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Organization of Suprachiasmatic Nucleus Projections in Syrian Hamsters (*Mesocricetus auratus*): An Anterograde and Retrograde Analysis

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Abstract

Circadian rhythms in physiology and behavior are controlled by pacemaker cells located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The mammalian SCN can be classified into two subdivisions (*core* and *shell*) based on the organization of neuroactive substances, inputs, and outputs. Recent studies in our laboratory indicate that these subdivisions are associated with functional specialization in Syrian hamsters. The core region, marked by calbindin-D_{28K} (CalB)-containing cells, expresses light-induced, but not rhythmic, clock genes. In the shell compartment, marked by vasopressinergic cells and fibers, clock gene expression is rhythmic. Given these findings, an important question is how photic and rhythmic information are integrated and communicated from each of these regions to effector areas. The present study used localized, intra-SCN iontophoretic injections of the anterograde tracer biotinylated dextran amine (BDA) to investigate intra-SCN connectivity and the neural pathways by which information is communicated from SCN subregions to targets. Intra-SCN connections project from the core to the shell compartment of the SCN, but not from the shell to the CalB region of the SCN. Retrograde tracing experiments were performed using cholera toxin- β (CTB) to determine more specifically whether SCN efferents originated in the core or shell using neurochemical markers for the rhythmic (vasopressin) and light-induced (CalB) SCN subregions. The combined results from anterograde and retrograde experiments suggest that all SCN targets receive information from both the light-induced and rhythmic regions of the SCN (albeit to varying degrees) and indicate that light and rhythmic information may be integrated both within the SCN and at target effector areas.

Indexing terms

efferent; circadian; tracing; SCN; rhythm

In mammals, circadian rhythms are generated by the paired suprachiasmatic nuclei (SCN) located in the anterior hypothalamus. Several lines of evidence indicate that the SCN is the locus of the circadian pacemaker in mammals. Lesions of the SCN abolish daily rhythms in

physiology and behavior (Moore and Eichler, 1972; Stephan and Zucker, 1972), whereas transplants of donor SCN tissue restore these rhythms (Lehman et al., 1987; Ralph et al., 1990). Likewise, circadian rhythms in neural firing rate persist in isolated SCN tissue maintained in culture (Groos and Hendriks, 1982; Green and Gillette, 1982; Shibata et al., 1982). In the absence of environmental time cues, animals with an intact SCN exhibit daily cycles in behavior and physiology with a period close to 24 hours. A direct retinohypothalamic tract (RHT), projecting from photosensitive retinal ganglion cells to the SCN, allows synchronization of these endogenous rhythms with the environmental light/dark cycle (Berson et al., 2002; Klein and Moore, 1979; Moore and Klein, 1974; Provencio et al., 2002).

Extensive research on the SCN demonstrates that the nucleus is heterogeneous in terms of the pattern of afferent input and the organization of phenotypically distinct neuronal cell types (Moore, 1996, 1997; Moore et al., 2002; van den Pol and Tsujimoto, 1985). It has been useful to conceptualize the SCN as having two subdivisions, termed the *core* (or the ventrolateral subdivision) and the *shell* (or the dorsomedial subdivision; reviewed in Moore et al., 2002). These subdivisions can be characterized by differences in chemoarchitecture, as well as by differences in afferent and efferent projections, and this relationship remains relatively stable among mammalian species (Leak and Moore, 2001; Moore et al., 2002). In general, the common feature of the mammalian SCN is that cells of particular phenotypes are topographically organized, although variation in peptide localization is seen among species (van den Pol and Tsujimoto, 1985; Mikkelsen and Fahrenkrug, 1994; Miller et al., 1996; Moore, 1997; Moore and Silver, 1998; Silver et al., 1999). The discrete organization of peptides and afferent and efferent projections of the SCN suggests important compartmentalization of function.

Analogous to the neurochemical organization of the SCN, several studies on the distribution of rhythmic and light-induced genes important for circadian rhythm generation indicate a functional specificity within subregions of the SCN. In rats (Miyake et al., 2000) and mice (Shigeyoshi et al., 1997), light-induced *Per1* expression is largely restricted to the SCN core, while endogenously rhythmic *Per1* mRNA is seen in the shell. In mice, light-induced *Per* gene expression is initially seen in the core of the SCN followed later by activation in the shell (Yan and Silver, 2002), suggesting that entrainment involves the communication of light information from the retinorecipient SCN to the dorsomedial shell.

In Syrian hamsters (*Mesocricetus auratus*), there is a small dense population (~250 cells; LeSauter et al., 1999) of calbindin-D_{28K} (CalB)-containing cells in the SCN core (Silver et al., 1996). *Per1* and *Per2* are light-induced, but not detectably endogenously rhythmic, in this SCN region. The fact that CalB cells are not rhythmic has been confirmed in studies of electrical activity (Jobst and Allen, 2002). In contrast, the shell, marked by vasopressin-containing cells, has rhythmic *Per1*, *Per2*, and *Per3* (Hamada et al., 2001). Importantly, lesions bilaterally destroying the CalB region of the SCN, while sparing a significant remainder of the nucleus, abolish locomotor rhythmicity in hamsters (LeSauter and Silver, 1999). We have proposed that the function of the CalB subregion is to serve as a “gate” that resets the phase of independent cellular oscillators in the shell (Antle et al., 2003). Together, these findings suggest an important compartmentalization of function within the SCN, with a light recipient zone necessary to coordinate oscillator cells in the region of the SCN expressing endogenous rhythmicity.

The goal of the present study was to investigate efferent projections from each of these distinct functional subregions of the SCN. The organization of efferent projections was investigated by using small anterograde tracer injections into the SCN as well as retrograde tracing from SCN targets. The regional distribution of projections from subdivisions of the

SCN identified using retrograde tracing was further characterized by evaluating overlap with CalB and vasopressin cells and fibers in order to demarcate the light-induced versus rhythmic compartments of the SCN, respectively.

MATERIALS AND METHODS

Animals

One-hundred and six (41 for anterograde studies and 65 for retrograde studies) adult male LVG hamsters (*Mesocricetus auratus*) were used in the present experiment. Animals were purchased from Charles River (Wilmington, MA) at age 4–5 weeks. All animals were housed in translucent polypropylene cages (48 × 27 × 20 cm) and provided with ad libitum access to food and water for the duration of the study. Animals were maintained in a colony room with a 24-hour light/dark cycle (LD 14:10) at 23 ± 1°C. All procedures were approved by the Columbia University Animal Care and Use Committee.

Anterograde tracing

Small iontophoretic injections of the anterograde tracer biotinylated dextran amine (BDA) were used to visualize SCN efferents. Prior to the injection, hamsters were deeply anesthetized with 60 mg/kg ketamine and 5 mg/kg xylazine. A glass micropipette (tip diameter 10–12 µm) was filled with 10% BDA (10,000 Mol. Wt., Molecular Probes, Eugene OR) in 0.01 M phosphate-buffered saline (PBS; pH 7.4). The head of the animal was shaved and positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), and the animal was prepared for aseptic surgery. Injections were aimed at the following coordinates: 0.8 mm anterior to bregma, 0.1 mm lateral to midline, and 7.9 mm below the dura. Iontophoretic injections were made using 5-mA positive current pulses (7 seconds on, 7 seconds off) for 7 minutes. Three minutes after the injection, the pipette was removed under negative current in order to prevent leakage of the tracer along the tract.

