Published in final edited form as:

J Comp Neurol. 2008 January 1; 506(1): 122–140. doi:10.1002/cne.21545.

Heterogeneous output pathways link the anterior pretectal nucleus with the zona incerta and the thalamus in rat

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Abstract

The anterior pretectal nucleus (APT) and the zona incerta (ZI) are diencephalic nuclei that exert strong inhibitory influence selectively in higher order thalamic relays. Besides the thalamus, APT is also known to project to ZI, but anatomical details of the APT-ZI projection have not been described.

In the present study, the efferent pathways of APT were examined in the APT-ZI-thalamus network using anterograde and retrograde tracings in combination with pre- and postembedding immunocytochemical stainings and in situ hybridization. The vast majority of APT fibers selectively innervated the parvalbumin-positive, ventral part of ZI, which contains ZI neurons with axons projecting to higher-order thalamic nuclei. The APT-ZI pathway consisted of both GABA-negative and GABA-positive components; 38.2 % of the terminals in ZI contained GABA, and 8.6 % of the projecting somata in APT were GAD67 mRNA-positive. The combination of parvalbumin immunostaining with retrograde tracing showed that strongly and weakly parvalbumin-positive as well as parvalbumin-negative neurons were all among the population of APT cells projecting to ZI. Similar heterogeneity was found among the APT cells projecting to thalamus. Double retrograde tracing from higher-order thalamic nuclei and their topographically matched ZI regions revealed hardly any APT neuron with dual projections.

Our data suggest that both ZI and the higher-order thalamic relays are innervated by distinct, physiologically heterogeneous APT neurons. These various efferent pathways probably interact via the rich recurrent collaterals of the projecting APT cells. Therefore, the powerful, GABAergic APT and ZI outputs to the thalamus are apparently co-modulated in a synergistic manner via dual excitatory and inhibitory APT-ZI connections.

Keywords

somatosensory; thalamocortical; GABA; ascending control; higher order relays; diencephalon

INTRODUCTION

The anterior pretectal nucleus (APT) is at the cross-road of major ascending and descending pathways in the caudal diencephalon. Its role in somatosensory functions, including antinociception and behavioural aversion, is well-established in rodents (Brandao et al., 1991; Foster et al., 1989; Terenzi et al., 1995; Villarreal et al., 2004). The APT contains morphologically and neurochemically diverse cell populations including a high

concentration of GABAergic neurons (Esclapez et al., 1994); (Benson et al., 1992). APT neurons display distinct firing patterns, which show different degrees of correlation with synchronously recorded cortical activity (Bokor et al., 2005). APT is innervated by several brainstem nuclei including the spinal trigeminal nucleus (Veinante et al., 2000) and the superior colliculus, and by somatosensory- and visual areas of neocortex (Cadusseau and Roger, 1991; Foster et al., 1989). Its descending projections reach the superior colliculus, the pontine nuclei and the medullary sensory nuclei (Terenzi et al., 1995; Zagon et al., 1995), whereas its ascending fibers have two main targets: the thalamus and the zona incerta (ZI) (Bokor et al., 2005; May et al., 1997).

The APT-thalamic pathway selectively innervates the so called higher-order thalamic nuclei (Bokor et al., 2005), mainly via giant, GABAergic terminals. These APT-thalamic boutons contact the proximal dendrites of relay cells via multiple synapses and exert profound inhibitory control on thalamic activity. Indeed, activation of a single APT-thalamic fiber is able to induce rebound bursts in a relay cell *in vitro* (Bokor et al., 2005).

The other major diencephalic target of the APT, the zona incerta, shares many inputs and outputs with the APT (for a review see Mitrofanis, 2005). ZI also contains a large population of GABAergic cells (Benson et al., 1992; Esclapez et al., 1994) and its ascending axons form giant GABAergic terminals selectively in higher-order thalamic nuclei, showing similar ultrastructure to APT terminals (Bartho et al., 2002). The activity of many ZI cells shows similar correlation to cortical slow oscillations than that of certain APT cells (Bartho et al., 2007) and ZI has been shown to exert strong inhibition on the relay of somatosensory inputs in higher-order thalamic nuclei *in vivo* (Trageser and Keller, 2004; Lavalley et al., 2005).

The considerable similarity between these two inhibitory (APT-thalamic and ZI-thalamic) pathways suggests that their effects on the thalamus could be synchronized. Indeed, a major pathway from APT to ZI in both cat and rat have already been described (Shammah-Lagnado et al., 1985; May et al., 1997). An APT-ZI projection has also been demonstrated in primates (Benevento et al., 1977; Johnson et al., 1992). Based on the ultrastructure of synapses, the APT-ZI pathway was proposed to be purely excitatory (May et al., 1997). However, postembedding GABA reaction was not performed in this latter study to unequivocally demonstrate the presence or absence of a GABAergic component among these fibers. Furthermore, it has not been examined whether the same APT neurons innervate the thalamus and the ZI, providing a possible route of synchrony between the APT-thalamic and ZI-thalamic activity. Finally, based on the combination of parvalbumin (PV) content and firing pattern, three populations of APT cells were distinguished recently, strongly PV-positive “fast bursting cells”, weakly PV-positive “tonic cells”, and PV-negative “slow rhythmic cells” (Bokor et al., 2005). This raises the possibility that APT-ZI and APT-thalamic neurons could be distinguished based on their PV-content and the correlated firing patterns.

In the present study we used anterograde tracing from APT, as well as retrograde tracing from ZI and from the higher-order thalamic nuclei. This was combined with immunocytochemistry and in situ hybridization to study the APT-ZI projection, and to compare it to the APT-thalamic pathway. This allowed us to formulate predictions about how the APT-thalamic and APT-ZI pathways are synchronized, and to form a functional hypothesis about the APT-ZI-thalamic network.

MATERIALS AND METHODS

Male Wistar rats were used (n=15, 50-60 days old, weight between 300-400 g; supplier Medical Gene Technology Unit, Institute of Experimental Medicine, Hungary). All experimental procedures were performed according to the ethical guidelines of the Institute of Experimental Medicine, Hungarian Academy of Sciences and approved by the Ethical Committee.

Anterograde tracing

Surgery, perfusion—Rats (n=7) were deeply anaesthetized by Equithesin (chlornembutal, 0.3 ml/100 g) and were mounted in a stereotaxic frame. The animals received iontophoretic injections of Biotinylated dextran amine (BDA 10000 MW, Molecular Probes, Leiden, The Netherlands) (n=7; injection parameters: 10% BDA in PB, 2 μ A, 2 s on/off duty cycle for 10 min) or *Phaseolus vulgaris* leucoagglutinin (n=7; PHAL, Vector Labs, Burlingame, CA, 2.5% in 0.1M phosphate-buffer [PB, pH 7.4]; 5 μ A, 7 s on/off duty cycle for 10 min) via a glass capillary (borosilicate, Shutter Instrument, 10-30 μ m outer diameter at the tip), at the following coordinates: 4.8-5.2 mm posterior, 1.7-2.0 mm lateral, and 4.5-5.5 mm ventral to the Bregma, according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). Since the APT-ZI projection has virtually no contralateral component, both hemispheres were injected to reduce the number of animals used. The hemispheres were handled separately and were treated as independent cases (Table 1). The thalami of some of these injections were used in our previous study (Bokor et al., 2005).

After a survival time of 5-7 days, rats were deeply anaesthetized by Equithesin, then perfused through the heart, first with physiological saline (3 min), then with 100 ml of a fixative containing 2% paraformaldehyde (TAAB, UK) and 0.5% glutaraldehyde (TAAB) in acetate buffer (pH 6.0; 5 min), and finally with 500 ml fixative containing 2% paraformaldehyde and 0.5% glutaraldehyde in borate buffer (pH 8.5; 50 min) (Berod et al., 1981).

Preembedding immunocytochemistry—Coronal sections (50-60 μ m thick) were cut on a Vibratome, washed, cryoprotected in 30% sucrose in 0.1 M PB overnight, and freeze thawed in aluminium foil boats over liquid nitrogen. Before the incubation with primary antibodies, the sections were treated with sodium-borohydride (1% in 0.1 M PB for 15 min). Bovine serum albumin (BSA, 3% in TRIS-buffered physiological saline, TBS) was used as blocking serum.

The position of the injection sites were verified with the help of the atlas of Paxinos and Watson (Paxinos and Watson, 1998) and by simultaneous, fluorescent visualization of the tracer and parvalbumin (PV), a marker for APT. In the case of PHAL injections, the sections at the level of APT were incubated first in rabbit anti-parvalbumin antibody (1:4000; together with biotinylated goat anti-PHAL (1:400) overnight, followed by Alexa 594-conjugated goat anti-rabbit F_{ab} fragment (GAR-A594; 1:500, 3 h; Molecular Probes) and Alexa 488-conjugated streptavidin (StA 488; 1:2000, 3 h; Molecular Probes).

