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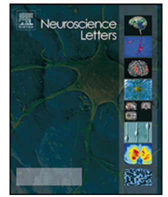

5-HT$_{1B}$ receptor in the suprachiasmatic nucleus of the common marmoset (*Callithrix jacchus*)

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ABSTRACT

Serotonin (5-HT) is involved in the fine adjustments at several brain centers including the core of the mammal circadian timing system (CTS) and the hypothalamic suprachiasmatic nucleus (SCN). The SCN receives massive serotonergic projections from the midbrain raphe nuclei, whose inputs are described in rats as ramifying at its ventral portion overlapping the retinohypothalamic and geniculohypothalamic fibers. In the SCN, the 5-HT actions are reported as being primarily mediated by the 5-HT1 type receptor with noted emphasis for 5-HT$_{1B}$ subtype, supposedly modulating the retinal input in a presynaptic way. In this study in a New World primate species, the common marmoset (*Callithrix jacchus*), we showed the 5-HT$_{1B}$ receptor distribution at the dorsal SCN concurrent with a distinctive location of 5-HT-immunoreactive fibers. This finding addresses to a new discussion on the regulation and synchronization of the circadian rhythms in recent primates.

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The circadian timing system (CTS) is a neural network responsible for the generation and modulation of the circadian rhythms. The CTS of mammals comprises an encephalic circuitry, in the center of which, the hypothalamic suprachiasmatic nucleus (SCN) is conceived as the main circadian pacemaker. Along the last decades, the SCN has been investigated in respect to its major hodological, neurochemical, and molecular features [34,37].

The rhythms generated by the SCN are synchronized by environmental cues, of which the light/dark cycle is the major synchronizing agent. Three main pathways reach the SCN influencing its activity: (1) the retinohypothalamic tract (RHT), from the retinal glutamatergic ganglion cells [7,17,20,26]; (2) the geniculohypothalamic tract (GHT) [18], formed by neuropeptide Y-positive fibers [14] from the thalamic intergeniculate leaflet [15]; and (3) the serotonergic midbrain projections from the median raphe nucleus [16,22,25]. In rats, all of these pathways are described as ramifying in an overlapping way at the ventral portion of the SCN [16,26,25,35], even though that in a previous study of a New World primate the common marmoset (*Callithrix jacchus*), serotonergic terminal plexus was described as reaching the most dorsal portion of the SCN in a complementary manner to the ventral RHT and GHT projections [5,31].

The serotonergic pathway is functionally implicated in both photic and non-photic modulation of the SCN [13,23,27,25,28,29]. The 5-HT actions are dependent on several serotonergic receptor subtypes, which can be pre or post-synaptically positioned. To date, the main subtypes of 5-HT receptors found in the SCN, characterized by biochemical and pharmacological approaches, belong to the 5-HT$_1$ family [26]. Also, in different species the subtype 5-HT$_{1B}$ seems to prevail [12,30,33]. 5-HT$_{1B}$ receptors can be found in the axon terminals of neurons (acting as a presynaptic modulator) or in the postsynaptic cellular membrane [21].

The presence of 5-HT fibers in the dorsal SCN of the marmoset [31] motivated us to search the presence of serotonergic receptor subtypes in the same species, so that, the aim of this study was to identify and map the 5-HT$_{1B}$ receptor distribution comparing with serotonergic and retinal innervations.

Four adult male marmosets (257–378 g) from the Primatology Center of the Federal University of Rio Grande do Norte (IBAMA register 1/24/92/0039-0), Natal, Rio Grande do Norte, Brazil, were used in this study. The animals were housed under natural lighting, temperature, and humidity conditions, with food and water freely available. The maintenance and minimal use of the experimental animals followed the guidelines of the Brazilian Society of Neuro-

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science and Behavior under recommendations of the Guide for the Care and Use of Laboratory Animals in Research (in accordance with the National Institutes of Health, NIH, USA).

