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Dual chemoarchitectonic lamination of the visual sector of the thalamic reticular nucleus

Zsolt B. Baldauf^{1,2}

¹Neurobiology Research Group, Hungarian Academy of Sciences at the Department of Anatomy, Histology and Embryology, Semmelweis University Medical School, Budapest, Hungary*

²Department of Anatomy, Histology and Embryology, Albert Szent-Györgyi Medical School, University of Szeged, Szeged, Hungary

Abstract

The chemoanatomical organization of the visual sector of the cat's thalamic reticular nucleus (TRN) - i.e. at the dorsal lateral geniculate nucleus (dLGN) and at the pulvinar nucleus (Pul) - was investigated with two novel cytoarchitectonic markers. The Wisteria floribunda agglutinin (WFA) binding reaction visualized the extracellular perineuronal net (PN) and the SMI 32 immunoreaction stained intracellular neurofilaments. Two distinct layers of the TRN could be detected, particularly by WFA- but also by SMI 32-staining. The outer tier outlined a canopy of labeling placed a bit detached from the diencephalon dorsolaterally, while the inner TRN tier is very tightly attached to the thalamic lamina limitans externa. The labeled neurons showed typically fusiform morphology with dendrites orienting in the plane of TRN.

Additionally, these chemoarchitectural reactions identified a chain of structures in the ventral diencephalon connected to the TRN tiers. One stained string is formed by the subthalamic nucleus bound laterally to the peripeduncular nucleus extending further dorsolateral into the outer TRN tier. The other chain laced up the field of Forel, the zona incerta, the ventral LGN, the perigeniculate nucleus (PGN) and the previously-overlooked peripulvinar nucleus (PPulN) and so formed the inner TRN tier. In the third most distanced TRN tier, in the perireticular nucleus, a very few WFA-binding presenting neuron were found. In addition to the PN possessing TRN neurons, WFA-reactive presumable interneurons were also labeled within the visual thalamus.

Following tracer injections into the feline Pul, two stripes of cells were retrogradely labeled in the neighboring visual TRN sector. The location of these reticular neurons coincided precisely with the chemoanatomically identified inner and outer TRN tiers. On the analogy of the PGN-TRN duality at the dLGN, the chemoanatomical and tract tracing findings strongly suggest a similar dual organization in the pulvinopprojecting TRN portion.

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Correspondence: Zsolt B. Baldauf, Department of Anatomy, Histology and Embryology, Albert Szent-Györgyi Medical School, University of Szeged, 40 Av. Kossuth Lajos, 6724 Szeged, Hungary, phone: 36-62-545-665, fax: 36-62-545-707, zsolt.baldauf@yahoo.com.

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Keywords

Wisteria floribunda agglutinin; SMI 32; pulvinar; peripulvinar nucleus; lateral geniculate nucleus; perigeniculate nucleus

The mammalian thalamic reticular nucleus (TRN) covers the thalamus rostrally and ventrolaterally (Jones, 1985). It consists of a thin sheet of neurons receiving collaterals of the traversing thalamocortical and corticothalamic axons; hence it readily obtains information about the thalamocortical interplay. Apart from its intrinsic connections, the lone projection of the TRN enters the thalamic nuclei (Minderhoud, 1971; Jones, 1975) and inhibits their neurons (Schlag and Waszak, 1970; Montero and Scott, 1981) the way the Scheibels originally proposed (Scheibel and Scheibel, 1966).

The TRN is organized in modality specific sectors lying in the vicinity of its innervated thalamic nucleus (Jones, 1975). Inside a TRN sector at least two parallel tiers of neurons were found chemoarchitectonically attaching to the external medullary lamina of the carnivore thalamus (Clemence and Mitrofanis, 1992). Their potentially different projection pattern to the thalamus is not yet known with the exception of the TRN tiers aside the dLGN. A and A1 laminae of the dLGN are innervated by the perigeniculate nucleus (PGN), while the overlying TRN 'proper' sends projection to the entire lateral geniculate complex (Cucchiari et al., 1990; Uhlich et al., 1991; Wang et al., 2001).

In Golgi material, the main neuronal type of these TRN layers was the fusiform cell spanning its mostly opposing neurites in the plane of the TRN organizing a strapping network; however, some multipolar neurons were also observed in the TRN (Scheibel and Scheibel, 1966; Spreafico et al., 1991) and also in the PGN (Uhlich et al., 1991). Three basic types of rodent TRN neurons were described differing in their soma size, dendritic arrangement and distribution (Spreafico et al., 1991). In intracellular filling experiments of various mammals, however, no obvious basis for neuronal classification in the TRN could be found, the majority of cells presented the characteristic spindle form and only some had distinctive multipolar cellular morphology (Lübke, 1993).

It has long been recognized that the TRN consists exclusively of GABAergic neurons (Houser et al., 1980; Spreafico et al., 1991). Although several chemoanatomical markers are available for inhibitory neurons, relatively few of them are able to specifically label the TRN. Calcium binding proteins (Celio, 1990) and neuropeptides, e.g. somatostatin (fibers: Graybiel and Elde, 1983; perikarya: Oertel et al., 1983) and neuropeptide Y (fibers: Coveñas et al., 1990; perikarya: Borostyánkői et al., 1999) have all been detected to date in the feline TRN, but they did not turn out specific to the TRN.

Various plant lectins - e.g. *Wisteria sinensis* - were extensively studied to characterize the surface of blood cells (Boyd and Reguera, 1949) and many of them proved to be a very promising tool in the nervous system detecting the perineuronal net (PN). This lattice-like structure wraps the soma and the proximal dendrites of certain neurons extracellularly consisting of several chondroitin sulfate proteoglycans and hyaluronic acid (Celio et al., 1998). The prevailing capacity of *Wisteria floribunda* agglutinin (WFA) to bind to the PN's sugar components - i.e. N-acetyl-D-galactosamine - was established (Kurokawa et al., 1976). It was originally suggested to be useful to study cell surface carbohydrates, since these sugar residues often occur in cellular membranes (Cheung et al., 1979). The extracellular neuronal labeling capacity of the WFA was successfully tested in the brain (Härtig et al., 1992) and the detailed distribution of these binding sites in the rat brain was soon described (Seeger et al., 1994). The presence of WFA-binding sites was also detected specifically in the TRN (rodents:

Seeger et al., 1994; carnivores: Boss et al., 2003; Baldauf, 2007; Bickford et al., 2008; primates: Preuss et al., 1998).

