Full-length review

The suprachiasmatic nucleus and the circadian time-keeping system revisited

Lisette (K.) E. van Esseveldt\textsuperscript{a}, Michael N. Lehman\textsuperscript{b}, Gerard J. Boer\textsuperscript{a,\textasteriskcommand}*

\textsuperscript{a}Graduate School Neurosciences Amsterdam, Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam ZO, The Netherlands
\textsuperscript{b}Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati College of Medicine, P.O. Box 670521, Cincinnati, OH 45267-0521, USA

Accepted 18 April 2000

Abstract

Many physiological and behavioral processes show circadian rhythms which are generated by an internal time-keeping system, the biological clock. In rodents, evidence from a variety of studies has shown the suprachiasmatic nucleus (SCN) to be the site of the master pacemaker controlling circadian rhythms. The clock of the SCN oscillates with a near 24-h period but is entrained to solar day/night rhythm by light. Much progress has been made recently in understanding the mechanisms of the circadian system of the SCN, its inputs for entrainment and its outputs for transfer of the rhythm to the rest of the brain. The present review summarizes these new developments concerning the properties of the SCN and the mechanisms of circadian time-keeping. First, we will summarize data concerning the anatomical and physiological organization of the SCN, including the roles of SCN neuropeptide/neurotransmitter systems, and our current knowledge of SCN input and output pathways. Second, we will discuss SCN transplantation studies and how they have contributed to knowledge of the intrinsic properties of the SCN, communication between the SCN and its targets, and age-related changes in the circadian system. Third, recent findings concerning the genes and molecules involved in the intrinsic pacemaker mechanisms of insect and mammalian clocks will be reviewed. Finally, we will discuss exciting new possibilities concerning the use of viral vector-mediated gene transfer as an approach to investigate mechanisms of circadian time-keeping. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Suprachiasmatic nucleus; Biological clock; Circadian rhythm; Clock genes; Vasopressin; Neurotransplantation; Adenoviral vector

1. Introduction

Since the discovery of the suprachiasmatic nucleus (SCN) of the hypothalamus as the site of the master circadian pacemaker in mammals, many scientists have tried to unravel the mechanism underlying its endogenous circadian rhythmicity. In particular SCN lesion and SCN lesion/neurografting in vivo experiments and in vitro SCN slice studies have provided firm evidence for its biological clock characteristics. Under intact conditions, its rhythm with a period length of approximately 24 h becomes apparent when an animal is kept under constant lighting conditions. Under daily light–dark (LD) conditions, the phase of the rhythm is adjusted and entrained to 24-h periods. Phase-shifts of the SCN pacemaker activity can be induced by light exposure; the direction and magnitude of the phase shift depends on the time point of light exposure during the circadian cycle. Light pulses given during the subjective day (circadian time CT0–12), have little effect, whereas light pulses during the start of the subjective night (CT12–14) will delay the clock, and light pulses at the end of the subjective night (CT18–24) will advance the clock. Other effective modulators of circadian rhythmicity are non-photic stimuli, such as circulating melatonin, locomotor activity and food availability. Some of these, like melatonin secretion and locomotor activity, have a circadian rhythm themselves and may play a role in feedback regulation of the clock.

Anatomical and physiological studies have provided a large quantity of data about the structural organization of the SCN, its transmitter and peptide content and afferent and efferent connections. Studies on circadian regulation of body functions and of phase shifting and entrainment of circadian rhythms to external cues, have resulted in an understanding of the input and output pathways of the SCN. Insights in the molecular mechanisms underlying the
pacemaking property of the SCN, however, have only recently been obtained using molecular techniques and the expanding knowledge about clock mechanisms in insect species.

This review will summarize how the different fields of neuroscience have contributed to the understanding of circadian pacemaker mechanisms of the SCN system. First, data concerning the anatomical and physiological organization of the SCN will be presented. Subsequently, it will discuss SCN grafting studies and the contribution of these transplantation results to knowledge of pacemaker function. Third, recent findings concerning genes involved in the intrinsic pacemaker mechanisms of insect and mammalian clocks are summarized, focusing on the possible clock mechanisms in the mammalian SCN. In the fourth and final part of this overview, the application of viral vectors for gene transfer and their possible new and future contribution to elucidation of circadian time-keeping mechanisms will be discussed.

2. Anatomy and physiology of the suprachiasmatic nucleus

2.1. Cellular organization of the SCN

The SCN is a paired nucleus, localized adjacent to the third ventricle and on top of the optic chiasm. The rat SCN contains about 16,000 small and tightly packed neurons [357]. Based on morphological differences, the SCN can be divided in a small rostral area and a large caudal area with a dorsomedial and ventrolateral part. The morphology of the SCN cells and their organization in the different parts of the rat SCN has been studied in detail by Van den Pol [357]. The dorsomedial part of the caudal SCN (dmSCN), also called the ‘shell’ of the SCN [188], is characterized by small elongated neurons with large nuclei and few cell organelles. The neurons are tightly packed and interconnected via somato-somatic appositions, resulting in chains of neurons, arranged in an antero-posterior direction. The ventrolateral part of the SCN (vlSCN), or ‘core’ SCN [188], is characterized by a lower density of spherical neurons which have a organelle-rich cytoplasm. The neuronal somata tend to be separated by glial cells, which completely enclose the synaptic junctions within the vlSCN [132], and communicate with each other through gap junctions [357,383]. Though morphological studies failed to show neuronal gap junctions in the SCN [357], tracer and electrical coupling of neurons have shown a low resistance communication pathway to exist [164]. In the SCN, complex synaptic arrangements have been found with axo-somatic contacts, as well as with dendro-dendritic appositions [132], which might, together with the somato-somatic appositions particularly in the dmSCN, enable cell–cell communication within the SCN by means of so-called ephaptic interaction [357]. Because of the large regions of cell–cell appositions and small extracellular volume in the SCN, changes in the composition of the extracellular fluid can alter the probability of firing of the SCN neurons.

Two different forms of possible ephaptic interactions are proposed [357]: when the membrane resistance is low, an action potential in one cell may either directly elicit an action potential in the apposed neuron, or it may change the extracellular ion and/or neurotransmitter concentrations so that it influence the excitability of the apposed neuron [358]. A role for astrocytes in synchronization of the SCN neurons has been mentioned in this respect. Astrocytes can regulate extracellular ion levels, particularly potassium and calcium levels, by their response to neurotransmitters [40,356]. Glial cells can also release substances, like nitric oxide or arachidonic acid, that influences neurons. Nitric oxide, for instance, has been shown to phase-shift SCN neurons in a slice preparation [95], and arachidonic acid reduces glutamate uptake from the extracellular space [22,358]. The possible importance of the astrocytes in functioning of the SCN is further strengthened by the finding that functional interference with gap junctions or glial metabolism can disrupt circadian rhythmicity of the SCN [270].

The proposed non-synaptic cellular communication within the SCN may provide an explanation for the synchronization of the activity of individual SCN neurons, which, following in vitro isolation, show an individual circadian rhythm in spontaneous electrical activity [37,382]. It is however not yet known to what extent ephaptic interactions, gap junction communication, synaptic transmission, or even neurohumoral signals from adjacent cells are involved in synchronizing SCN neuronal activity. Synchronization of the SCN neurons, both in vitro and in vivo, takes place in the absence of sodium-dependent synaptic transmission [39,98,301,315,382]. The application of tetrodotoxin to the SCN, for instance results in a transient arhythmicity of drinking behavior, without effect on the phase of the rhythm [304]. In SCN slice preparations kept under tetrodotoxin, LD differences in membrane properties are maintained [91]. Defined subpopulations of SCN neurons in slice preparations reveal a circadian rhythm in membrane potential and input resistance parallel to their rhythm in spontaneous firing, but a rhythm in membrane potential remains after inhibition of cell firing. Apparently, synaptic transmission between SCN neurons is not necessary for the immediate continuation of a synchronized rhythm in the SCN, but is necessary for the SCN to impose its rhythm on the animals’ (drinking) behavior.

2.2. Neuropeptide and neurotransmitter content of the SCN

The clear differences in morphology and arrangement of the neurons in the dmSCN and vlSCN suggest that these
parts play different roles in the generation and regulation of circadian rhythmicity. This notion is supported by the partially different neuropeptide content in these subareas. In the rat, most of the neurons in the dmSCN synthesize vasopressin (VP) [341,359,368], whereas neurons in the vlSCN synthesize vasoactive intestinal polypeptide (VIP) (Fig. 1), peptide histidine isoleucine (PHI) and/or gastrin releasing peptide (GRP) [65,66,232,326]. A smaller proportion of somatostatin (SOM)-producing neurons is found in between these two cell populations [66]. Other substances that have immunocytochemically been reported to be produced in perikarya of rat SCN neurons include angiotensin II [27,174,207], calcitonin gene-related peptide [256], corticotrophin releasing factor [87], dynorphin [400], enkephalin [296], galanin [328], substance P [231], thyrotrophin-releasing hormone [189], and VGF [253]. In the hamster SCN, moreover, a localized expression of calbindin-D_{28k} [324] and cholecystokinin (CCK) [228] is reported.

Several neuropeptides have been reported to colocalize in SCN neurons: VP colocalizes for instance with enkephalin [296], and dynorphin [375], while VIP and PHI, which are derived from a common precursor molecule, colocalize with GRP [252], and VIP colocalizes with corticotrophin releasing factor [87]. SOM does not colocalize with either VP or VIP [348], and thus marks a distinct cell group. γ-Amino-butyric acid (GABA) is present in most if not all SCN neurons [238], and thus colocalizes with all neuropeptides mentioned above.

2.2.1. γ-Amino-butyric acid

The abundant presence of GABA and GABA receptors suggests a prominent role for this transmitter in the SCN. Levels of mRNA of glutamic acid decarboxylase (GAD), the enzyme converting glutamate into GABA, shows a circadian rhythm in the SCN [151], and both GABA levels and GAD activity show a circadian fluctuation under light–dark (LD) and dark–dark (DD) conditions in dissected SCNs [7]. Administration of the GABA antagonist bicuculline abolished almost all inhibitory postsynaptic potentials [175] and elevated the mean discharge rate of SCN neurons in vitro during subjective daytime, indicative of a tonic inhibition by GABA under normal day conditions [218]. Application of GABA to the SCN gave however conflicting results. A diurnal action of GABA was reported with an inhibitory action at night and an excitatory action at day time, which was proposed to be caused by an oscillation in the intracellular chloride

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Fig. 1. Cytoarchitecture of the rat SCN showing the complementary distribution of VIPergic and VPergic neurons in the ventrolateral (vl) and dorsomedial (dm) aspects of the SCN. The VIPergic neurons are shown to receive the main input signals of the retinohypothalamic and the geniculohypothalamic tracts. Immunocytochemical staining for VIP and VP on near-adjacent 50 μm vibratome coronal sections. The VP staining photograph has been mirrored to emphasize the bilateral aspect of the SCN. III third ventricle, OC optic chiasm, bar 50 μm.
concentration [374]. An intrinsic rhythm in chloride levels in SCN neurons would thus form the basis for this dual action of GABA. This effect of GABA was however not found by other groups, who report an inhibitory action of GABA independent of the time of the day [209,210,218,249], in particular also when during L- and D-phase the same neuron is recorded for GABA responses [128].

2.2.2. Vasopressin

Although VP is the most extensively studied neuropeptide of the SCN, its precise role in the SCN is still unclear. VP levels [351,391] and VP mRNA levels [55,283,355] cycle in the SCN under both LD and DD conditions with peak levels during the L-phase of the circadian rhythm. The size of the VP mRNA poly(A) tail also shows a circadian variation in rats housed under LD conditions (Fig. 2), and in SCN explants kept under constant conditions [67,286]. This variation in poly(A) tail length implies a variation in mRNA stability [42,393], and translation efficiency [255], and is thus likely to contribute to the circadian rhythm in VP production in dmSCN neurons. The VP V1a receptor (V1aR), located on both VPergic and VIPergic SCN neurons, has also been shown to express a diurnal rhythm under LD conditions [397]. However, since both the rhythmicity in the size of the VP mRNA and the expression of the V1aR were only investigated under LD conditions, the rhythmicity of these parameters might have been imposed by the light cycle. Under LD, the expression of V1aR is 12 h out of phase from that of VP, with peak levels around midnight [397], probably accounting for the higher percentage of SCN cells that can be activated by VP in vitro during the night than during the day [208].

Despite its abundant expression in the SCN, a crucial role for VP in rhythm generation is not shown. It is for instance disputed by the near normal circadian sleep–wake rhythms [88] and motor and drinking activity rhythms in the VP-deficient Brattleboro rat [45,130,260]. The VP deficiency causes however slight differences in the amplitude and the entrainability of the rhythms [45,243,244] and the period length [130], more supporting a modulatory role for VP. For instance, the lower amplitude in the rhythm of electrical activity and mutant VP mRNA levels in Brattleboro SCN neurons may account for the lower amplitude of several behavioral rhythms [130]. Also studies in the CLOCK (CLK) mutant mouse (see Section 4.3.2) failed to show a primary control of VP on rhythm generation. This clk−/− mouse expresses a free-running period of locomotor activity shorter than wild type and becomes arrhythmic after 2–3 weeks when placed in DD [370]. Both VP peptide and mRNA levels in the SCN are low and non-rhythmic in LD [325]. Hence, VP circadian expression is absent in the presence of behavioral rhythm, though not under determined under DD (free-run) conditions. Finally, the tau mutant hamster with changed, not absent, periods and unusual phase-shifting responses to light, had significant lower peak levels of VP (and VIP) mRNA in the SCN [299].

A modulatory rather than a rhythm-generating role of VP expression has also been proposed on the basis of results of in vitro studies. Application of a VP antagonist on SCN cells in vitro resulted in a decrease in activity of about half of the VP-responsive neurons. The magnitude of this decrease in activity was moreover phase-dependent [230], suggesting a circadianly regulated tonic excitatory input to these neurons. This is again supported by the lower mean basal firing rates of SCN neurons of the Brattleboro rat [155]. Application of VP itself to SCN cells in vitro resulted in an increase of firing rate in about 50% of the neurons, which was moreover comparable between Brattleboro and Wistar SCN neurons [155,229]. Notably, the VP-responsive neurons showed a greater variation in basal activity between the L- and D-phase compared to the non-responsive neurons [155].

Although VP may be dispensable for the expression of circadian rhythmicity within the SCN, several studies show that the presence of the neurons that express VP is crucial. Elimination of the VPergic neurons reached by intracerebroventricular application of the immunotoxin, anti-VP antibody-ricin A conjugate, for instance resulted in the disappearance of circadian drinking rhythmicity of rat [318]. Several studies reported, moreover, a correlation between the number of VPergic neurons in the SCN and the strength and consistency of circadian activity rhythms. In different rat strains and mice selected for differences in nest-building behavior, the animals with the highest number of VPergic neurons in the SCN had the strongest unimodal activity pattern, whereas the animals with the lowest numbers of VPergic neurons had the weakest multimodal activity pattern [48,49,388]. The latter finding suggests that VP is involved in synchronization of activity of the SCN neurons. The reverse relation was however
reported for common voles, where the animals with the highest number of VPergic neurons had no circadian activity rhythms [119]. This was therefore speculated to result from a reduced VP release, rather than an increased VP synthesis [119]. Since rat strains with the weakest rhythm also had the highest amount of VPergic fibers in the SCN (structures from which VP is released), it may be important to study synthesis and release in relation to differences in the strength of the rhythms.

All these studies indicate the importance of the VPergic neurons for the overt behavior of rodents, but it is not clear whether VP plays a role in the generation of the rhythm itself, or serves as a modulator influencing the type and amplitude of circadian output signals [146,227,377]. In conclusion, several anatomical and physiological findings illustrate the importance of the VPergic SCN neurons for circadian rhythmicity, but the role of VP is still unclear. It is conceivable therefore, that another factor colocalized in the VPergic neurons might play a more important role in the generation and/or propagation of the endogenous circadian rhythmicity of the SCN.

2.2.3. Vasoactive intestinal polypeptide

Levels of VIP mRNA and protein show a diurnal rhythm in SCN neurons of the vlSCN under LD conditions with peak levels during the night, but remain constant under DD conditions [239,250,322,345], suggesting that VIP is not necessary to convey the rhythmic signal of the SCN to target areas and that light entrains the VIP mRNA rhythm. In the postnatal rat SCN however a circadian rhythm in VIP mRNA is also observed in DD [21]. When the SOM content of the adult rat SCN is experimentally depleted, a VIP rhythm emanated also under DD conditions with a peak during the subjective day (CT6) and a nadir during the subjective night [113]. Apparently, the elimination of the SOM message allows an endogenous VIP rhythm to appear in DD. A direct synaptic effect of SOM through SOM receptors in the vlSCN [43,86,217] might underlie its levelling effect on the VIP rhythm, since activation of these receptors leads to inhibition of VIP-stimulated cAMP accumulation [96] and modulation of potassium and calcium channels [156]. On the other hand an indirect action of SOM on VIP neurons (and the VIP rhythm), allowing them to become sensitive for the rhythmic activity of other SCN neurons, however, cannot be excluded. The absence of a rhythmic VIP mRNA and protein expression under DD conditions when mammals retain a circadian rhythm of behavior and the induced rhythm under LD conditions, suggest that VIP release does not play a crucial role in rhythm generation within the SCN. It is however very important for the translation in the SCN of external cues, such as photic input.

