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Circadian Trafficking of Calbindin-ir in Fibers of SCN Neurons

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Abstract

Calbindin-D28K (CalB) containing cells form a distinct cluster within the core of the hamster suprachiasmatic nucleus (SCN). These cells are directly retinorecipient but lack detectable rhythms in clock gene expression or electrical activity. In studies exploring SCN connectivity using double-label immunocytochemistry, we previously reported an absence of contacts among CalB fibers and vasopressin (VP) cells in animals sacrificed during the day. Here we explored circadian variations in CalB-immunoreactivity (-ir) and re-examined the connections between CalB and other SCN cell types at zeitgeber time (ZT) 4 and 14. The results reveal a circadian rhythm of CalB-ir in fibers of SCN cells with high expression during the night and subjective night and low expression during the day and subjective day. This circadian difference is not seen in the other brain regions studied. Significantly more appositions were detected between CalB fibers and VP cells during the night than during the day, while circadian variation in numbers of contacts was not seen between CalB fibers and VIP, CCK or GRP cells. There was no detectable variation in appositions from any peptidergic fiber type onto CalB cells. The present findings suggest that CalB cells relay photic information to VP oscillator cells of the SCN shell in a temporally gated manner.

Keywords

Suprachiasmatic; Calbindin-D28K; Hamster

Introduction

Calbindin-D28K (CalB)-containing cells are localized to the core region of the suprachiasmatic nuclei (SCN) of hamsters. These cells are c-FOS-positive in response to a light pulse (Silver et al. 1996) and double-label electron-immunocytochemistry studies indicate that they receive direct retinal input via the retinohypothalamic tract (Bryant et al. 2000). In previous work, we suggested that the SCN is comprised of two fundamentally different cell types with regard to circadian rhythmicity. One population, including CalB-containing cells of the hamster SCN, express *Per1* and *Per2* mRNA and their proteins in response to a light pulse, but lack a detectable rhythm in endogenous *Per1* and *Per2* mRNA expression or in electrical activity (Hamada et al. 2001; Jobst and Allen 2002). The second population oscillates on a circadian basis with respect to clock genes and electrical activity and is not directly retinorecipient. The

latter also differ from CalB-containing cells in that they do not express *Per* genes immediately after a light pulse (Hamada et al. 2001; Jobst and Allen 2002). A gate-oscillator model explores how this two-compartment SCN can sustain rhythmicity (Antle et al. 2003; Antle et al. 2007). In this model, non-oscillating gate cells provide a signal that maintains coherence among a population of independent oscillators, which themselves are reset by signals from gate cells. Feedback is required to control the state of the gate.

To understand the circuit organization of the SCN it is necessary to determine the relationship between oscillators and directly retinorecipient cells. Because CalB cells are on the input pathway from the retina to SCN oscillators, we were surprised at the absence of appositions between CalB and vasopressin (VP) cells (LeSauter et al. 2002). In the present study, we took into account the fact that we had previously examined efferent connections at only one time of day, zeitgeber time (ZT) 2-8 (LeSauter et al. 2002). In view of evidence of a circadian rhythm in localization of CalB protein within the soma of SCN neurons (Hamada et al. 2003), we sought here to explore the distribution of CalB in projections of these cells. The results indicate a diurnal and a circadian rhythm of CalB-ir within the fibers of CalB-containing SCN cells, and also reveal numerous appositions between CalB fibers and VP cells at times when fibers contain high levels of CalB.

Methods

Animals and housing

Adult male LVG hamsters (*Mesocricetus auratus*) were purchased from Charles River Labs (Wilmington, MA) or from Kyudo Company, Tosu, Saga, Japan at age 4-5 weeks. Circadian changes in fiber-ir and diurnal changes in appositions were analyzed in Syrian hamsters at Columbia University, USA. Diurnal changes in fiber-ir were analyzed in Kyushu University, Japan. Animals were housed in translucent propylene cages (48×27×20 cm) and provided with ad libitum access to food and water. The rooms were kept at 22±1°C. Animals were either kept in a 12:12 light-dark (LD) cycle, or transferred to constant darkness (DD) for 7-8 days. A dim red light which generates less than 1 lux (Delta 1, Dallas, TX) allowed for animal maintenance. The room was equipped with a white noise generator (91dB spl) to mask environmental noise. Animals housed in DD were sacrificed at 4 hour intervals and those housed in LD cycle were sacrificed at ZT4 or 14. All handling of animals was done in accordance with Institutional Animal Care and Use Committee guidelines of Columbia University and by the Committee of Animal Care of Kyushu University.

