Published in final edited form as:

Neuroscience. 2002 ; 111(3): 575–585.

Calbindin-D_{28K} Cells Selectively Contact Intra-Scn Neurons

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Abstract

Calbindin-D_{28K}-immunoreactive cells are tightly packed within a discrete region of the caudal aspect of the suprachiasmatic nuclei of hamsters. These cells receive direct retinal input and are Fos-positive in response to a light pulse. Knowledge of their afferent and efferent connections is necessary to understand suprachiasmatic nucleus organization. The first aim of the present study is to identify interconnections between calbindin and other peptidergic cells of the suprachiasmatic nuclei, using epi- and confocal microscopy and intra-suprachiasmatic nucleus tract tracing. The results indicate that essentially all calbindin cells receive numerous appositions from vasoactive intestinal polypeptide (VIP), neuropeptide Y and serotonin fibers and that most receive appositions from gastrin releasing peptide (GRP) and cholecystokinin (CCK) fibers. Reciprocal connections are seen from VIP, GRP and CCK cells but surprisingly, not from dorsomedial vasopressin cells. Injection of biotinylated dextran amine into the suprachiasmatic nucleus indicates that the ventrolateral suprachiasmatic nucleus projects to the entire nucleus, while the dorsal and medial regions of the suprachiasmatic nucleus project densely to most of the nucleus, except to the calbindin region. Analysis of colocalization of the peptides in the calbindin cell region shows that 91% of the substance P cells, 42% of the GRP cells and 60% of the VIP cells in the calbindin subnucleus coexpress calbindin-D_{28K}.

Our results reveal a highly specialized topographical organization of connections among suprachiasmatic nucleus cells.

Keywords

suprachiasmatic nucleus; calbindin; vasopressin; confocal microscopy; tract tracing

The suprachiasmatic nuclei (SCN) are the locus of the brain clock regulating circadian rhythms in mammals (reviewed in Silver and Moore, 1998). In rodents, these paired hypothalamic nuclei are each composed of about 8000 neurons that are heterogeneous in their content of neuronal antigens (van den Pol, 1980; van den Pol and Tsujimoto, 1985). Some of the neuropeptides that have been identified to date in the rodent SCN are vasopressin (AVP), vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine, gastrin releasing peptide (GRP), sub-stance P (SP), cholecystokinin (CCK) and somatostatin. We have reported that a subregion in the caudal SCN is characterized by the

presence of calbindin-D_{28K} (CalB)-containing cells (Silver et al., 1996). These cells are densely packed, receive direct retinal synaptic input (Bryant et al., 2000) and are responsive to photic stimuli, with about 80% of the cells showing Fos-like immunoreactivity (ir) following a light pulse given during subjective night (Silver et al., 1996). The foregoing evidence indicates these CalB cells are on the input pathway for photic stimuli reaching the SCN.

The function of this subregion of the SCN has been examined in hamsters. Animals with lesions that destroyed the CalB subnucleus, but spared other compartments of the SCN, lost their locomotor activity rhythm. Animals with partial bilateral SCN lesions sparing part of one or both CalB subnuclei remained rhythmic. Transplants of half-SCN grafts that contain cells of the CalB subnucleus restored locomotor rhythms in SCN-lesioned host animals, while transplants containing SCN tissue lacking cells of this subnucleus failed to restore rhythmicity (LeSauter and Silver, 1999). This indicates that the CalB subregion is essential for the maintenance of locomotor rhythmicity. Examination of clock gene expression indicates that there is no detectable rhythm of *Per1* and *Per2* mRNA in the CalB subregion, although high amplitude rhythmic expression is seen in other parts of the SCN (Hamada et al., 2001). Importantly, light induces *Per1* and *Per2* in the CalB subnucleus at night, but not during the day. Finally, *Per3* mRNA is constitutively high in the CalB subregion. These findings motivated the present examination of the intra-SCN connections of CalB cells of the SCN. We reasoned that if the CalB cells are part of the light entrainment pathway, it is important to study their connectivity.