The SMI 32, an antibody recognizing the intracellular intermediate neurofilament proteins (Sternberger and Sternberger, 1983) has also been proved a very powerful parcellating marker in the cat's central nervous system (van der Gucht et al., 2001; Baldauf, 2006). In the TRN of cats, intense SMI 32-immunoreactivity was found (Baldauf, 2006; 2007). However, the thorough descriptions of the localization of WFA and SMI 32 and the detailed cellular morphology of the reactive neurons in the feline TRN are lacking.

In this study, a comprehensive anatomical analysis of the WFA and SMI 32-containing elements in the visual TRN is given and the dual lamination of the TRN around the higher order visual thalamic nucleus, the pulvinar nucleus (Pul) is documented. The related structures of the TRN tiers in the prethalamus (Puelles and Rubenstein, 2003; called formerly ventral thalamus) are identified; where these tiers appear to connect or perhaps originate (Rose, 1942; Altman and Bayer, 1988). Furthermore, a detailed topographical analysis of the reticulopulvinar neurons of two former Pul injections (Wang et al., 2001; Baldauf et al., 2005a) is given further proving the bilaminar organization of the TRN as nicely demonstrated by Clemence and Mitrofanis (1992). Preliminary results of this study have been published earlier (Baldauf, 2007).

Experimental procedures

Seven adult cats about the age of 2 years were used in this study. Two cats receiving tracer injections into the pulvinar were also used in previous works and the experimental protocols had been described there in detail (Baldauf et al., 2005a; Wang et al., 2001). The other five animals were used for histochemistry. All experimental procedures on animals were conducted in agreement either with the European Communities Council Directive (86/609/EEC) or with the National Institute of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the University of Louisville Animal Care and Use Committee.

The cats were anesthetized with sodium pentobarbital (50mg/kg IV) then perfused transcardially as follows: The blood was washed out with 300mL physiological saline followed by 2000mL fixative containing either 4% paraformaldehyde and 0.2% picric acid or 1% paraformaldehyde and 2.5% glutaraldehyde dissolved in 0.1M phosphate buffer pH=7,4 (PB). Following the perfusion, the brains were left in place for 2 hours then the skull was fixed properly in the stereotaxic apparatus and the cranial vault was opened. A wide blade was attached to a manipulator arm and at antero-posterior 0.0 - according to Horsley-Clarke coordinates - and lowered to the brain cutting it on the frontal plane completely through. Then the brain was removed from the skull; thalamic blocks were trimmed and put overnight at 4° C in a freshly made fixative omitting glutaraldehyde on a shaker and then in 30% saccharose (dissolved in PB) until they were fully immersed through. After the cryoprotection, the formerly cut surfaces of the thalamic blocks were laid on the specimen holder of the freezing microtome (Reichert Jung 1205, Vienna, Austria) in order to get the accurate coronal plane and obtain precise stereotaxic coordinates. Then the blocks were cut into six parallel series of 50µm-thick frozen sections and collected serially in PB.

All sections processed for WFA or SMI 32 were first placed in 0.5% Triton-X solution dissolved in PB for 30 minutes then in a solution containing 3% hydrogen peroxide and 10% methanol in PB also for 30 minutes. After repeated rinsing in PB, sections reacted for detecting the WFA-binding were placed in 2% bovine serum albumin (BSA) for 30 minutes, then in a solution containing 2µg/ml of WFA (biotinylated lectin from *Wisteria floribunda*, L-1766, Lot# 26F8100; Sigma-Aldrich GmbH, Steinheim, Germany) and 0.1% BSA dissolved in PB

for 48 hours at 4°C. Sections processed for SMI 32 immunohistochemistry were placed in 10% normal horse serum (NHS) for 30 minutes then in a solution containing SMI 32 antibody (Lot#17; Sternberger Monoclonals Inc., Baltimore, MD, USA) at a 1:2000 dilution and 1% NHS dissolved in PB for 48 hours at 4°C. Afterwards, the sections were rinsed several times in PB then put in biotinylated anti mouse secondary antibody at a 1:300 dilution dissolved in PB overnight at 4°C.

Sections containing tissue-bound biotin were rinsed several times in PB then put in 1:100 dilution of ABC (Vector, Burlingame CA, USA) solution overnight at 4°C. The next day the sections were rinsed in PB, then in 0.05M Tris buffer pH=8.1 (TB) and the peroxidase binding was visualized with nickel-intensified diaminobenzidine containing 0.001% hydrogen peroxidase reacted for 10–15 minutes. Following the reaction, the sections were thoroughly rinsed in TB then were mounted onto chromium-gelatin subbed slides. After drying, they were dehydrated, cleared and coverslipped. The sections were photographed with an Olympus BX51 microscope connected to an Olympus DP50 digital camera system. Pictures were captured with Viewfinder Lite 3.0 (Pixera, Los Gatos, CA, USA). Finally, the figures were edited with Corel PhotoPaint 10.0 software (Corel Co. Ottawa, ON, Canada).

Either fluorescent dextran amine (FDA, Molecular Probes, Eugene, OR, USA) or a fluorescent latex microsphere (LumaFluor, Naples, FL, USA) were injected into the pulvinar as neuroanatomical tracers (described in details in Baldauf et al., 2005a and Wang et al., 2001, respectively). Thalamic sections originating from these experiments were reanalyzed in greater stereotaxic detail and the retrogradely labeled cell bodies in the TRN were plotted with a MD3 Microscope Digitizer system (AccuStage Co., Shoreview, MN, USA). Their distribution is shown on various antero-posterior levels and the plots were edited with Corel Draw 10.0 software (Corel Co. Ottawa, ON, Canada).