The parvalbumin antiserum kindly donated by Dr. Kenneth G. Baimbridge was raised against rat muscle parvalbumin extracted from isoelectric focusing gels of rat muscle extracts. Immunostaining of the hippocampus with this antiserum was completely prevented by preincubation of the antiserum (1:5000) with 10 ng/mL (Calbiochem) rat muscle PV (Sloviter, 1989). Further characterization of the antiserum, reported by Mithan et al. (1987) showed that in SDS-PAGE of rat brain and muscle soluble proteins the antiserum labels a single band, which corresponds to rat muscle PV. The antiserum was reported to have no cross-reactivity with other calcium binding proteins (Mithani et al., 1987). The antiserum

gave identical staining to another mouse parvalbumin antiserum against carp muscles (see below).

The affinity purified biotinylated goat anti-PHAL antiserum (Vector, Code No BA0224) was prepared against the plant protein Phaseolus vulgaris agglutinin, which is normally not present in the brain. Brain regions free of the tracer showed absolutely no immunostaining demonstrating the specificity of the antibody. In the case of BDA injection sites, parvalbumin was visualized as above, whereas BDA was visualized directly with StA 488. Only those injections were considered for the further observations that displayed no retrogradely-labeled neurons in zona incerta (n=10, see Table 1). Thus, the local collaterals of these cells were negligible in our samples.

To visualize BDA for light microscopic observations, sections were incubated with avidin biotinylated-horseradish peroxidase complex (ABC; Vector Laboratories; 1:300) in TBS for 2 hours, then developed with nickel intensified 3,3'-diaminobenzidine (DABNi) resulting in a black reaction product. Sections from animals injected by PHAL were first treated with biotinylated goat anti-PHAL (1:400) in TBS overnight, followed by ABC and then DABNi.

Double staining was used to examine the anterogradely labeled fibers and parvalbumin in ZI. Following the visualization of the tracer (see above) the sections were incubated in rabbit anti-PV (1:4000; in TBS) overnight, followed by biotinylated goat anti-rabbit (bGAR; 1:300; in TBS; 3 h) and developed by an ABC-DAB reaction, which results in a brown reaction product. Sections were treated with OsO₄ (1% in 0.1 M PB; 45 min) containing 7% glucose, dehydrated in ethanol and propylene oxide and embedded in Durcupan (ACM, Fluka, Buchs Switzerland). As a result, the color differences between the black (DABNi) and the brown (DAB) precipitations remained distinguishable.

Combination of pre- and postembedding immunocytochemistry for electron microscopy—Three injections were used for electron microscopic observations (see Table 1). In our experimental conditions, postembedding GABA immunogold labeling was not always reliable for quantitative purposes when DAB was used as a chromogen for the tracers. The DAB precipitate could not be etched properly from the heavily labeled terminals, which increased the chance of identifying a terminal as falsely GABA-negative. To overcome this difficulty, the tracer was visualized by the preembedding gold method as described before (Bokor et al., 2005). Briefly, for preembedding immunogold staining, in case of PHAL, first rabbit anti-PHAL (1:10000) was used, followed by biotinylated goat anti-rabbit and ABC. The affinity purified rabbit anti-PHAL antiserum (Vector, Code No AS 2300) was prepared against the plant protein Phaseolus vulgaris agglutinin, which is normally not present in the brain. Brain regions free of the tracer showed absolutely no immunostaining demonstrating the specificity of the antibody. In the case of BDA, sections were simply incubated with ABC. Following ABC, in both cases the signal was amplified by biotinylated tyramide reagent (1:50, 15 min; PerkinElmer Life Sciences, Boston, MA), then sections were incubated in 1 nm gold-conjugated streptavidin (1:50; Aurion, Wageningen, The Netherlands) dissolved in TBS, containing 0.8% BSA and 0.1% gelatine overnight, postfixed in 2% glutaraldehyde in TBS, then silver intensified with Aurion R-Gent intensification kit (17-20 min).

All sections were treated with OsO₄ (1% for 1 min and 0.5% for 20 min in 4°C), 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 minutes. Selected blocks containing labeled pretecto-incertal terminals were reembedded and 55-60 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. (Somogyi et al., 1985).

The rabbit anti-GABA antibody kindly donated by Prof. Péter Somogyi (MRC Unit Oxford) was raised against GABA bound to bovine serum albumin. The reactivities of the antibody to GABA and other structurally related compounds were tested by coupling these compounds to nitrocellulose paper activated by polylysine and glutaraldehyde and incubating the paper with the unlabeled enzyme. The antisera did not react with L-glutamate, L-aspartate, D-aspartate, glycine, taurine, L-glutamine, L-lysine, L-threonine, L-alanine, alpha-aminobutyrate, beta-aminobutyrate, putrescine or delta-aminolevulinate (Hodgson et al., 1985). Immunodot staining of GABA was completely abolished by adsorption of the sera to GABA coupled to polyacrylamide beads by glutaraldehyde (Hodgson et al., 1985). GABA immunostaining was abolished by solid phase adsorption to GABA but the staining was not affected by adsorption to glutamate, aspartate, taurine glycine, cholecystokinin or bovine serum albumine (Somogyi et al., 1985). GABA and GAD immunostaining labeled identical type of axon terminals in the cerebellum and the striate cortex. GAD immunoreactivity was weaker in the somata compared to GABA (Somogyi et al. 1985). Beside these regions, the specificity of this antiserum has been demonstrated in the following physiologically identified GABAergic pathways e.g.: septohippocampal (Freund and Antal, 1988), striato-nigral, pallido-nigral (von Krosigk et al., 1992) APT-thalamic (Bokor et al., 2005), incerto-thalamic (Bartho et al., 2002).

Incubations were performed on droplets of solutions in humid Petri dishes. Briefly, 0.5% periodic acid (H_2IO_6) for 5 min; wash in distilled water; three times 2 min in TBS; 30 min in 1% ovalbumin dissolved in TBS; three times 10 min in TBS containing normal goat serum (NGS); 1-2 h in a rabbit anti-GABA antiserum (1:3000 in NGS/TBS); two times 10 min TBS; 10 min in TB containing 1% bovine serum albumin, 0.05% Tween 20. 1% NGS; 2 h colloidal gold-conjugated goat anti-rabbit IgG (12 nm; Jackson, 1:20 or 15 nm, Amersham, Little Chalfont, UK, 1:20 in NGS/TBS) in the same solution as before; two times 5 min wash in distilled water; 30 min saturated uranyl acetate; wash in distilled water; staining with lead citrate; wash in distilled water.

Boutons were identified as labeled by the tracer only if they were found to contain silver-intensified gold particles in at least 3 consecutive sections ($n=76$). They were regarded as GABA-positive if they contained the GABA signal on at least 3 sections and the number of gold particles was at least five times higher than background.

The ultrathin sections were analyzed with a HITACHI 7100 and a JEOL JEM1011 electron microscope, electron micrographs were made by Megaview II (lower resolution, Soft Imaging System, Munster, Germany) and Cantega (high resolution, Soft Imaging System, Munster, Germany) digital cameras. Brightness and contrast were adjusted when necessary using Adobe Photoshop 7.0 applied to whole images only. Digital montages were created using the 'extended depth of field' function of Image-Pro Express 6.0 software (Media Cybernetics). The diameter of the APT-ZI terminals and their postsynaptic elements were measured using AnalySiS software. Standard deviation values were calculated using Microsoft® Excel 2002.

Retrograde tracing

Surgery, perfusion—The animals ($n=8$) were anaesthetized with ketamine (75 mg/kg) plus xylazine (5 mg/kg). BDA 3000 ($n=1$ for ZI, $n=4$ for Po; BDA 3000 10% in saline) and Fluorogold ($n=5$ for ZI, $n=4$ for Po; FG; Fluorochrome, Denver, Colorado, USA) were used as retrograde tracers. FG was injected with 300-500 nA positive current pulses (2 s on/off duty cycle, 25 min). BDA injections were made iontophoretically (5 μ A, 8 s on/off duty

cycle, 20 min) and subsequently by pressure (1 μ l) as well. Glass capillaries (borosilicate, inner diameter 20-50 μ m) were used for all injections. Injections into Po were made according to the coordinates of the atlas of Paxinos and Watson (Paxinos and Watson, 1998): 3.3 mm posterior, 2.5 mm lateral to the Bregma, and 5.3-5.9 mm ventral from the surface of the brain. For injections into ZI, the lateral and the antero-posterior coordinates were determined by the atlas of Paxinos and Watson (Paxinos and Watson, 1998); 4.3 mm posterior, 3.1 mm lateral to the Bregma. The exact dorso-ventral coordinate (6.1-7.1 mm ventral from the surface of the brain) of the injection in the zona incerta was determined by extracellular recording, identifying the characteristic firing activity of ZI cells (Bartho et al., 2007). In six animals, both Po and ZI were injected, ipsilaterally in 5 animals (using FG and BDA) and contralaterally in 1 animal (using FG) (see Table 2). In two animals, only Po was labeled unilaterally by FG. After 3-4 days, animals were anaesthetized with Equithesin and perfused through the heart (see above). In the experiments without *in situ* hybridisation (n=3 animals), we used the same 2-step fixative procedure described for the anterograde tracing, except that the fixatives did not contain glutaraldehyde. For the experiments (n=5) involving *in situ* hybridisation, we used physiological saline (3 min) and then 300 ml of a fixative containing 4% paraformaldehyde (pH 7.5, 30 min). Both solutions had been previously treated with diethyl pyrocarbonate (DEPC) to minimise RNA degradation. Sixty and 40 μ m thick coronal sections were cut at the level of the thalamus and ZI-APT respectively. For *in situ* hybridisation, APT blocks were sectioned in DEPC-treated 0.1 M PB and every other section was separated for hybridisation reactions. Slices were then cryoprotected and frozen either subsequently or following the GAD67 probe reaction (see below).

