

# Calcium-binding proteins in GABAergic calyciform synapses of the reticular nucleus

Bertalan Csillik<sup>a</sup>, Andras Mihaly<sup>a</sup>, Beata Krisztin-Peva<sup>a</sup>, Robert Fenyo<sup>a</sup> and Elizabeth Knyihar-Csillik<sup>b</sup>

Departments of <sup>a</sup>Anatomy and <sup>b</sup>Neurology, Albert Szent-Gyorgyi Medical and Pharmaceutical Center, University of Szeged, Hungary

Correspondence and requests for reprints to Bertalan Csillik MD, PhD, DSc, Department of Neuroanatomy, University Medical School, 40 Kossuth Lajos sgt, H-6701 Szeged, Hungary

Tel: +36 62 544 918; 36 62 545 665; fax: +36 62 545 707; e-mail: csillik@anatomy.szote.u-szeged.hu

Sponsorship: Supported by Grant 7/2003 from the Hungarian Medical Research Council.

Received 6 February 2006; accepted 8 February 2006

Large calyciform synapses in the rat reticular thalamic nucleus are characterized by the presence of  $\gamma$ -aminobutyric acid. Presynaptic terminals are also loaded with calcium-binding proteins such as parvalbumin, calbindin, calretinin and calcineurin. The number of calyciform terminals containing  $\gamma$ -aminobutyric acid and parvalbumin is 2005 in young adult rats; calbindin is present in 1500, calretinin in 850 and calcineurin in 560 calyciform terminals. Developmental studies revealed that  $\gamma$ -aminobutyric acid and

calcium-binding proteins are virtually absent from calyciform terminals at birth but their occurrence increased considerably during postnatal life, suggesting increasing regulation of presynaptic calcium signaling during postnatal life. It is concluded that synaptic activity of large calyciform  $\gamma$ -aminobutyric acid-containing synapses of the reticular thalamic nucleus is mediated, regulated or accompanied by calcium ions. *NeuroReport* 17:575–578 © 2006 Lippincott Williams & Wilkins.

**Keywords:** calcium-binding proteins,  $\gamma$ -aminobutyric acid, reticular nucleus, thalamus

## Introduction

The reticular thalamic nucleus (RTN) occupies a strategic position between the neocortex and the thalamus. By axon collaterals innervating  $\gamma$ -aminobutyric acid-containing (GABAergic) RTN cells, the RTN controls thalamo-cortical and cortico-thalamic functions via intrinsic cell-to-cell communications that are supposed to be mediated by dendrodendritic synapses [1].

Calcium ( $\text{Ca}^{2+}$ ) binding proteins (CBPs) were observed in the RTN first by Celio [2], and later by other investigators, but none of these authors noticed the large calyciform presynaptic structures described by us [3]. Parvalbumin (PV) as well as calretinin (CR), calbindin (CB), calmodulin (CM) and calcineurin (CN) are members of the large family of extended prefinger (EF)-hand CBPs; they function either as  $\text{Ca}^{2+}$  sensors or as  $\text{Ca}^{2+}$  buffers [4].

GABAergic large calyciform presynaptic complexes were recently identified in the rat RTN [5]. GABA is known to coexist with PV [6] and other CBPs in various parts of the central nervous system [7]; thus, we tried to locate CBPs in the calyciform synapses of the RTN. On the other hand, as Collin *et al.* [8] reported recently that developmental changes in PV regulate presynaptic  $\text{Ca}^{2+}$  signaling in the cerebellum, we studied numbers of CBP and GABA-containing calyciform terminals of RTN in the course of ontogeny. The results of these experiments suggest that  $\text{Ca}^{2+}$  ions and CBPs may play important parts in the function of GABAergic calyciform synapses in the RTN.

## Materials and methods

Investigations were performed on 12 young adult albino rats, on four newborn rats, on four 3-week-old rat puppies and on four adolescent (3-month-old) rats of both sexes, Wistar strain, obtained from the animal house of the Szeged University Medical School. Care of the animals complied with the guidelines of the Hungarian Ministry of Welfare; experiments were carried out in accordance with the European Communities Council Directive (24 November 1986; 86/609/EEC) and the University Guidelines for Ethics in Animal Experiments. For the histochemical demonstration of PV, a polyclonal anti-mouse PV antibody raised in rabbits was used (obtained from Sigma, St Louis, Missouri, USA) in a dilution of 1:3000. GABA immunoreactivity was detected by means of a GABA antiserum (Chemicon, Temecula, California, USA) obtained from rabbit, in a dilution of 1:500. Calbindin-28k (CB) was demonstrated immunohistochemically by a polyclonal anti-CB (H-50) serum, obtained from rabbit (Santa Cruz Biotechnology, California, USA); also, antisera against CR (N-18, goat polyclonal) and calcineurin (CN-PP1, rabbit polyclonal) were obtained from Santa Cruz. GABA has been detected with a Chemicon polyclonal antiserum, obtained from rabbit.

