Changes in Calbindin-D28K Immunoreactivity in the Organotypic Slice Culture of Mouse Hypothalamic Suprachiasmatic Nucleus

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Summary

The hypothalamic suprachiasmatic nucleus (SCN) is of the main circadian pacemaker in mammals. Calbindin-D28k (Calbindin) is a calcium-binding protein that is distributed widely in the mammalian brain. In vivo the localization of Calbindin immunoreactivity (CalB-ir) in mouse SCN was examined before. However, detailed information about the localization and developmental distribution of CalB-ir neurons in the mouse SCN slice culture is lacking. SCN derived from 3 day old mouse, were maintained in culture at the interface between air and a culture medium. CalB-ir neurons are detected within the general central SCN area and the surrounding region. CalB-ir neurons showed variations in morphology where the majority of neurons were stellate and round or oval cells with multipolar dendrites. The density of CalB-ir was clearly decreased during the SCN development in organotypic slice cultures at the level of the cell bodies and the fibers. These findings may provide new morphologic evidences for explaining the functional mechanism in the SCN.

Key words: Organotypic slice culture, Calbindin immunoreactivity, Hypothalamus, Immunohistochemistry, Developmental suprachiasmatic nucleus

Fare Hipotalamik Suprakiazmatik Nükleusun Organotipik Kesit Kültüründe Calbindin- D28K İmmunoreaktivitesindeki Değişiklikler

Özet


Anahtar Kelimeler: Organotipik kesit kültürü, Calbindin immunoreaktivitesi, Hipotalamus, İmmünohistokimya, Gelişimsel suprakiazmatik nükleus
INTRODUCTION
In mammals, the master clock located in the suprachiasmatic nucleus of the hypothalamus (SCN) is able to generate and distribute a rhythmic message to the whole body. These paired SCN in rodents are composed of about 8000 neurons and are divided anatomically into a ventrolateral, an intermediate and a dorsomedial part, or, as more recently proposed a core and a shell, corresponding to the ventral input site with larger neurons and the dorsal region with smaller neurons, respectively. Some of the neuropeptides that have been identified to date in the rodent SCN are vasopressin (VP), vasoactive intestinal polypeptide (VIP), gastrin releasing peptide, substance P, somatostatin and calbindin. Neurons synthesising vasoactive intestinal peptide, substance P and gastrin releasing peptide are located in the core of the SCN, whereas vasopressin neurons are found in the shell. Gamma-amino butyric acid (GABA), somatostatin (SS) producing neurons have been demonstrated in the core and shell. Calbindin-D28k (CalB) is a calcium-binding protein that is distribute widely in the rodent brains. Silver et al., reported the presence of CalB-containing cells in the caudal part of the SCN. CalB cells are densely packed and receive direct retinal synaptic input and respond to photic stimuli. Detailed information about the localization of CalB-ir in mouse SCN development is lacking. Therefore, we are showing the changes in the CalB-ir neurons and fibers in the SCN during the development in the organotypic slice culture of mouse.

MATERIAL AND METHODS
Animals
Organotypic slice cultures were prepared from CD1 mice (3 days old). The mice were bred in the department's facilities of the Tanta university under controlled conditions (12h : 12h light-dark cycle, lights on at 06:00 h with about 60% humidity and 25°C temperature).

Tissue culture
All procedures were approved by the local animal care committee and were in accordance with the law for animal experiments. Organotypic slice cultures from hypothalamus were prepared using the interface culture methods. Four hours after light on, animals were killed by decapitation, the skulls were opened with fine scissors and the brains were quickly removed into ice-cold artificial cerebrospinal fluid (aCSF, 124mM NaCl, 5mM KCl, 1.25mM KH2PO4, 1.3mM MgSO4, 26mM NaHCO3, 2.2mM CaCl2, 10mM Glucose, 10mM HEPES). Hypothalami were excised and 300 µm thickness coronal slices were made using a vibroslicer. Sections were transferred into ice-cold aCSF in a Petri dish and those presumed to contain the bilateral SCN and the PVN. Selected sections were trimmed dorsally at the level of the anterior commissure and laterally just before the supraoptic nuclei and kept in drops of aCSF for 30 minutes. The selected slice was then positioned over Millicell filter in Petri dish and incubated for 1 hour at 37°C in 5% CO2/95% air with a small amount of culture medium (DMEM/F12 supplemented with 10% fetal calf serum, 10mM HEPES, 100 U/ml penicillin and streptomycin and 100 µg/ml ascorbic acid). Then, the Petri dish was filled with culture medium. The culture medium must be not completely cover the Millicell filter. The culture medium was exchanged every 2–3 days.