Results

In general, the WFA staining presented a halo-like appearance of labeled neurons. Since this reaction product accumulates exclusively on the extracellular side of somata and proximal dendrites, nuisances in morphology - like spines or protrusions - can not be accurately observed. While the thalamus (former dorsal thalamus) presented a weak WFA-binding, the TRN - spanning from the 'dorsal' thalamic surface toward the prethalamus (former ventral thalamus) - has been intensely stained (Fig. 1). Moreover, the WFA staining in the TRN presented a well-discernible bilaminar pattern around the cat diencephalon.

The SMI 32-immunoreaction, however, resulted in a superb intracellular neuronal label detecting delicate details of cellular morphology. Contrary to the WFA-binding reaction, the SMI 32 antibody resulted in a strong label throughout the entire diencephalon, including structures of thalamic and prethalamic origin. The bilamination of the feline TRN could be easily recognized.

All the following figures show stained sections of the right hemisphere cut in the coronal plane. All sections were oriented on the figures as follows: dorsal to the top, lateral to the right.

Distribution of WFA binding sites in the TRN

In the cat's TRN two cytoarchitectonic layers revealed by WFA binding could clearly be observed (Fig. 1A,B). While the inner tier attached more closely to the external medullary lamina of the thalamus, the outer tier was separated a little farther forming a canopy of WFA label around the thalamus (Fig. 1A,B). The inner tier appeared to contain less WFA-binding sites than the outer one (Fig. 2A,B).

Similarly in the visual sector, i.e. by the Pul and the dLGN, a bilaminated TRN could be found (Fig. 2A,B; 4A,B). Within the inner tier, the direct continuation of the TRN layer at the lateral side of the Pul into the PGN could be unequivocally observed (Fig. 2A), therefore a separate name to this sector beside the Pul is proposed. The term peripulvinar nucleus (PPulN) would suit the best. The inner TRN tier contained characteristic fusiform cells (Fig. 4D) and additionally round multipolar neurons (Fig. 4D,E; 6G). Long spanning dendrites without ramification belonging to both cell types could be frequently seen.

In the outer tier of the TRN the WFA reaction product was much more (Fig. 2A,B; Fig. 4A,B). Similarly to the inner tier, the dominant cell type was the fusiform neuron in the outer tier (Fig. 4F; Fig. 6H). The soma size of the outer tier cells seemed a bit larger than that of the inner tier's. Alternatively, the cells may have presented more agglutinin binding sites making the cells appear larger. Similar to the neurons of the TRN inner tier, the dendrites of the outer tier's cells could be traced a long way.

Rostral from the visual TRN, this chemoarchitectonic bilamination - unveiled by the WFA-binding - continued to the anterior tip of the thalamus, where the duality could no longer be observed on a coronal section.

Distribution of WFA-binding sites in other prethalamic nuclei

The inner TRN tier began at the lateral side of the Pul (as PPulN), continued around the dLGN (as PGN) then joined the ventral LGN (vLGN) as revealed by the WFA-binding reaction (Fig. 1A; 2B; 4C). Quite numerous round WFA-reactive cells of small size were found in the vLGN, but some fusiform cells could also be observed (Fig. 4G).

Passing along the inner tier, a previously unnamed stripe of cells - running between the external medullary lamina and the TRN 'proper' - presented WFA-binding (Fig. 1B). These neurons extended from the vLGN to the lateral edge of the zona incerta (ZI) and both fusiform and multipolar cells were found in this area. The neurons were loosely organized and the dendrites formed a net-like structure leaving unstained patches between the intensely stained dendrites. Since this stripe of cells continued uninterruptedly between the vLGN and the zona incerta, and showed WFA-binding in a similar fashion, one can argue that this is also part of the inner tier of the TRN; however, it has been formerly unnoticed to the best of my knowledge. Since its neighboring thalamic nucleus is the first-order somatosensory nucleus, the ventrobasal nucleus, and the bilamination of the TRN is present, the somatosensory TRN sector may be similarly bilaminated as the visual TRN sector.

Ventrally, this area merged into the ZI and showed a well-demarcated border (arrow on Fig. 3A). The ZI presented a little more intense WFA binding in a reticular or patchy fashion (Fig. 5A). Its dorsoventral boundaries were quite easy to determine, dorsally the thalamic mass completely without WFA label, and ventrally the white matter of the lenticular fasciculus also void of WFA stain were present (Fig. 3A,B,E). In this nucleus, mostly multipolar neurons were observed orienting their dendrites rather unsystematically (Fig. 5A).

Lying more medial from the ZI, the Forel-field (FF) could be found with a noticeable border (arrow on Fig. 3E). The WFA labeled mostly multipolar neurons were scarcely distributed, therefore the staining seemed a bit lighter. The dendrites were quite thick and seemingly random running (Fig. 5B). Its medial end at the hypothalamus could also be easily observed (arrow on Fig. 3F), because the hypothalamus contained an even more transparent network of WFA-reactive neurons with similarly picturesque long dendrites.

The outer tier (the so-called TRN 'proper') also veiled the visual thalamus then turned ventral, while getting very thin, later became wider again and merged into the peripeduncular nucleus

(PPN; Fig. 1B). The PPN contained very intensely labeled, overwhelmingly large multipolar cells with thick and long spanning dendrites (Fig. 5C). As the PPN reached the cerebral peduncle, an ovoid area void of label connected with the cerebral peduncle was found (Fig. 3A). This region did not stain with other neurochemical markers either, so it is probably an accessory white matter of the cerebral peduncle.

A WFA-labeled thin lamina branched out from the PPN and continued further medial, dorsal from the cerebral peduncle and ventral from the lenticular fascicle (Fig. 3A,B). A few fusiform neurons were observed here presenting WFA-binding. Afterwards it reached the tail of the subthalamic nucleus (STN) and joined it (Fig. 3E). In the STN fairly numerous multipolar and also fusiform neurons were observed showing WFA label (Fig. 5D). Their dendrites were not as thick as in the PPN and did not extend as far.

