The presence of neuronal-specific nuclear protein (NeuN) in the circadian timing system of the capuchin monkey (*Cebus apella*)

A presença da proteína nuclear específica neuronal (NeuN) no sistema de temporização circadiano do macaco capuchinho (*Cebus apella*)

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ABSTRACT

Background and objective: The circadian timing system (CTS) is composed of a group of specialized neuronal structures that establish a temporal organization of physiological and behavioral processes within precise patterns. The central components of this system are the suprachiasmatic nucleus of the hypothalamus (SCN) and the intergeniculate leaflet of the thalamus (IGL). The objective of this study was to verify the presence of the neuron-specific nuclear protein (NeuN) in the circadian timing system of capuchin monkeys (*Cebus apella*) using immunohistochemical techniques. **Methods:** Capuchin monkeys (*Cebus apella)* were anesthetized and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, and then their brains were removed and frozen. A microtome was used to make 30 μm sections in the coronal plane. One of the series was used for Nissl staining (thionin) to demarcate the cytoarchitecture, and the remainders of the sections were processed for immunohistochemical detection of NeuN (ABC protocol). **Results:** NeuN-positive neurons were observed in the suprachiasmatic nucleus of the capuchin monkey. The pregeniculate nucleus (PGN), a structure equivalent to the ventral lateral geniculate nucleus (vLGN) and to the IGL in rodents, did not have any NeuN-positive neurons. **Conclusions:** In this primate species, only the suprachiasmatic nucleus neurons of the central structures of the circadian timing system express the NeuN protein.

Keywords: Circadian rhythm; Suprachiasmatic nucleus; Nuclear proteins; Immunohistochemistry/methods; Geniculate bodies; Neurons; Animals; Cebus

RESUMO

Introdução e objetivo: O sistema de temporização circadiana (do inglês circadian timing system, CTS) é composto por um conjunto de estruturas neurais especializadas que estabelecem uma organização temporal dos processos fisiológicos e comportamentos dentro de padrões precisos. Seus componentes centrais são o núcleo supraquiasmático (SCN) do hipotálamo e o folheto intergeniculado (IGL) do tálamo. O objetivo deste estudo foi verificar, através da técnica imunoistoquímica, a presença da proteína nuclear neuronal específica (NeuN) no sistema de temporização circadiana do macaco-prego (*Cebus apella*), um primata do Novo Mundo. **Métodos:** Os animais foram previamente anestesiados e submetidos à perfusão transcardíaca com solução salina heparinizada, seguida de solução de paraformaldeído a 4% em tampão fosfato 0,1 M. Os encéfalos foram removidos e submetidos à microtomia por congelação, obtendo-se secções coronais de 30 μm. Secções de uma série foram submetidas ao método de Nissl (Thionina) para demarcar a citoarquitetura e as outras secções foram processadas por imunoistoquímica (protocolo ABC) a fim de revelar a presença de NeuN. **Resultados:** Neurônios NeuN positivos foram observados no núcleo supraquiasmático do macaco-prego. O núcleo pré-geniculado (PGN), estrutura equivalente ao núcleo geniculado lateral ventral (GLV) e ao IGL dos roedores, não apresenta neurônios NeuN positivos. **Conclusão:** Nas estruturas centrais do sistema de temporização circadiana, somente os neurônios do núcleo supraquiasmático nesta espécie de primata expressam a proteína NeuN.

Descritores: Ritmo circadiano; Núcleo supraquiasmático; Proteínas nucleares; Imunoistoquímica/métodos; Corpos geniculados; Neurônios; Animais; Cebus

INTRODUCTION

The generation and regulation of circadian rhythms in mammals originate from a neural-specific system, the circadian timing system (CTS). This system utilizes a central pacemaker, which includes inputs such as retinal afferents that allow for the synchronization of environmental cycles and outputs that lead to behavioral effectors [1]. The supra-

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chiasmatic nucleus (SCN) and the intergeniculate leaflet (IGL) are considered to be the central components of the CTS [1]. The SCN is a small region localized at the anterior hypothalamus bilateral to the third ventricle and above the optic chiasm [2]. In the majority of mammalian species studied, the SCN is divided into two principal cell populations: the ventrolateral SCN, which contains neurons that secrete vasoactive intestinal polypeptide (VIP), and the dorsomedial SCN, which contains neurons that secrete vasopressin (VP) [2,3]. In addition to VIP and VP, the SCN also contains a large number of other neuroactive substances that can act as neurotransmitters or neuromodulators. The SCN contains neuropeptide Y (NPY), serotonin (5-HT), glutamate (Glu), bombesin (BBS), gastrin-releasing peptide (GRP), cholecystokinin (CCK), substance P (SP), angiotensin II, enkephalin (ENK), somatostatin (SS), tyrosine hydroxylase (TH), and calcium binding proteins such as calbindin (CB) and calretinin (CR) [2]. Because the SCN has a crucial role in the temporal organization of behavior, it is considered the central circadian pacemaker in mammals. SCN lesions result in a loss of behavioral circadian, endocrine, and physiological rhythms [4]. Arrhythmic animals with SCN lesions recover their circadian rhythms through fetal SCN transplant; however, these animals acquire the rhythm of the donor [5].