Immunocytochemistry (ICC)

Hamsters were heavily anesthetized (pentobarbital: 200 mg/kg), and perfused intracardially with 150 ml 0.9% saline followed by 300-400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Brains were post-fixed for 18-24 hrs at 4°C, cryoprotected in 20% sucrose in 0.1M phosphate buffer overnight. For quantification of CalB fibers, serial 20 µm coronal sections from animals in DD (N= 4-5 /time point) or in LD (N=4/time points) were cut and collected in individual wells. To control for possible variations in immunochemical runs, sections from animals at each experimental time point were processed simultaneously. The sections were processed with mouse monoclonal anti-CalB (1:20,000; Sigma, St. Louis, MO), using a modified avidin-biotin-immunoperoxidase technique, the chromogen used was either DAB or the SG substrate (Vector, Burlingame, CA). The sections were coverslipped with Permount. For fiber distribution, serial 40 µm coronal and sagittal sections from ZT 14 animals (N=4) were stained for CalB (1:20,000) using DAB. For appositions onto other cells serial 50 µm sections from ZT4 (N=9) and ZT 14 (N=15) animals were stained for double-label fluorescence for CalB (1:20,000) and VP (1: 5,000, made in guinea pig, Peninsula Laboratories, San Carlos, CA), VIP (1: 5,000, made in guinea pig, Peninsula) GRP (1:2000, made in rabbit,

DiaSorin, Stillwater, MN, USA), or CCK (1:3000, made in rabbit, DiaSorin). Sections were coverslipped with Krystalon (EMD Chemicals, Gibbstown, NJ), and coverglass N° 1 1/2.

Quantification of CalB fibers

CalB fibers were observed under a light microscope (Olympus BH-2) by two independent investigators, blind to the experimental conditions. In the SCN, fiber staining was assessed by counting fibers crossing the lines of 32 squares (8×4) on a 180 × 90 μm grid. The grid was placed on the SCN region dorsal to the CalB subnucleus in four adjacent sections (8 SCN)/animal (Similarly, the TS lab used a 32 square grid (8×4 squares) measuring 200 μm × 100 μm). We also determined whether differences in CalB-ir occurred in other brain regions at CT 4 and CT14. In the paraventricular nucleus (PVN) and in the amygdaloid nucleus CalB-ir fiber staining was quantified by relative optical density (ROD), measured as optical density of staining within the region of CalB fiber-ir minus optical density of background. The area to be measured was determined using the free-hand tracing tool in NIH image. Care was taken not to include cell bodies in the area analyzed. The staining for each animal was expressed as the average ROD in two brain sections for each nucleus. The time of day effects were analyzed by ANOVA followed by Fisher's PLSD or by unpaired t-test.

Tracing of CalB fibers

Images were captured using a Nikon Eclipse 800 epifluorescent microscope and a cooled CCD digital camera using Spot software (Diagnostic Instruments, Sterling Heights, MI). Images were loaded into Photoshop (Adobe, San Jose, CA) and tracings of fibers were made of the ZT 14 DAB-stained sections.

Appositions

Double-labeled sections were observed under a confocal microscope (Zeiss Axiovert 100TV with argon-krypton LSM 510 laser scanning microscope (Carl Zeiss, Thornwood, NY). The images were collected with a 63× Zeiss C-apochromat water immersion objective and digital image resolution of 1024×1024, as 1 μm multitract optical sections, with sequential excitation by each laser to avoid cross-talk between the two wavelengths. Red and green images were superimposed using LSM 3.95 software (Zeiss). Each cell was examined through its entirety in 1 μm steps to estimate the number of axosomatic and/or dendrosomatic appositions. Appositions among fibers were not evaluated. Each yellow profile (corresponding to signal from both red and green fluorescent dyes) was counted as one apposition regardless of its depth and area. Caveats to the present methods include: 1) dendrites and axons can not be differentiated; 2) synapses can not be evaluated, and 3) over or underestimation of the number of appositions due to ICC procedure, or 4) to missing fibers cut during sectioning, may occur. These caveats do not however affect comparison between time points, germane to the present study.