Retrograde tracing

Injections of retrograde tracer into monosynaptic SCN targets were used to confirm the organization of SCN projections identified using BDA. Animals received pressure injections of a 1% solution of low-salt CTB (List Biologicals, Campbell, CA). Each animal was anesthetized with 60 mg/kg ketamine and 5 mg/kg xylazine. The head was shaved and the animal placed in a stereotaxic apparatus (David Kopf Instruments). The animal was prepared for aseptic surgery. The head was positioned with the skull flat. The coordinates for each injection site were measured in the anterior-posterior and medial-lateral from bregma, and in the dorsal-ventral dimension, from the surface of the brain, as follows: medial preoptic area (MPO), +1.8 mm, +0.4 mm, –7.0 mm; anterior ventrolateral preoptic area (VLPO), +1.3 mm, +1.0 mm, –7.9 mm; lateral septum (LS), +1.8 mm, +0.4 mm, –4.0 mm; paraventricular nucleus of the thalamus (PVT), +0.7 mm, +0.2 mm, –4.9 mm; subparaventricular zone of the hypothalamus (SPVZ), +0.8 mm, –0.4 mm, –7.5 mm; dorsomedial nucleus of the hypothalamus (DMH), –0.5 mm, +0.4 mm, –8.0 mm. Injections were made with a 5-µl syringe (Hamilton, Reno, NV), volumes ranging from 20 to 300 nl were injected at a rate of 10–20 nl/min. The needle was left in the brain following injection for 5–10 minutes to prevent upward diffusion of tracer along the injection tract. Animals were sacrificed 3–4 days following injection. For the retrograde tracing experiments, alternate sections from another investigation were used (R.K. Leak, R. Silver, J. LeSauter, and R.Y. Moore, in preparation).

Perfusion and histology

Six days after the tracer injection, animals were deeply anesthetized with sodium pentobarbital (200 mg/kg) and perfused intracardially with 150 ml of 0.9% saline followed by 300 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.3). Brains were postfixed for 18–24 hours at 4°C and then cryoprotected in 20% sucrose in 0.1 M PBS overnight at 4°C. Coronal sections (25 µm) were cut on a cryostat and collected into 0.1 M PBS. The brain sections were processed as free-floating sections.

For simultaneous visualization of CalB and BDA, every fourth 25-µm section was double-labeled using fluorescence immunocytochemistry. For visualization of BDA, sections were washed in PBS, incubated in 1% H₂O₂, and then incubated in 0.4% Triton X-100 (PBT) for 1 hour. Sections were then incubated in avidin-biotin complex (ABC Elite Kit, Vector, Burlingame, CA; 1:1,000 in PBT) for 1 hour at room temperature. In order to amplify the BDA signal, sections were then incubated in biotinylated tyramine (0.6%) for 30 minutes at room temperature prior to incubation in fluorescein isothiocyanate (FITC)-conjugated streptavidin (1:200) for 60 minutes. Following labeling for BDA, sections were labeled using a monoclonal antibody directed against CalB (1:10,000, Sigma), with Texas Red horse anti-mouse (1:200) as the secondary antibody/fluorophore.

Brains injected with CTB were cut at 30 µm in the coronal plane on a freezing microtome and collected in 0.01 M PBS or, for long-term storage, in cryoprotectant at –20°C. Fluorescent double labeling of CTB-immunoreactive neurons was performed with antibodies raised against vasopressin and CalB. Sections were washed and placed in a solution containing both primary antibodies (anti-CTB plus either anti-vasopressin or anti-CalB; every fourth section) at concentrations of 1:2,000 each, with 0.3% Triton-X 100 and 1.5% normal donkey serum. Following overnight incubation at 4°C, sections were washed and incubated in a solution containing CY2 anti-goat (for CTB) and either CY3 anti-mouse (for CalB) or CY3 anti-guinea pig (for vasopressin) fluorophores (Jackson ImmunoReagents), at a dilution of 1:100 each, with 0.3% Triton-X 100 and 1.5% normal donkey serum. Following a 2-hour incubation at room temperature, sections were washed and mounted, dehydrated in ethanols, cleared in xylenes, and coverslipped with Krystalon (EM Diagnostics, Gibbstown, NJ).

Microscopy

Immunofluorescent labeling was examined on a Nikon Eclipse E800 microscope. Sections were excited using the standard wavelengths for Texas Red and FITC. Images were digitally captured in 8-bit grayscale using a cooled CCD camera (SPOT; Morrel, Meville, NY). Images were processed using Photoshop 5.5 (Adobe Systems, Mountain View, CA). In order to visualize fibers more clearly in the printed image, grayscale images were digitally inverted using Photoshop, so that BDA-labeled fibers were dark and the background light. All photomicrographs of BDA staining are presented in this format.

Injection sites

In order to evaluate injection size, every fourth tissue section was labeled with a peroxidase substrate kit (Vector SG) rather than a fluorescent label. This chromogen allowed for optimal visualization of the center of the injection site because fibers are more lightly stained compared with fluorescence labeling, allowing a more accurate evaluation of the assessment of the injection. In order to estimate the size of each SCN injection, all sections comprising the injection site in the SG-substrate-stained sections were measured. Injection sites were evaluated at 10× magnification by viewing microscopic images from an Olympus BH-2 microscope equipped with a Sony CCD camera of view on a Power Macintosh 7600 computer (model # XC-77). The injection site for each section was outlined and the two-

dimensional area was calculated using NIH Image 1.61. Injection size is represented as the sum of the two-dimensional areas from all sections (of every fourth section) comprising the injection site. To clarify the extent of labeling, fluorescent images of the injection site were also evaluated.

The appearance of the injection sites was similar for both BDA and CTB tracers. The injections resulted in a small central necrotic area surrounded by an ovoid, immunoreactive zone. Previous studies indicate that the area of uptake and transport of CTB and BDA encompasses the major portion of the immunoreactive zone including the entire diffusely stained region surrounding the small necrotic center (Leak and Moore, 2001). The assessment of the effective injection site was confirmed by comparing the pattern of retrograde labeling with that predicted by localized injections of anterograde tracer. In addition, injections missing, but directly surrounding the SCN, served as controls to assess the validity of this characterization further.

Evaluation of anterograde and retrograde tracing

For SCN injections with BDA, each target brain region was qualitatively characterized as having presumptive boutons and varicosities versus long thin branches bearing linear arrays of the en passant fiber type. Comparing different localized SCN injections with an injection of the entire SCN allowed the determination of whether or not a particular SCN target was innervated by the SCN core or shell. Retrograde tracing from targets was then used to evaluate the validity of this method of characterization and confirm the topography identified with anterograde tracer. Retrograde label was visualized as labeled neurons within the SCN (Table 1).

RESULTS

Anterograde injection locations

In five animals, the BDA tracer was largely confined to the SCN. For three other animals, tracer labeling occurred in both the dorsolateral aspect within the SCN and the adjacent hypothalamus. This paper reports on these eight animals. In the 33 remaining hamsters, injections were either outside of the SCN, in the third ventricle, or the tip of the pipette was clogged, preventing release of tracer into the brain. These animals are not described in the manuscript. Schematics showing the center of each injection site are shown in Figure 1.

