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J. Hannibal · J. Fahrenkrug

Neuronal Input Pathways to the Brain's Biological Clock and their Functional Significance

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Prof. Dr. F. **BECK**, Howard Florey Institute, University of Melbourne,
Parkville, 3000 Melbourne, Victoria, Australia, e-mail: fb22@le.ac.uk

Prof. Dr. B. **CHRIST**, Anatomisches Institut der Universität Freiburg,
Abteilung Anatomie II, Albertstr. 17, 79104 Freiburg, Germany
e-mail: christb@uni-freiburg.de

Prof. Dr. F. **CLASCÁ**, Department of Anatomy, Histology and Neurobiology,
Universidad Autónoma de Madrid, Ave. Arzobispo Morcillo s/n, 28029 Madrid, Spain
e-mail: francisco.clasca@uam.es

Prof. Dr. D. E. **HAINES**, Ph.D., Department of Anatomy, The University of Mississippi Med. Ctr.,
2500 North State Street, Jackson, MS 39216-4505, USA
e-mail: dhaines@anatomy.umsmed.edu

Prof. Dr. H.-W. **KORF**, Zentrum der Morphologie, Universität Frankfurt,
Theodor-Stern Kai 7, 60595 Frankfurt/Main, Germany, e-mail: korf@em.uni-frankfurt.de

Prof. Dr. W. **KUMMER**, Institut für Anatomie und Zellbiologie,
Universität Gießen, Aulweg 123, 35385 Gießen, Germany
e-mail: wolfgang.kummer@anatomie.med.uni-giessen.de

Prof. Dr. E. **MARANI**, Leiden University, Department of Physiology,
Neuroregulation Group, P.O. Box 9604, 2300 RC Leiden, The Netherlands
e-mail: e.marani@lumc.nl

Prof. Dr. R. **PUTZ**, Anatomische Anstalt der Universität München,
Lehrstuhl Anatomie I, Pettenkoferstr. 11, 80336 München, Germany
e-mail: putz@anat.med.uni-muenchen.de

Prof. Dr. Dr. h.c. Y. **SANO**, Department of Anatomy,
Kyoto Prefectural University of Medicine,
Kawaramachi-Hirokoji, 602 Kyoto, Japan

Prof. Dr. Dr. h.c. T. H. **SCHIEBLER**, Anatomisches Institut der Universität,
Koellikerstraße 6, 97070 Würzburg, Germany

Prof. Dr. K. **ZILLES**, Universität Düsseldorf, Medizinische Einrichtungen,
C. u. O. Vogt-Institut, Postfach 101007, 40001 Düsseldorf, Germany
e-mail: zilles@hirn.uni-duesseldorf.de

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With 22 Figures

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Jens Hannibal, MD, PhD, Dr. Sci
Jan Fahrenkrug
Department of Clinical Biochemistry
Bispjebjerg Hospital
Bispjebjerg Bakke 23
2400 Copenhagen NV
Denmark
e-mail: j.hannibal@inet.uni2.dk

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Abbrevations

3v Third ventricle
5-HT Serotonin
Aq Cerebral aqueduct
APTD Anterior pretectal nucleus, dorsal part
AVP Arginine-vasopressin
CKIε Casein kinase ε
CaMKII Ca^{2+}/calmodulin-dependent kinase
CREB cAMP response element binding protein
CRY Cryptochromes
CTb Cholera toxin subunit B
DGL Dorsal geniculate nucleus
DRN Dorsal raphe nucleus
EGFP Enhanced green fluorescent protein
ERK Extracellular signal-related kinase
GABA Gamma aminobutyric acid
GCL Ganglion cell layer
GHT Geniculo-hypothalamic tract
IGL Intergeniculate leaflet
INL Inner nuclear layer
IPL Inner plexiform layer
ipRGCs Intrinsic photosensitive retinal ganglion cells
LGN Lateral geniculate nucleus
LH Lateral hypothalamic area
LP Lateral posterior thalamic nucleus
MAPK Mitogen-activated protein kinase

MPT Medial pretectal nucleus
MRN Median raphe nucleus
NAAG *N*-acetylasparthylglutamate
Neu Neuron
NPY Neuropeptide Y
oc Optic chiasma
OPT Olivary pretectal nucleus
ONL Outer nuclear layer
OT Nucleus of the optic tract
PACAP Pituitary adenylate cyclase activating polypeptide
PAC1 PACAP receptor type 1
Per Period
PLi Posterior limitans thalamic nucleus
PPT Posterior pretectal nucleus
PRC Phase response curve
PRV-Bartha Pseudorabies virus of the Bartha strain
RGCs Retinal ganglion cells
RHT Retinohypothalamic tract
SC Superior colliculus
SCN Suprachiasmatic nucleus
SP Substance P
SPVZ Subparaventricular zone
τ TAU, endogenous period length
TIM Timeless
TRP Transient receptor potential
VGL Ventral geniculate nucleus
VIP Vasoactive intestinal peptide
VLPO Ventrolateral preoptic nucleus
VPAC1 VIP/PACAP receptor type 1
VPAC2 VIP/PACAP receptor type 2

1 Introduction

Rhythmic changes in physiology and behavior within a 24 h period occur in living organisms on earth to meet the challenges associated with the daily changes in the external environment. The circadian pacemaker responsible for the temporal internal organization and the generation of endogenous rhythms of approximately 24 h (24 h=circa one day, circa diem) is in mammals located in the hypothalamic suprachiasmatic nucleus (SCN; Reppert and Weaver 2001; Lowrey and Takahashi 2004). The endogenous period (τ) generated by the pacemaker is close to, but generally not equal to 24 h and the biological clock therefore needs to be daily adjusted (entrained) by external cues. The daily alteration of light and darkness due to the rotation of our planet on its axis in relation to the sun is the most prominent "zeitgeber" that adjusts the phase of the circadian rhythms to the astronomical day length, a process known as photoentrainment (Roenneberg and Foster 1997). In mammals, light is perceived only through photoreceptors located in the retina. Light information is directed to the SCN via the retinohypothalamic tract (RHT; Hannibal 2002a) by activation of the classical photoreceptor system of rods and cones and a more recently identified system of intrinsic photosensitive retinal ganglion cells (ipRGCs) using melanopsin as a photopigment (Berson 2003). Each of these systems is dispensable for light transmission and entrainment (Freedman et al. 1999; Panda et al. 2002; Ruby et al. 2002), but elimination of both rods and cones and melanopsin makes an animal visually and "circadian" blind (Jenkins et al. 2003; Jenkins et al. 2003; Hattar et al. 2003). The RHT also mediates information on seasonal changes in day length corresponding to the environmental photoperiod of summer and winter (Sumova et al. 2004; Oster et al. 2002). The SCN is also responsive to cues that are non-photic in nature such as arousal/locomotor activity, social cues, feeding, sleep deprivation, and temperature. The SCN shows distinct temporal responsiveness to photic and non-photic stimuli, which seem to be mutually antagonistic. For instance, in hamsters light attenuates the phase-shifting effects of non-photic stimuli and vice versa. The sum of stimuli interacts on phase resetting, leading to the overall stable entrainment of the clock (Challet and Pevet 2003).

The afferent pathways to the SCN mediating entrainment originates not only from the retina, but also from several areas in the brain including the limbic system, the hypothalamus, the raphe nuclei, the paraventricular thalamus, and the extraretinal subcortical visual system [e.g., intergeniculate leaflet (IGL) of the lateral geniculate nucleus and the pretectum; Moga and Moore 1997; Abrahamson and Moore 2001]. The neuronal pathways that participate in entrainment of the circadian timing system are well conserved among various mammalian species from rodents to man, even though some species variance can be found regarding neuronal phenotypes. Functional studies of entrainment are, however, restricted primarily to the three major input pathways to the SCN: the RHT; the geniculo-hypothalamic tract (GHT), which reaches the SCN from the IGL (Pickard 1985;

Moore 1995); and the median raphe projection (Fig. 1; Rea et al. 1994; Pickard et al. 1996, 1999; Meyer-Bernstein and Morin 1996; Meyer-Bernstein et al. 1997).

Light information reaches the SCN via the RHT and indirectly from the IGL via the GHT. The GHT also mediates, together with projections from the median raphe nucleus, non-photic information from the brain (Harrington 1997; Morin 1999; Rea and Pickard 2000). An important role of non-photic projections is to modulate the response of light stimulation by pre- and postsynaptic modulation of RHT input to the SCN (Morin 1999; Rea and Pickard 2000). The SCN is also influenced by humeral and metabolic inputs, but these regulatory systems will not be dealt with (Klein 2004; Challet et al. 2003).

In the present review we do not intend to give a comprehensive analysis of all input pathways to the SCN, but instead have focused on the more recent findings on the anatomical and functional significance of the three major pathways involved in entrainment of the circadian master clock—namely, the RHT, the GHT, and the midbrain raphe projection.

2
The "Master Clock": The Suprachiasmatic Nucleus

2.1
Neuroanatomy

The SCN, which in mammals consists of two paired nuclei, each containing 10–15,000 neurons, is located on either side of the third ventricle just above, and extending into the optic chiasm in the anterior hypothalamus. In a variety of mammals including rat, mouse, hamster, monkey, and human, the SCN can be separated into two distinct subdivisions designated "core" and "shell" (Moore et al. 2002). The term core refers to the ventral subdivision of the nucleus, always containing vasoactive intestinal polypeptide (VIP) and gastrin releasing peptide (GRP) (Fig. 2), but several other neurotransmitters can be identified in different species (Moore et al. 2002; Moore and Leak 2001).

The core receives direct input from the eyes via the RHT, secondary light input from the IGL via the GHT (Figs. 3, 4) and input from the median raphe nucleus (Figs. 3, 4). The SCN shell contains a large population of arginine-vasopressin (AVP)-containing neurons (Fig. 2) and this subdivision of the nucleus receives input from the limbic system, the hypothalamus, the paraventricular thalamic nucleus, and the medial raphe nucleus (Moga and Moore 1997; Abrahamson and Moore 2001; Moore et al. 2002; Moore and Leak 2001).

Neuronal phenotypes in the two distinct subdivisions of the SCN are well conserved among different species even though some variation exists (Moore et al. 2002; Moore and Leak 2001). The neurotransmitters of the major input pathways will be considered in detail below. The RHT contains the two neurotransmitters glutamate and the neuropeptide pituitary adenylate cyclase activating polypeptide

(PACAP; Hannibal 2002a; Fig. 2), the GHT, the inhibitory neurotransmitter gamma aminobutyric acid (GABA), and neuropeptide Y (NPY; Fig. 3; Harrington 1997) whereas the midbrain raphe projections contain the neurotransmitter serotonin (5-HT) and yet unidentified neurotransmitter(s) (Morin 1999; Fig. 4).

Receptors/receptor subtypes corresponding to the neurotransmitters of the input pathways are all found in the SCN (see Sects. 3.2.3, 4.1.2).

A complex cytoarchitecture exists within the paired SCN, with major projections mainly from the core to the shell and to the contralateral nucleus and only minor reverse projections from the shell to the core region (Moore et al. 2002; Moore and Leak 2001). Efferent projections from the SCN originate from both the core and the shell and reach many nuclei in the forebrain (Leak and Moore 2001; Abrahamson et al. 2001; Abrahamson and Moore 2001; Kriegsfeld et al. 2004; see also Moore et al. 2002 and Moore and Leak 2001).

2.2 The Endogenous Rhythm of the SCN

A fundamental property of the circadian clock is the ability to generate and sustain an endogenous rhythm of approximately 24 h. The endogenous rhythm of the SCN can be characterized at various levels such as the behavioral level, the cellular level, and the molecular level. At the behavioral level the classical measurement method used is of the running-wheel activity of an animal (usually hamster or mouse) collected over a 24 h period on consecutive days. Under the normal light/dark condition of 12 h light and 12 h darkness (12:12 LD) a nocturnal animal will entrain to this light paradigm and start running soon after darkness and continue at various times during the dark period and keep at rest for most of the light period. When external "zeitgebers" such as light are removed, then animals will "free-run" with a phase angle determined by the length of the endogenous period τ. When τ is shorter than 24 h, the phase of the circadian rhythm will gradually advance by a period length corresponding to the derivation of the τ from 24 h (Daan and Pittendrigh 1976a). When τ is longer than 24 h, the phase will gradually delay from the initial zeitgeber time. The physiological properties of the entrainment system is the ability of daily adjustment of the derivation of the endogenous period length to the astronomical 24 h day length (Daan and Pittendrigh 1976a). The τ is a unique property of the circadian pacemaker which is determined by genetic factors (see Sect. 2.3) and by the previously experienced light/dark conditions. Furthermore, τ varies within individuals and among species (Pittendrigh and Daan 1976; Summers et al. 1984; Wright, Jr. et al. 2001; see also Sect. 3.4.1). Fast pacemakers (short τ) are found in the mouse and slow pacemakers (long τ) are found in the rat and hamster (Daan and Pittendrigh 1976a).

At the cellular level, the neuronal population of the SCN exhibits oscillations in firing rate, with a τ close to 24 h using an "SCN island" preparation in which the SCN of rats were isolated by a knife cut. Peak activity was registered during the day and a trough at night (Inouye and Kawamura 1979). The SCN's circadian rhythm could

persist for about 35 d after isolation, whereas circadian rhythmicity in brain regions outside the island was lost (Inouye and Kawamura 1979). In an isolated coronal or horizontally sectioned brain slice containing the SCN, electrical firing rhythm was found to persist for two to three cycles (Green and Gillette 1982; Groos and Hendriks 1982; Gillette 1986). The electrical firing rhythm can persist in dissociated SCN cultured neurons for more than 30 d (Welsh et al. 1995). The circadian rhythm of electrical firing is not spontaneously synchronized in cell cultures (Welsh et al. 1995) but can be synchronized after treatment with GABA, an endogenous ligand found in almost all SCN neurons (Liu and Reppert 2000). Recently, individual cellular clocks of SCN organotypic cell cultures were shown to be synchronized by protein synthesis inhibitor cyclohexamide (CHX) (Yamaguchi et al. 2003). In this transgene model, the circadian "clock gene" (see Sect. 2.3) *mPer1* expressed in SCN cells is visualized by a *mper1*-promotor-driven luciferase reporter gene (*mper1-luc*). Due to a half life of the reporter luciferase of approximately one hour, the system gives adequate temporal resolution, and analysis shows a circadian rhythm of individual cells with a τ close to 24 h (Yamaguchi et al. 2003). It is generally accepted that the circadian rhythm of neuronal firing rhythm in the SCN is endogenous and that this rhythm is not driving the biological clock but rather is an expression of the clock. The mechanism behind the generation of the electrical firing rhythm and the synchronization of SCN clock cells are not fully understood, but both synaptic and non-synaptic transmission between the clock cells seems to be involved (Liu and Reppert 2000; Long et al. 2005; Colwell 2005).

2.3 The SCN Molecular Clock

Within the last ten years identification of the molecular components that constitute the circadian clock have increased the understanding of the molecular machinery behind the function of biological clocks including the mammalian clocks (Reppert and Weaver 2001; Okamura et al. 2002; Lowrey and Takahashi 2004; Hirota and Fukada 2004). In brief, the circadian clock is driven by a group of "clock genes", which in complex auto-regulatory self-sustained feedback loops control their own transcription/translation (Fig. 5).

A negative loop encompasses the rhythmic transcription of three mammalian orthologs of the Drosophila *Period* (i.e., *Per1, Per2, Per3*), two cryptochromes (i.e., *Cry1* and *Cry2*), the mammalian ortholog of the Drosophila TIMELESS (TIM), and the two transcription factors DEC1 and DEC2. The rhythmic expression of these genes is driven by a complex of BMAL1/CLOCK heterodimers that bind to so-called E-box elements in the promotor region of these genes (Fig. 5). After transcription/translation, the respective proteins (CRY1 and CRY2 and PER) translocate to the nucleus, where they inhibit the BMAL1/CLOCK complex thereby blocking their own transcription (Fig. 5, *red arrows*). Regulated degradation of PER and CRY proteins caused by phosphorylation by casein kinase ε (CKIε) leads to a restart of the cycle (Fig. 5, *light blue arrows*). A positive loop driven by tran-

scription of the positive regulator gene *Bmal1* is repressed by the orphan nuclear receptor REV-ERB, whose mRNA is activated by CLOCK-BMAL1 (Fig. 5, *green arrows*). Circadian oscillation of *Bmal1* peaks at subjective night and is in antiphase to *Per* expression, which peaks at subjective day (Fig. 5B). These two loops of negative and positive regulators are tightly coupled and constitute the core clock mechanism. Secondary loops driven by DBP and E4BP4 transcription factors regulate *Per1* gene expression (Fig. 5, *dark blue arrows*; Reppert and Weaver 2001; Okamura et al. 2002; Lowrey and Takahashi 2004; Hirota and Fukada 2004). The molecular clock cycles with a period length (τ) close to but often not exactly 24 h. There is evidence that photic as well as non-photic entrainment of the molecular clock is accomplished via regulation of the two light sensitive *Per* genes and *Bmal1*. So far, it has been shown that both the *Per1* and *Per2* and *Bmal1* are regulated by light (Reppert and Weaver 2001; Okamura et al. 2002; Lowrey and Takahashi 2004; Hirota and Fukada 2004) (Fig. 6; see also Sect. 3.4.5). *Per1* is also directly regulated by non-photic stimuli (see also Sect. 4.3). It seems, however, that each molecular element of the clock plays separate roles for the functional clock. This conclusion comes from studies in mice lacking one or more clock genes. Using running-wheel activity as parameter for circadian clock function, lack of different clock genes was found to alter or disrupt the endogenous rhythm. CLOCK mutant mice have a much prolonged τ compared to wild type animals and when exposed to constant darkness, these animals become arrhythmic (Antoch et al. 1997; King et al. 1997). Elimination of other clock genes has shown that mice lacking *Per1* and *Per2* have a significant shorter τ (Zheng et al. 2001). Furthermore, as observed for the CLOCK mutant mice, the *Per2* knock-out mice lose their clock-driven rhythms within a few days in constant darkness (Zheng et al. 1999; Albrecht et al. 2001; Antoch et al. 1997; King et al. 1997). Mice lacking *Cry1* have short τ (Vitaterna et al. 1999) whereas mice lacking *Cry2* have long τ (Thresher et al. 1998).

Naturally occurring mutation in the circadian system was described more than 15 years ago in the Syrian hamster. The circadian phenotype of this animal is characterized by a very short τ of only 20 h when present in homozygous form (Ralph and Menaker 1988). By the comparative genomics approach it was shown that this hamster has a single gene mutation in a gene encoding CKIε (Lowrey et al. 2000). In humans, a complementary phenotype to the tau hamster is found in a hereditary form of familial advanced sleep phase syndrome (FASPS) characterized by a nearly 4 h shorted endogenous period caused by a mutation in the CKIε binding region of *hPer2*. Affected individuals have a serine to glycine mutation within this region that causes hypophosphorylation by CKIε in vitro (Toh et al. 2001).

Studies on photic and non-photic entrainment that links anatomy, physiology, and molecular regulation of central core clock elements will be dealt with below.