Results

Circadian fluctuations in CalB-ir

Quantification of SCN fibers indicates a circadian rhythm in immunoreactivity with few fibers detected in the first half of subjective day and an elevation at the beginning of subjective night (Fig. 1 upper panel; $F(5-17) = 19.8, p < 0.0001$). Similar changes were seen in the SCN of animals housed in LD, with more fibers at ZT14 than ZT4 (Fig. 1 lower panel; $t(6) = -4.42, p < 0.005$). In the PVN and amygdaloid nucleus, no differences in fiber staining were seen between CT4 and CT14 ($t(7) = 0.006, p = 0.99$ and $t(7) = 0.34, p = 0.74$ respectively).

Fiber distribution

Representative photomicrographs of coronal sections stained for CalB show that more CalB fibers are detected at CT 14 than at CT 4 (Fig. 2 A, B respectively). Schematics of rostral to caudal coronal sections show the distribution of CalB-containing neurons and their fibers at ZT 14 (Fig. 2 C-J). The majority of fibers emanating from the CalB subnucleus extend dorsally within the SCN (Fig. 2, D-I). Some fibers course toward the dorsomedial region or the ventral SCN (Fig. 2, G & H). A few fibers are detected within the medial SCN (Fig. 2, E to J) where some are seen crossing the midline (Fig. 2 D, E, F). Few CalB fibers are seen in the rostral-most aspect of the SCN (not shown).

In sagittal sections, more fibers are seen extending further from the CalB subnucleus at ZT14 than at ZT4 (Fig. 3 A, B respectively). In schematics of lateral to medial sections (Fig. 3 C-F), few fibers are detected in the most lateral and most medial aspects of the SCN (Fig. 3C, F). At the level of the mid-SCN where CalB cells are densest, most fibers are seen coursing dorsally and dorso-rostrally within the shell (Fig 3 E).

Appositions among SCN cell types

We had previously studied contacts of CalB fibers onto other SCN cell types at ZT2-8 and found few appositions with VP cells (LeSauter et al. 2002). In view of the present observations of a circadian rhythm in CalB localization in fibers of SCN neurons, we reexamined contacts among SCN neurons of various peptidergic phenotypes at ZT4 and 14 (see Table 1 and Fig 4).

We counted appositions by evaluating separately those with 1-2 appositions, which might be false positives, and those with more than 3 appositions, which were unlikely to occur by chance, as has been done previously (LeSauter et al. 2002). The quantitative analysis of CalB fiber appositions on SCN somata indicates that the number of cells bearing ≥ 3 CalB-positive appositions is greater at ZT14 than at ZT4 and a difference among cell types [2-way ANOVA; time (df 1, $F=10.7$, $p=0.004$) $\times$ peptide (df 3, $F=5.2$, $p=0.01$)]. This time of day effect is significant for appositions onto VP cells (Tukey test, $p=0.003$), but not onto VIP, CCK and GRP cells. Similar to the previous report (LeSauter et al. 2002), CalB fibers make 1-2 appositions with few VP cells.

The quantitative analysis of ≥ 3 appositions from VP, VIP, CCK or GRP fibers onto CalB somata indicates no effect of time, but a difference between the different cell types [2 way Anova; time(df 1, $F=0.02$, ns) $\times$ peptide (df 3, $F=59.9$, $p<0.001$)]. VIP fibers appose more CalB cells than do VP, CCK or GRP fibers at both time points ($p<0.008$) and GRP fibers contact more CalB cells than do VP or CCK fibers at both times ($p<0.008$). VP and CCK fibers contact a similar number of CalB cells. In summary, there is dense innervation of the CalB subnucleus by VIP fibers, somewhat less dense GRP innervation and sparse VP and CCK innervation (Table 1).

