Comparative ultrastructural analysis of inhibitory thalamic afferents

PhD thesis

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INTRODUCTION

The diencephalon

The diencephalon contains three main parts: the epithalamus, the thalamus, and the hypothalamus. The thalamus and the cortex are highly interconnected, and this is called the thalamocortical system. This system is critical for all higher order brain function.

The thalamic relay cell

The main cell type in the thalamus is the glutamatergic relay cell, which is also called the thalamocortical cell. The relay cell's dendrites are radial, have no local collaterals, and their axons project to the cortex.

The main input of the relay cell is called "driver input." Driver input arrives from outside of the thalamus, forming large boutons with multiple synapses on the proximal dendrite of the relay cell. Every thalamic area also receives glutamatergic modulator input from the cortical layer VI. The terminals of this input are small, and they form single synapses on their postsynaptic elements, which is usually the relay cell's thin distal dendrite.

Organization of the thalamus - First and higher order thalamic nuclei

The original idea about the thalamus is that it is just a relay between subcortical structures and the cortex. Novel data shows that a large part of the thalamus receives driver input from the cortex, with the direction of the information transmission opposite of that previously believed.

The anatomical basis of this mechanism is the cortical layer V input, which morphologically and physiologically is very similar to the driver input arriving from the periphery. Based on the existence of this cortical driver, the thalamus can be divided into first and higher order thalamic nuclei. The role of this connection in the cortico-thalamo-cortical information transmission is not known.

GABAerg input to the thalamus

The thalamus receives three types of GABAerg input: local interneurons, the input from the nucleus reticularis (nRT), and inputs from outside the nRT.

The reticular input

The most well-known GABAerg input to the thalamus is that arriving from the nRT, which is a part of the thalamus that exclusively contains GABAergic neurons. The nRT cells send their axons to the thalamus and they receive input from the collaterals of the corticothalamic and thalamocortical axons (both of these inputs are glutamatergic). Both the corticoreticular and the reticulothalamic pathway are topographic, which suggests that the whole system is topographic. This input is important to generate receptive field properties, thalamocortical rhythms, and the synchronization of relay cells.

GABAerg input to the thalamus arriving from outside of the thalamus

The thalamus receives GABAergic inputs outside the nRT, but the origin and the properties of these are only partially known. The most famous are the output pathways of the basal ganglia, namely the substantia nigra reticularis (SNR) and the globus pallidus (GP) projection to the motor thalamus. Studies, carried out with retrogade and anterograde tracers by our group described two diencephalic areas which project to the thalamus, and contain mainly GABAerg elements.

One of these is the zona incerta ventralis (ZIV). This area projects with large GABAerg terminals with multiple synaptic contacts to higher order thalamic nuclei. The anterior pretectal nucleus (APT) also project similarly to particular higher order thalamic nuclei.

In this study our aim is to compare the terminals arriving from the nRT, the APT, and the SNR. We examined the SNR in both rat and monkey.

Variability in the structure of axonterminals

Examination of axon terminals shows that the dynamics of transmitter release and the kinetics of the postsynaptic response depend primarily on the number and organization of synapse and the size of terminal.

The GABAergic terminals are much less examined, but recent studies show small terminals with a single synapse and giant terminals with multiple synapses. The functional importance of multisite and single site terminals is not well understood.

AIMS

In this study our aim was to examine the ultrastructural properties of three GABAerg terminal types arriving to the thalamus from different origin. Anterograde tracing were used to selectively label the terminals in the thalamus. Electron microscopic serial sections and 3D reconstruction were used to define the qualitative and quantitative properties of the boutons. The anatomical analysis was followed with in vitro electrophysiological examination in collaboration with the laboratory of Professor Anita Lüthi.

The three main experiments were the following:

1, nRT-thalamic and the APT-thalamic terminals were labelled and their ultrastructural properties were compared.

