Anatomical and physiological characterization of a novel inhibitory thalamic pathway from the zona incerta

PhD theses

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Introduction

The thalamus is the major relay between subcortical structures and the neocortex. Except for olfaction, all sensory information passes through the thalamus, as well as motor information from the cerebellum and basal ganglia, and location information from the mamilary bodies. Also its higher order nuclei connect distant cortical areas with each other. It takes part in the genesis of cortical EEG, modulates information transfer ccording to the sleep-wake cycle, and its widespread lesion can lead to terminal loss of consciousness.

The thalamus contains two types of nuclei, first order and higher order. The former get their driver input from the periphery, the latter get it from layer V. of the cortex.

According to the traditional view, inhibition in the thalamus comes from the thalamic reticular nucleus (nRt), and the local interneurons. During our pilot experiments we have found large, GABAergic terminals in certain higher-order thalamic nuclei, that exhibited the characteristics neither of the nRt terminals (F1), nor that of the postsynaptic dendrites of the local interneurons (F2). From retrograde labelling results we suspected the zona incerta as the origin of these terminals.

Zona incerta, like nRt, developes from the P2 prosomere, and consists mainly of GABAergic cells. It has widespread connections with several brain regions, notably it gets input from the cortical layer V., and peripheral axons, that also innervate the higher order thalamus.

Aims of the study

Earlier retrograde experiments showed that zona incerta projects to the thalamus. The exact innervation pattern, the transmitter of the incertothalamic tract, the ultrastructural characteristics and postsynaptic targets of the terminals, however, is unknown. Also little

is known about the physiological characteristics of zona incerta cells, as well as their relation to cortical oscillations.

Our aims were :

- Mapping the termination pattern of the incertothalamic tract in different thalamic nuclei.
- Characterization of the incertothalamic tract according to terminal size, postsynaptic targets, number of active zones, and other ultrastrucural traits.
- Investigate the GABAergic nature of the projection by postembedding GABA immunostaining.
- Investigate the firing characteristics of zona incerta neurons in anesthetized animals, under different levels of anesthesia.
- Quantify the phase locking of zona incerta cells to ongoing cortical activity, and compare it to that of the thalamocortical cells.
- Investigate the light and electron microscopic characteristics of the cortico-incertal pathway, responsible for cortical control over the zona incerta.

Methods

Anterograde tracing with BDA and PHAL

Seventeen male Wistar rats (250±350 g) were used for the biotinylated dextran amine (BDA; 3000 MW, Molecular Probes, Leyden, The Netherlands; 10% in physiological saline) tracing, and seven rats for the Phaseolus vulgaris leucoagglutinin (PHAL; Vector, 2.5% in 0.01 PB pH 8.0) tracing experiments. BDA or PHAL were injected with a glass capillary under deep Equithesin anaesthesia. Coordinates for the zona incerta injections were anteroposterior 3.7-4.5 caudal to Bregma, lateral 2.4-2.6 mm from the midline, dorsoventral 6.9-7.1 mm. After 6-8 days survival time, the animals were perfused and processed for immunohistochemistry.

Pre-embedding immunostaining

For single immunostaining, consecutive sections were treated with rat anti-m2 (1 : 300; Chemicon International Inc., Temecula, CA, USA), rabbit anti-parvalbumin, rabbit anti-calbindin (both at 1 : 2000; Baimbridge & Miller, 1982) or rabbit anti-calretinin (1 : 5000 (Rogers, 1989) for two days at 4 °C. The second layer was biotin-SP conjugated antirat IgG (1 : 300, Jackson Immuno Research, West Grove, PA, USA) or biotinylated anti-rabbit IgG made in goat (1 : 300, Vector Laboratories, Burlingame, CA, USA) for 2 h followed by avidin-biotinylated horseradish peroxidase complex (ABC, Vector Laboratories, 1 : 300) for 2 h. All the washes and dilutions of antisera were done in 0.05 M Tris-buffered saline (TBS), pH 7.4. The immunoperoxidase reaction was developed with 3,3¢-diaminobenzidine (DAB) as a chromogen. The sections were treated with 1% OsO4 in 0.1 M PB for 45 min, dehydrated in ethanol and propylene oxide, and embedded in Durcupan (ACM, Fluka, Buchs, Switzerland). During dehydration the sections were treated with 1% uranyl acetate in 70% ethanol for 40 min.