The largest injection (#11; Fig. 2A, column 1) was centered in the ventrolateral subdivision of the mid-SCN. All other injections were localized to subregions within the SCN. The injection for #20 (Fig. 2A, column 2) was localized to the shell region of the SCN, whereas injection #17 (Fig. 2A, column 3) was highly localized to the medial SCN. Injections #34 (Fig. 2A, column 5) and #26 (Fig. 2A, column 4) were both localized to the core, although injection #34 was slightly more medial than that of #26. Representative high-power photomicrographs through the rostral and caudal SCN for a shell (#20) and core (#34) injection are shown in Figure 3. Each of these injections is discussed in more detail below.

Extra SCN targets characterized with anterograde tracing

Fibers of passage were generally distinguishable from terminal fields. Fibers of passage were typically thin projections with few varicosities. Terminal fields were associated with numerous presumptive boutons and varicosities. High-power photomicrographs of representative monosynaptic targets following entire, shell (#20), and core (#34) BDA injections are shown in Figure 4.

Efferent projections from a whole SCN injection—The largest injection (#11; 0.15 mm²) resulted in the most labeling of efferents and is used as a point of comparison for other injections (Fig. 2A, column 1). This injection was centered in the rostral third of the SCN, with dense labeling seen throughout the rostrocaudal extent of the nucleus. More specifically, dense reaction product completely filling the neuropil began approximately 50 μ m caudal to the rostral tip of the SCN. The injection extended to the caudal aspect of the SCN. Sparse neuron labeling was seen in the retrochiasmatic area at the level of the mid-SCN and the medial peri-SCN at more caudal levels. In this brain, fibers exiting the SCN occurred in the rostral, lateral, dorsal, and dorsocaudal directions. In each instance, a majority of projections were ipsilateral, yet for all projections, contralateral labeling was also seen. Efferent labeling for this and subsequent injections is discussed sequentially from the most rostral projection to the most caudal. The most rostral projections terminated in the ventral portion of the lateral septum (LSv, Fig. 2B, column 1). Fibers innervating the LSv exited the SCN and followed a medial course along the dorsal surface of the optic chiasm, turning dorsally and continuing around the anterior commissure to terminate in the LSv. An extensive projection was seen on the same plane as the LSv terminating in the medial preoptic area (MPO), directly surrounding and avoiding the OVLT (Fig. 2C, column 1). A dense plexus of terminals and fibers was seen in the contralateral MPO.

More caudally, the most extensive projection proceeded rostr dorsally, close to the third ventricle, and terminated in the anteroventral periventricular nucleus (AVPV; Fig. 2D, column 1). Slightly caudal, a dense projection was seen more ventrally and laterally innervating the ventrolateral preoptic area (VLPO; Fig. 2G, column 1). Further caudal, but still rostral to the SCN, a dense fiber bundle was seen coursing ventrally along the third ventricle. Projections from this fiber bundle innervated the medial preoptic nucleus (MPN; data not shown) and the AVPV (Fig. 2D, column 1). Innervation of the VLPO continued to be prominent at this level. Also at this level, fibers and sparse terminals could be seen more dorsally and laterally just ventral to the anterior commissure, including the ventral portion of the bed nucleus of the stria terminalis (BST) as well as portions of the medial and, to a lesser extent, lateral preoptic areas (MPO and LPO, respectively; Fig. 2C, column 1). Slightly further caudal a dense innervation of the anterior portion of the paraventricular portion of the thalamus (PVA, Fig. 2E, column 1) was seen. Staining was dense rostrally as fibers coursed around the parataenial nucleus (PT) and diminished more caudally. Staining became more sparse at the level of the habenula (Hb) in the posterior portion of the paraventricular nucleus of the thalamus (PVTp; data not shown).

At the level of the rostral SCN, a dense bundle of fibers left the SCN dorsally and innervated the parvicellular division of the anterior paraventricular nucleus of the hypothalamus (PVHap; Fig. 2F, column 1). Also at this level, fibers exited the SCN laterally and provided significant innervation of the anterior hypothalamic area (AHA; data not shown). These fibers continued laterally to innervate the supraoptic nucleus sparsely (Fig. 2I, column 1). More caudally, fibers exited the SCN dorsally and innervated the paraventricular nucleus of the hypothalamus (Fig. 2H, column 1). Terminals were diffusely spread throughout the PVH, with a dense projection to the medial parvicellular portion (PVHmp) of the nucleus (data not shown). The majority of fibers from this projection terminated in the region just ventral to the PVH, termed the subparaventricular zone (SPVZ; Fig. 2H, column 1). Dense innervation was seen caudal to the SCN in the dorsomedial nucleus of the hypothalamus (DMH), retrochiasmatic area, and tuberal hypothalamus (Fig. 2J, column 1). Fibers at this level primarily formed a “ring” around the ventromedial nucleus of the hypothalamus (VMH), although sparse innervation of the nucleus was apparent. This dense innervation of the DMH and tuberal hypothalamus continued at the level of the arcuate nucleus (Arc). Slightly further caudal, the intergeniculate leaflet (IGL) was densely labeled with many fibers and terminals, bordered by unlabeled dorsal and ventral lateral geniculate nuclei (Fig. 2K,

column 1). Finally, and most caudally, a sparse projection terminated in the periaqueductal gray (PAG; Fig. 2L, column 1).

Efferent projections from the dorsomedial SCN—In each of the remaining animals, injections were more localized to a subregion of the SCN. In all cases, labeling in target areas was less dense than that seen with injection #11. For each of these animals, comparisons with hamster #11 were made as a reference point for evaluation of the density of projections from smaller, localized injections.

Two localized injections were used to evaluate projections arising from the SCN shell. The first, injection #20 (0.094 mm^2) was centered in the shell of the mid-SCN (Fig. 2A, column 2), with scattered neuronal labeling in the core. The injection began approximately $100 \mu\text{m}$ caudal to the rostral tip of the SCN and ended slightly rostral to the caudal aspect of the SCN. Scattered neurons were labeled in the contralateral SCN. Virtually no terminal or fiber staining was seen in the LSv, whereas modest innervation of the MPO, surrounding the OVLT (Fig. 2B,C, column 2), at the same rostrocaudal extent was seen. Innervation was densest in the AVPV and MPN regions (Fig. 2D, column 2). The VLPO, PVH, and SPVZ received modest and diffuse innervation, with dense staining seen in the more medial aspect of the SPVZ (Fig. 2G,H, column 2). In the DMH, terminal and fiber staining was dense and in a pattern similar to that seen for animal #11 (Fig. 2J, column 1). Staining in the IGL was relatively sparse given the injection size, with few terminals compared with the extent of labeling in other target regions receiving dense innervation (Fig. 2K, column 1). All other regions labeled in #11 exhibited a similar pattern of efferents as seen in #20 that was commensurate to the size of the injection.