The marmosets were deeply anesthetized with sodium thiopental (Cristália, São Paulo, SP, Brazil, 40 mg/kg, i.p.), placed on a surgical table and received a topical application of tetracaine hydrochloride in the cornea followed by a unilateral intraocular injection of cholera toxin subunit B (CTb) (List Biological Laboratories, Inc., Campbell, CA, USA). A total of 80 μl of 1 mg/ml aqueous CTb solution containing 10% dimethylsulfoxide was pressure injected into the vitreous humor through a 30-gauge needle catheter attached to a micropump, introducing the solution at a rate of 1 μl/min. To minimize reflux and spread of the tracer to the extraocular muscles and to avoid postoperative local infection, the ocular surface was continuously cleansed with saline during surgery. The ocular surface was then rewashed with saline and an antibiotic ointment (Dexafenicol, Alergon, Brazil) was applied topically. After 5–7 post-injection days, the marmosets were re-anesthetized with sodium thiopental and transcardially perfused with 500 ml of phosphate-buffered saline, pH 7.4, containing 500 IU of heparine (Parinex, Hipolabor, Brazil) followed by 700 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed from the skull, postfixed for 2–4 h and transferred to a solution containing 30% sucrose in PB.

Brain blocks from a point of a few millimeters anterior to the optic chiasm through the midbrain were frozen and serially sectioned in the coronal plane (30 μm) on a sliding microtome. Sections were sequentially collected and kept in PB in six separate compartments. To establish the limits of the SCN, one of the six series of sections from each animal was stained with thionin and another was submitted to immunohistochemical reaction to calbindin (CB), a calcium-binding protein, which is reported to be a useful marker for identifying the SCN boundaries in this species [6]. Three other series were used for the immunohistochemical detection of the CTb, 5-HT and 5-HT_{1B} receptor. The remaining series were used to perform double labeling experiments combining 5-HT_{1B} receptor and CB labels to analyze the arrangement of this receptor in relation to the SCN cells and nuclear architecture.

For the immunohistochemical detection of CTb, free floating brain sections of the injected animal were incubated for 18 h at room temperature (ca. 24 °C) using goat anti-CTb IgG (List Biological Labs, Inc., Campbell, CA, USA), diluted at 1:5000, as primary antibody and 2% of normal donkey serum in 0.4% Triton X-100 in PB. The sections were then incubated with a biotinylated secondary antibody (donkey anti-goat IgG, Jackson Labs, Westgrove, PA, USA) diluted at 1:1000 for 90 min. The sections were subsequently incubated with an avidin–biotin–peroxidase solution (ABC Elite kit, Vector Labs., Burlingame, CA, USA) for 90 min. They were then reacted for peroxidase activity in a solution of diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, MO, USA) and 0.03% H_2O_2 in PB. The sections were washed with PB (5×5 min) between each step and at the end of the procedure. For detection of the CB, 5-HT and 5-HT_{1B} receptor, the same general procedure was adopted using as primary antibodies a mouse anti-CB (Sigma, St Louis, 1:5000), a rabbit anti-5HT (Immunostar, Hudson, WI, USA, cat # 20079, 1:5000), a guinea-pig anti-5HT_{1B} (PharMinger, San Diego, CA, USA, cat # 550470, 1:500) and the respective secondary antibodies all raising in goat (Jackson Labs, Westgrove, PA, USA, 1:1000). All of the incubations with primary antibody included 2% normal goat serum in 0.4% Triton X-100 in PB. Specificity tests and the controls for the 5-HT label can be found at the company site (www.immunostar.com). The anti-5-HT_{1B} was made in guinea pig using a synthetic peptide conjugated with bovine thyroglobulin corresponding to amino acids 273–287 with homologue correspondence in rodents, canines and primates. As for control procedures, in some sections all the antibodies were used except the primary antibody, which was substituted by normal serum or by incubation of the secondary antibody only.

