Research report

Direct vasoactive intestinal polypeptide-containing projection from the suprachiasmatic nucleus to spinal projecting hypothalamic paraventricular neurons

Rebecca Teclemariam-Mesbah a,b,*, Andries Kalsbeek a, Paul Pevet b, Ruud M. Buijs a

a Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam ZO, The Netherlands
b CNRS/URA 1332, Neurobiologie des Fonctions Rythmiques et Saisonnieres, Universite Louis Pasteur, Strasbourg, France

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Abstract

In mammals, photoperiodic information is conveyed from the retina to the pineal through a polysynaptic pathway, which includes the suprachiasmatic nucleus (SCN), the paraventricular nucleus of the hypothalamus (PVN), the spinal preganglionic neurons and, finally, the superior cervical ganglion. Precise data on the site in the PVN or which SCN transmitters are involved in the transmission of information in this pathway is lacking. In the present experiment we investigated whether SCN efferents containing vasoactive intestinal polypeptide (VIP) innervate PVN neurons that project to the spinal cord. A combination of retrograde tracing and immunocytochemistry with the aid of a confocal laser scanning microscope allowed us to assess possible interaction of SCN efferents and spinal cord projecting neurons in the PVN. Approximately 30% of identified autonomic projecting neurons in the dorsal PVN and 40% in the ventral PVN received VIP innervation mainly on their dendrites. These results provide further evidence for the involvement of SCN-derived VIP in the transmission of circadian information to the pineal.

Keywords: Hypothalamus; Suprachiasmatic nucleus; Paraventricular nucleus; Vasoactive intestinal polypeptide; Spinal cord; Light; Melatonin

1. Introduction

Circadian rhythms in mammals, including that of pineal melatonin release, are regulated by a neural pacemaker in the hypothalamic suprachiasmatic nucleus (SCN). The circadian rhythm in synthesis and release of melatonin is controlled via its sympathetic innervation, originating in the superior cervical ganglion (SCG). Until now, the anatomical pathway controlling melatonin secretion involving the SCN, the paraventricular nucleus of the hypothalamus (PVN), the spinal cord and the SCG, has been largely determined by lesions, which, at the level of either the SCN, the PVN or the SCG, abolish all rhythmic release of melatonin [24]. At present, only the transmitter involved in the last step, from the SCG to the pineal, has been established. Noradrenaline released from the SCG in the pineal stimulates melatonin synthesis [21]. The transmitters in the neurons contributing to the pathway are unknown. Preganglionic neurons projecting to the SCG have been localized in the intermediolateral column (IML) of the upper thoracic cord [30]. Hypothalamic descending projections to the IML originate from ventromedial and dorsal parvicellular PVN subnuclei [32,37]. The rhythmic pattern of melatonin secretion is thought to be controlled via a direct SCN input to these "autonomic" PVN subdivisions.

The SCN efferents have been studied by anterograde transport of radioactive amino acids [3,34,36], silver impregnation after lesion-induced degeneration [34] and by anterograde tracing with lectins such as Phaseolus vulgaris leucoagglutinin (Pha-L) [4,20,42]. The greatest part of SCN projections ends around the PVN. Specific regions reached are: the periventricular nucleus, the dorsal cap of the PVN and most extensively the zones immediately ventral and caudal to the PVN, i.e., the subparaventricular region (subPVN) and the dorsomedial hypothalamic nucleus (DMH). Thus, these regions may play a major role in relaying circadian signals to the rest of the brain. The subPVN is thought to be important for the integration of circadian and limbic information [41]. In the golden hamster and the rat, vasoactive intestinal polypeptide (VIP), but also vasopressin (VP), gastrin releasing-peptide (GRP)
and \(\gamma\)-aminobutyric acid (GABA) have been shown to be present in projections from the SCN to the PVN and subPVN [4,6,20]. We investigated the possible connectivity between the afferent input from the SCN and spinal cord projecting neurons in the PVN in more detail. Since the VIP and VP innervation of the PVN region have been shown to originate from the SCN exclusively, we employed VIP and VP immunocytochemistry in combination with retrograde tracing from the spinal cord to investigate the possibility of a direct projection from the SCN to PVN neurons projecting to the spinal cord. Possible contacts were further assessed with the aid of a confocal laser scanning microscope (CLSM).