3
Photic Regulation of the Circadian Rhythm

3.1
Retinal Input—Neuroanatomy

3.1.1
The Retinohypothalamic Tract

Three decades ago a novel retinofugal projection to the hypothalamus was demonstrated to innervate the SCN. Visualization of this pathway, named the retinohypothalamic tract (RHT) was performed by using injection of tritiated leucine or proline into the posterior chamber of the eye followed by autoradiographic visualization of the tracer in the SCN (Moore and Lenn 1972; Hendrickson et al. 1972). New and more sensitive anterograde and retrograde tracers have shown, due to higher resolution, that this projection also innervates other hypothalamic regions including the paraventricular hypothalamus, subparaventricular region, ventrolateral preoptic area and lateral hypothalamus (Johnson et al. 1988). Retinal projections also considered to be part of the RHT were shown to innervate the IGL, several pretectal nuclei, the superior colliculus and together, these projections constitute a non-imaging photoreceptive pathway to the brain (Hannibal and Fahrenkrug 2004a; Morin et al. 2003; Muscat et al. 2003; Gooley et al. 2003) (Fig. 7).The IGL, which participates in the control of circadian rhythmicity by integrating photic and non-photic cues (Harrington and Rusak 1986; Pickard et al. 1987; Johnson et al. 1989), will be dealt with below.

The RHT has been identified in all mammalian species examined including rat, hamster, mouse, ferret, sheep, monkey, and the blind mole rat by using the anterograde-tracing cholera toxin subunit B (CTb) injected into the eye (Pickard and Silverman 1981; Johnson et al. 1988; Levine et al. 1991; Murakami et al. 1989; Murakami and Fuller 1990; Mikkelsen 1992a; Tessonneaud et al. 1994; Hannibal et al. 1997, 2001a; Hannibal and Fahrenkrug 2004a). In all species the RHT bilaterally innervates the SCN, with a slight contralateral dominance in some species such as the rat, whereas in the hamster, mouse, and blind mole rat the contralateral and ipsilateral projections are approximately equal (Johnson et al. 1988; Levine et al. 1991; Mikkelsen 1992a; Abrahamson and Moore 2001; Hannibal and Fahrenkrug 2004a). The terminal field in the SCN of crossed and uncrossed projections from each retina has recently been analyzed in detail in the hamster using an elegant approach of coupling two fluorephores to the anterograde tracer CTb simultaneously injected into each eye. It was found that retinal projections from the eyes completely overlapped in the SCN, but within distinct subregions, input from one side predominated (Muscat et al. 2003). The functional implication of this finding remains to be clarified. In contrast, retinal projections to other hypothalamic regions, to the IGL and pretectum, have been found to be predominantly contralateral (Muscat et al. 2003; Hannibal and Fahrenkrug 2004a). Ganglion cells constituting the RHT contain the neurotransmitter PACAP together with a novel photopigment,

named melanopsin (see Sect. 3.3; Hannibal et al. 2002a; Hannibal and Fahrenkrug 2004a; Fig. 8). By using a PACAP immunostain as a marker for RHT projections in combination with CTb injected into the eyes, PACAP containing retinal projections to the SCN and other brain areas have been demonstrated in the rat (Hannibal et al. 1997, 2001a; Hannibal and Fahrenkrug 2004a), hamster (Bergström et al. 2003), mouse (Fig. 9), and, furthermore, PACAP-immunoreactive retinal ganglion cells (RGCs) and PACAP-immunoreactive nerve fibers most likely originating from the RGCs have been demonstrated in the SCN of the blind mole rat (Hannibal et al. 2002b) and in man (Hannibal et al. 2004).

Retrograde tracing from retinal target areas in the brain using CTb, FluoroGold and transsynaptic retrograde tracing with the pseudorabies virus of the Bartha strain (PRV Bartha) has extended these observations. Thus, it was shown that the SCN-projecting RGCs bifurcate and innervate both SCNs, the IGL, and parts of the pretectum including the olivary pretectal nucleus. It is likely that the RGCs play a broad role in the regulation of non-visual photoreception (Gooley et al. 2003; Morin et al. 2003; Pickard 1985).

3.1.2 Morphology of Retinal Ganglion Cells of the RHT

The RGCs of the RHT projecting to the SCN and other non-visual target areas in the brain are intrinsically photosensitive (ipRGCs; Berson et al. 2002; Warren et al. 2003) due to the expression of melanopsin (Hattar et al. 2002; Lucas et al. 2003; Panda et al. 2005; Qiu et al. 2005; Melyan et al. 2005; see Sect. 3.3). The ipRGCs, which resemble ganglion cells known as type III or W cells according to the classification by Perry (1979), are mainly located in the ganglion cell layer but a few are displaced to the inner nuclear layer (Fig. 8). In the rat, hamster, and mouse the RGCs of the RHT account for 1–2% of the total number of RGCs (Pickard 1980; 1982; Pickard and Silverman 1981; Murakami et al. 1989; Moore et al. 1995; Hattar et al. 2002; Morin et al. 2003; Sollars et al. 2003), in monkey 0.2% (Dacey et al. 2005), while in human it is approximately 0.8% (Hannibal et al. 2004). The ipRGCs are widely distributed in the retina with the majority of these cells in the superior half in rat, but not in other rodents (Hannibal et al. 2002a; Hattar et al. 2002; Sollars et al. 2003). The soma diameter is approximately 15 μm and 2–4 branching dendrites which are up to 500–800 μm long (Hannibal et al. 2002a; Hattar et al. 2002; Provencio et al. 2002; Belenky et al. 2003; Sollars et al. 2003; Warren et al. 2003; Hannibal et al. 2004) form an overlapping plexus in the outermost sublayer of the inner plexiform layer (IPL; Figs. 8 and 10). Functional studies have provided evidence (see Sect. 3.3) that the melanopsin-containing RGCs receive input from the rods and cones (Berson et al. 2002). In accordance, ultrastructural immunohistochemical studies have shown that the melanopsin-containing RGCs are innervated by bipolar and amacrine cells (Belenky et al. 2003).

3.2 Neurotransmitters in the RHT

3.2.1 Glutamate and PACAP

The excitatory amino acid glutamate is considered to be the primary neurotransmitter in the RHT based on anatomical and functional studies (for review see Ebling 1996). Glutamate immunoreactivity occurs in nerve terminals in the SCN. At the ultrastructural level glutamate immunoreactivity is found within pre-synaptic nerve terminals in the SCN of the rat and mice (van den Pol and Tsujimoto 1985; van den Pol 1991; Castel et al. 1993; Hannibal et al. 2000) in a significantly higher number of retinal nerve fiber terminals compared to intra-SCN terminals (De Vries et al. 1993). Glutamate has been shown to be co-stored with PACAP in RGCs and their terminals (Fig. 11) (Hannibal et al. 2000). PACAP is a neuropeptide of the vasoactive intestinal polypeptide (VIP)/secretin family of regulatory peptides widely distributed in the central (Hannibal 2002b) and peripheral nervous systems (for review see Vaudry et al. 2000; Arimura 1998). PACAP is found in two biologically active forms, the dominant form in tissue being PACAP38 (Hannibal et al. 1995; Fahrenkrug and Hannibal 1996, 1998, 2000; Hannibal et al. 1998). The existence of PACAP in the RHT was discovered in 1997 (reviewed by Hannibal 2002a) and recent studies demonstrate that PACAP is found in the RHT in several mammalian species including rat, mouse, hamster, blind mole rat, rabbit, cow, pig, sheep, monkey, and man (Figs. 8, 12; Bergström et al. 2003; Hannibal et al. 2001b, 2002b, 2004, and Hannibal unpublished). A question remaining to be fully addressed is whether PACAP is found in all RGCs projecting to the SCN. Tracing studies using a combination of anterograde (CTb) and retrograde (PRV-Bartha) tracing have shown that the major part (>90–95%) of RHT fibers projecting to the SCN store PACAP (Hannibal et al. 2001a; Hannibal and Fahrenkrug 2004a). All PACAP-containing RGCs express melanopsin and vice versa (see Sect. 3.3; Fig. 8; Hannibal et al. 2002a; 2004; Bergström et al. 2003; Hannibal and Fahrenkrug 2004b). However, anterograde tracing from the eye has disclosed that not all PACAP immunoreactive fibers in the SCN co-store CTb (Hannibal and Fahrenkrug 2004a). This observation raises the possibility that a non-PACAP/non-melanopsin-containing part of the RHT exists (Hannibal and Fahrenkrug 2004b). Studies in rodents using different approaches of tracer application into the SCN [direct injection (Gooley et al. 2003; Morin et al. 2003), and transsynaptic spread of virus via autonomic circuits from the brain to the SCN and contralateral retina (Sollars et al. 2003)] raise the possibility that a retinal projection to the SCN originates from ganglion cells lacking melanopsin (and PACAP). In the hamster, Sollars et al. used the PRV-Bartha virus modified to express enhanced green fluorescent protein (EGFP; Smith et al. 2000), which via autonomic circuits the Edinger-Westphal nuclei and the SCN infect a subset of SCN-projecting RGCs. Immunohistochemical visualization of melanopsin and EGFP showed that 10–20% of the EGFP labeled RGCs projecting to the SCN did not contain melanopsin (Sollars et al. 2003). Gooley et al. used a combination of

retrograde tracing from the brain in combination with melanopsin mRNA analysis in the retina and found that approximately 70% of the RGCs projecting to the SCN contain melanopsin (Gooley et al. 2003). Morin et al. found by injecting retrograde tracer into the SCN that only about 70–80% of the RGCs projecting to the SCN expressed melanopsin (Morin et al. 2003). The number of melanopsin-expressing RGCs that project to the SCN may be underestimated due to the tracing methods used. The findings suggest, nonetheless, the existence of a non-melanopsin-containing projection of the RHT. The functional implication for the existence of a non-melanopsin/non-PACAP-containing RHT projection will be considered below.

3.2.2 Other Neurotransmitters of the RHT

It has been suggested that apart from glutamate and PACAP, a number of closely related molecules and derivates may also function as neurotransmitters of the RHT. Thus, L-aspartate (Liou et al. 1986; Csaki et al. 2000; De Vries and Lakke 1995) and *N*-acetylasparthylglutamate (NAAG) have been reported to occur in retinal fibers projecting to the SCN (Moffett et al. 1990). Substance P (SP) has also been suggested as a neurotransmitter in the RHT (Takatsuji et al. 1991a; Mikkelsen and Larsen 1993) but a recent study in rat seems to exclude this suggestion (Hannibal and Fahrenkrug 2002).

3.2.3 RHT Neurotransmitter Receptors in the SCN

3.2.3.1 Glutamate Receptors

Both ionotropic (NMDA and AMPA/kainate) and metabotropic receptors are present in the SCN identified by radio-ligand binding and in situ hybridization histochemistry and immunohistochemistry (for review see Ebling 1996). The NMDAR1C subtypes are expressed in the entire SCN and the NMDAR2C is present in the dorso-medial SCN (Ebling 1996). Also mRNA encoding the AMPA (GluR1, GluR2, and GluR4) and kainate receptors (GluR6 and GluR7) as well as the metabotropic receptors (mGluR1 and mGluR5) have been demonstrated within the SCN (Ebling 1996).

3.2.3.2 PACAP Receptors

PACAP exerts it function via two classes of G-protein coupled receptors. (1) The PAC1 receptor is PACAP specific and coupled to adenylate cyclase and phospholipase C. (2) The VPAC1 and VPAC2 receptors bind PACAP and VIP with equal affinity and are coupled mainly to adenylate cyclase (Harmar et al. 1998). The PAC1

and VPAC2, but not the VPAC1, receptors have been demonstrated in the SCN. The VPAC2 receptor has a relatively restricted distribution within the CNS (Usdin et al. 1994; Vaudry et al. 2000; Sheward et al. 1995) and the highest expression is found in the SCN (Lutz et al. 1993; Sheward et al. 1995; Cagampang et al. 1998a; Kalamatianos et al. 2004). Double-labeled in situ hybridization histochemistry has revealed that the SCN neurons expressing VPAC2 mRNA are located mainly in AVP cells of the shell region and to a minor extent in the VIP cells of the core region (Kalamatianos et al. 2004). The PAC1 receptor is widely distributed in the brain and spinal cord (Vaudry et al. 2000; Shioda et al. 1997; Hashimoto et al. 1996). The PAC1 mRNA visualized by in situ hybridization is found in both shell and core region (Hannibal et al. 1997; Cagampang et al. 1998a, 1998b; Lutz et al. 1993; Sheward et al. 1995; Kalamatianos et al. 2004). Both the PAC1 and the VPAC2 receptor mRNA show circadian expression in the SCN with peak expression during subjective day and mid subjective night (Cagampang et al. 1998b) and mid to late subjective day and late subjective night (Cagampang et al. 1998a), respectively. Aging seems to influence the diurnal rhythm and the expression level of the VPAC2 receptor but not the PAC1 receptor in the SCN (Kallo et al. 2004).

3.3 Melanopsin—An Irradiance-Detecting Photopigment of the RHT

From the beginning of the early 1990s, the classical view of rods and cones as the only photoreceptors in the mammalian eye responsible for light perception to the brain began to change due to observations in some blind persons (Czeisler et al. 1995; Klerman et al. 2002; Lockley et al. 1997) and in mice lacking the rods (*rd/rd* mice; Foster et al. 1991) or both rods and cones (*rd/rd/cl* mice; Freedman et al. 1999; Lucas et al. 1999). Humans and mice are visually blind due to severe degeneration of the outer retina but have retained the ability to entrain to the light/dark cycle suggesting the existence of a photopigment located in the inner retina (Foster 2002). By the identification of melanopsin, an opsin-like molecule located in a subset of retinal ganglion cells in the mammalian retina (Provencio et al. 1998, 2000), a likely "circadian" photopigment was subsequently identified. The remarkable findings by Berson and colleagues (Berson et al. 2002) that the RGCs projecting to the SCN are intrinsically photosensitive (ipRGCs; see Sect. 3.4.2) even after anatomical and pharmacological isolation, and the demonstration that these ipRGCs contain melanopsin (Hattar et al. 2002; Warren et al. 2003), strongly indicate that melanopsin could be the predicted circadian photopigment. Within the last few years melanopsin has been shown to fulfill criteria for being the circadian photopigment. (1) Melanopsin is expressed in ipRGCs that project to the SCN and non-visual photoreceptive areas in the brain (Hannibal et al. 2002a, 2004; Hattar et al. 2002; Provencio et al. 2002; Belenky et al. 2003; Sollars et al. 2003; Morin et al. 2003; Gooley et al. 2001, 2003; Lucas et al. 2003; Bergström et al. 2003). (2) The ipRGCs are sensitive to light with wavelengths that correspond well with light-causing behavioral phase shifts (Berson et al. 2002; Dacey et al. 2005).

(3) Melanopsin expressing RGCs from melanopsin-null mutant mice are no longer intrinsically photosensitive (Lucas et al. 2003). (4) Melanopsin knockout mice have significantly attenuated response to light (e.g., light-induced phase shift, negative masking behavior, attenuated pupillary light reflex; Panda et al. 2002; Ruby et al. 2002). (5) Transfection of mouse melanopsin into *Xenopus* oocytes (Panda et al. 2005) or mammalian HEK293 cells (Qiu et al. 2005) makes these cell intrinsically light sensitive with an absorption spectrum similar to that of ipRGCs. Obviously, melanopsin plays an important role in irradiance detection and transmission to the brain including the circadian timing system. The observation that melanopsin-null mutant mice can still entrain to light suggests, however, the involvement of another light detection system as well. It has been suggested that the melanopsin system primarily plays a role in bright light detection whereas in light at lower intensities the rod–cone system prevails (Lucas et al. 2003; Mrosovsky and Hattar 2003; Dacey et al. 2005). There is now evidence that these two systems cooperate. Animals lacking both melanopsin and the rod–cone system have no pupillary reflex and they are unable to entrain to light/dark cycles, and show no masking behavior in response to light stimulation (Hattar et al. 2003; Panda et al. 2003). Thus, the rod–cone and melanopsin systems together seem to be responsible for all of the photic input involved in these accessory visual functions (Hattar et al. 2003; Panda et al. 2003).

3.4 Retinal Input—Physiology

3.4.1 Photic Regulation of the Circadian Rhythm

Photic stimulation of the circadian timing system has been studied most extensively in nocturnal (night active) animals and only to a minor extent in diurnal (day active) or crepuscular (twilight active) animals (Smale et al. 2003). Even though a general scheme can be drawn from these studies of nocturnal animals it is important to notice that some fundamental mechanisms may differ considerably with respect to the pattern of response when exposed to light or housed in constant light or darkness. One reason may be that light sensitive "settings" are different for diurnal animals which in their natural habits are exposed to higher light intensities than nocturnal animals. When nocturnal animals are exposed to constant light the endogenous period (τ) is lengthened (Aschoff 's rule; Aschoff 1979). In the diurnal *Octodon degus* much brighter light is needed to cause a similar τ change (Lee and Labyak 1997).

A fundamental property of the circadian timing system is the clock-dependent change in responsiveness to light over the circadian period. The phase response obtained after light pulse stimulation at various time points of the 24 h day/night cycle is called the phase response curve (PRC) to light (Daan and Pittendrigh 1976a; Fig. 13).

The PRC is generated by measuring the size of the phase shift of (running wheel) activity or temperature in response to a light pulse given at various time points during a 24 h period of subjective day and night when animals are kept in constant darkness. During such condition, nocturnal animals respond to light stimulation by a phase delay during the early subjective night. Light stimulation during late subjective night, on the other hand, cause a phase advance of the circadian rhythm whereas light during subjective day has little effect on the circadian phase (Daan and Pittendrigh 1976a; Fig. 13B). Similar responses to light are observed in diurnal animals and in humans but light seems also to affect the phase of the circadian rhythm in subjective day (Kas and Edgar 2000; Jewett et al. 1997; Daan 2000). The PRC in nocturnal rodents varies with species and seems to be a function of τ. Mice usually have a short τ (<24 h) and therefore need to adjust this advance daily. The mouse PRC is thus characterized by large phase delays in the early subjective night and a small phase advance during late subjective night. Hamsters usually have a long τ (>24 h) and their ability to entrain to light is ensured by a PRC characterized by a small phase delay in early subjective night and a larger phase advance during the transition between night and day (Daan and Pittendrigh 1976a). The human circadian pacemaker oscillates with an average intrinsic period of 24.18 h (Czeisler et al. 1999) and responds similarly to light as rodents, although humans do not seem to have a complete "dead zone" during subjective day (Khalsa et al. 2003; Cajochen et al. 2000). The magnitude of the phase shift is determined by the duration of the light pulse (Daan and Pittendrigh 1976b), light intensity (Takahashi et al. 1984), and the wavelength of the light (Takahashi et al. 1984; Meijer 2001). Also the time in darkness influences the magnitude of the phase shift. The dark adaptation that results in a larger phase shift with the extension of time in darkness is a process much slower than visual dark adaptation (in rodents up to three weeks vs. less that one hour; Refinetti 2001, 2003; Shimomura and Menaker 1994). In rodents, the light-sensitive photopigment responsible for light adjustment has a spectral sensitivity maximum near 500 nm (Takahashi et al. 1984; Nelson and Takahashi 1991). In humans, using light suppression of melatonin as an index, light between 460 and 477 nm seems to be most effective (Brainard et al. 2001; Thapan et al. 2001). The absorption maximum for rod photopigment is ~500 nm (Takahashi et al. 1984; Bridges 1959), for green cone opsin ~510 nm (Jacobs et al. 1991), and for melanopsin 480 nm (Panda et al. 2005; Berson et al. 2002). Two features characterize the photoreceptive system that mediates entrainment: (1) the threshold of the response is high, and (2) the reciprocal relationship between intensity and duration holds for extremely long durations (up to 45 min). These results are consistent with the observation that the photoreceptive system mediating entrainment is markedly different from that involved in visual image formation (reviewed in Foster and Hankins 2002).