Distribution of WFA in the dorsal lateral geniculate complex

WFA binding of small cells was found in the dLGN (Fig. 6A,C), and also in the medial intralaminar nucleus (Fig. 6B,D) as reported by Bickford et al. (2008). These cells had mostly fusiform morphology. Apparently, the intensity of the WFA-binding reaction in the dLGN always remained below the level that was observed in the TRN. In addition, very few WFA-reactive cells, presumably interneurons, were found in other thalamic nuclei, e.g. in the Pul (Fig. 6E), in the medial geniculate nucleus and in the ventrobasal nucleus.

In the outermost tier of the TRN - in the perireticular nucleus (PRN) - only very few WFA-reactive neurons could be found in adult animals (Fig. 6F). Supporting its proposed thalamocortical organizing role (Mitrofanis and Baker, 1993), the cell emitted a neurite toward the cortex and another toward the thalamus.

Distribution of SMI 32 immunostaining in the TRN

Both laminae of the TRN presented intense perikaryal and dendritic SMI 32 staining in the cat (Fig. 1C,D). Two unstained stripes divided the TRN layers, once from the thalamic mass (external medullary lamina) and additionally between the two TRN tiers. Hence, the separate TRN tiers could be simply recognized (Fig. 2C, 7A,B). The inner tier constituted the PPuN starting below the 'dorsal' thalamic surface of the PGN by the dLGN, both showing similar SMI 32 staining pattern (Fig. 7A,B). The little farther placed TRN 'proper' covered both thalamic nuclei then the two TRN tiers run parallel further ventral (Fig. 1A,B). The major cell type of the TRN 'proper' was the fusiform neuron (Fig. 7F), but few larger multipolar neurons were also observed. In contrast, the main morphological type of the inner visual TRN tier was the multipolar cell, both in the PPuN (Fig. 7D) and in the PGN (Fig. 7E), but fusiform neurons were also observed. No further regularity in the distribution of different cellular forms could be observed. The characteristic fusiform cells distributed rather unevenly in the TRN; they formed nests from SMI 32-immunoreactive (-ir) dendrites. A great many SMI 32-ir dendrites and fibers without a parent perikarya falling in the thickness of the section could be also observed; some run parallel to the TRN, some perpendicular to the TRN (Fig. 7F), resembling the course of thalamocortical/corticothalamic axons.

As described earlier, the feline dLGN contained a lot of SMI 32 immunostained relay cells and some of their dendrites infiltrated the PGN (Fig. 7E; see also Fig. 13C Baldauf, 2006). Similarly, some SMI 32-ir Pul cells emitted intensely immunostained dendrites protruding into the inner TRN tier beside the pulvinar, i.e. in the PPuN (Fig. 7D).

Rostral from the visual TRN, this chemoarchitectonic bilamination -- revealed by the SMI 32-immunoreaction -- continued to the anterior tip of the thalamus, where the duality could no

longer be observed on a coronal section. Moreover, no SMI 32-immunoreactivity (-ir) was found in the PRN.

Distribution of SMI 32 immunostaining in other prethalamic nuclei

The vLGN - remaining in continuation of the PGN (Fig. 2D) - showed a very intense SMI 32-ir staining (Fig. 7C,G). Many labeled neurons of a relatively smaller size (10–15µm) and an entangled network of fibers running with various orientations and no apparent order among them could be seen. Some fibers were quite thick leaving an impression of dendrites; on the other hand some SMI 32-ir fibers were rather thin appearing like axons. Similar to what on WFA stained sections was observed, a thin layer of cells stretched from the vLGN until the ZI covered the ventrobasal nucleus. As mentioned before, it may be the bilaminated somatosensory sector of the feline TRN.

The ZI showed a quite different picture. This cerebral region - lying beneath the thalamus between the white matter of the lenticular fascicle (inferiorly) and the thalamic fascicle (superiorly) - was observed to contain a few, but very intensely labeled SMI 32-ir fusiform neurons with long running dendrites parallel with the thalamic border (Fig. 8A). Some cells were quite large (20–25µm in diameter).

Further medial from ZI, in the field of Forel, a somewhat lighter SMI 32-immunostaining could be observed (Fig. 8B). The organization of SMI 32-ir elements presented a grossly reticular character, stronger stained bundles of fibers alternated with lighter stained patches in between (Fig. 3E). Few SMI 32-ir perikarya were also found possessing fusiform appearance with rather long, straight and non-ramifying dendrites. Like in ZI, thinner SMI 32-ir fibers - possibly axons - could also be observed.

Passing the level of vLGN, the outer TRN tier is first penetrated by the optic tract then continued as the peripeduncular nucleus (Fig. 1C,D). Its SMI 32 architecture resembled very closely the TRN 'proper' by the visual thalamus. Groups of fusiform neurons got wedged in the white matter; their two opposite dendrites ran mainly parallel to the myelinated axons. Similarly to the FF, clear SMI 32-immunonegative zones and more intensely stained bundles of dendrites determined its appearance (Fig. 8C). A strangely unstained area could be delineated, embedded in the PPN (Fig. 3C). As discussed earlier, this can be part of the cerebral peduncle.

Above the cerebral peduncle, a very thin lamina continued with a few SMI 32-ir fusiform cells to the medial end of the peduncle then joined the subthalamic nucleus presenting a characteristic ovoid form (Fig. 3D,E,G). Small SMI 32-ir cells of very high density and an intensely stained network of fibers composed of thin dendrites could be observed in the STN (Fig. 8D).

Distribution of reticulopulvinar cells

Fluorescent dextran amine (case #1; presented with permission, published originally in Baldauf et al., 2005), and green fluorescent latex microspheres (case #2; presented with permission, published originally in Wang et al, 2001) were injected into the Pul of the cats' right hemispheres (Fig. 9). The tracer remained inside the Pul on all sections and no tracer leakage could be observed at the Pul. In the first case (Fig. 9A), two streaks of retrogradely labeled cells in the adjoining TRN sector could be seen. In the second case, the fluorescent power of the tracer was only intense enough to be seen at the injection site at low magnification (Fig. 9B). Here, precise topographical plotting of the injection sites and of the labeled reticulopulvinar cells were given (Fig. 10, 11) on representative antero-posterior levels and were projected on a thalamic map drawn from the SMI 32-immunostained sections. This map of the Pul-lateral posterior complex with its TRN portion was meticulously compared with Updyke's one (1983) and the two maps largely matched to one another.

