Anterograde tracing of retinal afferents to the tree shrew hypothalamus and raphe

Stefan Reuss*a,b,*, Eberhard Fuchs*a,b

*aDepartment of Anatomy, School of Medicine, Johannes Gutenberg-University, Saarstr. 19-21, D-55099 Mainz, Germany
bDepartment of Neurobiology, German Primate Center, D-37077 Göttingen, Germany

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Abstract

The anterograde neuronal transport of Cholera toxin B subunit (CTB) was used in this study to label the termination of retinal afferents in the hypothalamus of the tree shrew Tupaia belangeri. Upon pressure-injection of the substance into the vitreous body of one eye, a major projection of the retinohypothalamic tract (RHT) was found to the hypothalamic suprachiasmatic nuclei (SCN). Although the innervation pattern was bilateral, the ipsilateral SCN received a somewhat stronger projection. Labeling was also found in the supraoptic nucleus and its perinuclear zone, respectively, mainly ipsilaterally as well as in the bilateral para- and periventricular hypothalamic regions without lateral predominance. In the raphe region, scattered fibers and terminals were seen in the dorsal and median raphe nuclei. CTB-immunoreactive structures were observed neither in the locus ceruleus nor in vagal nuclei. Our results, partly in contradiction to earlier studies using different tracing techniques in another tree shrew species (Tupaia glis), reveal that hypothalamic nuclei, in particular the SCN, are contacted by retino-afferent fibers which are thought to mediate the effects of light to the endogenous ‘clock’ and to parts of the neuroendocrine system.

Keywords: Anterograde neuronal tracing; Cholera toxin B subunit; Hypothalamus; Paraventricular nucleus; Retina; Suprachiasmatic nucleus; Supraoptic nucleus, Locus ceruleus, Tupaia belangeri, Vagal nuclei

1. Introduction

The hypothalamic suprachiasmatic nucleus (SCN) is regarded as the major pacemaker for circadian rhythms of mammalian body functions [27,38]. The nucleus itself is influenced by internal and external factors. The most important of the latter is light which is transmitted from the retina to the hypothalamus via retino-afferent fibers, the so-called retinohypothalamic tract (RHT). The RHT conveys photoperiodic information to the SCN (the endogenous ‘clock’ in mammals) and thus mediates the entrainment of circadian and seasonal rhythms (cf. [21,33] for review).

The RHT, first observed in the last century (cf. [33]), was characterized in 1972 by autoradiographic techniques [15,28] and since then was investigated in a variety of mammalian species. The topography of its termination pattern in the hypothalamus is a major research item of neuroanatomical investigations of the circadian system. Many studies, however, have been conducted in rats and in hamsters. To extend our respective knowledge of nonrodent mammals, the present study was designed to investigate the retinal projection pattern to the hypothalamus in the tree shrew (Tupaia belangeri). Phylogenetically, tree shrews are considered as intermediates between insectivores and primates and serve as a suitable animal model for the study of bio-behavioral consequences of psychosocial stress (cf. [14]).

Although the visual system of tree shrews is relatively well-investigated [3], the existence and termination of the RHT in Tupaia is a matter of controversy since some authors did not find evidence for such a projection in Tupaia glis [1,18,43], while others identified degenerated fibers of passage in the contralateral ventral hypothalamus upon unilateral eye enucleation [2]. A predominantly contralateral projection pattern was also found in a horseradish peroxidase (HRP) tracing study [30]. However, autoradiography studies revealed the bilateral innervation of the SCN [4,5,26].

We therefore decided to study the projection pattern of
retinal afferents in *Tupaia belangeri* by using Cholera toxin B subunit (CTB) as an anterograde neuronal tracer. CTB was shown in previous studies to exhibit the best results in RHT tracing compared to Fast blue, Phaseolus vulgaris-Leucoagglutinin, biocytin or Fluoro-Gold [34,35]. We concentrated on the analysis of the hypothalamus but also studied sections of further brain regions known to receive retinal afferents or to be involved in mediation of stress responses, i.e. the raphe nuclei, locus ceruleus and the vagal nuclei [9,10].

2. Materials and methods

2.1. Animals and treatment

The experiments were performed on adult tree shrews (*Tupaia belangeri*) of either sex with body weights of 400–500 g. The animals stem from the breeding colony of the German Primate Center, Göttingen, Germany, and were held under long-day conditions (light:dark 16:8 h) with food and water ad libitum (for details see Ref. [13]). All animal experimentation was conducted in accordance with the European Communities Council Directive of Nov. 24, 1986 (86/EEC) and was approved by the Government of Lower Saxony, Germany.

