

Comparison of numbers of interneurons in three thalamic nuclei of normal and epileptic rats

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ABSTRACT

The inhibitory sources in the thalamic nuclei are local interneurons and neurons of the thalamic reticular nucleus. Studies of models of absence epilepsy have shown that the seizures are associated with an excess of inhibitory neurotransmission in the thalamus. In the present study, we used light-microscopic gamma-aminobutyric acid (GABA) immunocytochemistry to quantify the interneurons in the lateral geniculate (LGN), ventral posteromedial (VPM), and ventral posterolateral (VPL) thalamic nuclei, and compared the values from normal Wistar rats and genetic absence epilepsy rats from Strasbourg (GAERS). We found that in both Wistar rats and GAERS, the proportion of interneurons was significantly higher in the LGN than in the VPM and VPL. In the LGN of Wistar rats, 16.4% of the neurons were interneurons and in the GAERS, the value was 15.1%. In the VPM, the proportion of interneurons was 4.2% in Wistar and 14.9% in GAERS; in the VPL the values were 3.7% for Wistar and 11.1% for the GAERS. There was no significant difference between Wistar rats and the GAERS regarding the counts of interneurons in the LGN, whereas the VPM and VPL showed significantly higher counts in GAERS. Comparison of the mean areas of both relay cells and interneuronal profiles showed no significant differences between Wistar rats and GAERS. These findings show that in the VPL and the VPM

there are relatively more GABAergic interneurons in GAERS than in Wistar rats. This may represent a compensatory response of the thalamocortical circuitry to the absence seizures or may be related to the production of absence seizures.

Keywords: immunohistochemistry; interneuron; relay neuron; thalamus; GABA

INTRODUCTION

There are two types of cells in the thalamus: interneurons and relay cells. The interneurons have axons that do not leave the thalamus, whereas the axons of the relay cells project beyond the thalamus. The relay cells use the excitatory amino-acid glutamate and the interneurons use the inhibitory gamma-aminobutyric acid (GABA) as neurotransmitter^[1].

The GABAergic control of thalamocortical neurons is via local interneurons and the cells of the thalamic reticular nucleus (TRN)^[2–8]. Earlier studies have shown that in some mammals GABAergic interneurons are absent or only sparse in many thalamic nuclei. In members of the primate and carnivore orders, interneurons represent 20–30% of the total neuronal population in all the thalamic nuclei, but are sparse or absent in rodents, except for the lateral geniculate nucleus (LGN)^[9–16], where they represent 20–25% of neurons.

Studies have shown that thalamic nuclei receive

additional GABAergic inputs from the zona incerta, anterior pretectal nucleus, and substantia nigra pars reticulata in the rat. These extrathalamic inputs project preferentially to the higher-order thalamic relays, not to first-order-relays^[17–22]. A light-microscopic study has shown that the GABAergic terminal groups innervating first-order and higher-order nuclei are dissimilar in the cat^[23] and another study has shown that higher-order thalamic nuclei receive some larger GABA-positive terminals that have been described as 'driver-like' and are not present in the first-order nuclei^[24].

The TRN cells and interneurons both send inhibitory axons to relay cells^[25, 26]. However, they have distinct developmental origins: the dorsal thalamus gives rise to the interneurons whereas the ventral thalamus gives rise to the cells of the TRN. Further, the two cell types differ in their connections. How the TRN cells relate to the interneurons is not clearly defined, although the presence of putative inhibitory inputs onto interneuronal dendrites suggests that the TRN cells innervate interneurons and that the two inhibitory systems are closely related^[27].

Many studies have shown that the GABAergic system is important in generating absence seizures and spike-and-wave discharges (SWDs)^[28–33]. The genetic absence epilepsy rats from Strasbourg (GAERS), a selected inbred strain of Wistar rats, is a well-validated genetic model of typical absence epilepsy for neurological, behavioral, and pharmacological studies^[34, 35].

In this study, we used light microscopic immunocytochemistry with a GABA immunogold marker to compare the ratios of GABAergic interneurons to relay cells in the LGN, the ventral posteromedial (VPM) and the ventral posterolateral (VPL) thalamic nuclei of Wistar rats and GAERS.