The intergeniculate leaflet (IGL), along with the ventral lateral geniculate nucleus (vLGN) and the dorsolateral geniculate nucleus (dLGN), form the lateral geniculate complex of the thalamus in non-primate species [6]. The IGL is a thin layer of neurons containing NPY, interposed between the vLGN and the dLGN, and its function is to modulate the SCN [7]. The organization of the lateral geniculate complex is different in primates in relation to rodents. Although the primate dLGN corresponds to the rodent dLGN, the primate pregeniculate nucleus (PGN), which is a wedged-shaped cellular grouping that dorsomedially encircles the dLGN, is considered equivalent to the rodent vLGN [8,9]. NPY-immunoreactive neurons in the PGN have been found in *Rhesus* monkeys [8], common marmosets (*Callithrix jacchus*) [10], and *Cebus* monkeys [9]. Thus, it can be assumed that the PGN of primates also contains a rodent-equivalent IGL . A new neuronal marker that has been utilized to identify nerve cells is the neuronal-specific nuclear protein (NeuN), which is expressed in both the nucleus and cytoplasm of the majority of vertebrate nervous system cells [11]. This protein has been utilized in studies on nervous system development [12], in morphometric studies as a postmortem neuronal marker of human cerebral tissue [13], in histopathology diagnosis as a marker of neuronal differentiation in cerebral tumors, and in studies of neurodegenerative disorders [14]. Although this protein has been identified in the majority of nerve cells, there are some NeuN-negative neurons. In development,

Cajal-Retzius neurons of the first layer of the cerebral cortex, the medullar inferior olivary nucleus, cerebellar Purkinje cells, mitral cells of the olfactory bulb, retinal photoreceptors, and glial cells are all negative for NeuN [11,15].

Considering the importance of the CTS in mammals and the quantity of neuroactive substances present in the central components of this system, the objective of this study was to identify NeuN-protein-immunoreactive neurons in the central components of the CTS of the capuchin monkey (*Cebus apella*).

METHODS

For this study, we utilized two young adult capuchin monkeys (*Cebus apella*) obtained from the Júlio Mesquita Filho Primate Center of Universidade Estadual Paulista, Araçatuba (SP), Brazil. The experimental procedures were in compliance with the guidelines for the care and use of mammals in neuroscience and behavioral research. The capuchin monkeys were housed in individual cages under natural humidity, temperature, and lighting conditions and were fed with a standard fruit and vegetable diet.

The animals were anesthetized with sodium thiopental (30 mg/kg, i.p.) and transcardially perfused with 800 mL of 0.9% saline containing heparin (Hipolabor Laboratories, 5000 IU/mL). The saline was followed by 1500 mL of 4% paraformaldehyde in 0.1 M acetate buffer (pH 6.5) and subsequently with 1500 mL of 4% paraformaldehyde in borate buffer (pH 9.0). The brains were exposed and sliced into blocks utilizing stereotaxic equipment. The blocks were removed from the cranium and placed in cryoprotectant solution composed of 10% glycerol and 2% dimethylsulfoxide in 0.1 M borate buffer (pH 9.0) at 4ºC. After three days, the blocks were transferred to a similar solution containing 20% glycerol for four more days of cryoprotection. Next, the blocks were sliced into 30-μm coronal sections with a cryomicrotome and collected in an anti-freezing solution. One of the series was used in Nissl staining for the cytoarchitecture study, which utilized thionin as a dye. Another series was pretreated for antigen recovery utilizing 1% sodium borohydrate. After the pretreatment, the slices where treated immunohistochemically with an anti-NeuN primary antibody (1:1000, Chemicon) in 0.4-% Triton X-100 and 2-% normal rabbit serum (1:50) at room temperature for 24 hours. Next, slices were incubated with secondary biotinylated antibody (1:200, Sigma) for two hours. Next, the slices were incubated in avidin-biotin immunoperoxidase complex (Elite ABC kit, Vector Laboratories) for two hours and exposed to chromogenic 2.5% 3,3'–diaminobenzidine tetrahydrochloride (DAB) (Sigma, St Louis, MO, USA) diluted in 0.1 M phosphate buffer (pH 7.4) for 15 minutes. Between incubations, the slices underwent a sequence of eight 5-minute washes each in 0.1 M phosphate

buffer (pH 7.4). Lastly, the slices were mounted on gelatinized glass slides, intensified in osmium tetroxide, dehydrated in a series of alcohols, cleared by xylene, and coverslipped for optic microscopy analysis.