VIP2 receptors are abundantly expressed in the rat SCN and a comparable biphasic pattern can be observed in LD and DD with peaks at CT10 and CT22 [54]. The expression of VIP2 receptors is thus under control of the pacemaker system in a different way than that of VIP. Their abundant presence support a role of VIP within the SCN and their phase-related expression supposes a phase-dependent actions of VIP to occur.

The circadian expression pattern of PHI in the SCN has not been investigated, but transcript levels of propro-VIP/PHI, the precursor of which VIP and PHI are derived, show a diurnal rhythm under LD with night-time peak levels [11].

2.2.4. Gastrin-releasing peptide

Levels of GRP, which is found to be colocalized with VIP/PHI in the vlSCN, also show a diurnal rhythm under LD conditions, which is however 12 h out of phase with the VIP rhythm [250]. The ratio of VIP/PHI to GRP available for release from SCN neurons thus varies over the day–night cycle, and might act as a timing signal. This notion is supported by in vivo studies, which show that a microinjection of a cocktail of VIP, PHI and GRP in the SCN, but not of the single peptides, results in phase delays when applied at the beginning of the activity period [10]. A second study shows, however, phase delays and advances after a microinjection of GRP or VIP alone, and after an injection of a cocktail of VIP, PHI and GRP [266]. Since VIP and GRP neurons also project outside the SCN, the ratio of these peptides might communicate information about the LD cycle to areas outside the SCN.

2.2.5. Somatostatin

Like for VP, the circadian expression of SOM in the SCN during LD remains present under DD conditions [114,320]. SOM marks a separate group of cells located in the ventral aspect of the dmSCN, more or less located in between the large pools of VPergic and VIPergic neurons [348]. These latter neurons are shown to function as the efferent system of the SCN (see Section 2.4), whereas the innervation field of the SOMergic neurons is confined within the SCN [86] with its receptors mainly present in the vlSCN [43]. They serve therefore a role within the SCN, perhaps on other cells of the ventrolateral aspect of the SCN, that is still not known. The inhibitory role of SOM modulating the rhythmic VIP expression [113] supports such an intrinsic role. The function in the rhythm system of this subtype of neurons is still not elucidated. The high degree of connectivity with other SCN neurons suggest an important role.

2.3. Input pathways to the SCN

2.3.1. Three main input pathways

Based on the afferent input to the vlSCN, this part is generally thought to act as a relay station, integrating the various inputs to the SCN. Neurons in the vlSCN or core SCN, thus mostly the VIPergic neurons, receive glutamatergic input from the retina via the retina-hypothalamic tract (RHT) [237], neuropeptide Y (NPY) input from the
intergeniculate leaflet (IGL) of the lateral geniculate nucleus (LGN) via the geniculo-hypothalamic tract (GHT) [343], and serotonergic (5-hydroxytryptamine, 5-HT) input from the raphe nuclei [38,111]. Neurons in the dmSCN or shell SCN receive modest non-photic input from the cortex, basal forebrain and hypothalamus [235].

The RHT conveys information about the external lighting conditions to the SCN, as well as to adjacent areas of the SCN and to other nuclei in the brain [240]. The exact projection area in and around the SCN reveals inter-species variability. The main target in the rat SCN is the ventrolateral division, although the dmSCN also receive retinal input from retinal ganglion cells that send collaterals to the IGL [261,353]. Postmortem tracing studies in human have identified a RHT which mainly innervated the anteroventral SCN [84]. Circadian rhythms have been described at different levels of the photic input pathway to the SCN. The outer segments of the retinal photoreceptors, for instance, undergo a periodic shedding of membrane from their distal tips even under DD conditions [125,186]. Pacemaker activity has also been reported in the rodent retina, evidenced by a circadian release of melatonin from cultured neural retinas. These rhythm could be entrained by LD cycles and were free-running in under DD conditions [352]. This rhythm is thus independent of the rhythm generated by the SCN and might modulate entrainment of the SCN to light by regulating its retinal input. Processes have been reported that indeed act to modulate the retinal input [93,317]. Moreover, a circadian rhythm has been described in the number of RHT synapses in the SCN. Presynaptic receptors for BDNF (brain-derived neurotrophic factor) have been described on RHT terminals [204]. The diurnal rhythm in BDNF content in the cells in the vlSCN was subsequently proposed to induce a diurnal rhythm in synaptic function of the RHT terminals on the SCN neurons [205]. The serotonergic input was also shown to influence the photic input to the SCN via 5-HT receptors on the retinal axon terminals [265]. Such processes might be the basis of the diurnal sensitivity of the SCN to phase shifting effects of light (see Section 2.3.1).

A second indirect pathway through which photic information reaches the SCN, is formed by a projection of the retina to NPY-immunoreactive neurons in the IGL, which project through the GHT to the SCN. A circadian variation has been reported for levels of NPY in the vlSCN under LD conditions, with two peaks corresponding to the L-to-D and D-to-L transitions, while under DD conditions, the NPY rhythm is virtually absent [63,321,322]. The overlap in SCN projection areas of the RHT and the GHT [337] suggest that the IGL is involved in modulating the responses of the circadian system to light. Important in this respect is the finding that the IGL and the SCN are innervated by the same set of retinal ganglion neurons [261,353], and that the retinorecipient neurons in the SCN are more likely to respond to application of NPY than the other neurons [314]. Application of NPY antiserum to the SCN was indeed able to enhance the phase advance in response to a light pulse at CT18 [25]. Besides conveying photic information to the SCN, the IGL and GHT are also involved in conveying non-photic information (see Section 2.3.2).

The serotonergic input from the median raphe to the SCN, represents the third main input pathway to the SCN. The 5-HT content of the SCN also exhibits a circadian rhythm. Peak 5-HT level was found during the light period in LD conditions. Under DD conditions, the rhythm appeared to be differently phased, with peak 5-HT levels around CT16 [53]. The activity of the 5-HT input to the SCN may therefore regulated both by the SCN and by photic input. Manipulations of the brain 5-HT system, for instance by chemical depletion or agonist application, affect the phase and period of circadian locomotor rhythms in rat and hamster under LD or constant lighting conditions [81,143,241]. Depletion of 5-HT under LD conditions through systemic injections of p-chlorophenylalanine moreover results in a reduction of the L-phase nadir levels of VIP mRNA, but has no influence on the D-phase peak mRNA levels [173]. These data are consistent with peak 5-HT activity during the day time. The effect of 5-HT depletion on VIP mRNA levels might be explained by a modulation of retinohypothalamic neurotransmission at two different levels: by presynaptic 5-HT1B receptors on the RHT axons in the SCN, influencing the glutamate release, or by postsynaptic 5HT1A or 5HT2 receptors on the SCN neurons [263].

The proposed effect of 5-HT on the light-induced glutamate release from RHT terminals might underlie the finding that manipulations of the 5-HT system change light-induced phase shifts. Destruction of the 5-HT system results in a 50% increase in size of phase delays after a light pulse given during mid-subjective night [41], whereas stimulation of the median raphe, or application of the 5-HT precursor tryptophan, or of 5-HT agonists results in an inhibition of light-induced phase delays and phase advances [123,264,265,380]. The reported serotonergic modulation of retinohypothalamic neurotransmission is achieved via postsynaptic 5-HT5 receptors on the SCN neurons and via presynaptic 5-HT receptors on retinal axon terminals in the SCN [262,263]

### 2.3.1. Photic phase shifts

As light is the main entraining factor of the SCN, its effect on the SCN and its output has been extensively studied. The signalling pathway from the retina to the SCN comprises the activation of photoreceptors in the retina and a glutamate release in the SCN from terminals of the RHT [57,211] and subsequent resetting of the pacemaker. Activation of glutamate receptors results in a calcium influx in the SCN neurons, leading to activation of calcium-dependent kinases, proteases and transcription factors, amongst others [390]. Recently, controversial new data has suggested that, in addition to the RHT, extraocular
photoreceptors are capable of mediating photoentrainment in humans [216]. Specifically, it was reported that exposure of the human knee joint for 3 h to a light source of 50 lux resulted in phase shifts of body temperature and melatonin rhythms. However, a subsequent study found that a similar light pulse of a much higher intensity (14,000 or 67,500 lux) applied to the back of the knee was unable to suppress night time melatonin levels. Thus, at present, the existence of extraocular photoreceptors by which light information may entrain the SCN of humans and other mammals remains to be confirmed.

Light-induced phase shifts (Fig. 3) are marked by an induction of immediate early gene (IEG) expression, of which C-fos has most extensively been investigated [17,76,99,181,276,290]. This induction of IEG expression is mediated through NMDA receptors, since blockade of these receptors blocks C-fos induction [3,102,278]. A NMDA receptor-mediated phosphorylation of the cAMP response element-binding protein (CREB) was proposed to play a role, since CREB phosphorylation has been found to occur simultaneously with C-fos induction [121], whereas the C-fos promoter contains a CREB-responsive element [312]. A specific role for C-fos mRNA induction in the phase-shifting effect of light was inferred from its localiza-

tion in the retinoreipient area of the SCN, the vLSCN [71,277,288,324], as well as the resemblance between the circadian timing and light sensitivity of both processes [181,182]. C-fos induction seems to be a prerequisite for phase shifting by light, since the intracerebral application of antisense oligonucleotides for C-fos was shown to block light-induced phase shifts in the wheel-running rhythm of rats [389]. However, the increase of C-fos levels per se does not necessarily lead to phase shifting [77].

Several other neurotransmitters, like 5-HT [41,122,212,233,264,265,272] and substance P [2,316] have in addition been reported to influence the C-fos induction and light-induced phase shifting. A role for GABA in the regulation of photic input is illustrated by the finding that GABA_A and GABA_B agonists applied in vivo can block the photic induction of C-fos in the SCN [77], and reduce both light-induced phase advances and delays [120,275]. A direct effect of the neurotransmitters on the expression of C-fos is however questionable. The effect of 5-HT on C-fos expression, for instance, is likely to be mediated via the receptors on the RHT terminals, and a regulation of the glutamate release from these terminals.

Apart from C-fos, the expression of several other IEGs in the SCN, such as jun-B, appeared to be induced by a light pulse. Different members of the fos and jun family form heterodimers, the so-called AP-1 complexes, which can induce the transcription of late response genes [312]. The circadian-phase-related expression of IEGs in SCN cells results in a time-related change in composition and abundance of the AP-1 complexes in the SCN [182,346]. These circadian changes in the AP-1 complexes might cause a circadian regulation of transcription of genes that contain an AP-1 site in their promoter region, such as the preprotachykinin gene, from which substance P is derived [69,346].

Besides the induction of IEG expression, other processes have been implicated in the phase shifting effect of light. Several findings suggest that a calcium-mediated stimulation of nitric oxide synthase (NOS) is involved, resulting in a cascade of production of NO from L-arginine, activation of soluble guanylyl cyclase, increase of cGMP levels and activation of cGMP-dependent protein kinase [95]. Evidence for a role of the NO-cGMP pathway in photic phase shifting comes from the finding that cGMP can induce phase advances of wheel-running rhythms in hamsters [213,271]. The NO-cGMP pathway probably mediates only phase advances, since light- or glutamate-induced phase advances, but not delays, are blocked by inhibition of cGMP-dependent protein kinase [94,220,379].

Several reports have implicated the calcium channel ryanodine receptor (RyR) in the signalling pathway in phase shifting of the SCN. RyRs are present in neurons, where they regulate calcium mobilization from internal stores [225]. The first indications for a role of RyRs in phase shifting came from in vitro studies which showed that modulators of RyRs could phase shift circadian

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Fig. 3. Schematic representation of a circadian rhythm as normally entrained to a 24 h light–dark cycle (LD), or as free-running pattern in constant darkness (DD). The dark bars indicate the active (movement, drinking, eating for rodents) periods on successive days (the onset of activity is arbitrarily chosen), presented in a so-called double-plotted actogram. The free-running rhythm is phase-delayed by a light pulse at the beginning of the active period (CT12–18), and phase-advanced by a light pulse at the end of the active period (CT18–23). A lesion of the suprachiasmatic nucleus (SCN) results in a fragmented activity pattern in rodents.
adrenocortical activation has been suggested \[338\]. \[188\] proposed the following hypothesis for their respec-
tion, but a role for serum cortisol through arousal-induced between the two subdivisions of the SCN, Moore et al.
Non-photic stimulation neither effect CREB phosphoryla-
Based on the differences of input and output pathways
[166,183,280,302,305,306], all point to VP as an important
measured in the vicinity of the SCN \[167\] and in the CSF
Furthermore ensures a major role for the retinal input on
and thalamic brain structures, and a rhythmic VP release
their localization in the dmSCN, the VPergic projections
projections, allow modi®cations of the output of the shell
nerve input to the SCN on non-photic phase shifts. Destruction
be a potent non-photic phase shifting stimulus. An increase of the activity level of a nocturnal animal during its
inactive period, for instance by a dark pulse, forced wheel-
running or a benzodiazepine injection, results in large phase
advances \[115,161,366,386\]. A common structure involved in the non-photic phase shifts appears to be the
IGL, with NPY as the main neurotransmitter acting in the
SCN. Application of NPY to the SCN or electrical and
chemical stimulation of the GHT, induced phase shifting in a phase-dependent manner \[25\], while application of NPY
antiserum blocks activity-induced phase shifts \[26\], and
ablation of the IGL abolishes the phase advance in hamsters after a benzodiazepine injection \[224\] or after
novelty-induced wheel-running \[162,385\].
Some controversy exists concerning the role of 5-HT
input to the SCN on non-photic phase shifts. Destruction or depletion of the 5-HT input to the SCN was shown to
disrupt the phase shifting effect of triazolam \[80,258,259\],
while 5HT1/2 receptor antagonists could attenuate arousal-
induced phase shifts \[339\]. On the other hand, 5-HT
depletion did not have an effect on the magnitude of phase
shifts after novelty-induced wheel-running \[29\].
The mechanism of phase shifting by a non-photic
stimulus is largely unknown. An injection of triazolam does not induce C-fos expression in the SCN \[226,407]\.
Non-photic stimulation neither effect CREB phosphoryla-
tion, but a role for serum cortisol through arousal-induced
adrenocortical activation has been suggested \[338\].
2.3.2. Non-photic phase shifts
Arousal or induced physical activity has been shown to
be a potent non-photic phase shifting stimulus. The efferent connections of the SCN in the brain have
been elucidated using VIP or VP immunocytchemistry,
combined with lesion and tracing techniques
\[146,376,378\]. The most prominent projections from the
rat and hamster SCN (Fig. 4) run dorsally and ends in the
subparaventricular zone (sPVz) below the hypothalamic
paraventricular nucleus (PVN) with a small portion of
fibers extending into the paraventricular nucleus of the
thalamus (PVT) and the dorsomedial and ventromedial
nuclei of the hypothalamus (DMH and VMH). As for the
input pathways, also for the output pathways, a segregation
is seen between the vLSN or core SCN and the dmSN or
shell SCN. The core appears to project to the lateral sPVz,
while the shell projects to the medial sPVz and the DMH
\[188\]. Smaller projections are found anterior in the medial
preoptic nucleus (MPN), the anteroventral nucleus, the
periaqueductal gray and the raphe nuclei \[377,378\]. Retro-
grade tracing in most of these projection areas has shown
that they are also innervated by the peri-SCN and by the
subparaventricular zone \[377\]. Thus, multiple pathways
couple the SCN to its target areas. The projections of the
SCN to the LGN and the raphe nuclei might enable the
SCN moreover to modulate its own input through these
feedback loops. The main output pathways and the above-
mentioned input pathways from the retina and the IGL of the
rat SCN are schematically shown in Fig. 4. Tracing and
immunocytchemical studies in the human brain have
shown that the human SCN has a more prominent projec-
tion to the ventral part of the PVN as compared to the rat
and hamster SCN, but that the projections are otherwise
comparable \[82,83\].
Based on the differences of input and output pathways
between the two subdivisions of the SCN, Moore et al.
\[188\] proposed the following hypothesis for their respec-
tive function within the clock. Circadian rhythmicity
generated in the core neurons is synchronized through multiple local and commissural connections, and is en-
trained by direct and indirect retinal input. The response of the core pacemaker to retinal input is modified by the other
input pathways. Projections from the core to the shell
neurons ensures synchronization of all SCN neurons. The
input pathways to the shell, from the limbic system,
amongst others, and the virtual lack of shell-to-core
projections, allow modifications of the output of the shell
neurons with respect to the amplitude and the waveform of
the signal. The relative lack of shell-to-core projections
furthermore ensures a major role for the retinal input on
the pacemaker, independent of internal signals sent to the

2.4. Output pathways of the SCN

The circadian rhythm that is generated in the SCN is
imposed on many physiological functions, including body
temperature, locomotor activity, sleep, oestrous cycle,
oxogen utilization, water and food intake, adrenal corticos-
terone production, pineal melatonin synthesis and receptor
densities. Several characteristics of the VP neurons, like
their localization in the dmSN, the VPergic projections
from the SCN to various target areas in the hypothalamic
and thalamic brain structures, and a rhythmic VP release
measured in the vicinity of the SCN \[167\] and in the CSF
\[166,183,280,302,305,306\], all point to VP as an important
candidate as output signal from the SCN. However, it
should be noted that the circadian rhythm of VP levels in
the CSF is not likely to act as a timing signal for the brain,
since chronic delivery of VP inducing elevated non-
rhythmic levels in the CSF had no influence of the
circadian sleep/wake rhythm of rats \[183\].