MATERIALS AND METHODS

Animals

Adult (6–12 months old) Wistar albino control rats ($n = 5$) and GAERS ($n = 5$) weighing 250–300 g were used. The animals were housed in Plexiglas cages in a temperature-controlled room ($20 \pm 3^\circ\text{C}$) under a 12-h light/dark cycle, with standard laboratory chow and tap water *ad libitum*. All procedures were approved by the Animal Care and Use Committee of Marmara University.

Tissue Preparation

Rats were deeply anesthetized with intraperitoneal ketamine (100 mg/kg) and chlorpromazine (1 mg/kg) and perfused transcardially with 4–5 mL heparin solution (2 mL heparin in 8 mL 0.1 mol/L HEPES, pH 7.6) and a fixative containing 2.5% glutaraldehyde, 0.5% paraformaldehyde, and 0.1% picric acid in 0.1 mol/L HEPES at pH 7.6. After fixation, the animals were decapitated and the brains removed. The entire brain was immersed overnight in the same fixative solution at 4°C , then washed several times in 0.1 mol/L HEPES, pH 7.3. Coronal sections were cut at 300 μm on a vibratome (Leica VT 100S). Cylindrical portions of the LGN, VPL, and VPM nuclei were isolated from the sections (Fig. 1). Specimens were post-fixed in 1% osmium tetroxide and 1.5% potassium ferricyanide (1:1) for 30 min at room temperature. The samples were then washed several times in deionized water and stained with aqueous 0.5% uranyl acetate for 30 min at room temperature in the dark. The tissue was then dehydrated in a graded series of ethanol, cleared in propylene oxide, and embedded in Epon 812 for 24 h at 60°C . Semi-thin sections (1 μm) were cut on a Leica Ultracut R ultramicrotome, stained with toluidine blue, and viewed under a Nikon Eclipse 90i light microscope to check the fixation quality and to select an area for immunocytochemistry. All well-fixed tissues were included in the study.

Immunocytochemistry

The semi-thin sequential 1- μm sections from the Epon blocks were cut using a Leica Ultracut R ultramicrotome and collected on Silane-treated slides. Then they were dried in a 37°C oven for 2 h. To remove osmium and some of the resin the Epon sections were treated with a saturated solution of sodium ethoxide diluted 2-fold with absolute ethanol, followed by two changes of an absolute ethanol rinse, brought to distilled water and washed in Tris-buffered saline (pH 7.6) containing 0.1% Triton X-100 and 10% bovine serum albumin (BSA) (TBST 7.6). The slides were incubated with the primary anti-GABA antibody (1:2 000 in 5% BSA in TBST 7.6) overnight in a moist chamber at 4°C . Then they were washed several times in TBST 7.6 and incubated for 3 h with goat anti-rabbit IgG (Sigma G7277) conjugated to 5-nm gold, which was diluted 1:50 in TBST 7.6 containing 1% BSA. The sections were then washed in TBST 7.6 and then with deionized water and subjected