3.4.2
Putative Neurotransmitters of the RHT

Glutamate is considered the principal neurotransmitter mediating phase shifts of the endogenous rhythm. Injection of the glutamate agonist NMDA into or adjacent to the SCN in vivo induces phase shifts in running-wheel activity similar to the changes observed after light stimulation (Mintz and Albers 1997; Mintz et al. 1999). Studies using coronal or horizontal section of the hypothalamus (Gillette 1986) in which the SCN neurons maintain the temporal organization of electrical firing rhythms (Gillette 1991; see also Sect. 2.2) and gene expression (Nielsen et al. 2001, 2002) for three days, also support a role for glutamate. Thus, application of NMDA in vitro phase shifts the electrical activity rhythm with a pattern similar to the phase shift induced by light in vivo (Ding et al. 1994; Shibata et al. 1994). Furthermore, 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, see also Ebling 1996). PACAP, which is stored together with glutamate in the same nerve terminals in the retinorecipient SCN (Fig. 11; Hannibal et al. 2000), also causes phase shift of the circadian rhythm of running-wheel activity similar to light when injected in vivo (intracerebroventricular or intra SCN; Harrington et al. 1999; Piggins et al. 2001; Bergström et al. 2003) and in the electrical firing activity when added in vitro (Fig. 14; Harrington et al. 1999). The effect of PACAP in vitro is dose dependent, showing a maximal effect on the phase in nanomolar concentration, whereas at micromolar concentration the peptide has no effect on the phase during subjective night either in vivo (Harrington et al. 1999) or in vitro (Hannibal et al. 1997). Application of micromolar concentration of PACAP in vitro, however, modulates glutamate-induced phase shifts in both early and late subjective night (Chen et al. 1999; Fig. 15, see also Sect. 3.4.6). A role for PACAP in light signaling to the clock comes from studies of mice lacking the PAC1 receptor (Hannibal et al. 2001b) or PACAP (Kawaguchi et al. 2003; Colwell et al. 2004).

Both animal models show various degrees of impaired responses to light stimulation at night. PACAP can regulate the response of SCN neurons to glutamatergic stimulation (see Sect. 3.4.6); loss of PACAP or PAC1 receptor might change the sensitivity of the circadian system to photic stimulation mediated by glutamate released from the RHT.

3.4.3
Photic Activation of the RGCs of the RHT

It has been known for more than a decade that retinal illumination stimulates retinal cells including ganglion cells despite lack of the classical photoreceptors, the rods and cones (reviewed in Foster 2002). In rod-and-coneless mice light induces the immediate early gene *c-fos* in a subpopulation of retinal ganglion cells, and it has been hypothesized that these light responsive RGCs are part of the RHT (Masana et al. 1996). Thus, it has been shown in rats that RGCs of the RHT containing PACAP express *c-fos* when exposed to light (Fig. 9; Hannibal et al. 2001a). Interestingly,

in contrast to other retinal cells in which Fos immunoreactivity disappears within 2–3 h, the PACAP-containing RGCs continue to express Fos immunoreactivity for as long as light is on. These observations could indicate that the RGCs respond to light stimulation with a sustained ON response corresponding to the light stimulus (Hannibal et al. 2001a). That this seems to be the case is proven by the elegant observation of Berson et al. showing that RCGs of the RHT are intrinsically light sensitive and respond to light stimulation with a sustained ON response in contrast to the hyperpolarization found in ganglion cells connected to the classical photoreceptors (Berson et al. 2002). RGCs of the rat projecting into the RHT were identified by retrograde labeling from the SCN with rhodamine beads, and their neuronal activity was analyzed using a whole-cell current clamp on isolated flat mounts. By intracellular recordings it was shown that light tonically depolarizes the RCGs innervating the SCN. This response persists as long as light is turned on even if synaptic inputs from rods and cones are chemically blocked or the cells are microsurgically isolated (Berson et al. 2002). It was also shown that the sensitivity, spectral tuning, and slow kinetics of the light response matched those of the photic entrainment system, giving further evidence that these ganglion cells are the primary photoreceptor-bearing cells for this system (Berson et al. 2002; Warren et al. 2003).

The photopigment responsible for the intrinsic photosensitivity is most likely melanopsin, since RGCs of melanopsin-null mutants have lost intrinsic photosensitivity (Lucas et al. 2003). In addition, studies in which murine or human melanopsin are transfected into *Xenopus* oocytes (Panda et al. 2005), HEK293 cells (Qiu et al. 2005) or Neuro-2a cells (Melyan et al. 2005) have demonstrated that melanopsin is a genuine photopigment with functional characteristics corresponding to that of the ipRGCs. Furthermore, melanopsin seems to function more like an invertebrate opsin (Hardie and Raghu 2001) by having photoisomerase activity and by signaling via Gq/G11 and the transient receptor potential (TRP) subfamily C (TRPC) cation channel (Panda et al. 2005; Qiu et al. 2005). Functional studies have shown that the intrinsically photosensitive RGCs (ipRGCs) receive input from rods and cones (Berson 2003; Dacey et al. 2005). This response is in accord with the neuroanatomical demonstration of synapses between amacrine and bipolar cells and the melanopsin-containing RGCs (Belenky et al. 2003).

The functional significance of the melanopsin-containing RGCs has been studied in newborn rats. Photoentrainment is established in early postnatal life (Munoz et al. 2000; Leard et al. 1994; Weaver and Reppert 1995) before a functional outer retinal segment is developed (Ratto et al. 1991). Melanopsin was found to appear in RGCs before birth in rodents (Fahrenkrug et al. 2004; Tarttelin et al. 2003) and showed an adult pattern of expression from the tenth days after birth (Fahrenkrug et al. 2004). From the day of birth melanopsin-containing RGCs are light responsive, as evidenced by the induction of Fos. At this time the RHT is also functional, mediating light-induced Fos expression in the SCN (Hannibal and Fahrenkrug 2004b).

3.4.4
Electrophysiological Responses of SCN Neurons to Photic Stimulation

The electrophysiological response of SCN neurons to light has been studied, mainly in nocturnal animals, to a minor extent in diurnal animals. In both groups light changed the membrane potential and discharge rate in neurons located mainly in retino-recipient areas. The total portion of light-responsive neurons seems much lower in diurnal than in nocturnal animals and the threshold intensities required to stimulate light-responsive neurons are several hundred lux higher in diurnal animals (Meijer and Schwartz 2003). The light sensitive or illuminance-coding SCN neurons differ in their response from light-sensitive cells in visual brain areas by having (1) a sustained response as long as the illumination changes are maintained, and (2) a sustained firing dependent on the level of light intensity (Groos and Mason 1978; Meijer et al. 1986; Meijer 2001; Aggelopoulos and Meissl 2000). In nocturnal animals retinal illumination increases firing rate in a large majority of light-sensitive neurons (up to 70%) and decreases firing rate in a minority of such neurons. In diurnal animals these proportions are altered or reversed (Jiao et al. 1999; Jiao and Rusak 2003). Both light-activated and light-suppressed neurons code for light intensities within a small range ideal for discrimination around dusk and dawn. In rat and hamster the thresholds are of 0.1 and 1 lux, respectively, which are much higher than for vision (Meijer et al. 1986; Aggelopoulos and Meissl 2000). The light sensitivity of SCN neurons is higher during the night than during the day (Meijer et al. 1996; Nakamura et al. 2004; Meijer et al. 1996, 1997). The mechanisms regulating these different responses in the light-sensitive SCN are not fully understood. To be most sensitive for a given change in light intensity above or below background illumination (without being affected by the sudden change in illumination), the "light coding" system must be able to smoothen the local fluctuations in order to obtain reliable measures of light levels —and hence time of day. One way to achieve this would be to use a long sampling or integration time to gather photons. This sampling may occur both in the retina and in the SCN (Roenneberg and Foster 1997; Meijer 2001). The neurotransmitters of the RHT, glutamate and PACAP, are believed to be released by light and after electrical stimulation of the optic nerve (Liou et al. 1986; Burgoon and Gillette 2000; Shibata et al. 1984, 1986; De Vries et al. 1994; Jiao and Rusak 2003; Burgoon and Gillette 2000) and their effects on the clock are temporarily restricted (see Sect. 3.4.6). The excitatory signaling is mediated via glutamate and NMDA receptors whereas the inhibitory signaling within the SCN after in vitro stimulation of the optic nerve seems to be regulated by GABA (Jiao and Rusak 2003; see also Sect. 3.4.5). It is possible that PACAP, a modulator of glutamate signaling in the SCN, may be involved in the regulation of light-induced suppression of SCN neurons (see also Sect. 3.4.6).

3.4.5
Signal Transduction and Gene Expression in SCN Neurons After Photic Stimulation

Light-induced resetting of the circadian clock is dependent on transcriptional activation and gene expression. Activation of these signaling pathways is temporally restricted to distinct time domains occurring during the circadian day and night (Gillette and Mitchell 2002). When initiated, phase shifts occur within few hours after light onset (Best et al. 1999; Watanabe et al. 2001; Asai et al. 2001) and is dependent on protein synthesis (Watanabe et al. 2001).

The signaling pathways involved in light-induced phase shifts (delays or advances) are initiated by the release of glutamate from RHT terminals, leading to activation of NMDA receptors, to be followed by depolarization and Ca^{2+} influx (Ding et al. 1994; Mintz et al. 1999; Moriya et al. 2000; see also review by Ebling 1996). Depolarization is an important determinant for Ca^{2+} influx because at resting potential, the channel is blocked by extracellular Mg^{2+}. Upon depolarization, the Mg^{2+} block is removed by a process which seems to involve the AMPA receptor, and Ca^{2+} influx through the NMDA receptor can occur. The signaling transduction cascade involves the production of nitric oxide (NO; Ding et al. 1994; Mintz et al. 1999; Mintz and Albers 1997; Rea et al. 1993), Ca^{2+} release via rhyanodine-sensitive receptors (Ding et al. 1998), and/or cGMP/protein kinase G (PKG, the c-GMP-dependent kinase; Prosser et al. 1989; Weber et al. 1995; Ding et al. 1994; 1997) and the Ca^{2+}/calmodulin-dependent kinase (CaMKII; Agostino et al. 2004; Nomura et al. 2003), and extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK; Obrietan et al. 1998; Butcher et al. 2002; Coogan and Piggins 2003). Recent studies indicate that nighttime restricted gating of the ERK/MAPK signaling pathway via NMDA activation is regulated by Dexras1, a RAS-like G-protein (Cheng et al. 2004; Graham et al. 2002; Fang et al. 2000). MAPK stimulation activates the phosphorylation of cAMP-response-element-binding protein (P-CREB; Ginty et al. 1993; Gau et al. 2002; Ding et al. 1997; Obrietan et al. 1998; 1999; Tischkau et al. 2002), which stimulates *c-fos* (review in Kornhauser et al. 1996; Rea 1998) and the light responsive 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; Nielsen et al. 2001a; Tischkau et al. 2002). Induction of *c-fos* strongly correlates with phase shifts of the overt rhythm in rats (Travnickova et al. 1996), and there is a direct proportionality between the induction of Fos immunoreactive neurons in the SCN and the numbers of photons, rather than between irradiance and duration of the light pulse (Dkhissi-Benyahya et al. 2000). Fos is a useful parameter for light activation of cells within the SCN, even though a distinct role in light entrainment is not obvious (Honrado et al. 1996). Light induction of *Per1* also strongly correlates with the light-induced phase shifts (Shigeyoshi et al. 1997) and in contrast absence of *Fos* (Honrado et al. 1996), blocking the *Per1* gene by anti-sense DNA-oligonucleotides, annuls light and glutamate-induced phase shifts in mice (Akiyama et al. 1999; Wakamatsu et al. 2001) and rats (Tischkau et al. 2002). Furthermore, mice lacking *Per1* and/or *Per2* have an altered response to light stimulation at night (Albrecht

et al. 2001). Null-mutant *Per1* mice do not advance the clock phase in response to light, and *Per2* knockout mice do not show a light-induced delay in the circadian phase of early night (Albrecht et al. 2001). These observations indicate that the *Per1* and *Per2* genes are important for the light-induced resetting of the clock, and the expression of both genes is a useful endpoint parameter when studying the effects of light or neurotransmitters mediating/modulating the effect of light.

3.4.6 PACAP Interacts with Glutamate Signaling During Light-Induced Phase Shift

The functional significance of the co-existence of PACAP and glutamate in the RHT is not fully understood, but increasing evidence indicates that PACAP modulates the clock sensitivity to glutamatergic signaling. When applied together with glutamate in early night, PACAP in micromolar concentration potentiated the glutamate-induced phase delay whereas the specific antagonist PACAP6–38 blocked glutamate-induced phase shift (Chen et al. 1999; Fig. 15). In contrast, when co-administered with glutamate during late subjective night, PACAP (in micromolar concentration) blocked glutamate-induced phase advance, whereas co-administration of PACAP6–38 potentiated the glutamate-induced phase shift (Chen et al. 1999; Fig. 15).

These in vitro studies are supported by in vivo studies of intraventricular injection of a specific PACAP antibody followed by a light pulse resulting in potentiation or attenuation of the light-induced phase advance (Chen et al. 1999) and delay (Bergström et al. 2003), respectively. The mechanism behind this interaction remains to be clarified but PACAP seems to modulate glutamate signaling via several different mechanisms. In primary SCN culture, PACAP seems to amplify glutamate-dependent calcium increase by interacting with AMPA/kainate signaling (Kopp et al. 2001). Activation of AMPA/kainate receptors induces a depolarization in neurons which releases the voltage-dependent Mg^{2+} blockade of the NMDA-receptor cation channel and thus allows glutamate to go directly to the NMDA receptor. On the other hand, PACAP reduces/inhibits calcium increase elicited by glutamate acting on metabotropic receptors (Kopp et al. 2001). The study by Kopp et al. does, however, not address the question whether the PACAP-sensitive interaction with glutamate is clock regulated. Neither is it known from the present data whether the stimulatory and/or inhibitory effect of PACAP stimulation is/can be initiated in the same SCN neuron, or whether different SCN neurons are "activators" and other are "inhibitors" due to PACAP stimulation (Kopp et al. 2001). Since SCN neurons in the ventral retinorecipient SCN either can be characterized electrophysiologically as excitatory or inhibitory (see Sect. 3.4.4), it is likely that the PACAP-sensitive cells represent different subtypes of SCN neurons.

Another mechanism by which PACAP may modulate glutamate/NMDA receptor signaling in the SCN is via *Homer-1a*. This is an immediate early gene product whereas other members of the *Homer* family, *Homer-1b/c*, *2a/b/c*, and *Homer-3*, are constitutively expressed. *Homer-1a* is a C-terminally truncated form

of *Homer-1*; it modulates the property of the long forms and participates in activity-dependent control of glutamatergic neurotransmission by binding to metabotropic glutamate receptors type 1 and 5, inosol-triphosphate receptors, and the shank/GKAP/PSD-95/NMDA receptor complex, thus forming a link between NMDA receptors, metabotropic glutamate receptors, and intracellular calcium signaling (Xiao et al. 2000). Light has been shown to induce *Homer-1a* in the SCN (Park et al. 1997). Studies using the in vitro brain-slice model have shown that glutamate induces *Homer-1* mRNA in the SCN during early but not late subjective night (Nielsen et al. 2002). In contrast, PACAP in nanomolar concentrations, on one hand induces *Homer-1* mRNA in both early and late subjective night (Nielsen et al. 2002; Fig. 16). On the other, application of PACAP in micromolar concentrations blocks glutamate-induced *Homer-1* expression during early night, whereas PACAP and glutamate applied together induce *Homer-1* expression during late night (Nielsen et al. 2002; Fig. 16).

The above-mentioned data indicate that the interaction between glutamate and PACAP in the SCN is complex. The role of PACAP and glutamate in the regulation of the two clock genes, *Per1* and *Per2*, has also been examined. In vitro application of glutamate induces both *Per1* and *Per2* in the SCN during subjective night (Nielsen et al. 2001; Tischkau et al. 2002; Asai et al. 2001). As observed for the phase shift and the induction of *Homer-1* mRNA, PACAP in a concentration-dependent manner also regulates the expression of both *Per* genes during late subjective night (Nielsen et al. 2001; Fig. 17).

In nanomolar concentrations PACAP significantly induces both *Per1* and *Per2* mRNA in the SCN (Fig. 17). In micromolar concentrations, PACAP blocks glutamate-induced *Per* gene expression in the SCN (Fig. 17; Nielsen et al. 2001). These observations seem to provide the molecular substrate for the above-mentioned electrophysiological findings of the ability of PACAP to modulate glutamate-induced phase shift in late subjective night (Chen et al. 1999). A further support for an interaction between PACAP signaling and NMDA activation/signaling is provided in a recent study. In vivo application (icv) of PACAP in early subjective night induces a moderate phase delay followed by induction of *Per1* in the SCN (Minami et al. 2002). Both the PACAP-induced phase shift and *Per* gene induction are blocked by pretreatment with the NMDA receptor antagonist MK-801 (Minami et al. 2002). PACAP exerts its effect via three types of G-protein-coupled receptors (see Sect. 3.2.3.2), and studies in mice lacking the PACAP-specific PAC1 receptor indicate that activation of this receptor is important for light-induced phase shift and induction of *c-fos* and the *Per* genes in the SCN (Hannibal et al. 2001b). When exposed to light during early subjective night, these mice show altered phase response, and compared to the wild-type mice, *Per1* and *Per2* expression in the SCN of PAC1-deficient mice is markedly attenuated (Fig. 18; Hannibal et al. 2001b).

4
Non-photic Regulation of Circadian Rhythms

The daily cycle of light and darkness is the principal zeitgeber for the circadian system, but non-photic cues such as cycles of feeding, temperature, social interactions, behavioral activity, or arousal and sleep deprivation interact and often antagonize in a phase-dependent manner the effect of light on the circadian rhythm, and vice versa (Mrosovsky 1996; Hastings et al. 1998a; Mistlberger et al. 2000; Challet and Pevet 2003). For instance, non-photic phase shift can block or modulate light or glutamate-induced phase shift at night whereas light attenuates the phase-shifting effect of non-photic stimulation during subjective day at the behavioral level (Biello and Mrosovsky 1995; Prosser 2001, see also review by Mistlberger et al. 2000).

Phase shift of the circadian rhythm by non-photic cues was originally described by Mrosovsky who observed that free-running hamsters kept in constant darkness phase shift their circadian rhythm after cage change or when pairs of animals had social interaction during their normal resting phase (Mrosovsky 1988). These innocuous procedures awaken the hamster and stimulate activity/arousal. A phase response curve for non-photic stimulation due to novelty-induced wheel running activity established that such stimulation resulted in phase advances during the subjective day and small delays during the late subjective night (Fig. 19; Reebs and Mrosovsky 1989; Bobrzynska and Mrosovsky 1998, see also Mrosovsky 1996).

It soon turned out that other measures that either induce activity or arousal, such as systemic injection of triazolam, morphine, hypertonic saline, or sleep deprivation, resulted in phase shift as seen following novelty-induced wheel running (Morin 1999; Mistlberger et al. 2000; Challet and Pevet 2003). High activity during wheel running seems to be important for the phase shift of the circadian rhythm (Reebs and Mrosovsky 1989; Marchant and Mistlberger 1995), but it does not seem to be the activity per se that causes the phase shift, but rather the arousal at a time when the animal normally sleeps. Thus, sleep deprivation during subjective day induces a similar phase shift with running wheels locked as with unlocked wheels (Antle and Mistlberger 2000; Mistlberger et al. 2002).