The top row of Fig. 4 shows photomicrographs of sections (50 μ m) double-labeled for CalB (red) and VP, VIP, CCK or GRP (green), taken at the level of the mid-SCN where CalB cells are densest, from animals sacrificed at ZT14. The middle row of confocal optical slices (2 μ m) shows CalB fiber appositions onto VP, VIP, CCK or GRP cells. The bottom row of confocal slices shows VP, VIP, CCK or GRP contacts onto CalB cells. In the first column, CalB fibers are seen extending dorsally and dorsomedially toward the region of VP cells (top panel) and make numerous appositions onto VP cells (middle panel). VP fibers are detected within the CalB subnucleus and make a few appositions onto CalB cells (bottom panel). The second column shows the interrelationship of VIP and CalB. The ventral VIP cells send fibers throughout the SCN (top panel). In the ventral SCN, CalB fibers make appositions onto VIP cells (mid panel). Many VIP fibers make numerous appositions onto the CalB cells (bottom

panel). The third column reveals the distribution of CCK and CalB cells and fibers. The CCK cells form a ring within the borders of the SCN (top panel). Most CCK cells receive a few appositions from CalB fibers (mid panel). Some CCK fibers contact CalB cells, making few appositions in most instances (bottom panel). The fourth column shows the distribution of GRP and CalB cells and fibers. CalB cells and GRP fibers are dense within the SCN core (top panel). CalB-ir fibers make many appositions onto the GRP cells and vice-versa (mid and bottom panels).

A schematic representing the connections to and from the CalB-ir cells to other SCN peptidergic cells is shown at the bottom of Fig. 4 for both ZT4 and ZT14.

Discussion

Light is the most salient stimulus adjusting the circadian clock to the local light-dark cycle, though resetting occurs only at specific times of the day. Light-induced phase shifts of behavior occur during the night (or subjective night) and not during the day (or subjective day). The mechanisms mediating such gating of photic effects are not well understood. The retinorecipient SCN contains CalB-expressing cells and the protein appears to influence the responses to photic cues as indicated by studies using CalB-antisense oligonucleotides (Hamada et al. 2003). The CalB protein is contained within SCN cells that are directly retinorecipient (Bryant et al. 2000) and cells of this core region are thought to be important for relaying photic signals to those SCN cells that are not directly retinorecipient. Thus it had been a surprise to find, in a previous study, that CalB cells did not make contacts with VP cells (LeSauter et al. 2002). The present findings go a long way to explaining that puzzle.

While overall CalB protein is not rhythmic in the SCN (Cayetanot et al. 2007; LeSauter et al. 1999), the results show a marked circadian rhythm of CalB-immunoreactivity in fibers of SCN cells. The number of detectable fibers peaks at night around ZT/CT 14 and is low at ZT/CT 2-6. This rhythm is associated with spatial localization of CalB in the soma, with nuclear content highest during the day and lowest at night [hamster, (Hamada et al. 2003); mouse lemur, (Cayetanot et al. 2007)]. CalB-ir fibers make numerous appositions onto VP cells at night, while few such contacts are detected during the day. In contrast, circadian changes in the numbers of contacts were not seen between CalB fibers and VIP, CCK and GRP cells. The absence of differences in CalB fiber-ir in the PVN and in the amygdala suggests that the circadian expression of CalB in fibers is specific to the SCN.

The cause of changes in CalB-ir in fibers may reflect local variation in amount of CalB protein, or may be the result of changes in the amount of bound vs. unbound CalB, or of conformational changes in the CalB protein and associated variation in antibody recognition (Winsky and Kuznicki 1996). Whatever the cause, the present findings suggest a temporally gated signaling pathway between the retinorecipient CalB cells and the oscillators of VP cells. As the present study cannot differentiate between axons and dendrites, such gating may modulate the axonal release of neurotransmitters from the CalB cells by changing the amount of free calcium ions or it may modify dendritic signaling from oscillator cells to CalB cells. In biocytin-filled cells, we have shown that both axons and dendrites of GRP cells extend into the shell and contact VP cells (Drouyer In press)