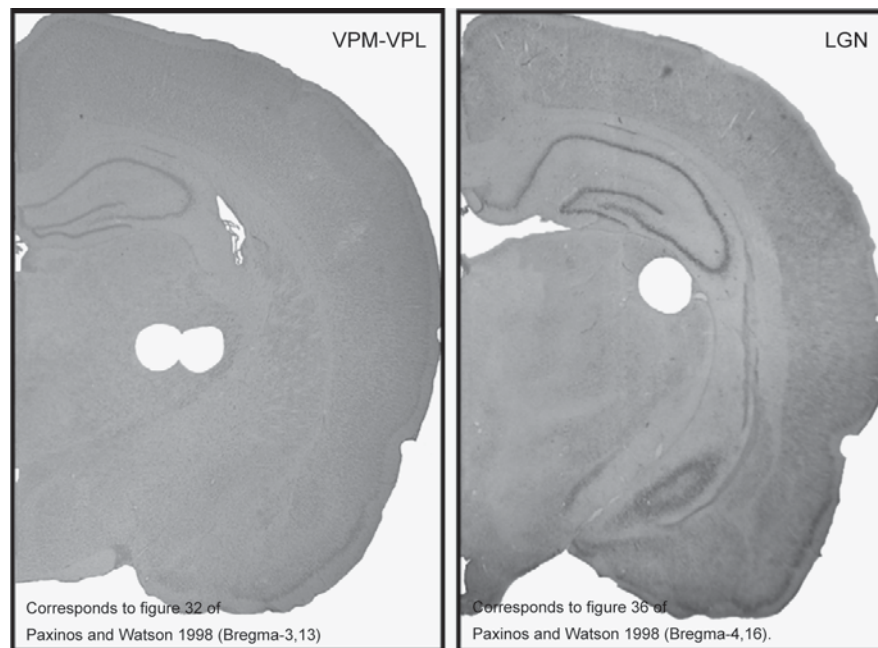


Fig. 1. Coronal vibratome sections cut at 300 μm , showing thalamic areas sampled for the study of the lateral geniculate (LGN), ventral posteromedial (VPM) and ventral posterolateral (VPL) nuclei. The section chosen for each sample was neither the most rostral nor the most caudal of sections through the relevant nucleus. The LGN sample section was published in our previous manuscript^[36], for which the same tissue blocks were used.

to a silver enhancement procedure by incubation with the LI Silver enhancement kit (LI Silver 2013, Nanoprobes) for 15–20 min at room temperature in the dark. After several washes with deionized water, sections were counterstained with Fast Green. The sections were mounted in Entellan (Merck, Darmstadt, Germany), examined under an Olympus BX51 photomicroscope and then photographed.

Neuron Counting

Due to the higher probability of including a relay cell (large cells) than an interneuron (small cells) in a section, the disector method was used to overcome the underestimation of interneurons. Sections through the Epon-embedded samples were serially cut at 1 μm . Then, every fourth and fifth sections were used as a pair for the counts. A total of ten pairs of sections from each animal were processed for GABA immunocytochemistry. All the relay cells and interneurons that had a visible nucleus in the section were counted. Cells whose nuclei appeared in consecutive sections were not counted, but cells with a nucleus in only one of the two sections were included in the counts. All parts of the section were included in the counts, and the

area of each section was measured using the Image J program. The counts of interneurons and relay cells were expressed as the number of cells/ mm^2 . Then the mean for 5 animals and the SEM for each nucleus were calculated. The GABA-positive cells and relay cells were counted at $\times 200$ final magnification. Sections for the quantitative study were selected by a systematic random sampling procedure. The relative proportion of interneurons was expressed as a percentage of the total population. The cells were counted by an observer who was blinded to the nuclei and groups.

Neuron Area Measurement

The average areas of the profiles of the interneurons and relay cells were calculated in each of the LGN, VPM, and VPL thalamic nuclei for each animal. For relay cells, the average area of profiles was calculated from 20 randomly-selected relay cells from each nucleus of each animal. Area measurements were made at $\times 200$ final magnification. All the interneurons present in the sample were used to calculate the average area of interneuronal profiles. Area measurements were made using Image J. The measurements were made by an observer who was blinded

Table 1. The average number and percentage of relay cells and GABA-positive interneurons in 1mm² area of LGN, VPL and VPM thalamic nuclei.

| | Total No. of relay cells | Av. No. of relay cells/mm ² (± SEM) | % of relay cells | Av. area of relay cell profiles (μm ²) | Total No. of interneurons | Av. No. of interneurons/mm ² (± SEM) | % of interneurons | Av. area of interneuronal profiles (μm ²) | Total neuron numbers (relay + interneuron) counted |
|---------------------------|--------------------------|--|------------------|--|---------------------------|---|-------------------|---|--|
| LGN Wistar (<i>n</i> =5) | 1220 | 246.8 ± 38.9 | 83.6 | 239.6 ± 33.9 | 241 | 48.4 ± 12.1 | 16.4 | 100.5 ± 20.3 | 1461 |
| LGN GAERS (<i>n</i> =5) | 1106 | 289.2 ± 41.0 | 84.9 | 231.6 ± 23.4 | 197 | 52.2 ± 14.4 | 15.1 | 91.4 ± 19.6 | 1303 |
| VPM Wistar (<i>n</i> =5) | 1217 | 304.3 ± 16.6 | 95.8 | 372.3 ± 27.1 | 53 | 13.3 ± 0.6 | 4.2 | 111.0 ± 26.4 | 1270 |
| VPM GAERS (<i>n</i> =5) | 676 | 166.0 ± 6.6 | 85.0 | 365.1 ± 39.1 | 119 | 29.3 ± 1.6 | 14.9 | 105.2 ± 24.6 | 795 |
| VPL Wistar (<i>n</i> =5) | 601 | 150.3 ± 17.5 | 96.3 | 352.51 ± 47.6 | 23 | 5.9 ± 1.1 | 3.7 | 71.3 ± 11.5 | 624 |
| VPL GAERS (<i>n</i> =5) | 477 | 106.7 ± 27.47 | 88.9 | 334.9 ± 47.9 | 60 | 13.3 ± 4.9 | 11.1 | 102.1 ± 34.7 | 537 |