Animals were subjected to transcardial perfusion in deep chloral hydrate anesthesia with Zamboni's aldehyde fixative. Cryostat sections were obtained in the paramedian sagittal plane, 3–4 mm from the midline, or in coronal sections. Endogenous peroxidase activity was blocked by

the application of 0.3% hydrogen peroxide diluted in methanol (10 min), followed by three successive rinses in 0.1 M phosphate buffer. Free-floating sections were pre-treated with blocking serum (0–1.0 M phosphate-buffered saline), 10% normal goat serum, 1% bovine serum albumin and 0.3% Triton X-100) on a shaker plate at room temperature for 1 h, and then transferred into the primary antibody. Incubation was carried out at 4°C on a shaker for 36 h, followed by three rinses in 0.1 M phosphate buffer. To detect the bound primary antibody, we used the avidin-biotin peroxidase method. Kits were obtained from Vector Laboratories (Burlingame, California, USA). The secondary antibody, biotinylated anti-mouse immunoglobulin, was applied for 90 min at room temperature. Three more rinses in 0.1 M phosphate buffer were followed by incubation in the avidin-biotinylated-peroxidase complex for 60 min at room temperature. After three rinses in 0.1 M phosphate buffer, peroxidase activity was visualized by a histochemical reaction involving diaminobenzidine-tetrahydrochloride and hydrogen peroxide (3 µl of 30% H<sub>2</sub>O<sub>2</sub> to 10 ml 1% diaminobenzidine). After three rinses in 0.1 M phosphate buffer, free-floating sections were dehydrated in a graded series of ethanols, cleared in xylene and coverslipped with Permount.

Specificity of the immunohistochemical reactions was assessed by means of the following treatments: (1) omission of the first specific antiserum; (2) use of normal rabbit or mouse serum instead of anti-PV antiserum; (3) treatment according to the avidin-biotin complex method, from which one of the steps had been omitted; (4) preabsorption of the specific antibodies with the appropriate blocking peptides (sc7447P and sc7448 for PV, sc11644P for CR, sc7691P for CB and sc443 for CN). None of these specimens showed any reaction.

For electron microscopic immunohistochemistry, 50-µm-thick free-floating vibratome sections were processed like the samples for light microscopy, except that Triton X-100 was omitted. Having visualized the peroxidase activity, the free-floating sections were postfixed in an osmic acid solution (2% OsO<sub>4</sub> in phosphate buffer) for 1 h and dehydrated in a graded series of ethanol. Sections were flat embedded in Durcupan (Fluka, Buchs, Switzerland) on Liquid Release-treated slides, and polymerized for 36 h at 56°C. Selected regions of the brain were excised and remounted. Ultrathin serial sections were obtained on a Reichert Ultratome (Reichert, Vienna, Austria), using a diamond knife. Sections were stained with lead citrate according to Reynolds, and analyzed and photographed on a JEOL 1010 electron microscope (JEOL, Tokyo, Japan).

Determination of the number of PV, GABA, CM and CR-immunoreactive terminals was obtained with the stereological technique of the optical fractionator method of West *et al.* [9]. On the basis of immunohistochemically stained coronal serial sections, the number of calyciform terminals was determined in uninterrupted coronal section series of RTN. In a young adult rat, in whom the RTN extends anteroposteriorly for 3 mm, 30 sections were analyzed; in a newborn rat, in whom the RTN extends anteroposteriorly for 1.5 mm, 15 sections were analyzed, in a 3-week-old animal in whom the RTN extends anteroposteriorly for 2 mm, 20 sections were analyzed, and in a 3-month-old rat, in whom RTN extends anteroposteriorly for 2.5 mm, 25 sections were analyzed. Stereological analysis was performed with the aid of an Olympus microscope (Olympus,

Tokyo, Japan) equipped with a video camera and stepping motors, to move the section to predetermined *x-y* positions. The number of calyciform terminals was determined using a 100 × oil immersion lens. Counting frames were superimposed on video images of the microscopic fields. Differences between the numbers of immunoreactive calyciform terminals were determined statistically with the paired *t*-test; *P* value was set at 0.05 for significance.