Preembedding immunocytochemistry—The position of the injection sites were checked by double fluorescent stainings. BDA was visualized with StA 488 (1:1000 in TBS; 3 h). FG was developed by rabbit anti-FG antibody (raFG, 1:10000 in TBS; overnight), followed by GAR-A488 (1:500 in TBS; 3 h). The raFG (Chemicon, Code No AB153) was raised against Fluorogold, which is normally not present in the brain. Brain regions free of the tracer showed absolutely no immunostaining demonstrating the specificity of the antibody. Area of the posterior thalamic nucleus was visualized by its characteristic cellular marker, calbindin-D-28k (CB), which was labeled with mouse anti-CB (1:4000, overnight; Swant; Bellinzona, Switzerland) and then Alexa 594-conjugated goat anti-mouse (GAM-A594; 1:500; 3 h). Injections into ZI were localized by means of parvalbumin staining (mouse anti-parvalbumin, 1:4000; overnight) followed by GAM-A594 (1:500; 3 h).

The mouse anti-parvalbumin (Swant, Code No 235) and the mouse anti-CB antibodies (Swant, Code No 300) were prepared by the hybridization of mouse myeloma cells with spleen cells from mice immunized with parvalbumin from carp muscles and calbindin D-28k purified from chicken gut, respectively.

The parvalbumin antibody reacts specifically with parvalbumin in cultured nerve cells and in tissue originating from human, monkey, rabbit, rat, mouse chicken and fish. It specifically stains the 45Ca-binding spot of parvalbumin (MW 12'000 and IEF 4.9) in a two-dimensional "immunoblot". In RIA set up the antibody measures parvalbumin with a sensitivity of 10 ng/assay and an affinity of 7.9×10^{12} L/M (Celio and Heizmann 1981).

The calbindin antibody reacts specifically with calbindin D-28k in tissue originating from human, monkey, rabbit, rat, mouse and chicken (but probably not fish). This antibody does not cross-react with calretinin or other known calcium binding-proteins. This monoclonal antibody specifically stains the 45Ca-binding spot of calbindin D-28k (MW 28'000, IEP 4.8) in a two-dimensional gel. In radioimmunoassay it detects calbindin D-28k with a sensitivity of 10 ng/assay and an affinity of 1.6×10^{12} L/M.

In the cases when FG was injected into the ZI and BDA into the thalamus, triple fluorescence was used, visualizing BDA using StA 488, parvalbumin as above, and FG using its intrinsic fluorescence. Using our fluorescent filters (see below) FG emits blue fluorescent light without any treatment and thus PV, BDA and FG could be examined together (see Table 2 and Supplementary figure 1 / A-G).

Double fluorescent staining was used to simultaneously visualize the thalamus-projecting and the ZI-projecting APT neurons. BDA was developed by Alexa 488-conjugated StA, FG was incubated with rabbit anti-FG antibody followed by Alexa 594-conjugated GAR. In 1 animal, beside BDA and FG, the neuronal marker NeuN (Mouse Anti-Neuronal Nuclei, 1:2000) was also visualized by Alexa 350-conjugated goat anti-mouse (see Supplementary figure 2 / A-C) to determine the proportion of all APT cells that were labeled from either of the two nuclei in an overlapping area. The mouse NeuN antibody (Chemicon, MAB377) was raised against purified cell nuclei from mouse brain. Staining was localized only to the nuclei of neurons. The antibody recognizes 2-3 bands in the 46-48 kDa range and possibly another band at approximately 66 kDa. For simultaneous observation of the parvalbumin-containing APT cells and the ZI- or Po-projecting cells, only one of the two tracers was developed on a given section, using green fluorescent Alexa 488 chromogen, while PV was visualized by red Alexa 594 dye.

Sections for fluorescent microscopy were covered by Vectashield (Vector) after mounting and were examined using a Zeiss Axioscope (the wavelength for filter sets absorption and emission in nm: 365 bandpass / 420-460; 450-490 / 512-542; 540-546 / 578-643). Digital micrographs were made with a digital camera (Olympus Optical, DP 70, Tokyo, Japan).

Combination of retrograde tracing and in situ hybridization—A segment of the rat GAD67 coding sequence (GenBank accession number: gi:204227, 764 bp from 589 to 1352; numbering of the nucleotide positions starts from the beginning of the open reading frame) was amplified by RT-PCR from cDNA derived from a Wistar rat hippocampal total mRNA sample (forward primer 5' - ATT GGT TTA GCT GGC GAA TG, reverse primer 5' - GCC TTG TCC CCT GTA TCG TA). The primers were designed using the Primer3 software (Rozen and Skaletsky, 2000). The PCR product was cloned into the SmaI site of pBluescript II SK- (Fermentas UAB, Lithuania). The integrity and orientation of the clone was verified by sequencing. The GAD67 probe was linearized by HincII and XbaI digestion for the antisense and sense probe, respectively. The linearized template DNA was gel-extracted, precipitated, resuspended in DEPC-treated H₂O at a concentration of 1 µg/µl and stored at -20 °C. *In vitro* transcription was carried out for 2h at 37 °C in a total volume of 20 µl containing 1 µg of template DNA, 1 x transcription buffer, 1 x DIG RNA Labeling Mixture, 40 units RNase Inhibitor, 20 units of T3 or T7 RNA polymerase, which was adjusted to 20 µl using DEPC-free double-distilled H₂O. All components were purchased from Roche Molecular Diagnostics, Germany. Labeled riboprobes were DNase-treated and purified using the RNeasy MinElute Cleanup Kit (Qiagen, Germany). Finally, the integrity and quantity of the riboprobes were determined using gel electrophoresis.

All solutions used for *in situ* hybridization were first treated with 0.1% DEPC for one hour and then autoclaved. Chemicals were purchased from Sigma Aldrich Kft, Hungary, if otherwise not indicated. Incubation of the slices was carried out in a free-floating manner in RNase-free sterile culture wells for all steps. First, the sections were washed in phosphate-buffered saline (PBST, containing in mM: NaCl, 137; KCl, 2.7; Na₂HPO₄, 10; KH₂PO₄, 2; and 0.1% Tween-20 pH 7.4) three times for 20 minutes. Hybridization was then carried out overnight at 55 °C in 0.6 ml of hybridization buffer containing the digoxigenin-labeled riboprobe (0.6 µg/ml). Hybridization buffer consisted of 50% formamide, 5 x SSC, 1% sodium dodecyl sulphate (SDS), 50 µg/ml yeast tRNA and 50 µg/ml heparin in DEPC-

treated H₂O. During the overnight incubation and the following three washing steps, the sections were continuously incubated on a shaker within a humidified chamber. After incubation, the sections were first washed for 30 minutes at 55 °C in Wash Solution 1 (containing 50% formamide, 5 x SSC, 1% SDS in DEPC-treated H₂O) and then twice for 45 minutes at 55 °C in Wash Solution 2 (containing 50% formamide, 2 x SSC in DEPC-treated H₂O). The sections were then washed for 5 min in 0.05 M Tris-buffered saline containing 0.1% Tween-20 (TBST), pH 7.6 and then blocked in TBST containing 10% normal goat serum (TBSTN) for 1 hour, both at room temperature. Next, sections were incubated overnight at 4 °C with sheep anti-digoxigenin F_{ab} fragment conjugated to alkaline phosphatase (Roche Molecular Diagnostics, Germany) diluted at 1:1000 in TBSTN. The next day, the sections were washed three times for 20 minutes in TBST and then developed with freshly prepared chromogen solution in a total volume of 10 ml, containing 3.5 µl 5-Bromo-4-chloro-3-indolyl-phosphate and 3.5 µl Nitro blue tetrazolium chloride (NBT-BCIP) dissolved in chromogen buffer (containing in mM: NaCl, 100; Tris-Cl, 100, pH: 9.5; MgCl₂, 50; (-) tetramisole hydrochloride, 2; and 0.1% Tween-20). The sections were gently rinsed in 1 ml of the above developing solution in the dark for 4-6 hours and the reaction was stopped using PBST. Finally, the sections were washed in 0.1 M PB three times for 10 min and were processed for immunostaining.

BDA or FG was visualized in pale brown by DAB, or in light red by NovaRed (Vector) chromogen as described above. Sections were cleared in xylene and covered by Depex.