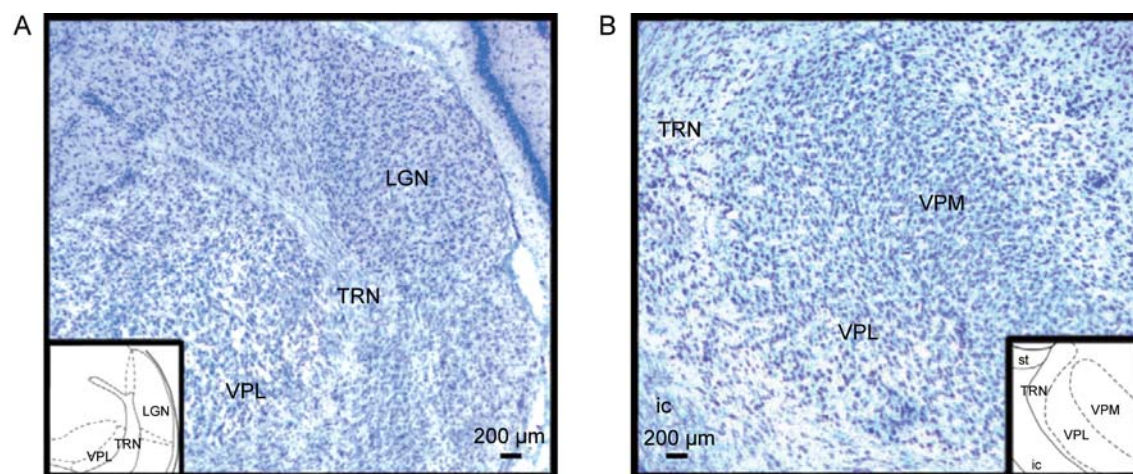


Fig. 2. Nissl-stained coronal sections of the LGN, VPM, and VPL nuclei of Wistar rats. **A:** The LGN showed a homogenous cell distribution throughout the nucleus with no distinct lamination. **B:** The cells of the VPM nucleus were more densely packed than in the VPL and an apparent border between the VPM and VPL was formed by compact cells lying parallel to the long axis of the nuclei. Inserts are schematic illustrations from Paxinos and Watson rat brain atlas^[37]. ic: internal capsule. LGN, lateral geniculate thalamic nuclei; TRN, thalamic reticular nucleus; VPL, ventral posterolateral thalamic nuclei. VPM, ventral posteromedial thalamic

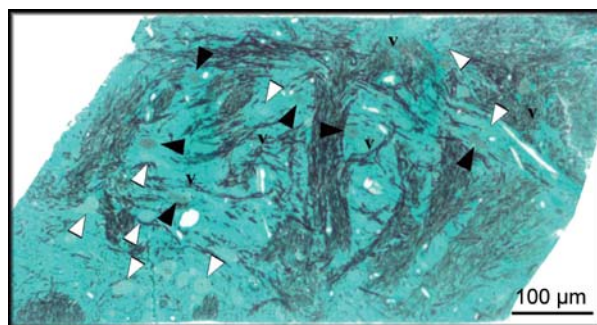


Fig. 3. GABA-immunolabeled interneurons (black arrows) and non-labeled relay cells (white arrows) in the LGN. v: vessel.

to the thalamic nuclei and the groups. Data were analyzed with the Mann-Whitney U test in Graphpad PRISM.