## Results

The RTN of the young adult rat is a shell-shaped structure, semicircular in a coronal section and hook-like in the paramedian plane. The size of the RTN is 3.2 mm in the sagittal plane (from lateral 0.9 to lateral 4.1). In the coronal plane, it extends from bregma -1.3 mm (interaural 7.7) to bregma -3.8 mm (interaural 5.2) [10].

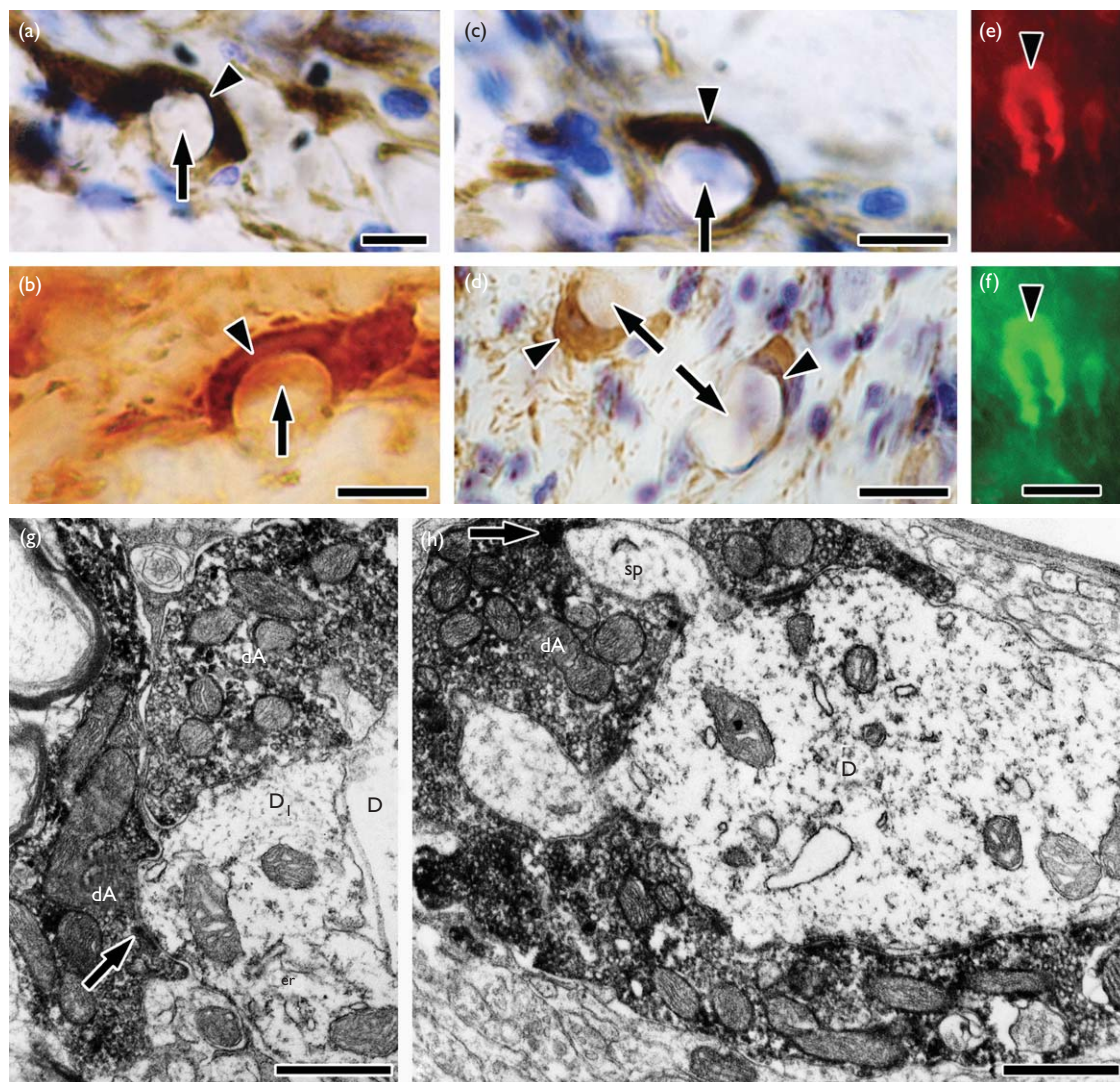
In the RTN, numerous GABAergic calyciform terminals were found that also exhibited PV immunoreactivity. At the light microscopic level, both PV and GABA were found in calyciform structures that surrounded immunonegative dendritic bulbs (Fig. 1a and b). CB and CR were found in similar localizations in the calyciform nerve endings, surrounding large, immunonegative dendritic profiles (Fig. 1d and e); the same was true for CN. The identity of PV and GABA-immunoreactive terminals was shown by fluorescence microscopy (Fig. 1f and g). At the electron microscopic level, GABA (Fig. 1h) and PV (Fig. 1i) were seen to be present in calyciform terminals, presynaptic to large dendritic bulbs. In the terminals, a large number of spheroid synaptic vesicles were observed. The presynaptic and the postsynaptic membranes exhibited approximately equal thickenings, but presynaptic membranes were sometimes more dense than postsynaptic ones.