Injection #17 was a very small (0.043 mm^2) medial injection spreading slightly ventromedial (Fig. 2A, column 3) at the level of the mid-SCN. Dense reaction product was seen approximately $200 \mu\text{m}$ rostral and caudal to the center of the injection. The injection site did not extend beyond the caudal aspect of the SCN. In the medial/lateral plane, the injection did not spread more than $100 \mu\text{m}$ from the border of the third ventricle. Dorsally, the injection reached the ventricular ependyma and labeled a few cells in the peri-SCN. This injection resulted in sparse labeling in most areas labeled in #11 (Fig. 1). Very sparse label was seen in the SON of this animal (Fig. 2I, column 3). Notably, for an injection of this size, well-defined labeling of fibers and terminals was seen in the MPO (around the OVLT), PVT, VLPO, PVHap, SPVZ, PVH, and IGL (Fig. 2, column 3).

Efferent projections from the ventral SCN—Projections arising from more core locations were evaluated by using injections #34 and #26. Injection #26 (0.12 mm^2) was centered in the core of the caudal aspect of the SCN, with sparse neuronal labeling seen throughout the SCN (Fig. 2A, column 4). Dense reaction product was first seen approximately $200 \mu\text{m}$ caudal to the rostral aspect of the SCN. Some minor neuron labeling was seen in the retrochiasmatic area immediately adjacent to the SCN. The injection extended approximately $100 \mu\text{m}$ caudal to the SCN. In contrast to #11 (whole SCN) and #20 (dorsomedial injection), most projections were diffuse, with some notable exceptions. Dense terminal fields were seen in the PVHap, SPVZ, DMH, and IGL (Fig. 2F,H,J,K, column 4). It is noteworthy that, although the lateral SPVZ received dense innervation, the PVH remained virtually unlabeled (Fig. 2H, column 4). Although #20 was an injection of similar size to that of #26, fibers and terminals were not concentrated in the AVPV to the same extent as that seen for #20 (or #11) (Fig. 2D, column 4). In addition, contralateral projections were almost as extensive as ipsilateral projections for each target, excluding targets with dense staining (i.e., PVH, SPVZ, DMH, and IGL) (Fig. 2, column 4).

Number 34 represents the smallest injection (0.008 mm^2) centered in the core of the caudal half of the SCN (Fig. 2A, column 5). Dense label extended approximately $150 \mu\text{m}$ rostral and caudal to the center of the injection site. The majority of labeled cell bodies were seen in the core of the nucleus. Minor labeling was seen in the dorsal portion of the optic chiasm directly beneath the core of the injection. Most projection sites labeled in #11 were sparsely labeled in #34, with several exceptions. Although the SPVZ shows modest labeling given the size of the injection, the MPO, AVPV, and PVH were more sparsely innervated (Fig. 2C,D,H, column 5). Likewise, the VLPO remained unlabeled in #34 (Fig. 2G, column 5). In relation to the size of the injection, terminals and varicosities were present in the most caudal projections of the IGL and PAG (Fig. 2K,L, column 5). Consistent with the fact that part of the injection labeled the optic chiasm, other portions of the geniculate had fiber labeling including the ventrolateral geniculate (VLG) and the dorsolateral geniculate (DLG; Fig. 2K, column 5).

Although #26 labeled cells sparsely throughout the entire SCN (albeit most densely in the ventral SCN), because #34 was highly localized to the SCN core, the combination of these two injections could be used to evaluate ventral projections accurately compared with a dorsomedial injection. As an example, a large shell injection (#20) resulted in virtually no labeling in the LSv (Fig. 2B, column 2). However, #26 (a large core injection) resulted in dense label in the LSv (Fig. 2B, column 4), whereas #34 (a very small core injection) resulted in sparse label in the LSv (Fig. 2B, column 5). Together, a comparison of these three injections indicates that this projection arose from a more core location.

Efferent projections from the peri-SCN region—Injection #19 (Fig. 5) was a small injection that extended beyond the borders of the SCN, centered in the caudal extent of the SCN, at the dorsolateral edge of the nucleus, spreading into the adjacent hypothalamus and SPVZ. Dense reaction product was first seen approximately $350 \mu\text{m}$ caudal to the rostral tip of the SCN. The injection extended approximately $150 \mu\text{m}$ past the caudal aspect of the SCN into the adjacent hypothalamus. As with #26, although projections were seen in all regions labeled for #11, dense innervation (relative to injection size) was not seen in the AVPV or MPN (Fig. 5). Likewise, only a few terminals were apparent in the VLPO. However, dense projections terminating in the rostrocaudal extent of the PVT and the DMH were seen. Projections to the LVs and PAG were as dense as those seen for #11. Unlike #11, extensive terminals and fibers were seen in the VMH.

Injection # 21 was centered in the anterior hypothalamic nucleus just dorsal to the caudal aspect of the SCN (Fig. 6). This injection extended into the SPVZ in addition to labeling cells in the most lateral (and caudal) portions of the SCN. Dense reaction product was first seen at a brain level approximately $100 \mu\text{m}$ caudal to the rostral tip of the SCN extending to a region just rostral to the DMH. As with injection #19, few terminals were seen in the AVPV or MPN. Some areas were densely innervated relative to injection size including the LS, MPO, VLPO, and PVHap. In contrast to injections largely confined to the SCN, the DMH was sparsely labeled following this injection. Also unlike SCN injections, but similar to results seen following injection #19, a large concentration of terminal fields was seen in the VMH.

Injection #14 was centered just lateral to the SPVZ dorsolateral to the SCN (Fig. 7). Dense reaction product was seen at a brain level approximately $200 \mu\text{m}$ past the rostral tip of the SCN and extended about $50 - 100 \mu\text{m}$ caudal to the SCN. Labeled cells extended into the peri-SCN and retrochiasmatic area but did not label cells in the SCN proper. Most notably, the MPO was not innervated in this animal, whereas the LPO received significant innervation. Likewise, the AVPV, directly lateral to the third ventricle, and the MPN were only sparsely innervated. The LS, VLPO, PVT, and PVHap were densely innervated for an

injection of this size. As with #19 and #21, significant innervation of the VMH was seen. In contrast to #21, but similar to #19, the DMH demonstrated significant labeling of terminals and varicosities. However, the most medial portions of the DMH were sparsely labeled.

Extra SCN targets characterized with retrograde tracing

To confirm and extend the results of anterograde labeling, CTB injections were made into the lateral septum (LS), preoptic area (POA), ventrolateral preoptic area (VLPO), paraventricular thalamic nucleus (PVT), paraventricular nucleus of the hypothalamus (PVH), dorsomedial hypothalamus (DMH), and subparaventricular zone (SPVZ). Of the animals injected, 18 injections were localized to the targets listed above. All other injections either missed the intended target or spread significantly to surrounding brain regions. The locations of these 18 injections reported in this manuscript are shown schematically in Figure 8. Representative examples depicting injections resulting in both localized SCN staining and more widely distributed CTB labeling in the SCN are shown in Figure 9. A summary outlining the relative proportion of CTB cells overlapping the vasopressin (rhythmic; shell) and CalB (light-induced; core) SCN subregions is given in Table 2. For the purposes of this evaluation, these subregions are defined as both cellular and fiber labeling, as are the functional subdivisions (e.g., Hamada et al., 2001).

Preoptic area and septal region—For injections that were within the septal complex and strial area with little extension into the POA (H25; Figs. 8, 9), retrograde label was seen primarily in the CalB region of the SCN, with a large number of double-labeled cells in the anterior division of the population of CalB cells. Rostral and caudal portions of the SCN had retrograde label spreading into the vasopressin region with few cells double-labeled with vasopressin and CTB. An injection localized to the *dorsal* septum (H66; Fig. 8) did not result in retrograde label in the SCN.