RESULTS

Anterograde tracing of the APT-ZI pathway

Characterization of the injection sites—For light microscopical observations, 5 injections of BDA and 5 injections of PHAL were used. The diameter of the injection sites were approximately 0.5 mm (see Figure 1/A), i.e. approximately one third of the widest extension of APT, sectioned coronally. Rostral and caudal APT were injected in 2 cases each. Within the middle part of the APT, dorsal areas were injected in 2 cases, a ventral area in 1 case, medial parts in 2 cases and a lateral part of the APT in 1 case (see Figure 1/A-E and Table 1). Double staining of the tracer with PV demonstrated that in 8 out of the 10 cases the injections sites did not reach the surrounding pretectal nuclei, superior colliculus or the thalamus. We used the higher-order thalamic nuclei as controls to confirm that the injections were localized within the APT. In all 10 cases, a characteristic pattern of labeled terminals was found in the expected thalamic nuclei, as described before (Bokor et al., 2005). In 1 case (injection in the dorsal APT by PHAL), the injection site encroached upon thalamic areas as well. This was indicated by a few labeled terminals in the nucleus reticularis thalami (nRT). In another animal the injection site was at the medial border of APT. These two injections were only analyzed at the light microscopical level to examine the topography of the pathway.

Light microscopical features of the APT-ZI projection—The BDA and PHAL injections in different areas of the APT resulted in a very dense terminal region within the ventral part of the zona incerta (vZI) and much sparser labeling in the dorsal part. Following a single APT injection, the dense APT-ZI terminal region did not fill the entire vZI but formed an elongated slab with dimensions of 0.5-1 mm in the medio-lateral and 0.3-0.6 mm in the antero-posterior directions (see Figure 1/F-J and Table 1). Only a very loose topography could be observed. Thus, injection sites in different areas of the APT could have overlapping target zones in the ventral ZI and injection sites in matched regions of APT could display distinct terminal fields. More dorsal and rostral injections (the visual part of APT) tended to innervate the lateral (visual) sector of vZI (Mitrofanis, 2005), whereas

progressively more ventral and caudal injections (the somatosensory APT) produced terminal fields concentrated in the central (somatosensory vZI) and medial (motor vZI) sectors (Mitrofanis, 2005).

Simultaneous visualization of the tracers and PV was performed in 5 out of 10 injections. In all cases the dense zone of the APT terminals was located within the strongly PV-positive region of the ventral ZI, both in the antero-posterior and in the dorsoventral directions (see Figure 2/A-E). Other areas of ZI displayed much fewer terminals (see Figure 2/A-C, D). Earlier studies demonstrated that the PV-positive region of ZI contains all the thalamus-projecting cells in ZI (Power et al., 1999; Lavalley et al., 2005; Trageser et al., 2006).

The GABA-content and ultrastructural properties of the APT-ZI boutons—In one BDA 10000- and two PHAL-injected animals, 76 labeled axon terminals were examined at the electron microscopical level in serial sections. Each terminal was observed in at least 8 sections but often more.

We found that 60.5 % (n=46) of the APT terminals within the ZI were GABA-negative (see Figure 3), 38.2 % (n=29) were GABA-positive (see Figure 4) and 1.3 % (n=1) was unidentified. The diameter of GABA-negative and GABA-positive terminals were similar; GABA-negative boutons had a mean diameter of 1.2 μm (SD 0.34; range 0.5-2.0 μm), and GABA-positive boutons had a mean diameter of 1.1 μm (SD 0.30; range 0.5-1.8 μm). The main target of both GABA-positive and GABA-negative APT-ZI terminals were medium sized dendrites (Figures 3 & 4), with an average diameter of 0.84 μm (SD 0.38; range 0.3-2.0 μm) for GABA-negative terminals and 0.90 μm (SD 0.36; range 0.3-1.7 μm) for GABA-positive terminals. Only 4% of the GABA-negative terminals and none of the GABA-positive boutons innervated somata in our sample. Most terminals formed synapses onto only one postsynaptic element, but a few contacted two elements (13 % of the excitatory and 7 % of the inhibitory terminals) e.g. a dendrite and a spine emanating from it. In some cases GABA-positive and GABA-negative APT boutons formed synapses onto the same ZI dendrite. GABA-negative axon terminals more frequently established multiple (2 or 3), mainly asymmetrical synapses (Figure 3) than GABA-positive boutons, which formed symmetrical (Figures 4) or in some cases asymmetrical synapses. Only a few (1 or 2 per terminal) punctum adhaerens-like structures were observed in both excitatory and inhibitory boutons. In one third of the GABA-negative terminals, remarkably long (around 1 μm) active zones were observed.

Retrograde tracing of the APT-ZI and the APT-thalamic pathways

Characterization of the injection sites—The location of the injection sites within the ZI were confirmed by double fluorescent visualization of the tracer and PV (Figure 5 /A and Figure 6 /A), the characteristic marker of the vZI (see above). In five cases FG and in one case BDA were injected into the ZI. The FG injections involved either vZI alone (3 cases), or both vZI and dZI (2 cases). The diameter of these injection sites was around 0.4-0.5 mm. The lack of retrogradely labeled cells in the nRT and in layer VI of the neocortex confirmed that the FG injections did not involve thalamic regions. Besides APT, retrogradely labeled neurons were found throughout the brainstem down to the spinal trigeminal nucleus, as well as in layer V of the neocortex in all cases, in accordance with earlier results (Kolmac et al., 1998; Mitrofanis and Mikuletic, 1999; Nicoletis et al., 1992); (Lavalley et al., 2005). The single BDA injection was restricted to dZI alone, which resulted in very few retrogradely labeled cells in the APT. This confirmed the results of the anterograde tracing and demonstrated the preferential innervation of vZI by APT fibers (see above). This injection was used as a control and was not analyzed further.

Four FG and four BDA injections were successfully made into the higher-order thalamus, each time involving the posterior thalamic nucleus (Po). FG injections had an average diameter of 0.4 mm, while BDA injections were larger (0.8 mm wide on average). The BDA injections spread to visual higher-order nuclei dorsal to Po (laterodorsal and lateral posterior nuclei). This did not interfere with the results since the dorsal part of APT is known to also project to these higher-order nuclei (Bokor et al., 2005). The injection site in Po was verified by calbindin or parvalbumin fluorescent immunolabeling (see Figure 6 /B). After each injection into higher-order thalamic nuclei, labeled cells were found in the nRT and layers V and VI of the neocortex. Back-labeled ZI neurons were always located within the PV-positive region of vZI in accordance with earlier studies (Power et al., 1999); (Lavalley et al., 2005); (Trageser et al., 2006) (Supplementary figure 1/A, C, D, F).

Distribution of retrogradely labeled cells—Injections into both ZI and higher-order thalamus labeled numerous APT cells which were spread in a rather large portion of the nucleus. Indeed, following a single injection labeled cells were found throughout almost the entire APT, with one or two more densely packed aggregates. This demonstrates only a weak topography in both the APT-ZI and APT-thalamus pathways (Figure 6/F-H) confirming the results of the anterograde tracing ((Bokor et al., 2005) and this study). We did not observe any APT regions that contained retrogradely labeled cells from only one of its diencephalic targets.

The GAD content of the APT-ZI projecting neurons—To confirm our electron microscopic results and to examine the distribution and proportion of GABAergic APT-ZI cells, we combined retrograde tracing with *in situ* hybridization.

Confirming earlier results, APT contained a much higher concentration of glutamic acid decarboxylase 67 (GAD67) mRNA-expressing neurons than the surrounding diencephalic and mesencephalic areas (Esclapez et al., 1994), (Benson et al., 1992). The distribution of FG-labeled ZI-projecting cells was similar as described above. We found that 8.6 % of the APT cells (n=1883 cells, n=2 animals) labeled from ZI contained GAD67 mRNA (Figure 5 / A-F). These double-stained cells were located evenly throughout the APT (see Figure 5 /D-F).

Double retrograde tracings of the APT-ZI and APT-thalamic pathways—In the four cases used for these experiments, FG was injected into ZI and BDA into the ipsilateral thalamus. In two out of the four cases, the cell populations retrogradely-labeled from the thalamus and from the ZI overlapped in the APT. In these cases, the ZI injections were located in exactly the sector of vZI that contained the ZI neurons back-labeled from the thalamus (see Supplementary figure 1 /A-F). In a third case, Po-projecting and ZI-projecting populations were also largely intermingled within the APT (see Figure 6 / F-H), but the ZI neurons labeled from the thalamus were fully separated from the location of the ZI injection site (see Table 2). In the fourth case, the injection site in the vZI did not overlap with the thalamus-projecting population and the two retrogradely labeled populations were separated within the APT.

We considered only those cases when the two retrogradely-labeled APT cell populations were largely intermingled. The number of cells labeled from both target areas was small (see Figure 6 / A-H): only 10 out of the 823 retrogradely labeled cells contained both tracers (1.2 %; n=3 animals) (Supplementary figure 2 / D-F). This represented 2.1 % of the ZI-projecting cells (n=466 cells) and 2.7 % of the thalamus-projecting cells (n=367 cells). In the case of 6 neurons the presence of one or the other tracer could not be unequivocally identified.