Pre-embedding double immunostaining

In this case, the first tracer was visualized by nickel-intensified DAB (DAB-Ni) instead of DAB. This yields a black reaction product and allows a second reaction to be developed with DAB (brown). Following visualization of the tracer with DAB-Ni, sections were incubated with rabbit anticalbindin (1 : 2000 Bainbridge and Miller, 1982) for 2 days, then with goat anti-rabbit IgG (1 : 2000; Sterberger Monoclonals Inc., Luthervill, MD) for 2 h, followed by rabbit peroxidase-anti-peroxidase complex (1 : 400 Sternberger) overnight, and developed with DAB.

Postembedding immunostaining for GABA

Ultrathin sections were cut on an ultramicrotome and mounted on nickel grids. Postembedding GABA immunostaining was carried out on the grids according to the protocol of Somogyi et al. (1985). Profiles containing at least five times higher density of gold particles than neighbouring asymmetrical (presumed glutamatergic) synaptic terminals in 2-3 serial sections were considered as GABA-positive. The electron micrographs were taken on a Hitachi 7100 electron microscope.

In vivo *electrophysiology*

Experiments were conducted under urethane anesthesia [20% urethane (Sigma-Aldrich, St. Louis, MO) in saline (0.12– 0.15 g/100 g body weight; *n*=30)] in Wistar (*n*=15) or Sprague Dawley (*n* =14) rats. To achieve large-amplitude cortical local field potential (LFP), additional doses of ketamine (10 mg/kg, i.m.; Henry Schein, Melville, NY) were given to the rats (*n* = 21). Monopolar tungsten electrodes (*in vitro* impedance, 0.8 –1.2 MΩ; FHC, Bowdoinham, ME) were implanted in layer V of the primary somatosensory cortex (2 mm posterior, 4 mm lateral from bregma; 1.5mm deep from the cortical surface) to monitor cortical LFP and multiunit activity (MUA). A stainless steel reference screw was placed above the cerebellum.

An extracellular broadband signal was amplified with 1000x gain, filtered between 0.1 and 5 kHz (BioAmp; Supertech, Pecs, Hungary) and digitized at 16.6 kHz (micro 1401 mkII; Cambridge Electronics Design, Cambridge, UK). Cortical MUA was derived from the bandpass-filtered (0.8–5 kHz) LFP recordings by detecting deflections from baseline exceeding 3.5 SDs.

Pipettes for juxtacellular recordings of 20–60 MΩ were pulled from borosilicate capillary glass (1.5 mm outer diameter, 0.86 mm inner diameter; Sutter Instruments, Novato, CA) filled with 0.5 M NaCl or 0.5 M CH3COONa and 1.5–3% Neurobiotin (Vector Laboratories, Burlingame, CA) or 2–3% biocytin (Sigma-Aldrich).

Electrodes were lowered into the thalamus and the ZI (3.6–4.3mmposterior, 2.5mmlateral from bregma; 4–6.2 and 6.8 –7.2 mm deep for the thalamus and ZI, respectively) by a piezoelectric microdrive (6000 ULN; EXFO Burleigh, Quebec, Quebec, Canada). Neuronal activity was amplified by aDCamplifier (Axoclamp 2B; Molecular Devices, Foster City, CA) with 1000x gain, filtered between 0.1 and 5 kHz by a signal conditioner (Supertech) and digitized at 16.6 kHz (Cambridge Electronics Design). Recordings were made in the "extracellular" position for 30 min to 2 h, after which the capillary was advanced to reach the "juxtacellular" position for labeling (Pinault, 1996). Depolarizing current pulses of sufficient amplitude (0.5–8 nA) were applied to drive the cell firing for 2–10 min.

Data analysis

Spike detection and data analysis were performed in Matlab (Mathworks, Natick, MA). Power spectra were estimated using Welch's method (Matlab pwelch function), with 2.44 Hz resolution. Autocorrelograms and cross-correlograms were computed using 10 ms time bins To assess the degree of cortical modulation in ZI cells, two indices were used. To calculate the MUA index, the cortical MUA–ZI single-unit cross-correlogram was normalized to unit area and smoothed using a 55 ms Hamming window. The MUA index was defined as the SD of the smoothed and normalized cross-correlogram. The STA index was computed as the amplitude of the central trough of the normalized STA of the cortical LFP triggered by the firing of a ZI unit.

Results

Anterograde tracing from zona incerta

After tracer injections into the zona incerta large, varicose fibers were found in higher order thalamic nuclei. e following nuclei recieved afferent fibers: posterior , angular, ethmoid, rethroethmoid, and posterior triangular nuclei (higher order somatosensory nuclei), suprageniculate, posterior limitans, and scattered fibres in the medial part of medial geniculate body (higher-order auditory nuclei); the ventral and medial aspects of the laterodorsal nucleus and a few fibres in the mediorostral and dorsal part of lateral posterior nucleus (higher-order visual nuclei). From the higher-order intralaminar group the centrolateral, paracentral, central medial, parafascicular and posterior intralaminar nuclei were labelled, whereas from the midline group only the reuniens nucleus contained considerable numbers of fibres. No labeled fibres were found in first order nuclei.