Injections concentrated in the POA (H42, H34; Fig. 8) resulted in retrogradely labeled cells concentrated in the vasopressin-rich region of the SCN throughout the rostral-caudal extent of the nucleus, although a few double-labeled cells were observed. Scattered retrograde label was seen in the CalB region of the SCN in both cases, with a few double-labeled cells.

Two injections were localized to the VLPO, with minimal spread into the surrounding POA (H56, H70; Fig. 8). These two injections resulted in sparse retrogradely labeled cell bodies in the SCN located predominately in the dorsomedial vasopressin region of the SCN, with some scattered cells in the CalB region also being labeled (Fig. 9).

Subparaventricular zone and paraventricular hypothalamic injections—

Consistent with the fact that the lateral SPVZ was heavily labeled following injections of anterograde tracer into the ventral SCN, injections of CTB into the lateral SPVZ (H4; Fig. 8) with minor spread into the medial aspect resulted in dense retrograde label seen in the ventral and lateral SCN, with only scattered CTB label seen in the vasopressin subregion. An injection slightly more lateral to the SPVZ (H3, Fig. 8) resulted in considerably fewer double-labeled cells, primarily in the ventral SCN. One injection into the PVN with minor spread into the SPVZ (H5; Fig. 8) resulted in dense retrograde label in the CalB subregion, with a considerable number of double-labeled cells. Retrograde labeling was also seen in the vasopressin-rich region with some double-labeled cells being observed.

Midline thalamus—Injections into the PVT with little spread to the adjacent thalamus (H16, H18, H20; Fig. 8) resulted in retrograde label in both the CalB and vasopressin regions of the SCN, with substantive double-label seen in both subregions. Staining was slightly more dense in the CalB region than that of the vasopressin subregion.

Dorsomedial and ventromedial hypothalamic nuclei—Several injections into the DMH (H7, H48, H60, H63; Fig. 8) with varying degrees of diffusion dorsally resulted in labeling throughout the rostral-caudal extent of the SCN, with slightly denser labeling in the rostral portion of the nucleus. Labeling was not differentially concentrated in either the CalB or vasopressin subregion, with each region expressing double-labeled cells.

Intra-SCN targets investigated using anterograde tracing

For all intra-SCN injections of BDA, considerable labeling of the contralateral SCN was seen, with the extent of labeling being directly proportional to the size of the injection. Ipsilateral SCN staining was more extensive and dense than that of contralateral projections. An interesting result seen in #20 (small shell injection) was that dense fiber staining was seen both ipsilaterally and contralaterally, except in the region of the SCN containing CalB. Here, virtually no staining of fibers and terminals occurred either ipsilateral or contralateral to the injection site, essentially “ringing” the CalB subnucleus. A similar pattern, with a more robust outlining of the CalB nucleus, was seen in #17 (small medial and ventromedial injection; Fig. 10A–E). Injection #26 (small core injection) resulted in extensive ipsilateral and contralateral SCN staining, filling the rostrocaudal extent of both nuclei, including the region containing densely packed CalB neurons. Injection #34 (small core injection) resulted in significant staining throughout both halves of the SCN, including the region containing CalB (Fig. 10F–J). The most lateral portions of both the ipsilateral and contralateral SCN remained unlabeled in this animal. In the ipsilateral SCN, fibers and terminals were more abundant in the ventral half of the CalB subnucleus than in the dorsal portion. A similar pattern of labeling was apparent in the contralateral SCN. Data for the general ipsilateral pattern of intra-SCN projections have been previously presented in part (LeSauter et al., 2001).

DISCUSSION

Neural output from the SCN has been extensively investigated in rats and hamsters using tract tracing techniques (e.g., hamster, Kalsbeek et al., 1993; Morin et al., 1994; rat, Leak and Moore, 2001; Stephan et al., 1981; Watts and Swanson, 1987; Watts et al 1987). However, the organization of intra-SCN communication and the neural targets of specific SCN subregions have been minimally investigated in hamsters (for rats; Buijs et al., 1993 [projections to PVN]; Leak and Moore, 2001 [major SCN targets]; Teclemariam-Mesbah et al., 1997 [projections to PVN]; Van der Beek et al., 1997 [to GnRH neurons]; Vrang et al., 1995a [projections to PVN]). New data on the functional organization of the mammalian SCN, with the hamster SCN being composed of both rhythmic and nonrhythmic cells in the shell and core, respectively, underscore the importance of determining more precisely the topographical organization of both intra- and extra-SCN communication.

As specified above, previous findings in Syrian hamsters indicate that the SCN shell (defined by vasopressin) is rhythmic in terms of *Per1*, *Per2* and *Per3*, whereas the region of the SCN marked by CalB expresses light-induced, but not rhythmic, *Per1*, *Per2*, and *Fos* (Hamada et al., 2001). Concordant findings in rats and mice (Shigeyoshi et al., 1997; Miyake et al., 2000) suggest that this functional organization may be common to the mammalian circadian timing system more generally. Together, these data highlight the importance of identifying the means by which light and rhythmic information are integrated and communicated to effector areas to regulate the vast array of circadian rhythms in behavior and physiology controlled by the SCN. As a result, the present study investigated intra- and extra-SCN neural communication with specific emphasis on the organization of cells projecting from the two functionally distinct compartments demarcated by vasopressin and CalB.

Technical considerations

As with all neuroanatomical tracing studies, the results must be interpreted with a degree of caution. BDA has been shown to be equally as sensitive as *Phaseolus vulgaris* leucoagglutinin (PHA-L), but it might exhibit a slightly greater amount of retrograde labeling (Dolleman-Van der Weel et al., 1994). This is a common problem among most anterograde tracers including horseradish peroxidase (HRP), PHA-L, biocytin, and Neurobiotin (reviewed in Veeman et al., 1992). In theory, this should be a minor problem because collateral labeling should be minor in comparison with anterograde labeling. Likewise, in most instances, retrogradely labeled neurons probably do not project to the same target areas as the SCN (with the exception of the SPVZ). In addition, a common problem with all tracers is the potential uptake by fibers of passage (particularly axons that have been damaged by the injection; Wouterlood et al., 1993). These caveats are abated by the fact that the results of the present study were confirmed with retrograde injections.

The present results suggest that uptake by fibers of passage by SCN projections destined for more distant targets did not confound the present interpretation. For example, injections of CTB into the lateral SPVZ labeled cells predominantly in the SCN core, confirming results with anterograde tracer injections. Had fibers of passage destined for the PVT been partly responsible for the pattern of SCN labeling following lateral SPVZ injections of CTB, dense label would have been seen in SCN shell as well as core. Similarly, had POA injections labeled fibers en passant to the LS, significant label would have been seen in SCN core as well as shell.

For anterograde injections with BDA, our estimate of the effective injection site was confirmed with corroborating evidence. For example, had injections in the dorsomedial SCN been taken up by cells in the peri-SCN or SPVZ, patterns of labeling similar to injections #21, #19, or #14 would have been seen. For example, injections #19, #21, and #14 led to significant labeling of the VMH, whereas both dorsomedial injections (#20 and #17) did not label the VMH. These data suggest that regions directly dorsal to the SCN project heavily to the VMH and also that injections characterized as being in the dorsomedial SCN did not spread significantly dorsal. Likewise, the fact that the pattern of retrograde label confirmed anterograde results in all instances lends further support to the definition of the BDA injection sites in the present study.