Effect of changes in CalB

CalB is a high-affinity calcium-binding protein, and is implicated in the regulation of Ca^{2+} homeostasis by acting as a cytosolic Ca^{2+} buffer and possibly as a Ca^{2+} sensor (Berggard et al. 2002; Schmidt et al. 2005; Schwaller 2009; Schwaller et al. 2002). This function suggests several possible mechanisms whereby CalB cells of the SCN might act to modulate the gating of photic input. In axon terminals of the caudate-putamen nuclei, CalB contributes to the

immobilization of calcium and has been implicated in terminating GABA release (Pickel and Heras 1996). In contrast, in postsynaptic spines, CalB acts as a shuttle, allowing Ca^{2+} ions to travel greater distances (Schmidt and Eilers 2009; Schmidt et al. 2007). Another possible mechanism for the gating effect of CalB could be through voltage dependent Ca^{2+} currents [reviewed in (Schwaller 2009)]. The absence of CalB is linked to an increase in Ca^{2+} dependent inactivation of voltage dependent Ca^{2+} currents in CalB knockout animals (Klapstein et al. 1998), and in patients with Ammon's horn sclerosis (Nagerl and Mody 1998). Similar mechanisms may apply in the SCN. Voltage-gated calcium channels play a crucial role in phase shifting (Kim et al. 2005). The presence of CalB in fibers at night may cause a decrease in Ca^{2+} -dependent inactivation of voltage-dependent Ca^{2+} current. During the day, on the other hand, when CalB is absent from the terminals, there would be inactivation of voltage dependent Ca^{2+} currents.

Diversity of CalB-containing cells

CalB cells coexpress other peptides, including gastrin releasing peptide (GRP) and substance P (SP) that can themselves phase shift oscillators. About 40% of CalB cells contain GRP (LeSauter et al. 2002). GRP induces phase shifts in locomotor (Albers et al. 1995; Piggins et al. 1995a) and electrical activity (McArthur et al. 2000). GRP induces c-FOS, MAPK, *Per1* and *Per2* mRNA in cells of the SCN shell, and impairment of the MAPK pathway by inhibiting ERK1/2 phosphorylation attenuates GRP-induced phase shifts (Antle et al. 2005). About 65% of the CalB cells contain SP and more than 90% SP cells are CalB-positive (LeSauter et al. 2002). SP induces phase shifts in electrical activity in rat SCN cells at night (Shibata et al. 1992). SP increases firing rate in most hamster SCN cells in vitro (Piggins et al. 1995b) but does not phase shift hamster activity rhythms (Piggins and Rusak 1997). The SP receptor neurokinin-1 is located mostly at the dorsal and dorsolateral border and outside of the SCN. Administration of the SP receptor antagonist spantide attenuates light-induced c-FOS in most of the SCN, including the dorsomedial region of VP cells, but not in the region of CalB cells (Abe et al. 1996).

CalB and the maintenance of circadian rhythms

CalB has an important role in the generation/maintenance of rhythmic circadian outputs. While CalB cells lack detectable rhythms in electrical activity (Jobst and Allen 2002) or gene expression (Hamada et al. 2001) they may be essential for maintaining synchronicity among the oscillator cells in order to generate a rhythmic output. Mice lacking CalB show low amplitude behavioral rhythms or become arrhythmic in constant conditions (Kriegsfeld et al. 2008). The suprachiasmatic nucleus of CalB knockout mice has reduced levels of PER2 and VP (Kriegsfeld et al. 2008). The CalB subregion of the hamster SCN is essential for the maintenance of rhythmicity as its ablation results in arrhythmicity even if large parts of the shell remained intact (Kriegsfeld et al. 2004; LeSauter and Silver 1999).

The role of core cells in synchronization of SCN oscillators has been demonstrated. Mice lacking the VIP receptor VPAC2, show low amplitude rhythms or arrhythmicity, abnormal entrainment (similar to that seen in mice lacking CalB) and desynchronization among oscillators. Synchronization is restored by VIP agonists (Aton et al. 2005; Aton et al. 2006) or by GRP administration (Brown et al. 2005; Maywood et al. 2006). This could occur through axosomatic signaling or through dendritic release as has been described in other hypothalamic regions (Ludwig and Leng 2006). SCN oscillator cells may also contribute to temporal regulation of CalB cells through somatodendritic communication.

Hamster-mouse comparison

Species comparisons can reveal general principles of SCN function, though these give rise to terminological and perhaps conceptual problems. The terms core and shell were initially

introduced by Miller, Morin, Schwartz and Moore (Miller et al. 1996) to characterize the hamster SCN and their use has been extended, even though the precise localization of these compartments differs in other species (Morin and Allen 2006). In contrast to the hamster, the adult mouse SCN lacks a cluster of CalB in the SCN core (Ikeda and Allen 2003; Kriegsfeld et al. 2008; Silver et al. 1996). However, we have recent evidence of coexpression of CalB and GRP in the perinatal mouse (Drouyer In press) and like the hamster, these core cells respond to light at night but are not rhythmic in gene expression (Karatsoreos et al. 2004) or electrical activity (LeSauter 2005). These findings suggest the possibility of topographical and functional similarity in SCN organization among species.