2.5. Phase advances
Phase advances are induced by both a physical and
chemical stimulation of the SCN, and are visually
mediated. The main input pathways to the SCN are

- **Light:** Light is the strongest stimulus for phase advances, with short-wavelength light being more effective.
- **Chemical Stimulation:** Chemical stimulation with serotonin (5-HT) can also induce phase advances.
- **Drugs:** Drugs such as triazolam can act as a non-photic phase shifting stimulus.

Phase advances are mediated by a number of different pathways, including:

- **Projections from the SCN to the Ventrolateral Preoptic Area (vLPOA):** These projections are involved in the regulation of sleep and wakefulness.
- **Projections to the Suprachiasmatic Nucleus (SCN):** These projections are involved in the synchronization of the circadian rhythm.
- **Projections to the Hypothalamus:** These projections are involved in the regulation of energy and water balance.
- **Projections to the Amygdala:** These projections are involved in the regulation of emotional behaviors.

Phase advances are also associated with changes in the activity of certain neurons, such as the activity of the ventral pallidum (Vp) neurons and the activity of the preoptic area (POA) neurons. The activity of these neurons is modulated by a number of different neurotransmitters, including dopamine, norepinephrine, and serotonin.
shell neurons. Thus, the reported segregation in input and output pathways of the core and shell enables the SCN to differentially modulate the output neurons projecting to different target areas. Whether the core and shell dichotomy described in the rat represents a general mammalian plan for the organization of the SCN remains to be seen.

Functional studies have to some extent confirmed a role of VP in signalling circadian rhythm to the SCN target areas via its efferent projections, but there is relatively little evidence that VIP serves a similar role. However, as several transmitters in particular GABA (see Section 2.2.1) are colocalized in the SCN neurons containing VP and VIP, the release of a particular cocktail of colocalized transmitters might be more crucial to the signalling role of the SCN efferents, than just the presence of VP or VIP only. SCN projections to the sPVz, the MPN, the PVN and the DMH, may allow the SCN to modulate many neuroendocrine and autonomic functions [46].

Circadian neuroendocrine control pathways have been proposed for melatonin, corticosterone and the gonadotrophins. The best described of these is a multisynaptic pathway by which the SCN controls the diurnal synthesis and secretion of the pineal hormone melatonin. Through a multisynaptic pathway, consisting of a GABAergic projection from the SCN to PVN [168], projections from the PVN to the intermediolateral column of the spinal cord, and further connections to the superior cervical ganglion, the SCN influences melatonin secretion from the pineal gland. Melatonin can feedback by inhibition of SCN neuronal firing [219,313] and can entrain the phase of the rhythm [70,168,202]. The role of melatonin in circadian behavior varies among species and no uniform global view can be given. For instance in hamsters the hormone mediates seasonal variation in reproductive behavior [126,279], in human it has a role as ‘sleep-promoting hormone’ [396,408,409].

Second best described are the pathways controlling corticosterone plasma rhythms [46]. Several routes of control seem incorporated: direct synaptic contacts of SCN neurons on the corticotroph releasing factor(CRF)-producing neurons of the PVN and an indirect input on these neurons via the DMH. In both instances the release of adrenocorticotrophic hormone (ACTH) from the anterior lobe of the pituitary may be controlled in a rhythmic fashion leading to rhythmic production and release of corticosterone from the adrenal. In addition however, a multisynaptic pathway via the PVN and the intermediolateral column neurons of the spinal cord to the adrenal exists as demonstrated by transneuronal virus tracing from the adrenal [47]. The functional significance of this SCN-adrenal connection was demonstrated by a light-induced fast decrease in plasma corticosterone that could not related to a decrease in ACTH, and was not observed in SCNX rat. VP and VP antagonists delivered by reversed microdialysis in the rat DMH indeed revealed a pronounced inhibitory role of SCN-derived VP for the circadian activity of the hypothalamus–pituitary–adrenal axis, but also to the existence of an as yet unidentified stimulatory factor [169,170].

The VIPergic neurons of the SCN synapses in a gender-
specific way on the gonadotrophin-releasing hormone-synthesizing neurons of the preoptic area [147,360]. This pathway may thus play a role in the circadian regulation of the oestrous cycle in the female rat.

The precise functions of the many other neural efferents of the SCN are much less well established, although it has been speculated on the basis of anatomical connectivity that the projections to the PVN play a central role in nearly all circadian active autonomic functions, whereas via projections to the MPN and PVT, the SCN can exert its control over fluid homeostasis, reproduction and thermoregulation, respectively sleep and activity rhythms [46]. Pathways possibly involved have recently been explored by retrograde transneuronal labeling of the rat SCN using viral tracers [47,185,354]. Injections of Pseudorabies virus in the superior cervical, stellate and celiac ganglia, and, following lesion of the sympathetic input, in organs like pancreas, submandibular gland, liver and adrenal, have demonstrated connections of the SCN with diverse types of sympathetic as well as parasympathetic systems. Simultaneous use of different tracers, moreover, revealed connections of single SCN neurons to multiple autonomic systems. Tracing from liver and adrenal showed the dorsal cap of the PVN and the DMH as site of second and third order neurons, respectively, in these multisynaptic pathways [47,185]. Both areas are innervated by SCN neurons (see above). Labeling of SCN neurons was usually first seen in the dmSCN, which included labeled VPergic neurons (20%). This was followed within one day by spread to the entire SCN including the vlSCN where VIPergic (3%), SOMergic (10%) and GRPergic neurons (4%) became labeled [354]. Output neurons of the dmSCN therefore receive input from these subpopulations of the vlSCN, which, in turn, are driven by environmental Zeitgebers (the core neurons, see above). Thus autonomic outputs of the SCN arise from the dmSCN neurons, also from its VPergic cell population. The observation that dmSCN neurons are capable of transmitting multiple autonomic outputs signals support the notion of the SCN as a universal timekeeper.

2.5. Significance of peptides

Given limited evidence as to the role of SCN peptides in endogenous rhythmicity or the transfer of rhythmic activity to other parts of the brain (output), it is difficult to escape the conclusion that the peptides may be of little importance to pacemaker functions of the SCN. In contrast, intrinsic GABA release, which affects membrane potential and intracellular molecular mechanisms of the SCN neurons, probably plays a more important role. Indeed, studies of dissociated SCN cell culture on fixed multielectrode plates, show that GABA, acting through GABAA receptors, can both phase shift and synchronize the electrical activity of the SCN clock cells [215]. Peptide expression of the SCN neurons, however, still serve as a useful marker to distinguish subtypes of neurons with respect to their specific role as input and output stations of the biological clock system. For example, recent evidence suggest that the small population of calcium-binding protein calbindin-D28K (CaBP)-synthesizing neurons in the hamster SCN play a critical role in controlling locomotor rhythms [199,324], indicating the importance of neuronal subtyping to unravel the many functional aspects on the biological clock system.

3. Transplantation of the suprachiasmatic nucleus

The first SCN transplantation experiments were carried out years after the first lesion experiments of Stephan and Zucker [336] and Moore [236], and contributed to the proof of the pacemaking capacities of the nucleus. When transplanted in the brain of SCN-lesioned (SCNX) arrhythmic rats and hamsters, fetal or early postnatal SCN tissue (Fig. 5) is able to restore circadian rhythmicity in some behavioral parameters (Fig. 6) [193]. The most frequently used rhythmic outputs examined in transplantation studies are drinking behavior in rats and wheel-running activity in hamsters. The specificity of the action of the fetal SCN graft in this restoration of behavioral rhythms was initially questioned by the finding that transplants of fetal occipital cortex tissue could also reinstate a circadian drinking rhythm in SCNX rats [116]. However, in this study, graft recipients were only rhythmic under LD conditions, suggesting a masking effect of light, an effect which was also observed in SCNX rat with ineffective SCN transplants (Fig. 6A) [32]. Moreover in the study with cortical grafts, the completeness of the SCN lesions was not verified by immunocytochemical detection of SCN neuropeptidergic cells, so that indirect effects of the fetal tissue on the host SCN cannot be excluded. Other studies using non-SCN tissue as a control have never observed rhythm restoration [92,196,222]. Since then, many studies have characterized the properties of SCN transplants with respect to cell survival and neuropeptide content, and the formation of connections with the host brain, in order to elucidate the mechanism of rhythm restoration (Fig. 6).

Topics that have been investigated by using SCN transplantation can roughly be categorized as follows: (a) intrinsic properties of the SCN; (b) communication between the transplanted SCN and the recipient brain; (c) communication between two independent pacemakers and their combined behavioral output; and (d) changes in the circadian timing system related to aging. The main results of SCN transplantation studies, and their contribution to the understanding of circadian time-keeping mechanisms will be discussed for each of these topics.

3.1. Intrinsic properties of the SCN

The potential ability to manipulate the donor SCN prior
Fig. 5. Typical example of the appearance of a SCN-containing fetal hypothalamic transplant in the IIIrd ventricle of a rat hypothalamus at a survival time of 6 weeks and immunostained for its VPergic cell moiety. In this particular example, fetal E17 SCN tissue was grafted in the VP-deficient Brattleboro rat to visualize the neurite extensions of the VPergic neurons of the SCN in the host brain. (A) The intraventricularly placed transplant is present beneath the paraventricular nucleus (PVN) of the host and revealed the presence of two SCN cell clusters of which the morphology and density was comparable to that of the in situ situation (cf. Fig. 1). It also shows the lack of efferent growth to the recipient brain when the ependymal layer of the IIIrd ventricle is intact. Bar 50 μm. (B) Extensive VPergic efferent growth from the transplant can be observed when graft and host tissue fused at the ependymal layer-free ventricular wall in the more ventro-caudal aspect of the IIIrd ventricle of the recipient brain. Dashed line indicate the transplant-host interphase. Bar 125 μm.

to grafting makes SCN transplantation an elegant method to study properties of the SCN that are important for rhythm generation, such as the composition and organization of the SCN. Furthermore, transplantation in SCNX host animals offers the opportunity to determine which features of circadian rhythmicity are intrinsic to the transplanted SCN. This is best illustrated by the transplantation studies using the tau mutant hamster, of which the heterozygote form has a circadian period length of about 22 h and the homozygote a period of about 20 h. Donor SCN obtained from a homozygous tau mutant hamster re-instated a 20 h rhythm in an arhythmic SCNX host animal with a 24-h rhythm prior to lesion and vice versa [273], showing that the restored rhythm is always derived from the donor SCN. The ability of these and other [36] cross-genotype transplants, as well as cross-species grafts [330], to restore rhythms with a period corresponding to the donor tissue provides strong evidence that circadian period is an intrinsic property of the SCN and not dependent on the rest of the brain.

A characteristic feature of the in situ SCN is its compact organization and the clear distinction between dorsolateral and ventromedial components containing neurons that express different neuropeptides. Independent of the brain site of implantation, transplants of rat E17 SCN or hamster E15 SCN have been shown to develop a similar neuropeptide organization within the transplant, an organization that includes adjacent, and hardly overlapping populations of VIPergic and VPergic neurons [31,129,195,287,387]. In the rat the SOMergic cell portion of the grafted fetal SCN, however, often does not appear adjacent to the VIP-/VPergic cell complex, but instead develops at a somewhat distant site in the transplant [32,35,129]. Thus the development of the SCN complex may not entirely be normal when grafted in the adult host brain. The presence of VPergic and VIPergic neurons in the transplant is reported
Fig. 6. Rhythm restoration, and masking and entrainment phenomena in SCN-lesioned arhythmic rats following intraventricular transplantation of fetal E17 SCN tissue. (A) Double plotted actogram of drinking activity of the SCN-lesioned arhythmic rat that received a SCN neurograft, but failed to recover its circadian drinking pattern. Following a shift from the constant dark condition (DD) to a 12 h light/12 h dark cycle (LD) and a reversed LD cycle (rLD), drinking activity immediately follows the D phase, indicating a strong masking effect, which can also be seen in SCN-lesioned animals without a graft or a control cerebral cortical graft. Upon return to DD the rats showed arhythmia again. (B) Double plotted actogram of a rat that showed re-instatement of circadian drinking rhythm as well as entrainment of the SCN graft-induced rhythm. A recovered rhythm becomes visible in the actogram by about 3 weeks and has a period length of 23.7 h. Following the introduction at day 70 post-transplantation of a 12 h/12 h LD condition, which is 12 h out of phase with the re-instated free running rhythm, the drinking pattern adapts within a week to this external cue. When again DD is introduced at a time point chosen to be 12 h out of phase with the free-running rhythm in the first DD period (see line indicating the beginning of the activity in the post-grafting free run period in DD), the rhythm continues to show its 23.7 h free-running period but it was shifted by 12 h shift compared to the pre-LD period. The phenomena at both transitions (DD to LD and LD to DD) strongly indicate that the re-instated rhythm as a result of SCN neurografting can be entrained by external light, and may indicate that the retino-hypothalamic tract has re-innervated the donor SCN.
a prerequisite for rhythm restoration in most transplantation studies, since these cells are used as markers for the presence of the biological clock in the transplant. However, the presence of VP in the transplanted SCN neurons, appeared not necessary for rhythm restoration since SCN grafts dissected from VP-deficient homozygous Brattleboro fetuses were equally able to restore circadian drinking rhythm in arhythmic SCNX Wistar rats [36]. This result suggests that VP is not essential for the generation and expression of circadian rhythm in the SCN system (see also Section 2.2.2). Since the dmSCN cells that normally express VP are still present in the Brattleboro SCN, this evidence does not exclude the possibility that other activities of these neurons do play such a role. Interestingly, homotopic SCN transplants derived from homozygous and (control VP-synthesizing) heterozygous Brattleboro fetuses restored a circadian rhythm with a similar difference in period length (24.3 vs. 23.8 h) as reported for the intact adult Brattleboro genotypes. This is yet another example of the donor SCN determining the period of the reinstated circadian rhythm, as shown by the cross-genotype and cross-species neurotransplantation studies mentioned above.

In hamster, but not in rat (K.E. van Esseveldt, unpublished observation), a compact subnucleus of CaBP-positive cells is present in the core of the SCN that respond to light pulses by c-fos expression [324]. That these cells are pacemakers controlling circadian locomotor rhythms is suggested by evidence that locomotor rhythms are retained after partial lesion of the SCN if this region is spared but not if this region is ablated. In addition locomotor rhythms are restored in SCN-grafted arhythmic SCNX hamster when SCN-specific CaBP-positive cells are present in the transplant [199]. It would be of interest to see whether these cellular subgroup is also involved in regulating other circadian rhythms, such as in autonomic or endocrine functions.

So far, little attention has been paid to the role of the SOMergic cell population in rhythm restoration of SCN-grafted SCNX rat and hamster. The presence or absence of a close association of the SOMergic neurons with the component organisation of VIPergic and VPergic neurons, as seen in the in situ SCN, could not be related to the capacity of the transplanted SCN to restore circadian rhythmicity in rat [35,36,129]. Immunocytochemical detection of other neuropeptides of the SCN showed their presence in the transplanted SCN, but a detailed 3-dimensional cytoarchitectonical study with multiple stainings has never been performed.