The two major neuronal pathways involved in non-photic regulation of the circadian system are (1) the serotonergic projections which originate in the midbrain raphe nuclei (i.e., the median and the dorsal raphe) and (2) the geniculohypothalamic tract (GHT) which originates from the IGL. The GHT in all mammalian species contains NPY and GABA, and also enkephalin in hamsters (but not in rat). The anatomy and functional implication of these pathways on the regulation of circadian rhythm will be dealt with below.

4.1 Midbrain Raphe Input—Neuroanatomy

4.1.1 The Median Raphe Projection to the SCN

Serotonin (5-HT)-containing projections from the midbrain raphe nuclei to the SCN and IGL have been known for many years, based on radioautographic and lesion studies combined with determination of the content of 5-HT in various brain areas (Azmitia and Segal 1978; Moore et al. 1978; van de Kar and Lorens 1979). A detailed anatomical picture of midbrain raphe projections in rodent species is now well established, based on retrograde and anterograde tracing studies from the SCN, the dorsal raphe nucleus (DRN), and the median raphe nucleus (MRN) and, further, by injections of the serotonin selective neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT) into the raphe nuclei (reviewed in Morin 1999). A dense innervation is found in the ventral core region of the SCN overlapping with RHT and GHT projections (Ueda et al. 1983; Morin et al. 1992; Goel et al. 1999; Sanggaard et al. 2003; Fig. 3). In the rat, 5-HT immunoreactive nerve fibers are distributed throughout the rostrocaudal part of the ventrolateral SCN, together with RHT terminals (van den Pol and Tsujimoto 1985; Sanggaard et al. 2003; Fig. 3). Both terminal fields make synaptic (axo-dendritic and axo-somatic) contacts with neurons containing VIP (Bosler and Beaudet 1985; Francois-Bellan and Bosler 1992). A sparse projection is also found in the dorsal (shell) subdivision (Sanggaard et al. 2003). The projections to the SCN originate exclusively from the MRN in the hamster (Meyer-Bernstein and Morin 1996; Leander et al. 1998; Morin and Meyer-Bernstein 1999). In the rat, serotonergic projections originate primarily from the median raphe nuclei, but a few neurons located in the DRN send sparse projections to the SCN (Moga and Moore 1997; Hay-Schmidt et al. 2003). Neurotoxic lesion studies of the MRN and DRN have confirmed the tracing studies. After application of 5,7-DHT into the MRN but not into the DRN, 5-HT immunoreactive fibers in the SCN are eliminated in the hamster. In contrast, 5-HT immunoreactive fibers in the IGL, another major projection area to the SCN, are eliminated when 5,7-DHT is injected into the DRN, but not after injection into the MRN (Meyer-Bernstein and Morin 1996). Double immunohistochemistry of 5-HT and retrograde tracer (injected into the MRN and/or DRN) in the SCN and IGL revealed, however, that only about 50% of the neurons in the MRN projecting to the SCN contain 5-HT, whereas about 40% of the neurons in the DRN projecting to the IGL are serotonergic (Leander et al. 1998; Hay-Schmidt et al. 2003; see also Morin 1999). These observations indicate that the midbrain raphe projections most likely contain unidentified neurotransmitter(s) innervating the SCN and IGL. In man and monkey a dense plexus of serotonergic fibers terminals has been shown to overlap with that of the RHT and to innervate VIP-containing nerve cell bodies. Thus, the distribution in primates is similar to that found in rodent species (Moore and Speh 2004).

4.1.2
Serotonin Receptors in the SCN

The existence of multiple 5-HT receptor subtypes, grouped into several families, has been demonstrated in the SCN by anatomical methods and binding assays, and these observations have been supported by pharmacological experiments using subtype-specific 5-HT receptor agonists and antagonists in vivo and in vitro. By in situ hybridization histochemistry the presence of substantial 5-HT_{1C}-receptor mRNA has been demonstrated in the SCN, while relatively little mRNA for the 5-HT_{1A} and 5-HT_{1B} and 5-HT_2 are reported (Roca et al. 1993). Also the 5-HT_7 receptor has been found in the SCN (Heidmann et al. 1998; Rea and Pickard 2000), a finding supported by binding studies using the 5-$HT_{7/1A}$ radioligand [^{3}H]-8-OH-DPAT (Lovenberg et al. 1993; Rea and Pickard 2000; Duncan et al. 1999; Prosser et al. 1993). Incubation with [^{3}H]-8-OH-DPAT in the presence of ritanserin, a specific 5-HT_7 receptor agonist, and pindolol, a selective 5-HT_{1A} agonist, are consistent with the presence of both functional 5-HT_7 and 5-HT_{1A} receptors in the SCN of the hamster (Rea and Pickard 2000). Binding studies using various selective 5-HT_{1B} radioligands also support the presence of this receptor subtype in the SCN of rat (Manrique et al. 1999; Prosser et al. 1993) and hamster (Pickard et al. 1996; Duncan et al. 1999). Some discrepancy between 5-HT_{1B}-receptor binding and the expression of 5-HT_{1B} mRNA in the SCN suggests that this receptor may be localized mainly presynaptically on projections to the SCN, a notion supported by radioligand binding studies on bilaterally enucleated rats (Manrique et al. 1999) and hamsters using the 5-HT_{1B} receptor-specific ligands (Rea and Pickard 2000). In hamster, bilateral enucleation results in a 35% reduction in specific ^{125}I-iodo-cyanopindolol (^{125}I-ICYP) binding within the SCN (Rea and Pickard 2000), suggesting that approximately 35% of 5-HT_{1B} receptor binding is on RHT terminals (Rea and Pickard 2000), whereas the majority of 5-HT_{1B} receptors are found on non-retinal terminals many of which contain GABA (Barnard et al. 2004). Immunohistochemical studies at the light and at the electron microscopic levels are consistent with this notion (Pickard et al. 1999; Belenky and Pickard 2001). Visualization of retinal afferents by CTb injected into the eye in mice demonstrate that 5-HT_{1B} receptor immunoreactivity is found in retinal and non-retinal axons (which may be GHT afferents from the IGL; Manrique et al. 1999) and to a lesser extent in post-synaptic SCN processes and somata (Belenky and Pickard 2001; Pickard et al. 1999). In contrast, 5-HT_7 receptor immunoreactivity is found in non-retinal processes and in somata containing VIP, GABA, and arginine–vasopressin immunoreactivity (Belenky and Pickard 2001).

4.2
Serotonin (5-HT) and Regulation of the Circadian System

Substantial evidence indicates that 5-HT is a neurotransmitter in non-photic regulation of the circadian system. This evidence is based on: (1) anatomical findings;

(2) measures of 5-HT in the SCN after electrical stimulation of the raphe nucleus; (3) lack of function due to neurotoxic destruction of 5-HT projection to the SCN and IGL using 5,7-dihydroxytryptamin (5,7-DHT) injected into the dorsal and median raphe nuclei; (4) studies using specific 5-HT receptor agonists and antagonists; and (5) studies on 5-HT-receptor-deficient mice. Two main conclusions can be drawn: (1) Serotonin can phase advance the circadian rhythm during the subjective day, a time when light has little or no phase-shifting capacity although light can block or modulate the response to serotonergic stimulation. (2) Serotonin can block or modulate light-induced resetting of circadian rhythm in subjective night but has little or no phase shifting capacity itself (Morin 1999; Challet and Pevet 2003; Rea and Pickard 2000; Hastings et al. 1998a). Given the anatomical characteristics of the raphe (5-HT) innervation of the SCN and IGL, 5-HT may: (1) directly influence the clock cells in the SCN, (2) affect RHT and/or GHT terminals in the ventrolateral SCN, (3) act on target areas projecting to the SCN, (4) or a combination of (2) and (3).

4.2.1 Serotonin in Non-photic Phase Shifting of the Circadian Rhythm

4.2.1.1 Serotonin Is Released in the SCN In Vivo

It is well documented that activity and arousal are strongly related to the release of 5-HT in various brain regions (Schwartz et al. 1989; Rueter and Jacobs 1996; see also review by Jouvet 1999). Within the SCN, increased release of 5-HT has been demonstrated by microdialysis studies and concentrations of 5-HT were found to correlate positively with locomotor activity in blind rats (Shioiri et al. 1991) and in hamsters (Dudley et al. 1998; Mistlberger et al. 2000). In hamsters circadian release of 5-HT peaks at the transition time between day and night, a time point when motor activity generally is high in nocturnal animals (Glass et al. 1992; Dudley et al. 1998). Electrical stimulation of both the median and the dorsal raphe nuclei increases the 5-HT content in the SCN indicating that both nuclei contribute to the serotonergic activity within the SCN (Dudley et al. 1999; Glass et al. 2003). It seems, however, based on systemic and/or local microinjections of 5-HT receptor agonists and antagonists, that different functional pathways exist for these nuclei in the regulation of 5-HT output within the SCN (Glass et al. 2003). Microinjections into the MRN of the 5-$HT_{1A/7}$ receptor agonist 8-OH-DPAT causes significant inhibition of 5-HT release in the SCN, whereas the injection of the antagonist WAY 100635 into the MRN stimulates 5-HT release in the SCN (Dudley et al. 1999). Both drugs have substantially less effect in the DRN. These differential drug actions indicate that somatodendritic 5-HT_{1A} autoreceptors on median raphe neurons may provide the prominent raphe autoregulation of 5-HT output in the SCN, possibly by 5-HT input from the dorsal to the median raphe nuclei (Dudley et al. 1999; Glass et al. 2003).

Sleep deprivation causing increased arousal at the end of subjective day also provokes release of 5-HT in the SCN and a shift in the behavioral rhythm (when animals were kept in dim red light), most likely via the DRN (Glass et al. 2003). On the other hand, when animals are kept in incandescent white light, the size of the phase shift is reduced indicating that light can modulate the effects of serotonin during the day (Grossman et al. 2000, see also Sect. 4.2.1.4).

4.2.1.2
Behavioral Effects of Neurotoxic Destruction of 5-HT Projection to the SCN

A role of 5-HT in the regulation of the circadian system is supported by in vivo studies performed to reduce or eliminate 5-HT levels in the SCN. From the mid 1970s in vivo studies were initiated using various neurotoxins such as 5,7-DHT and p-chloroamphetamine injected into or close to the SCN or into the DRN or MRN of hamster, mouse, and rat (Morin 1999; Mistlberger et al. 2000). These treatments did not generally eliminate circadian rhythms, but the rhythms became more irregular and had lower overall amplitude. Detailed analysis showed that the rhythms usually exhibited an advance in phase angle, which means that the locomotor activity onset (used to determine the circadian phase) usually began 30–45 min earlier compared to normal, and that the off-set of activity is delayed resulting in a prolonged activity period without an increase in the total activity. Thus, 5-HT plays a modulatory role that seems to enhance the overall stability of rhythmicity (reviewed in Morin 1999). The lesion studies also demonstrated that the MRN and not the DRN projects to the SCN. Furthermore, a lesion of the MRN produced by 5,7-DHT yielded a pattern of activity rhythm response similar to that found after icv injection of 5,7-DHT (Meyer-Bernstein and Morin 1996), whereas no effects were observed after lesion in the DRN (Meyer-Bernstein and Morin 1996). These studies also demonstrated that 5,7-DHT lesion of the MRN, but not the DRN, blocks triazolam-induced phase shifts while, phase shifts due to novelty-induced wheel running is not blocked after these lesions. These observations indicate that the MRN, but not the DRN, contribute to the triazolam-induced phase shift and suggest the presence of separate pathways mediating the phase response of these two different stimuli (Meyer-Bernstein and Morin 1998).

4.2.1.3
Effects of 5-HT on the Circadian Rhythm During the Subjective Day

Non-photic phase shift of the circadian rhythm is seen after novelty-induced wheel running and after injection of triazolam or hypertonic saline during subjective day (Fig. 19). A similar phase shift is obtained during subjective day but not subjective night after icv or systemic injection of putative 5-HT agonists in rats and hamsters (Tominaga et al. 1992; Edgar et al. 1993; Cutrera et al. 1994; Bobrzynska et al. 1996; Mintz et al. 1997). The areas responsible for action of 5-HT on circadian rhythms have not been fully identified but the SCN and/or the midbrain raphe nuclei or the

IGL seem to be involved (reviewed in Morin 1999). Recent studies indicate the DRN regulates the 5-HT tonus in the MRN which subsequently regulates 5-HT output in the SCN (Ehlen et al. 2001; Antle et al. 2000; Glass et al. 2000). During the subjective day in vitro application of 5-HT (Medanic and Gillette 1992), the 5-$HT_{1A/7}$ agonist 8-OH-DPAT, the $5HT_{1A}$ receptor agonist 5-carboxamidotryptamine (5-CT; Medanic and Gillette 1992; Prosser et al. 1993; Prosser 2003), or the $5HT_{1A/7}$ receptor agonist quipazine (Prosser et al. 1990) all produce similar phase advances in the electrical firing rhythm in SCN neurons in the brain slice preparation. Pretreatment of the brain slice with pindolol, a potent $5HT_{1A}$ receptor antagonist, or the 5-HT_7 receptor antagonist ritanserin reveals that phase shift during the day induced by 5-HT most likely is via the 5-HT_7 receptor (Prosser 2000).

4.2.2
Serotonin Modulates Light-Induced Phase Shift During Subjective Night

Anatomical findings and functional in vivo and in vitro studies have documented 5-HT as a modulator of photic input to the circadian system. Electrical stimulation in the median raphe nuclei of hamsters releases 5-HT in the SCN (Dudley et al. 1999) and blocks light-induced phase shift in early and late subjective night (Rea and Pickard 2000). The results conform to the observation that light-induced Fos immunoreactivity is markedly attenuated after electrical stimulation of the DRN and the MRN (Meyer-Bernstein and Morin 1999). Light-induced phase shift in hamsters is attenuated by novelty-induced wheel running (Mistlberger and Antle 1998). On the other hand, depletion of 5-HT-containing nerve fibers in the SCN by 5,7-DHT injected treatment in mice or hamsters increases the light-induced phase shifts compared to controls, accompanied by an increase in SCN *fos* expression, suggesting the existence of a 5-HT tonus in SCNs light-responsive neurons (Bradbury et al. 1997; Morin and Blanchard 1991).

The contribution of different 5-HT receptor subtypes in the modulation of light input to the SCN has been studied using receptor-specific agonists and 5-HT antagonists injected in vivo (systemic or directly into the SCN) or in vitro. Stimulation of the optic nerves attached to the SCN in a horizontal brain slice preparation (Bennett et al. 1996) produces phase shifts in the circadian rhythm similar to light (Jagota et al. 2000), and this model has been shown useful for studies on the interaction between retinal input and various neurotransmitters in the SCN. Furthermore, studies on 5HT-$_{1B}$-receptor-deficient mice indicate that this receptor is important for the effects of 5-HT on light-induced phase shift.

4.2.2.1
Activation of 5-$HT_{1A/7}$ Receptors Modulates Light Signaling in the SCN

Pretreatment of hamsters with both systemic and local application of the 5HT receptor agonists 8-OH-DPAT and 5-CT attenuates light-induced phase shifts at night, whereas the 5HT-$_{2A/2C}$ receptor agonist DOI has no effect (Fig. 20). On the

other hand, the blocking effect of 8-OH-DPAT on light-induced phase advance is completely reversed by application of the 5-$HT_{7/2}$ receptor antagonist ritanserin and by WAY100,135, a selective 5-HT_{1A} receptor antagonist (Rea and Pickard 2000). WAY100,135 injected alone, however, increased the light-induced phase delay, but not the phase advance (Smart and Biello 2001). These results indicate that 5-HT modulates light-induced phase shift via post-synaptically located 5-$HT_{1A/7}$ receptors. But systemic administration of the 5-HT_{1A} receptor agonist 5-3-[((2S)-1,4-benzodioxan-2-ylmethyl)amino]-propoxy-1,3-benzodioxole HCl (MKC-242) potentiated light-induced phase shift in late night, which may suggest that 5-HT_{1A} autoreceptors in the MRN mediate the effects on light of this compound (Moriya et al. 1998). The results also emphasize that various 5-HT agonists or antagonists may have different targets in the brain (Smart and Biello 2001). In rats, but not in hamsters, some studies have shown that also the 5-$HT_{2A/2C}$ receptor plays a role in the interaction between 5-HT and light (Kennaway 2004).

Electrical stimulation of the optic nerves attached to a brain slice preparation produces excitatory postsynaptic currents (EPSCs) in voltage-clamped SCN neurons. Stimulation-produced short-latency EPSCs are inhibited by application of 8-OH-DPAT, consistent with activation of 5-HT_7 receptors in the SCN (Pickard et al. 1999). In cultured SCN neurons, glutamate-induced calcium increase is markedly attenuated after application of 5-HT and/or 8-OH-DPAT, and this effect is blocked by the 5-$HT_{7/2}$ receptor antagonist ritanserin (Quintero and McMahon 1999a). Similarly, glutamate-induced EPSCs in mouse SCN are reduced after treatment with 8-OH-DPAT, an effect that is minimally attenuated by WAY100,135, but significantly reduced by ritanserin, consistent with activation of 5-HT_7 receptors during light/glutamate stimulation of the SCN (Smith et al. 2001).

4.2.2.2
Activation of 5-HT_{1B} Receptors Modulates Light Signaling in the SCN

Light signaling to the SCN is controlled also by 5-HT_{1B} receptors located presynaptically on RHT nerve terminals, and to a minor extent postsynaptically. Pretreatment of hamsters with both systemic and local application of the 5-HT_{1B} receptor agonists TFMPP and CGS 12066 inhibits light-induced phase shifts at night (Fig. 20; Pickard et al. 1996). TFMPP also attenuates light-induced *Fos* expression in the SCN (Pickard et al. 1996).

Furthermore, TFMPP administered after in vitro stimulation of the optic nerve reduces the amplitude of the EPSCs in the SCN in a dose-dependent manner, an effect unaffected by simultaneous application of the 5-HT_{1A} receptor antagonist WAY100,135 or the 5-HT_7 antagonist ritanserin (Pickard et al. 1999). Glutamate-induced EPSCs are, however, unaffected by application of TFMPP, supporting a presynaptic mechanism of 5-HT_{1B} receptor activation (Pickard et al. 1999). Systemic treatment with TFMPP failed to alter photic phase shift (Pickard et al. 1999) or electrically evoked EPSCs (Smith et al. 2001) in 5-HT_{1B} receptor knockout mice at doses that fully block light-induced phase shift or the EPSCs in littermate control

animals (Pickard et al. 1999; Smith et al. 2001). The 5-HT_{1B} receptor knockout mice have an altered sensitivity to light since they have a significantly longer τ when placed in constant light, as compared to wild type animals (Sollars et al. 2002). However, the 5-HT_{1B}-receptor-deficient mice re-entrain to a 6-h phase advance similar to the wild type mice suggesting that other mechanisms are involved in re-entrainment (Sollars et al. 2002).