A low occurrence of colocalization upon double-retrograde tracing might occur when only a small proportion of the projecting cells are labeled. Thus, in one case we checked the colocalization of APT cells labeled by the two tracers when injected together. Using NeuN immunostaining, we calculated that over half of all APT neurons were labeled by FG or BDA (see Supplementary figure 2 /A-C). This suggested that the small number of double-projecting cells was not due to an insufficient extent of retrograde labeling.

Thus, the Po-projecting and the ZI-projecting cells form two largely segregated efferent pathways. Next we asked if the APT-thalamic and the APT-ZI cell populations can be distinguished by their parvalbumin content, since parvalbumin is known to label APT cells with distinct firing patterns (Bokor et al., 2005).

The parvalbumin content of Po-projecting and ZI-projecting cells in the APT—

The parvalbumin content of ZI-projecting APT cells (n=3 animals injected with FG) and thalamus-projecting APT cells (n=3 animals, 2 FG and 1 BDA injections) were examined by fluorescent microscopy. The distribution of parvalbumin-positive, -negative and weakly positive cells were similar to our earlier results (Bokor et al., 2005).

Among the ZI-projecting cells (n=420) most neurons (77.3 %) proved to be PV-negative, 19.2% showed weak but unequivocally positive PV-fluorescence, and 3.5 % were strongly PV-positive (see Figure 7/A-F). In the thalamus-projecting cell population (n=447) the proportion of strongly PV-positive cells was higher (28.3 %), but still over half of the cells (54.5 %) were PV-negative. The number of weakly PV-positive cells was similar to the ZI-projecting cell group (17.2 %) (Figure 7 /G-L). Thus, APT-ZI and APT-thalamic cells can not be unequivocally distinguished based on their parvalbumin content though the ratio of the three populations is different within the two projecting cell class. .

DISCUSSION

In this study we demonstrated that (Figure 8): i) APT preferentially innervates the PV-positive ventral subregion of ZI that contains all the thalamus-projecting cells, ii) this APT-vZI projection contains both GABA-negative and GABA-positive components, iii) the thalamus- and ZI-projecting cell populations are intermingled within the APT, but only a very small proportion project to both targets, iv) parvalbumin content did not distinguish ZI- and thalamus-projecting APT cells.

The dual nature of the APT-ZI pathway

An earlier electron microscopic study (May et al., 1997) described that the APT-ZI terminals form asymmetrical synapses in cats. Our electron microscopic observations have shown, however, that the APT-ZI pathway is more complex in rats. A larger proportion of the APT terminals established asymmetrical synapses in ZI and were GABA-negative (60.5 %), but in addition, a considerable cohort of the boutons (38.2 %) were GABA-positive. The synapses in this latter population could not always be unequivocally classified as symmetrical or asymmetrical, and thus a postembedding GABA reaction was absolutely necessary to identify the inhibitory nature of these boutons. The presence of an inhibitory APT-ZI component that we describe here was not mentioned in May's earlier study (May et al., 1997), possibly because this inhibitory component is missing in the cat. However, a more plausible explanation is that a quantitative analysis of the transmitter content of APT-ZI terminals was not among the aims of this previous study, and examination based on the ultrastructure alone may have missed the numerically smaller GABAergic component. This explanation is further supported by our results indicating that the inhibitory and excitatory terminals could not be distinguished by their size or targets.

We found that GABA-positive- and GABA-negative APT terminals can converge in close proximity onto the same ZI dendrite, which suggests a dual excitatory/inhibitory influence of APT. Brain areas innervating target regions via both excitatory and inhibitory fibers are not uncommon in the nervous system. For example, the medial septum sends both excitatory cholinergic (Frotscher and Leranth, 1985; Muller and Misgeld, 1986) and inhibitory GABAergic (Freund and Antal, 1988; Toth et al., 1997) fibers to hippocampus. A possible functional interpretation of the synaptic connections described here is that the APT exerts a diffuse excitation on ZI, structured by temporally patterned inhibition. Indeed, an APT neuron, which established symmetrical synapses in ZI, fired in a regular tonic manner (Bokor et al., 2005), which may indicate a GABAergic pacemaker function.

Excitatory inputs in the zona incerta

The ultrastructure of two other major excitatory inputs to ZI has been described recently. These afferents arise from the interpolaris nucleus of the trigeminal complex (SpVi) and the primary somatosensory cortex (S1) (Bartho et al., 2007; Lavalley et al., 2005). The size of the SpVi terminals (1-5 μm) is well above the size of APT-ZI terminals described here, and they can establish up to 18 release sites on their targets. The type of their postsynaptic elements are also different, as they form synapses onto the proximal dendrites and somata of GABAergic vZI neurons (Lavalley et al., 2005). The cortico-ZI terminals were also somewhat larger (0.7-3.3 μm diameter, 1.9 μm in average) than those originating in the APT, but the average diameter of the postsynaptic targets were similar (0.90 vs. 0.98 μm). However, the targets of cortical terminals in ZI consisted of two separate classes. Around half targeted thicker dendrites (average diameter 1.4 μm), whereas the rest established synapses on spines (average diameter 0.6 μm) (Bartho et al., 2007). These data suggest that SpVi, cortical and APT terminals largely innervate different dendritic domains in the vZI with a certain degree of overlap. SpVi terminals target thick, proximal dendritic regions, cortical inputs innervate mid-calibre dendrites and spines, whereas APT boutons synapse mainly on mid- to distal dendrites.

Based on morphological and electrophysiological criteria, excitatory terminals were classified as “drivers” or “modulators” (Sherman and Guillery, 2006; Sherman and Guillery, 1998), which refers to their impact on their postsynaptic target. Using our previous and present data, SpVi terminals are likely to be drivers in ZI (which is also suggested by the fast whisker responses of ZI cells), whereas APT and cortical terminals represent modulators, since SpVi establishes contacts via a large number of release sites onto proximal dendrites, whereas the latter have fewer release sites and target more distal dendrites. However, cortico-ZI terminals can form unusually large (up to 0.95 μm) active zones (Bartho et al., 2007), and we often observed similar-sized APT-ZI terminals. Since the number of postsynaptic receptors are usually proportional to the size of the active zone (Nusser et al., 1998) cortico-ZI and APT-ZI projections might also exert a strong effect on ZI activity by means of their large active zones. Also, distal synapses can be more effective in ZI cells due to the morphology of the ZI neurons, since these cells have sparsely branching dendrites with long dendritic segments (Bartho et al., 2007). This morphology suggests that postsynaptic potentials arising at distal segments show little attenuation before reaching the soma (Bartho et al., 2007). Indeed, the activity of many ZI cells showed strong correlation to cortical slow oscillations (Bartho et al., 2007), suggesting a strong cortical influence via terminals with similar morphology to APT-ZI terminals. Thus, APT terminals, via only a few release sites per terminal, may also be able to significantly affect the activity of ZI cells.

Inhibitory inputs in the zona incerta

The size and targets of GABA-positive APT-terminals were similar to GABA-negative APT-ZI terminals, but were different from the GABA-positive APT-thalamic boutons described before (Bokor et al., 2005). APT terminals in Po are very large (2-6 μ m) and form several (3-16) synapses onto the proximal dendrites of relay cells (Bokor et al., 2005), whereas GABAergic APT terminals in ZI are smaller (1.1-1.2 μ m) and form one or two synapses (present study). This cannot be treated as evidence that they originate from different cell populations in the APT, since a single neuron can have different types of terminals (Acsady et al., 1998), and indeed the local terminals of APT-thalamic cells are small (Bokor et al., 2005). The results of our double retrograde tracing experiments, however, demonstrated that the APT-thalamic- and APT-ZI pathways are segregated; thus these terminals originate from different cell groups (see below).

Using *in situ* hybridization we confirmed that, in addition to the major excitatory component, there is a GABAergic projection from the APT to the ZI. However, only 8.6 % of the projecting cells were positive for GAD67, compared to 38.2 % GABA-positive terminals among the anterogradely-labeled terminals in our electron microscopical experiments. This discrepancy can be explained by preferential uptake or transport of either the anterograde- or the retrograde tracer. Such preferential transport had been described earlier (Freund and Antal, 1988). A further alternative is that a subpopulation of GABAergic APT cells expresses no detectable level of GAD67 mRNA, only GAD65, and thus the proportion of the GABAergic component might have been underestimated in our retrograde labeling experiments. Finally, inhibitory APT cells may possess a more extensive axon arborization in vZI than the excitatory cells. In any case, the two tracing studies convincingly demonstrate the presence of an inhibitory component in the APT-ZI pathway.

What could be the role of an inhibitory input to ZI? Previous studies demonstrated that both the ongoing tonic and the sensory-evoked responses of ZI effectively suppress the relay function of thalamocortical cells in Po (Lavallee et al., 2005). Thus, it was hypothesized that disinhibition (e.g. suppressing ZI activity) may be the rule of operation in this circuit. Indeed, cessation of ZI activity was observed before saccades in monkeys. The interpretation was that in order to allow the motor commands of oculomotor cells to be performed, it is necessary to suppress the inhibition exerted by ZI on the superior colliculus, another major target of GABAergic ZI cells (Ma, 1996). APT receives both subcortical- and cortical inputs that are necessary to time ZI activity. Thus, it may be one component in the disinhibitory circuit that suppresses ZI, provided that these excitatory inputs target the GABAergic APT-ZI cells.