RESULTS

General Summary

The total numbers of relay cells and interneurons and their corresponding percentages in each thalamic nucleus are shown in Table 1. The Nissl-stained sections showed that the cells of the LGN were homogenous with no distinct lamination (Fig. 2A); the cells in the VPM nucleus were

more densely packed than in the VPL (Fig. 2B). An apparent border was detectable between the VPM and VPL nuclei, formed by compact cells lying parallel to the long axis of the two nuclei (Fig. 2B). Fig. 3 shows a low-magnification sample of GABA-immunolabeled interneurons and non-labeled relay cells in LGN of the thalamus. Representative GABA-positive interneurons and unlabeled relay cells from the three thalamic nuclei in both Wistar and GAERS animals are shown at a higher magnification in Fig. 4.

Quantification of Relay Neurons and Interneurons

GABA-positive interneurons were in the minority in the LGN, VPM, and VPL nuclei of both Wistar rats and GAERS (Table 1). In the LGN, the interneurons constituted 16.4% of the neurons in Wistar rats, and in the GAERS it was 15.1%; in the VPM of Wistar rats the interneurons formed 4.2%, while in the GAERS it was 14.9%; in the VPL of Wistar rats the interneurons formed 3.7%, while in the GAERS it was 11.1%. The number of interneurons per unit area was

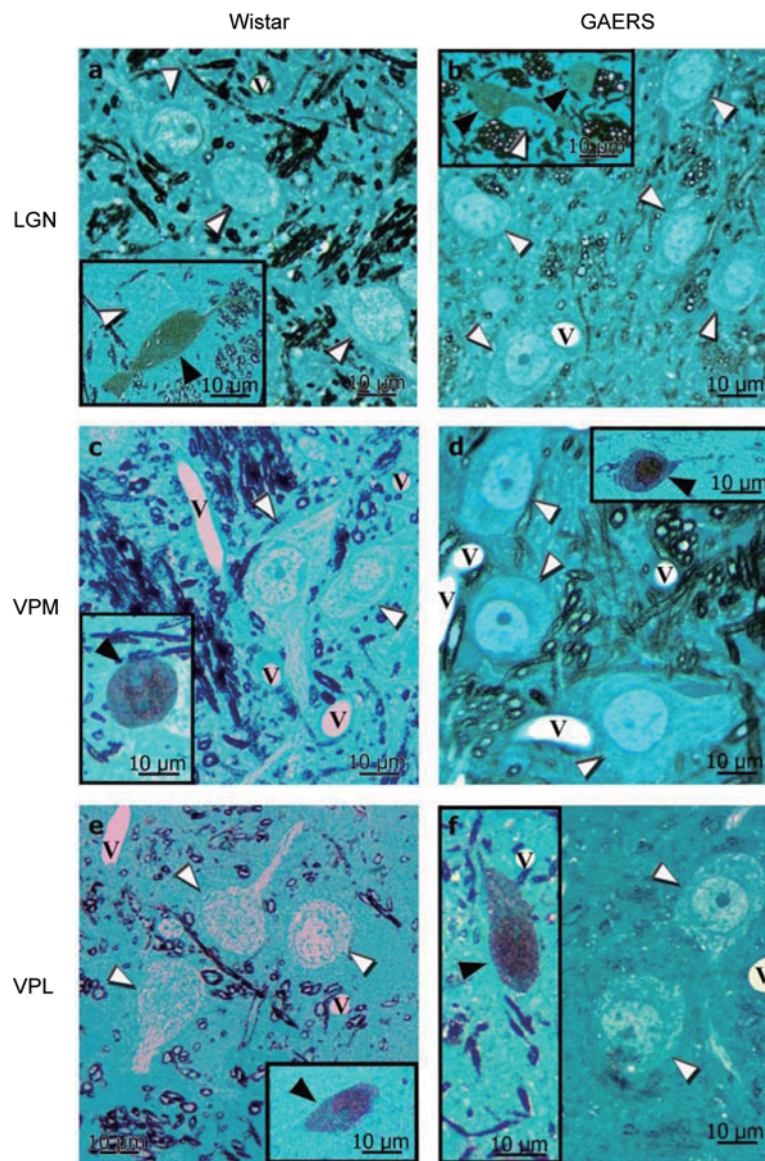


Fig. 4. Samples of GABA-immunolabeled interneurons in LGN, VPM, and VPL of Wistar (a, c, e) and GAERS (b, d, f) rats. White arrowheads indicate relay cells and black arrowheads indicate interneurons. V, vessel. LGN, lateral geniculate thalamic nuclei; VPL, ventral posterolateral thalamic nuclei. VPM, ventral posteromedial thalamic nuclei.