It has been estimated in rats that individual SCN neurons make as many as 1000 synapses within the nucleus (Moore and Bernstein, 1989). Based on silver chromate impregnation of axons within the rat SCN, electron microscopic studies have shown that the majority of axons terminate within the boundaries of the SCN (van den Pol, 1980). Following surgical isolation of the adult rat SCN from the adjacent hypothalamus, up to 80% of the synaptic endings survive (cited in van den Pol, 1991), again showing that the afferent circuits of the SCN are mostly intrinsic (unless new synapses had formed by collateral sprouting). Glutamate decarboxylase (GAD)-ir in the hamster SCN also indicates an extensive and dense neural network that fills the whole SCN (Card and Moore, 1984). However, the precise pattern of these intrinsic connections is not well elucidated.

While interconnections among SCN neurons have not been studied in the hamster, there are reciprocal interconnections between all the peptidergic cell types studied in rats. Specifically, reciprocal appositions have been described between cells bearing the following peptides using double-label immunocytochemistry: AVP, VIP and somatostatin (Daikoku et al., 1992); GRP and GAD; VIP and GAD (van den Pol and Gorcs, 1986); AVP, VIP, GRP, peptide histidine isoleucine and somatostatin (Romijn et al., 1997); AVP and VIP (Jacomy et al., 1999).

Connections made between CalB-containing SCN cells and other peptidergic cell types of the SCN have not been studied. This study focuses on the interconnections between CalB cells and VIP, AVP, GRP, and CCK cells of the hamster SCN and neuropeptide Y (NPY) and serotonin (5-hydroxytryptamine or 5-HT) afferent fibers, using double-label epi- and confocal microscopy. Injection of tracer into discrete zones within the SCN was used to confirm the patterns of connections seen in the confocal double-label studies. Finally, colocalization of these antigens within individual cells of the SCN was also examined.

Experimental Procedures

Animals and housing

Subjects were LVG hamsters (*Mesocricetus auratus*) obtained from Charles River Laboratories. Animals were housed in translucent polypropylene cages (48 × 27 × 20 cm) and provided with *ad libitum* access to food and water. They were kept in a 14:10 light:dark cycle, with lights on at 08.00 h. The room was kept at 21±1°C. All handling of animals was done in accordance with Institutional Animal Care and Use Committee guidelines of Columbia University.

Perfusion

Hamsters were heavily anesthetized with sodium pentobarbital (200 mg/kg) between 10.00 and 16.00 h, when some of the peptides known to be rhythmic in the SCN (AVP, NPY) are expressed at high levels (Inouye, 1996). They were perfused intracardially with 150 ml of 0.9% saline followed by 300–400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. Brains were post-fixed for 18–24 h at 4°C. For frozen sectioning (but not for vibratome sections), brains were cryoprotected in 20% sucrose in 0.1 M PB overnight, and were then transferred to PB.

Antisera for immunocytochemistry

The following antibodies were used: mouse monoclonal anti-CalB (1:20000) from Sigma (St. Louis, MO, USA); rabbit polyclonal antisera against VIP (1:5000), NP or AVP (1:5000), CCK (1:3000), GRP (1:2000), SP (1:5000), NPY (1:5000), goat polyclonal antisera against 5-HT (1:2500) from DiaSorin (Stillwater, MN, USA).

Confocal microscopy

Sections (50 µm) were cut on a vibratome and stained for double-label fluorescence. CalB was visualized with avidin-conjugated fluorescent Texas Red and the other peptides with fluorescein isothiocyanate (FITC) conjugated to the secondary antibody. The sections were mounted and coverslipped with gelmount and coverglass No. 1 1/2. They were observed under a Zeiss Axiovert 100TV fluorescence microscope (Carl Zeiss, Thornwood, NY, USA) with a Zeiss LSM 410 laser scanning confocal attachment. The sections were excited with an argon–krypton laser using the standard excitation wavelengths for Texas Red (568 nm) and fluorescein (488 nm). The images were collected as 1-µm multitract optical sections (with sequential excitation by each laser to avoid cross-talk between the two wavelengths). Using the LSM 3.95 software (Zeiss), red and green images of the sections were superimposed. Each cell was examined through its entirety in 1-µm steps and axo/dendro-somatic appositions were counted.