RESULTS

The SCN of *Cebus apella* is localized in the anterior hypothalamus dorsal to the optic chiasm and bilateral to the third ventricle. The Nissl staining showed that the SCN cells neighbored the wall of the third ventricle (Figure 1A). This cellular group is triangular in the rostral level but becomes more rounded as it reaches the caudal levels. NeuN immunoreactivity was observed in the three regions (rostral, intermediate and caudal) of the SCN. The largest number of NeuN-positive cells was observed in the intermediate region; however, the staining of these cells was pallid when compared to the lateral hypothalamus, which presented robustly stained cells (Figure 1B).
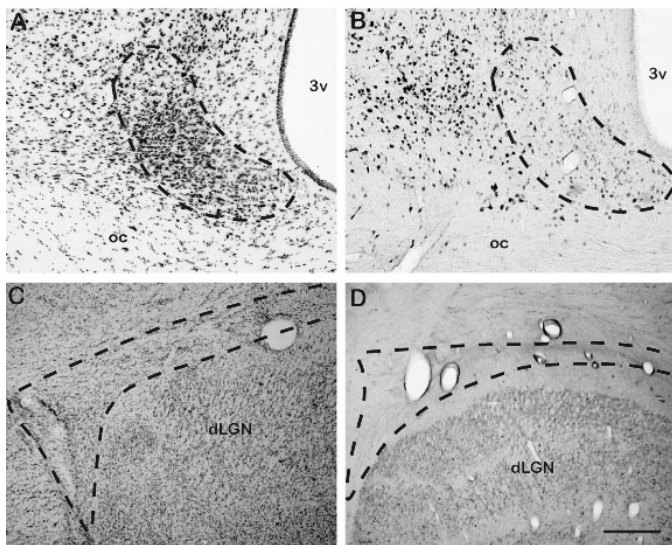
oc: optic chiasm; 3v: third ventricle; dLGN: dorsal lateral geniculate nucleus.
The scale bar is equivalent to 70 µm in A, C and D, and to 85 µm in B.

Figure 1: Photomicrographs of brain sections of *Cebus apella* showing the components of the CTS in bright field. (A) SCN of *Cebus apella* stained by Nissl staining. (B) Panel showing the immunoreactivity for NeuN in the SCN of the *Cebus apella* monkey. (C) PGN of the *Cebus apella* monkey stained by Nissl staining. (D) Panel showing the negative reaction for immunoreactivity against NeuN in the PGN of the *Cebus apella* monkey.

The Nissl-stained sections demonstrated that the PGN is localized dorsomedially to the dLGN (Figure 1C). It was observed that the PGN cells in *Cebus apella* do not present NeuN immunoreactivity (Figure 1D). However, we did confirm that the immunohistochemical process was success-

ful in dLGN neurons, thus representing a positive control (Figure 1D).

DISCUSSION

The SCN is composed of oscillatory neurons that control a series of effector systems, including those that regulate the activity and rest cycles, body temperature, neuroendocrine function, and psychomotor performance. Its role as the central pacemaker of the CTS is modulated by its inputs, which are topographically organized within itself [16,17]. The rodent IGL or the PGN in primates, along with the SCN, are considered the central components of the CTS because they are the first participants in the modulation of photic and non-photic circadian responses [1].

The present study demonstrated that the SCN of *Cebus Apella* is elongated in the rostro-caudal axis. Neurons strongly stained with thionin were localized in the anterior hypothalamus above the optic chiasm and bilateral to the third ventricle. Furthermore, the PGN had less intense thionin staining and was localized dorsomedially to the dLGN. This result is in accordance with our previous studies in *Cebus* monkeys [9,18]. The SCN and PGN cells presented a high level of homogeneity in the Nissl staining method. Neuron-specific nuclear protein is considered to be a nuclear regulatory molecule specific to the nervous system, and it has been increasingly used in central nervous system studies [11-13]. The present study demonstrated that SCN neurons in *Cebus apella* were immunoreactive for NeuN in the whole rostro-caudal extension. The greatest number of NeuN-positive cells was found in the intermediate level of the SCN. The more robustly stained cells were observed in the lateral portion, the most pallid were present in the medial portion of the SCN, but this result could be explained by immunoreactivity variability. A study with adult rats demonstrated that NeuN expression in the SCN was low, particularly in the dorsal region when compared to the neighboring hypothalamic neurons. However, detectable levels of NeuN were observed in the ventral portion of the SCN when it was co-localized with doublecortin, which is a microtubule-associated protein that is also considered a marker for neurogenesis [19]. The NeuN immunohistochemical labeling in the PGN of *Cebus apella* was negative.

In summary, our study demonstrated that the localization of the SCN and the PGN of *Cebus apella* is similar to other primate species. In addition, SCN neurons were immunoreactive for NeuN, whereas the PGN did not exhibit labeled neurons. Taking into consideration that some neurons of the nervous system are NeuN negative [18,15], it still remains to be determined whether *Cebus apella* SCN contains specific subpopulations of neurons that are not immunoreactive for NeuN. The data in this study are preliminary, but provide a perspective

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for the comparative studies between primate and non-primate species when it comes to understanding the role of NeuN as a neuronal marker in this physiological system.

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