

# Neuropeptidergic Organization of the Suprachiasmatic Nucleus in the Blind Mole Rat (*Spalax ehrenbergi*)

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**ABSTRACT:** The blind mole rat, *Spalax*, is a subterranean rodent with atrophied, subcutaneous eyes. Whereas most of the visual system is highly degenerated, the retino-hypothalamic pathway in this species has remained intact. Although *Spalax* is considered to be visually blind, circadian locomotor rhythms are entrained by the light/dark cycle. In the present study we used anterograde tracing techniques to demonstrate retinal afferents to the suprachiasmatic nucleus (SCN) and immunohistochemistry to examine the distribution of neuropeptides that are known to be involved in the regulation or expression of circadian rhythmicity. Based on the localization of retinal afferents and neuropeptides, the SCN can be divided into two subdivisions. The ventral region, which receives retinal afferents, also contains vasoactive intestinal polypeptide (VIP)-containing neurons, and fibers that are immunopositive to neuropeptide Y (NPY) and serotonin (5-HT). The dorsal region contains vasopressinergic neurons, but this latter cell population is extremely sparse compared to that described in other rodents. The dorsal region is also characterized by numerous VIP-immunoreactive fibers. The presence of NPY and 5-HT fibers suggests that the SCN receives afferent projections from the intergeniculate leaflet and from the raphe nuclei, respectively. These neuroanatomical results, together with previous studies of behavior, visual tract tracing, and immediate early gene expression, confirm that an endogenous clock and the capacity for light entrainment of circadian rhythms are conserved in the blind mole rat. © 1997 Elsevier Science Inc.

**KEY WORDS:** Circadian system, Neuroanatomy, Immunohistochemistry, Serotonin, Vasoactive intestinal polypeptide, Neuropeptide Y, Vasopressin, Subterranean mammal, Rodent.

## INTRODUCTION

The blind mole-rat (*Spalax ehrenbergi*, Nehring) is a subterranean rodent that displays a mosaic of morphological and physiological adaptations to underground existence [54,55]. This *Spalax* super-species complex includes several chromosomal species ranging from the Northern part of Africa, through the near-east to Turkey and Southern Russia [54]. The subcutaneous atrophied eye measures less than 700  $\mu\text{m}$  in axial length [14,16,65], leading to a retina that is highly reduced in size but appears normally constituted with thin internal and external plexiform and nuclear layers [14,20]. The photoreceptors are extremely small but contain a rod and/or cone-like opsin [3,19,65]. The reduction in retinal surface area is correlated by a reduction in the total number of ganglion cells and optic nerve fibers (less than 900) [16,17,27,28]. Ganglion cells form a single morphological class [17], and resemble the

gamma type ganglion cell described in the cat [85]. In contrast to other rodents the optic nerve is composed entirely of unmyelinated fibers [27,28]. This ocular atrophy has resulted in a severe regression of retino-thalamic and retino-tectal projections, although the retinohypothalamic tract (RHT) has remained intact [5,17].

Although *Spalax* was initially considered to be completely blind [22,24], light entrains circadian locomotor activity [61,62] and induces expression of the proto-oncogene *c-fos* in the suprachiasmatic nucleus (SCN) [83], demonstrating that the photic system effectively transmits light information to the biological clock. In addition, thermoregulatory capacities in *Spalax* are photoperiod dependent [24], and perception of photoperiodic changes by the eye and melatonin are involved in the response [58].

Although the neuropeptidergic organization of the SCN has been studied in a number of rodents (rat [80,81]; hamster [51]; mouse [15]) the mole rat hypothalamus has not been examined. The neuropeptides and connections of SCN neurons are considered to be essential components for the generation and regulation of circadian rhythms by the endogenous clock. For example, many neuropeptides show quantitative endogenous variations, are influenced by the external light cycle, or affect the activity of the SCN. For example, the liberation of vasoactive intestinal polypeptide (VIP) varies with the external light cycle [68,69], whereas vasopressin (VP) content shows an endogenous rhythm in the SCN [19,36]. Afferent serotonergic (5-HT) projections from the raphe nucleus and neuropeptide Y-containing fibers (NPY) from the intergeniculate leaflet (IGL) also regulate the activity of the SCN [8,21]. The objective of the present study was to characterize the neuropeptide content of the SCN in the blind mole rat.

