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Research paper

Different distributions of calbindin and calretinin immunostaining across the medial and dorsal divisions of the mouse medial geniculate body

E. Lu [a], D.A. Llano [a,b,c,*], S.M. Sherman [a]

[a] Department of Neurobiology, University of Chicago, USA
[b] Department of Neurology, University of Chicago, USA
[c] Abbott Laboratories, Abbott Park, IL, USA

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ABSTRACT

We studied the distributions of calretinin and calbindin immunoreactivity in subdivisions of the mouse medial geniculate body and the adjacent paralaminar nuclei. We found that the vast majority of labeled cells in the dorsal division of the medial geniculate body were immunoreactive for calbindin-only, whereas most of the remaining labeled cells were double-labeled. Very few calretinin+ only cells were observed. By contrast, we observed significant proportions of calbindin+ only, calretinin+ only and double-labeled cells in the medial division of the medial geniculate body. Further, the distributions of calbindin-only, calretinin-only and double-labeled cells did not differ between the medial division of the medial geniculate body, the suprageniculate nucleus, the peripeduncular nucleus and the posterior intralaminar nucleus. We found essentially no somatic staining for either calbindin or calretinin in the ventral division of the medial geniculate body. These data suggest that there are distinct neurochemical differences between the two non-lemniscal auditory thalamic nuclei. In addition, these data extend previous observations that the medial division of the medial geniculate body shares many properties with the paralaminar group of nuclei.

1. Introduction

The presence or absence of several calcium-binding proteins, including calbindin (CB), parvalbumin (PV) and calretinin (CR), has been used to delineate different functional cell types in the neocortex, hippocampus, cerebellum and thalamus (Jones, 1998; Hof et al., 1999; Bastianelli, 2003; Jinno and Kosaka, 2006). The specific roles of these proteins in shaping neuronal activity have yet to be established, though it has been proposed that the differential total calcium-binding capacity and kinetics observed in these proteins can preferentially modulate specific types of calcium currents (Schwaller et al., 2002; Meuth et al., 2005).

Within the thalamus, the distribution of CB and PV are strikingly complementary, and these distributions have been used in the formulation of models of thalamic organization (Jones, 2001). For example, primary sensory thalamic nuclei (lateral geniculate nucleus, ventral posterior medial nucleus, ventral posterior lateral nucleus and the ventral division of the medial geniculate body, MGBv) demonstrate immunostaining for PV, with label found in both somata and in the neuropil. CB staining in these regions is weak or non-existent. Non-primary sensory nuclei, such as the lateral posterior–pulvinar complex, posterior medial nucleus and the dorsal and medial subdivisions of the medial geniculate body (MGBd and MGBm, respectively) show strong somatic immunoreactivity for CB and poor to non-existent PV immunoreactivity (Rausell et al., 1992; de Venecia et al., 1995; Morel et al., 1997; Jones, 1998; Cruikshank et al., 2001). More recently, CR-immunoreactivity was demonstrated in the thalamus in several species, and was shown to generally have a similar distribution as CB in most thalamic nuclei (Arai et al., 1994; Fortin et al., 1998; Hof et al., 1999; FitzGibbon et al., 2000; Münkle et al., 2000; González et al., 2002), though CR positivity appears to be particularly prominent in the intralaminar and midline groups of nuclei (Arai et al., 1994; Oda et al., 2004; Uroz et al., 2004). Therefore, it appears that both CB and CR may be markers for the non-primary sensory thalamic nuclei.

This differential distribution of thalamic CB/CR and PV corresponds to differences in the presumed roles of these nuclei. For example, neurons in non-primary sensory thalamic nuclei receive large-terminal afferents, in part, from cortical layer 5 and have been referred to as "higher-order" nuclei (Sherman and Guillery, 2002). It has been proposed that higher-order thalamic nuclei

Abbreviations: CB, calbindin; CR, calretinin; PIN, posterior intralaminar nucleus; PP, peripeduncular nucleus; PV, parvalbumin; MGBd, dorsal division of the medial geniculate body; MGBm, medial division of the medial geniculate body; MGBv, ventral division of the medial geniculate body; PBS, phosphate-buffered saline; SG, suprageniculate nucleus

* Corresponding author. Address: Department of Neurobiology, University of Chicago, 549 E. 58th Street MC 0926, Chicago, IL 60637, USA. Tel.: +1 773 834 8764.
E-mail address: a.daniel.llano@gmail.com (D.A. Llano).

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receive receptive field information from one cortical area and relay it to another (Guillery, 1995). In contrast to the higher-order nuclei, primary sensory nuclei receive receptive field information from the sensory periphery and relay this information to the cortex, and have been referred to as "first-order" nuclei. For further discussion of first and higher-order thalamic nuclei, see (Sherman and Guillery, 2005). Thus, it appears that in the sensory thalamic nuclei, CB/CR and PV positivity may correspond to higher-order and first-order thalamic nuclei, respectively.