For CTB injections, the effective injection site was evaluated by examining the pattern of cell labeling within the SCN compared with the pattern predicted by anterograde tracing experiments. In all cases, the pattern of retrograde label was accurately predicted by the topography of afferent labeling. For example, injection #3 (lateral SPVZ) labeled cells predominantly in the SCN core. Had CTB label spread to the adjacent medial SPVZ, significant labeling of the shell (or peri-SCN) would have been seen.

Organization of SCN projections

The results from the present investigation suggest a degree of organization of SCN efferents, such that both SCN subregions project to all SCN targets, albeit to varying degrees (see Fig. 11 for summary). Although the methods used do not permit precise quantification, the results suggest a pronounced projection from the shell that densely innervates the AVPV (Fig. 2D, column 2); projections from the core to this target were less extensive. The projections to the VLPO also appeared to arise primarily from the shell (Fig. 2G, columns 1–5). Likewise, projections to the PVH came most extensively from the shell, with comparatively few projections seen after a core injection (Fig. 2H, columns 1–5). The core (and possibly shell and peri-SCN) projected heavily to the IGL, whereas the shell projected less extensively (Fig. 2K, columns 1–5). Targets of the SCN that were densely innervated

(i.e., DMH, PVHap, PVT, SPVZ), received equally dense projections from all regions of the SCN. For the SPVZ, however, the medial portion of the SPVZ received dense innervation from the SCN shell, whereas the more lateral SPVZ received projections from the core (Fig. 2H, columns 1–5). In general, these results in hamsters are in agreement with previous studies on the organization of extra-SCN projections to the LS, SPVZ, PVH, POA, and PVT in rats (Buijs et al., 1993; Vrang et al., 1995a; Teclemariam-Mesbah et al., 1997; Van der Beek et al., 1997; Munch et al., 2002). However, one previous study in rats has shown that projections to the DMH arose from the SCN shell, whereas the present results in hamsters suggest that projections arose from both core and shell (Leak and Moore, 2001). The organization of SCN projections in other brain areas investigated (VLPO, IGL, PAG) in the present study has not been previously characterized in any species.

The SCN afferent organization identified in the present study is strikingly similar to results obtained in mice (Abrahamson and Moore, 2001). In this previous study, projections from SCN AVP (shell) and VIP (core) cells were characterized. In all cases, excluding projections to the DMH, the same general patterns of core and shell projections were seen. In mice, it appeared that AVP projected more densely than VIP to the DMH, whereas both core and shell projected densely to DMH in the hamsters in the present investigation. These results suggest two possibilities: either the SCN innervates the DHM of mice and rats differently, or core peptides other than VIP project significantly to the DMH in mice and labeling of these additional peptides would have revealed a more robust core projection. Nonetheless, these similarities between hamster and mouse projections suggest that the organization seen in SCN projections in the present study may be common to other rodents (and potentially other mammals).

The organization of all projections revealed in retrograde tracing studies is in agreement with those suggested by anterograde tracing using small SCN injections in the present investigation. For example, injection of CTB into the medial and lateral POA and VLPO resulted in neuronal labeling primarily in the vasopressin cell and fiber-rich shell of the SCN. CTB injections into the LS resulted primarily in core staining overlapping with CalB cells and fibers (Fig. 9), with scattered staining in the vasopressin subregion. Likewise, CTB injections into the PVT labeled neurons equally in both the core and shell. CTB injections into the DMH labeled neurons in both the core and shell (Fig. 9). For all other retrograde injections into SCN targets, the pattern of SCN labeling was accurately predicted by the anterograde tracing results.

The present findings for monosynaptic SCN efferents are in agreement with data on the organization of projections to multisynaptic central and peripheral targets revealed by transneuronal tracers such as pseudorabies virus (PRV). For example, injections of PRV into the pineal gland of rats (*Rattus norvegicus*) labeled the SCN shell following the appearance of infected neurons in the dorsal and lateral parvocellular subdivision of the PVH (reviewed in Card, 2000; Larsen, 1999; Larsen et al., 1998). The SCN core was labeled only after longer survival times and following infection of the medial parvocellular division of the PVH. Taken together, these findings suggest that the SCN shell in rats projects monosynaptically to dorsal and lateral PVH whereas the core projects to either the medial PVH and/or the SCN shell. This finding is consistent with investigations on the organization of SCN projections to PVH (Buijs et al., 1993; Vrang et al., 1995b; Teclemariam-Mesbah et al., 1997). As with rhythmic regulation of pineal function, several recent studies indicate more widespread multisynaptic control of numerous peripheral processes (e.g., corticosterone secretion, Buijs et al., 1999; Ueyama et al., 1999; lipid mobilization, Bamshad et al., 1998; 1999; Bartness et al., 2001; ovarian function, Gerendai et al., 2000; thyroid function, Kalsbeek et al., 2000) via PVH projections to the sympathetic nervous system.

Organization of intra-SCN communication

The principle novel finding concerning *intra*-SCN projections is that shell neurons do not appear to project to the subregion of the SCN marked by CalB-containing cells either ipsilaterally or contralaterally. However, neurons in the ventral SCN project to all regions of the ipsilateral and contralateral SCN. Because the retina projects, in part, to the CalB subregion of the SCN in hamsters (Silver et al., 1996; Bryant et al., 2000), this finding suggests a unidirectional flow of light information from the retinorecipient region of the SCN to the remainder of the SCN (although specific projections from the CalB region to the dorsal SCN require empirical investigation). Recent results from our lab using double-label immunohistochemistry and confocal microscopy lend support to this finding; VIP (i.e., ventral core) projected heavily to the CalB region of the SCN, whereas vasopressin (i.e., shell) did not (Le-Sauter et al., 2001). The present finding is consistent with studies in rats, in which core projected to shell, but shell did not project to core (Leak et al., 1999). In rats, projections from one-half of the SCN target homologous populations of neurons in the contralateral SCN (Leak et al., 1999; Buijs et al., 1994). In contrast, results from the present study suggest that the SCN of hamsters does not seem to project to homologous populations of neurons in the contralateral SCN, but instead projects more diffusely throughout the ipsilateral and contralateral SCN.

Functional considerations

It is noteworthy that *both* the shell and core of the SCN (including the CalB-ir region receiving retinal input) gave rise to projections to virtually all identified SCN targets (but to varying degrees). Together with previous findings on the functional organization of the hamster SCN (Hamada et al., 2001), these findings suggest that both light and rhythmic information reaches effector areas, indicating that phase information may also be integrated at the level of the target (Fig. 12). This finding is in agreement with one recent study indicating that a sub-population of light-responsive neurons from the SCN projected to the SPVZ (De la Iglesia and Schwartz, 2002) and suggests that this communication may be common to all monosynaptic SCN targets. The fact that the gating of light responsiveness occurs in the CalB-ir, retinorecipient SCN in hamsters, a region not endogenously rhythmic (Hamada et al., 2001), requires that rhythmic information be communicated to this SCN subregion to regulate the gated response to light. The present findings demonstrate that the SCN shell does not communicate via monosynaptic connections to the region of the SCN marked by CalB. This finding suggests that rhythmic information is either 1) communicated from the dorsomedial to the ventral SCN directly, via non-neural means, or 2) because all identified SCN targets also project back to the SCN (e.g., Moga and Moore, 1997), rhythmic information may feed back from effector areas to the CalB region of the SCN.