Animals were anesthetized with tribromoethanol (0.3 g/kg b.wt., i.p.) and received two pressure-injection of 0.5 μl each of the anterograde tracer substance Cholera toxin B subunit (CTB) into one eye. The tracer was applied to the vitrous body at the lateral and dorsal pole of the eye by using a glass micropipette connected to a Hamilton syringe. CTB (List Biological, Campbell, CA, USA) was diluted in phosphate-buffered physiological saline (PBS) to a final concentration of 0.5%.

After 1 week, the animals received an overdose of the anesthetic i.p. and were perfused transcardially with 250 ml of phosphate-buffered 0.9% saline (PBS), to which 15,000 IU heparin/l were added, at room temperature (RT) followed by 500 ml of ice-cold fixative (4% paraformaldehyde, 1.37% L-lysine, 0.21% sodium-periodate in phosphate buffer; according to [22] with a constant rate of 20 ml/min. The right atrium was opened to enable venous outflow. All animals were killed at the middle of the light period. The eyes and the brain were removed, postfixed for 2 h, and stored at 4°C in phosphate-buffered 30% sucrose. The brains were marked on one side and cut at 40 μm thickness on a freezing microtome in the frontal plane.

2.2. Section treatment

For CTB immunohistochemistry, sections were incubated free-floating for 24 h at RT in goat CTB-antiserum (List Biological), diluted 1:1000 in PBS to which 1% normal swine serum and 0.3% Triton-X 100 were added. After three rinses in PBS, the sections were incubated in biotinylated mouse anti-goat IgG (1:100 in PBS; Dianova, Hamburg, Germany) for 90 min at RT, rinsed three times, and incubated in streptavidin (SA) coupled to Cy3 (1:100 in PBS; Amersham, Braunschweig, Germany). Sections were mounted onto gelatin-coated slides, briefly dried, coverslipped with Merckoglas (Merck, Darmstadt, Germany) and analyzed using a Leitz Orthoplan microscope with a Ploemopak epifluorescence unit through filter set N2. The retinas were excised, sectioned in the sagittal plane and treated in the same way as the brain sections.

In control experiments in which CTB was applied to the extrabulbar space, we observed retrograde neuronal tracing of the nucleus of the optic tract but no retino-afferent fibers. Specificity studies were carried out by omitting primary or secondary antisera. The specificity of the CTB antiserum was demonstrated by the absence of staining in animals that did not receive injections.

After taking microphotographs of selected regions, the coverslips were removed and the sections stained with Cresyl violet.

3. Results

In frontal brain sections containing the hypothalamus, retino-afferent fibers and terminals were seen upon anterograde neuronal transport of CTB injected into one eye. The major hypothalamic projection pattern was found to the bilateral SCN (Fig. 1). The comparison of both sides showed that the nucleus ipsilateral to the injection site receives a somewhat stronger innervation. In the anterior parts of the SCN (Fig. 1A,B), retinal innervation was concentrated in ventral aspects of the nuclei. In sections of the medial SCN (Fig. 1C,D), labeling was distributed over the dorso-ventral extension of both nuclei but was concentrated in the ventral two-thirds. In higher magnification (Fig. 1E,F), it is seen that labeled terminals are concentrated over, or surrounding, SCN perikarya. The innervation pattern of the SCN was found to be dense in particular in its central portion (Fig. 1D). The fibers, however, were of fine caliber and exhibited small varicosities (Fig. 1F).