According to stereological investigations, the number of calyciform terminals exhibiting GABA and PV immunoreactivity in young adult rats was 2005 in the RTN. The number of CB-immunoreactive terminals was 1530, the number of CR-immunoreactive terminals was 850 and the number of CN-immunoreactive terminals was 560. Virtually no difference was observed between RTNs on the right and left sides.

We found hardly any PV-containing calyciform terminals in newborn rats; according to stereological determinations, their occurrence was limited to 8. In 3-week-old rats, the number of PV-immunopositive terminals was 134. The corresponding values for CBPs and GABA are shown in Table 1.

## Discussion

Impulses of specific thalamic nuclei are known to be transmitted to the cerebral cortex via GABAergic relay systems in the RTN. Although the PV-immunoreactive presynaptic structures bear a deceptive resemblance to PV-immunoreactive cell bodies equipped with large empty nuclei, our earlier investigations [3,5], just like the present ones, prove that these structures are either axons or dendrites studded with synaptic vesicles. Electrical stimulation of the RTN results in expression of *c-fos* protein in the ipsilateral retrosplenial cortex, while electrical stimulation of the retrosplenial cortex results in *c-fos* expression in the ipsilateral RTN [11].



**Fig. 1** Immunohistochemical analysis of calyciform terminals in the reticular nucleus of the thalamus. (a)  $\gamma$ -Aminobutyric acid (GABA), (b) parvalbumin, (c) calbindin and (d) calretinin in calyciform terminals. Cross sections: arrowheads indicate the calyciform terminals; arrows point at the cross sections of the dendritic bulbs. Scale bar, 10  $\mu$ m. (e) Fluorescence microscopic appearance of GABA and parvalbumin (f) of the same presynaptic structure in the reticular thalamic nucleus, cut longitudinally. Arrowhead points at the horseshoe-like presynaptic structure surrounding an immunonegative dendrite. Anti-GABA marked with streptavidin peroxidase-labeled CY3 (Jackson, West Grove, Pennsylvania, USA), anti-parvalbumin marked with fluorescein isothiocyanate (Vector Laboratories, Burlingame, California, USA). Scale bar, 10  $\mu$ m. (g) Two dendritic profiles (D<sub>1</sub> and D) surrounded by GABA immunoreactive dendraxonal calyciform terminals (dA) in the reticular thalamic nucleus. er, Endoplasmic reticulum in the dendritic cytoplasm. Arrow points at presynaptic thickening in the dendraxon. Scale bar, 1  $\mu$ m. (h) Parvalbumin immunoreactivity of a calyciform terminal in the reticular thalamic nucleus. dA, Calyx formed by a dendraxon; D, dendrite; sp, dendritic spines. Arrow points at presynaptic thickening in the dendraxon. Scale bar, 1  $\mu$ m.

**Table 1** The numbers of PV, CB, CR, CN and GABA-containing calyciform terminals in the RTN during ontogenetic development of the rat

	PV	CB	CR	CN	GABA
Newborn	8 ± 3	7 ± 3	6 ± 2	7 ± 3	8 ± 3
Three weeks old	134 ± 33	80 ± 25	75 ± 22	66 ± 21	134 ± 33
Young adult	2005 ± 285	1530 ± 280	850 ± 78	560 ± 40	2005 ± 285

PV, parvalbumin; CB, calbindin; CR, calretinin; CN, calcineurin; GABA,  $\gamma$ -aminobutyric acid; RTN, reticular thalamic nucleus.