This material was also analyzed to determine the proportion of cells colocalizing CalB and other peptides.

Iontophoretic injections

Small iontophoretic injections of the anterograde tracer, biotinylated dextran amine (BDA), were used in order to visualize intra-SCN connectivity ($n=41$ hamsters). Animals ($n=4$) in which BDA injections were confined to a small part of the SCN are reported here. 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, USA) in 0.01 M phosphate-buffered saline (PBS, pH 7.4). The head of the animal was shaved, positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and 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 μ A positive current pulses (7 s on, 7 s off) for 7 min. Three minutes after the injection, the pipette was removed under negative current in order to prevent leakage of the tracer along the needle tract. Animals were killed 6 days later.

Tract tracing

For single label of BDA tracer, alternate 25- μ m free-floating frozen sections were incubated in 1% H_2O_2 , washed and then incubated in avidin–biotin complex (ABC-Elite kit, 1:250 in 0.1% PB–Triton X-100). Alternate sections were stained using the SG peroxidase kit as a chromogen (Vector Laboratories, Burlingame, CA, USA).

For simultaneous visualization of CalB and BDA tracer, the second set of sections were double-labeled using fluorescence immunocytochemistry. For visualization of BDA, sections were washed in PBS, then incubated in ABC (ABC-Elite kit; 1:1000 in PB–Triton X-100) for 1 h at room temperature. In order to amplify the BDA signal, sections were incubated in biotinylated tyramine (0.6%) for 30 min at room temperature prior to incubation in FITC-conjugated streptavidin for 30 min. CalB immunoreactivity was detected as described above, using Texas Red horse anti-mouse as the secondary antibody.

In order to determine the size and spread of the BDA injections, sections stained with the SG chromogen or with the FITC fluorophore were examined. The SG chromogen stains fewer fibers than a fluorescent label and allows optimal visualization of the spread of BDA. The injection site is outlined with a dashed line (Fig. 4).

All data are presented as mean $\pm$ S.E.M.

Results

Intra-suprachiasmatic CalB connections

CalB cells make selective intra-SCN connections. Figure 1 is a schematic representing the distribution of cells and fibers and the intra-SCN interconnections between the CalB cells and the VIP, AVP, CCK or GRP cells. The SCN is represented at four different levels from rostral to caudal.

Figure 2 shows photomicrographs of representative parts of the SCN, double-labeled for CalB and VIP, AVP, CCK or GRP. The top row shows light microscopic images of 50 μ m thick sections through the CalB subregion of the SCN stained for CalB in red and other peptides in green. The middle row shows confocal 1- μ m optical slices showing the afferent contacts onto the CalB cells. The bottom row shows confocal slices of the CalB fibers making appositions onto the other peptidergic cell types. Table 1 indicates the proportion of cells that receive appositions from other peptidergic fibers.

Interconnections between CalB and VIP—The first panel of Fig. 1 represents the interrelationship of VIP and CalB. The VIP cells located within the ventral part of the SCN send fibers throughout the SCN. Many VIP fibers make appositions with the CalB cells. As seen in the top panel of Fig. 2, the VIP/CalB double label indicates extensive innervation from VIP onto the CalB cells. Some cells are also double-labeled. The middle panel, VIP to CalB, shows the many appositions of VIP fibers onto each CalB cell. The VIP fibers make numerous (at least three and often many more) appositions onto all the CalB cells.

Most CalB cells are located in the posterior aspect of the mid-SCN region (Fig. 1). Some sparsely distributed CalB cells are also present in the rostral and dorsal SCN. CalB fibers

emanating from the CalB subnucleus course dorsally within the dorsolateral SCN. A few CalB fibers are also detected within the ventral SCN. These ventral fibers make appositions with the VIP cells (Fig. 1, second column and Fig. 2, CalB to VIP panel). All VIP cells receive appositions from CalB fibers with a third receiving three or more appositions and two thirds receiving one or two appositions (Table 1).