higher in the LGN than in the VPM and VPL of both Wistar rats (LGN-VPM $P = 0.0003$, LGN-VPL $P = 0.0001$) and GAERS (LGN-VPM $P = 0.03$, LGN-VPL $P = 0.003$) (Fig. 5). Further, there was no statistically significant difference in the density of interneurons per unit area in the LGN between Wistar rats and GAERS ($P = 0.68$) (Fig. 5), while the VPM ($P = 0.0001$) and VPL ($P = 0.003$) in GAERS had higher values for interneurons than Wistar rats (Fig. 5).

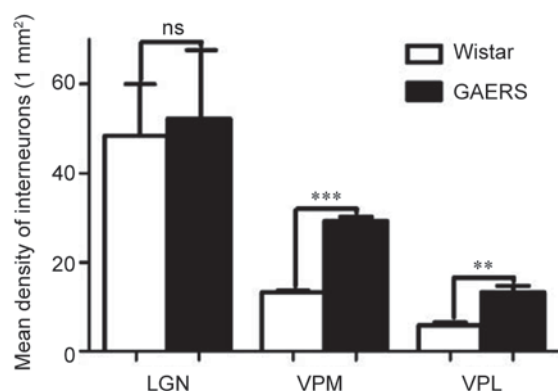


Fig. 5. Average number of interneurons per mm² in the LGN, VPM, and VPL nuclei of Wistar and GAERS animals. The results are expressed as mean \pm SEM. LGN, lateral geniculate thalamic nuclei; VPL, ventral posterolateral thalamic nuclei; VPM, ventral posteromedial thalamic nuclei.

Area Measurements of Relay Cells and Interneurons

The average areas of neuronal soma of the interneurons and the relay cells in the LGN, VPM, and VPL nuclei of Wistar rats and GAERS are shown in Table 1. The mean areas of the relay cells were almost three times of those of interneurons in all the three thalamic nuclei. However, there were no significant differences between the Wistar rats and GAERS in any of the nuclei (Table 1).

DISCUSSION

The present study confirmed earlier reports of the relative abundance of interneurons in the LGN of normal rats^[10, 14, 38]. However, we showed a slightly higher proportion of interneurons in the VPM and VPL than previously reported and also provided a novel observation regarding the significantly greater proportion of interneurons in the VPM and VPL parts of the ventrobasal (VB) nucleus in the GAERS compared to the Wistar rats.

General Considerations

Earlier studies have shown that the relative proportion of GABAergic interneurons varies in thalamic nuclei and in mammalian species^[15, 39]. The highest proportions of interneurons are found in the LGN of the human and monkey^[14] (Table 2). Arcelli *et al.*^[14] reported that in the rat and mouse the GABA-positive neurons in the LGN make up 15–20% of the total neuronal population. Further, Spreafico *et al.*^[10] found that 16–21% of the neurons in the VPL nucleus of the cat are interneurons. Here, we showed that interneurons accounted for 16.4% in the LGN, 4.2% in the VPM, and 3.7% in the VPL of the total neuronal population in Wistar rats. The figures for the LGN confirm earlier studies. However, the proportion of interneurons was slightly higher in the VPM and VPL than reported earlier^[14, 38].

Differences between LGN, VPM, and VPL

The variation in the distribution of interneurons among the LGN, VPM, and VPL in some rodents suggests a major difference in the organization of the inhibitory circuits between the thalamic nuclei. Nothing is known about the processing of visual *versus* somatosensory inputs in any of these species that might account for such marked differences, and we do not know the extent to which TRN cells may replace interneurons where their proportions are low. Montero^[43], and Sanchez-Vives *et al.*^[44] described a special class of interneurons in the interlaminar region of the LGN of the cat and ferret, which resemble the cells of the TRN, and proposed that these cells had migrated from the TRN, suggesting that there may be a close developmental relationship between interneurons and TRN cells, with one cell-type perhaps able to functionally replace the other, but at present there is no evidence to support such a view.