The experiments described above suggest that GABAergic calyciform synaptic terminals in the reticular nucleus of the rat thalamus use Ca<sup>2+</sup> in their signaling mechanism.

Neurons are known to have numerous Ca<sup>2+</sup> channels in different parts of the nerve cell to carry out separate functions. N-type and P/Q-type voltage-operated channels are known to trigger the release of neurotransmitters at synaptic endings [12]; it is reasonable to assume that such channels take part in the Ca<sup>2+</sup> release in the calyciform synapses. In contrast, L-type voltage-operated channels on the cell body and proximal dendrites are involved in providing Ca<sup>2+</sup> signals inducing gene activation, and functioning as kinetic filters responding to small depolarizations [13]; these are unlikely to play a part in the Ca<sup>2+</sup> release in calyciform synapses. The involvement of receptor-operated channels [14] responsible for Ca<sup>2+</sup> signals in

dendritic spines cannot be ruled out as such spines can be observed to be associated with the calyciform terminals. Most plausible, however, is the participation of store-operated  $\text{Ca}^{2+}$  channels that are supposed to open in response to depletion of internal  $\text{Ca}^{2+}$  stores. Even though the identity of such channels is uncertain [12], it appears that these channels are the main participants in the  $\text{Ca}^{2+}$  release operating in the function of the calyciform synapses.

Release of  $\text{Ca}^{2+}$  after supramaximal stimulation has been demonstrated with histochemical techniques more than 40 years ago [15]. It appears that a similar  $\text{Ca}^{2+}$  release also takes place in the large calyciform GABAergic terminals in the RTN. Therefore, it is small wonder that the calyciform synapses are loaded with a battery of CBPs.

Presence of PV and CB was first reported in neurons of RTN by Celio [2]. Later it was discovered that several PV-immunoreactive structures in the RTN are presynaptic elements [16]. The studies of Schwaller *et al.* [4] revealed that PV coexists with GABA in the cerebellum as in several other areas of the central nervous system [6,7]. Apparently, the same is also true for the presynaptic calyces of the RTN. Whether the calyciform terminals, surrounding large dendritic bulbs, correspond to dendrites, or rather to axons or even to parts of the perikaryal cytoplasm, may depend on the length of the neck-like process protruding from the perikaryon. In the absence of a 'neck', the coupling of the two neurons is brought about by a somato-dendritic synapse [3]. When a long 'neck' connects the neurons, the junction becomes dendro-dendritic or even axo-dendritic [5]. Therefore, the term 'dendraxo' seems to fit the presynaptic elements of these structures.

According to Steriade [17], the GABAergic RTN functions as a pacemaker for normal and paroxysmal thalamocortical oscillations; it is assumed that dendro-dendritic synapses are instrumental in this function [18]. Indeed, in a rat model of absence epilepsy [16], a selective increase of T-type  $\text{Ca}^{2+}$  conductance was observed in neurons of the RTN.

According to our ontogenetic studies, development of PV-containing calyciform terminals in the RTN occurs during the first months of postnatal life of rats. It seems that maturation of the terminals occurs mainly during the second and third postnatal months. This is in agreement with the studies demonstrating that thalamic neurons undergo a postnatal cytochemical maturation [19]. Recent investigations [8] also arrived at similar results in the cerebellar cortex, suggesting that developmental changes in PV concentration regulate presynaptic  $\text{Ca}^{2+}$  signaling. We assume that developmental maturation of PV-containing calyciform terminals may play a similar role in the function of the RTN.

## Conclusions

On the basis of the results of these studies and on recent literature data [20], it seems that the large, GABA and CBPs containing calyciform presynaptic complexes in the RTN may play a part in the process of establishing synchronized thalamo-cortical oscillations [21,22], and may subserve the goal of coincidence detection, mainly in regulating attention and distraction of nociceptive impulses. Synaptic activity of large calyciform synapses seems to be mediated, regulated or accompanied by the release of  $\text{Ca}^{2+}$  ions.

## Acknowledgements

Our thanks are due to Mrs Valeria Szell for skillful immunohistochemical assistance. The text has been edited by Dr Karoly Balogh, Boston, Massachusetts, USA.