2, Nigrothalamic terminals were examined and compared to the APT-and nRT-thalamic terminals.

3, Nigrothalamic terminals of rats and monkeys were compared.

The morphological properties thought to be important in transmitter release (e.g. size of the terminal, number and placement of synapses on a terminal) were examined. In addition the number and size of a target elements/bouton, and synapses number/target were defined. Other ultrastructural properties able to affect the transmitter diffusion like glial coverage or number and placement of PA were also studied.

MATERIALS AND METHODS

All experimental procedures were carried out on rats and monkeys (Macaca mulatta).

Rat surgery and perfusion

In case of rats three pathways (the Anterior pretectal nucleus to the thalamus, the Thalamic reticular nucleus to the thalamus, and the Substantia nigra pars retikulata to the thalamus) were labeled with the anterograde tracer biotinylated dextran amine or Phaseolus vulgaris leucoagglutinin. Coordinates were defined relative to Bregma 0 point according to the atlas of Paxinos and Watson (1998).

Adult male Wistar rats (250-300 g, n=7) were deeply anaesthetized and iontophoretic injections of anterograde tracers were made. After a survival time rats were deeply anaesthetized, then perfused through the heart.

Pre-embedding and post-embedding immunocytochemistry for light and electron microscopy

Coronal sections (50-60 μm thick) containing the thalamus and the SNR were cut with a Vibratome. Injection sites and labeled fibers were visualized with nickel intensified 3,3'-diaminobenzidine (DABNi) reaction resulting in bluish-black reaction product.

Double labeling was carried out to map the co-distribution of calbindin and the labeled terminals in the thalamus. After visualizing the tracer with the bluish black DABNi, sections containing anterogradely labeled terminals were incubated in calbindin antiserum and the immunoperoxidase reaction was developed using 3,3'-diaminobenzidine (DAB) as chromogen, which gave brown reaction product.

Combination of pre- and postembedding immunocytochemistry for electron microscopy the tracer was visualized by the preembedding gold method as described before. Sections were dehydrated in ethanol and propylene oxide and embedded in Durcupan.

Selected blocks containing labeled nigrothalamic terminals were reembedded and 60 nm thick (silver color) ultrathin sections were cut with an Ultramicrotome (Reichert), and alternate sections were mounted on copper or nickel grids. Postembedding GABA immunostaining was carried out on nickel grids according to the protocol of Somogyi et al. (1985).

Monkey

In order to minimize the number of animals necessary for this work the thalami of three adult female macaque monkeys (Macaca mulatta), which received cortical injections in the contralateral hemisphere for other experiments, were used for this study. The monkeys were perfused through the heart. 50 μm thick coronal sections were cut from the anterior part of the thalamus. Alternating sections were processed for type-2 vesicular glutamate transporter (vGLUT2) immunostaining or postfixed in 1% OsO_4, dehydrated and embedded for electron microscopic analysis. vGLUT2 was visualized using DABNi. Postembedding GABA immunostaining was performed on ultrathin sections, cut from the unstained material.

Three-dimensional reconstruction and measurements

Three dimensional reconstructions of the bouton membranes, the synapses, puncta adhaerentia (PA) and the glial sheathes covering the terminals were made using the AnalySIS software (Olympus, Tokyo, Japan). The volume, the surface, the largest diameter of the reconstructed boutons and the intersynaptic distances were measured using AnalySIS.

RESULTS

Morphology of the nRT-thalamic and the APT-thalamic terminals on the light microscopic level

Tracer injection into the "head" of the nRT and the middle part of APT labeled axon terminals in overlapping regions of Po, confirming earlier results. The nRT axons branched profusely and were studded with a large number of small varicosities distributed evenly along the individual branches. In contrast, APT terminals were medium to large-size and long, bouton-free segments alternated with small clusters of terminals, resulting in uneven inter-varicose distances.