We found a loose topographical organization of the incertothalamic pathway. In one animal (B39), which received PHAL injection into the ZI at the level of the intermedioventral thalamic commissure, and where the diameter of the injection site was < 1 mm in the anteroventral and mediolateral dimensions, labelled fibres were observed from the anterior (ventral anterior and reuniens nuclei) to the caudal end of the thalamus (posterior triangular and posterior limitans nuclei). More caudal injections did not appear to systematically change the number of innervated nuclei though the relative density of fibres among the nuclei was altered. More lateral injections resulted in a higher number

of fibres in the higher-order visual nuclei. Only few injections were localized more or less selectively to the dorsal or ventral ZI. The density of labelled fibres appeared to be lower following dorsal ZI injections.

Co-distribution of incerto-thalamic fibres and calbindin-positive relay cells

Higher order nuclei contain a higher concentration of calbindin-positive relay cells than first order nuclei. When examined in double-immunostained sections the distribution of incerto-thalamic fibres showed close correspondence to the arrangement of calbindin-positive relay cells in several higher-order nuclei. BDA- and PHAL-labelled fibres were frequently found to establish multiple contacts on the proximal dendrites of calbindin-positive relay cells. In the first-order ventrolateral nucleus the oblique band of incerto-thalamic fibres exactly matched the strip of calbindin-positive relay cells which traverses this nucleus throughout its antero-posterior extent. Incerto-thalamic fibres, however, were also found in certain parts of the parafascicular, laterodorsal and angular nuclei, which contain few calbindin-positive relay cells. On the other hand, in the caudal thalamus large regions were immunoreactive for calbindin and yet incerto-thalamic terminals were restricted to certain patches only.

Light-microscopic features of incerto-thalamic terminals

Incerto-thalamic terminals showed significant heterogeneity in size and shape. Most of them were fusiform or elongated, but irregularly shaped or spherical terminals were also encountered. The majority of them were en passant, whereas others had a short stalk. The terminals were distributed unevenly along the axons. Long, terminal-free, intervaricose segments alternated with clusters of 4-8 closely spaced terminals. In many cases, clusters of large terminals formed by several axons appeared to outline immunonegative structures which resembled the size and shape of the thick proximal dendrites of relay cells. The largest of these terminals reached 9 mm along their long axis, but the majority of the terminals were in the range of 2-7 mm (major axis) by 1-3 mm (minor axis).

Ultrastructural characteristics of incerto-thalamic terminals

The size, shape and synaptic organization of incerto-thalamic axon terminals varied considerably. Confirming light microscopic observations, some of them reached a giant size (minor axis 1-2 mm, major up to 6-8 mm). Incerto-thalamic terminals established almost exclusively symmetrical synapses, frequently with multiple release sites. Perforated synapses with multiple active zones targeting single postsynaptic elements were commonly observed. In other cases, single boutons established synapses on different postsynaptic targets.

The electron microscopic study revealed that the clustering of large terminals observed at the light microscopic level indeed occurs around a single postsynaptic element. In many cases several large incerto-thalamic terminals surrounded the thick proximal dendrite of relay cells, each establishing multiple release sites. Punctum adherens-like specialisations, which are also called filamentous contacts were a common feature of the incerto-thalamic large terminals and they were generally close to synapses.

The large incerto-thalamic terminals were many times in close proximity to large principal afferents. Occasionally the entire complex, i.e. the principal afferent, incerto-thalamic terminal and the proximal dendrite, were surrounded by astrocytic processes in a glomerulus-like manner. Smaller incerto-thalamic terminals established multiple release sites and filamentous contacts less frequently than their large counterparts.

The major postsynaptic elements of the incerto-thalamic terminals were thick proximal dendritic shafts of relay cells or exceptionally somata. We compared the minor diameter of 34 dendrites postsynaptic to incerto-thalamic terminals in Po with the minor diameter of 100 randomly selected dendrites. The two populations were significantly different (Mann-Whitney U-test, $P < 0.01$). Incerto-thalamic terminals selectively innervated thick dendrites (mean diameter 1.34 mm), and largely avoided dendrites thinner than 1 mm, which were the most abundant (mean diameter for the random sample, 0.65 mm). Dendrites thicker than 1 mm are known to receive principal afferent input and are mostly ignored by corticothalamic terminals originating in layer VI.