One novel SCN projection site in hamsters was identified in the present study (Fig. 2G, column 1, the sleep-active VLPO). This result was confirmed with retrograde tracing in the present series of studies and is in agreement with recent findings in rats reporting projections from the dorsomedial SCN to the VLPO (Chou et al., 2002; cf. Novak and Nunez, 2000). The projection to VLPO characterized in the present study appears to be more dense than that of rats. However, it is difficult to make quantitative comparisons among tracing studies due to different injection sizes, tracers, and staining techniques. The VLPO has been implicated in the regulation of both rapid-eye movement (REM) and non-REM (NREM) sleep (Sherin et al., 1996; Lu et al., 2000). Lesions of the VLPO disrupt both REM and NREM sleep without affecting circadian oscillations in body temperature (Lu et al., 2000). These findings, along with those from the present study, suggest that sleep is regulated by a distinct subregion of the preoptic area and that the VLPO is modulated by direct input from the SCN.

Anterograde tracing results in the present study suggest that the SCN may project to the IGL in hamsters. However, previous retrograde studies in hamsters report equivocal results for SCN projections to IGL; some studies report sparse projections from SCN to IGL (e.g., Goodless et al., 1989) whereas others report projections only from the peri-SCN and retrochiasmatic area (e.g., Morin et al., 1992). Because the present study did not apply retrograde tracer to the IGL, it is not possible to verify whether or not cell bodies projecting to IGL were in the SCN proper or nearby in the retrochiasmatic area.

Given the extensive neural SCN projections, an important question is why some behavioral rhythms persist after connections from the SCN have been severed. Circadian rhythms in locomotor behavior do not require neural output from the SCN; these rhythms can be supported by a diffusible signal in intact animals and by transplants in SCN-lesioned animals (Silver et al., 1996; Kramer et al., 2001; Cheng et al., 2002). These findings suggest that neural and diffusible communication may represent redundant signals from the SCN in some cases, and diffusible SCN signals can maintain some rhythms in the absence of neural connections. Alternatively, some rhythms may be regulated by diffusible SCN signals even when neural output is intact. In contrast, other rhythms such as endocrine rhythms require neural output; endocrine rhythms are abolished after knife cuts severing SCN fiber and are not restored in transplanted animals (Silver et al., 1996; Nunez and Stephan, 1977; Meyer-Bernstein et al., 1999). Although the projections from the SCN to the VLPO seen in the present study may be important for modulating the sleep-wake cycle, previous studies suggest that diffusible signals acting either in the VLPO or in other brain regions regulating sleep (e.g., the reticular formation) are sufficient to maintain rhythms in the activity-rest cycle.

Taken together, the present findings identify the potential neural mechanisms by which different functional compartments of the SCN communicate rhythmic and phase information to effector areas in the brain. The results from intra-SCN tracing suggest that the SCN core may be responsible for receiving and communicating light information to the remainder of the SCN, whereas the portion of the core marked by CalB represents a more specific subregion that does not receive input from the rhythmic SCN shell. Thus, outputs from the region of the SCN marked by CalB are probably directly regulated by light, whereas outputs from the shell are modified by phase information from the retinorecipient region of the SCN. The present findings outline the organization of hamster intra- and extra-SCN projections that suggest meaningful segregation of function, as is now being revealed for rodent SCN (e.g., Lu et al., 2001; Hamada et al., 2001), and offer a novel means by which rhythmic and phase information may be communicated to target brain areas. These results also underscore the utility of peptidergic markers within the SCN for defining and evaluating functional subdivisions within the circadian clock.

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Abbreviations

aco	anterior commissure
AHA	anterior hypothalamic area

ARC	arcuate nucleus
AVPV	anteroventral periventricular nucleus of the hypothalamus
BST	bed nucleus of the stria terminalis
BDA	biotinylated dextran amine
CalB	calbindin-D _{28K}
CCK	cholecystokinin
DBB	diagonal band of Broca
DMH	dorsomedial nucleus of the hypothalamus
fx	fornix
GHT	geniculohypothalamic tract
GRP	gastrin-releasing peptide
Hb	habenula
5-HT	serotonin
IGL	intergeniculate leaflet
LGN	lateral geniculate nucleus
LPO	lateral preoptic area
LSv	lateral septum, ventral
LV	lateral ventricle
MPN	medial preoptic nucleus
MPO	medial preoptic area
och	optic chiasm
OVL	organum vasculosum of the lamina terminalis
POA	preoptic area
PHI	peptide histidine isoleucine
PAG	periaqueductal gray
PT	parataenial thalamic nucleus
PVA	paraventricular nucleus of the thalamus, anterior
PVH	paraventricular nucleus of the hypothalamus
PVHap	paraventricular nucleus of the hypothalamus, anterior parvicellular portion
PVHmp	paraventricular nucleus of the hypothalamus, medial parvicellular portion
PVT	paraventricular nucleus of the thalamus
PVTp	paraventricular nucleus of the thalamus, posterior
RHT	retinohypothalamic tract
SCN	suprachiasmatic nucleus
SON	supraoptic nucleus
SPVZ	subparaventricular zone

VIP	vasoactive intestinal peptide
VLPO	ventrolateral preoptic area
VMH	ventromedial nucleus of the hypothalamus
V3	third ventricle

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Fig. 1.

Schematic diagrams depicting the location of BDA injections for eight animals. Black regions show the center of the injection site, and gray areas represent the “halo” comprising the less densely stained surrounding immunoreactive zone. 3V, third ventricle.

Fig. 2.

A–L: Medium-power photomicrographs showing the injection site and labeled brain regions from entire, shell, and core SCN injections of BDA. In the top row, brain sections show the center of the injection site (outlined with a dashed white line) defined by fluorescent labeling. Each subsequent row depicts a different target area of the SCN. Each column corresponds to the injection depicted in the top row of the same column. For the PVH/SPVZ, the arrow points to the general location of the SPVZ. For abbreviations, see list. Scale bar = 200 μ m in A–D; 100 μ m in E–L.

Fig. 3. High-power photomicrographs showing the rostral (A,B) and caudal (C,D) SCN for representative injections of BDA localized to either the dorsomedial (A,C) or the ventral (B,D) SCN. Note that a fold in the tissue in the rostral SCN for the ventral injection (B) makes it appear as if the tracer spread more dorsally than the actual tracer deposit. Scale bars = 100 μ m.

Fig. 4. High-power photomicrographs of a subset of SCN targets following injections in the entire, dorsomedial, or ventral SCN. Presumptive boutons are present in all micrographs demonstrating innervation by both core and shell subregions, albeit to varying degrees. For abbreviations, see list. Scale bar =100 μ m.

Fig. 5.