## MATERIALS AND METHODS

### Immunohistochemistry

The animals used in this study ( $n = 8$ ) originate from the Anza population in northern Israel. Animals were caught in the field and included adult males and females. Animals were anesthetized with a lethal intraperitoneal injection of sodium pentobarbital and perfused through the heart with warm (37°C) saline, followed by cold (4°C) Zamboni's fixative [87]. The brains were removed and sunk in 30% sucrose solution in phosphate buffer (0.1 M, pH 7.4). The brains were sectioned on a freezing microtome at 40  $\mu\text{m}$  thickness. All immunohistochemical reactions were carried out at 4°C. Endogenous peroxidase was first suppressed by a 30-min incubation in a 50% ethanol–50% saline solution containing 3%  $\text{H}_2\text{O}_2$ . Sections were then rinsed in phosphate buffer containing 0.3% Triton,

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0.1% sodium azide, and 0.9% sodium chloride (PBSTA). Following a 30-min incubation in 10% normal sheep serum in PBSTA, sections were rinsed in PBSTA and incubated for 3–5 days with the primary antibody diluted in PBSTA, containing 1% bovine and 1% human albumin sera. Dilutions depended on the antibody employed (VIP, 1/2000; VP 1/5000; NPY 1/10.000; and 5-HT 1/15.000). In most cases one series of SCN sections were incubated with two different antibodies on alternate sections. After three rinses in PBSTA, sections were incubated in the secondary antibody (sheep anti-rabbit, diluted 1/300) for 24 h, rinsed in PBSTA and incubated in rabbit peroxidase antiperoxidase (PAP, DAKO) diluted 1/2000 for 24 h. Sections were rinsed in Tris buffer (pH 7.0, 0.05 M). Sections were then reacted in a 3-3' diaminobenzidine solution (DAB, 0.02%) containing 0.5% nickel ammonium sulfate and 0.003% H<sub>2</sub>O<sub>2</sub> for 5–20 min. The specificity of the antibodies has been reported elsewhere by replacing the first and second antibodies by normal rabbit serum and sheep gamma-globulin or by incubating the first antibody with saturating amount of the homologous antigen prior to use [78]. Cell counts were made visually or using computer-assisted image analysis (Biocom, Les Ulis, France).

#### Retinal Injections

Six animals received an intraocular injection of a 2% solution of wheat-germ agglutinin horseradish peroxidase conjugate (WGA-HRP) or a mixture of 2% WGA-HRP and 0.2% cholera toxin horseradish peroxidase conjugate (CT-HRP). Animals were first anesthetized with an intraperitoneal injection of ketamine hydrochloride (30–40 mg/kg) and xylazine (2 mg/kg). Total volume injected in the eye varied from 0.5–1.0  $\mu$ l. Injections were made using a 50  $\mu$ m tipped glass pipette sealed to the needle of a Hamilton syringe. Animals survived for periods of 48–72 h. For fixation, animals first received a lethal dose of anesthetic and were subsequently perfused through the heart with 300 ml of warm saline (0.9%) followed by 1 liter of cold 4% paraformaldehyde in 0.1 M phosphate buffer. After 20 min this was followed by a postfixation rinse of 10% sucrose in the same buffer. The brain was subsequently removed and placed overnight in 30% sucrose prior to sectioning. The brains were sectioned on a freezing microtome in the coronal plane at a thickness of 30–40  $\mu$ m. The anterogradely transported HRP was reacted using tetramethylbenzidine (TMB) as a chromagen, according to the method of Mesulam [46] as modified by Gibson et al. [23]. Alternate sections were also used for cytoarchitectural study (Nissl stain).

All the experiments were carried out in agreement with the ethical national and European guidelines, and the necessary permits for animal housing and experimentation were obtained.

## RESULTS

Because the cytoarchitecture and retinal projection to the SCN have been described in detail elsewhere [16,17], only a brief description will be given here. The cytoarchitecture and size of the SCN is comparable to that of other rodents. In Nissl-stained coronal sections, the SCN appears as a compact aggregate of densely stained, small-sized cells located on each side of the third ventricle (Fig. 1B). Cell density is greater in the ventral as compared to the dorsal part of the nucleus. The nucleus receives a bilateral projection from the retina, with the greatest density of retinal terminals located in the ventral region (Fig. 1A). The distribution of neuropeptides allows division of the nucleus into two distinct regions.