The interneurons in the thalamic nuclei are important for the functional arrangement of its synaptic organization since they provide the morphological basis for an intrinsic modulatory mechanism that adds complexity to the synaptology and circuitry of these nuclei. The presence of a greater proportion of GABAergic neurons in one thalamic nucleus may suggest an increased need for an intrinsic inhibitory modulation, but we have no information about how the numbers of interneurons relate to the complexity of the inhibitory interactions in any one nucleus. The low proportion of interneurons in the VPM and VPL thalamic nuclei may still be sufficient to form some of the

Table 2. Percentage of interneurons previously reported for the lateral geniculate (LGN) and ventrobasal (VB) thalamic nuclei of different species.

| | Rat/Mouse (%) | | Cat (%) | | Guinea Pig (%) | | Monkey (%) | | Human (%) | |
|---|------------------------|-------|---------|-------|----------------|-----|------------|-------|-----------|-----|
| | VB | LGN | VB | LGN | VB | LGN | VB | LGN | VB | LGN |
| LeVay & Ferster ^[40] | | 20–25 | | | | | | | | |
| Penny <i>et al.</i> ^[3] | | | | 30 | | | | | | |
| Spreafico <i>et al.</i> ^[10] | | | 16–21 | | | | | | | |
| Fitzpatrick <i>et al.</i> ^[25] | | | | 25 | | | | | | |
| Madarász <i>et al.</i> ^[11] | | | 33–27 | | | | | | | |
| Montero & Zempel ^[42] | | | | | | | 33 | | | |
| Rinvik <i>et al.</i> ^[12] | | | | 20–30 | | | 20–30 | | | |
| Spreafico <i>et al.</i> ^[38] | | | 19–21 | | 15 | 20 | | | | |
| Arcelli <i>et al.</i> ^[14] | | 15–20 | 24–27 | 24–27 | 14 | 20 | 24–27 | 24–27 | 40 | 40 |
| Present study 2013 | 3.7 (VPL) 4.2 (VPM) | 16.4 | | | | | | | | |

Note that the corrections used for counting errors differed and are not included here.

complex synaptic arrangements necessary for the intrinsic organization of the nucleus. Although the proportion of interneurons can be <5% in the VPM and VPL nuclei, a richly-branched arbor of interneuronal processes with several hundred terminals could still allow each relay cell to interact with the processes of a few interneurons.

These findings point out an apparent dissociation between the behavioral needs of these animals and the occurrence of GABAergic cells in the LGN, VPM, and VPL nuclei, and suggest that the numbers of thalamic local circuit neurons are not directly related to an animal's ability to perform a specific sensorimotor task. Also, the physiological properties of one thalamic nucleus (e.g. LGN) differ from those of others (e.g. VPM and VPL), and the same nucleus can vary among species.

Differences between Wistar Rats and GAERS

An important further result of the present study was the significantly greater numbers of interneurons in the VPM and VPL nuclei in GAERS than in Wistar rats. However, the interneurons in the LGN showed no such difference. Our recent electron microscopic (EM) study quantifying the three major terminal types; 'RL'—round vesicles, large terminals (glutamatergic), 'RS'—round vesicles,

small terminals (glutamatergic) and 'F'—flattened vesicles (GABAergic) in several thalamic nuclei of Wistar rats and GAERS, showed that the GABA-immunoreactive profiles in the LGN are similar in both strains (9.4% and 10.5%, respectively) whereas for the VB nucleus they are 7.5% in the Wistar rats and 19.2% in the GAERS^[36, 41]. This is in accord with the present results that an unexpectedly high number of GABAergic interneurons occurred in the VPM and VPL nuclei of the GAERS animals relative to the Wistar rats. However, both the present results and the earlier EM study show that the inhibitory circuits in the LGN are not abnormal in GAERS animals. These differences in GAERS may be related to either the production of absence seizures or represent a compensatory response of the thalamocortical circuitry to these seizures. In accord with the present findings, there is evidence that the somatosensory pathways (barrel fields and the VB nucleus) are involved in the initiation of SWDs, but not the LGN and visual cortex^[34, 45, 46].

