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

Keywords PACAP · Glutamate · Substance P · Melanopsin · Suprachiasmatic nucleus · Circadian rhythm · Entrainment

Introduction

The mammalian biological clock is located in the hypothalamic suprachiasmatic nuclei (SCN), which in the rat consist of a heterogeneous group of approximately 16,000 neurons (van den Pol 1980). The SCN drives diurnal changes in physiology and behaviour, such as hormone secretion, temperature and the sleep-waking cycles, in a predictable manner thereby preparing the body for oncoming events and demands (Klein et al. 1991). When examined under constant conditions (constant darkness or constant light), the endogenous rhythms driven by the clock oscillate with a period length close to 24 h (Latin *circa* + *dies* = circadian). Consequently, the clock needs daily adjustment (entrainment) to be synchronized with the astronomical day length. Without entrainment, the endogenous rhythms will be “free running” resulting in a daily shift in rhythmicity depending on the length of the endogenous period. Two types of “zeitgebers” (photic and non-photic cues) act on the clock and are important for its daily entrainment. The most powerful zeitgeber known is the environmental light/dark cycle that arises because of planetary rotation. Photic information is processed by the retina and reaches the brain via the optic nerves. The signalling pathway to the circadian timing system mediating the light entrainment of the clock is, however, anatomically and functionally different from the neural pathway used for vision. The retinal projection innervating the circadian pacemaker originates from a subset of retinal ganglion cells and is known as the retinohypothalamic tract (RHT; Moore and Lenn 1972; Moore et al. 1995). Lesion of the optic nerves results in free running of the circadian rhythm and blindness (Morin and Cummings 1981), whereas selective lesions of the RHT fibres to the SCN result in “circadian blindness” but not in loss of vision.
(Johnson et al. 1988a). Retinal projections also reach other parts of the circadian timing system, such as the intergeniculate leaflet (IGL) of the lateral geniculate complex (Pickard 1985). Cells in the IGL integrate photic and non-photic information and project to the SCN providing feedback regulation of the pacemaker via the geniculo-hypothalamic tract (Moore 1995). Photic information from the RHT is also modulated within the SCN by non-photic input via neural projections originating mainly from the median raphe nucleus of the midbrain (Rea et al. 1994; Pickard et al. 1996, 1999; Meyer-Bernstein and Morin 1996; Meyer-Bernstein et al. 1997). At least one of the photoreceptors mediating photic information to the circadian timing system is functionally different from the classical photoreceptors used for vision (von Schantz et al. 2000). These observations are based on findings in mice lacking the classical photoreceptors, i.e. rods (rd/rd mice) or both rods and cones (rd/rdcl mice). These mice strains are visually blind as a result of severe degeneration of the retina but retain the ability to entrain to the light/dark cycle (Foster et al. 1991; Freedman et al. 1999) most likely due to an intact RHT (Provencio et al. 1998). The photopigment mediating light information to the clock is not known (see Bellingham and Foster 2002) but a good candidate for a “circadian photopigment” is a recently identified opsin, melanopsin (Provencio et al. 2000), which is exclusively expressed in the ganglion cells of the RHT (Hannibal et al. 2002; Hattar et al. 2002; Gooley et al. 2001). Until recently, the primary neurotransmitter of the RHT was considered to be the excitatory amino acid glutamate (for reviews, see Ebling 1996; Rea 1998). A few years ago, the widespread neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP; Vaudry et al. 2000) was found to be co-stored with glutamate in the rat RHT (Hannibal et al. 2000). Functional studies have provided evidence that PACAP alone or in concert with glutamate is involved in light signalling to the clock (Harrington et al. 1999; Chen et al. 1999; Nielsen et al. 2001).

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

### Neuroanatomical studies

#### Identification of the RHT

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

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