4.2.2.3
Signal Transduction and Gene Expression Associated with 5-HT Stimulation

Relative to knowledge about receptor subtypes, little is known about the signal transduction pathways and target gene in the SCN mediating the effects of 5-HT on the phase response of the circadian rhythm. In vitro application of cAMP, cAMP analogs, or substances like PACAP found in the RHT, to hypothalamic brain slices produces phase advance in the electrical activity rhythm similar to the behavioral phase response obtained after non-photic stimulation (Prosser and Gillette 1989; Hannibal et al. 1997). Phase advances in the electrical activity rhythm obtained after application of the 5-HT agonists 8-OH-DPAT and quipazine can be blocked by protein kinase A (PKA) inhibitors and by blocking K^+ channels, indicating that cAMP/PKA signaling pathways and K^+-dependent channels mediate 5-HT phase resetting during the subjective day (Prosser et al. 1994). Detailed knowledge on target genes involved in non-photic phase shift is missing but the two clock genes *Per1* and *Per2* seem to be involved. The *Per* genes both oscillate over the 24 h LD cycle with peak expression found at mid subjective day and at the transition between day and night for *Per1* and *Per2*, respectively (Fig. 5; Reppert and Weaver 2002). Systemic application of 8-OH-DPAT during subjective day significantly phase advances the circadian rhythm and depresses the amplitude of both *Per1* and *Per2* mRNA time dependently in the SCN. On the other hand, no effect of 8-OH-DPAT on the clock gene expression is found in subjective night (Horikawa et al. 2000). Non-photic stimuli such as novelty-induced wheel running also down-regulates *Per1* and *Per2* expression in the SCN (Maywood et al. 1999). Studies using antisense oligodeoxynucleotides to suppress *Per1* expression in the SCN show that the decrease in *Per1* is the cause rather than a consequence of non-photic phase shift (Hamada et al. 2004). It is likely that the altered *Per* gene expression during non-photic stimulation involves 5-HT signaling, even though it remains to be shown that 5-HT antagonists can block non-photic *Per* gene suppression.

Non-photic and photic responses are mutually antagonistic in a phase-dependent manner. (Biello and Mrosovsky 1995; Prosser 2001; Mistlberger et al. 2000). At the molecular level the effect of light during subjective day seems to involve *Per1* and *Per2* genes, since exposure of a 30-min light pulse almost completely abolishes the down regulation of both *Per* genes in the SCN caused by novelty-induced wheel running (Maywood and Mrosovsky 2001).

Light exposure causing phase shift of the circadian rhythm induces a number of "light responsive" genes in the retino-recipient zone of the SCN (see Sects. 3.4.6 and

3.4.7), including the immediate early gene *c-fos* and the clock genes *Per1* and *Per2*. Although a functional significance of light-induced *c-fos* expression in the SCN remains obscure, the response has been used as a marker for photic activation of SCN neurons (Kornhauser et al. 1996). Systemic injection of 8-OH-DPAT in a concentration that blocks light-induced phase shifts significantly attenuates light-induced *c-fos* in the ventral SCN when injected prior to light stimulation (Rea et al. 1994, see also Morin 1999, Table 3). At night, non-photic stimuli phase-dependently modulate light/glutamate-induced phase shifts (Morin and Blanchard 1991; Selim et al. 1993; Smith et al. 2001; Pickard et al. 1996; 1999; Ying and Rusak 1997; Quintero and McMahon 1999b). Systemic application of the 5-HT_{1A} receptor agonist MKC-242 has been shown to potentiate light-induced phase shift, probably via 5-HT_{1A} autoreceptors in the MRN (Moriya et al. 1998). Systemic application of MKC-242 before a light pulse at night potentiates light-induced *Per* gene expression in the SCN (Takahashi et al. 2002), a response most likely due to a reduced 5-HT tonus within the SCN (Takahashi et al. 2002). Interaction at the molecular level between 5-HT signaling and light signaling accomplished by the two RHT neurotransmitters, glutamate and PACAP (Hannibal 2002a), has recently been studied in the SCN. It was shown, using a rat in vitro brain slice preparation and quantitative in situ hybridization histochemistry, that 5-HT alone has no effect on *Per1* or *Per2* mRNA levels when applied during early subjective night. At this time point, however, 5-HT blocks glutamate-induced *Per1* and *Per2* expression (Fig. 21), an effect most likely mediated via postsynaptically located 5-$HT_{1/7}$ receptors (Sanggaard et al. 2003).

In late subjective night, 5-HT application alone induces *Per1* mRNA expression significantly but has no effect on *Per2* mRNA level. On the other hand, no effects of 5-HT on glutamate-induced *Per1* mRNA expression are seen, whereas glutamate-induced *Per2* mRNA expression is significantly attenuated (Sanggaard et al. 2003). Serotonin does not influence PACAP induced *Per* gene expression in late night (Sanggaard et al. 2003). These observations support the notion that the *Per* genes involved in light-induced phase shift at night (Shigeyoshi et al. 1997; Zylka et al. 1998; Albrecht et al. 1997; Yan et al. 1999; Field et al. 2000; Akiyama et al. 1999; Wakamatsu et al. 2001) are targets for 5-HT modulation of the circadian phase.

4.3
The Intergeniculate Leaflet and the Geniculohypothalamic Tract–Neuroanatomy

The intergeniculate leaflet (IGL) of the thalamus was first described by Hickey and Spear (1976) as a thin zone of neurons intercalated between the dorsal and the ventral lateral geniculate nuclei in the thalamus. The IGL is bilaterally innervated from the eye, and there is no evidence of a topographical organization to its retinal input (Harrington 1997; Holcombe and Guillery 1984). The retinal afferents in rats have been shown to terminate on neuropeptide Y (NPY)- and/or enkephalin-immunoreactive neurons (Takatsuji et al. 1991b). A considerable plexus of PACAP immunoreactive nerve fibers, which overlap extensively with the distribution of retinal afferents, has been demonstrated in the IGL. After eye enucleation the

PACAP-containing fibers in the IGL almost completely disappeared (Hannibal et al. 1997). The findings indicate that the retinal afferents to the IGL originate in the above-described melanopsin-containing RGCs. The IGL contains several types of small to medium-sized neurons that project to a number of target areas including the SCN, and most of the cells have their dendritic arborization within the nucleus (Morin 1994; Moore and Card 1994). The neurochemical phenotypes of the IGL neurons have been examined in several rodent species. The IGL in rat, hamster and mouse is characterized most clearly by cells immunoreactive for NPY (Card and Moore 1989; Harrington et al. 1985; Laemle et al. 1993; Morin et al. 1992), and some researchers have attempted to define the IGL by detailing the extent of NPY-immunoreactive neurons in this area. There is, however, evidence that the most abundant neurotransmitter in the IGL is gammaaminobutyric acid (GABA) since virtually all IGL neurons in rats are immunopositive for the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD; Moore and Speh 1993). Many of the GABA-containing cells also store either NPY or enkephalin (Ohara et al. 1983; Moore and Card 1994). The enkephalin-immunoreactive cells in hamster, but not rat, IGL project to the SCN (Card and Moore 1989). On the other hand in rat, but not in hamster, the enkephalin-containing neurons project to the opposite IGL (Mantyh and Kemp 1983; Wadhwa et al. 1990; Moore and Card 1994; Card and Moore 1989) via the optic chiasma and posterior commissure (Mikkelsen 1992b). The GHT is the name for the monosynaptic nervous pathway that originates from nerve cells in the IGL and the immediately adjacent VLG and terminates primarily in the ventral part of the SCN (Card et al. 1981; Mikkelsen 1994; Moore et al. 1984; Ribak and Peters 1975; Swanson et al. 1974). Anterograde tracing of GHT cells has provided evidence that laterally located IGL cells project to the ventrolateral SCN, while medial IGL cells terminate in both lateral and medial SCN (Mikkelsen 1990). The GHT arises from IGL neurons that storing GABA and NPY as well (Card and Moore 1989; Harrington et al. 1985, 1987; Moore and Speh 1993; Morin et al. 1992). In the rat NPY-containing cells also project extensively within the ipsilateral IGL (Moore and Card 1994). In the macaque monkey and in man, a large portion of the pregeniculate nucleus contains NPY-immunoreactive neurons, and this nucleus is considered homologous to the rodent IGL (Moore 1989). In the monkey SCN a dense plexus of NPY immunoreactive fibers is found in a location corresponding to the ventral SCN of rodent species, whereas in the human SCN a less dense plexus is found together with NPY-containing neurons (Moore 1989; Moore and Leak 2001). These observations suggest that the human SCN differs in organization from other mammals by a local NPY-containing circuit within the SCN (Moore 1989; Moore and Leak 2001). GABA-immunoreactive nerve fibers and cell bodies visualized using antibodies directed against glutamate decarboxylase (GAD) have been demonstrated in the SCN of rodents (Francois-Bellan et al. 1990; Abrahamson and Moore 2001). Nerve fibers in the SCN storing both NPY and GAD have been identified and they seem to innervate VIP-containing cell bodies in the ventral SCN (Francois-Bellan et al. 1990). Functionally the IGL is considered a structure in which photic and non-photic inputs related to resetting of the circadian clock

can be integrated (Harrington 1997). The GHT thus provides an indirect route by which photic information from the retinal ganglion cells could have access to the circadian clock in the SCN.

4.4 Effects of NPY on Circadian Rhythm During Subjective Day

Neurotransmitters associated with non-photic input mediated by the GHT, such as NPY, GABA, and enkephalin, are capable of phase advancing the SCN master oscillations when applied during subjective day in vivo and in vitro (Hastings et al. 1998b; Harrington and Schak 2000). A role for geniculo–hypothalamic inputs to the SCN as possible mediators of non-photic phase shifting comes from GHT lesion studies and observations that electrical stimulation of the IGL can cause phase shifts similar to those induced by wheel running (Harrington 1997). Whereas the effects of GABA in the SCN are complex (Yannielli and Harrington 2004), probably due to the presence of GABA in SCN neurons as well as in multiple input pathways, NPY has been shown to induce a dose-dependent phase advance in circadian rhythm when applied during subjective day in hamsters, both in vivo and in vitro (Biello and Mrosovsky 1996; Albers and Ferris 1984; Harrington and Schak 2000). Phase shift induced by wheel running can be blocked in vivo by microinjection of antiserum to NPY into the SCN, supporting a role for the peptide in mediating this function of GHT (Biello et al. 1994). Application of NPY together with other afferent neurotransmitters in the SCN demonstrates mutual interactions with photic and non-photic neurotransmitters. Application of NPY together with 5-HT or PACAP in subjective day blocks the 5-HT and/or PACAP induced phase advance in vitro most likely by blocking a cAMP-mediated pathway (Prosser 1998; Harrington and Hoque 1997). Application of NPY together with glutamate during subjective day blocks NPY induced phase advance (Biello et al. 1997a), corresponding to the effects of light on running-wheel-induced phase advance (Zhang et al. 1993).

NPY exerts its effects by activation of one or more of five subtypes of receptors, and the phase shifting of rhythms during subjective day seems to be via the Y2 receptor (Golombek et al. 1996; Huhman et al. 1996; Soscia and Harrington 2005). Anatomical studies, however, have revealed abundant Y1 and Y5 receptor mRNA present in the rat and hamster SCN while the Y2 receptor mRNA is surprisingly scarce (Larsen and Kristensen 1998; Yannielli et al. 2004).

The signal transduction pathways leading to phase shifts induced by NPY during the subjective day are not fully known but seem to depend on protein kinase C activation (Biello et al. 1997b). Dexras1, a member of the RAS superfamily of small G proteins and which is involved in light signaling to the clock, also seems to be a key molecule in non-photic signaling. Besides abnormal light entrainment, mice lacking Dexras1 show enhanced responsivity to non-photic stimulation in vivo and in vitro, most likely due to an enhanced sensitivity to NPY (Cheng et al. 2004). Dexras1 in the SCN could be a link between photic and non-photic signal transduction to the circadian system, acting as a positive regulator of a photic-

input pathway and a negative modulator of non-photic signaling (Cheng et al. 2004).

The mechanism by which NPY influences the circadian system appears to be related to the basic genetic machinery of the clock. During subjective day NPY can decrease the normal mid-day rise in *Per1* and/or *Per2* (Maywood et al. 2002; Fukuhara et al. 2001). Light can interact with the NPY-mediated non-photic phase advance during the subjective day and, in accordance, NPY-induced suppression of *Per1* and *Per2* genes in the SCN is attenuated when a light pulse is delivered immediately after NPY infusion (Biello and Mrosovsky 1995; Maywood et al. 2002).

4.5 NPY Modulates Light-Induced Phase Shifts During Subjective Night

During the subjective night NPY itself is unable to influence the circadian rhythm (Biello et al. 1997a), but the peptide can block the phase-resetting effects of light. Thus, it has been shown in hamsters in vivo that NPY inhibited light-induced phase advances but not delays (Weber and Rea 1997), and in later studies it was established that NPY could reduce both advances and delays (Lall and Biello 2003; see Fig. 22). In vitro NPY can also inhibit the phase shifting effect of glutamate and NMDA (used to mimic the effects of light in the SCN slice preparation), and NPY applied in vitro can block the effect of in vivo-delivered light pulses (Biello et al. 1997a,b; Yannielli and Harrington 2001, 2001a, 2001b).

This negative interaction of NPY has been studied at the molecular level and it has been shown that NPY inhibits the rapid light induction of both *Per1* and *Per2* (Brewer et al. 2002; Prabakar et al. 2004). Although NPY can cause a striking and prolonged depression of *Per2* mRNA, the *Per1* mRNA level was found to rebound quickly to equal the unsuppressed *Per1* induction peak at 1 h and mirrored the control light induction pattern thereafter. The inhibitory effects of NPY on light-induced phase shift in vitro could be blocked by a selective Y5 antagonist, RJW-57926 (Yannielli and Harrington 2001a, 2001b). Subsequently, several Y5 antagonists have been shown to counteract the inhibitory effect of NPY on phase shift to NMDA measured in vitro and phase shift in vivo induced by light (Lall and Biello 2002; Yannielli et al. 2004). It has also been shown that immunoneutralization of released NPY with NPY antiserum administered directly into the SCN caused a potentiation of light-induced phase advances (Biello 1995). In recent hamster studies performed both in vivo and in vitro it was also shown that blockade of the NPY5 receptor increases circadian responses to light (Yannielli et al. 2004). The finding that NPY Y5 receptor antagonism potentiates the phase shifting effects of light, especially late in the subjective night, might imply the existence of an endogenous tone of NPY with some sort of physiological role in the circadian system.

5 Summary

Circadian rhythms of physiology and behavior are generated in mammals by the hypothalamic suprachiasmatic nucleus (SCN). Due to deviation from the 24 h astronomical day and night, these rhythms need to be synchronized (entrained) daily by environmental photic and non-photic cues. The present review describes the anatomy and functional characteristics of the three major input pathways to the circadian clock mediating entrainment, the retino–hypothalamic tract (RHT), the geniculo–hypothalamic tract (GHT), and the midbrain raphe projection. Light signaling, which is the principal "zeitgeber" for the circadian timing system, is dependent on functional photoreceptors in the eyes. A dual light detecting system consisting of the classical rod and cone photoreceptor system (primarily image-forming) and a system of photosensitive retinal ganglion cells (non-image-forming), using melanopsin as a photopigment and glutamate and the neuropeptide PACAP as neurotransmitters, mediate light information to the brain. Via complex, not fully known pathways, glutamate and PACAP interact on SCN neurons regulating the expression of central molecular core clock components, *Period 1* (*Per1*) and *Period 2* (*Per2*). The SCN is also responsive to cues that are non-photic in nature such as arousal/locomotor activity, social cues, feeding, sleep deprivation, and temperature. Non-photic information is mediated via the GHT using NPY/GABA as neurotransmitters and serotonin (5-HT)-containing projections from the median raphe nucleus. NPY and 5-HT signaling seems to converge on the SCN neurons also regulating the *Per* gene expression. The distinct temporal responsiveness of the SCN to photic and non-photic cues, which seem to be mutually antagonistic, ensures the overall stable entrainment of the clock.

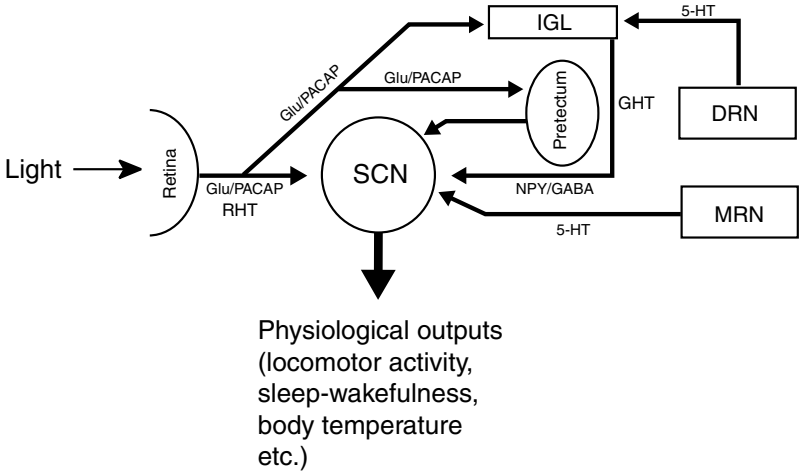


Fig. 1 Schematic diagram of the major input pathways to the brain's biological clock, the suprachiasmatic nucleus (*SCN*). For abbreviations, see list

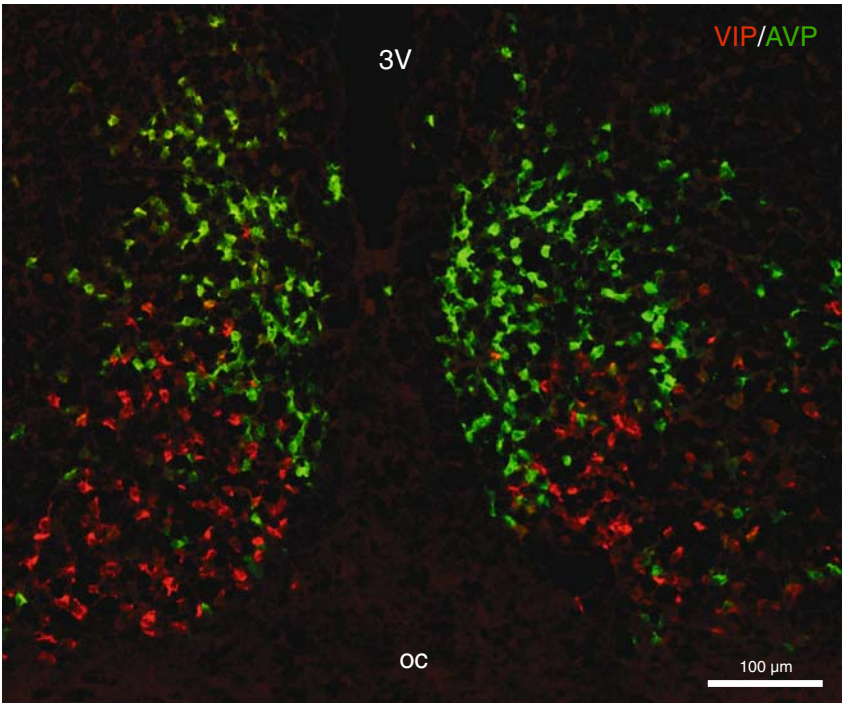


Fig. 2 Neurons in the core and shell subdivision of the rat SCN. Double fluorescence in situ hybridization histochemistry using cRNA probes for VIP and AVP on coronal section through the mid SCN. VIP-expressing neurons are visualized by Alexa-592 (*red*) and AVP expressing neurons are visualized by Alexa-488 (*green*) in the ventral core region and dorso-medial shell region of the SCN, respectively. *3v*, third ventricle; *oc*, optic chiasm