As mentioned before, the synchronization of the individual rhythmic neurons of the SCN has been proposed to be based on the close apposition of the SCN neurons and the intermingling glial cells. In vitro cultures of dissociated SCN cells gave contradictory results, however, with respect to this proposition. A synchronized circadian release of both VP and VIP has been reported for cultures of dissociated SCN cells, at a time before neural connections had been formed [144]. Other studies however, indicated independent and even opposed rhythms of firing rates of adjacent SCN neurons in 3-week-old suspension cultures, even in the presence of numerous synapses [382]. The importance of the structural organization of the SCN has been investigated by transplanting a dissociated cell suspension of fetal SCN cells into the brains of SCNX rat and hamster. SCN cell suspensions transplanted in the medial thalamus or medial hypothalamus of SCNX hamsters could reinstate a circadian wheel-running rhythm within a few days after transplantation [323]. As in the case of solid tissue SCN grafts, the presence of VIPergic SCN neurons appeared to be a prerequisite for rhythm restoration, but the number of SCN neurons was very low in these suspension grafts. These data indicate that the intrinsic structural organization of the SCN including close apposition among SCN neurons, is not a prerequisite for rhythm restoration in hamster. Whether synchronization occurs between the individual SCN neurons of the transplant which are possibly connected via synapses, remains unclear, but apparently the rhythmic signal from these neurons is strong enough to drive a rhythmic activity pattern of the host animal. In rat, SCN cells transplanted as a cell suspension displayed ample VP and VIP immunoreactivity [33], but such grafts were so far never placed in SCNX rat to investigate the re-instatement of rhythm. Transplantation of a fetal rat SCN as a suspension of minced tissue (mesh between 50 and 100 μm) rather than a cell suspension resulted in restoration of a circadian drinking rhythm with a comparable success rate as with solid tissue blocks (41% and 44% for groups of 18 and 22 SCNX rats, respectively) [362]. Histological analyses of these transplants revealed aggregation of the minced tissue containing up to five separate clusters of SCN cells. Whether the reinstated drinking rhythm results from activity from one or several SCN cell clusters that have synchronized their activity, is unclear. Synchronisation of VP release may be lacking, since the occurrence of a circadian rhythm in CSF VP levels at 3–6 month post-grafting negatively correlated with the number of VPergic cell clusters in the transplant [365]. Curiously there was no relationship with re-instatement of circadian drinking between either parameters.

In principle, the ability of SCN cell suspension grafts to restore rhythmicity in hamsters (and rats) offers the possibility of investigating the functional roles of the different subpopulations of SCN cells in rhythm restoration and generation by selecting or eliminating a certain population of SCN cells prior to transplantation. Separation of the glial cells and neurons, for instance, can be achieved by cell culture under conditions that favor neuronal or glial survival, but selection of the neurons on basis of their neuropeptide content requires more sophisticated methods. Shimuzu and colleagues [318] report in vivo elimination of VP and VIP cells after application of
anti-VIP antibody-ricin-A chain conjugate. This method appeared to be not reliable, however, in elimination of one of these cell populations. Another approach to selectively eliminate specific SCN cell subpopulations would be to use viral vectors containing a specific neuropeptide promoter driving the expression of a lethal gene. The potential application of this approach is discussed in Section 5.2.

3.2. Communication between the SCN and other brain areas

In general, transplantation studies have focused on the issue of what extent integration of the transplanted cells with the host brain is needed for functional recovery to occur. In SCN transplantation studies, several approaches were used to study the degree of afferent and efferent connectivity of the graft with the host brain. With regards to the input, immunocytochemical staining for NPY and 5-HT both revealed fiber ingrowth from the host into the transplant [6,32,129,222,287]. Some studies report that NPY and 5-HT fibers innervate the region of the SCN cells in the transplant, while other studies report the incoming fibers to avoid the donor SCN. Whether NPY or 5-HT afferents that innervate the transplant derive from the IGL and the dorsal raphe respectively, has not been investigated. Moreover, since cell bodies containing NPY and 5-HT are present in the vicinity of the hypothalamus [74,381], it is possible that these may have sprouted into the grafted mass. Behavioral studies additionally indicate no functional connections between the IGL and the transplanted SCN, since rhythms restored by SCN grafts do not appear to be phase-shifted by non-photic stimuli such as a triazolam injection or increased locomotor activity [64]. Tract tracing analyses in the grafted animals revealed retinal innervation of grafts located above the optic chiasm [5,195,222]. Occasionally, the retinal fibers innervate the area of the SCN, but in hamster this innervation is apparently not sufficient to restore entrainment to a LD cycle [222]. However, recent preliminary rat studies however, using a paradigm of phase-timed DD–LD transitions for SCN-grafted SCNX rats, revealed that entrainment of the re-instated rhythm can sometimes be noticed (Fig. 6B).

Cross-species transplantation has been used to determine the extent of the outgrowth of the graft towards host target areas. In these studies, species-specific antibodies revealed a massive outgrowth into the host brain [330,331]. For the most part, however, these studies did not distinguish between efferents derived from the SCN neurons or from the other neurons in the graft, so that a specific reinnervation pattern by the transplanted SCN could not be judged. This problem was partially circumvented by the use of SCN micropunch grafts that contained minimal extra-SCN tissue, so that most efferent neurite growth is derived from the transplanted SCN. Transplantation of rat micropunch grafts in the third ventricle of intact hamsters resulted in innervation of host areas that normally are innervated by the intact SCN, like the medial preoptic area, the PVT and the sPVz [192,332,387]. The pattern was however variable and dependent on the location of the transplant and its site of attachment to the ventricular wall. Surprisingly, transplants of fetal cortex were found to reinnervate the host brain in a pattern similar to that of SCN grafts [192]. Cross-species transplantation of micropunch grafts does not allow the identification of specific neuropeptide efferents derived from the transplanted SCN, unless double labeling is performed. Outgrowth of VIPergic fibers has been investigated, however, by using the VP-deficient Brattleboro rat as a host. Implantation of a fetal SCN in the third ventricle of a Brattleboro rat results in a similar innervation pattern as seen in the normal situation, but, again, considerable variability was present in the precise host targets innervated [30,31,387].

The innervation of the host brain by SCN transplants has been assumed to be a key factor in functional recovery, since in the intact situation, the extensive efferents to hypothalamic and non-hypothalamic areas are thought to be responsible for transmission of the endogenous circadian rhythm of the SCN (see Section 2.4). Transplantation studies have however come up with conflicting results concerning the necessity of neural efferents into the host brain for rhythm restoration. In most transplantation studies, both in hamster and rat, efferent growth is reported but it is usually limited in density and extent. Due to difficulties in identifying graft-derived SCN fibers in the host brain, no clear correlation could be found between efferent growth and rhythm restoration, except for one study in which a correlation was reported between VIPergic innervation of the PVT and restoration of circadian wheel-running rhythms in SCNX hamsters [332]. These findings are however challenged by several other findings in hamster studies. Graft-derived innervation of target areas was for instance reported to be of no predictive value for rhythm restoration in a study by LeSauter et al. [197]. Moreover, in some cases the rhythm was restored within a few days after transplantation, a time window in which reinnervation is unlikely to occur [195]. Moreover, rhythm restoration has also been shown when transplants are located in the lateral ventricle or foramen of Monro, sites from which appropriate neural connections with targets in the thalamus and hypothalamus are unlikely [5,195]. Finally, SCN transplants that are encapsulated in a semi-permeable polymer capsule, which prevents neural connections with the host brain, are nevertheless still able to restore circadian locomotor rhythms to SCNX hamsters [412]. These latter findings point to the existence of a diffusible factor that is sufficient to restore rhythmicity to SCNX hamsters. A recent report showing rhythm reinstatement of SCNX rats using immortalized SCN cells also proposed a diffusible factor to exert this function [100], but the absence of graft-host brain connectivity was not shown. Whether a soluble factor is also involved in the
intact situation when neural efferents are present, remains to be determined.

If diffusible signals are indeed sufficient for rhythm restoration, the observation that many rat SCN transplants do not restore a circadian rhythm in SCNX rats despite the presence of numerous VPergic and VIPergic cells in an SCN-like organisation in the transplant [32,36,129,365], is at least curious. These transplants, which are microscopically indistinguishable from successful transplants, are apparently not able to transmit their circadian rhythm to the host brain. Immunocytochemical analyses of VP- and VIPergic fibers could not reveal a relation between rhythm restoration and efferent growth towards the host brain [32,129]. Also a lack of rhythmic activity of the transplanted SCN as an explanation for the failure to restore circadian rhythmicity is unlikely, since non-functional transplants still release VP in a circadian fashion into the CSF of the host brain. In hamster studies, one also finds instances of grafts that contain VP-/VIPergic cell clusters but fail to reinstate rhythmicity [194]. Apparently the conditions by which a homotopic SCN graft will become functional are more strict in rat than in hamster [36].

SCN transplantation studies carried out in SCNX rat and hamster have in some aspects led to different results. The most striking difference concerns the success rate of SCN grafts to restore circadian rhythm to arhythmic hosts. Recovery rates in hamster are usually between 80 and 100% [172,194,195,197,332], whereas in rat they are variable, but rarely exceed 60% [32,36,129,362,365]. In addition, the success of the transplantation in rat appears to depend more on the location of the transplanted SCN than in hamster [362]. For example, in rats, SCN transplants implanted in the lateral ventricle are unable to restore rhythmicity [32,129,295], whereas in hamster, rhythm recovery could also be achieved by grafts in the anterior part of the lateral ventricle, and even in the foramen of Monro [5,195,198] In rat, grafts in the third ventricle appear to be most successful for rhythm restoration [4,32,129,294]. In hamster though, rhythm recovery of less accuracy was observed with increasing distance between the sites of the implanted donor SCN and the site of the SCN lesion of the recipient [197]. The discrepancy between the results might partly be explained by methodological differences (in rat studies drinking activity is usually monitored, while in hamster studies wheel-running is the output most frequently monitored), or by differences in methods (age of donor, graft dissection, rhythm criteria), but may also be the result of species differences in the organization of the SCN clock system. For instance calbindin is specifically expressed in cells clustering in the core of the hamster SCN [16] but rather diffusely distributed in rat (unpublished results) and mouse SCN [325], whereas for calretinin-positive neurons the opposite is noticed [325]. Whether the observed differences in the results of rat and hamster SCN transplantation studies partially reflect a real species difference remains however to be investigated. Either a study in which both species are subjected to the same procedures and evaluation criteria, or hamster-to-rat xenotransplantations could shed light on the background of the difference.

3.3. Communication between two independent pacemakers

Synchronization of two independent pacemakers and their output on locomotor activity has been investigated by transplanting a fetal SCN in host animals with a weakened rhythm, such as that seen in partially SCNX or aged hosts. Using the tau mutant hamster, the rhythms of the partially lesioned wild type host SCN and the mutant donor SCN could both be recognized in the wheel-running actograms [371]. The two clocks appeared not to influence each other, but both had their own impact on the observed complex pattern of wheel-running activity. Rhythmic components with periods of both host and donor oscillators can be recognized after grafting. However, interactions take place at the output level. Time windows of various patterns of rhythmic wheel-running activity and inactivity can be detected, leading to postulate stimulatory as well as inhibitory inputs from the circadian system to the brain areas controlling locomotor behavior. If activity-stimulating circadian outputs from host and donor are employed simultaneously, both periods become visible, but when the two activity-suppressing outputs work in parallel, the period becomes intermediate by masking of one of the suppressive outputs [371]. Transplantation of a fetal SCN in aged hamsters mainly revealed the same results, with complete dominance by the donor SCN, relative coordination between the two pacemakers, or spontaneous switching between the host and donor phenotype [153]. The lack of interaction between the two pacemakers in these studies is not explained. A difference in period length could make entrainment difficult to reach or it may be difficult to establish input connections with the (adult) recipient SCN.

Other studies using pacemakers with similar period lengths, indeed report a synchronization of a transplanted SCN with the host SCN when the graft is implanted in the third ventricle of an intact rat or hamster, respectively [6,309]. In one study, the glucose metabolism of the transplant and the electrical activity of the graft were synchronized to the endogenous SCN within 6 weeks after transplantation [6]. In another study, donor and host hamster were used 12 h out of phase with each other at the time of implantation. Within 2 weeks after grafting, the rhythm of \((1^{14}C)2\text{-deoxyglucose uptake of the transplanted SCN was synchronized to the rhythm of the endogenous hamster SCN [309]. Location was reported to be of importance for synchronization, since only grafts located in the third ventricle were synchronized, while grafts in the lateral ventricle were not [310]. The grafted SCN was thus entrained by the host, possibly though a diffusible humoral
3.4. Changes in the circadian timing system related to aging

Aging of rat and hamster results in a decrease in amplitude of behavioral rhythms, in a less accurate onset of activity periods and in a loss of responsiveness to phase-shifting stimuli, such as light pulses and activity-inducing stimuli [44,406]. Transplantation of a fetal SCN has been shown to reverse some of these changes [203,367,369]. For example, weak rhythms in old ratsjuveniled following E16 fetal SCN grafting in the third ventricle [203]. In aged hamster the mean free-running period lengthened to young values after transplantation [369]. In addition phase shifts in response to a triazolam injection are normally decreased in 18–25-month-old hamsters, and this decrease was reversed by a fetal SCN transplant [367]. Since the phase-shifting effect of triazolam is mediated through an increase in activity, age-related decreases in monoaminergic levels, which play a central role in the regulation of activity, have been thought to underlie the decreased responsiveness to triazolam. The effect of the transplanted SCN was therefore proposed to be mediated through an alteration in monoaminergic activity in the host SCN [367].

The phase-shifting response to a light pulse during the subjective night time is blunted with aging, while concurrent increases in C-fos and jun-B expression in the SCN are also lower. The normal diurnal rhythm of C-fos expression in the SCN is moreover disturbed, showing a premature rise during the dark, while the light-induced increase of C-fos is delayed. Transplantation of a fetal SCN in middle-aged rats was shown to restore the diurnal C-fos pattern to that of a young animal, and the light-induced responses of C-fos and jun-B to levels of young rats [58,61]. The authors give several possible explanations for this finding: entrainment of the host SCN by the donor SCN, improvement of host rhythms by the donor SCN, which feedback on the endogenous SCN, or rejuvenation of the host SCN by trophic factors originating from the donor SCN.

In aging rats, circadian rhythms in CRF mRNA in the dorsomedial PVN, and pro-opiomelanocortin (POMC) mRNA in the anterior pituitary disappear [60]. Both rhythms can be restored by fetal SCN transplants, although the timing of the CRH mRNA rhythm was somewhat different, and the amplitude of the POMC mRNA rhythm lower than in young rats [59]. Apparently, the aging effect on the rhythms of the neuroendocrine system involves changes in the timing signal from the SCN, and does not result from a loss in responsiveness of the system to cues derived from the SCN. Introduction of a young SCN with appropriate time cues appears to be sufficient to restore the CRH and POMC mRNA rhythms to a more or less young pattern.

4. Genetic determinants of circadian rhythmicity

The fast evolving field of molecular biology has provided new tools in the search for the pacemaking mechanism of the SCN. The electrophysiological identification of circadian rhythm in single isolated SCN cells [382] as well as the observation that blockade of electrical signals within the SCN does not affect the phase of circadian activity [98,304] and that immortalized SCN cells keep their property to generate robust rhythms in metabolic activity [100], already indicated the pacemaker rhythm to be of molecular origin at the cellular level. The identification of genes which appear to be crucial for circadian rhythmicity ('clock genes') in the fruit fly Drosophila melanogaster and the fungus Neurospora crassa, has prompted many investigators to search for mammalian homologs [297,340,349,370,410]. The result indicated that also in mammals a similar mechanism underlies the circadian clock. In the mean time, mutagenesis studies in mouse resulted in the identification of a clock gene Clock(clk) [370] that vice versa has its homolog in the Drosophila pacemaker system, and have a similar function. Finally behavioral screens have resulted in the recognition of individual variations in rhythmic parameters in several species. A variation was observed, for instance, in the strength of activity rhythms in a population of common voles [119] and in different mice and rat strains [48,49,388], whereas deviating rhythmic parameters were also observed in naturally occurring mutants, such as the obese Zucker rat [242], the VP-deficient Brattleboro rat [45,130], and the tau mutant hamster [274]. Moreover, changes in circadian activity were observed in transgenic animals, like the hyperactive Wocko mouse [333], the prion protein knockout mouse [350], the C-fos-deficient mice [145] or Cry1 and Cry2 single and double mutant mouse [361]. Not all of these alterations in rhythmic behavior will necessarily derive from a change in the pacemaker itself, but could reflect a change in the input or output pathways of the SCN, or represent an effect only on the behavioral parameters that were studied. The exception is the CRY mutant, since expression of this protein is pivotal for circadian rhythm [184]. In general, clock genes are expected to satisfy the following criteria [18]: (a) the absence of the clock protein must lead to arrhythmicity; (b) the protein must have a circadian rhythm, even in the absence of 'Zeitgebers'; (c) the rhythm must be phase-shifted by these 'Zeitgebers'; and (d) manipulation of protein levels must change the phase of the rhythm. In all latter rodent mutants, except for the CRY mutant mouse, the possible aberrations or changes in the expression of the clock genes are still to be investigated.
Using circadian rhythms of locomotor activity and hatching from the pupa in the fly as output of the clock, mutations in \( \text{per} \) were shown to result in arhythmicity (\( \text{per}^0 \) null mutants) or to alter the period length under DD conditions [97]. Further studies revealed that \( \text{per} \) fulfills the criteria for a clock gene. Arhythmicity in DD in \( \text{per}^0 \) flies correlates with the absence of cycling per mRNA levels [134]. In wild type flies, \( \text{per} \) mRNA and PER protein levels fluctuate with a circadian periodicity in LD cycles and in free-running conditions [104,134,327,405], whereas the period length of \( \text{per}^+ \) or \( \text{per}^- \) mutant mRNA and protein rhythms become shorter or longer in line with the respective fly behavior [134,405]. PER rhythms can be phase-shifted by light [405] and an induction of \( \text{per} \) expression can phase shift the fly behavioral rhythm [103].