Heterogeneous output pathways in the APT

It had been shown previously that the firing pattern of APT cells and their parvalbumin content is correlated (Bokor et al., 2005). Thus, our results concerning the PV content of APT-ZI and APT-thalamic cells enable us to make predictions about the temporal pattern of the APT-ZI and APT-Po activity.

In this study, we have demonstrated that only a few of the ZI-projecting APT cells are strongly PV-positive (3.5 %), whereas their number is much higher (28.3 %) among the thalamus-projecting neurons. In our previous study, all strongly PV-positive neurons were characterized by high frequency bursts. Two physiologically and morphologically identified fast bursting cells, which had ascending axons reaching the thalamus, but not ZI, also displayed strong PV-positivity (Bokor et al., 2005).

A similar number of weakly PV-positive APT neurons projected to ZI and thalamus (19.2% vs. 17.2%). These cells fire in a tonic manner *in vivo* and their firing shows no correlation to

the neocortical EEG activity. An APT neuron which established symmetrical synapses in the ZI also belonged to this class (Bokor et al., 2005).

PV-negative APT cells, on the other hand, display rhythmic activity and their firing is strongly correlated with neocortical slow oscillations (Bokor et al., 2005). However, since no positive marker was found for this cell class, it cannot be excluded that PV-negative cells include more than one cell type (see below). In this work we have found that these cells compose the vast majority (77.3 %) of the ZI-projecting population, but a large number of PV-negative cells can also be found among the thalamus-projecting neurons (54.5 %).

These data allow us to conclude that ZI- and thalamus-projecting APT neurons cannot be distinguished based on their firing pattern. Apparently, both ZI and thalamus receive a temporally-complex heterogeneous input from APT, including cortically-modulated rhythmic and arousal-dependent tonic activity.

Our preliminary data suggest that strongly parvalbumin-positive cells (“fast bursting”) are possibly all positive for GAD67, whereas among PV-negative cells there are GAD67 positive and negative neurons (Giber, Acsády unpublished). Though triple labeling was not attempted, based on our data many PV-negative cells should be GAD-negative as well, since a large proportion of the APT-ZI cells were GAD67-negative (91.4 %) and PV-negative (77%). The GAD67 content of weakly parvalbumin-positive APT neurons could not be established, but it probably also represents a mixed population. Namely, PV-negative APT-ZI cells (77.3 %) do not account for the entire GAD67-negative population (91%), thus weakly PV-positive APT-ZI cells should also be among the GAD67-negative cells. However, all 20 of the examined synapses of a weakly PV-positive tonic neuron labeled previously *in vivo* (Bokor et al., 2005) were symmetric thus this neuron was probably GABAergic.

To summarize, aside from the GABAergic fast bursting cells, which largely target thalamus but not ZI, tonic and rhythmic activity may be conveyed via excitatory and inhibitory output channels of APT to both thalamus and ZI.

The APT-ZI-thalamus network, functional implications

Our anterograde tracing results demonstrated that the vast majority of APT fibers terminated preferentially in the same PV-immunoreactive subregion of ZI where the ZI-thalamic pathway originates from (Power et al., 1999; Lavalée et al., 2005; Trageser et al., 2006). This suggests the existence of an indirect APT-ZI-thalamic pathway in addition to the direct APT-thalamic route, and confirms similar observations in cats (May et al., 1997).

We found that only a very small proportion of APT cells project to both ZI and thalamus. If APT neurons were found to project to both targets a double-projecting GABAergic cell in APT would directly inhibit the thalamus, while at the same time it would release the ZI-thalamic inhibition. A double-projecting, excitatory APT cell would excite the thalamus by a direct excitation, but would subsequently induce inhibition via the activated ZI cells. This arrangement would indicate a competition between direct APT-thalamic, and indirect APT-ZI-thalamic pathways. Our data does not support this scenario. Rather, based on our data, we propose an alternative, synergistic view of the APT-thalamic and APT-ZI pathways.

The majority of APT cells provide rich recurrent collateral fibers within the APT (Bokor et al., 2005). These enable the various APT cell populations to interact with each other and to control the direct and indirect APT-thalamic pathways. We assume that the excitatory APT-ZI cells, besides innervating the inhibitory, thalamus-projecting ZI cells, also innervate the inhibitory APT-thalamic cells by their local collaterals. Increased activity of excitatory

APT-ZI cells would result in a synergistic increase of inhibition in the thalamus via the direct and indirect pathways. On the other hand, inhibitory APT-ZI projecting cells could synchronously suppress inhibition in the thalamus via reducing the strength of ZI-thalamic inhibition and via the local collaterals which suppress the direct inhibitory APT-thalamic pathway. In this way the activity of the two strong GABAergic inputs to the thalamus could be regulated together.

In summary, APT is likely to influence its higher-order thalamic target nuclei not only in a monosynaptic way, but indirectly as well, by means of the modulation of vZI. The co-modulation of direct and indirect pathways would result in a synergistic co-operation, simultaneously amplifying or diminishing the APT- and vZI inhibition exerted on the thalamus. The large heterogeneity found in the parvalbumin content of both ZI- and thalamus-projecting APT cells suggests a complex output activity of multiple firing patterns. As a result, we can assume that the interaction between the direct and indirect APT-thalamic pathways is important for the precise, complex, GABAergic regulation of the higher-order thalamus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support: This work was supported by the Wellcome Trust (A.L. is in receipt of a Wellcome Trust International Senior Research Fellowship), the Hungarian Scientific Research Fund (OTKA 49100, 64184), Institute du Cerveau et de la Moelle épinière and the National Office for Research and Technology (Program Öveges).

OTHER ACKNOWLEDGEMENTS: We thank Krisztina Faddi, Katalin Lengyel, Gabriella Urbán and Gyözö Goda for their excellent technical assistance.

List of Abbreviations

ABC	Avidin biotinylated-horseradish peroxidase complex
APT	Anterior preteectum
BDA	Biotin-dextran amine
Br	Bregma point
CB	Calbindin
cp	Cerebral peduncle
DAB	Diaminobenzidine
DABNi	Nickel intensified 3,3'-diaminobenzidine
DEPC	Diethyl pyrocarbonate
DLG	Dorsolateral geniculate nucleus
DpG	Deep gray layer of superior colliculus
DpMe	Deep mesencephalic nucleus
Eth	Ethmoid thalamic nucleus
FG	Fluoro-gold
fr	Fasciculus retroflexus

GAD67 mRNA	Glutamic acid decarboxylase 67 mRNA
LD	Laterodorsal thalamic nucleus
LM, ml	Medial lemniscus
LP	Lateral posterior thalamic nucleus
LPMC	Lateral posterior thalamic nucleus, mediocaudal part
LPMR	Lateral posterior thalamic nucleus, mediorostral part
MG	Medial geniculate nucleus
MGD	Medial geniculate nucleus, dorsal part
NBT-BCIP	Nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl-phosphate
NeuN	Mouse Anti-Neuronal Nucleus
nRT	Reticular thalamic nucleus
OPT	Olivary pretectal nucleus
OT	Nucleus of the optic tract
PAG	Periaqueductal gray
PF	Parafascicular thalamic nucleus
PHAL	<i>Phaseolus vulgaris</i> leucoagglutinin
PIL	Posterior intralaminar thalamic nucleus
Po	Posterior thalamic nucleus
PoT	Posterior thalamic nuclear group, triangular part
PPT	Posterior pretectal nucleus
PV	Parvalbumin
SC	Superior colliculus
SG	Supragenulate thalamic nucleus
StA 488	Alexa 488-conjugated streptavidin
STh	Subthalamic nucleus
SubG	Subgenulate nucleus
VLG	Ventral lateral geniculate nucleus
VM	Ventro-medial thalamic nucleus
VPL	Ventral posterolateral thalamic nucleus
VPM	Ventral posteromedial thalamic nucleus
ZI	Zona incerta
dZI	Dorsal zona incerta
vZI	Ventral zona incerta