Interconnections between CalB and AVP—The second panel of Fig. 1 represents the interrelationship of AVP and CalB cells and fibers. AVP cells are located in the rostral and dorsomedial SCN. Most of the AVP fibers course dorsally, with no AVP-ir fibers seen descending from the dorsomedial region toward the CalB subnucleus. Very few AVP fibers (Fig. 2, second column) are detected among the CalB cells. These AVP fibers seem to originate from the ventrolateral AVP cells (Fig. 1, AVP to CalB, mid-posterior SCN). As seen in Table 1, these fibers make at most a single apposition with CalB cells.

Most CalB fibers course dorsally through the dorsolateral SCN, and only rarely enter the dorsomedial portion of the mid-SCN (Fig. 2). In some instances, CalB fibers course dorsomedially, then turn toward the dorsolateral SCN. The rare CalB-ir fibers and appositions (seen in two of 40 cells) in the AVP region seem to originate from CalB cells located within the same region. For one of these appositions the fiber originated from one of the few CalB cells lying in the dorsal SCN.

Interconnections between CalB and CCK—The third panel of Fig. 1 represents the distribution of CCK and CalB cells and fibers. The CCK cells are located near the edge of the SCN, with very few cells at the dorsolateral border. The CCK fibers form a ring within the borders of the SCN. CCK fibers seemed to contact each of the CalB cells (Fig. 2, column 3), making few appositions in most (81.3%) instances (Table 1). Most CCK cells receive contacts from CalB fibers in the ventral part of the central SCN (Fig. 2; Table 1), though these are few in number.

Interconnections between CalB and GRP—The fourth panel of Fig. 1 summarizes the distribution of GRP and CalB cells and fibers. The GRP cells are located in the same region as the CalB cells. A dense plexus of GRP fibers course dorsolaterally within the SCN, while sparse GRP fibers are detected throughout the SCN (Fig. 1). There are numerous GRP-ir fibers in the CalB subregion contacting most of the CalB cells (Fig. 2, column 4). Almost two thirds of the CalB cells receive many GRP appositions, while one third receive one or two appositions, and conversely, 57% of the GRP cells receive many CalB appositions (Table 1). Some cells colocalize CalB and GRP (Fig. 2, column 4).

Appositions of extra-SCN afferents on CalB (Fig. 3; Table 1)

A dense plexus of NPY fibers is seen in the ventral and mid-SCN (Fig. 1, panel 5). All CalB cells are surrounded by NPY fibers and receive many NPY-ir appositions (Fig. 3, column 1). Sparse 5-HT fibers are seen throughout the SCN, while a dense plexus is found in the ventral and ventromedial and caudal regions (Fig. 3, column 2). It is noteworthy that all CalB cells receive at least three appositions from the sparse 5-HT fibers (Fig. 3, 5HT to CalB). Although all the CalB cells receive some appositions from both NPY and 5-HT fibers, the number of appositions to each CalB cells is much greater for NPY than for 5-HT fibers.

Tract tracing

BDA injections in the SCN were localized as follows: two animals had an injection in the ventral SCN, one animal in the medial SCN and the fourth, in the dorsal SCN. Injection of the anterograde tracer BDA into the dorsal or medial region of the SCN confirms the

sparsity of projections from this region to the CalB subregion. Indeed, BDA-ir fibers from these two injections are intensely distributed throughout the SCN, but generally avoid the CalB subnucleus. In contrast, injections of BDA in the ventral SCN label efferent fibers throughout the SCN, including the CalB subregion (Fig. 4).

Colocalization of the CalB with SP, GRP and VIP

The CalB-containing cells colocalize SP, GRP and VIP. This is shown in representative photomicrographs (Fig. 5) and quantitatively (Fig. 6). The SP- and GRP-ir cells lie within the CalB subnucleus, although some GRP cells extend outside the boundaries of the CalB subregion. Most SP cells also contain CalB, while about 40% of the GRP cells contain CalB. Most VIP cells (60%) located within the CalB subnucleus co-express CalB. VIP cells in the ventral SCN along and within the optic chiasm do not colocalize CalB. As mentioned above, the CCK cells are located near the edge of the SCN while the CalB cells are located more centrally. No cells double-labeled for CCK and CalB were observed.