Ultrastructure of nRT and APT terminals based on single section and 3D analysis

In single electron microscopic sections, both nRT and APT terminals established conventional symmetrical synapses, containing flattened, pleomorph vesicles and several mitochondria, as previously reported. In the case of the APT terminals, multiple synapses were often encountered even in single sections. These terminals were also characterized by large numbers of PA and a glial cover.

APT terminals were at least five times larger than the nRT terminals. The median volume of nRT and APT boutons was 0.47 μm^3 and 2.4 μm^3 respectively. The size difference was statistically highly significant both for the volume and for the longest diameter.

3D electron microscopic reconstruction demonstrated a striking difference between the nRT and the APT boutons with respect to the number of synapses a given terminal establishes with its postsynaptic partner. The median number of active zones in case of nRT terminals was 2 (mean, 2.1). 26.8% of the nRT terminals had single active zones (hence obviously a single target), and the rest had 2 – 5 active zones. Most of these (80%) contacted more than one postsynaptic partner (2 – 4). As a result, the majority of the dendrites (80.6%) postsynaptic to nRT terminals were innervated by a single synapse. The mean number of synapses a nRT terminal established with a given target was 1.21 (median 1).

Compared to nRT terminals, the number of active zones/terminal was several-fold higher for APT terminals (mean 7.55, median 7). Furthermore, all synapses of single APT terminals converged on the same postsynaptic profile. Hence, the mean number of active zones a given terminal possessed equals the mean number of synapses a terminal established with a single partner. This means that a single APT terminal forms, on average, seven times more synapses on a target than a nRT terminal. The synapses of APT boutons were organized

in a circular manner around a centrally localized network of PA; a network such as this was never observed with nRT terminals. Almost all APT synapses were localized onto dendritic shafts. The number of synapses correlated with the volume of the terminals in both terminal types, suggesting that terminal size is proportional to synaptic strength (Xu-Friedman and Regehr, 2004).

3D reconstruction revealed that the synapses of nRT terminals contacting different dendrites were separated by glial lamellae. In contrast to nRT boutons, almost all intersynaptic spaces of APT terminals were free of glia but the entire outer surface of the APT terminals had complete glial cover. nRT terminals never displayed complete glial sheath.

The median value of the nearest neighbor distances between APT synapses was 204 nm and 77.8% of the nearest neighbor distances were below 300 nm. On average, every synapse had at least 5 neighboring synapses (5.32) within 1 μm distance (Fig. 3F).

The anatomical data show strikingly different morphological organizations of the two GABAergic pathways. The small nRT terminals innervate the entire dendritic tree mostly via single synapses, whereas the large APT terminals innervate proximal dendrites via multiple synapses.

Light microscopic analysis of nigrothalamic terminals in rat

SNR axons had dual terminal fields in the thalamus as described previously. The majority of the terminals were localized to the ventromedial nucleus (VM), whereas another contingent of fibers innervated the intralaminar nuclei and the paralamellar part of the mediodorsal nucleus. All terminal fields consisted of a dense central core surrounded by scattered terminals. At the light microscopic level individual nigrothalamic axons had medium to large size terminals, which were distributed unevenly along the axon, resulting in highly variable intervaricose intervals.

Ultrastructural features and 3D reconstruction of rat nigrothalamic terminals

Nigrothalamic terminals in the VM were medium to large sized (see below for details), contained large number of mitochondria and flattened or pleomorph vesicles. Multiple synapses were frequently evident even in single sections. The terminals were also characterized by large number of PA, (also known as filamentous contacts) and by a glial envelope around the membrane surface not opposed to the postsynaptic cell. Nigrothalamic terminals followed from end-to-end in serial electron microscopic sections established

symmetrical synaptic contacts with their target elements via multiple, distinct synapses. The median number of synapses established by a single terminal was 8.5. In each case, all synapses of a single terminal converged on a single postsynaptic target.