Most of the labeled neurons - apart from the pretectum - were concentrated on the TRN and on the vLGN. Laterally from the Pul, in the inner TRN tier, large numbers of labeled reticulopulvinar neurons were found and the outer TRN tier was also heavily packed with retrogradely filled cells. Additionally, few cells were observed further away in the TRN, i.e. in the PPN and in the ZI (Fig. 10,11). The labeled cells had an overwhelmingly fusiform shape in the TRN and mostly round in the vLGN. Judging from the chemoarchitectonically defined TRN tiers of the two labeled cell groups in the pulvinopprojecting sector, the outer one placed a bit further lateral and may correspond to the TRN 'proper', while the one closely adjoining the Pul is presumably the inner tier of the TRN, i.e. the PPuLN.

Discussion

The main finding of this work concerns the extension of the concept of bilaminated TRN alongside the dLGN to the Pul based on chemoarchitectonical and tract tracing observations. The WFA and the SMI 32 - as cytological markers - visualized the tiers unequivocally at the two visual thalamic nuclei. Both TRN tiers beside the Pul projected upon it, the same way as the TRN proper and the PGN innervated the dLGN. Based on these chemoanatomical and tract tracing evidences presented here, the inner TRN tier lateral from the feline Pul is proposed to call as "peripulvinar nucleus".

Chemoanatomy of the TRN

Some neurons in the central nervous system possess PN, some do not, but its presence indisputably implies different functions. Although the thalamus is rather deficient in PN, an early study of visualizing the distribution of the N-acetyl-D-galactosamine binding sites of the PN with a cohort of lectins (but not WFA) in the rat brain mentioned the TRN and the PPN as reactive structures but their appearance has not been photodocumented (Nakagawa et al., 1986). The WFA-binding in the TRN was first detected in the rat (Seeger et al., 1994), but it has not been further investigated. The Cat-301 is a widely used antibody against neuronal surface antigens (Hockfield and McKay, 1983). The Cat-301-ir showed a strikingly similar distribution to the WFA-binding pattern in the feline thalamus owing to its ability to recognize the core protein of the chondroitin sulphate proteoglycan, which is an integrant part of the PN (Zaremba et al., 1989). All diencephalic structures of presumed prethalamic origin were labeled (TRN, interlaminar zone, ventral lateral geniculate nucleus, ZI, STN) and so were the intralaminar thalamic nuclei (Hendry et al., 1988), which were otherwise unlabeled with WFA. Interestingly, multiple TRN layers have been described by means of Cat-301-immunoreaction in the auditory reticular sector of cats (Fig. 1B,C of Crabtree, 1998). Until now, numerous further antibodies were generated against neuronal surface epitopes and some of them specifically recognized thalamic reticular neurons (Bertolotto et al., 1991; Pimenta et al., 2001).

In the study of Bickford et al. (2008), the presence of WFA-binding was shown in the diencephalon of cats. That paper, however, did not notice the separate TRN tiers, but this was candidly beyond the scope of that study. Nevertheless, earlier attempts documented the presence of WFA-binding in the feline visual TRN tiers (Boss et al., 2003; Baldauf, 2007).

Although the presence of PN seems universal in the TRN, it shows slight variations in its appearance among different species. In the murine diencephalon, no bilamination of the TRN can be revealed with WFA (Seeger et al., 1994; Ajmo et al., 2008) similar to tree shrews (Baldauf, unpublished). However, the split TRN could be clearly observed in Old World monkey macaque tissue reacted with WFA (Preuss et al., 1998; see also the WFA binding reaction of *Macaca fascicularis* by EG Jones at www.brainmaps.org; Baldauf, unpublished), but not in New World monkey marmosets (see also the WFA binding reaction of *Callicebus moloch* by EG Jones at www.brainmaps.org; Baldauf, unpublished).

This is the first report concerning the presence of PN in the feline vLGN, FF and ZI, although in rats it has been previously detected (Seeger et al., 1994). In the STN, no report is available in any species about the presence of PN-coated neurons.

The neurofilaments are important constituents of the dynamic neuronal cytoskeleton occurring only in subsets of cells in the visual system. These structural polypeptide complexes help to maintain the often asymmetrical shape of cells, assist in the movements of intracellular organelles and also sway cellular motility by effecting microtubular and actin filament functions. An antibody (SMI 32) against the heavy neurofilament subunit was successfully generated (Sternberger and Sternberger, 1983) and is still in wide use today to sort out reactive neurons in the central nervous system. However, it has been more often used for cortical parcellation (rat: Kirkcaldie et al., 2002; hamster: Boire et al., 2005; squirrel: Wong and Kaas, 2008; tree shrew: Jain et al., 1994; cat: van der Gucht et al., 2001; marmoset: Baldauf, 2005b; vervet monkey: Chaudhuri et al., 1996; macaque: Hof & Morrison, 1995) than for subcortical areas (Gutierrez et al., 1995; Bickford et al., 1998; Baldauf, 2006).

Few studies have been done concerning the distribution of neurofilaments in the feline diencephalon. One exception is feline dLGN, where the SMI 32-ir has been found to specifically stain the Y-class cells (Bickford et al., 1998). In another study providing a detailed distribution of neurofilaments in the visual system of the cat, no SMI 32-ir neurons or faintly labeled ones were reported in the vLGN, in the TRN and in the PGN (van der Gucht et al., 2001); nevertheless, in their Fig. 13A they actually showed SMI 32-ir structures in these nuclei. Contrary to this report, I found intense perikaryal SMI 32 label in these structures, as well as in the visual thalamic nuclei. Furthermore, SMI 32 staining in the prethalamic region was only reported in humans, however, only on low-power photomicrographs (Morel et al., 2002).

Here, the capacity of the SMI 32 antibody and the WFA binding reaction to distinguish among the tiers of the feline TRN is presented. Their detailed distribution in the prethalamus is also reported for the first time in the cat brain showing a very similar pattern of labeling.