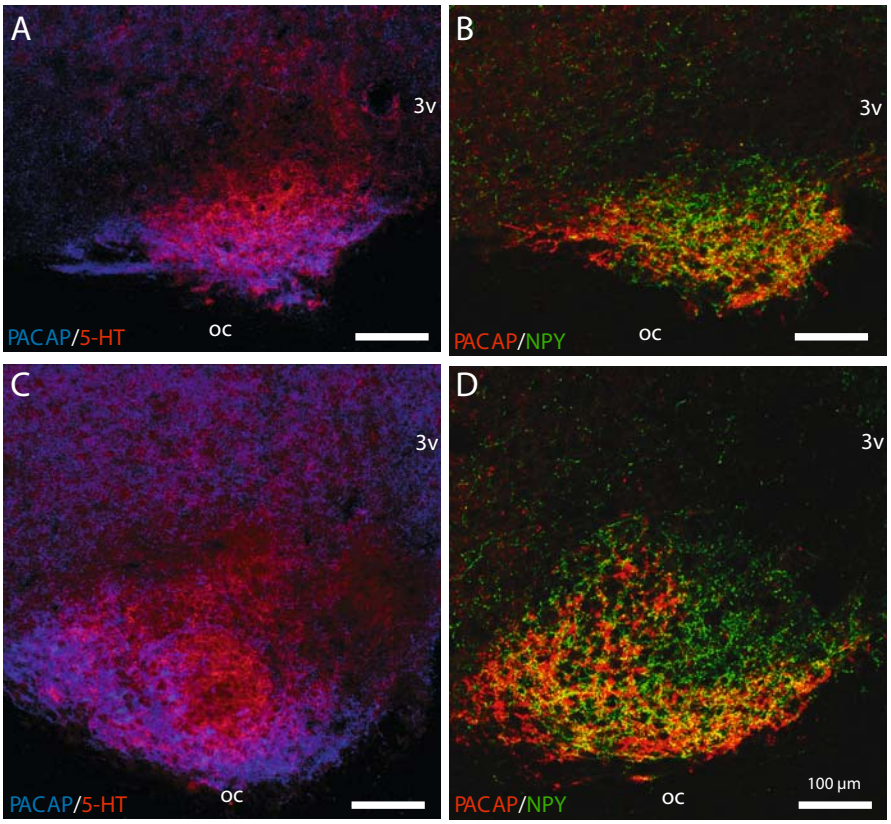
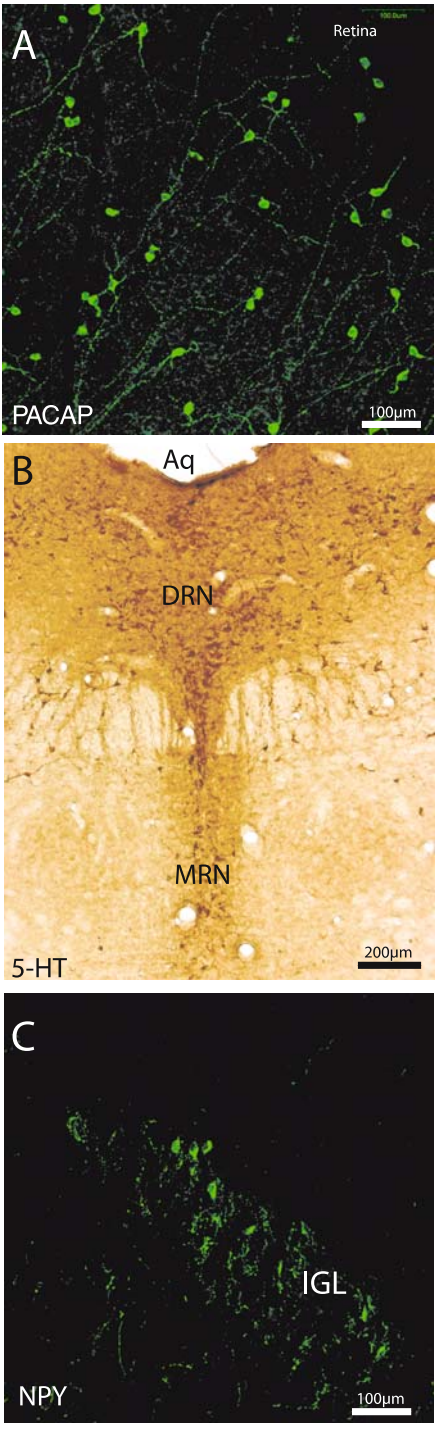


Fig. 3A–D The three major input pathways to the ventrolateral (core) region of the rat SCN visualized by immunohistochemistry and confocal microscopy of the rostral (**A** and **B**) and middle part (**C** and **D**) of the SCN. RHT projections are visualized by PACAP immunostaining (*blue* in **A** and **C**, *red* in **B** and **C**). GHT projections are visualized by NPY staining (*green* in **B** and **D**) and projections from the MRN are visualized by immunostaining for serotonin (5-HT; *red* in **A** and **C**). *3v*, third ventricle; *oc*, optic chiasm

Fig. 4A–C Origin of the three major input pathways to the SCN visualized in the rat by immunohistochemistry and confocal (**A** and **C**) and light microscopy (**B**). **A** A subpopulation of retinal ganglion cells containing PACAP constitute the RHT. **B** Serotonin-containing neurons in the MRN, but not in the DRN, project to the SCN. **C** NPY-containing neurons in the IGL constitute the GHT. For abbreviations, see list


A
Retina
PACAP
100µm
B
Aq
DRN
MRN
5-HT
200µm
C
IGL
NPY
100µm

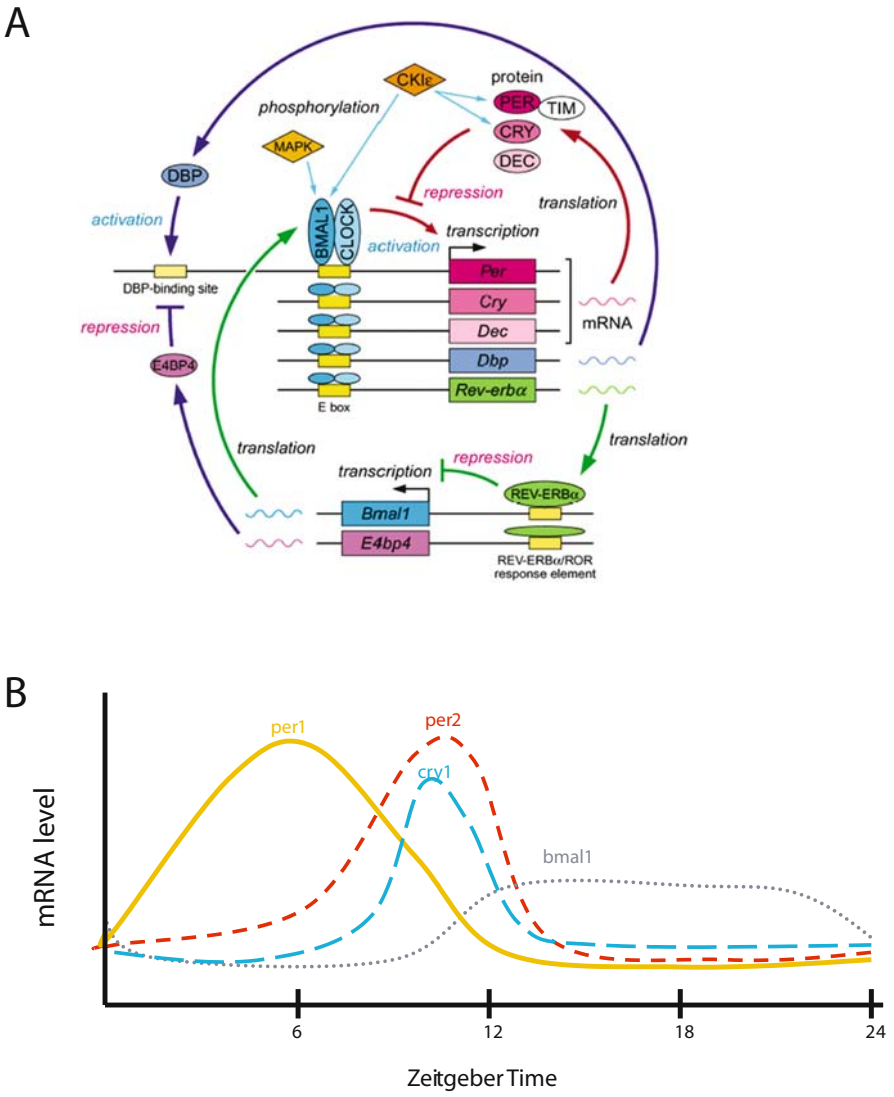


Fig. 5 A Schematic model of the mammalian circadian clock (from Hirota and Fukada 2004). See text (Sect. 2.3) for details. **B** Circadian oscillation of central core clock elements. *Bmal1* which is in antiphase with *Per1* peaks in subjective night whereas *Per1* expression peaks at mid subjective day. *Per2* and the *Cry*s peak at subjective dusk. (Redrawn based on Reppert and Weaver 2001; Okamura et al. 2002; Lowrey and Takahashi 2004; Hirota and Fukada 2004)

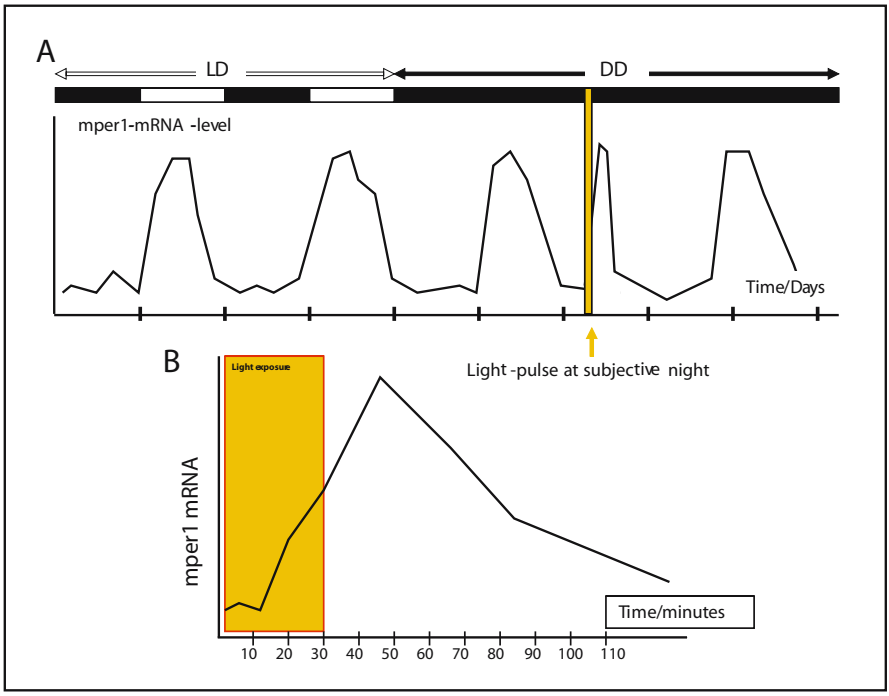


Fig. 6A, B Exposure to light at night induces *mPer1* mRNA expression in the SCN. **A** Circadian oscillation of *mPer1* mRNA in the mouse SCN kept in 24 h light/dark (*LD*). Cycles continue to oscillate when light is turned off (*DD*) and *mPer1* mRNA is induced by a light pulse at subjective night which is followed by a phase shift in rhythmicity. **B** A 30-min light pulse induces *mPer1* gene expression in the SCN within 15 min and peak expression is reached at 60 min. (Redrawn from Shigeyoshi et al. 1997)

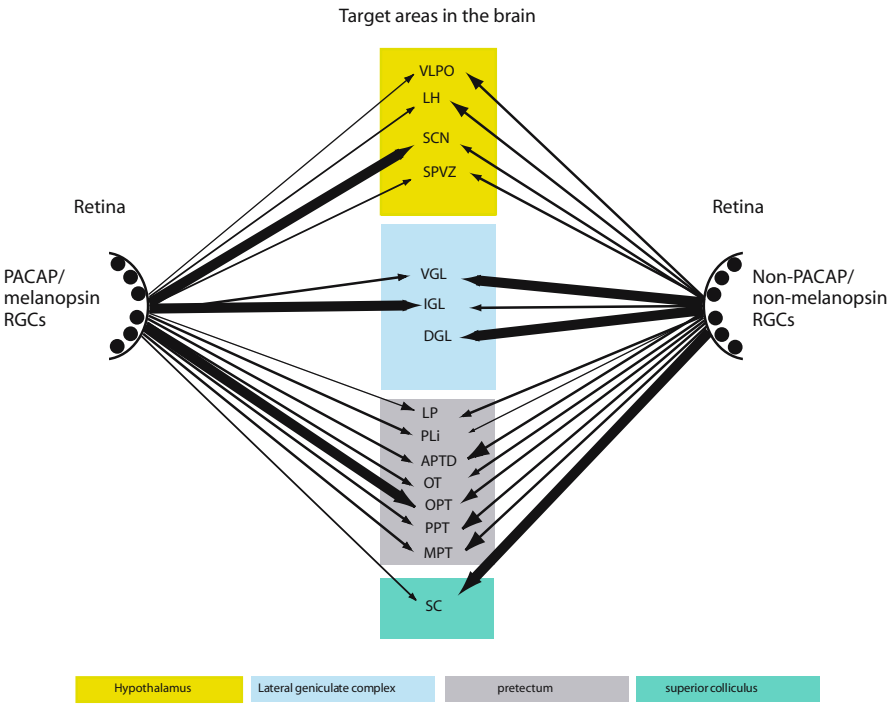
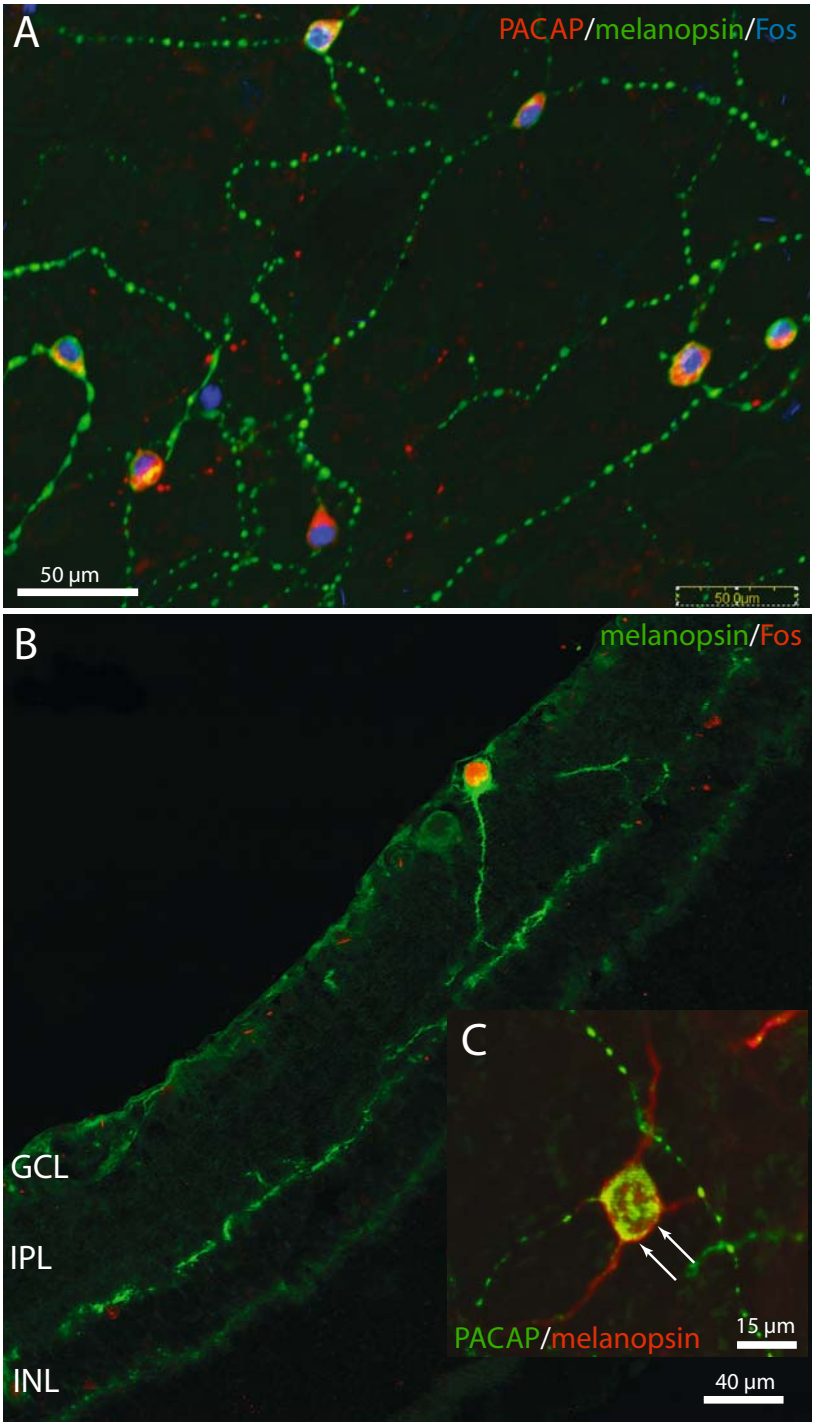


Fig. 7 Schematic diagram showing the target areas in the brain reached by projections from PACAP/melanopsin-containing retinal ganglion cells and from non-PACAP/non-melanopsin retinal ganglion cells. The density of the retinal projections is roughly indicated by thickness of the *arrows*. The retinal projections are mainly contralateral (for clarity the ipsilateral projections are not shown). For abbreviations, see text. (From Hannibal and Fahenkrug 2004a)

Fig. 8A–C Intrinsically photosensitive retinal ganglion cells (ipRGCs) of the rat RHT (shown in flat mount preparation in **A** and **C** and in cross section in **B**). The cells express the photopigment melanopsin (*green* in **A** and **B** and *red* in **C**) in the membrane of soma and dendrites (*arrows* in **C**) and the neurotransmitter PACAP (*red* in **A** and *green* in **C**). Light exposure induces the immediate early gene *c-fos* in the melanopsin/PACAP-containing ipRGCs (blue in **A** and red in **B**). For abbreviations, see text

Fig. 9A–C (on page 39) PACAP is stored with the anterograde tracer CTb in the mouse retinohypothalamic tract. Confocal microscopy of ChB (**A**) and PACAP (**B**) immunoreactivity in a coronal section through the mouse SCN. The retinorecipient SCN contains RHT projecting nerve fibers which store both CTb and PACAP as shown in merged images (**C**). *oc*, optic chiasm; *3v*, third ventricle


A
PACAP/melanopsin/Fos
50 µm
B
melanopsin/Fos
C
GCL
IPL
INL
PACAP/melanopsin
15 µm
40 µm