The volume of the nigrothalamic boutons displayed large variability (median, 1.76 μm^3; min-max, 0.32 – 6.06 μm^3;). The long diameter of the terminals was most frequently in the range of 2 − 4 μm (median, 2.77 μm). The vast majority of the synapses were situated at the outer part of the opposing membrane surfaces in a circular manner, whereas the middle part was occupied by a network of PA. Most of the nigrothalamic terminals (21 out of the 22) were covered with a complete glial sheath on the outer surface of the terminal Serial reconstruction revealed that more than one nigrothalamic terminal of the same axon can innervate the same dendrite. Systematic analysis of the number of terminals/target is beyond the scope of the present account.

Ultrastructural features of large GABAergic terminals in the nigro-recipient thalamus of macaques

Previous data demonstrated that nigrothalamic terminals can be unequivocally identified in the macaque monkey based on the following observations:

1) The only synaptic population in the somata and first order dendrites of relay cells, and the vast majority of the boutons contacting second order dendrites, originate in the SNR. The diameter of these dendrites is thicker than 1 μm

2) The GABA-positive nigrothalamic terminals are characterized by large size, multiple PA, numerous mitochondria and a lack of postsynaptic specialization (Kultas-Ilinsky and Ilinsky, 1990). These features clearly distinguish them from the other two types of vesicle containing GABAergic elements of the primate thalamus i.e. interneuron dendrites and terminals from the reticular thalamic nucleus.

The distribution of vGLUT2-positive terminals in the anterior (motor) portion of the thalamus closely matched the that occupied by cerebellar afferents described in previous tract tracing studies. vGLUT2 has also been used as a marker for cerebellar-recipient thalamic territory in the motor thalamus (not shown). For the electron microscopic analysis the nigrothalamic block was reembedded rostral to the most anterior vGLUT2-positive patches close to the internal medullary lamina, avoiding both pallidal and cerebellar territories (Figure 5A).

Confirming earlier results, all GABAergic boutons contacting the somata and dendrites in our sample displayed the ultrastructural features of nigrothalamic terminals. The ultrastructural characteristics of these terminals were similar to rat nigrothalamic boutons.

Comparison of the 3D ultrastructure of the rat and macaque terminals

Three-dimensional reconstruction revealed that all macaque GABAergic terminals in our sample established multiple synapses on the postsynaptic GABA-negative relay cells, similar to the SNR boutons in rat. The average number of synapses/terminal median (8.5), were surprisingly similar to the rat data. All synapses were established on a single target, like in rat. Two terminals, however, also contacted very thin dendrites with 2 synapses on each. One of these dendrites proved to be a GABA-positive interneuron dendrite.

The arrangement of synapses and PA of the macaque terminals replicated the pattern found in rat nigrothalamic boutons, i.e., a centrally located network of PA surrounded by laterally spaced synapses Glial ensheathment was identified in all cases. The glial processes covered the entire non-opposing surface. The opposing surface, however, was almost completely free of glia.

In case of the GABAergic macaque terminals, the volume (median, 2.91 μm^3) and the length of the longest diameter (median, 2.96 μm) were in the same range as that of the nigrothalamic terminals of rats.

Analysis of the inter-synaptic distances in rat and monkey

Transmitter diffusion and spillover among the synapses significantly affects the magnitude and kinetics of postsynaptic responses. Thus, we measured the inter-synaptic distances in the 3D reconstructed rat and monkey terminals.

The data were analyzed in three ways. First, all of the synaptic distances the median value of all intersynaptic distances were highly similar between rats (900 nm) and monkeys (796 nm). Next we examined the average number of synapses within increasing distances from any given. The rat and monkey data showed very close correspondence. The average number of synapses within 0.5 μm was 1.94 in rats, and 2.11 in monkey. Finally, we examined the nearest neighbor synaptic distance. The median values of the nearest neighbor distances between synapses were 169 nm in the case of rat and 178 nm in the monkey. In both species 80% of the nearest neighbor distances were below 300 nm.