Low-power photomicrograph of a coronal brain section showing the core of the injection site for #19 defined by fluorescent labeling (**A**). The center of the injection is outlined with a dashed white line. The remainder of the figure (**B–H**) depicts coronal sections through the brain of animal #19 showing the distribution of BDA-positive fibers at the level of LSv, AVPV, PVHap, VLPO, PVT, IGL, and PAG. Note the high density of staining in the LSv (**E**) and PVT (**G**) relative to the more diffuse staining seen at the level of the AVPV (**E**), and VLPO (**F**). For abbreviations, see list. Scale bar = 200 μ m, in **A**; 100 μ m in **B–H**.

Fig. 6.

Low-power photomicrograph of a coronal brain section showing the core of the injection site for #21 defined by fluorescent labeling (**A**). The center of the injection is outlined with a dashed white line. The remainder of the figure (**B–H**) depicts coronal sections through the brain of animal #21 showing the distribution of BDA-positive fibers at the level of LSv, MPO, AVPV, VLPO, PVT, PVHap, and VMH. Note the high density of staining in the VLPO (**E**), PVHap (**G**), and VMH (**H**) relative to the more diffuse staining seen at the level of the AVPV (**E**) directly surrounding the third ventricle. For abbreviations, see list. Scale bar = 200 μ m in A,H; 100 μ m in B–G.

Fig. 7.

Low-power photomicrograph of a coronal brain section showing the core of the injection site for #14 defined by fluorescent labeling (**A**). The center of the injection is outlined with a dashed white line. The remainder of the figure (**B–H**) depicts coronal sections through the brain of animal #14 showing the distribution of BDA-positive fibers at the level of LSv, MPO, AVPV, VLPO, PVT, PVHap, and VMH. Note the high density of staining in the VLPO (**E**), PVHap (**G**), and VMH (**H**) relative to the more diffuse staining seen at the level of the AVPV (**E**) directly surrounding the third ventricle, and the paucity of labeling in the MPO. For abbreviations, see list. Scale bar = 200 μ m in A (also applies to H; 100 μ m in E (applies to B–G)).

Fig. 8. Schematic diagrams in the coronal plane showing the location of CTB injections. Images proceed from rostral to caudal. For abbreviations, see list.

Fig. 9.

Representative photomicrographs from brains of animals injected with CTB in either the LS (A–C), DMH (D–F), or VLPO (G–I). Column 1 shows the injection sites labeled with CTB in red. Columns 2 and 3 show double-label fluorescence immunohistochemical staining for CTB (green) and CalB (red, column 2) or CTB (green) and AVP (red, column 3). Note that an injection in the LS resulted in retrograde label concentrated in the core region of the SCN (overlapping with CalB), whereas CTB injection in the DMH resulted in more diffuse retrograde label in both the core and shell (AVP-ir) SCN. In contrast, injection of CTB in the VLPO resulted in labeling predominantly in the vasopressin-rich SCN shell, with scattered neuronal label in the core. Green=AVP or CalB, Red=CTB. Insets show high-power images of cells pointed to in low-power micrographs. Arrows for VLPO point to retrogradely labeled cells in either the CalB core (H) or AVP shell (I). For abbreviations, see list. Scale bar = 200 μm in A (also applies to D,G); 100 μm in B (also applies to C,E,F,H,I).

Fig. 10.

Low- and high-power photomicrographs of coronal sections showing the distribution of intra-SCN BDA-positive fibers in relation to the subregion of the SCN expressing CalB. CalB and BDA were double-labeled in the same brain section, but CalB and BDA are shown separately (A,B,F,G) and together (C,H) for visibility. The two animals shown here represent examples of the two patterns of staining seen with localized intra-SCN injections of BDA. The top figures (A–E) represent the general pattern of staining seen with a dorsal or medial injection (#20 and #17); the bottom photomicrographs (F–J) represent the general pattern of staining seen with a ventral injection (#34 and #26). High-power photomicrographs of the CalB region of the ipsilateral (D, I) and contralateral (E, J) SCN are shown to highlight differences between dorsomedial (D, E) and ventral (I, J) SCN projections to these CalB cells. Scale bar = 100 μ m, in F (also applies to A–C,G,H; low-power photomicrographs); 50 μ m in I (also applies to D,E,J; high-power photomicrographs).

Fig. 11.

Summary of results from both anterograde and retrograde tracing studies. **Top:** Schematic drawing in the sagittal plane summarizing the efferent projections arising from primarily from either the shell (solid line) or core (dashed line) SCN subdivisions. For the SPVZ, the medial (M) and lateral (L) divisions are depicted separately. **Bottom:** Summary of projections to SCN targets. Projections from the shell (vasopressin) SCN are on the left, and projections from the core (CalB) are on the right. The thickness of the lines projecting to the target regions represents the density of each projection. For abbreviations, see list.

Fig. 12.

Theoretical model of photic entrainment, rhythmicity generation, and communication to effector areas based on available findings and results from the present study. According to this model, light information is received by the SCN core and transmitted via neural connections to the “rhythmic” shell. Projections to targets of the SCN arise from both the core (CalB) and shell (vasopressin) subdivisions, suggesting that phase and rhythmic information is integrated at target sites in addition to within the SCN. For abbreviations, see list.

TABLE 1
Summary of SCN Projections After Precise Microiontophoretic Injections of BDA¹

Injection no.	LSv	BST	AVPV	MPN	MPO	LPO	VLPO	PVT	AHA	SON	PVHap	SPVZ	DMH	ARH	PAG	IGL
11 (entire SCN) 0.15 mm ²	**	**	****	****	***	**	***	****	***	***	****	****	****	***	**	***
20 (dorsomedial) 0.094 mm ²	Ø	↓	***	***	**	↓	↓	***	**	**	***	***	***	**	↓	↓
17 (ventromedial) 0.043 mm ²	↓	↓	↓	↓	↓	Ø	**	**	↓	Ø	**	**	0	↓	—	**
26 (ventral) 0.012 mm ²	**	↓	**	**	**	↓	**	**	↓	***	***	***	***	***	***	***
34 (ventral) 0.008 mm ²	Ø	↓	Ø	Ø	Ø	Ø	Ø	↓	↓	↓	↓	**	↓	↓	↓	**
19 (peri-SCN)	***	↓	**	**	**	↓	↓	****	**	↓	***	***	***	***	***	***
21 (peri SCN/ISPVZ)	**	↓	↓	*	*	*	**	***	**	↓	***	**	↓	↓	**	**
14 (peri SCN/RCA)	***	↓	↓	↓	Ø	**	***	***	**	↓	***	**	**	**	**	**

¹ For abbreviations, see list. Ø, very sparse; ↓, sparse; **, dense; ***, more dense; ****, extensive; —, tissue not available.

TABLE 2

Summary of the Relative Proportion of Retrogradely Labeled Cells Overlapping With Either the CalB or Vasopressin Subregions of the SCN¹

Brain region	CalB	AVP
LS	**	*
POA	*	**
VLPO	↓	*
PVH	*	**
l-SPVZ	***	*
PVT	**	**
DMH	**	**

¹ After injection into the LS, POA, VLPO, PVH, lateral SPVZ, PVT, or DMH. These values are qualitative and based on CTB cells within regions defined by CalB and vasopressin peptidergic staining, not the proportion of double-labeled cells. ↓, sparse; *, dense; **, more dense; ***, extensive. For abbreviations, see list.