Under LD conditions \( \text{per} \) flies show a 24-h rhythm, Fig. 7. Expression patterns of \( \text{per} \) mRNA and PER protein in Drosophila indicating that masking takes place [384].

The phase response curve (PRC) illustrates the relation of the comparable Zeitgeber time (ZT) at which a light pulse is given under DD free-running conditions, with the magnitude and direction of the resulting phase shift of the activity rhythm. A light pulse induces a breakdown of TIM and thereby indirectly of PER due to lower protection for intracellular breakdown. A light pulse given during the accumulation phase of PER thus results in a lower production of protein and hence in a phase delay, while a light pulse given when PER levels are high results in an accelerated breakdown, and thus in a phase advance. See also scheme of Figs. 7 and 8.

4.1. Identification of clock genes in Drosophila

4.1.1. The period gene

Mutagenesis studies in Drosophila resulted in identification and isolation of the clock gene period (\( \text{per} \)) [23,180].

Fig. 8. Schematic representation of genes of proteins involved in the Drosophila clock and their homologs identified in mammals. Functional domains in the nucleotide sequences are indicated by shaded or black boxes. Bar lengths indicate the relative sizes of the DNA sequences. Acidic: acidic region that might act as activator domain; ATP: ATP-binding site; BHLH: basic helix-loop-helix; CLD: cytoplasmic localization domain; DEDD: tetrapeptide sequence; NLS: nuclear localization signal; PAS: PAS domain (indicated are both PAS A and B domains); PB: PER binding sequence; PER-C: C-terminal region of PER; poly Q: glutamine-rich activator domain; ST: serine-threonine kinase catalytic domain; TG: threonine-glycine repeat. Note that for the \( \text{tim} \) gene the PER binding sites have not yet definitively identified (see referring text).
translocator (ARNT), and with Drosophila single minded (SIM) [150]. The PAS domain spans approximately 260 amino acids and contains two repeats of about 50 amino acids each (PAS A and PAS B motifs). The bHLH region, which is known as a DNA-binding region, is lacking in PER protein. The PER C-domain can auto-interact with the PER PAS domain to form an intramolecular bond [149], resulting in a ‘closed PER monomer’ (Fig. 9). This intramolecular bond appears to prevent its nuclear entry during the day phase, since a PER fusion protein without a PAS-containing region, and thus without intramolecular bond, was constitutively nuclear.

The timing of the per mRNA and PER protein peaks, the homology with several transcription factors, and its predominantly nuclear localization suggest that PER might negatively regulate its own transcription. However, since PER does not contain any known DNA-binding domain, this feedback must be mediated by a second factor with DNA-binding capacities.

### 4.1.2. The timeless gene

A second Drosophila clock gene *timeless (tim)* has been isolated following mutagenesis [117,291,307,372] and shown not only to be crucial for rhythmicity in fly behavior, but also for circadian rhythmicity of *per* [269,307] and its nuclear entry [372]. *Tim* mRNA and TIM protein levels show circadian variations, which are in phase with *per* mRNA and PER protein rhythms, respectively [152,281,308]. TIM does not contain a PAS domain or a known DNA-binding domain, but a basic region in the protein could act as a nuclear localization signal (NLS), and an acidic region as activator domain [247] (Figs. 8 and 9). PER and TIM form heterodimers with sequences within the PAS and cytoplasmic localization domains (CLDs) of
PER and PB1 and PB2 domains (PER-binding sites) of TIM [293,297]. The heterodimer can enter the nucleus to inhibit transcription of their respective genes [117,404]. The absence of one of the binding partners results in the absence of a rhythm of the other: per<sup>0</sup> flies lack a tim rhythm, whereas tim<sup>0</sup> flies lack a per rhythm. For heterodimerization with PER, TIM has to compete with the PER C-domain for binding with the PAS domain [149]. In tim<sup>0</sup> flies, PER remains in the cytoplasm, whereas a PER fusion protein without a PAS-containing region, and thus without intramolecular bond, was constitutively nuclear [372].

The period-lengthening effect of the per<sup>L</sup> mutant with a mutation in the PAS domain [24], results from a temperature-sensitive defect in PER binding to TIM [117], and thus a slower built-up of PER–TIM complexes [149]. In the short period per<sup>S</sup> mutation, the timing of PER nuclear entry is unaltered consistent with an intact PAS domain, but an earlier phosphorylation peak of PER occurs mediating an accelerated breakdown of nuclear PER [104]. Based on a negative feedback of PER and TIM on their own transcription, several mathematical models have been developed to determine how such a sequence of fast reactions can result in a 24-h oscillation, and what properties of the timing system determine its phase response [124,201,300].

4.1.3. The double time gene

The recent identification of the Drosophila clock gene double time (dbt), clarified the role of phosphorylation of PER in the timing of its nuclear entry and breakdown [178]. DBT is a structural homolog of the human casein kinase le with 86% identity in the kinase domain (Fig. 8). DBT is constitutively expressed in wild type flies in the same regions of the adult Drosophila head as per and tim, and can bind to cytoplasmic monomers of PER. It thereby mediates phosphorylation, ensuring breakdown of monomeric PER proteins [178,268] (Fig. 9). Absence of DBT leads to an accumulation of hypophosphorylated PER in the cytoplasm [268]. PER is then more slowly degraded, and not dependent anymore for its stability on the formation of heterodimeric complexes with TIM, which consequently leads to arrhythmicity. A ‘long period’ mutation in dbt appeared the result of a lengthened presence of PER in the nucleus as compared to wild type flies, a ‘short period’ mutation of dbt coincides with a premature disappearance of PER and TIM in an LD cycle, and maximal phosphorylated forms of PER appear earlier than in wild type [268].

4.1.4. Resetting of the Drosophila rhythm by light and the Cry gene

Breakdown of TIM by light exposure provides the mechanism by which the per and tim cycles entrain to the LD cycle. Since PER is unstable without TIM under normal phosphorylation conditions [269], and TIM levels are thus reduced [152,190,246,404], the PER/TIM complex is at low concentration in the L-phase whereby transcription of both per and tim is disinhibited. However both proteins can only start to accumulate at the beginning of the night, when TIM is not broken down anymore. In situ PER and TIM levels start to build up at Zeitgeber time (ZT) 15–16 in the cytoplasm, whereas a sudden nuclear entry is noted at ZT18–19 [79]. If a light pulse is given during this accumulation phase, TIM’s breakdown results in a phase delay. On the other hand the clock is phase-advanced when the light pulse is given in the second half of the night: it accelerates the breakdown of the proteins (see Figs. 3 and 9).

The mechanisms of light-mediated TIM degradation are only recently better understood. Several older findings revealed a strict correlation between TIM decrease and behavioral responses during phase shifts studies and tests on wavelength dependence and light sensitivity [396]. Strong evidence has now been obtained for the involvement of the photoreceptor gene Cry in light-mediated TIM degradation [72]. CRY is a light-absorbing protein originally shown to control daily rhythms in plants, but homologs are also expressed in flies, among which Drosophila, as well as throughout the animal kingdom (see Section 4.3.3.2). In Drosophila the single gene has a 50% homology with hCry1 and hCry2 genes [105]. Expression of dCry is rhythmic under LD and DD conditions and has a peak at CT1–7 and a trough between CT17–19. Per<sup>o</sup> and tim<sup>o</sup> mutant flies have no circadian expression of dCry, thus expression is under circadian control. CRY protein expression in LD is rhythmic as well, but strikingly different from its mRNA rhythm, indicating strong translational and/or posttranslational effects. In DD however, expression of CRY is constant and thus under inhibitory control of light [105]. CRY-overexpressing flies manifest hypersensitive circadian responses to light [105], whereas Cry<sup>o</sup> mutant flies had a poor synchronization to light [334]. CRY therefore acts as light-sensitive receptor setting the circadian rhythms. The light-triggered TIM degradation is moreover most sensitive to light in the blue range, CRY’s speciality as well. The direct interaction with the PER/TIM complex was most recently shown by Ceriani et al. [72], CRY is present in both the nucleus and the cytoplasm and binds to TIM in a light-dependent way, probably by conformational changes. It thereby reduces the level of the PER/TIM complex and therefore its autoregulation on transcription. Thus, CRY acts as a circadian photoreceptor by directly interacting with the core component TIM of the circadian pacemaker (Fig. 9).

4.1.5. The clock and cycle genes

The questions concerning the lack of DNA-binding domains of PER and TIM to explain their inhibitory effect on per and tim transcription, seems to be solved by the identification of two genes in Drosophila mutants which code for proteins with DNA-binding properties. One of these genes appeared the Drosophila homolog [13] of the
mouse clock (clk) gene (see Section 4.3.2) with homology especially high in the functional domains. The second gene is cycle (cyc) [292], which appeared to be the Drosophila homolog of the human gene bmal1 [141,142,154], which codes for a variety of protein products, some of which resemble CYC. Cyc shares 68% homology and 55% identity with this human gene, of which the function was not known until a role was suspected in circadian rhythmicity. Clk and cyc both encode bHLH-PAS transcription factors but CYC lacks a C-terminal glutamine-rich activator domain (Fig. 8). Recently, a bimodal rhythmic expression pattern with peak levels at ZT5 and ZT23 was reported under LD conditions for clk mRNA in Drosophila brain [89]. Whether this rhythm is also present dimers with CLK, respectively. Consequently PER and ZT23 was reported under LD conditions for clk mRNA in Drosophila brain [89]. Whether this rhythm is also present at the protein level, and whether cyc is rhythmically expressed is unknown.

Homozygous clk and cyc mutants lose rhythmicity in DD and have difficulties maintaining a rhythm under LD conditions [13,292], in contrast to clear 24 h behavioral rhythms of per0 and tim0 flies under these entrainment conditions [384]. The arhythmic phenotype of the homozygous clk and cyc mutants (clk0 and cyc0) results from constant and low levels of per, tim [384] and Cry expression [105]. Heterozygous clk mutant flies have a longer period than wild type flies and in 50% of the cases they lose their rhythms in DD. Heterozygous cyc mutant flies also have a slightly longer period length, but their rhythms are robust with no hint of arhythmicity. The observed behavioral rhythms in both heterozygote cases parallel per and tim rhythms. In the heterozygous clk mutant per and tim mRNA and protein levels fluctuate with a 50% reduced amplitude, whereas per and tim cycle at normal levels in the heterozygous cyc mutants [292]. Above data indicate that clk and cyc are involved in transcriptional regulation of per and tim, as well as in the retinal signal to the clock.

The identification of an enhancer sequence in the per promoter [133], provided the mechanism through which clk and cyc might regulate per transcription. The circadian enhancer (CE) contains a so-called E-box (CACGTG), a known binding site for some bHLH transcription factors including bHLH-PAS transcription factors [78,245,342]. In vitro experiments indicate that BMAL1 can form a heterodimer with mCLK, which subsequently binds to and activates transcription from per-like E-boxes [142]. The above cyc mutation indeed affects the activity of the per transcriptional enhancer CE when driving a reporter gene [292]. The per E-box appeared indispensable for a high-level expression of per, however not for its rhythmic expression [133].

Based on above findings, the following model has been proposed for the role of clk and cyc in the generation of circadian rhythmicity (cf. Fig. 8). CLK and CYC form a heterodimer, which binds to the E-box within CE of both the per and tim promoters, thereby promoting expression of PER and TIM [89]. The mechanism by which PER inhibits the transcriptional activity of the CLK/CYC heterodimers remains to be established. A PER-dependent sequestration of one of the binding partners, or a PER-dependent repressor have been suggested [118]. The finding that coexpression of PER and TIM in cultured cells is sufficient to inhibit CLK-dependent gene activation suggests a direct mechanism [89]. CRY seems to play no role, since CLK/CYC effects on tim expression are not modulated by an enhanced Cry expression [72]. The rhythmicity in clk0 and cyc0 Drosophila mutant flies arise from the elimination of the C-terminal glutamine-rich activator domain of CLK, and the elimination of the PAS B domain of CYC, necessary for the formation of heterodimers with CLK, respectively. Consequently PER and TIM are hardly expressed.

4.1.6. Location of the Drosophila clock

In order to localize the clock responsible for circadian locomotor rhythmicity in Drosophila, several strategies have been used. In transgenic flies driving a reporter gene under the control of the per promoter, cyclic transcription has been observed in photoreceptors in the eye, in several distinct neurons in the brain, and in peripheral body parts, such as the proboscis, antennae, legs, wings, thorax and abdomen [171,267,373,405]. However, when placed in DD, the amplitude of most rhythms declined, except for the rhythm in the brain which maintained the same amplitude [267]. This expression pattern was confirmed by PER immunocytochemistry [107,112,214,327,405]. PER is expressed in approximately 20 neurons in the dorsal part of Drosophila brain, and in approximately 30 neurons in the lateral part, as well as in many glial cells throughout the adult CNS [107]. Expression of PER in the lateral brain neurons (LNs), appeared to be necessary for locomotor rhythmicity in the fruit fly, although also neighboring glial cells might participate in rhythm generation [107]. The LNs show cycling PER and TIM levels, but interestingly, these neurons are the only neurons that show cycling of per and tim in the larval stage, and continue to do so throughout metamorphosis [171].

The photoreceptor nuclei of the Drosophila eyes appeared to contain an autonomous clock. When the eyes are placed in tissue culture, they maintain a circadian rhythm in LD, which however dampens in DD [267]. Strikingly, cycling of PER is observed in Drosophila eyes even when a constant level of per mRNA expression is induced [73,373]. The presence of an autonomous clock in the eye appears to be a common feature of a wide variety of species. Pacemaker activity in the eye has been reported in the mollusks Bulla and Aplysia [206,285], in the Xenopus [56] and in the beetle and cockroach [109,254]. Observation in Drosophila suggest that the observed clock in the eyes is probably not involved in the regulation of overt behavior of the fly, but might regulate local properties of photoreception over the 24-h cycle. Drosophila mutants without eyes and optic lobes, still show circadian
locomotor rhythmicity, and can even be entrained to LD cycles [106,137], indicating that the eyes are dispensable for photic entrainment of the Drosophila clock.

4.2. Identification of clock genes in other insect species

Based on the sequence of Drosophila per, homologs of this clock gene were identified in other insect species. In the giant silkmoth Antheraea pernyi, two other moth species, i.e. Hyalophora cecropia and Manduca sexta, and in the American cockroach Periplaneta americana, per homolog have been identified [282]. Especially the amino terminus, the PAS A region, the region downstream of PAS B, and the region surrounding the site of perS mutation in Drosophila show high identity among these species [282]. Similar to Drosophila, per mRNA and PER protein have a circadian rhythm in the silkmoth head and eyes, with peak mRNA levels at night [282,298]. In the silkmoth brain, expression is restricted to 8 neuroendocrine neurons in the dorsal lateral protocerebrum, in which TIM-like immunoreactivity is colocalized, and these cells are the origin of eclosion and flight rhythms [298].

Unlike for Drosophila, PER and TIM remain cytoplasmic in the silkmoth cells throughout the LD cycle, except in the photoreceptor cells, in which nuclear PER is observed. Moreover, peak levels of PER coincide with per mRNA peak levels, precluding the negative autoregulation of per expression in the silkmoth, as seen in Drosophila [298]. Per antisense RNA has been reported in the silkmoth, cycling in antiphase with per mRNA specifically in the eight neuroendocrine cells in the brain thought to contain the clock. Silkmoth transcriptional regulation of PER might therefore be differently organized than in Drosophila, and involve regulation by the formation of RNA–RNA duplexes [298]. Despite these differences between the fruit fly and silkmoth, a similarity in function of the PER proteins in both species is illustrated by the finding that expression of silkmoth cDNA in arrhythmic per0 flies can rescue circadian behavior [200].