 Immunoreactive structures were further observed in the region of the supraoptic nuclei (SON), paraventricular nuclei (PVN), and periventricular nuclei (PeVN). In the SON (Fig. 2), fibers and terminals were seen mainly in sections of the ipsilateral anterior (Fig. 2B) and middle part of the nucleus (Fig. 2E), dorsally and laterally to the optic chiasm. Although some immunoreactive structures were observed within the borders of the SON, they were predominantly found in the perinuclear region. Fibers in the peri-SON were of fine- to medium-sized caliber and the varicosities mainly were larger compared to those of the SCN. In the higher magnifications from both regions, immunoreactive fibers and terminals were seen at sites where, in the Nissl-stain, neuronal cell bodies were observed (Fig. 2C,D and F,G).
Fig. 1. Frontal sections of the tree shrew hypothalamus. The left panels depict the Cresyl violet-stain and the right panels show CTB-immunoreactivity upon injection of the tracer into one eye and anterograde neuronal transport. Each pair of panels was taken from the same section. The right sides of the sections are ipsilateral to the injection site. The termination of the retinohypothalamic tract in the anterior SCN of a female tree shrew is demonstrated in (A,B). A section through the mid-SCN at the level where the major projection pattern was found is shown in (C,D). A region from the left ventral SCN is shown in higher magnification in (E,F). Dense accumulations of labeled fibers are seen in close vicinity of small SCN neurons. The asterisks mark the same region in both panels. Abbreviations: oc=optic chiasm, V=third ventricle. Magnification bars=100 μm (A–D); 20 μm (E,F).
Fig. 2. Frontal section of the tree shrew hypothalamus showing the region of the ipsilateral supraoptic nucleus (SON) in the Cresyl violet-stain (A,C,G) and CTB-immunoreactivity in the same sections (B,D,E,F) upon injection of the tracer into the ipsilateral eye. A relatively dense retinal innervation of the SON and its perinuclear regions is seen at the level of the anterior SON (A,B). The higher magnifications (C,D) demonstrate that CTB-immunoreactive fibers and terminals are seen in close vicinity to neuronal perikarya. In the peri-SON (E), many fibers are seen. The comparison of CTB-immunolabeling and Cresyl violet-stain in higher magnification (F,G) reveals that fibers are present at neuronal cell bodies. Abbreviations: l=lateral, m=medial, ot=optic tract. Bars=50 μm (A,B,E); 20 μm (C,D,F,G).
In the PVN, we observed immunoreactive fibers mainly in its medial subdivision, predominantly in the dorsal parts (Fig. 3A–C). The paraventricular innervation was found to be bilateral with no apparent dominance. Fiber caliber and varicosity size appeared larger than those seen in the peri-SON. In the bilateral periventricular nuclei (PeVN), scattered fibers and terminals were seen in the ventral (Fig. 3D–F) and dorsal (Fig. 3G–I) aspects. In PVN, PeVN and peri-SON, the amount of fibers and terminals traced was relatively large.

In sections of the raphe nuclei (Fig. 4A), we observed few fibers and scattered terminals in the dorsal raphe nuclei (Fig. 4C), in its ventral part (between the bilateral medial longitudinal fasciculus; Fig. 4B) and in the median raphe nucleus (Fig. 4D).

Immunoreactive fibers and terminals were also seen, predominantly contralateral, in the lateral geniculate nucleus (LGN), mainly in its dorsal subdivision but also in the ventral part, and in the intergeniculate leaflet. Scattered fibers were also observed in the anterior central gray, mainly dorsally to the aquaeduct without preference to either side, and in the accessory optic nuclei. In the superior colliculus, intensive labelling was observed predominantly contralateral to the injection site, in particular in its laminated (input) structure (data not shown).

In the locus ceruleus and in vagal nuclei (dorsal nuclei of vagus nerve, nuclei of solitary tract and nucleus ambiguus), no CTB-immunoreactivity was observed.

The examination of retinal sections showed that ganglion cells exhibited CTB-immunoreactivity. Signs of necrosis were not seen (data not shown).

4. Discussion

The visual system of tree shrews is well-investigated [3], however, comparatively little knowledge is available as yet that deals with the RHT of these small diurnal mammals which are considered as intermediates between insectivores and primates. Early reports contradict one another with regard to the existence of the tree shrew RHT. Laemle [18] and Abplanalp [1] did not comment upon a retinal projection to the *Tupaia glis* hypothalamus, while Tigges [43] mentioned that no evidence was found for a retino-hypothalamic projection in the same species upon unilateral eye enucleation and application of the Nauta-Gygax technique to identify degenerated fibers. In contrast, enucleation was described to result in degenerating fibers appearing as fibers of passage in the contralateral ventral *Tupaia glis* hypothalamus [2]. An HRP tracing study in the same species pointed to a predominantly contralateral projection to the SCN [30]. In addition, retinal projections traced with radioactive amino acids and/or fucose and visualized by autoradiography revealed that labeled fibers enter both the ipsi- and contralateral SCN in *Tupaia glis* [4,5,26].