Discussion

Connections of CalB-immunoreactive cells within the SCN

The molecular machinery that generates rhythmicity at the cellular level is being elucidated (Dunlap, 1999; King and Takahashi, 2000; Reppert and Weaver, 2001); however, the mechanisms of entrainment, coupling between entraining cues and oscillators and output are poorly understood. Knowledge of SCN organization is necessary to understand the generation and control of circadian rhythmicity in mammals.

This study focuses on the connections of the CalB cells of the hamster which has several specialized features. The CalB cells are part of the entrainment pathway. They receive synaptic contacts from the retinal fibers (Bryant et al., 2000). Most (79%) of the CalB cells within the CalB subnucleus express Fos in response to a light pulse (Silver et al., 1996). *Per1* and *Per2* mRNA are induced by light in the CalB subregion (Hamada et al., 2001). Finally, the CalB subregion seems necessary for the maintenance of circadian rhythms of locomotor activity (LeSauter and Silver, 1999).

The present study shows that in addition to retinal afferents, the hamster CalB cells receive many appositions from NPY and some from 5-HT fibers (Fig. 3) which are also implicated in entrainment (reviewed in Miller et al., 1996; Morin, 1999). The results also reveal that interconnections are not made among all SCN cell types. Compared to the other peptidergic cell groups such as AVP, VIP, GRP, somatostatin and GAD that show reciprocal interconnections in rats (Daikoku et al., 1992; Jacomy et al., 1999; Romijn et al., 1997; van den Pol and Gorcs, 1986), hamster CalB cells appear to have a more specialized pattern of interconnections. The differences go from no or very few (CalB and AVP) to moderate (CalB and CCK; CalB and GRP) to numerous (VIP and CalB, NPY and CalB) interconnections. This is depicted in the schematic in Fig. 7. This figure indicates that the retinal (Silver et al., 1996; Bryant et al., 2000), NPY and 5-HT afferent pathways, as well as the ventral VIP cells, contact all or nearly all the CalB cells.

A caveat in the present study is that only somatic appositions were counted. While the varicosities terminating onto the cell bodies, on large fibers, or axon hillocks near the cell bodies are quite clear, it is very difficult to determine whether varicosities seen on fine fibers are appositions, fibers crossing each other, or small artifacts resulting from the immunocytochemistry procedure. Therefore, no attempt was made to estimate the number of appositions on fibers.

Especially interesting is the lack of reciprocal communication between the CalB and AVP cells. This result is confirmed by tract tracing to determine the pattern of neural communication between the dorsomedial SCN and the CalB subnucleus. The anterograde tracer BDA injected into the medial or dorsal SCN leaves the CalB subregion relatively empty of efferent fibers. This indicates that it is not only the AVP cells that do not send efferents to the CalB subregion, but that in general, the medial/dorsomedial SCN does not have direct contact with the CalB subnucleus. The immunocytochemistry and tract tracing studies fit together to indicate heterogeneity in intra-SCN coupling organization.

Colocalization of peptides in the CalB subnucleus

Many of the CalB cells colocalize other peptides. All these cells may be part of the light entrainment mechanism in hamsters. CalB (Bryant et al., 2000) and GRP (Aioun et al., 1998) cells receive retinal terminals and presentation of a light pulse during the night induces c-Fos in about 80% of the CalB cells (Silver et al., 1996), 78% of the GRP cells and 60% of the VIP cells (Aioun et al., 1998). It is also known that administration of GRP (McArthur et al., 2000) or coadministration of VIP, peptide histidine isoleucine and GRP (Albers et al., 1991) induce phase-dependent resetting of the SCN that resembles the light-induced phase response curve. Finally, administration of CalB antisense blocks light-induced phase shifts (Hamada et al., 2000). SP has also been implicated in photic entrainment, possibly through glutamate release (Abe et al., 1996; Hamada et al., 1999; Kim et al., 1999), although it is not known whether this is due to SP from the retinohypothalamic tract or SP within the SCN (Otori et al., 1993; Takatsuji et al., 1991).

It is not known whether hamster CalB cells expressing (or lacking) SP or GRP, or the VIP cells expressing (or lacking) CalB, have different functions. While it is possible that the chemoarchitecture of the SCN neurons is related to specific functions of discrete subpopulations, these remain to be identified.