For the double labeling experiments, the tissue was simultaneously incubated with an antibody guinea pig anti-5-HT_{1B} receptor (PharMinger, San Diego, CA, USA) and a mouse-anti-CB (Sigma Chemicals, St Louis, MO, USA), both at a dilution of 1:250 during 24 h. The labeling was developed using secondary anti-guinea pig fluorescein-labeled and anti-mouse rhodamine-labeled antibodies all made in donkey (Sigma, St Louis, MO, USA) at a 1:100 dilution in PB with 0.4% Triton X-100. Proper immunofluorescence controls were performed by the omission of the primary and produced lack of label. Material was examined under bright-field illumination in an Olympus microscope for the single-label experiments and in a Nikon E-800 epifluorescence microscope for the double-label experiments. The images were captured using a CCD camera (Optronics Magnafire, Goleta, CA, USA) and the composite images were mounted with the aid of Adobe Photoshop (Adobe Software). The distribution of labeled elements and staining intensity were subjectively evaluated.

The SCN of the marmoset displays a triangular shape located over the optic chiasm bilateral to the third ventricle as evidenced in Nissl-stained coronal sections (Fig. 1A). The SCN CB-containing neurons exhibit a clear label with visible and well delimited cell bodies containing some apparent neuropil contrasting with adjacent hypothalamic areas (Fig. 1B). Retinal CTb-immunoreactive fibers in the common marmoset SCN were visualized in an unambiguous placement at its entire ventral portion with a noticeable enlargement in the middle portion (Fig. 1C). This pattern appears to be consistent from rostral to caudal levels showing a slight decrease in intensity as the SCN extends toward the caudal direction. Fine 5-HT-immunoreactive fibers/terminals were identified in an apparently scattered distribution in the SCN of the marmoset (Fig. 1D). However, a more detailed observation shows that these 5-HT positive fibers exhibit a preferential distribution medially around the ventricle wall at the upper dorsal limit of the SCN (Fig. 1D). In general, the disposition of the CTb-containing fibers overlap the 5-HT containing fibers on the ventral portion of the SCN, but the site of CTb exuberance coincides with the absence of 5-HT in an apparent complementary position in relation to RHT. As for CTb, the 5-HT immunopattern appears to be constant from rostral to caudal levels showing a slight decrease in intensity as the SCN moves toward the caudal position.

5-HT_{1B} immunoreactive (5-HT_{1B}-IR) neurons show a broad and apparent and homogeneous distribution in the hypothalamus of the marmoset (Fig. 2A). In the entire rostro-caudal SCN the distribution for the 5-HT_{1B}-IR was more evident dorsally concentrated in the medial contour near the ventricle wall, corresponding to higher concentration of 5-HT innervation and letting an immunoreactive free zone, which corresponds to the location of the bulk of RHT fibers (Fig. 2B and C). Most of the CB-positive neurons of the dorsal district appear to be encircled by the dotted 5-HT_{1B} label (Fig. 2D and E).

The 5-HT_{1B} immunoreactivity pattern in the SCN differs from that of midbrain, which resembles postsynaptic neurons or autoreceptors since they appear to immunolabel the entire cell in the dorsal raphe nucleus used now as a positive control label (Fig. 3A and B). No label was observed in the control experiments (Fig. 3C and D). For all parameters evaluated the results were similar in all cases with only minor variations.

Evidences implicating 5-HT as a key neurotransmitter modulating the effects of light on circadian rhythmicity is recurrent. For example, the depletion of serotonergic input to the SCN of rodents provokes a change of the circadian rhythms with an elongation of the activity phase and disruption in constant light [22] and it was proposed that this effect was mainly mediated by the 5-HT_{1B} receptor [30]. This subtype was observed to be associated with the