2. Materials and methods

Eight adult male Wistar rats (200–300 g) housed under 12:12 h light/dark conditions with food and water ad libitum were anaesthetized with hypnorp (1 ml/kg). Unilateral pressure injection of 0.2 \(\mu\)l of fluorogold 5% (Fluorochrome, Englewood, CO) or of a suspension of rhodamine-coated latex beads 10% (Molecular Probes) were made in the thoracic (T1 to T4) spinal cord. Following a survival period of 4 to 7 days, the animals were anaesthetized again with pentobarbital and perfused through the heart with 50 ml saline followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered 0.9% saline (PBS) pH 7.4. The brain and spinal cord were removed and stored in vials containing the same fixative. After further overnight fixation, 35-\(\mu\)m sections through the hypothalamus and the spinal cord were cut on a vibratome. Spinal cord sections were mounted on glass slides and examined under fluorescence illumination to verify injection placement. Only small injections that included the IML and a restricted part of the grey matter were studied. Injections that did not include the IML or completely covered the grey matter unilaterally or more were not considered. Hypothalamic sections of animals with accurate fluorogold injections were incubated in rabbit anti-fluorogold (Chemicon) 1:6000 overnight, then incubated with a biotinylated goat anti-rabbit (Vector) for 1 h followed by avidin-biotin-peroxidase complex (Vector). Peroxidase was reacted with diaminobenzidine (DAB) 0.025% in TBS with 0.015% \(\text{H}_2\text{O}_2\). They were then incubated overnight with rabbit anti-VIP (Viper, Netherlands Institute for Brain Research, NIBR) or rabbit anti-VP (Truus, NIBR) followed by the same protocol; only 0.5% nickel ammonium sulfate in TBS was used in the last step. Sections were mounted on gelatin-coated slides, dehydrated in graded ethanol, cleared in xylene and coveredslipped with Entellan. Hypothalamic sections of animals with rhodamine coated latex beads injections were processed for immunofluorescence. The sections were incubated with rabbit anti-VIP (Viper, NIBR) 1:2000 overnight. They were then incubated with a donkey anti-rabbit antibody labeled with FITC (Jackson) 1:400 for 1 h. All antibodies were diluted in TBS with 0.5% Triton and all rinses between incubations were done 3 \(\times\) 10 min in TBS. The sections were mounted on gelatin coated glass slides and coverslipped in a slow fading medium (Vectashield). Sections were analyzed with a Zeiss CLSM microscope and scanned with an Argon blue laser (488 nm) and a red laser (633 nm). Scanning time was 2 s averaged four times. By simultaneous scanning we could observe the possibility of contacts between labeled cells and VIP or VP fibers.

3. Results

The spread of the tracer was limited with both fluorogold and rhodamine beads. In three cases, the center of the injections covered the IML. Only these cases are considered in this study. After injection of fluorogold in the spinal cord, retrogradely labeled, large elongated or round cells with extensive dendritic arborization were observed in the rostral PVN (PVN,) and small elongated or comma-shaped cells in the dorso-medial parvcellular PVN (PVNap) (Fig. 1A, B). Both comma-shaped small cells and large elongated cells were observed in the ventral medial parvicellular PVN (PVNvp) (Fig. 1A). Few labeled cells could be seen in the periventricular area, the subPVN, the zona incerta, the nucleus reuniens or the dorsomedial hypothalamic nucleus (DMH).