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Figure 1.

Bar graphs describe the circadian rhythm in number of grid crossing of CalB-positive fibers in the SCN region dorsal to the CalB subnucleus in animals maintained either in constant darkness (A; $*p < 0.0001$) or in a light-dark cycle (B; $*p < 0.005$).

Figure 2.

Photomicrographs show the difference between (A) subjective night at CT14 and (B) subjectiveday at CT4 in detectable CalB-ir fibers in coronal SCN slices (20 μm). Exposure time was set identically for both images but overexposed to optimize visualization of fibers. A dense fiber plexus can be seen extending dorsally from the CalB containing cells at CT14. Scale bar: 100 μm. (C-J:) Tracings of CalB-positive neurons showing the distribution of immunoreactivity in rostral to caudal coronal sections at ZT14.

Figure 3.

Photomicrographs show the difference between (A) subjective night at CT14 and (B) subjective day at CT14 in detectable CalB-ir fibers in sagittal SCN slices (40 μ m). Exposure time was set as in Figure 2. As in the coronal sections, a dense fiber plexus can be seen extending dorsally from the CalB containing cells ZT14. Scale bar: 100 μ m. Tracings of CalB-containing cells show the distribution pattern of fibers at ZT14 in sagittal (C-F: lateral to medial) sections of the SCN.

Figure 4.

Top: Photomicrographs of double-labeled coronal SCN sections. The top row of photomicrographs show the distribution of CalB-containing cells in relationship to VP-, VIP-, CCK- and GRP-ir (left to right, respectively) in sections of the caudal SCN (50 μ m). The CalB cells form a dense cluster in the ventrolateral region, and some scattered cells are seen in the dorsal and the peri-SCN region. Scale bar 100 μ m. The second and third rows are confocal sections (2 μ m optical slice). The second row shows CalB fibers within the region of VP- (dorsomedial SCN), VIP- (ventral SCN), CCK- (medial SCN) and GRP- (ventromedial SCN) immunoreactivity. CalB fibers make numerous appositions with VP and VIP cells. Sparse CalB-ir fibers are seen among the CCK-ir cells with some contacts on soma. CalB-ir fibers make appositions onto GRP-ir cells. Scale bar 10 μ m. The third row shows VP-, VIP-, CCK- and GRP-immunoreactivity within the CalB subregion. As can be seen, VIP makes numerous appositions with the CalB cells, while VP and CCK make few appositions. GRP cells are distributed among the CalB cells and GRP fibers contact CalB cells. Scale bar 10 μ m. The bottom row is a schematic representation of the connections between the CalB cells and other peptidergic cell types. The arrow thickness and the percentages indicate the proportion of cells receiving 3 or more appositions.

Table 1

Quantification of appositions among peptidergic cell types during night and day.

	ZT 14					ZT 4				
	% cells with ≥3 appositions	% cells with 1-2 appositions	# animals	# cells	% cells with ≥3 appositions	% cells with 1-2 appositions	# animals	# cells	% cells with 1-2 appositions	# cells
CalB to VIP	64.1±8.2	29.4±4.6	4	306	0	12.9±0.3	2	62		
VP to CalB	24.7±7.7	62.5±12.9	4	291	18.8±0.9	71.8±6.8	2	117		
CalB to VIP	32.5±2.5	53.3±3.8	3	77	29.2±1.2	56.9±2.1	2	65		
VIP to CalB	91.4±2.8	8.2±2.7	4	233	95.1±1.5	4.9±1.5	3	183		
CalB to GRP	61.1±5.7	27.8±3.4	4	36	50.0±3.9	35.0±4.3	2	20		
GRP to CalB	59.1±7.6	25.1±4.3	4	291	53.7±5.4	33.9±8.3	2	121		
CalB to CCK	26.7±2.9	57.3±10.5	5	75	20.0±4.7	62.9±9.0	3	36		
CCK to CalB	19.3±3.3	80.0±3.2	5	290	19.8±3.9	77.5±2.3	3	182		