GABA-mediated Mechanisms in the Production of Spike-and-Wave Discharges

Previous studies have shown that GABA neurotransmission plays a critical role in the generation and control of SWDs^[46, 47].

Drugs enhancing GABA function exacerbate absence seizures^[47]. In GAERS, intraperitoneal administration of a GABA agonist induces a dose-dependent increase in the duration of SWDs^[47, 48]. Similar results were obtained when the same drugs were injected bilaterally into thalamic relay nuclei^[48]. Further, Liu *et al.*^[49] and Hosford *et al.*^[50] concluded that absence seizures constitute a particular form of epilepsy that may be associated with an excess of GABAergic inhibition within the thalamus, and this fits well with our current findings. The greater proportion of interneurons in the VPM and VPL nuclei in GAERS than in Wistar rats in the present study and the higher proportion of GABAergic inhibitory terminals (with flattened vesicles) in our previous study strongly suggest the involvement of the GABAergic system in the absence epilepsy mechanism^[50]. However, Champier *et al.*^[51] made *in vivo* intracellular recordings from layer V cortical neurons in the facial motor cortex and from thalamocortical neurons in the VPL and VPM in GAERS and showed no rhythmic GABA_B IPSPs and low-threshold Ca²⁺ potentials during seizures. This suggests that during seizures there is an abnormal corticothalamic input to the relevant thalamic nuclei even if no burst firing is recorded in the cells.

Possible Role of VPM and VPL in Absence Epilepsy

Electrophysiological studies have shown that the cortex and thalamus underlie absence seizures^[29, 31]. There is considerable evidence that thalamic nuclei, primarily the VB nucleus, are involved in the propagation and regulation of seizures^[31, 52]. Previous findings using a microdialysis method also showed increased levels of extracellular GABA in the VB thalamus of GAERS compared to non-epileptic control Wistar rats^[53]. Danober *et al.*^[34] reported that the VB nucleus is critical in controlling the oscillatory thalamocortical activity that underlies generalized epileptic seizures such as absence seizures. Vergnes and Marescaux^[46] produced lesions by unilateral injection of KCl into the superficial layer of the lateral frontoparietal cortex (including the barrel cortex). SWDs were immediately suppressed, not only in the injected cortex but also in the ipsilateral VB thalamus. Further, cortical SWDs are suppressed by VB thalamic lesions in GAERS^[45]. In GAERS, bilateral injections of a GABA_B agonist into the VB nuclei induces dose-dependent increases in SWDs. In contrast, injection of GABA_B antagonists into the VB

suppresses SWDs in this model of absence seizures^[48]. Further, a specific role of the somatosensory cortex, in particular the peri-oral region of S1, in the initiation of SWDs has been reported^[52]. Inactivation of the peri-oral region suppresses SWDs in the cortex and VPM nucleus of the thalamus^[54]. The results summarized above suggest that the VPM and VPL nuclei and the somatosensory cortex are crucially important in initiating SWDs, and this is in accord with our results.

CONCLUSION

Each thalamic nucleus has a characteristic pattern of GABAergic synaptic inputs and the relative density of GABA-immunoreactive interneurons varies from one nucleus to another. The local inhibitory circuits are formed by incoming afferents as well as by intrinsic GABAergic cells, and both contribute to shaping the output activity in thalamocortical projection neurons. Our results showed that in the VPM and VPL nuclei but not in the LGN, there were significantly more GABAergic interneurons in GAERS than in Wistar rats, and this relates to our previous quantification of the terminals and synapses in the LGN and VB that provides evidence that in the GAERS there is a significant increase of modulatory inhibitory terminals and synapses in the VB but not the LGN. The increase of GABAergic interneurons in the VPM and VPL and the increase of GABAergic inhibitory terminals in the VB nucleus may well both be related to the production of absence seizures, or may represent a compensatory response of the thalamocortical circuitry to absence seizures.

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