LITERATURE CITED

- Acsady L, Kamondi A, Sik A, Freund T, Buzsaki G. GABAergic cells are the major postsynaptic targets of mossy fibers in the rat hippocampus. *The Journal of Neuroscience*. 1998; 18(9):3386–3403. [PubMed: 9547246]
- Bartho P, Freund TF, Acsady L. Selective GABAergic innervation of thalamic nuclei from zona incerta. *Eur J Neurosci*. 2002; 16(6):999–1014. [PubMed: 12383229]
- Bartho P, Slezia A, Varga V, Bokor H, Pinault D, Buzsaki G, Acsady L. Cortical control of zona incerta. *The Journal of Neuroscience*. 2007; 27(7):1670–1681. [PubMed: 17301175]
- Benevento LA, Rezak M, Santos A. An autoradiographic study of the projections of the pretectum in the rhesus monkey (*Macaca mulatta*): evidence for sensorimotor links to the thalamus and oculomotor nuclei. *Brain Research*. 1977; 127(2):197–218. [PubMed: 405084]
- Benson DL, Isackson PJ, Gall CM, Jones EG. Contrasting patterns in the localization of glutamic acid decarboxylase and Ca²⁺/calmodulin protein kinase gene expression in the rat central nervous system. *Neuroscience*. 1992; 46(4):825–849. [PubMed: 1311814]
- Berod A, Hartman BK, Pujol JF. Importance of fixation in immunohistochemistry: use of formaldehyde solutions at variable pH for the localization of tyrosine hydroxylase. *J Histochem Cytochem*. 1981; 29(7):844–850. [PubMed: 6167611]
- Bokor H, Frere SG, Eyre MD, Slezia A, Ulbert I, Luthi A, Acsady L. Selective GABAergic control of higher-order thalamic relays. *Neuron*. 2005; 45(6):929–940. [PubMed: 15797553]
- Brandao ML, Rees H, Witt S, Roberts MH. Central antiaversive and antinociceptive effects of anterior pretectal nucleus stimulation: attenuation of autonomic and aversive effects of medial hypothalamic stimulation. *Brain Research*. 1991; 542(2):266–272. [PubMed: 2029634]
- Cadusseau J, Roger M. Cortical and subcortical connections of the pars compacta of the anterior pretectal nucleus in the rat. *Neurosci Res*. 1991; 12(1):83–100. [PubMed: 1721119]
- Celio MR, Heizmann CW. Calcium-binding protein parvalbumin as a neuronal marker. *Nature*. 1981; 293:300–302. [PubMed: 7278987]
- Celio MR, Baier W, Scharer L, Gregersen HJ, de Viragh PA, Norman AW. Monoclonal antibodies directed against the calcium binding protein Calbindin D-28k. *Cell Calcium*. 1990; 11:599–602. [PubMed: 2285928]
- Esclapez M, Tillakaratne NJ, Kaufman DL, Tobin AJ, Houser CR. Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms. *J Neurosci*. 1994; 14(3 Pt 2):1834–1855. [PubMed: 8126575]
- Foster GA, Sizer AR, Rees H, Roberts MH. Afferent projections to the rostral anterior pretectal nucleus of the rat: a possible role in the processing of noxious stimuli. *Neuroscience*. 1989; 29(3):685–694. [PubMed: 2472580]
- Freund TF, Antal M. GABA-containing neurons in the septum control inhibitory interneurons in the hippocampus. *Nature*. 1988; 336(6195):170–173. [PubMed: 3185735]
- Frotscher M, Leranth C. Cholinergic innervation of the rat hippocampus as revealed by choline acetyltransferase immunocytochemistry: a combined light and electron microscopic study. *The Journal of Comparative Neurology*. 1985; 239(2):237–246. [PubMed: 4044938]
- Hodgson AJ, Penke B, Erdei A, Chubb IW, Somogyi. Antisera to gamma-aminobutyric acid. I. Production and characterization using a new model system. *J Histochem Cytochem*. 1985; 33:229–39. [PubMed: 3973378]
- Johnson JC, Hoskins GA, Mihailoff GA, May PJ. Morphology of incertotectal and incertopretectal neurons in macaques. *Society for Neuroscience*. 1992 T.P. M. Volt. 18.
- Kolmac CI, Power BD, Mitrofanis J. Patterns of connections between zona incerta and brainstem in rats. *J Comp Neurol*. 1998; 396(4):544–555. [PubMed: 9651011]
- Lavallee P, Urbain N, Dufresne C, Bokor H, Acsady L, Deschenes M. Feedforward inhibitory control of sensory information in higher-order thalamic nuclei. *J Neurosci*. 2005; 25(33):7489–7498. [PubMed: 16107636]
- Ma TP. Saccade-related omnivectoral pause neurons in the primate zona incerta. *Neuroreport*. 1996; 7(15-17):2713–2716. [PubMed: 8981453]

- May PJ, Sun W, Hall WC. Reciprocal connections between the zona incerta and the pretectum and superior colliculus of the cat. *Neuroscience*. 1997; 77(4):1091–1114. [PubMed: 9130790]
- Mithani S, Atmadja S, Baimbridge KG, Fibiger HC. Neuroleptic-induced oral dyskinesias: effects of progabide and lack of correlation with regional changes in glutamic acid decarboxylase and choline acetyltransferase activities. *Psychopharmacology*. 1987; 93:94–100. [PubMed: 2888156]
- Mitrofanis J. Some certainty for the “zone of uncertainty”? Exploring the function of the zona incerta. *Neuroscience*. 2005; 130(1):1–15. [PubMed: 15561420]
- Mitrofanis J, Mikuletic L. Organisation of the cortical projection to the zona incerta of the thalamus. *J Comp Neurol*. 1999; 412(1):173–185. [PubMed: 10440718]
- Muller W, Misgeld U. Slow cholinergic excitation of guinea pig hippocampal neurons is mediated by two muscarinic receptor subtypes. *Neuroscience Letters*. 1986; 67(2):107–112. [PubMed: 3014395]
- Nicolelis MA, Chapin JK, Lin RC. Somatotopic maps within the zona incerta relay parallel GABAergic somatosensory pathways to the neocortex, superior colliculus, and brainstem. *Brain Res*. 1992; 577(1):134–141. [PubMed: 1521138]
- Nusser Z, Hajos N, Somogyi P, Mody I. Increased number of synaptic GABA(A) receptors underlies potentiation at hippocampal inhibitory synapses. *Nature*. 1998; 395(6698):172–177. [PubMed: 9744275]
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. Academic Press; London: 1998.
- Power BD, Kolmac CI, Mitrofanis J. Evidence for a large projection from the zona incerta to the dorsal thalamus. *J Comp Neurol*. 1999; 404(4):554–565. [PubMed: 9987997]
- Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol*. 2000; 132:365–386. [PubMed: 10547847]
- Shammah-Lagnado SJ, Negrao N, Ricardo JA. Afferent connections of the zona incerta: a horseradish peroxidase study in the rat. *Neuroscience*. 1985; 15(1):109–134. [PubMed: 4010931]
- Sherman, S.; Guillery, R. Exploring the Thalamus and Its Role in Cortical Function. The MIT Press; London: 2006.
- Sherman SM, Guillery RW. On the actions that one nerve cell can have on another: distinguishing “drivers” from “modulators”. *Proc Natl Acad Sci USA*. 1998; 95(12):7121–7126. [PubMed: 9618549]
- Sloviter RS. Calcium-binding protein (calbindin-D28k) and parvalbumin immunocytochemistry: localization in the rat hippocampus with specific reference to the selective vulnerability of hippocampal neurons to seizure activity. *The Journal of Comparative Neurology*. 1989; 280:183–196. [PubMed: 2925892]
- Somogyi P, Hodgson AJ, Chubb IW, Penke B, Erdei A. Antisera to gamma-aminobutyric acid. II. Immunocytochemical application to the central nervous system. *J Histochem Cytochem*. 1985; 33(3):240–248. [PubMed: 2579123]
- Terenzi MG, Zagon A, Roberts MH. Efferent connections from the anterior pretectal nucleus to the diencephalon and mesencephalon in the rat. *Brain Res*. 1995; 701(1-2):183–191. [PubMed: 8925282]
- Toth K, Freund TF, Miles R. Disinhibition of rat hippocampal pyramidal cells by GABAergic afferents from the septum. *The Journal of physiology*. 1997; 500(Pt 2):463–474. [PubMed: 9147330]
- Trageser JC, Burke KA, Masri R, Li Y, Sellers L, Keller A. State-dependent gating of sensory inputs by zona incerta. *Journal of Neurophysiology*. 2006; 96(3):1456–1463. [PubMed: 16775205]
- Trageser JC, Keller A. Reducing the uncertainty: gating of peripheral inputs by zona incerta. *J Neurosci*. 2004; 24(40):8911–8915. [PubMed: 15470158]
- Veinante P, Jacquin MF, Deschenes M. Thalamic projections from the whisker-sensitive regions of the spinal trigeminal complex in the rat. *J Comp Neurol*. 2000; 420(2):233–243. [PubMed: 10753309]
- Villarreal C, Kina V, Prado W. Antinociception induced by stimulating the anterior pretectal nucleus in two models of pain in rats. *Clin Exp Pharmacol Physiol*. 2004; 31(9):608–613. [PubMed: 15479168]
- von Krosigk M, Smith Y, Bolam JP, Smith AD. Synaptic organization of GABAergic inputs from the striatum and the globus pallidus onto neurons in the substantia nigra and retrorubral field which

project to the medullary reticular formation. *Neuroscience*. 1992; 50:531–549. [PubMed: 1279463]

Zagon A, Terenzi M, Roberts M. Direct projections from the anterior pretectal nucleus to the ventral medulla oblongata in rats. *Neuroscience*. 1995; 65(1):253–272. [PubMed: 7753399]

Figure 1. Distribution of APT terminals in ZI

A) A low power micrograph of a PHAL injection site in the APT. The section is also immunoreacted for PV. The PV-positive APT is depicted by small arrows. The same injection site is shown in C and D labeled by horizontal stripes. **B-E)** The extent of 6 injection sites within the APT at four different antero-posterior levels **F-J)** ZI sectors containing dense aggregates of anterogradely labeled fibers shown in five subsequent antero-posterior levels. Injection sites and corresponding terminal fields are labeled by the same hatchings. Note the loose topography and patchy termination preferentially in the vZI. The outlines of the nuclei are modifications of Paxinos and Watson (1998). Scale (A) 1 mm.