Neuronal organization of the mammalian TRN

To date, a great specificity of the TRN - formerly considered as a nonspecific thalamic nucleus - has been established. Topographical degeneration in the TRN has been found following various cortical ablations (monkey: Chow, 1952; rabbit: Rose, 1952; Carman et al., 1964) pointing toward the presence of segregated TRN portions connected to its corresponding cortical area. Later, accurate retinotopy was observed in the visual corticoreticular projection of the rabbit (Montero et al., 1977). Specific accumulations of isotopes in different TRN sectors have also been detected following their injection into various thalamic nuclei (Jones, 1975). Up to now, several TRN sectors have been described in the cat's diencephalon, e.g. the visual (Sanderson, 1971; FitzGibbon, 1999), auditory (Rouiller et al., 1985; Crabtree, 1998) and somatosensory (Yen et al., 1985; Crabtree, 1992; 1996).

Three layers in the feline TRN have been distinguished chemoarchitectonically (Clemence and Mitrofanis, 1992): An inner small-celled region ('ISCR') and the outer 'main body' of the TRN appear to correspond to the inner and outer TRN tiers of the present study. Additionally, a third outermost layer, the so-called perireticular nucleus embedded in the internal capsule has also been described. Here, I can unequivocally confirm the existence of the two inner TRN tiers with the WFA and SMI 32 labeling, and traces of the third could also be observed. Similarly to the original descriptions of Berman and Jones (1982) and Steriade et al. (1984), Clemence and Mitrofanis (1992) proposed that the ISCR is the dorsal extension of the ZI. Such a kinship can also be verified here chemoarchitectonically.

Three types of reticular innervation of the sensory thalamic nuclei are known. The first splits the TRN into medial and lateral tiers. The lateral tier innervates the first order nucleus, whereas the medial one projects to the higher order nucleus (rabbit: Crabtree and Killackey, 1989; rat: Pinault et al., 1995; Coleman and Mitrofanis, 1996; Galago: Conley and Diamond, 1990). Individual TRN axons following juxtacellular labeling can also branch and innervate both the first order ventrobasal nucleus and the higher order posterior thalamic nucleus (Pinault et al., 1995). The second archetype can be seen in marmosets, where no laminar segregation of TRN neurons was seen. A rostrocaudal gradient has been observed in the innervation of the visual thalamic nuclei - the rostroventral TRN portion was found to innervate the dLGN, whereas the more caudally placed TRN region established interconnections with the Pul (FitzGibbon et al., 2007). Finally, the third type of innervation can be seen in cats, where the nucleus specific sectors are spatially separate localizing on the dorsolateral margin of the dLGN and on the lateral margin of the Pul, accordingly. In addition, the TRN sectors consist of two tiers both innervating the appropriate thalamic nucleus, e.g. the TRN 'proper' and the PGN both project to the first order dLGN. Furthermore, the TRN 'proper' and the PPulN both innervate the higher order Pul, as the present study describes.

In the cat, the two thalamic reticular tiers - the TRN 'proper' and the PGN - influence the dLGN (Ahlsén et al., 1982; Uhlich et al., 1991), while the pulvinoprotecting TRN sector has not yet been characterized. Following injections of retrograde tracers into the subnuclei of the Pulateral posterior complex two rows of cells were labeled (Rodrigo-Angulo and Reinoso-Suárez, 1988; Baldauf et al., 2005a). The reticular projection to the Pul was ascribed to be very sparse and lacking a strict reciprocity (Rodrigo-Angulo and Reinoso-Suárez, 1988; FitzGibbon, 1994; FitzGibbon et al., 1995; Wang et al., 2001). Wang et al. (2001) also showed a plot (their Figure 1.), on which this sort of duality following retrograde tracer injection into the Pul appears to be present beside the Pul, but they did not discuss it. Nonetheless, based on the large number and the accurate localization of the retrogradely labeled reticular cells in a novel TRN sector beside the Pul observed in the present study, I argue for a stronger and more precise projection of the TRN to the Pul. Moreover, Steriade et al. (1984) presented plots of labeled TRN neurons following retrograde tracer injection into the feline centrolateral and centromedian nuclei and two parallel rows of labeled cells seemed to be present in the rostral tip of the TRN (their Figures 4. and 5.). The possibility of dual reticular innervation of feline thalamic nuclei has not been known earlier and no attention has been paid to it.

Origin of the mammalian TRN

It has long been known that the TRN originates from the cellular zone demarcating the hypothalamus from the thalamus (Droogleever Fortuyn, 1912; Rose, 1942; Altman and Bayer, 1988). Today, we learn from genetic mapping studies that the TRN develops from the first prosencephalic neuromere, the prethalamus (Puelles and Rubenstein, 2003), formerly called ventral thalamus. Based on molecular embryological data and the present chemoanatomical results, the inner TRN tier appears to originate in the prethalamic FF and ZI, continues laterally through its tail and gradually becomes the inner TRN tier of the ventrobasal nucleus. Then this cellular flow - drawing a bent range alongside the thalamus - reaches the vLGN then runs into the PGN and finally to the PPulN. The outer TRN tier, however, emerges from the subthalamic nucleus. This cellular flow seems to spread out further laterally, first forming a thin layer above the cerebral peduncle, then flowing into the peripeduncular nucleus, then turning dorsalwards right into the TRN 'proper', ending at the third cerebral ventricle at the dorsal thalamic surface close to the caudate nucleus.

An intriguing aspect of the diencephalic development is veiled in the fate of the reticular and thalamic interneurons. Similar physiological and morphological properties have been described for the PGN and the interlaminar zone interneurons (Montero et al., 1989; Sanchez-

Vives et al., 1996) and the idea of their common origin has been raised. Recent molecular biological evidences showed that cells in the TRN, in the PGN cells and in the interlaminar zone (perhaps the PN coated ones in the medial intralaminar nucleus and in the Pul, as well) all derive from the embryonic caudal ganglionic eminence and not from the postnatally appearing intrinsic thalamic interneurons (Hayes et al., 2003). The selective and mutual expression of transcription factor *Islet1* in the PGN and in the interlaminar zone further confirm the observation of the common origin of these neurons (Kawasaki et al., 2004). The present study also substantiates this notion, showing that both of these cells are coated by PN.