#### Vasoactive Intestinal Polypeptide (VIP)

In the SCN, VIP-immunoreactive cells are located in the ventral region of the SCN (Fig. 1C). Immunoreactive VIP fibers extend from these neurons to fill the entire dorsal region, and partly extend beyond the dorsal border of the nucleus to adjacent hypothalamus. This distribution of VIP cells and fibers is found throughout the entire rostrocaudal extent of the nucleus. The total number of VIP-positive cells in the SCN is  $552 \pm 251$ . VIP neurons are round in shape (Fig. 2A) and of relatively small size ( $12.65 \mu\text{m} \pm 1.22$ ). The fibers that extend into the dorsal region are of fine diameter and contain few varicosities. VIP cells are absent from other regions of the hypothalamus.

#### Vasopressin (VP)

Relatively few ( $68 \pm 23$ ) vasopressinergic cells are found in the SCN (Fig. 1D). These cells are restricted to the dorsal part of the nucleus, and were only observed in the rostral region. VP-containing fibers are absent from the ventral region of the SCN. The VP-containing neurons are larger in size ( $15.30 \mu\text{m} \pm 2.28$ ) than the VIP cells, show a simple dendritic arborization (Fig. 2B), and are often located around small blood vessels.

VP cells are abundant in other regions of the hypothalamus, and in particular, the anterior hypothalamus, the supraoptic nucleus, and the hypothalamic paraventricular nucleus.

#### Neuropeptide Y (NPY)

NPY-containing fibers are located in the ventral region of the SCN (Fig. 1F), distributed in the same part of the nucleus occupied by VIP neurons. These NPY fibers form a dense plexus and contain numerous large-sized varicosities (Fig. 2D). The dorsal and peripheral regions of the SCN contain relatively few, fine-diameter fibers. No NPY-positive cells are present in the SCN. Adjacent regions of the anterior and lateral hypothalamus, as well as the paraventricular nucleus and the borders of the third ventricle, show a dense meshwork of NPY fibers. In coronal-stained sections, the periphery of the SCN is outlined by a distinct region in which NPY fibers are entirely absent.

#### Serotonin (5-HT)

Fibers immunoreactive to 5-HT are present throughout the entire SCN, but the density is clearly greatest in the ventral region (Fig. 1E). The 5-HT fibers show numerous medium-sized varicosities (Fig. 2C). This region of greatest density corresponds to the distribution of NPY-containing fibers and VIP-containing neurons. 5-HT fibers are absent from the part of the nucleus containing VP neurons. A few 5-HT fibers are also present in the paraventricular nucleus.

## DISCUSSION

The SCN in rodents and in other mammals is divided into two distinct regions according to the distribution of neuropeptides, connections, and retinal innervation [47,76,81]. For example, in rodents the ventral region is characterized by VIP neurons and afferent fibers containing NPY, 5-HT, and substance P, whereas the dorsal region contains VP and somatostatin-containing neurons. Except for a brief description of the presence of VIP of the SCN of another subterranean mammal, the Japanese mole [42], the present results show that the SCN in *Spalax* is also organized according to these two fundamental subdivisions.

The distribution of VIP neurons in the ventral region of the SCN in *Spalax* is comparable to that of other rodents [81] and the insectivore Japanese mole [42]. In contrast, the total number of