DISCUSSION

In this study, we examined four inhibitory pathways to the thalamus: the nRT-thalamic pathway, the APT- nRT-thalamic pathway, the nigrothalamic pathway in rat, and the nigrothalamic pathway in a primatem the Rhesus Macaque. The nRT-thalamic and the APT-thalamic pathway ended on the same thalamic relay cell in nucleus posterior, allowing examination of the physiological properties of these two GABAergic inputs, which can then be compared with the morphological data.

Organization of the four examined thalamic pathways

The APT-thalamic terminals, the rat and monkey nigrothalamic terminals are very similar to each other. In all three cases we found large terminals with multiple synaptic contacts contacting a single postsynaptic element, which is the proximal dendrites, and rarely the soma, of a thalamic relay cell. Synapses were closely spaced, not separated with glia, and surprisingly, the other side of the terminal was completely covered by glial sheets. The nRT-thalamic terminals are strikingly different, being small, contacting more than one postsynaptic target and mostly establishing a single synapse per postsynaptic target. Punctum adhaerens were rare, and synapses made onto different dendrites were usually separated by astrocytic processes.

Multisite boutons and transmitter spillover

Our data on nigrothalamic pathway in monkey and in rat, the APT-thalamic pathway and the other thalamic inputs, and data from the literature based on single section analysis and 3D analysis suggest that the morphology discovered by this study are common in the brain, and evolutionary conserved across species.

In the case of the three pathways arriving from extra-thalamic sources that we studied, the nearest neighbour synaptic distances were in the range of 200 nm. Closely spaced synapses create favourable condition for transmitter spillover. Spillover among synapses results in larger charge transfer, due to the slower decay of the synaptic currents. This reduces the variability of synaptic transmission. Cross-talk among synapses limits vesicle depletion-based synaptic depression at the GABAergic cortico-nuclear synapses in the cerebellum during prolonged high frequency stimulation. Interestingly, this effect was mediated by multisite terminals of the Purkinje cells. The available data on the activity of SNR neurons supports high baseline firing rates (50-100 Hz) in awake behaving monkeys.

Our previous *in vivo* data on APT terminals suggests that high frequency bursting exists in anesthetized rat, with intraburst frequency reaching 600 Hz .

The receptors or other molecules on the SNR terminals are potential drug targets for treating Parkinson's disease. Molecules affecting transmitter spillover also would be able to modify the function of SNR terminals. The similarity between the monkey and rat nigrothalamic pathway terminals suggests that information transmission depends critically on the morphology we described in this study.

A novel GABAergic terminal type (F3) in the thalamus

Recently, several large GABAergic terminals of extrathalamic origin were characterized as having similar ultrastructural features to APT and SNR boutons in various thalamic nuclei of the rat and monkey brain. GABAergic terminals of the thalamus have been classically subdivided into F1 or F2 terminals (F referring to flattened vesicles). Any axon terminals establishing symmetric synapses were regarded as F1 terminals, whereas the dendritic terminals of interneurons, which participate in serial synapses, were termed F2 terminals. Based on the substantially different morphological features, which apparently characterize several major GABAergic pathways of the thalamus, here we propose to define the "F3" terminal. The distinguishing features of F3 terminals are large size with multiple synapses converging on one target, a meshwork of PA, a complete glia sheet, and a preferential innervation of proximal dendrites.

The extrareticular system

Based on our data, there is another inhibitory system in the thalamus beside the well known reticular system. This extrareticular inhibition arrives from diencephalic centers (APT, ZI) to the sensory thalamus, but to the motor thalamus extrareticular inhibition originates from the basal ganglis output nuclei (SNR, GP). Some parts of this extrareticular system are interconnected, for example APT and ZI. There are several differences among the extrareticular pathways and they originate from several different places, but their thalamic endings are very similar. Since all originate from outside the reticular nucleus, we refer to them collectively as the extrareticular system.

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