4.3. Identification of clock genes in mammals

Two different strategies have been applied in the search for genes that might form the core of the mammalian pacemaker. The widespread presence of per homologs in a number of lower animal species suggests that the mechanism of the pacemaker might be evolutionary well conserved, and perhaps similarly organized in mammals. Screening for per DNA sequence homologies in mammalian brain tissue, in particular the SCN, therefore appeared a logical step. A forward genetic approach, screening for deviations in rhythmic behavior after a mutagenesis treatment, has however been introduced as well, and has resulted in the identification of the murine clk gene. Finally, due to the general interest in the molecular biology of circadian rhythmicity, mutant rodents and transgenic mice made for various reasons have often been tested for circadian behavior and possible anomalies appeared in some cases to correlate with the genomic changes.

4.3.1. Genetic screening

Using the per PAS region or the per repeat sequence as a template, a reverse genetic screening approach indeed resulted in the identification of several mammalian per homologs. Studies established their role as genuine mammalian circadian clock genes. Homologs for the Drosophila tim gene have now also been reported, but mammalian TIM protein appears to play a different role in the circadian pacemaker system of the SCN, since it does not behave as a genuine clock gene, not even as a clock-controlled gene.

4.3.1.1. Period repeat homologs. In mice and rat brain, two different homologs of Drosophila per have been identified using the per repeat sequence as a probe. The DNA sequence pp2.5 shares a 6 base pair repeat with Drosophila per encoding (Thr–Gly)n [159,160,221], whereas the DNA sequence mp41 contains a 5 base pair repeat encoding (Ser–Thr–Ala–Gln–His)n [158]. Outside these repeat sequences, the homology of pp2.5 and mp41 with Drosophila per is low. Both, pp2.5 and mp41 mRNA are rhythmically expressed in the rat SCN under LD and DD conditions with peak levels during subjective day-time and nadir levels during subjective night-time [158,160,221]. The levels of both mRNAs increase after a light stimulus, but only when applied during the subjective night time, with maximal levels within 30 min after the stimulus, similar to that of above described C-fos induction [158,221]. The highest levels for the transcripts are found in the SCN, but both are also detected in various brain regions and in peripheral organs. Notably, the brain regions containing the two per repeat homologs do not overlap: pp2.5 mRNA is present in the brain in the diagonal band of Broca, the tuberculum olfactorium, striatum, pineal gland and hippocampus [160], whereas the transcript of mp41 is detected in relatively high levels in the subfornical organ, the supraoptic nucleus and the primary olfactory cortex, and in somewhat lower levels in the PVN, anterior hypothalamic nucleus, caudate putamen, cortex, amygdala and nucleus lateral olfactory tract. Both, the pp2.5 and the mp41 transcript, are moreover found in the liver, whereas mp41 is also found in the pancreas, uterus, ovary, adrenal gland, kidney, intestine and spleen [158].

Since functional studies have not yet been performed, a possible role for these genes in rhythm generation can only be anticipated. The genes might be involved in light entrainment of the pacemaker, since the transcripts are similarly upregulated as C-fos, an IEG of which a role in entrainment has been shown [389]. However, a role for the mammalian per repeat-containing genes in rhythm genera-
tion is only based on the presence of the per repeat sequence, a part of the Drosophila per gene which appears not to be crucial for locomotor rhythms. Deletion of the per repeat sequence in Drosophila results in only slight changes in the circadian rhythm [97,134,399], and the length of the per repeat is not highly conserved among Drosophila strains [75,399]. This per repeat might be involved in the 1 min rhythm of the Drosophila male courtship song, which shows interstrain differences, possibly related to the variation in per repeat length [399]. The identified mouse genes containing this per repeat sequence thus might still be just clock-associated genes rather than real clock components.

4.3.1.2. Period PAS homologs. Using the more relevant PAS domain of Drosophila per as a probe, two independent groups isolated and cloned a per homolog from mouse and human brain which were originally named m-RIGUI and h-RIGUI [340] but now described as mper and hper [349]. Hper and mper share 92% homology with each other, and about 50% homology with per in each of five stretches. These stretches lie within the NLS, PAS, CLD, Per-C and Per-repeat regions. A database search for human genes related to the cloned hper gene resulted in the recognition of the human gene KIAA0347 [248] which appeared to share 47% identity and 70% homology with hper, and was named hper2. Based on the sequence of this gene, another mouse homolog was identified by RT-PCR on mouse brain cDNA [12,311]. The PCR product showed 81% identity with the human KIAA0347 gene. The complete cDNA was sequenced and the gene, named mper2, revealed 70% homology and 61% identity in the PAS domain and 47% overall identity with the initially cloned mouse homolog, mper1 [12]. Recently, a third mouse and human homolog has been cloned [411]. Overall, mPER3 shows about 36% amino acid identity to mPER1 and mPER2, which show 46% identity with each other. The highest homology between the three genes is observed in the PAS domain and several small regions outside the PAS region (Fig. 8). Notably, mPER3 lacks several small conserved regions shared by mPER1 and mPER2 [411] The presence of a bHLH motif, which would qualify mPER1 and mPER2 as transcription factors, is questionable, since the number of basic residues in the basic region of both per homologs is quite low (2 or 3) as compared to those in other bHLH-based transcription factors (5 to 7) [12]. Sequential analysis of mper3 suggested the presence of a HLH region at the same site as the putative HLH of mper1 [411]. So, not all aspects of the Drosophila PER can be observed in the mPER molecules.

In situ hybridisation showed the expression of all 3 PER homologs in the SCN of mouse. These levels of mper1, mper2 and mper3 mRNA cycle under LD and DD conditions [165,311,317,411], and mper1 and mper2 rhythms can be entrained to a new LD cycle [12,317]. Peak transcript levels of all three genes are reached around CT6 to CT9 [12,311,317,340,349,411], with a relative delay in phase of mper2 expression in LD (ZT9 vs. ZT3–6) [165]. mPER1 and mPER2 protein levels are rhythmic as well [108,136]. When mice are kept in LL, mper1 mRNA levels are generally elevated, but still a circadian rhythm can be observed [317].

In addition to the strong homodimeric interactions of mPER1 and mPER2 both in vitro and in vivo, all three mPERs were found to interact with each other [108,410]. The discovery of TIM homologs in mouse and human, and the observation that expression of mtim and mTIM in the SCN has no circadian rhythm [108,297,410], makes this observation of importance in describing the differences between the pacemaker mechanisms of Drosophila and mammals (see Sections 4.3.1.5 and 4.3.3.2).

4.3.1.3. Localization of the per homologs. As expected and mentioned above, all PAS homologs of the Drosophila clock gene per are present at high levels in the mammalian pacemaker, the SCN. A detailed histological study in the mouse SCN revealed mper1 transcription throughout the nucleus at the time of maximal expression (CT4–8). Thereafter levels first decrease in the vIscN at CT8, and by the time that the total mper1 expression levels were low (CT12), mper1 levels had decreased all over the SCN [317]. Expression of mper2 peaks at CT8, thus approximately 2–4 h later [411,347]. Immunocytochemical studies in mice [108,136] and hamster [223] revealed mPER1 and mPER2 protein levels to follow mRNA fluctuations with a lag-time of 4–6 h, similar to that the time lag between mRNA and protein seen in Drosophila. A high degree of colocalization of both PER1 and PER2 was observed in the mouse SCN, and both proteins fluctuate more or less parallely, and are localized in the nuclei, not in the cytoplasm of SCN cells [108]. Only when PER protein levels were at their nadir, per expression start to increase. This temporal relationship of rhythms is consistent with the role of mPER in the negative feedback in the circadian clock loop. mPER3 protein of mouse SCN cycled as well with a suggested peak in Western blots at CT10 (immuno- cytochemical staining of mPER3 could not be established) [108].

In rat SCN under normal circadian profile, rper1 and rper2 expression fluctuate with peak and trough levels at respectively CT/CT4 and CT/CT18, and CT/CT8 and CT/CT0 [394], thus also showing a 4 h-shifted rhythm pattern between per1 and per2 expression. Also in the hamster SCN such patterns are reported with peak values at ZT4 and ZT7 for per1 and 3–4 h later for per2 [223]. Thus, per1 and per2 expression profiles in rodents appeared not entirely in phase. In rat, the circadian profile of rper1 and rper2 mRNA oscillation in LD and DD occurred strongly in neurons of the dmSCN but weakly in neurons of the vIscN [394].

Besides the SCN as circadian pacemaker, recent findings have identified the mammalian retina also as a site for the
presence of an autonomous clock. Hamster retina cells kept in tissue culture secrete melatonin in a circadian fashion [352]. In tau mutant hamster retinal cells this period length interestingly follows the shorter free running period of the behavioral rhythm of these animals [127]. Synchronous rhythms in mper1, mper2 and mper3 mRNA expression was reported in the mouse eye with peak levels lagging 3 h behind peak levels in the SCN [311,340,411]. Notably, per mRNA expression rhythm in the eyes of Drosophila does not lag behind the rhythm in the brain, but has an identical phase [403,405].

Mper1 expression is seen in a number of forebrain regions of mice and hamsters, with high expression in the piriform cortex, olfactory bulb, hippocampal CA zones, striatum, thalamus and adenohypophysis [12,136,223,297,311,349]. mPER1 and mPER2 proteins are found in these sites as well, but levels do not oscillate under LD and DD conditions [108,136,223]. A rhythmic expression of mper1 mRNA under LD conditions is however reported in Purkinje neurons of the cerebellum and in the adenohypophysis [12,340]. The phase of the rhythm in the Purkinje neurons resembled the phase of the rhythm in the retina, with peak levels around ZT12, whereas the rhythm in the pars tuberalis was opposite phased with peak levels around ZT24 [340]. Mper2 is also constitutively expressed at relatively high levels in the piriform cortex, olfactory bulb and CA regions of the hippocampus [12,340]. Mper3 is highly expressed in the hippocampus, piriform cortex and cerebellum, with lower levels in the neocortex [411].

In the periphery, mper1, mper2 and mper3 are rhythmically expressed under DD conditions in the mouse liver, skeletal muscle and testis, with peak levels lagging a few hours behind the peak levels in the SCN [411]. Remarkable is the recent finding that a rhythmic expression of rper1 and rper2 can be induced by a serum shock in in vitro Rat-1 fibroblasts. A rhythmic expression was also induced for a number of other transcription factors, that are rhythmically expressed in most rat or mouse tissues under conditions of strong transcription and each shows reciprocity between light intensity and duration. Such extended reciprocity has been described previously in mammalian circadian systems [344] and is one of the unusual features of the photoreceptive system that mediates entrainment [110]. Considering the fast induction of mper1 expression after a light pulse, the activation of the mper1 gene is probably not mediated by the immediate early genes C-fos or jun-B, whose induction has also been related to the phase shifting properties of light pulses [389]. Recent studies confirm the immediate responses of mper1 and mper2, not of mper3 transcription in the SCN following light pulses at ZT14, but there was no detectable effect on mPER1 and mPER2 protein staining within the critical 2-h interval for resetting of circadian rhythm [108]. Accumulation of mPER1 became visible only after 9 h, i.e. in the nocturnal nadir of the protein. Only following a strongly resetting light pulse (12 h extension of the light phase), an increase in abundance of mPER1- and mPER2-immunopositive nuclei was observed at ZT24, confirming that nocturnal light can sustain high levels of both per mRNA and PER protein.

Induction of mper1 and rper1 by light during the subjective night is reported to occur initially in the retinorecipient vlSCN [12,394]. The first report suggested that after light exposure, mper1 expression is thereafter extended throughout the entire SCN [12]. Later studies indicate an enhanced expression of mper1 and mper2 only in the vlSCN [307], and this finding is meanwhile also confirmed for rper1 and rper2 in rat SCN [394]. Strongly resetting 12 h extension of light in mice also produced a clear spatially specific effect on mPER1 and mPER2 protein expression [108]. For mPER1 at ZT24, the staining in the dmSCN disappeared, but now cells in the vlSCN displayed nuclear staining far above the low level in control animals. For mPER2, a strong expression resulted throughout the ventral SCN, but also the staining in the nuclei of the dmSCN increased. In addition dendritic staining was observed, indicating cytoplasmic localization

4.3.1.4. Photic and non-photic regulation of per homologs. Expression of per1 and per2 in the mouse and rat SCN can be induced by a light pulse during the subjective night [12,317,394], in contrast to mouse per3, which is like Drosophila per not inducible by light [152,404,411]. The time course of the per1 and per2 mRNA increase differs, however.

Levels of mper1 and rper1 mRNA rapidly increased after a 15 or 30 min light pulse [12,311,317,394]. Several other features of light-induced mper1 mRNA [317] indicate that mper1 might be involved in the phase shifting action of light in the mouse: (a) the degree of induction of mper1 expression is dependent on the phase of the clock, (b) mper1 expression is induced at time points when a light pulse can shift the clock, (c) the degree of induction is directly related to the size of the phase shift (mper1 induction is also seen in the subjective light period, but the increase is weak and not associated with a strong behavioral resetting), and finally, (d) induction of mper1 expression and phase shifting appear to have the same threshold level of the light pulse and the same saturation level (similar phase response curve). The dose responses for the latter two processes are quantitatively very similar and each shows reciprocity between light intensity and duration. Such extended reciprocity has been described previously in mammalian circadian systems [344] and is one of the unusual features of the photoreceptive system that mediates entrainment [110]. Considering the fast induction of mper1 expression after a light pulse, the activation of the mper1 gene is probably not mediated by the immediate early genes C-fos or jun-B, whose induction has also been related to the phase shifting properties of light pulses [389]. Recent studies confirm the immediate responses of mper1 and mper2, not of mper3 transcription in the SCN following light pulses at ZT14, but there was no detectable effect on mPER1 and mPER2 protein staining within the critical 2-h interval for resetting of circadian rhythm [108]. Accumulation of mPER1 became visible only after 9 h, i.e. in the nocturnal nadir of the protein. Only following a strongly resetting light pulse (12 h extension of the light phase), an increase in abundance of mPER1- and mPER2-immunopositive nuclei was observed at ZT24, confirming that nocturnal light can sustain high levels of both per mRNA and PER protein.

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to be present as well. This topographic difference of light-induced expression of per genes and of PER proteins occurs therefore in primarily, but not exclusively, in the region of retinal input of the SCN, i.e. where (VIPergic) light-regulated peptidergic neurons are located (see Section 2.3).

Comparison of photic induction of the three mper homologs by a 15 min light pulse of 400 lux at CT14 or CT23 revealed a remarkable response difference between the homologs [411]. At both time points, mper1 mRNA was rapidly induced and returned to control levels within 3 h after exposure. Mper2 mRNA showed a slow induction at CT14, lasting for about 6 h, and a small and brief induction at CT23. Mper3 mRNA levels showed no induction at any time point. The apparent differential regulation of the 3 mper genes suggest that they have distinct regulatory elements, and that they serve different functions within the SCN. In the rat SCN, light-induced responses of rper1 and rper2 also revealed difference in duration of effect at CT16, when normal expression levels are low: light pulses gave a 5-fold peak response for rper1 within 60 min and a 1 h-delayed nearly 2-fold response for rper2 expression [394]. This difference and the phase dependent type of photic induction of mper2 might relate to the phase-delaying or phase-advancing effect of a photic stimulus, but this suggestion needs further investigation.

Taken together rodent SCN per and PER expression profiles under normal circadian and light-induced conditions, the studies indicate a strong autonomous expression ability with limited light response in the dmSCN neurons and a strong light responsiveness with a weak autonomous expression in the vlSCN neurons. The referred subpopulations of neurons fit the core and shell dichotomy of SCN neurons [188].

A single report exist on the non-photic resetting of per expression in the SCN. Confinement of hamsters to a running wheel that generally elicits considerable activity and arousal, and phase-shifts of the clock, results in an acute down-regulation of per1 and per2 mRNA levels [223]. Thus, the per genes are a common target for both photic and non-photic resetting cues. In vivo photic and glutamate-induced phase-shifts can be blocked by intracerebroventricular application of mper1 antisense oligonucleotides [8], stressing further the crucial role of the per genes.

4.3.1.5. Timeless analogs. Starting on the basis of structural identities of dtim in expressed tag sequence databases of mouse and human DNA, the mtitim and httitim homologs have recently been identified [297,410]. Homology between the two was substantial (82% identity at the nucleotides, 84% at the amino acid level), but also major differences between the mammalian and Drosophila tim sequences were observed (Fig. 8). Regions of homology were found with identities between 18 and 56% and similarities between 30 and 67%, but the mammalian gene is shorter and several functional domains are differently localized on the sequence. A DEDD sequence in the CLD at the C-terminus of dtim is preserved in the mammalian tim sequences (Fig. 8). The PER binding site PB1 of dtim carrying a NLS has a shorter homolog in mtitim and httitim, whereas the sequence of the PB2 site is interrupted by a sequence of no homology [297,410]. Mtim and httitim have more acidic stretches at different locations than the single one observed in dtim.