Functional organization of the SCN

There is substantial evidence of functional specificity of SCN subregions from experiments using tract tracing or light induction of immediate early genes. Specifically, retinal input is much denser in the ventrolateral than in the dorsomedial rat SCN (Speh and Moore, 1993). Light-induced Fos expression in hamsters is concentrated in the CalB subregion, sparse in the dorsolateral area, and very low or not expressed in the dorsomedial SCN (Aronin et al., 1990; Guido et al., 1999; Hastings et al., 1996; Kornhauser et al., 1990; Rusak et al., 1990; Silver et al., 1996). More recently, Schwartz et al. (2000) have shown light-induced expression of c-Fos, Fra-2 and Fos-B in the ventromedial, but not in the dorsomedial SCN of rat. In contrast, endogenous circadian rhythms of c-Fos and Fra-2 expression occurred in the dorsomedial SCN, but not in the ventromedial SCN. The study of gap junctions between SCN neurons also shows division of the rat SCN (Colwell, 2000). Clusters of coupled cells were restricted to either the dorsomedial or the ventrolateral regions. That is, cells in one region were not coupled to cells in the other region.

It is not known how signals are communicated within the SCN. The present study suggests that information from CalB cells does not reach AVP cells through direct monosynaptic contact. It should be noted, however, that despite the absence of direct communication between CalB and AVP cells in hamster SCN, there could be indirect communication through those VIP or GRP cells which do contact the AVP cells (Daikoku et al., 1992; Jacomy et al., 1999; Romijn et al., 1997 for rats; unpublished observations in hamster from our laboratory).

It is also of interest to understand how signals from individual SCN pacemakers are transmitted to their respective targets. CalB cells in hamster SCN project to both intra- and extra-SCN regions (Fig. 2; LeSauter et al., 1999). AVP, VIP and GRP neurons are also projection neurons, as SCN lesions decrease the number of fibers in their respective targets (Kalsbeek et al., 1993). In summary, studies of SCN microcircuitry beg the question of how pacemaker cells are coupled and how they communicate their temporal information to their targets.

Acknowledgments

Supported by NIH Grants NS 37919 to R.S. and DK 07328 to L.J.K.

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Abbreviations

5-HT	5-hydroxytryptamine
ABC	avidin–biotin complex
AVP	vasopressin
BDA	biotinylated dextran amine
CalB	calbindin-D _{28K}
CCK	cholecystokinin
FITC	fluorescein isothiocyanate
GAD	glutamate decarboxylase
GRP	gastrin releasing peptide
-ir	-like immunoreactivity
NPY	neuropeptide Y
PB	phosphate buffer
PBS	phosphate-buffered saline
SCN	suprachiasmatic nuclei
SP	substance P
VIP	vasoactive intestinal polypeptide

Fig. 1.

Schematic representing the average distribution of cells and fibers of the peptides studied in the rostral, mid-anterior, mid-posterior and caudal aspects of the SCN. Each panel shows the fiber distribution between CalB and VIP, AVP, CCK, GRP, NPY or 5-HT. The left columns in the first four panels represent the distribution of the different peptidergic cells and fibers (gray) in relation to the CalB cells (black), the right columns represent the distribution of CalB cells and fibers (gray) in relation to the other peptidergic cells (black). The last two columns represent the distribution of afferent NPY and 5-HT fibers (gray) in relation to the CalB cells (black).

Fig. 2.

Photomicrographs of double-labeled coronal brain sections through the SCN. The top row are light microscopy photomicrographs showing the distribution of CalB- and of VIP-, AVP (VP)-, CCK- and GRP-ir in 50 μ m thick sections of the mid-posterior SCN. The CalB cells form a dense subnucleus in the ventrolateral region, some sparse CalB cells are also seen in the dorsal SCN. The second and third rows are 1- μ m confocal sections. The second row shows the presence (or absence) of VIP-, AVP-, CCK- and GRP-like immunoreactivity (left to right, respectively) within the CalB subregion. VIP makes numerous appositions with the CalB cells. AVP and CCK make few appositions. GRP cells are distributed among the CalB cells and GRP fibers contact CalB cells. The third row shows the presence (or absence) of

CalB fibers within the region of VIP- (ventral SCN), AVP- (dorsomedial SCN), CCK- (medial SCN) and GRP-like (ventromedial SCN) immunoreactivity (left to right, respectively). Arrows indicate some of the appositions. Some CalB fibers make appositions with VIP cells. In the second column, no CalB-ir fibers are seen in this section of the dorsomedial AVP region. CalB-ir fibers are seen among the CCK-ir cells and some contact the cells. CalB-ir fibers make appositions with GRP-ir cells, and some cells contain both CalB and GRP.