Calbindin immunoreactivity:
Immunohistochemical labeling was carried out by using the indirect fluorescence method. SCN slice cultures were fixed in 4% Paraformaldehyde in phosphate buffered saline (0.1M PBS, pH 7.4) for 36 hours at 4°C and then cryoprotected in 30% sucrose in PBS at 4°C for 72 hours. Brain slices were sectioned with a freezing
microtome into 20 µm coronal sections and mounted on gelatine-coated slides for calbindin immunohistochemistry. Sections were blocked for 1 hour in a solution containing 10% NGS, 1% bovine serum albumin (BSA) and 0.5% Triton- X-100 in PBS. Sections were incubated in primary antibody diluent (3% NGS, 1% BSA and 0.5% Triton-X-100 in PBS) prior to incubation in rabbit anti-Calbindin D28k primary antisera (1:2000, Sigma) for overnight at room temperature. Sections were rinsed in 0.1M PBS and then incubated for 1 hour (in dark room) in a goat anti-rabbit as secondary antibody (1:500). Then they were rinsed for 10 minutes in PBS and mounted and cover slipped with vectashield antifading mounting medium and examined under epifluorescence for CalB-ir. All fluorescent specimens were viewed by using a Leica TCS fluorescence microscope and a digital camera (Cannon 620) captured images. Each region was imaged under high magnification (X 40) and labeled cells were individually marked and manually counted. For analysis, the mean number of CalB-ir cells was calculated from the counts of four alternate images showing the highest number of labeled cells. Group means were derived from these values and analyzed using one-way ANOVA followed by Tukey Compromise test. Brightness and contrast of the images were adjusted using Adobe Photoshop software.

**RESULTS**

The detection and distribution of CalB-ir neurons in the hypothalamic slice cultures are illustrated figures 1 and 2. Numerous intensely labelled CalB-ir neurons and fibers were evident throughout the hypothalamic slice cultures. Most of CalB-ir labeled neurons detected within the general central SCN area and the surrounding region. CalB-ir labelled neurons showed variations in morphology within the SCN slice culture where the majority of neurons were stellate and round or oval cells with multipolar dendrites. On the 1st day in vitro (1DIV), CalB-ir neurons could be detected at all three rostrocaudal levels of SCN. CalB-ir neurons were scattered in the whole SCN (core and shell) at the rostral level. At the medial level, they were primarily located in the intermediate zone and more lateral at the caudal level. CalB-ir labeled neurons detected in the SCN/PVN complex (Fig. 1A, 2). Labeling CalB-ir neurons outside SCN was associated mainly with the PVN and scattered periventricular magnocellular neurons. A small number of CalB-ir cells that either bipolar or multipolar were found in the area of lateral hypothalamus (Fig. 2C, 2D) and in PVN along the third ventricle as well as in the magnocellular subdivision of the PVN with clear magnocellular and paravocellular immunopositive neurons (Fig. 2D). No clear CalB-ir neurons or fibers found in the optic chiasm. A few CalB-ir fibers detected within the ventral SCN. Heavily labelled CalB-ir fibers detected in the SCN that projected dorsally to the PVN. At early developmental stage of SCN (0DIV - 8DIV) of slice cultures, CalB-ir neurons observed in the dorsomedial SCN than the ventrolateral SCN (Fig. 2A, 2B). After the seventh day in vitro, the intensity of CalB-ir in the SCN area was gradually decreasing (Fig. 1B), both at the level of the cell bodies and the fibers. On the 14DIV, numerous CalB-ir fibers leave SCN dorsally adjacent to the ependyma of the third ventricle (Fig. 1C). The intensity

**Abbreviations**

SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; 3V, 3rd ventricle; OC, optic chiasm; LH, lateral hypothalamus; aCSF, artificial cerebrospinal fluid; PBS, phosphate buffered saline; DIV, day in vitro; CalB, calbindin; -ir, immunoreactive; CalB-ir, calbindin immunoreactivity; AH, anterior hypothalamus; VP, vasopressin; VIP, vasoactive intestinal polypeptide; GRP, gastrin releasing peptide; SP, substance P; SS, somatostatin; GABA, Gamma- amino butyric acid; NGS, normal goat serum; BSA, bovine serum albumin.
of CalB-ir neurons in the SCN area were more decrease with increasing the immunopositive fibers in the area of SCN/PVN complex in the following days in vitro (Fig. 1D). After 21DIV, a few CalB-ir neurons observed in the SCN area and the intensity of CalB-ir fibers decreased in both SCN and SCN/PVN (Fig. 1D).

**Fig 1:** Photomicrographs of immunoreactive CalB neurons and fibers in the hypothalamic organotypic slice culture (A: 1DIV; B: 7DIV; C: 14DIV; D: 21DIV) showing the development of mouse SCN in the slice cultures (3V: third ventricle, OC: optic chiasm).