## References

1. Pinault D. The thalamic reticular nucleus. *Brain Res Brain Res Rev* 2004; **46**:1–31.
2. Celio MR. Calbindin D-28k and parvalbumin in the rat nervous system. *Neuroscience* 1990; **35**:375–475.
3. Csillik B, Palfi A, Gulya K, Mihaly A, Knyihar-Csillik E. Somato-dendritic synapses in the nucleus reticularis thalami of the rat. *Acta Biol Hung* 2002; **53**:33–41.
4. Schwaller B, Meyer M, Schiffmann S. 'New' functions for 'old' proteins: the role of the calcium-binding proteins calbindin D-28k, calretinin and parvalbumin, in cerebellar physiology. Studies with knockout mice. *Cerebellum* 2002; **1**:241–258.
5. Csillik B, Mihaly A, Krisztin-Peva B, Chadaide Z, Samsam M, Knyihar-Csillik E, Fenyo R. GABAergic parvalbumin-immunoreactive large calyciform presynaptic complexes in the reticular nucleus of the rat thalamus. *J Chem Neuroanat* 2005; **30**:17–26.
6. Lewis DA, Akil M. Axon terminals of the chandelier class of cortical GABA neurons. *Mol Psychiatry* 1998; **3**:466–467.
7. Tamminga C, Hashimoto T, Volk DW, Lewis DA. GABA neurons in the human prefrontal cortex. *Am J Psychiatry* 2004; **161**:1764.
8. Collin T, Chat M, Lucas MG, Moreno H, Racay P, Schwaller B, Marty A, Llano I. Developmental changes in parvalbumin regulate presynaptic  $\text{Ca}^{2+}$  signaling. *J Neuroscience* 2005; **25**:96–107.
9. West MJ, Slomianka L, Gundersen HJ. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat Rec* 1991; **231**:482–497.
10. Paxinos G, Watson C. *The rat brain in stereotaxic coordinates*. Sydney, New York, London, Paris, San Diego, San Francisco, Sao Paulo, Tokyo, Toronto: Academic Press; 1982.
11. Knyihar-Csillik E, Chadaide Z, Mihaly A, Krisztin-Peva B, Csillik B. Effect of electrical stimulation of the reticular nucleus of the rat thalamus upon c-fos immunoreactivity in the retrosplenial cortex. *Ann Anat* 2005; **187**:245–249.
12. Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signaling. *Nat Rev Mol Cell Biol* 2000; **1**:11–21.
13. Mermelstein PG, Bito H, Deisseroth K, Tsien RW. Critical dependence of cAMP response element-binding protein phosphorylation on L-type calcium channels support a selective response to EPSPs in preference to action potentials. *J Neurosci* 2000; **20**:266–273.
14. Berridge MJ. Neuronal calcium signaling. *Neuron* 1998; **21**:13–26.
15. Csillik B, Savay G. Release of calcium in the myoneural junction. *Nature (London)* 1963; **198**:399–400.
16. Tsakiridou E, Bertolini L, de Curtis M, Avanzini G, Pape HC. Selective increase in T-type calcium conductance of reticular thalamic neurons in a rat model of absence epilepsy. *J Neurosci* 1995; **15**:3110–3117.
17. Steriade M. The GABAergic reticular nucleus: a preferential target of corticothalamic projections. *Proc Natl Am Sci USA* 2001; **98**:3625–3627.
18. Pinault D, Smith Y, Deschenes M. Dendrodendritic and axoaxonic synapses in the thalamic reticular nucleus of the adult rat. *J Neurosci* 1997; **17**:3215–3233.
19. De Biasi S, Amadeo A, Arcelli P, Frassoni C, Spreafico R. Postnatal development of GABA immunoreactive terminals in the reticular and ventrobasal nuclei of the rat thalamus: a light and electron microscopical study. *Neuroscience* 1997; **76**:503–516.
20. Llinás RR, Leznik E, Urbano FJ. Temporal binding via cortical coincidence detection of specific and non-specific thalamocortical inputs: a voltage-dependent dye-imaging study in mouse brain slices. *Proc Natl Acad Sci USA* 2002; **99**:449–454.
21. Steriade M, McCormick DA, Sejnowski TJ. Thalamocortical oscillations in the sleeping and aroused brain. *Science* 1993; **262**:679–685.
22. Jones EG. Thalamic circuitry and thalamocortical synchrony. *Philos Trans R Soc Lond B Biol Sci* 2003; **357**:1659–1673.