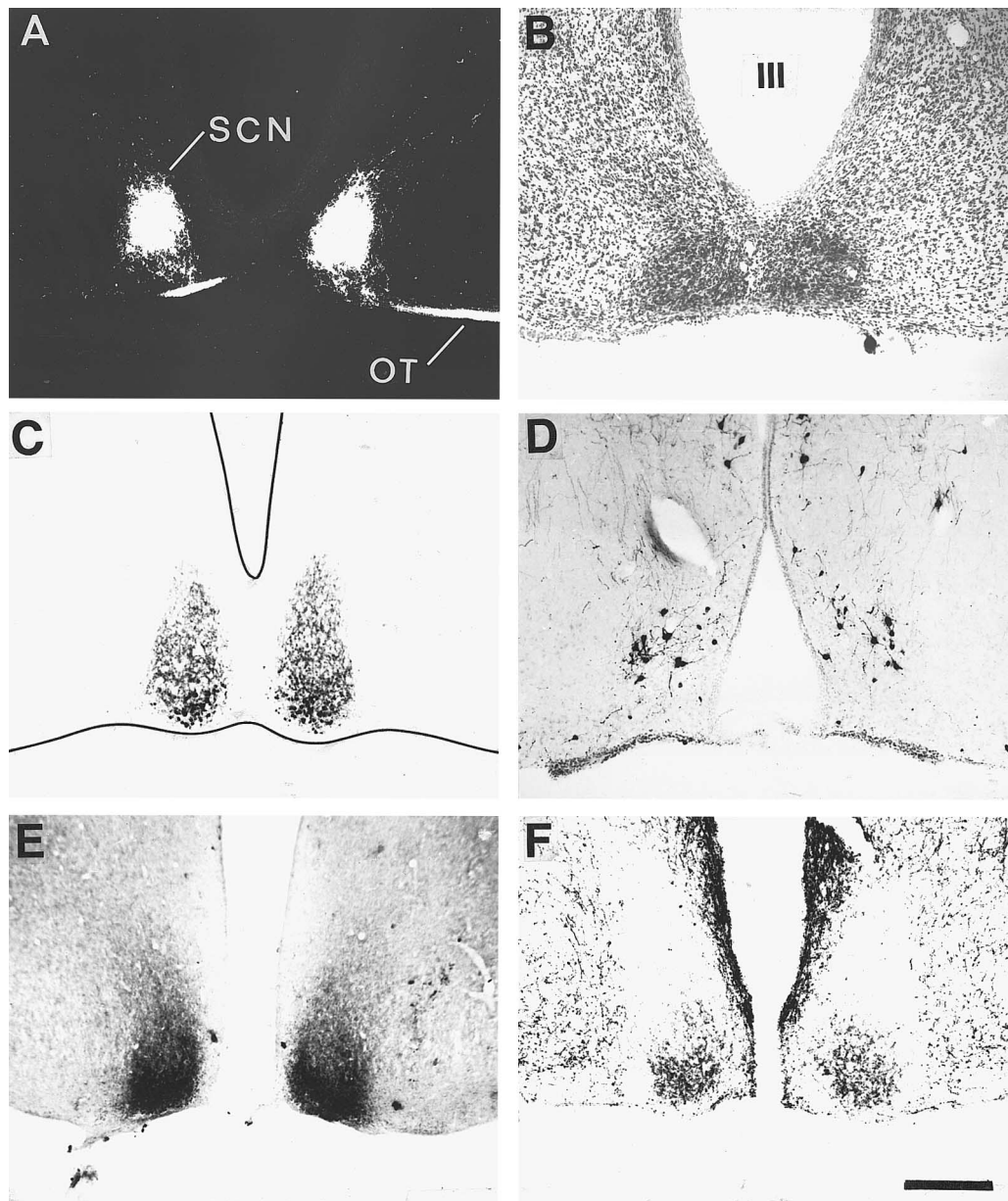


FIG. 1. Retinal projections to the suprachiasmatic nucleus (SCN) are shown in A. Note the small size of the optic tract (OT). The cytoarchitecture of the SCN is shown in a Nissl-stained coronal section in B. The distribution of neuropeptides illustrates the two subdivisions of the SCN in *Spalax*. The ventral region contains VIP cells (C) and NPY (F) and 5-HT (E)-containing fibers. The dorsal region contains VP cells (D) and VIP fibers (C). Scale in F = 400  $\mu$ m. III, third ventricle. (The borders of the section in C have been drawn in for clarity).

VIP-positive cells is significantly less than in the rat (552 cells in *Spalax*, over 2000 in the rat [48]). Previous studies have shown that the ventral part of the nucleus where the VIP neurons are located represents a region of convergence of different inputs. Afferent fibers from the raphe nucleus (5-HT), the IGL (NPY), and the retina make synaptic contact with VIP neurons in this region [30,34,35]. In contrast, VIP neurons are mainly involved in local circuits within the SCN. For example, VIP neurons establish synapses with VP neurons of the dorsal region, or with other cells immediately adjacent to the SCN border [4,18,35,40,44,75].