Possible function of the bilayered visual TRN

A very influential idea has been raised recently as to whether the TRN can act both globally and locally on the thalamus (Guillery and Harting, 2003). While certain afferents (arising in the brainstem and in the basal forebrain) may modify TRN's activity globally, the collaterals of traversing thalamocortical and corticothalamic axons following retinotopic order could act more locally. It is possible that these global and local effects ensue at the same time through the outer and inner TRN tiers, accordingly.

The feline TRN has two tiers at the dLGN having different connections and functions (Ahlsén et al., 1982). Morphologically, axons of the PGN confine to the A laminae of dLGN (Uhrich et al., 1991), while the TRN innervates not only the whole lateral geniculate complex but the Pul-lateral posterior complex, as well (Cucchiari et al., 1990; Wang et al., 2001). Functionally, the PGN can inhibit locally on retinotopically matching places in dLGN, while the TRN 'proper' could mainly be concerned about the global rhythmical en gross inhibition of the thalamus as a 'neuronal oscillator' and also with the regulation of sleep-wake cycle (Schlag and Waszak, 1970; Mülle et al., 1986; Steriade et al., 1986). In addition to this global job of the TRN, it is able to convey information from the first-order unto the higher-order thalamic nucleus (Crabtree et al., 1998). Furthermore, physiological studies described cells with different properties in the TRN tiers: Numerous bursting neurons were observed in the PGN responding to visual stimuli - that effect can be alternatively explained with the conveyed retinal excitation via the intruding geniculate dendrites to the inner TRN tier - whereas neurons in the TRN 'proper' were unresponsive to peripheral stimulation, but fired to cortical stimulation (Ahlsén et al., 1982; Pollin and Rokyta, 1982). Similarly to cats, electrophysiologically different neurons have also been identified in the rat visual TRN forming two subdivisions, but not tiers (Sumitomo et al., 1988; Lee et al., 2007). Additionally, the PN coating of the highly active TRN neurons can protect the cell from rapid ion changes (Brückner et al., 1993).

Based on chemoanatomical and tract tracing data, it is suggested that among visual thalamic nuclei not only the first order nucleus (Ahlsén et al., 1982), but the higher order Pul-lateral posterior complex is innervated by two TRN tiers possibly exerting different functions. While the PPuLN projects specifically to the Pul, implying a local effect, the pulvinopprojecting cells in the outer TRN tier are distributed much broader, implicating a more general effect on the Pul and probably on larger thalamic territory.

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Abbreviations

dLGN	dorsal lateral geniculate nucleus
FF	Forel field
LP	lateral posterior nucleus
PGN	perigeniculate nucleus
PN	perineuronal net
PPN	peripeduncular nucleus
PPuIN	peripulvinar nucleus
Pul	pulvinar nucleus
STN	subthalamic nucleus
TRN	thalamic reticular nucleus
vLGN	ventral lateral geniculate nucleus
WFA	Wisteria floribunda agglutinin
ZI	zona incerta

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Figure 1.
(two-column wide)

Low power photomicrographs illustrate WFA-binding (A, B) and SMI 32-immunostaining (C, D) of the cat's diencephalon at two rostrocaudal coordinates (Horsley-Clarke A6.8, A, C; Horsley-Clarke A8.0, B, D). Particularly the WFA staining shows the two tiers of the thalamic reticular nucleus (TRN) and it is also well-discernible on the SMI 32 staining. The thalamus is almost free of WFA-binding, while it is strongly labeled with SMI 32. Scale bar = 1mm (A-D).

Figure 2.

(two-column wide)

The pairs of figures of matched anatomical locations show the WFA-binding (A, B) and the SMI 32-immunostaining (C, D) in the visual thalamic reticular nucleus (TRN). Both markers visualize the bilaminar TRN tiers by the pulvinar nucleus (Pul; A, C) and by the dorsal lateral geniculate nucleus (dLGN; B, D). The ventral lateral geniculate nucleus (vLGN) is also intensely stained with the cytoarchitectonic markers (B, D) and its direct continuation in the inner TRN tier can be nicely seen, while the outer tier wraps the geniculate complex (B). Whereas, the dLGN contains numerous SMI 32-ir neurons (D), the WFA labeled cells can only faintly be seen (B). Scale bar = 200µm (A-D).

Figure 3.

(two-column wide)

Top 4 landscape figures: These pairs of figures of matched anatomical locations illustrate the prethalamus reacted for WFA (A, B) and for SMI 32 (C, D). The outer tier is contiguous with the well-discernible peripeduncular nucleus (PPN), then with the subthalamic nucleus (STN) through a thin layer of stained cells above the cerebral peduncle (CP) that abridges the gap between the two nuclei lying parallel with the lenticular fascicle (LF). Laterally from the CP, an unstained area with both markers can be seen (A, C). The inner TRN tier by the thalamic ventrobasal nucleus (VB) merges directly into the zona incerta (ZI). This transition can be well recognized (arrow on A). Bottom 4 portrait figures: These pairs of figures of matched

anatomical locations show the WFA- binding (E, F) and the SMI 32-immunostaining (G, H) in the medial part of the prethalamic region. The subthalamic nucleus (STN) having an ovoid shape connects to the outer tier of the thalamic reticular nucleus (TRN) presents a distinct WFA- (E) and an intense SMI 32-label (G). The prethalamic mass giving off the outer TRN tier seems to begin here. In the continuation of the inner TRN tier, the border between the zona incerta (ZI) and the field of Forel (FF; arrow on E) is well-visible. Further medial lays the hypothalamus (HTh) with more loosely organized WFA-labeled neurites. Its lateral cytoarchitectonic boundary from the prethalamus can be easily recognized (arrow on F) and medially the border is more unequivocal from the white matter of the fasciculus retroflexus (FR; F, H). Scale bar on D = 200 μ m (A-H)

Figure 4.

