glutamate on calcium signalling in cultured SCN neurons and showed that PACAP modulates glutamate signalling via different mechanisms. On the one hand, PACAP amplified glutamate-dependent calcium increases by interacting with AMPA/kainate signalling. On the other hand, PACAP reduced/inhibited calcium increases elicited by glutamate acting on metabotropic receptors. The latter action was mimicked by cAMP (Kopp et al. 2001). The interaction between PACAP and glutamate signalling seems to involve the *per* genes, since our recent findings show that in vitro application of PACAP in nanomolar concentrations induces *per1* and *per 2* gene expression in the SCN at late night, whereas micromolar concentrations of PACAP block glutamate-induced *per* gene expression in the SCN (Nielsen et al. 2001).

"Non-photic" modulation of photic signalling to the SCN

Although light is the principal zeitgeber to the mammalian circadian system, efferent projections from different parts of the brain to the SCN mediating behavioural states, such as arousal and/or sleep deprivation and metabolic changes, act together with humoral factors in the entrainment process by modulating light-induced phase shifts during subjective night. Signalling pathways and neurotransmitters mediating these non-photic cues are beyond the scope of this review but have been reviewed elsewhere (Hastings et al. 1998a, 1998b; Yannielli and Harrington 2001; Morin 1999; Rea 1998). However, some projections and transmitters/modulators that presynaptically act on the RHT terminals in the SCN should be considered. The serotonergic (5-HT) pathway from the median raphe nucleus (Leander et al. 1998; Meyer-Bernstein and Morin 1996) has been shown to block or modulate light-induced phase shift at both early and late night (Rea et al. 1994). 5-HT on the other hand has little effect on the phase of the clock at night (Meyer-Bernstein et al. 1997). Anatomical and functional data indicate that 5-HT modulates light signalling to the clock via 5-HT_{1B} receptors located on RHT terminals (Pickard et al. 1996, 1999; Pickard and Rea 1997; Belenky and Pickard 2001). These findings are supported by studies in mice lacking the 5-HT_{1B} receptor; these mice have an increased sensitivity to light compared with wild-type mice (Pickard et al. 1999). It is possible that a behavioural state such as sleep deprivation (Mistlberger et al. 1997; Challet et al. 2001) causes release of 5-HT in the SCN acting on presynaptic receptors controlling the release of transmitters from RHT terminals within the SCN.

Conclusions

Anatomical and functional data indicate that both PACAP and glutamate are neurotransmitters of the RHT, mediating light signalling to the clock, whereas the evidence for other putative transmitters is less convincing. It remains to be determined whether the phase-shifting effect of PACAP during subjective day in vitro is of physiological relevance. A detailed analysis of the release of both PACAP and glutamate during various lighting conditions should increase the functional understanding of their interaction within the circadian timing system.

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