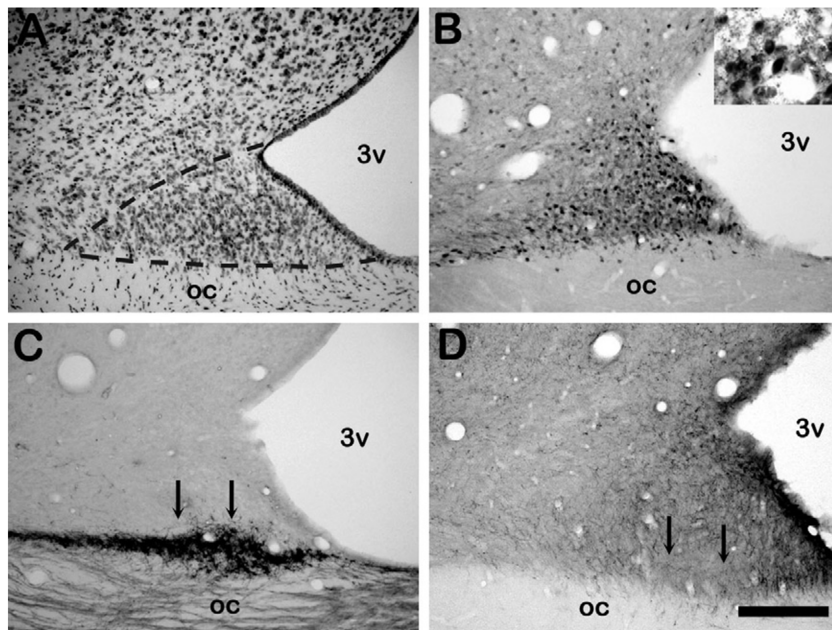

Fig. 1. Digital images of coronal sections at hypothalamic level of the marmoset brain showing (A) the triangular shaped SCN at Nissl stained sections; (B) CB-immunostained neurons filling all sectional area of the SCN; (C) distribution of CTb-retinal projections in the ventral portion (arrows) of the SCN; (D) 5-HT positive fibers ramifying in the medial and dorsal SCN, depriving the ventral area (arrows). The upper image in B (B′) is a detail of the CB-positive labeling in the SCN. Note the rounded nature of the small neurons (average of 10 μm). 3v, third ventricle; oc, optic chiasm. Scale bar: 180 μm in A, 100 μm in B–D, 50 μm in B′.