Fig. 3. Photomicrographs of coronal brain sections double-labeled for NPY and CalB (left) and 5-HT and CalB (right). The top panels show the distribution of the NPY and 5-HT fibers within the mid-posterior SCN at the level of the CalB sub-nucleus. The bottom panels are 1-μm confocal sections showing the NPY fibers surrounding and making appositions onto the CalB cells and the sparse 5-HT fibers contacting the CalB cells. Although both NPY and 5-HT fibers contact all the CalB cells, the contacts on each cell were more numerous for NPY than for 5-HT.

Fig. 4.

Light microscopy photomicrographs of 25- μ m sections showing the injection sites of BDA in the first column, stained with the FITC fluorophore. The size of the injection is represented by dashed lines drawn on alternate sections stained with SG chromogen. BDA-ir efferent projections are shown in the second column. The same section as in the second column double-labeled for CalB is shown in the last column. First row: BDA was injected in the ventral SCN. BDA-ir fibers innervate the whole SCN. Second row: An injection in the medial SCN results in fibers that densely innervate the whole SCN except the CalB subregion which receives only sparse fibers.

Fig. 5. Confocal microscopy photomicrographs of 1- μ m optical sections of the CalB subnucleus stained for CalB (first column) and for SP-, GRP- and VIP-like immunoreactivity (second column), and the overlay of the two laser wavelengths (third column). The arrowheads indicate some of the double-labeled cells. For VIP, the captured images are from the ventral part of the CalB subnucleus. The proportions of double-labeled cells are shown in Fig. 6.

Fig. 6.

Bar graphs showing the proportion of cells double-labeled within the SCN. For SP, the numbers refer to cells within the SP subnucleus only. For VIP, the numbers refer to cells within the ventral CalB subnucleus only. (A) Percentage (mean $\pm$ S.E.M.) of CalB cells that also colocalize each peptide. The counts were done on 74 CalB cells in three animals for SP, 154 CalB cells in eight animals for GRP and 40 CalB cells in three animals for VIP. (B) Percentage (mean $\pm$ S.E.M.) of peptidergic cells that colocalize CalB. The counts were done on 48 SP cells in three animals, 122 GRP cells in eight animals and 25 VIP cells in three animals. In four animals, 40 AVP (VP)-ir cells and 26 CalB-ir cells were sampled in the rostral and dorsomedial SCN, and no cell colocalized AVP and CalB.

Fig. 7.

Schematic representing the connections between the CalB cells and CCK, AVP, GRP, NPY, VIP and the retina. The arrow thickness and the percentages are the proportion of cells receiving three or more appositions (Table 1). For retinal afferents, the thickness of the arrow is derived from previous data (Bryant et al., 2000; Silver et al., 1996).

Table 1
Proportions of cells receiving appositions from other peptidergic fibers

	Percent cells with > 3 appositions	Percent cells with 1–2 appositions	Number of animals	Number of cells
VIP to CalB	98.4 ± 1.6	1.6 ± 1.6	3	40
CalB to VIP	38.9 ± 20	61.1 ± 20	3	16
AVP to CalB	0	66.7 ± 19.3	3	26
CalB to AVP	0	7.8 ± 5.9	4	40
CCK to CalB	18.8 ± 18.8	81.3 ± 18.8	3	12
CalB to CCK	14.6 ± 11.6	75.7 ± 13.2	3	36
GRP to CalB	61.8 ± 19.6	34.8 ± 17.7	6	87
CalB to GRP	57.4 ± 19.7	39.3 ± 18.5	6	57
NPY to CalB	100	0	2	69
5-HT to CalB	100	0	3	101