The staining intensity of VP-containing fibers originating from the SCN within the PVN appeared too weak compared with the staining intensity of PVN VP fibers and cell bodies to investigate reliably close appositions with retrogradely labeled cells. The VIP-containing fibers could be seen leaving the SCN dorsally towards the PVN where they could be demonstrated unambiguously in several parts of the PVN. They appeared thin with small varicosities. They innervated mainly the subPVN but a mediocre density of fibers could be seen also in the PVN,, the periventricular PVN and the PVNap (Fig. 1A).

In the PVNap and in the PVNvp, terminal boutons of VIP-containing fibers could be seen in close apposition to retrogradely labeled cells sometimes at the somatic level but more often at the dendritic level (Fig. 1B). Some neurons received VIP innervation on several dendrites and on the soma (Fig. 1B). Isolated portions of retrogradely labeled dendrites in the PVN,, probably from cell bodies in the PVNvp, were also seen to receive occasional innervation (data not shown). To investigate to what extent spinal cord projecting PVN neurons received VIP innervation, we employed two criteria to decide a contact at the light microscopic level: (1) the labeled cell and the VIP fiber are in the same plane; (2) at least one bouton contacts the labeled PVN neuron. In the PVN,, all retrogradely labeled neurons received VIP innervation. Approximately 30% of retrogradely labeled neurons in the PVNap received VIP innervation. This proportion was higher, approximately
Fig. 1. VIP-containing innervation on spinal projecting PVN neurons. A: labeled PVN neurons after fluorogold injection in the upper thoracic cord (brown) are mainly located in the medio-ventral (PVNm) and dorsal (PVNd) parvocellular subdivisions of the PVN. VIP-immunoreactive fibers, in black, originating from the SCN (arrow) overlap, at least partly, this distribution. Scale bar = 200 μm. B: higher magnification of the dorsal part of the PVN (PVNd) shown in A. Several labeled neurons receive VIP innervation (arrowheads). Scale bar = 72 μm. C, D, E: CLSM generated optical sections of retrogradely labeled neurons in the PVN after rhodamine latex beads (red) injection in the upper thoracic cord receiving VIP innervation (green). C: projection of 12 consecutive 1-μm-thick optical sections of a labeled neuron in the dorsal PVN. Scale bar = 5 μm. D: projection of 10 consecutive 1-μm-thick optical sections of a labeled neuron in the ventromedial PVN. Scale bar = 5 μm. E: projection of eight consecutive 1-μm-thick optical sections of a labeled neuron in the lateral PVN. Scale bar = 5 μm.
40%, in the PVN<sub>mp</sub>. In the caudal PVN, approximately 25% of retrogradely labeled neurons received VIP innervation.

The retrograde filling of rhodamine-latex beads neurons in the PVN was not as complete as with fluorogold. However, rhodamine-latex beads allowed a correct outline of the soma and proximal dendritic roots. Therefore, a confirmation of possible VIP innervation on distal dendrites was not possible. Nevertheless, with the aid of the CLSM, close appositions on somas and proximal dendrites could still be confirmed in series of 1-μm-thick sections (Fig. 1C, D, E).

4. Discussion

Our present results show that approximately 30% of spinal cord projecting neurons in the PVN<sub>dp</sub> and approximately 40% in the PVN<sub>mp</sub> receive VIP innervation. This VIP innervation originates exclusively from the SCN, because it was absent in SCN-lesioned animals [20]. VIP input from the SCN to the PVN is mainly directed toward the subPVN, and only a small contingent of VIP-containing fibers innervates the PVN<sub>dp</sub> and the PVN<sub>mp</sub> [4,20]. Experiments of anterograde tracing from the PVN to the spinal cord [17] have shown that apart from the IML, PVN projection areas comprised neurons in the central autonomic area (CA or lamina X) and an area connecting the IML and CA. The use of conventional tracers does not allow us to restrict our injection in the spinal cord to only preganglionic neurons. Our injections cover the IML and a small part of the region between CA and the IML. Retrograde labeling from these injections should therefore include most spinal cord projecting neurons of the PVN. Moreover, indications from transneuronal tracing with viral particles from for example the adrenal [35] have shown that the areas of the PVN which are labeled by the virus are the same as in our study.