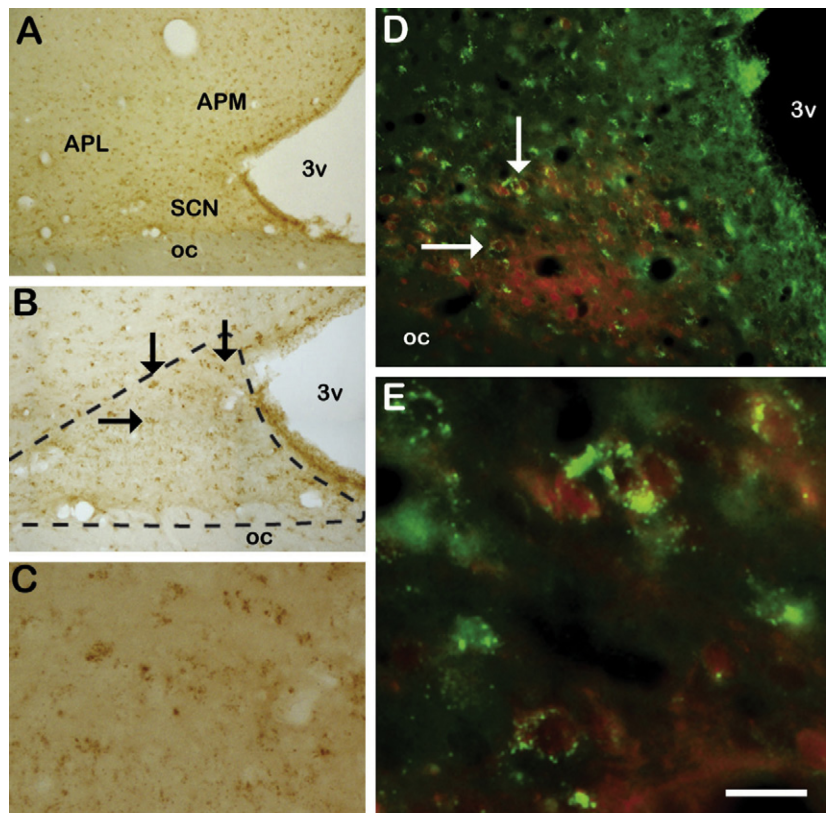

Fig. 2. Digital images of coronal sections of the marmoset brain at the SCN level showing (A) 5-HT_{1B}-immunoexpression in all anterior hypothalamus, including the SCN; (B) higher magnification of A showing 5-HT_{1B}-positive structures with the dorsal portion containing the richest labeling (arrows); (C) higher magnification of the region delimited by arrows in B showing the typical dot-like label pattern of 5-HT_{1B}; (D) the merged exposure of CB-containing neurons visualized by TRITC-conjugated secondary antiserum (red) and 5-HT_{1B}-positive structures detected by FITC-conjugated secondary anti-serum (green); (E) higher magnification of the neurons indicated by arrows in D. APL, preoptic lateral area; APM, preoptic medial area. Scale bar: 200 μm in A, 100 μm in B, 70 μm in C and D, 15 μm in E. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

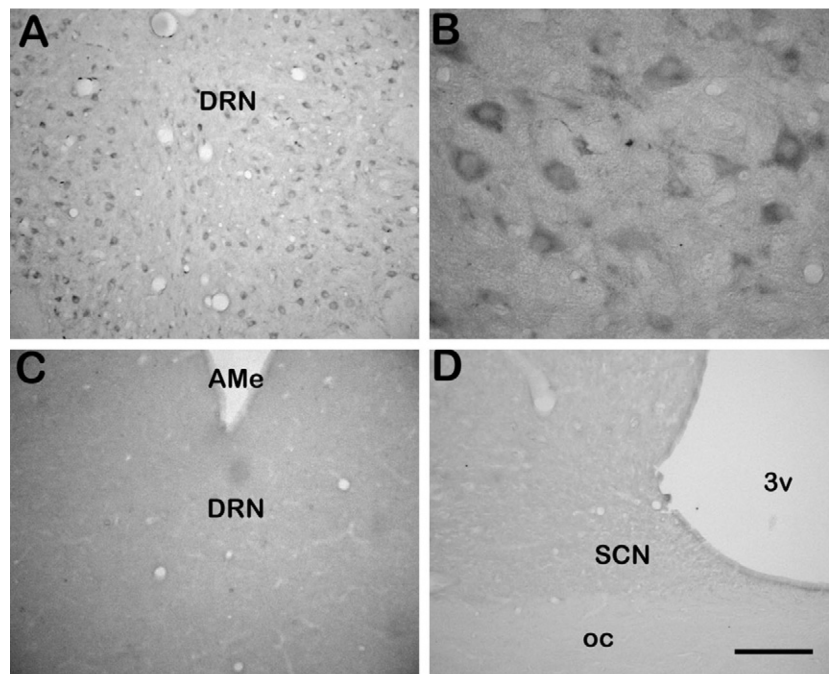

Fig. 3. Digital images of coronal sections of the marmoset brain showing 5-HT_{1B} label in DRN neurons (A) delimiting the neuronal body better evidenced at high magnification (B). Absence of labeling was evidenced in control experiments at DRN (C) and SCN (D). AMe, mesencephalic aqueduct. Scale bar: 200 μm in A and C, 30 μm in B, 100 μm in D.

membrane of retinal terminals in the SCN suggesting that by this presynaptic mechanism 5-HT can modify the response of the SCN to light [30,29]. More recently, 5-HT_{1B} binding sites were massively identified in nonretinal terminals in the SCN, and combined pharmacological and electrophysiological assays indicated that these receptors could be actually located on GABAergic terminals [3]. Thus, the 5-HT_{1B} influence on SCN neurons activity might be provided by inhibition of a GABA release in an intrinsic circuitry, most likely in the vicinity of afferent retinal fibers.