Whereas Sangoram et al. [297] reported httitim to interact with dPER, and to translocate in the nucleus of Drosophila cells and to inhibit CLK-BMAL1-induced activation of the mper1 promoter, Zylka et al. [410] and Field et al. [108] could not detect mPER–mTIM interactions in mice. Mtim is expressed in the mouse SCN, but mRNA and protein levels do not oscillate in neither LD and DD [108,136,297,410]. TIM mRNA and protein levels moreover remained unchanged in response to light at both ZT/CT14 and ZT22/CT23 [108,410]. Hence it became clear that mTIM (and possibly httitim) do not act as mammalian orthologs of dtim. Mammalian tim can therefore not been described as a clock gene perhaps not even as a clock-related gene (Fig. 10). As mPER expression alone could inhibit the CLK-BMAL1-induced activation of expression [297], PER–PER interactions rather than PER–TIM interactions have been suggested to play the necessary feedback autoregulatory role in the pacemaker system of the mammalian SCN [108,410]. Though mTIM levels in the SCN are low, the protein is still mainly present in the nuclei of SCN neurons [136], so that it is entirely possible that mTIM is a nuclear cofactor for negative regulation of CLK/BMAL1-mediated transcription. mTIM may complex with mCLK (see Section 4.3.2), which would fit with the ability of mTIM to shut down CLK/BMAL1-mediated transcription in vitro [311,410].

Mammalian tim is expressed in brain tissue (and remarkably high in the anterior pituitary), and many other organs such as the spleen, pancreas, thymus, testis, liver, heart and placenta, and with lower expression in lung and kidney [297,410]. In the anterior pituitary of mouse, the mtitim expression did not oscillate either [410,136]. The pattern of expression of mtitim and mper in the non-neuronal tissues of mouse is significantly different [410], suggesting that other binding partners for PER and TIM might be present.

4.3.2. Mutagenesis approach: identification of the ‘Clock’ gene

A mutagenesis approach in mammals has so far resulted in the identification of a single genuine mammalian clock gene of which the involvement in the generation of circadian rhythmicity is functionally established. In 1994, the group of Takahashi [370] reported a mutation in the mouse, ‘Clock’, which lengthens the period length of circadian wheel-running rhythms by 1 h in DD in heterozygotes, and by 3–4 h in homozygotes. The rhythm
Fig. 10. Model for the clock mechanism in mammalian SCN pacemaker cells proposed on the basis of the clock mechanism in Drosophila and reports of mammalian homologs of Drosophila clock and clock-related genes, including the involvement of CRY and VP. Three PERIOD proteins PER1, PER2 and PER3 are active in mammalian species, which can form either homodimers or heterodimers (with an homologous or heterologous PER or with CRY1 or CRY2) that inhibit expression of the per genes in a similar way as described for the PER/TIM complex in Drosophila (negative feedback loop; see also Fig. 9). These dimers enter the nucleus and interact by an as yet unknown negative mechanism with the CLK/BMAL1 complex that normally stimulates per expression through attachment to the E-box site. CRYs can also inhibit per transcription without the presence of PER. PER and PER/CRY dimers have a similar negative effect on the expression on VP and CRY and perhaps also on other clock-controlled genes (CCGs). TIM has no rhythmic expression levels in the SCN. The absence of a circadian rhythm in tim expression in the mouse SCN and the in vivo absence of PER/TIM complexes point to different yet unestablished role for TIM than that described in the Drosophila clock system (though PER/TIM complexes are shown to inhibit per and VP gene expression in vitro). CRYs are rhythmically expressed and the Cry/CRY loop may at the heart of the mammalian clock acting as the ‘mammalian Drosophila TIM’, but the loop is blind to light as photic input does not affect Cry expression. The per/PER loop of negative feedback is sensitive to light (via a yet unknown molecular pathway) and thus may confer photic sensitivity to the Cry/CRY loop. VP plays no key role in process of the endogenous mechanism of the mammalian pacemaker of the clock system, its expression is clock-controlled. However through the reported effect of the activation of the V1a receptor (also rhythmically expressed, but in reverse phase) on intracellular calcium levels, VP can influence transcription or post-transcriptional processing of clock proteins (dashed arrow lines), thereby influencing or modulating the clock mechanism.

in the latter group is however unstable and disappears after a few weeks in DD, but can be reinstated by a 6-h light pulse. In LD, the onset of wheel-running activity was more variable in the homozygous mice, while the distribution of activity over the day differed from the distribution of wild type and heterozygous mice [370].

Recently, the gene of mutation has been identified as a large unit of 24 exons (~100 kb) with predicted amino acid sequence motifs that suggest a transcription factor: a bHLH domain for binding to DNA, a PAS domain for protein–protein interactions, and a glutamine-rich (Q-rich) carboxyl terminus important for activating transcription [176]. The mutation in the ‘Clock’ mice is a A to T point mutation in the so-called clock gene (clk gene, see Section 4.1.5) by which exon 19 was skipped and a 51 amino acid sequence deleted from the glutamine-rich region, but leaving both the bHLH and PAS domains intact. Based on this sequence, the mutant protein can be expected to dimerize with other proteins and bind to DNA normally, but would be less able to activate transcription. Meanwhile
also hclk has been cloned and showed 89% homology with the mclk and 96% homology for the proteins [335] (Fig. 8).

Compelling evidence for the role of mCLK in rhythm generation comes from the rescue of the mutant phenotype by in vivo complementation with bacterial artificial chromosome (BAC) clones expressing the clk gene [15]. Induction of transgene mclk expression in otherwise wild-type mice resulted moreover in shortening of the circadian period [15]. The clk−/− mutant mice manifest a low amplitude mRNA circadian rhythm for each of the mper genes in the SCN in LD, and on the first day of DD [165], in line with CLK regulating the amplitude of per transcription. In the mouse, mclk mRNA expression was only found at high levels in the SCN, but also in the eyes, the pyriform cortex and hippocampus. In addition, expression was found in the supraoptic nucleus (SON), PVN, PVT and the medial habenula, and in most peripheral organs, such as the testes, ovaries, liver, heart, lung and kidney [15,176]. Clk mRNA levels reveal a circadian rhythm in the rat SCN, with the highest expression levels at ZT6 [1]. Levels of clk mRNA increased, moreover, after a 30-min light pulse with a similar phase dependence to the mper1 mRNA. mCLK protein has now also been immuno-cytochemically localized in the hamster SCN [191]. It is particularly expressed in the subset of VPergic SCN cells, not in VIPergic or CaBP-positive neurons and is rhythmically expressed. This seems to imply that disruption of circadian rhythm in the CLK mutant mouse may be due to disruption of pacemaker function in this subset. The human clk gene is expressed at relatively high levels in the human SCN and in the cerebellum [335].

The fact that several mammalian homologs have been found for Drosophila clock genes (per, tim, cyc and clk homologies), indicates that parts of the circadian time-keeping mechanisms are preserved through evolution (cf. Figs. 9 and 10). Recent findings support that mCLK act similar as in Drosophila: it binds to BMAL-1, the mammalian homolog of Drosophila CYC, and activates transcription from E-box elements in the mouse per1 gene [118]. The expression pattern of bmal1 RNA coincides moreover with the expression pattern of clk and mper1 RNA in mice, with high expression in the SCN and retina. Though these findings seem to illustrate the preservation of the parts of the clock mechanism, the above described observations on the absence of a critical role of TIM indicate a clear evolutionary dissociation between TIM’s role in the Drosophila and mouse (or mammalian) circadian feedback loop (see below) and cf. Figs. 9 and 10.

4.3.3. Clock genes in other rhythmically aberrant animals

Of the several mutant and transgenic animals that revealed changes in the circadian timing system in behavioral studies, only a few have been explored for the molecular pacemaker system, but new possible clock or clock-related genes have been identified.

4.3.3.1. Tau mutation hamster. First of all the tau mutant hamster strain, bred by selection for period length from golden hamster, has a reduced period length of body temperature and locomotor rhythms of about 22 h in heterozygotes and 20 h in homozygotes [274]. Several findings suggest that the shortened period length results from a speeding up of the clock. When animals are exposed to a light pulse, the relative duration of the phase delay and advance portions of the phase response curve (PRC) are equivalent, but differences exist in the magnitude of the response between wild type and mutant. Overall, the magnitude of the phase shifts of the mutant hamster are greatly enhanced after a prolonged period in DD, although two reports show conflicting results about an increase in the magnitude of the phase delay [131,319]. The short period length in the tau mutant hamster may concern a mutation in a real clock gene. The lack of an adequate genetic map in the hamster has impeded molecular analysis of the tau mutation and the identification of a clock gene.

4.3.3.2. CRY-deficient mice. Mice mutated through gene targeting in embryonic stem cells for expression of Cry1 and Cry2 show remarkable changes in their circadian activity pattern and photic responses [361]. Mammalian CRY proteins are members of the family of plant blue-light receptors (cryptochromes) and DNA repair photolyases (photoreactivation). They were first recognized in human cDNA, but lack detectable photoreactivating activity, and therefore called Cry. Two genes, Cry1 and Cry2, were found with 73% homology and with about 50% identity with dCRY [148]. Also in mouse, rat and hamster these two genes were identified [179]. In Drosophila, CRY indeed act as a photoreceptor interfering with the PER/TIM autoregulatory loop of circadian pacemaker activity (see Section 4.1.4 and Fig. 8). Expression of mCRY is high in the retina as well as SCN of the mouse [234], but a photoreceptor role in the SCN seems unlikely. Mammalian CRY1 is reported to be mainly present in mitochondria and CRY2 in the nucleus [179], but more recently both mCRY1 and mCRY2 were found to reside in the nucleus [184]. Mice lacking Cry1 or Cry2 expression display shorter (22.5 h) or longer (24.6 h) free-running period length respectively compared to wild type (23.8 h), whereas the double knockout Cry1−/−Cry2−/− mice are completely arrhythmic in DD [361]. Recent observations demonstrated that both mper1 and mper2 expression oscillate robustly in the Cry1−/− and Cry2−/− animals and that oscillations are absent in the double mutant [251]. Thus, the data provide a clear indication of Cry expression to be an indispensable component of the core oscillator.

All mutants have however a 24-h rhythm under LD conditions. In the totally CRY-deficient mutant, photic
stimulation by changes in the light regimes indicate light to promptly activate wheel-running behavior, masking the defective biological clock [361]. Light pulses induced a normal per expression response in the SCN, indicating mCRY is not essential for light-induced phase shifts of the biological clock [251]. The mutant deficient for one allele of Cry2 (Cry1/−−Cry2+−−) revealed initially a free-running rhythm upon exposure to DD (21.8 h), but loses periodicity after some time. Mutant mice with one allele for Cry1 remain rhythmic under DD (24.3 h). Apparently the expression of Cry2 is crucial but not sufficient to keep the clock running.

The nature of mammalian CRYs as clock-regulating genes has recently been investigated in wild type mouse [108,184,234,251]. Both Cry genes are expressed in the SCN, the level of mCry1 mRNA being several-fold higher. Expression of mCry1 is reported to oscillate in DD (peak between CT8–12). Expression of mCry2, while originally reported to be constitutive [184,234], now appears to oscillate as well [251]. The latter observation fits with the synchronous oscillating levels of both mCRY1 and mCRY2 proteins having a peak between CT12–16, paralleling the pattern of mPER accumulation in the SCN [108,184]. No immediate response of Cry expression is observed following light pulses [108,251], again indicating mCry not to be a target for resetting pulses. mCRY1 and mCRY2 appear active essential components of the negative limb of the circadian feedback loop. Both proteins interact with all mPERs as well as with mTIM to form heterodimers and can form also associates with each other as mCRY1/mCRY2 complexes [108]. Following translocation from cytoplasm to nucleus these complexes or their components blocks the CLK/BMAL1-induced transcription of mPER (and of VP; see Section 4.4). As the expression of Cry is rhythmic, and mCRY moves mPER to the nucleus, mCRY may act as the "mammalian dTIM" though without direct light responsiveness (Fig. 10).

The presence of PER appears to be no prerequisite for CRY nuclear translation: both CRY1 and CRY2 alone can inhibit CLK/BMAL1-induced transcription of mper and VP genes. The apparent lack of synergism on the CLK/BMAL1-mediated transcription of pairwise expression of the mCry1 and mCry2 genes and that of mper1, mper2 or mper3 in co-transfection experiments using NIH3T3 cells [184], seems to indicate independent effects of mPER and mCRY on the transcriptional machinery. The rhythm of mCry mRNA is moreover dependent on CLK as expression of mCry1 and mCry2 is reduced in the clk−− mouse and circadian rhythm of mCry1 is lost under free-run conditions. From this result it was concluded that mCRY translocation is not dependent on mPER–mCRY interactions and that the effect of CRYs (either alone or as complexes) must occur through direct or indirect interaction with the CLK/BMAL1-E box complex because this is the only complex common to both the VP and mper reporters [184]. mCry1 thus may play a dominant oscil-

lator role, explaining why mCry1+/−mCry2−− mice remain rhythmic whereas mCry1−−mCry2+−− mice become slowly arrhythmic under DD [361]. The mutual but different function of mCry1 and mCry2 in the time-keeping system still needs further investigations. For instance, it will be interesting to examine the effect of CRY1 and CRY2 mutations on the rhythmicity of individual SCN neurons as observed at the electrophysiological level.

Expression of mCry is also found in other parts of the brain, in the retina and in various peripheral organs like heart, lung, muscle, kidney, liver and testis [184,234,251]. The ratio of Cry1 and Cry2 expression differs in these tissues and rhythmic expression is not standard. In the mouse retina for instance the ratio is in favour of mCry2 and no circadian rhythms are present [234,251]. Outside the SCN neither a rhythm for expression is found. In muscle however, both mCry1 and mCry2 transduction exhibited a daily rhythm in LD and DD, with the peak of the mCry2 rhythm preceding that of mCry1 by 6 to 9 h, and the mCry2 rhythm delayed by several hour relative to the phase of its expression in the SCN [184]. This compares fully with the peripheral oscillations for the mRNA rhythms of the mpers [411]. The significance of peripheral rhythms of the clock or clock-related genes remains to be elucidated.

4.3.3.3. Other mutations. In any of the other mutant rodents mentioned above to have aberrations in circadian rhythms (4.3.3), studies on the molecular background have not been performed. Only for the C-fos null mutation mouse which has a normal periodicity of rhythm but deviates in its entrainability to LD cycles [145], a molecular basis can be put forward. The behavioral observations are in accordance with the known role of C-fos in photic phase-shifting. This is an example that mutant animals with aberrant circadian rhythmicity might reveal genes that do not have a direct role in the clock itself, but might turn out to be important genes involved in its input or output pathways.

4.4. Vasopressin and the mammalian clock genes

As VP is the most well-studied peptide of the SCN and its exact modulatory role is still unclear, it is challenging to speculate on its role in a Drosophila-like clock mechanism. It is clear that VP does not fulfill the criteria for a clock gene. The absence of VP does not lead to arrhythmicity (Brattleboro rat, see Section 2.2.2). Moreover, unlike Drosophila PER and TIM, VP has no negative feedback on its own transcription. Whereas mutations in the per or tim genes halt the rhythm of their mRNAs, the mutation in the VP gene in the Brattleboro rat only results in a somewhat lower amplitude of the mutant VP mRNA rhythm [355,398]. Several characteristics of VP expression in the SCN suggest that it is regulated by the circadian mecha-
nisms of the SCN. In particular, the persistence of VP mRNA and VP rhythms in the SCN under DD conditions show that the VP gene must be considered a ‘clock-controlled gene’. VP gene expression parallels the mper1, mper2 and mper3 expression in mouse SCN [165]. Like dper and dtim also the VP gene has an E-box element within its promoter region, to which the CLK-BMAL1 complex can bind and stimulate transcription [165]. Indeed the oscillation of VP mRNA and peptide expression is abolished in the clk−/− mouse SCN [165,325]. Co-expression with either mper1, mper2, mper3, mtim and mCry [165,184] all inhibited CLK/BMAL1-induced transcriptional activity (Fig. 10). This observation is consistent with the proposal that mPER–mCRY and mPER–mPER interactions are important regulators of the negative limb of the mammalian clock mechanism. It would be of interest to see whether VP in the SCN of the arhythmic Drosophila mutants with an altered period length are the result of either direct effects on the level of clock proteins (perS and perF, and timS and timF mutants) or indirect effects on post-translational processes which influence the stability of the clock proteins (dbt mutants) [24,104,268].

In mammalian SCN, the presence or absence of VP activity may also influence the levels of clock proteins. The increase in calcium levels following VP receptor activation might for instance affect enzymes involved in the processing of the clock proteins, such as casein kinase, the mammalian homolog of Drosophila DBT which phosphorylates PER, thereby mediating its breakdown and influence period length.