Incerto-thalamic terminals are GABAergic

A random sample of 73 boutons labelled by the anterograde tracer BDA or PHAL from three different animals were examined for GABA immunoreactivity by the postembedding immunogold method. Only profiles which were immunoreactive on three consecutive sections were considered positive. In the first animal, receiving a larger injection of BDA, 20 out of 25 (80%) labelled terminals in Po were GABA-positive. In the second animal, receiving smaller BDA injection, 29 out of 29 (100%) anterogradely labelled boutons showed GABA immunoreactivity in Po. In the third animal, we examined 19 PHAL-labelled boutons in the centrolateral nucleus, of which 17 were GABA-positive (89.4%). In summary, 66 of 73 labelled incertothalamic terminals were found to contain GABA (90.4%). The postsynaptic targets were in all cases GABA-negative.

Local field potential states vary under urethane anesthesia

Under urethane anesthesia, the depth of anesthesia varied spontaneously. Three LFP stages were distinguished in our study.

In the first stage, typically present during light anesthesia, the LFP was characterized by low-amplitude fast oscillations in the beta and gamma frequency ranges. In this state, the neocortex did not express significant rhythmic activity (except in the low-amplitude gamma frequency range.

In the second stage the LFP showed large amplitude, irregular oscillations in the delta frequency range (1–4 Hz).

The third state was characterized by large amplitude, highly regular 3.5 Hz oscillations, as high-voltage spindles (HVSs). This pattern arose 20–30% of the animals.

Firing pattern of ZI cells under urethane anesthesia

Thirty-five ZI cells were recorded extracellularly, followed by the juxtacellular labeling procedure.

The firing rate of ZI cells varied widely among different neurons between 0.08 and 41.67 Hz (mean, 10.58±11.07 Hz). During fast oscillation-dominated epochs, the cells typically fired single spikes in a tonic or irregular manner. Occasional high-frequency spike

transients reached the frequency of 70 Hz, but this activity was rarely observed.. The discharge activity during this state demonstrated no apparent relationship to the cortical LFP or cortical MUA.

Twenty ZI neurons were examined under synchronized LFP states together with well discriminated cortical MUA. With the emergence of slow waves, the tonic firing was replaced by rhythmic single spikes or clusters of spikes, which coincided with the slow cortical LFP waves in the majority of ZI neurons
(16 of 20). The magnitude of modulation differed from cell to cell. The maximum firing rate during upstate-associated spike clusters never exceeded 50 Hz, which is clearly distinct from the burst firing mode of TC cells characterized by high-frequency (400–500 Hz) spike clusters during slow cortical oscillation.

During slow oscillations, the discharge frequency of ZI neurons was slower (1.53 ±1.53 Hz) compared with fast oscillation-dominated LFP states (6.33±0.79 Hz) . However, a small number (*n* = 3) of ultraslow ZI neurons (firing rate below 0.5 Hz) actually increased their firing rates as they locked their activity to the 1–3 Hz slow waves

ZI neurons, the activity of which was modulated by the slow waves, fired preferentially during the depth-negative phase (the "active" phase or "up state") of the slow LFP oscillation. The firing rate of modulated neurons during the up states approached he firing rate observed during he fast oscillation-dominated LFP states 3.88±2.79 Hz vs 6.33±4.79 Hz, respectively).

To examine the timing of ZI euronal activity relative to the firing of ortical cells, the peak of the crosscorrelograms between ZI single-cell activity and MUA was calculated. The average peak of the cross-correlogram was at 1.09 ms with little variability (±1.76 ms), which suggests the coupling of ZI neuron firing to cortical MUA.

Four of the 20 ZI neurons recorded during slow cortical oscillations did not change their tonic firing pattern with the occurrence of slow waves. These tonic were characterized by flat STA and ZIunit-MUA cross-correlogram

Neurons that were strongly modulated, and those that were not modulated by cortical activity could be recorded in the same track within the ZI relatively close to each other. The firing frequency of tonic cells was significantly higher during the fast oscillation-dominated LFP states (19.8 ±8.81 Hz) than that of the rhythmic cells

(6.33 ±4.79) ($p < 0.0001$, two-sample t test), and they reacted with a smaller decrease in the firing rate with the emergence of slow waves in the LFP (16.9%) than rhythmic cells (75.8%).