Our study, using the reliable anterograde neuronal tracing properties of CTB following injection of the substance into one eye, now confirms the presence of retino-afferent projections to hypothalamic nuclei in tree shrews and provides a detailed description of the distribution of RHT termination of the tree shrew *Tupaia belangeri*. The SCN receives the strongest innervation. Although the projection pattern is bilateral, it shows a light ipsilateral predominance which was previously described to be the case in primates including man [7,12,39], in contrast to the greater contralateral projection as found in many rodents [29,42]. It is generally agreed that the RHT is the morphological substrate for the regulatory effect of light on the SCN and thus on the circadian timing system (cf. Refs. [29,33]). The widespread distribution of labeled fibers and terminals reveals that several neuronal subpopulations of the *Tupaia* SCN (not yet identified) are contacted by retinal afferents. The fibers and varicosities in the SCN were of relatively fine diameter compared to those observed in the PVN (see below). Similar results were previously described in a detailed report on target-specific differences of hamster retinal projections [20].

Of further interest is the relatively dense innervation of neuroendocrine nuclei, in particular of the supraoptic nucleus and the peri-SON region. This projection may be involved in the photic effects on mammalian physiology that are not mediated by the circadian timing system. Degenerating fibers in the contralateral SON region were found previously upon unilateral eye enucleation in two studies [2,18] but not by other groups investigating tree shrews. Our data reveal that the SON and its perinuclear region receives a relatively dense bilateral innervation from the RHT; however, the projection to the ipsilateral SON area was seen to be far greater than that to the contralateral nucleus. Retinal projections to the ventromedial SON are present in humans [7], and studies in rats have previously shown that a retina-supraoptic projections exists [19,24]. Electrical recordings from SON neurons while stimulating the optic nerve also revealed that a direct monosynaptic pathway between the RHT and the SON or its perinuclear zone, respectively, exists [6]. It is thus conceivable that the retinal input to the SON area is responsible for daily rhythms of oxytocin and vasopressin release as found in rats [31,44].

A retino-afferent innervation of the bilateral para- and periventricular nuclei was observed in the present study. In the dorsal aspects of the PVN, in particular, many labeled fibers were seen. Scattered fibers and terminals were also present in the ventral PVN and in the PeVN at the lateral borders of the third ventricle. The presence of a bilateral PVN innervation was previously observed in dogs and rabbits [17], hamsters [45] and humans [40]. Since there is experimental evidence that the PVN is a functional component of the retina-pineal gland pathway [32,36,37], it seems possible that photic information is transmitted to the pineal also bypassing the SCN and the sympathetic system.
Fig. 3. Frontal section of the tree shrew hypothalamus showing the contralateral paraventricular and periventricular regions in the Cresyl violet-stain (A,C,F,I) and CTB-immunoreactivity in the same sections (B,D,E,G,H) upon injection of the tracer into one eye. Fibers are seen in higher magnification in (B), while (C) shows the Cresyl violet-stain of the same region. The ventral periventricular region (vPeVN) is shown in (D), in higher magnification in (E) and the same region in the Cresyl violet-stain (F). Panels (G–I) depict scattered CTB-immunoreactivity in the dorsal periventricular area (dPeVN), in (H) in a higher magnification from (G), and in (I) as the Cresyl violet-stain of the region shown in (H). Abbreviations: l=lateral, m=medial. Bars= 300 μm (A); 50 μm (D,G); 20 μm (B,C,E,F,H,I).
We also observed CTB labeling of retinal afferents in the LGN, IGL, SC and in some other retinorecipient nuclei, in accord to previous observations on the tree shrew visual pathways (cf. [3]). It should be noted that retrogradely stained perikarya were observed neither in the hypothalamus nor in other brain regions known to receive retinal afferents. Retinopetal projections in mammals are still a matter of controversy (cf. [29] for review). We have, so far, only occasionally seen retrogradely labeled neuronal perikarya in the basal hypothalamus, but not within the borders of the SCN, upon Fluorogold injection into the eye of the dwarf hamster [34].

The present study has also demonstrated a retinal projection to the dorsal raphe nuclei (DRN). Fibers and terminals containing CTB were detected in the anterior dorsal and ventral parts of the DRN, and in the median raphe nuclei. These data are in accordance with previous and recent reports of direct retina-raphe projections in cat, rat and gerbil [8,11,16,41]. It is conceivable that this connection provides an indirect visual pathway to the circadian pacemaker since the dorsal and median raphe nuclei innervate the SCN [16,25] and both are capable of modulating the phase of the circadian rhythm [23].

In conclusion, the present study using anterograde tracing of retinal afferents by CTB reveals that the retinohypothalamic tract in *Tupaia belangeri* provides a strong innervation of the bilateral SCN, and weaker projections to the area of the ipsilateral SON, the bilateral PVN and PeVN regions, and the raphe nuclei. The discrepancies found between the present and previous
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