5. Genetic manipulation of the suprachiasmatic nucleus-adenoviral vectors

The preceding sections gave an overview of the vast amount of data available on the function and mechanisms of the SCN as generator of circadian rhythms in mammals. Many features of the SCN have been discovered using established anatomical and physiological techniques like immunocytochemistry, electrophysiology and behavioral tests. Pharmacological studies, applying neuroactive substances to the SCN, have greatly contributed to the elucidation of the role of these substances in the circadian time-keeping mechanisms. The fast discovery of genes involved in rhythm generation illustrates the high potential of modern molecular techniques. In addition to identification of clock genes, these techniques can also be applied to manipulate expression levels of clock and other genes in SCN cells to investigate further aspects of the clock.

An approach which appears promising in this respect, is gene transfer by means of viral vectors. In this paragraph, a short overview will be given of the technique, with special emphasis on the use of adenoviral vectors in the central nervous system. Furthermore, possibilities for the use of these vectors will be discussed for SCN studies.
using in vitro cultures, or transplantation and intact animals.

5.1. An introduction to adenoviral vectors

Viral vectors make use of the innate capacity of viruses to introduce their DNA in the nucleus of a host cell. This capacity make viruses efficient tools to introduce exogenous DNA into a wide range of cell types. Since the 1980s, several recombinant viral vector systems have been developed, each with their own advantages and limitations. An extensive review about these viral vector systems and their application in the nervous system has recently been published [140].

The adenoviral vector, based on adenovirus type 5, was developed in the early 1990s to transduce non-dividing cells, such as neurons, with a higher efficiency and less pathogenicity than the previously used Herpes simplex viral vectors. The wild type adenovirus contains a linear double-stranded DNA genome of approximately 36 Kb. The genome is packaged in a icosahedral capsid of 80 nm diameter, consisting of penton proteins and fibers proteins. Adsorption of an adenovirus particle to a host cell is initiated through a receptor interaction with the knob of the fiber protein. The penton base protein then binds to membrane-bound integrins whereafter the virus particle is internalized by receptor-mediated endocytosis. The viral nucleocapsid is released from endosomes and transported to the nucleus, where the viral genome comes free in the nucleus of the cell and remains episomal. The wild type viral genome encodes early (E) and late genes (L). The E1 gene is transcribed immediately upon nuclear entry, and encodes a series of transcriptional activators, promoting gene expression of the other E genes. The E1 gene is also essential for viral DNA replication, and, through activation of other E genes, also for expression of the L genes. The L genes encode capsid proteins, necessary for the capsidation of the viral DNA copies and thus for the production of new viral particles.

For the production of adenoviral vectors (Fig. 11), the inverted terminal repeat (ITR) containing the Ad5 origin of replication, the packaging signal (the ‘ψ’ sequence), and the E1 gene (first generation vectors) or more genes of the wild type viral genome are excised (second and higher generation vectors) [138,140]. The transgene, under the control of a constitutive viral promoter (e.g. of human cytomegalo virus CMV, or Rous sarcoma virus RSV) or a cell type specific promoter (e.g. of neuron-specific enolase NSE, of glial fibrillary acidic protein GFAP) is cloned in a targeting plasmid, thereby flanked by the adenoviral ITR, the packaging signal, and a fragment of the Ad5 genome. The plasmid is cotransfected together with the truncated Ad5 genome for homologous recombination in a producer cell line which provides the E1 function for multiplication of the recombinated genome. The recombinant genome is then packaged by these cells in capsid proteins transcribed

![Fig. 11. Generation of a recombinant adenoviral (Ad) vector. A transgene of interest is cloned in a targeting plasmid, flanked by the adenoviral inverted terminal repeat (ITR) and a fragment of the Ad5 genome. The targeting plasmid is co-transfected with the adenoviral genomic DNA (Addl309) after removal of the ITR and the packaging signal (ψ) of the E1 region by enzymatic cleavage. Homologous recombination occurs in a cell line genetically modified to contain the Ad E1 region (293 or 911 cells) and results in a recombinant replication-defective adenoviral vector containing the transgene.](image-url)
from the truncated Ad5 genome. The resulting adenoviral vector lacks the E1 gene which renders the vector replication-deficient and greatly reduces activation of transcription of the viral E and L genes.

Adenoviral vectors were shown to infect the main cell types of the CNS, viz. neurons, astrocytes, oligodendrocytes and ependymal cells, both in vitro and in vivo [9,19,62,90,138,187,284]. The majority of the studies using adenoviral vectors in the CNS concern studies on degeneration and regeneration of the CNS. These studies have been focussing on prevention of cell loss by providing genes for neurotrophic factors, on stimulation of regeneration by providing genes for growth-associated proteins or guidance molecules, and on supplementation of lost functions by providing genes for neurotransmitter precursors or deficient enzymes [140]. Recently developed viral vectors, based on the adeno-associated virus and the lentivirus, have shown to be potentially effective vectors as well [28,177,257], but were not yet introduced in the field of rhythm research.

5.2. Potential use of adenoviral vectors in SCN research

One of the evident applications of adenoviral vectors in the SCN, is to modify expression levels of proteins that are regarded important in the functioning of the SCN. For instance expression levels of a neuropeptide in SCN cells might be enhanced or decreased, or the relative levels of colocalized neuropeptides might be changed, and the consequences for the circadian rhythm investigated. An important application of adenoviral vectors can be anticipated in the field of the clock genes. Most of the candidate mammalian clock genes were identified on the basis of homology with Drosophila clock genes. Adenoviral vector-mediated gene transfer of these genes offers the possibility to facilitate the screening of their role in the generation of circadian rhythmicity. Expression of these genes may even be restricted to certain populations of SCN cells by selecting a specific promotor. Expression in neurons or glial cells only can be achieved by using the neuron-specific enolase (NSE) promotor or the (GFAP) promotor, respectively [289], whereas expression in a subpopulation of neurons can be achieved by using a promotor of a neuropeptide which is selectively expressed in that population [14]. Application of the VP promotor may, for instance, result in a rhythmic expression of the transgene, since VP is rhythmically expressed itself. A selective elimination of a population of SCN neurons may moreover be achieved when a lethal gene is driven by the neuropeptide promotor. The feasibility of these theoretical possibilities however depends on the efficiency of gene transfer and the duration of transgene expression in SCN cells in vitro.

5.2.1. In vitro cultures of the SCN

Previous in vitro studies have shown that all cell types of the CNS are transduced with a high efficiency when cultured as a cell suspension [62,138,284]. This offers the opportunity to apply adenoviral vectors to suspension cultures of the SCN and to study the effect of changes in expression levels of any protein on rhythmicity on the cellular level, for instance by electrophysiological recordings. SCN cell suspension cultures where indeed shown to be transduced with high efficiency by adenoviral viral vectors carrying the marker gene LacZ [392] (Fig. 12), although the identity of the transduced cells as SCN cells was not confirmed by double staining for SCN specific peptides.

Expression of e.g. specific neuropeptides or clock proteins could either be enhanced by using a adenoviral vector encoding the specific gene, or could be decreased by a vector encoding the antisense RNA for this gene. Using a constitutively active promotor, such as the CMV promotor, expression of a factor may dampen or mask rhythm and may result in arrhythmia of the (transduced) SCN cell. An out-of-phase rhythm may be induced by using the promotor of a normally rhythmic expressed gene. Finally, the

Fig. 12. SCN cell suspension prepared from E17 fetal rat and transduced with an adenoviral vector for the reporter gene LacZ (AdLacZ) revealed staining for the transgene product β-galactosidase after 10 days in vitro. Large number of glial and neuronal cells became transduced (Arens et al., unpublished observations). Bar 100 μm.
use of an inducible promotor may be used to temporally increase expression levels during a specific phase of the circadian rhythm. Effects of such manipulations in SCN cells could moreover be investigated in slice cultures of the SCN. In this model, the compact organization and circadian rhythmicity of the SCN remains intact [37,101], and viral vectors can be used to study a range of features of the SCN.

5.2.2. SCN transplantation

Viral vectors have been frequently used to produce genetically modified cells for neurotransplantation, for instance to replace lesioned dopaminergic cells in a model for Parkinson’s disease, or to provide neurotrophic factors in degenerative brain areas. Previous SCN transplantation studies have been focussing on the question what feature of the transplanted SCN is important for rhythm restoration of the SCNX host animal. Especially the contribution of the different populations of SCN neurons, and their afferent and efferent connections, to rhythm restoration seems important to establish. These issues might be investigated using adenoviral vectors which either eliminate expression of a certain neuropeptide, or eliminate the cells expressing the neuropeptide. Grafts of SCN cell suspension, which are capable of restoring a circadian wheel-running rhythm in SCNX hamsters [323], seem very suitable for this intervention, since the transduction efficiency is high in cell suspensions and cell populations can be eliminated before implantation. Also viral vector-mediated expression of neurotrophic factors in the fetal SCN graft may be introduced in an attempt to enhance SCN cell survival and to improve neurite growth and synaptic integration with the host brain.

The use of adenoviral vectors have meanwhile been explored in fetal rat grafts of solid SCN tissue. Transduction of cells in solid SCN grafts appears well feasible by ex vivo application of adenoviral vectors encoding the marker gene LacZ [34,364], and results in expression of reporter protein β-galactosidase for at least 7 months (Fig. 13), with ample signs of an host immune response [34,363]. The transduced cells in the transplant were of various origin: glial cells, neurons and ependymal cells, and were scattered throughout the transplants. The transduction of the transplant did not hamper the maturation of the VPergic and VIPergic cell populations of the SCN. However, hardly any cells of the transplanted SCN appeared infected by the adenoviral vector [34,363,364]. The compact organization of the SCN might be cause for this low infection rate, since it could have hampered a good penetration of the viral vectors following the ex vivo overnight immersion infection. On the other hand, the transduced SCN neurons may not have survived. It is therefore important to see whether application of the vector in in vitro SCN primary or slice cultures indeed can transduce specific SCN neurons without compromising their survival.

Fig. 13. Long-term survival of β-galactosidase-immunopositive cells following ex vivo transduction of a SCN graft placed in the IIIrd ventricle of rat. Two near-adjacent section showed the surviving SCN VIPergic cell cluster and the transduced cells at 7 month post-implantation. Note that the number of transduced cells within the SCN cell cluster is minimal. Bar 100 μm.

Though the limited infection grade of the grafted SCN by adenoviral vectors precludes the possibility to introduce foreign genes in order to study the effect on circadian rhythmicity, the method can still induce expression of functional peptides and proteins within the transplant. Expression of neurotrophins, even by a limited number of
non-SCN cells might be sufficient for the protein to exert an effect on SCN neurons in the transplant. Fetal rat SCN express the neurotrophin-3 (NT-3)-sensitive TrkC receptor [135]. SCN transplants that were ex vivo transduced with an adenoviral vector encoding for NT-3 and in which a number of cells express the factor, were larger in size as compared to transplants that were either mock-infected or transduced with an adenoviral vector encoding for the reporter protein β-galactosidase [362]. The CPP (i.e., VP)- and VIP-immunostained volume area of the transplants increased following NT-3 gene transfection, with no changes in staining density [Boer et al., unpublished], suggesting enhanced survival of the fetal SCN neurons. NT-3-transduced fetal SCN grafts placed homotopically in VP-deficient SCNX Brattleboro rats showed increased VPergic fiber innervation of the SCN target areas of the anterior hypothalamus and the DMH [362, Boer et al., unpublished]. In a preliminary experiment in which mock and AdNT-3 SCN grafts were placed in small groups of arrhythmic SCNX rats, restoration of circadian activity, drinking and eating rhythms occurred in 2 out of 7 and 3 out of 5 animals, respectively [362]. Thus, NT-3 production within the graft may enhance functional capacity of the donor SCN, so that the circadian rhythm of SCNX hosts is more efficiently reestablished. However, there was only no significant correlation of rhythm restoration and either VP and VIP immunostained volumes (though near significant for VIP, Fig. 14) or graft-to-host VP- and VIPergic neurite connections [Boer et al., unpublished].

Thus, the enhanced presence of VPergic and VIPergic neurons is not likely to have contributed to functional recovery. In stead, NT-3 may have stimulated yet other factors or subgroups of cells in the donor SCN.

5.2.3. Intact animal

The use of an intact animal to study the functioning of the SCN has clear advantages over the application in vitro SCN cultures and in vivo SCN transplantation studies. In order to establish the role of e.g. putative clock genes, and to fully appreciate the effect of an increase or decrease of their expression levels in the SCN on overt rhythmicity of an animal, manipulation of expression in the intact animal remains necessary. The generation of transgenic or knock-out animals for all of these genes is a laborious and time-consuming approach. In the case of the clock genes encoding transcription factors, the generation of transgenic animals might even result in phenotypes that are difficult to interpret, or that are not viable at all. As an alternative, a localized injection of adenoviral vectors containing these putative clock genes could potentially answer the same questions. By using an inducible promotor, the onset of the expression could even be controlled, so that rhythmic behavior of individual animals can be compared with and without expression of the gene of interest.

The spread of viral particles upon direct injection in the brain and the area in which transduced cells are found is dependent on the location of the injection. Injections in the hippocampus results in a horizontal spread of the viral particles.
particles with transduced cells present along the horizontal layers of the hippocampus. An injections in the facial nucleus results in transgene expression in virtually all cells within the boundary of the nucleus, but not in cells outside the nucleus [138]. The relative numbers of neurons and glial cells which are transduced appear to be variable among the different sites of the brain, although so far no attempt has been made to quantify the proportion of neurons and glial cells that express the transgene. Information on the infectability of SCN cells in vivo is not yet available.

Injection of adenoviral vectors in the brain is shown to be an efficient method to direct expression of a gene of interest to a high number of cells. However, the applicability of the adenoviral vectors in the CNS appears to be limited due to direct toxic effects and indirect immunological effects. The presence of a high number of viral particles in a small area was shown to be toxic for the cells in that area. Lowering of the concentration of viral particles in the injection solution and a slower infusion to increase the spread of the viral particles, could somewhat decrease the direct cytopathic effect. A second process resulting in the loss of transduced cells is a cellular immune response. An injection of the vector in the brain is associated with infiltration of cytotoxic T-lymphocytes. Due to this immune response, most transduced cells are eliminated within several weeks to a few months after transfection [50–52,85,138,139,395]. Suppression of the immune system, or the use of immunodeficient animals resulted in a slight increase in the duration of transgene expression, but proved to be no definite solution to the problem. In order to solve this problem, new generation adenoviral vectors were created, bearing less viral genes, to reduce the expression of viral proteins, which activate the host’ immune system. In the mean time, other viral vectors based on other viruses are developed, like the adeno-associated viral vector, and the lentiviral vector. As vectors in vitro or in transplantation studies are numerous. These viral vectors are shown to elicit hardly any or no immune response at all, these vectors might enable a long-term transgene expression in the brain. Infection characteristics of these new vectors have to be established so that they can be applied to further unravel the intrinsic properties of the SCN.

6. Concluding remarks

Great progress has been made in elucidating circadian time-keeping mechanisms since the 1970s. Initial studies focused on describing the role of the SCN in rhythm generation, and on describing its anatomical organization and neurotransmitter content. Functional studies were performed by lesioning input and output pathways of the SCN and investigating the consequence for behavioral rhythmicity. At that time, the SCN itself was more or less regarded as a black box, whose reaction to photic or other input was investigated and described in terms of behavioral rhythms. Using electrophysiological techniques and pharmacological interventions, the reaction of the SCN neurons to several stimuli, and the effect of several neurotransmitters on their individual circadian rhythms could be investigated more directly.

The introduction of SCN transplantation in the 1980s provided a new tool to study several aspects of the SCN, such as the importance of the compact cellular structure of the SCN and the nature of the communication pathways between the SCN and the rest of the brain. Although extrapolation of data from transplantation studies to the intact situation should be performed with great precautions, some features of the SCN could be established in transplantation studies as summarized in above. A great advantage of SCN transplantation is the possibility to manipulate the SCN prior to transplantation, which seems to make it a suitable model to study in the future the role of for instance putative mammalian clock genes.

The search for the intrinsic pacemaker mechanism of the SCN has benefited from the development of new molecular techniques in the 1990s and the extending knowledge of geneticists of clock mechanisms in invertebrate models. The recent identification of many putative mammalian clock genes necessitates further studies on their role in the SCN. At the same time, since many of these genes also show circadian rhythmicity in peripheral tissues, the question as to whether peripheral oscillators driven by the pacemaker cells in the SCN should receive great attention.

As a powerful new tool, genetic manipulation might prove to be useful to study the role of a variety of proteins in the SCN by manipulating their expression levels. In the last paragraph of this introduction, the potential of viral vectors to manipulate SCN neurons was summarized. The use of viral vectors in vivo awaits optimization of the technique, but the current possibilities for using viral vectors in vitro or in transplantation studies are numerous.

Acknowledgements

The authors would like to thank Drs. D.F. Swaab, J. Verhaagen and C. Pennartz for their valuable contribution in reading critically the manuscript. Partly supported by Grant 903-47-006 to K.E.v.E. from NWO-MW, Den Haag, The Netherlands.

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