The effect of paroxysmal events on ZI activity

Six of the 14 Sprague Dawley rats exhibited epochs of HVS activity spontaneously or after ketamine injection,during which the activity of seven ZI neurons was recorded. HVSs are characterized
by highly synchronous discharges (3.5 Hz) of cortical neurons, followed by their virtual silence. Five of seven ZI neurons showed significant modulation by these paroxysmal events. Three of the affected neurons increased their firing activity considerably during HVS.Two cells were more active on the positive phase of HVS, whereas the remaining two cells were not affected. Interestingly, these latter two cells were entrained by the cortical activity when HVSs were replaced by normal slow LFP oscillations.

Comparison of incertal and thalamic unit activity during synchronized cortical oscillations

TC neurons are known to be involved in cortical rhythm genesis. For the sake of comparison, we recordedfrom 21 TC cells under the same experimental conditions.
TC cells exhibitedtonic firing under fast oscillation-dominated LFP states with no apparent relationship to cortical activity. The firing frequency during these states in different cells varied between 4.29 and 16.72 Hz (mean, 11.51±4.92 Hz; SD). Ten TC neurons were recorded under spontaneous or ketamine-induced synchronized LFP states. Under this condition, the TC cells switched to burst firing mode.
TC cells were moderately synchronized to the LFP activity. During slow oscillations, the firing frequency decreased to 1.41 Hz.
The magnitude of synchronization between ZI neurons and cortical activity was assessed by two separate measures: ZI (or TC) spike-triggered LFP average index (STA index) and single unit (ZI or TC) versus cortical multiunit cross-correlation index (MUA index) (see Methods), generated from the respective data.

These measures enabled us to compare the synchrony of ZI and TC neurons to the LFP waves. Because both parameters depend on the relationship between the unit and the cortical activity, the two indices were correlated. The four tonic ZI cells (20% of the ZI cells in this analysis) had the lowest synchrony values. Eleven of the 16 modulated ZI cells (55% of the cells) had synchrony values comparable to TC cells, whereas the remaining five modulated ZI neurons (25% of all cells) far exceeded those of the TC neurons. These data demonstrate that in these conditions, the activity of a large proportion of ZI cells are similarly or better synchronized to cortical slow oscillation thanTC cells.

Conclusions

- Zona incerta projects to higher order thalamis nuclei.
- Incerto-thalamic terminals are large in size, they form multiple symmetric synapses on the thick proximal dendrites of thalamic relay cells.
- The incerto-thalamic projection is GABAergic.
- Zona incerta cells fire without burts in a tonic manner, their firing frequency is highly variable.
- We have distinguished two types of zona incerta cells; phasic cells synchronize to cortical slow oscillations, whereas tonic cells fire independently of the cortex.
- The degree of synchronization with the cortex of the phasic cells was comparable or larger than that of the thalamic relay cells.
- The cortico-incertal projection runs parallel to the dendrites of zona incerta cells. It forms asymmetrical synapses on the thick dendrites and spines of zona incerta neurons.

List of publications

Publications used in the thesis

Bartho P, Freund TF, Acsady L.
Selective GABAergic innervation of thalamic nuclei from zona incerta.
Eur J Neurosci. 2002 Sep; 16(6): 999-1014

Bartho P, Freund TF, Acsady L.
Differential distribution of the KCl cotransporter KCC2 in thalamic relay and reticular nuclei.
Eur J Neurosci. 2004 Aug;20(4):965-75.

Barthó P, Slézia A, Varga V, Bokor H, Pinault D, Buzsáki G, Acsády L.
Cortical control of zona incerta.
J Neurosci. 2007 Feb 14;27(7):1670-81.

Other publications

Csicsvari J, Henze DA, Jamieson B, Harris KD, Sirota A, Bartho P, Wise KD, Buzsaki G.
Massively parallel recording of unit and local field potentials with silicon-based electrodes.
J Neurophysiol. 2003 Aug; 90(2): 1314-23

Hirase H., Creso J., Singleton M., Bartho P., Buzsaki G.
Two-photon imaging of brain pericytes in vivo using dextran conjugated dyes
Glia. 2004 Apr 1;46(1):95-100.

Bartho, P., Hirase, H., Monconduit, L., Zugaro, M., and Buzsaki, G.
Characterization of neocortical principal cells and interneurons by network interactions and extracelluar features
J Neurophysiol. 2004 Mar 31

Hirase H., Qian L., Bartho P., Buzsaki G.
Calcium dynamics of the cortical astrocytic network in vivo
PLoS Biol. 2004 Apr;2(4):E96. Epub 2004 Apr 13.

Luczak A, Barthó P, Marguet SL, Buzsáki G, Harris KD.
Sequential structure of neocortical spontaneous activity in vivo.
Proc Natl Acad Sci U S A. 2007 Jan 2;104(1):347-52. Epub 2006 Dec 21.