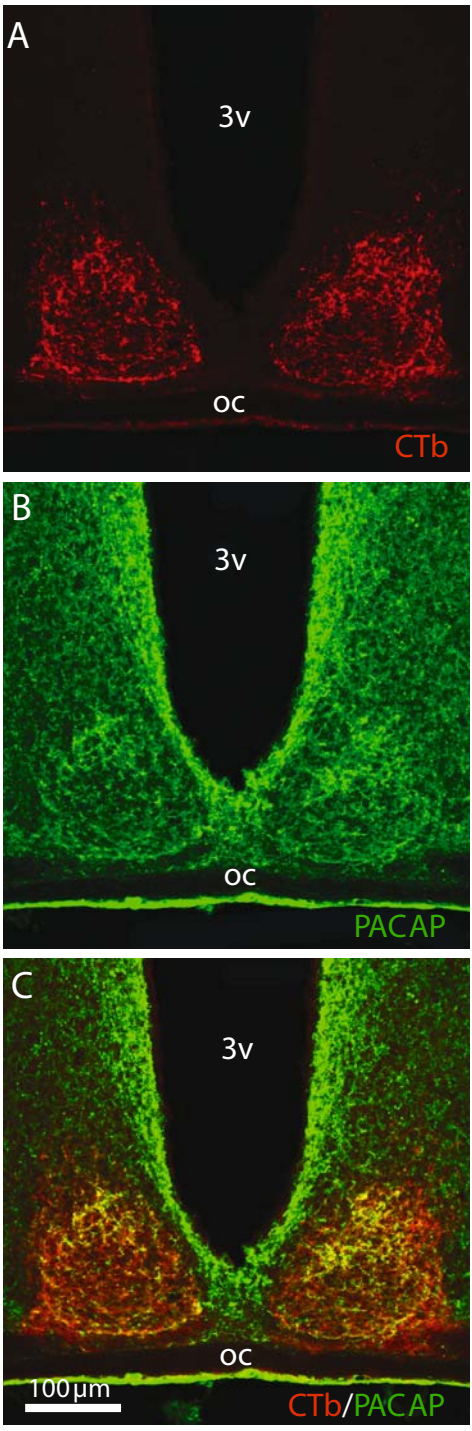
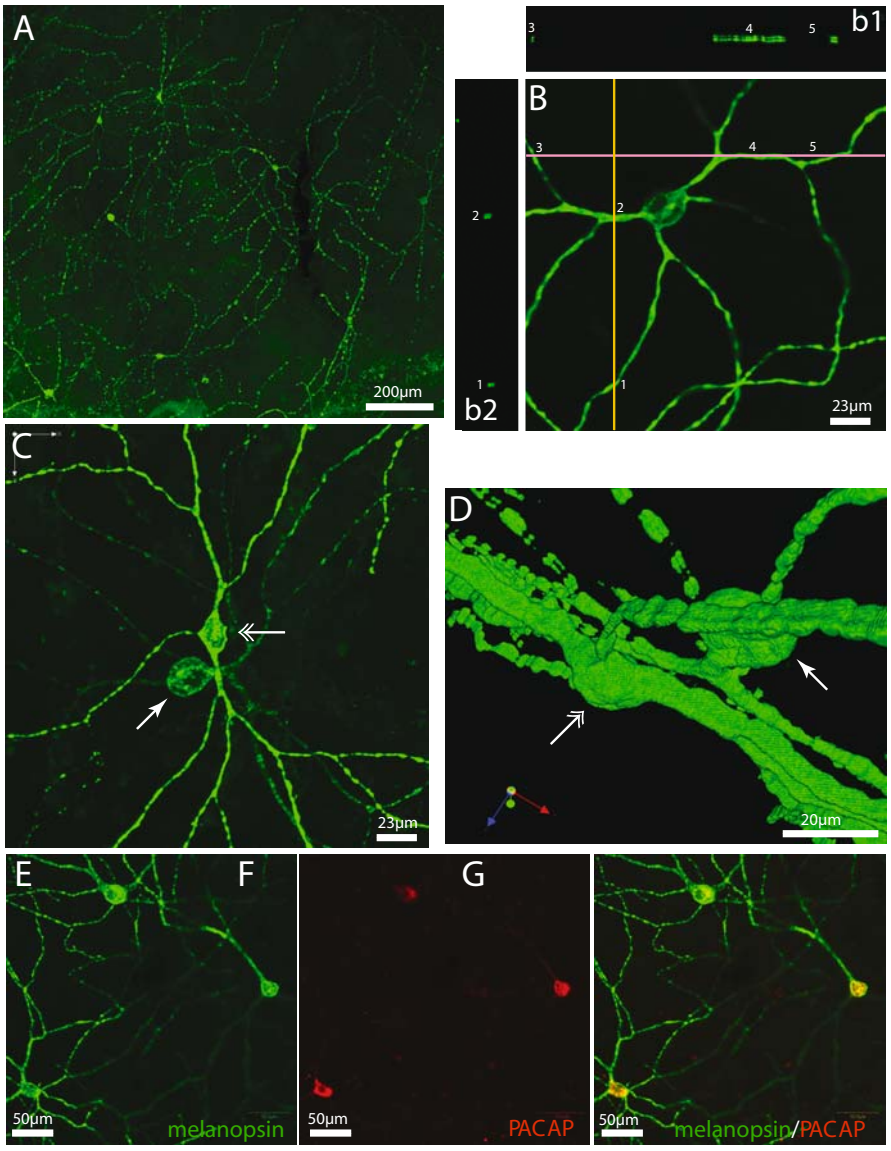
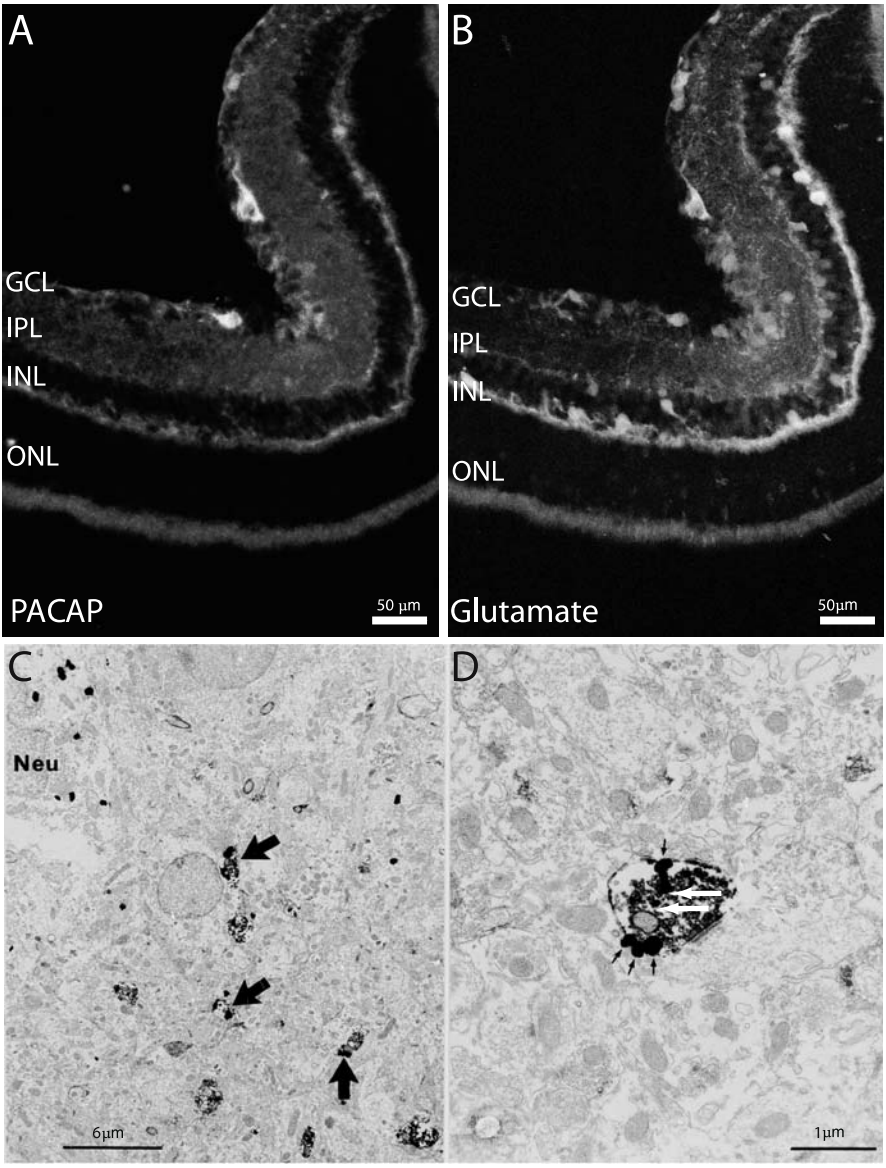

A
3v
oc
CTb
B
3v
oc
PACAP
C
3v
oc
100 µm
CTb/PACAP

Fig. 10A–G Melanopsin and PACAP immunoreactivities in the human retina. Confocal photomicrographs of melanopsin immunoreactivity in a normal part of the retina obtained from an eye removed due to malignant choroidal melanoma. **A** Melanopsin is found in a subset of retinal ganglion cells having a widespread dendritic network. **B** A single melanopsin-containing RGC analyzed in X–Y and X–Z planes. This image contains the extended focus of 181 sections of 0.2 μm thickness. **b1** shows a computer generated image in the X–Z plan corresponding to the *purple line* in **B**. The numbers (*1* to *5*) in **B** correspond to the same area marked by *numbers* in **b1**. Note the localization of melanopsin immunoreactivity in the dendritic membrane. A similar finding is demonstrated in **b2,** which shows the Y–Z plane corresponding to the *yellow line* in **B**. In **C** two melanopsin immunoreactive cells located in the ganglion cell layer (indicated by *arrow*) and in the INL (indicated by double arrow) are demonstrated. These two cells are reconstructed 3D in **D**. All the melanopsin-containing RGCs of the human RHT also store PACAP (melanopsin is shown in *green* in **E**, PACAP is shown in *red* in **F**, and the merged images in **G**)

Fig. 11A–D (on page 42) The neurotransmitters glutamate and PACAP are both stored in the RHT. Fluorescence photomicrograph showing double immunostaining of PACAP (**A**) and glutamate (**B**) in a sagittal section of rat retina. PACAP immunoreactivity is localized with glutamate in a subpopulation of glutamate-immunoreactive ganglion cells. Glutamate-positive cells are also located in the inner nuclear layer (*INL*) from which processes were observed to project toward the outer plexiform layer (in **B**). Electron micrographs (**C** and **D**) show double immunostaining for PACAP and glutamate in the suprachiasmatic nucleus of the rat. PACAP is visualized by horseradish-peroxidase-labeled antibodies with tyramide amplification. Glutamate is demonstrated using 1 nm gold-labeled antibodies. The gold particles have later been silver intensified in **C. A** Low-power electron micrograph of PACAP-immunoreactive nerve terminals. Several of these nerve terminals are also immunoreactive for glutamate. **D** High-power electron micrograph of a double-labeled nerve terminal making an axodendritic synapse. Several silver-intensified gold particles are indicated by *black arrows*, PACAP staining by *white arrows*


A
200µm
b1
3
4
5
B
3
4
5
2
2
1
b2
1
23µm
C
23µm
D
20µm
E
F
50µm
melanopsin
G
50µm
PACAP
50µm
melanopsin/PACAP


A
GCL
IPL
INL
ONL
PACAP
50 µm
B
GCL
IPL
INL
ONL
Glutamate
50µm
C
Neu
6µm
D
1µm

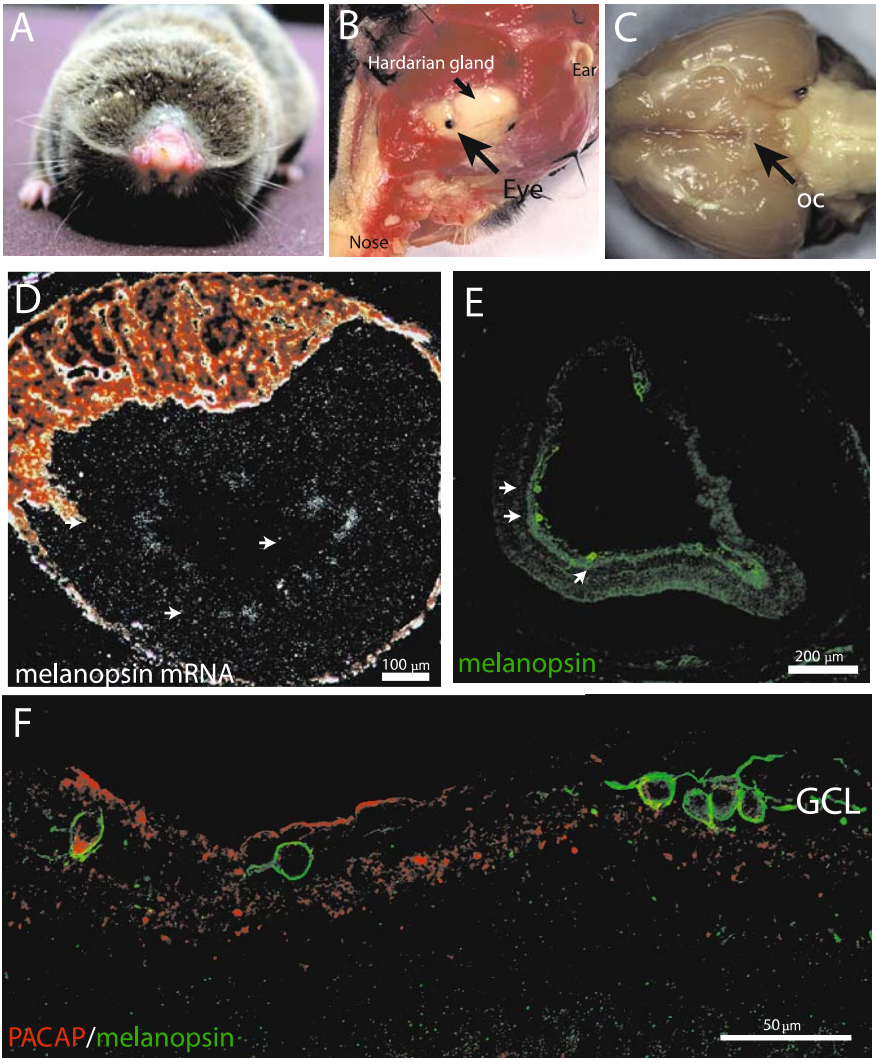


Fig. 12A–F The subterranean mole rat, *Spalax judaei* (Nevo et al. 2001), belongs to the superspecies *Spalax ehrenbergi,* (**A**). Despite severe degeneration of its subcutaneous eyes, it is able to adjust circadian rhythms to the environmental light/dark cycle due to a conserved RHT (the degenerated optic chiasma is indicated by an *arrow* in **C**). In **B** the skin of the head of the *Spalax* has been removed and the degenerated eye with a diameter of approximately 1 mm is seen in-layered in a hypertrophic Hardarian gland. Ganglion cells of the *Spalax* retina express the photopigment melanopsin mRNA (*arrows* in **D**) and melanopsin immunoreactivity is found corresponding to the mRNA positive RGCs (**E**). Melanopsin is expressed in retinal ganglion cells that also store PACAP, a neurotransmitter of the RHT (melanopsin immunoreactivity is shown in *green* in **F** and PACAP in *red*)

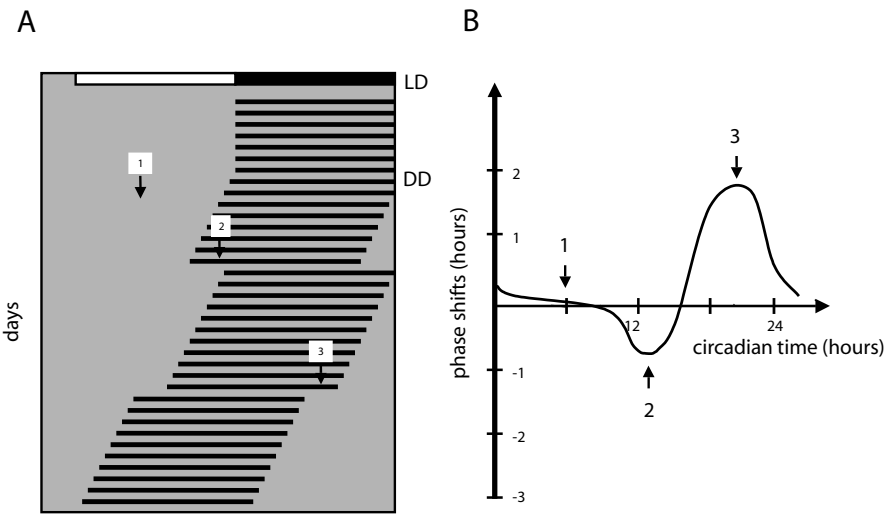


Fig. 13 A Schematic presentation of the activity rhythm of a nocturnal animal where *each horizontal line* represents the activity of the animal in one day (24 h). The animal is entrained to a light/dark photoperiod (*LD*) as represented *on the top of the record*. The animal is then released into constant darkness (*DD*) and the activity rhythm is now "free-running". During the free-running paradigm the animal experiences light pulses during subjective day (*1*), early subjective night, (*2*) and late subjective night (*3*). The light pulse given during the day has little or no effect on the phase of the endogenous rhythm. A light pulse given in early subjective night results in a phase delay of the overt rhythm (indicated by *2* in both **A** and **B**) and a light pulse given during late subjective night results in a phase advance of the overt rhythm (indicated by *3* in both **A** and **B**). A complete phase-response curve to light stimulation during a 24 h period is drawn in **B**. The phase delays are *plotted in the negative direction* and phase advances are *plotted in the positive direction*. The horizontal axis in **B** represents one circadian day. (Modified from Meijer and Rietveld 1989 and Daan and Pittendrigh 1976a)

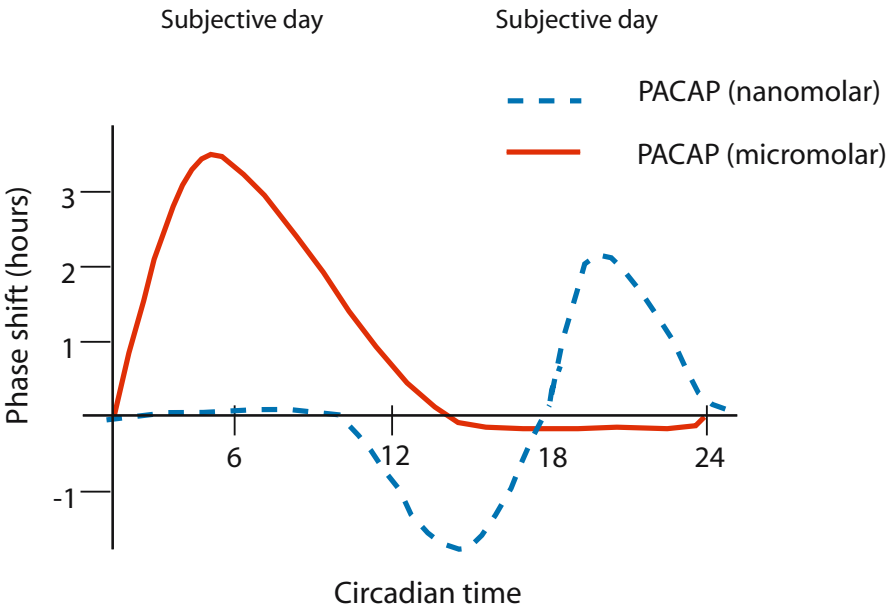


Fig. 14 PACAP phase shifts the circadian electrical firing rhythm in vitro in a concentration-dependent manner. In micromolar concentrations the effects of PACAP are similar to non-photic stimulation with induction of phase shift in subjective day but with no effects in subjective night (*red line*). In nanomolar concentrations PACAP induces phase shift similar to that of light with phase delays of the electrical firing rhythm during early subjective night and phase advances of the electrical firing rhythm during late subjective night (*blue line*). (Data are redrawn, based on Harrington et al. 1999, Hannibal et al. 1997, and Chen et al. 1999)