VIP secretion in the SCN is functionally linked to both light

exposure and feed-back from the circadian pacemaker [36]. The liberation of VIP in the SCN shows daily variations in relation to the light/dark cycle [1,50,68,74]. In constant light conditions, VIP content decreases over time, in constant darkness VIP levels remain constant, whereas a pulse of light will cause a decrease in VIP content during the subjective night [36,70]. However, microinjection *in vivo* of VIP alone in the SCN does not cause a phase shift in locomotor activity, and *in vitro* does not modify the pattern of electrical discharge [1].

In other rodents, VP is typically the most abundant neuropeptide in the dorsal SCN [9,13,48,81], although the SCN of

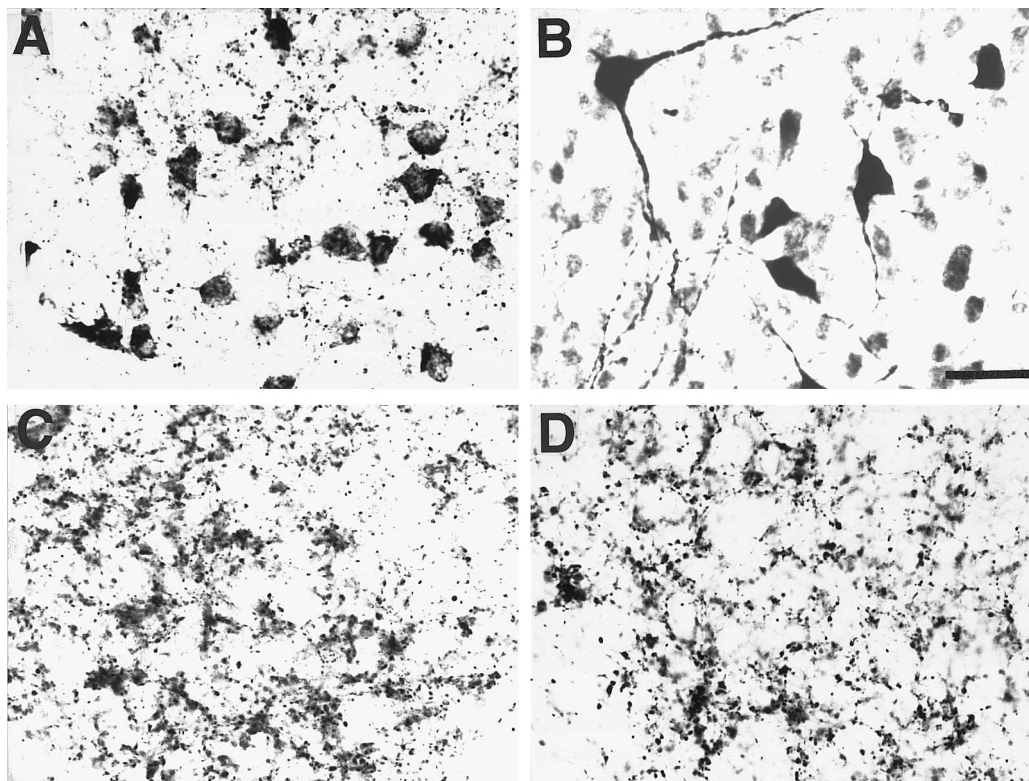


FIG. 2. High-power magnification of immunopositive label of the neuropeptides shown in Fig. 1. Immunopositive VIP neurons from the ventral region and VP neurons from the dorsal region of the SCN are shown in A and B, respectively. The photographs of immunopositive NPY (D) and 5-HT (C) fibers are from the ventral region of the nucleus. Scale in B = 30  $\mu$ m.

*Spalax* contains few VP and fibers. We find less than 70 VP cells in the SCN of *Spalax*, compared to over 3000 in the rat [48]. In addition, VP fibers are typically abundant in the dorsomedial and ventrolateral regions of the SCN in other mammals [77,81]. VP neurons are considered to represent the main efferent system of the SCN [10,18] and, for example, send a dense projection to the paraventricular and supraoptic nuclei of the hypothalamus [32,73,81,82,84]. In *Spalax*, these two nuclei contain numerous VP cells and are linked together by a dense network of VP fibers.