Currently it is assumed that there be a correlation between 5-HT and RHT innervations with 5-HT_{1B} in the ventral SCN, but in this study we identified the occurrence of 5-HT_{1B} receptors in the SCN of the common marmoset more concentrated in the dorsal portion concurrent with the predominant location of 5-HT immunoreactive terminals. Our present experimental approach does not allow us to determine the precise elemental location of 5-HT_{1B} receptors, but our data indicate that they could be acting as a presynaptic modulator like in rodents. Thus, 5-HT_{1B} immunoreactivity seemingly around the cell body together with the lack of cytosolic label and the absence of membrane contour strongly suggest its presynaptic position. Furthermore, the dot-like appearance and the topography of the 5-HT_{1B} immunoreactivity coincide with the presence of 5-HT terminal fibers. We observed that, although scarce, 5-HT_{1B}-immunoreactivity was also detected in the marmoset ventral SCN, and we speculate that they are connected with putative GABAergic ventral interneurons as reported in rodents [3]. However, it must be pointed out that the predominant labeling of the non-retinorecipient dorsal SCN be a remarkable difference in our results. At this time we are unable to provide a definitive explanation for this finding, however, it is possible that in the marmoset the 5-HT modulates the SCN in an alternative way, in that we cannot discard a non-photic role. Pharmacological manipulation suggests the participation of the 5-HT_{1A} and 7 subtypes in the phase advance in behavioral circadian rhythms in hamster [8,24] that was independent of light inputs. This also reinforces the participation of additional subtypes conveying the 5-HT transmission [2].

In addition to the median raphe nucleus (MRN)–SCN pathway, the dorsal raphe nucleus (DRN) projects to the IGL (DRN-IGL pathway) and these two pathways appear to exhibit different functional attributes in the mammal CTS. 5-HT fibers from the MRN facilitating the synchronization of the animal to the light–dark cycle and the disruption of the DRN-IGL do not influence this response [22]. The reception of photic information by the raphe mesencephalic complex through the retinal projections is well characterized and fully recognized in some species [9–11] attributing to this system a pivotal role for 5-HT in circadian rhythmicity by indirect modulation in response to light [23,27,28]. Furthermore, some data show that the function of 5-HT in circadian rhythmicity goes beyond the light response. This includes participation in sleep–wake cycle control and food synchronization suggesting that the serotonergic system takes part in the general organization of the photic-independent mechanism [13,29]. The well-known rhythmic discharge rate of the raphe neurons may have a tonic influence on the CTS, besides the light influence. We also cannot visualize if the double shaped 5-HT innervation in the marmoset SCN (present study) comes from distinct raphe nuclei. Whereas we cannot provide a prompt response for that, it is already known that 5-HT receptors immunoreactivity show a circadian rhythm in its expression in different rat brain areas [1], as well as the 5-HT levels as a neurotransmitter, verified both in the raphe complex [32] and in the innervation over the SCN [4]. The SCN also exhibits an inherent capability to respond to the light–dark cycle [36], which reinforces the power of intrinsic SCN circuitries in order to maintain and adjust endogenous rhythmicity according to environmental clues, not always exclusively light-dependent. Lesions in the MRN and DRN or the use of serotonergic blockers produce similar alterations in their targets, such as a loss of inhibition and increased discharge of SCN neurons [3,30]. In agreement with this, 5-HT_{1B} knockout mice display altered photic synchronization and a reduced but not abolished light-induced phase shift [33].

Until now, the occurrence of 5-HT_{1B} has been more linked to ventral SCN serotonergic innervation, and in our data we show that in the marmoset this receptor appears to be preferentially distributed on CB-enriched neurons in the medio-dorsal portion of the SCN. Even though the functional significance remains unknown, our data imply that the 5-HT_{1B} may influence the dorsal SCN seroton-

ergic path, raising the possibility of a parallel feature in humans. The 5-HT_{1B} has been the focus of clinical studies on psychosomatic pathologies related to biological rhythms. An example is the seasonal affective disorder that affects patients living in short day regions being consequently exposed to little sunlight [19]. Understanding the functional attributes of 5-HT_{1B} in the CTS seems to be essential for investigation and possible future pharmacologic management of these disorders.

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