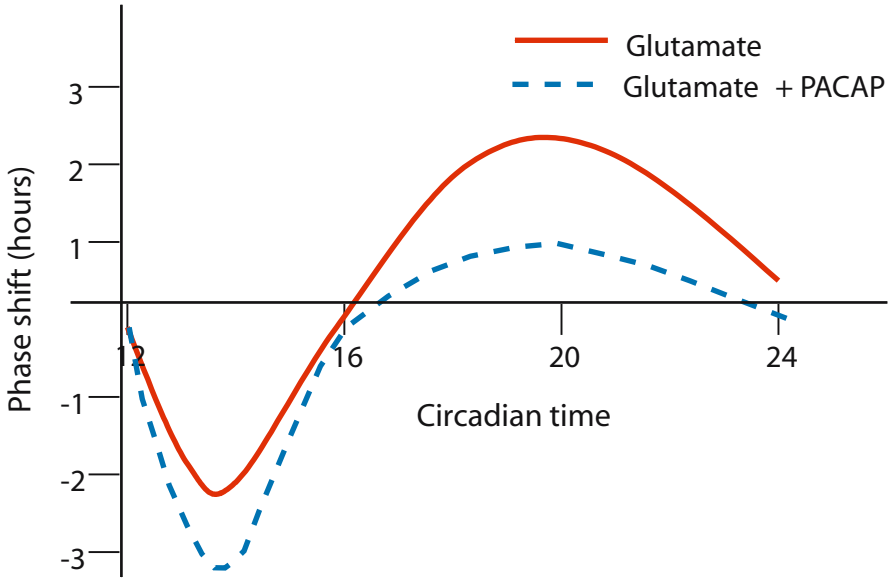


Fig. 15 Glutamate-induced phase shift of the circadian electrical firing rhythm in vitro is modulated by PACAP in micromolar concentrations. During early subjective night PACAP potentiates the effects of glutamate resulting in a larger phase delay than does glutamate alone. During late subjective night PACAP attenuates the glutamate-induced phase advance more than glutamate alone. (Data are redrawn based on Chen et al. 1999)

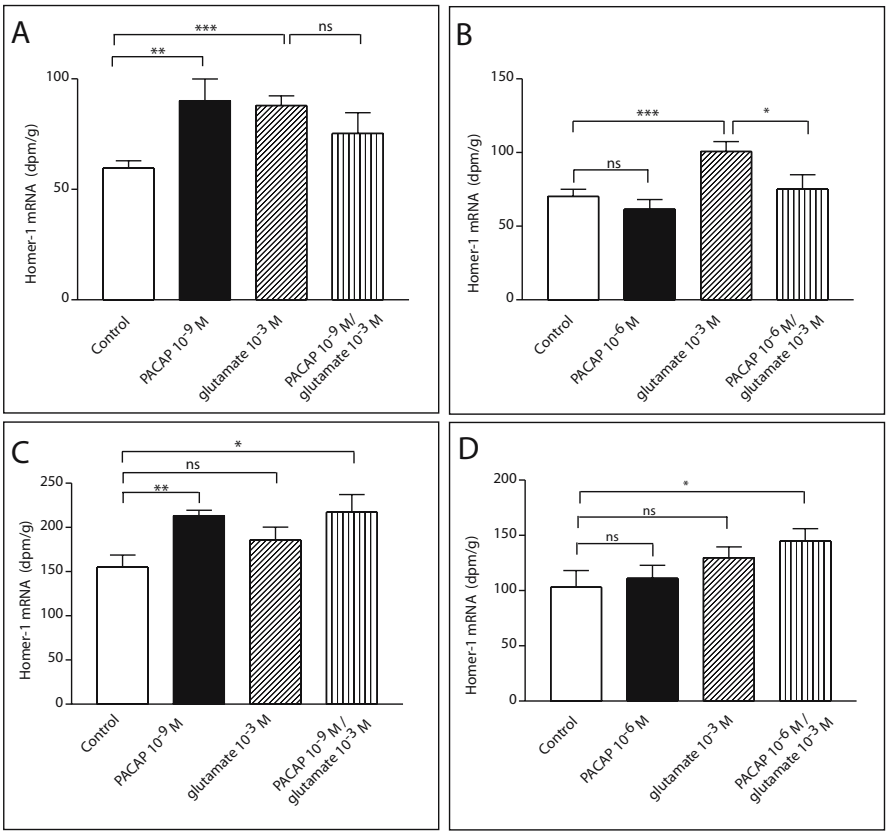


Fig. 16A–D *Homer-1a* gene expression is regulated by the RHT neurotransmitters glutamate and PACAP. Effects of PACAP and glutamate alone or in combination on r*Homer-1a* gene expression in early night (ZT14; **A** and **B**) and late night (ZT 19; **C** and **D**) in the rat SCN in vitro. SCN brain slices received either PACAP (*black columns*) 10^{-9} M (**A** and **C**) or 10^{-6} M (**B** and **D**), glutamate (10^{-3} M; *hatched columns*), or PACAP (10^{-9} M or 10^{-6} M) 10 min before glutamate (10^{-3} M; *lined columns*). Control slices received media (*white columns*) during the stimulation period. *Each bar* represents mean±SEM; (n=8–10), * P<0.05, ** P<0.005, *** P <0.001, *NS*=not statistically significant. (From Nielsen et al. 2002)

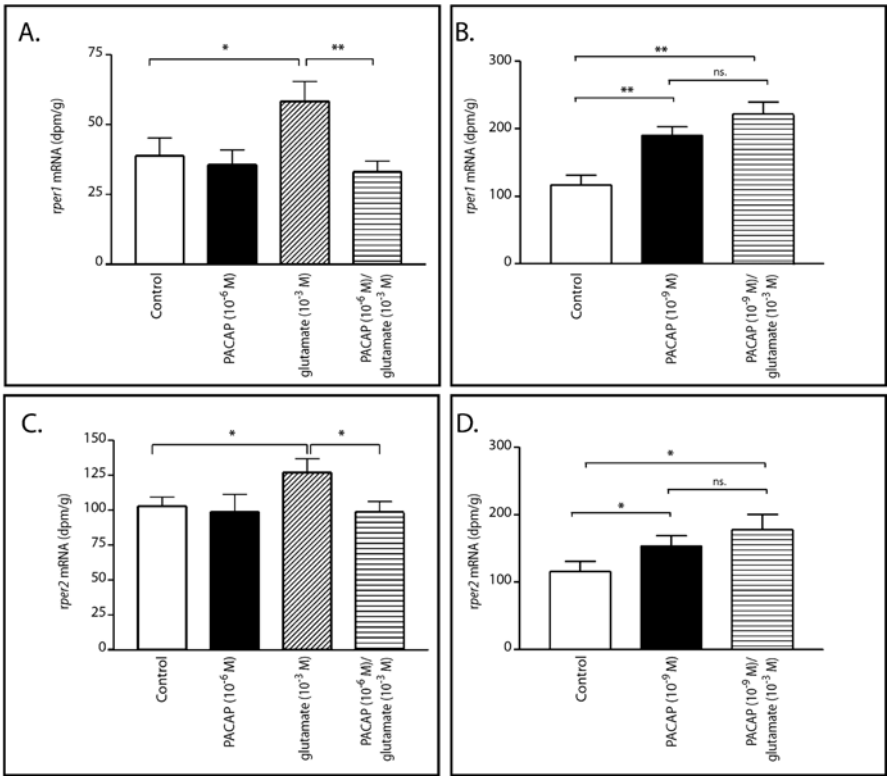


Fig. 17A–D PACAP modulates glutamate-induced *Per* gene expression in the SCN in vitro. Effect of PACAP on the glutamate-induced *rPer1* (**A**, **B**) and *rPer2* (**C**, **D**) gene expression at ZT19 in the rat SCN. SCN brain slices were treated with either PACAP (10^{-6} or 10^{-9} M; *black columns*) or glutamate (10^{-3} M; *hatched columns*) alone, or with PACAP (10^{-6} M or 10^{-9} M) applied 10 min before glutamate (*lined columns*). Control slices received media (*white columns*) during the stimulation period, **A**, **C**. PACAP (10^{-6} M) alone had no effect on *rPer1* and *rPer2* expression, whereas PACAP applied before glutamate completely blocked the glutamate-induced *Per1* gene expression in the SCN. In contrast, PACAP (10^{-9} M) significantly increased the level of both *rPer1* and *rPer2* mRNAs (**B**, **C**), and glutamate together with PACAP did not increase the mRNA level of *rPer1* or *rPer2* any further. *Each bar* represents mean ± SEM; (n=8–10 animals). *P<0.02, **P<0.01, *NS*=not statistically significant (Mann-Whitney U-test). (From Nielsen et al. 2001)

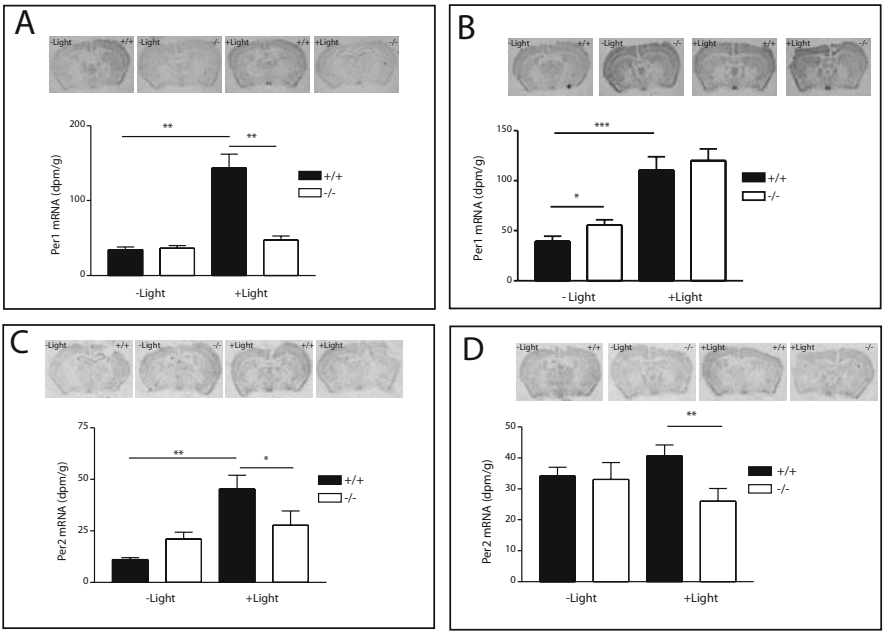


Fig. 18A–D Light-induced *Per* gene expression is altered in PAC1-receptor-deficient mice in early (**A** and **C**) and late subjective night (**B** and **D**). A 30-min light pulse (>300 lux) administrated at CT16 induces gene expression of *mPer1* (**A**) and *mPer2* (**C**) in the SCN of wild-type mice (*black bars*) whereas almost no effect was seen in*PAC1*$^{-/-}$ mice (*white bars*). Representative in situ hybridization signals for *mPer1* and *mPer2* in the SCN at each time point are shown *on top* of *each panel*. During late subjective night (CT23) a 30-min light pulse (>300 lux) induced *Per1* (**B**) gene expression of wild-type mice (*black bars*) and *PAC1*$^{-/-}$ mice (*white bars*), whereas a light-induced inhibition of *mPer2* gene expression in *PAC1*$^{-/-}$ mice was observed (**D**). Representative in situ hybridization signals of *mPer1* and *mPer2* in the SCN at each time point are shown *on top* of *each panel*. Values are given as means±SEM; (n=6–8 animals). *, P <0.05, **, P <0.01, *** P<0.001, (Mann-Whitney U test). *ns*=not statistically significant. (Modified from Hannibal et al. 2001b)

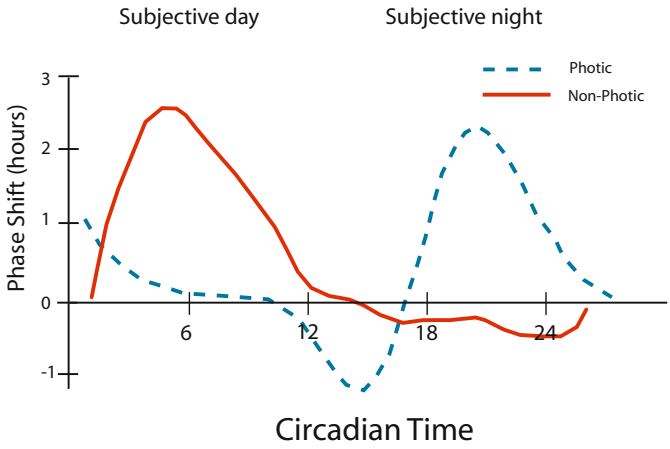


Fig. 19 Phase-response curve for photic (*blue*) and non-photic (*red*) stimulation. Light induces phase delays in early subjective night and phase advances in late subjective night. Non-photic stimulation induces phase advance during subjective day and small phase delays during late subjective night

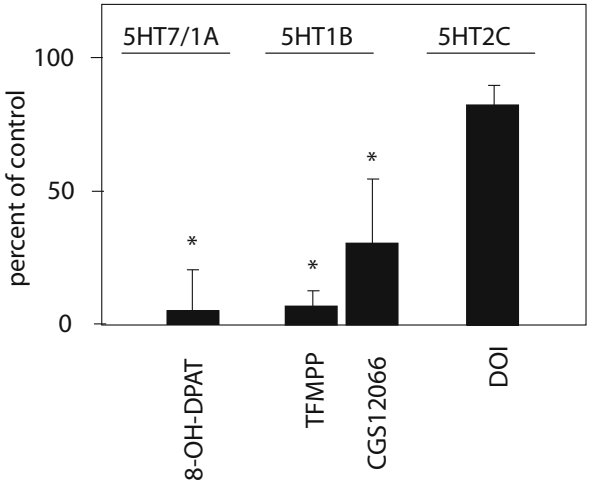


Fig. 20 Activation of 5-$HT_{1A/7}$ and 5-HT_{1B} receptors modulates light-induced phase advance. Effects are seen of systemic administration of selective serotonin receptor agonists on the magnitude of light-induced phase advance of the hamster activity rhythm. Data are expressed as percentage of the control (vehicle + light) values. For abbreviations, see list. (From Rea and Pickard 2000)

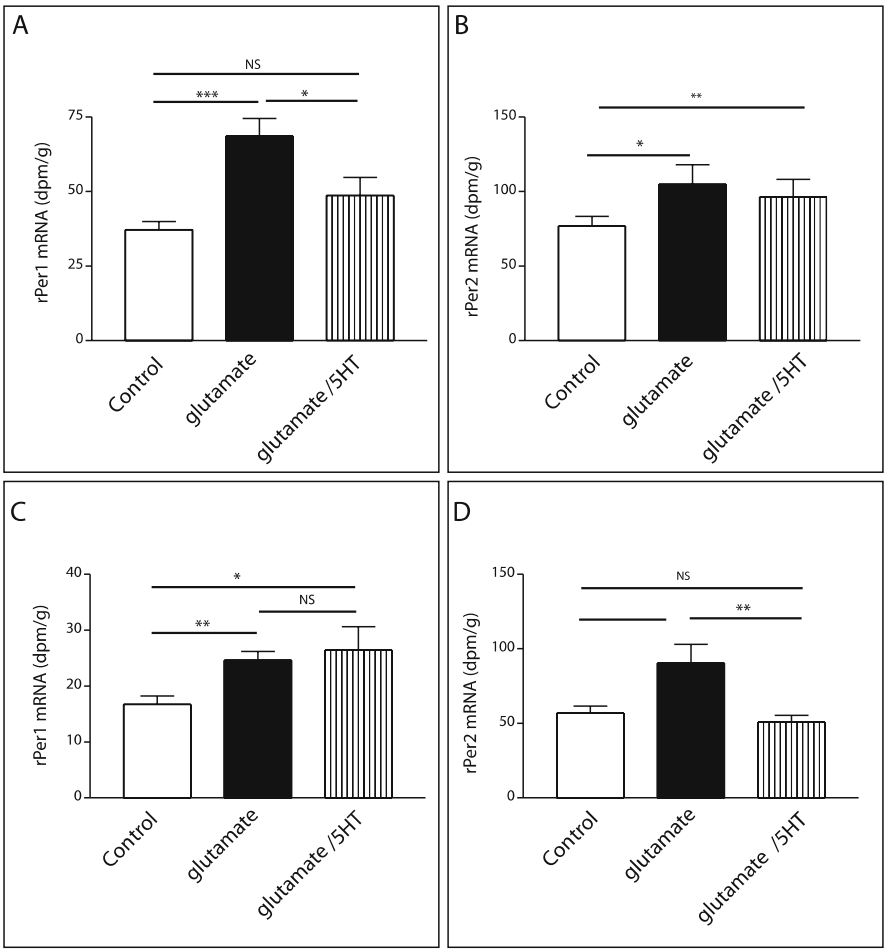


Fig. 21A–D Serotonin modulates the glutamate-induced *Per* gene expression in the SCN in vitro. Effect of 5-HT on the glutamate-induced *rPer1* (**A** and **C**) and *rPer2* (**B** and **D**) gene expression in early (*ZT 14*; **A** and **B**) and late night (*ZT 19*; **C** and **D**) in the rat SCN in vitro. SCN brain slices received either 100 µl of glutamate (5×10^{-3} M; *black columns*) for 10 min or 100 µl serotonin (10^{-6} M) 10 min before glutamate (*lined columns*). Control slices received medium (*white columns*) during the stimulation period. *Each bar* represents mean±SEM; (*n*=8–22). ; * $P<0.05$; ** $P<0.01$; *** $P<0.001$ (two-tailed Mann-Whitney test); *NS*=not significant. (From Sangaard et al. 2003)

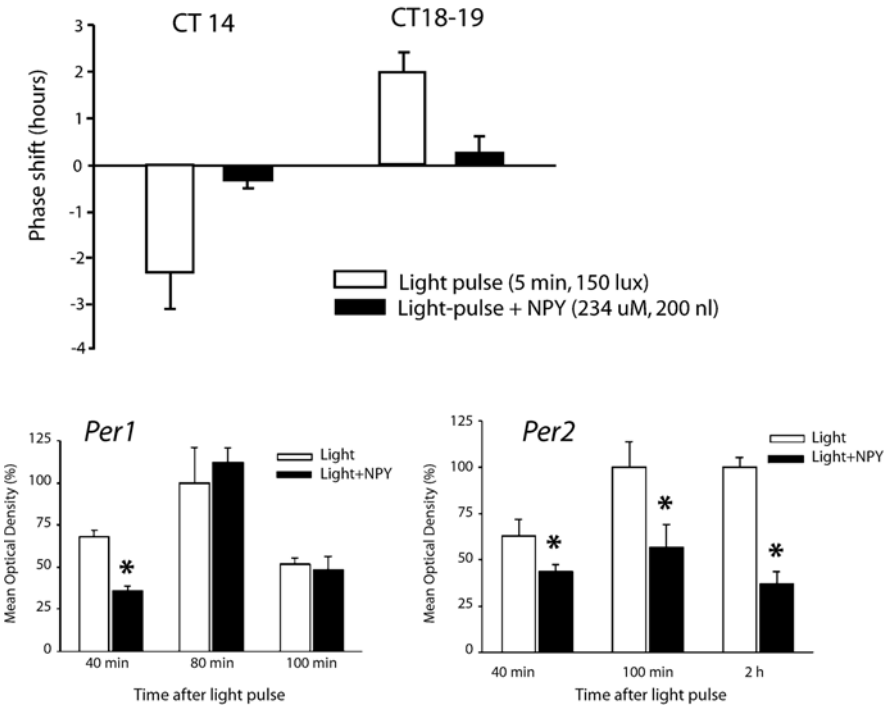


Fig. 22 Effects of NPY on light-induced phase shift and *Per* gene expression. *Upper panel* shows that NPY applied in vitro blocks the in vivo light induced phase shifts on the electrical activity of the hamster SCN as recorded in vitro (Yannielli and Harrington 2000). *Lower panel* shows that NPY applied to the hypothalamic slice preparation suppresses in vivo light induced expression of *Per1* and *Per2* mRNA in hamster SCN. (Adapted from Brewer et al. 2002)

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