Figure 2. Preferential innervation of the parvalbumin-positive vZI by APT-ZI terminals
A-C) Distribution of APT-ZI terminals (blue dots) in relation to PV-positive ZI cells (brown) shown in low power light micrographs at three different antero-posterior levels. Note the absence of fibers anterior to the PV-positive zone (A). **D-F)** Preferential innervation of vZI as opposed to dZI. Sparse terminal labeling within dZI (D; boxed area D, magnified from panel B), is contrasted by the dense meshwork of fibers within the vZI (E; boxed area E in panel B), shown at two different magnifications (E-F). Scale (A-C) 100 μ m, (D-F) 20 μ m.

Figure 3. Ultrastructure of GABA-negative terminals within vZI, anterogradely labeled from APT

A-C) High power electron micrographs of a PHAL-labeled terminal (b_1), filled with silver-intensified gold precipitate in A_1 - A_2 . The corresponding adjacent ultrathin sections (B - C) were immunoreacted for GABA using the immunogold method (small gold particles). Terminal b_1 is negative for GABA. Note that the silver signal is etched during the postembedding reaction. Terminals labeled by b_2 and b_3 are PHAL-negative terminals which are GABA-positive and negative respectively. The anterogradely labeled terminal establishes an asymmetrical synapse (arrowhead in A and B) on a thick dendrite (d). **D-E)** A similar pair of images showing a GABA-negative APT-ZI terminal contacting a soma. **F-G)**

A PHAL-labeled GABA-negative terminal establishes a synapse on a small calibre dendrite. Small arrows indicate puncta-adherens-like contacts. Scale (A-G) 500 nm.

Figure 4. Ultrastructure of GABA-positive terminals within vZI, anterogradely labeled from APT

Electron micrographs of two terminals labeled by BDA (b in A-D) or PHAL (b₁ in E-H) as in Figure 3. Postembedding GABA-reaction on the corresponding adjacent sections (C-D, G-H) demonstrate that both are GABA-positive. Terminal b₂ in E-H is a GABA-negative terminal unlabeled by the tracer. Note that the silver signal is etched during the postembedding reaction. The anterogradely-labeled terminals establish symmetrical synapses on thick (A-D) and medium sized (E-H) dendrites (d). Synapses are indicated by arrowheads, whereas puncta-adhaerentia-like contacts are shown by small arrows. Scale (A-H) 500 nm.

Figure 5. APT-ZI neurons include GAD67 mRNA-positive and negative subpopulations

A: Fluorescent micrograph demonstrating an overlay of double immunostaining for the FG injection site in ZI (green fluorescence) and for PV (red fluorescence), a marker of vZI. **B:** Light micrograph showing the APT after retrograde tracing from ZI (FG-labeled cells displayed by brown DAB chromogen) and the visualization of the GAD67 mRNA-positive neurons by *in situ* hybridization (blue colored cells developed by NBT-BCIP chromogen). The area of the APT (black arrows) is indicated by a large density of GAD67 mRNA-positive cells. **C:** The same labeling at higher magnification depicting GAD67-negative (small black arrow) and -positive (double arrowhead) projecting cells and GAD67-positive

cells without tracer (arrowhead). **D-F:** Distribution of GAD67-positive (red squares) and -negative (black triangles) ZI-projecting APT cells at three antero-posterior levels. Note that there is no spatial segregation of the two populations. FG-negative GAD67-positive neurons are labeled as green dots. Scale (A) 1mm, (B) 200 μ m, (C) 20 μ m, (D-F) 500 μ m.

Figure 6. Double retrograde tracing from the thalamus and ZI. The labeled neurons co-distribute in APT but double projection is rare

A-B: Overlay of double fluorescent micrographs showing the injection sites in ZI (A) and in Po (B) (both in green) and parvalbumin (red). The tracers were FG and BDA respectively. **C-E:** High power fluorescent micrographs showing the neurons (white arrowheads) within the APT retrogradely-labeled from the higher-order thalamus (C, green) and from vZI (red D, white arrows). In the overlay (E) it is clear that APT-ZI and APT-Po cells are intermingled, but none of them are double-labeled. **F-H:** Distribution of APT neurons retrogradely-labeled from the vZI (green dots) and from the ipsilateral Po (white dots) shown at three antero-posterior levels. The maps of the neurons were matched to the

micrographs of the adjacent PV-immunostained sections. Despite the large spatial overlap of the two APT cell populations, only a single double-projecting neuron was found (G, blue dot). Note the spreading of the retrograde cell population to almost the entire APT. Scale (A-B, F-H) 1 mm, (C-E) 50 μm .

Figure 7. The parvalbumin-content of APT cells retrogradely-labeled from vZI and higher-order thalamus

A-C and D-F: High power fluorescent micrographs showing two sites within the APT after simultaneous fluorescent visualization of PV (red fluorescence E and F) and FG-labeled ZI-projecting cells (green fluorescence, A and D). **G-I and J-L:** Co-distribution of PV-positive cells (red, H and K) and the BDA-labeled Po-projecting cells (green fluorescence, G and J). Pictures C, F and I, L are overlays. Many neurons are PV-negative (small arrows) in both projections, but weakly PV-positive (double arrowheads) and strongly PV-positive (arrowheads) cells are also present. Scale (A-L) 50 μ m.

Figure 8. Summary diagram showing the connectivity between the thalamus, the zona incerta (ZI) and the anterior pretectum (APT)

Inhibitory and excitatory elements are black and grey respectively. The thalamus is represented by n. posterior (Po) which is innervated by both APT and vZI. The vast majority of APT-thalamic (pathway 1) and vZI-thalamic (pathway 2.) fibers are GABAergic as described before (Bartho et al., 2002; Bokor et al., 2005). The present study demonstrated excitatory inputs from APT to vZI (pathway 3), thus APT, beside the direct inhibitory effect on thalamus may induce indirect inhibition via activating vZI. However, a significant proportion (38%) of the APT-vZI terminals was GABA-positive (pathway 4). Furthermore, the APT-thalamic (pathway 1) and APT-vZI (pathway 4) fibers were found to arise from distinct GABAergic cell populations. Since APT cells have rich local axon collaterals (pathway 5) and the three APT cell populations are spatially not segregated APT-vZI GABAergic cells are in position to simultaneously decrease the inhibition of the thalamus by inhibiting the vZI-thalamic (pathway 2) and the APT-thalamic (pathway 1) GABAergic routes. The neurons are juxtacellularly labeled cells in the APT, vZI and Po. For simplicity a single APT cell is shown three times for the three pathways. The Po cell is the work of Didier Pinault.

Table 1

Characterization of the Anterograde Injections to the APT1[/]

Tracing no.	Tracer used	Position of the injection site within the APT		Position of the densely labeled area within the vZI			Used for EM	Used for PV-tracer double staining
		Rostrocaudal position	Mediolateral and dorsoventral position	Rostrocaudal position	Mediolateral position			
1	BDA	Very rostral	Dorsal	Rostral	Very Lateral		No	No
2	BDA	Rostral	Middle	Middle	Lateral		No	Yes
3	BDA	Middle	Dorsal	Middle	Lateral		Yes	Yes
4	PHAL	Middle	Dorsal	Middle	Lateral		No	Yes
5	PHAL	Middle	Lateral	Rostral	Lateral		Yes	No
6	BDA	Middle	Medial	Rostral	Middle		No	No
7	PHAL	Middle	Medial	Middle	Middle		No	Yes
8	PHAL	Middle	Ventral	Rostral	Lateral		No	No
9	PHAL	Caudal	Dorsolateral	Caudal	Lateral		Yes	Yes
10	BDA	Very caudal	Medial	Rostral	Medial		No	No

[/]For abbreviations, see list.

Table 2
 Characterization of Retrograde Injections in the Higher Order Thalamus and Zona Incerta¹

Animal no.	Injection to the thalamus	Injection to the zona incerta	Ipsilateral or contralateral injections	ZI injection site and ZI cell population labeled from thalamus overlapped or not	APT cell populations labeled from thalamus and ZI overlapped or not
1	Fg in Po	None	—	—	—
2	Fg in Po	None	—	—	—
3	Fg in Po	FG in ZI	Contralateral	—	—
4	Fg in Po	BDA in dZI	Ipsilateral	Not	No labeled cells from dZI
5	BDA in Po, LD, LP	FG in vZI	Ipsilateral	Not	Not
6	BDA in Po, LD, LP	FG in vZI	Ipsilateral	Not	Overlapped
7	BDA in whole thalamus	FG in vZI	Ipsilateral	Overlapped	Overlapped
8	BDA in whole thalamus	FG in vZI	Ipsilateral	Overlapped	Overlapped

¹For abbreviations, see list.