**Fig 2:** Photomicrographs of immunoreactive CalB neurons and fibers in the mouse hypothalamic organotypic slice culture. Fig. 2A: Photomicrograph of CalB-ir showing the CalB-ir neurons within SCN. Fig. 2B: High power micrograph displaying CalB immunoreactive neurons and fibres in the unilateral SCN. Fig. 2C: Photomicrograph of CalB-ir. Magnocellular neurons in the PVN can be seen above to SCN. Thick CalB-ir axons from magnocellular neurons can be seen extending between the PVN and FX, and thin axons from paravocellular neurons can be seen dorsal to SCN, some of the latter extend from SCN into LH and sub PVN zone. Fig. 2D: High power micrograph of positive immunoreactive CalB in the Magnocellular and paravocellular cells.
DISCUSSION

Calbindin-D28k belongs to a large class of eukaryotic proteins that bind calcium (Ca\textsuperscript{2+}) to a specific helix-loop-helix structure. To date, this protein was mainly linked to brain, kidneys, and pancreas\textsuperscript{(3,2,6,10,17)}. In rats, CalB-ir neurons are more prevalent in the ventral SCN, but are not as tightly clustered as in the hamster\textsuperscript{(15)}. In the mouse, the CalB cells are densely packed within a discrete region in the ventrolateral SCN\textsuperscript{(1,16,25)}. The CalB cells are part of the entrainment pathway. They receive synaptic contacts from the retinal fibers\textsuperscript{(5)}. These cells receive afferent fibers containing neuropeptides like substance P, VIP and GRP, but not AVP\textsuperscript{(14,32)}. SCN cells that produce CalB seem to be very important in the genesis and control of the locomotor activity rhythm. In vivo, most of the CalB cells within the CalB subnucleus express Fos in response to a light pulse\textsuperscript{(25,26)}. Partial lesions of the SCN that destroyed the CalB region yielded animals with arrhythmic locomotor activity. In contrast, if the lesions spared a portion of one or both CalB regions, behavioral rhythmicity was maintained\textsuperscript{(11-13)}.

In vitro, the circadian rhythmicity in the hypothalamus depends on the presence of an intact SCN. Completely lesions of SCN from the mouse hypothalamus slice culture showing circadian rhythmicity in the other hypothalamic regions\textsuperscript{(30)}, but bilateral ablation of the SCN completely abolished all rhythms in the PVN and in adjacent hypothalamic areas.

Our results showing that, the CalB-ir neurons are present within the general central SCN area, the surrounding region and generally throughout the entire nucleus. The density of CalB immunopositive neurons in SCN after one week in vitro has the same characteristics as that in vivo situation\textsuperscript{(25,26)}. In vitro, the level of the cell bodies and the fibers, the intensity of CalB-ir were decreased. Results also revealed CalB-immunoreactive SCN neurons sending efferent fibers to the PVN, consistent with the in vivo SCN or rat SCN slice cultures prepared using similar methods\textsuperscript{(5,25)}.

Does the transplantation-induced decrease in calbindin immunoreactivity reflect a decrease in calbindin expression? CalB immunoreactivity can be influenced by calcium levels. In biochemical experiments the immunoreactive signals for CalB in blots from cytosolic extracts of rat cerebellum were directly proportional to calcium concentration in the incubation buffer; formalin fixation reduced, but did not eliminate, calcium effects\textsuperscript{(33)}. The number of CalB-ir cells increases after axotomy of sympathetic ganglion cells\textsuperscript{(23)}. In hippocampal slices opposite results have been obtained; slices preincubated in low calcium showed enhanced immunoreactivity, where as those in high calcium showed decreased immunoreactivity\textsuperscript{(6,9)}. Changes in CalB immunoreactivity during the SCN development reflect the state of the protein because of altered intracellular calcium levels rather than an actual decrease in protein synthesis or stability. The reduction of CalB-ir neurons in the developmental SCN showed parallel to RHT formation. Our findings support the idea that Calbindin presumably by regulating intracellular calcium concentrations in retinorecipient neurons, influences photic signaling within the SCN\textsuperscript{(12)}. Furthermore, they are consistent with evidence that the effect of light within the SCN involves activation of calcium-requiring transduction mechanisms\textsuperscript{(10,17)}. It is worth noting that a high proportion of CalB-positive neurons in the SCN express the transcriptional regulatory protein, Fos, in response to light\textsuperscript{(26)}, and there is evidence to suggest that Fos is involved in the mechanism underlying clock resetting by light\textsuperscript{(34)}. Our results support this view of SCN organization and extend it to specify a role for CalB protein in the transmission
of photic information from the ventral SCN to pacemakers in the dorsal SCN.

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