We have shown that there is a VIP-containing input from the SCN to part of the spinal cord projecting neurons in the PVN, thus providing the anatomical basis for a direct influence of the SCN on autonomic centers.

VIP-containing neurons in the SCN have been shown to receive both a direct retinal input [18] and an indirect one via the intergeniculate leaflet [15]. Light not only entrains the rhythm of melatonin secretion, but it also results in an immediate and acute inhibition of melatonin synthesis in the pineal [16], which is mediated through the SCN [26,40].

The daily rhythm of immunoreactivity and mRNA expression for VIP in SCN neurons peaks during the night [2,25,27,38,44,43]. Changes in retinal input can alter this VIP rhythm [1,22,28,33], which is abolished in constant dark conditions [33,39]. The VIP rhythm is modulated by photoperiod [8]. It is not known whether these changes in mRNA and VIP levels observed in the SCN are paralleled by changes at the release site. However, the study of Morin et al. [25] suggests that in certain target areas, e.g., PVN, this increased synthetic activity results in an increased release of VIP. In normal conditions, the peak of VIP mRNA coincides with the peak of melatonin synthesis [21]. Therefore, VIP is a good candidate for the mediation of photoperiodic information from the SCN to the PVN. Our results provide the basis for a direct route from the SCN to the PVN comprising VIP-containing fibers that would therefore transmit a 'photoperiod' signal to these autonomic neurons in the hypothalamus.

Recently, a VIP rhythm in the SCN was observed under constant dark conditions, after somatostatin depletion [11] or the blockade of the serotoninergic input to the SCN [29]. A VIP mRNA rhythm was also observed in animals reared in the dark [12]. It appears thus that VIP neurons may be able to oscillate endogenously, but in intact conditions these oscillations are under strong control of light and/or other afferent inputs. Therefore it is unlikely that VIP is part of the output of the circadian clock.

Induction of c-fos expression in the SCN after a light pulse showed that only few [31] or no [9] VIP-containing neurons expressed Fos. Particularly GRP- and peptide histidine isoleucine (PHI)-containing neurons expressed c-fos [9,31]. Although VIP and PHI are processed from the same precursor, here, Fos was observed mainly in neurons expressing only PHI and not VIP. It was suggested that PHI-containing neurons would be more prominently involved in light-induced phase delays and GRP with the VIP/PHI neurons in the light-induced phase advances [31]. VIP might therefore be only a minor part of the acute transmission of light to the pineal. Microdialysis studies show that the GABA-containing projection from the SCN to the dorsomedial hypothalamus (PVT–DMH) complex transmits a fast inhibitory signal on melatonin secretion [19]. A GABA-containing projection from the SCN to the PVN has been demonstrated by a combination of tracing and immunocytochemistry [5]. Electrophysiological data confirm these anatomical and physiological data showing that there is a monosynaptic transmission from SCN to PVN [13] involving GABA and glutamate [14]. In addition to amino acids, the effects of peptides such as VIP on PVN neurons after SCN stimulation cannot be ruled out [14], probably because in contrast to amino acids, peptides are released preferentially or only from axon terminals at times of increased neuronal activity [7,10,23]. Determining the role of peptides in the inhibitory effect of light is therefore not possible at the moment. The fast GABA- or amino acid-mediated and slow peptide-mediated transmissions could account for two types of output information from the SCN: (1) an acute effect of light mediated by GABA that immediately inhibits the synthesis of melatonin in the pineal, and (2) a circadian effect of the SCN mediated by peptides such as VIP, which would determine the melatonin synthesis at the beginning of the night and adjust it to the photoperiod.
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References