In contrast to VIP, the level of VP in the SCN shows an endogenous oscillation, which is similar under both light/dark conditions and under continuous darkness. These results suggest that the level of VP in the SCN is not directly affected by light, but is under the control of the circadian pacemaker [36]. The endogenous rhythm of VP liberation in the SCN is also the origin of the circadian variation of this neuropeptide in the cerebrospinal fluid [63,66,67]. The functional role of VP is thus considered to be the mediation of neuronal and endocrine output of the SCN [36].

Despite the importance of VP as an output system of the SCN, the absence of VP-containing cells does not prevent the expression of circadian rhythms. An absence of VP neurons is observed in the mink [45], a species that nevertheless expresses circadian locomotor activity [43]. Furthermore, the VP-deficient Brattleboro rat does not display major deficits in circadian behavioral or physiological rhythms [57]. Likewise, *Spalax* also manifests entrained circadian rhythms [61,62].

The ventral region of the SCN in *Spalax* contains a moderate density of NPY fibers compared to other rodents [9,11,13,81]. This

difference in density may be correlated with the fact that NPY fibers mainly originate from the IGL in rodents [8,25,26,48,51], while the geniculate complex is greatly reduced in size in *Spalax* [16] and the putative IGL region contains relatively few NPY cells (personal unpublished observations). The presence of NPY cells in the geniculate complex and NPY fibers in the SCN argues in favor of the presence of a geniculo-hypothalamic tract in *Spalax*.

The possible implications of the reduction of the IGL and of NPY innervation in the SCN of *Spalax* are unclear. In other rodents, the IGL and geniculo-hypothalamic tract play an important role in feedback regulation for both photic and nonphotic phase shifts [2,11,12,29,38,39,51,59]. Microinjection of NPY in the SCN, or microstimulation of the IGL, will both cause phase advances or delays depending on circadian phase. The phase response curve for the phase shifting effects of NPY are different from that caused by light [2,33,60,64]. In addition, the level of NPY in the SCN shows two peaks at the day/night and night/day transition periods [7,37,68,71], which suggests that the change in light intensity (increase or decrease) may be a more important parameter than the actual level of light intensity.

The distribution of 5-HT fibers in the SCN of *Spalax* is comparable to that observed in other rodents [81]. These fibers are colocalized in the ventral region of the nucleus with VIP cells, NPY fibers, and retinal afferents. The 5-HT fibers originate from the dorsal and medial regions of the raphe nuclei [21,31,41,49], and the SCN receives the densest innervation of all brain structures [41,56]. Although the precise role of 5-HT in the SCN is unclear, this monoamine may act in the SCN by directly modulating photic information conveyed to the nucleus from the retina. For example,

5-HT modifies the effects of light on circadian locomotor activity [52,53], and inhibits the photic responses of SCN neurons in a dose-dependent manner [86]. Under constant light conditions, serotonin-depleted hamsters have longer circadian periods and a more severe rhythm disruption than normal hamsters [51,53,72]. In addition, the endogenous clock may influence 5-HT secretion by the raphe nuclei, because these levels in the SCN show an endogenous circadian variation [6].

In conclusion, our results show that, as in other rodents, the SCN in *Spalax* contains two subdivisions. The ventral region receives the majority of retinal afferents, and also contains VIP cells, and NPY and 5-HT fibers, features shared with other species. In contrast, the dorsal region contains a scarce population of VP neurons, although this characteristic is not unique to *Spalax*. The presence of NPY and 5-HT fibers also suggests that the SCN receives afferent projections from the IGL and from the raphe nuclei, respectively. Finally, the combined neuroanatomical, behavioral, gene expression, and tract tracing results confirm that the capacity for light entrainment of circadian rhythms is conserved in the blind mole rat.

#### ACKNOWLEDGEMENTS

We would like to thank Christel Merrouche for help with histology, and Ghislaine Claine for care of the animals. The research was funded by grants from Human Frontiers (RG95/68), NATO (#950334), ENP (#185), and BIOMED2 (PL/962327).

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