(two-column wide)

The panel illustrates the WFA-binding in the thalamic reticular nucleus (TRN; A, B) and in the ventral lateral geniculate nucleus (vLGN; C). The bit lightly stained inner (peripulvinar nucleus, PPuIN; D) and darker outer (F) TRN tiers by the pulvinar nucleus (Pul) can be seen unequivocally divided (A); and the dichotomy between inner (perigeniculate nucleus, PGN; E) and the outer TRN tiers by the dorsal lateral geniculate nucleus (dLGN) looks fairly similar (B). The vLGN presents round and small WFA-reactive cells (C, G). Scale bar on C = 100 μ m (A-C); on G = 50 μ m (D-G)

Figure 5.
(one-column wide)

The panel demonstrates the neuronal morphology of WFA-binding in the prethalamus, where the dendrites can be followed a long way. The zona incerta (ZI) presents loosely organized dendrites (A), the same as the field of Forel (FF; B), but in this latter structure the cells occur scarcer. In the peripeduncular nucleus (PPN) the dendrites are organized parallel to the thalamus, like in the thalamic reticular nucleus (C). In the subthalamic nucleus (STN) both fusiform and multipolar neurons can be seen (D). Scale bar on D = 100 μ m (A-D).

Figure 6.
(two-column wide)

The panel illustrates the WFA-binding in the visual thalamus in a cat. On a low power photomicrograph, lightly stained neurons can be seen localized mostly to the interlaminar zones (A) and to the medial interlaminar nucleus (MIN; B) in the geniculate complex. In the interlaminar zone (ILZ), the WFA-reactive fusiform neurons tend to group (C), and similarly to the interlaminar zone in the medial interlaminar nucleus the neurons often emit their dendrites in the dorso-ventral axis of the nucleus (D). In the pulvinar nucleus (Pul) a few WFA-reactive cells can be seen (E), while in the outermost tier of the thalamic reticular nucleus (TRN) in the perireticular nucleus (PRN) a very rarely occurring neuron was found (F). Its two

opposing neurites run toward the cortex and the thalamus organizing the thalamocortical pathway. In the perigeniculate nucleus (PGN) the proximal dendrites of two neurons show very close apposition perhaps forming a gap junction (G). The outer TRN tier is dominated by the typical fusiform cells (H). Scale bar on A = 200 μ m (A, B); on H = 50 μ m (C-H).

Figure 7.

(two-column wide)

The panel shows the SMI 32-immunoreactivity (-ir) in the thalamic reticular nucleus (TRN) and in the ventral geniculate nucleus (vLGN). The peripulvinar nucleus (PPuN) is delineated by two white matter streaks (A), medially from the pulvinar nucleus (Pul) and laterally from the outer TRN tier. Between the perigeniculate (PGN) and the dorsal lateral geniculate nuclei (dLGN) a thin white matter can be seen (B) and a thicker between the outer TRN tier and the PGN. Note the SMI 32-ir thalamic dendrite protrude the PPuN (D) and also the PGN (E). A typical fusiform SMI 32-ir neuron on F is shown. The vLGN presents a dense network of SMI 32-ir dendrites (C,G). Scale bar on C = 100 μ m (A-C); on G = 100 μ m (D-G)

Figure 8.
(one-column wide)

The panel demonstrates the cellular morphology of SMI 32-immunoreactive (-ir) elements in the prethalamus on high power photomicrographs. Moderate amount of SMI 32-ir cells with mostly fusiform shape can be found in the zona incerta (ZI; A). In the field of Forel (FF) the SMI 32-ir dendrites are more loosely organized (B). Fusiform SMI 32-ir neurons can be found in the peripeduncular nucleus (PPN) shaping bundles of unstained areas (C). In the subthalamic nucleus (STN) numerous multipolar SMI 32-ir neurons can be seen (D). Scale bar on D = 100 μ m (A-D).

Figure 9.
(one-column wide)

The panel illustrates the injection sites of fluorescent dextran amine (A) and fluorescent latex microsphere (B) into the pulvinar. The picture captured with transmitting light (A) represents case#1, while the dark-field microphotograph (B) shows the fluorescing tracer in case#2. The tracer stays inside the injected nucleus. The Horsley-Clarke coordinates on top left indicate the locus of the images. Abbreviations: dLGN, dorsal lateral geniculate nucleus; H, habenula; LP, lateral posterior nucleus; Pul, pulvinar nucleus. Scale bar on B = 1mm (A,B).

Figure 10.
(two-column wide)

Plots of reticulopulvinar cells are shown in cat #1 on nine consecutive levels of 400 μ m intersection distance. The injected area with the retrograde tracer spans between A7.6 and A8.8 according to the Horsley-Clarke coordinates. The backfilled perikarya form two layers in the thalamic reticular nucleus (TRN) laterally from the pulvinar nucleus (Pul) consequently on numerous sections. Apart from the heavy labeling in the pretectum (PT), neurons can be seen in the ventral geniculate nucleus (vLGN), peripeduncular nucleus (PPN) and occasionally in the zona incerta (ZI). Scale bar = 5mm. Abbreviations: CP, cerebral peduncle; dLGN dorsal LGN; H, habenula; LPI, lateral portion of the lateral posterior nucleus; LPM, medial portion of the lateral posterior nucleus; OT, optic tract; STN, subthalamic nucleus.

Figure 11.
(two-column wide)

Plots of reticulopulvinar cells are shown in cat #2 on five consecutive levels of 1000 μ m intersection distance. The epicenter of the retrograde tracer injection is on A8.0. The backfilled perikarya form two layers in the thalamic reticular nucleus (TRN) laterally from the pulvinar nucleus (Pul). Additionally, the pretectal nuclei and the ventral geniculate nucleus are heavily labeled. Scale bar = 5mm. Abbreviations: APN, anterior pretectal nucleus; CG, central gray; CP, cerebral peduncle; dLGN dorsal LGN; H, habenula; LPI, lateral portion of the lateral posterior nucleus; LPM, medial portion of the lateral posterior nucleus; NOT, nucleus of the optic tract; OT, optic tract; PPuIN, peripulvinar nucleus.