Though CR and CB have been observed in similar groups of higher-order nuclei, it is not known if CB and CR colocalize to the same population of neurons, or if separate populations of CB- and CR-positive neurons exist. The answer to this question has potentially important implications on our understanding of the organization of higher-order thalamic nuclei, since there is evidence for connectional and functional heterogeneity within higher-order thalamic nuclei. For example, many higher-order thalamic nuclei receive large-terminal afferents from both cortical and subcortical structures, raising the possibility that higher-order circuits, driven by cortical inputs, may reside in the same nuclei of first-order circuits, driven by subcortical inputs. In addition, though the prototypical projection of thalamic principal neurons is to layers 4 and 6 of neocortex, many thalamic cells in higher-order nuclei project to layer 1 of neocortex (Rockland et al., 1999) or to other subcortical structures, such as the basal ganglia or amygdala (Harting et al., 2001; Cheatwood et al., 2003). This projection pattern is particularly prevalent among neurons in the intralaminar and the adjacent "paralaminar" nuclei (Herkenham, 1980), such as the suprageniculate (SG), posterior intralaminar nucleus (PIN) and the peripeduncular nucleus (PP, (Ryugo and Killackey, 1974; Ottersen and Ben-Ari, 1979; Clugnet et al., 1990)). The degree to which this heterogeneity may be reflected in different distributions of calcium-binding proteins has not yet been addressed.

In the current study, we take advantage of the tripartite organization of the auditory thalamus, which contains a PV-rich MGBv, and two CB/CR rich nuclei: the MGBd and MGBm. The MGBd receives large-terminal afferents from both the auditory cortex and external nuclei of the inferior colliculus and sends projections to layers 1, 4 and 6 of the secondary auditory cortical fields and layer 1 of the primary AC (Llano and Sherman, 2008). The MGBm receives input from the inferior colliculus, superior colliculus and spinal cord, and projects to layers 1 and 6 of cortex as well as to the basal ganglia and to the amygdala (Calford and Aitkin, 1983; Bordi and LeDoux, 1994; Linke, 1999). The MGBm also displays significant heterogeneity with respect to the intrinsic properties such that some neurons do not display bursting and there are subpopulations of neurons in the MGBm with large, reticular, non-bushy morphology (Winer and Morest, 1983; Smith et al., 2006). Given the functional and connectional heterogeneity in these nuclei, we hypothesized that distinct populations of CB-positive and CR-positive cells would be found in the MGBd and MGBm, and that the relative proportion of each cell type would differ.

2. Methods

2.1. Tissue processing

Adult (60-day or older) Balb/c mice of both sexes were used for this study. All surgical procedures were approved by the Institutional Animal Care and Use Committee at the University of Chicago, and animals were housed in animal care facilities approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Every attempt was made to minimize the number of animals used and to reduce suffering at all stages of the study. Mice were sacrificed through deep anesthesia with ketamine hydrochloride (100 mg/kg), and xylazine (3 mg/kg) and perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were postfixed overnight in perfusate. Brains were then cryoprotected in a series of increasing sucrose concentrations, up to 30% sucrose in PBS.

Frozen 30-μm-thick sections were cut by using a sliding microtome. After being allowed to equilibrate in PBS (pH 7.4 in all instances) for at least 60 min, the sections were immersed in PBS containing 0.3% Triton-X to increase membrane permeability and prepare them for immunostaining. Sections were then blocked with 3% goat serum in 0.3% Triton-X for 30 min. Primary antibody solution consisted of a mix of 1:1000 CR (Swant rabbit anti-CR, code number 7699/4), and 1:1000 CB monoclonal antibodies (Sigma, product number C9848) in the goat serum solution. The anti-CR antibody was generated by immunizing rabbits with human calretinin. Specificity of this antibody has been established by the manufacturer, who has demonstrated the absence of binding in calretinin knock-out mice (http://www.swant.com/pfd/Rabbit%20anti-calretinin%207699-4.pdf). The anti-CB antibody was generated against purified bovine kidney calbindin D-28k. Per the manufacturer, this monoclonal antibody does not reactwith other members of the EF-hand family such as calbindin-D-9K, calretinin, myosin light chain, parvalbumin, S-100a, S-100b, S100A2 (S100L), or S100A6 (calcyclin). Each lot was tested by using an immunoblot with bovine kidney cell extract as antigen and yields a single band at 28 kDa. For both anti-CR and anti-CB, we have run controls by eliminating the primary antibody and seen no signal.

Sections were incubated in the primary antibody solution overnight at room temperature. Afterwards, sections were washed three times using the Triton-X solution. Secondary antibody solution consisted of 1:200 goat biotinylated anti-rabbit antibody (Vector labs, BA-1000) for the CR-specific primary antibody and 1:100 Cy-2-conjugated anti-mouse antibody (AffiniPure Goat Anti-Mouse IgG conjugated to Cy-2, Jackson Immunoresearch, catalog number 115-225-174) for the CB-specific primary antibody. The washed sections were incubated in the secondary antibody solution for 60 min at room temperature. After secondary antibody incubation, the sections were again washed three times in the Triton-X solution, and then incubated in a 1:100 streptavidin–alexafluor 594 conjugate (Invitrogen, catalog number S11227) solution for 60 min. Finally, the sections were washed thoroughly in PBS. All sections were mounted on gelatin-coated slides, air-dried, and coverslipped using anti-fade solution (Vectashield, Vector labs).

2.2. Imaging and analysis

All images were captured using a Retiga 2000R digital camera mounted to a Leica DM5000B microscope using a 100 W Mercury lamp with fluorescence optics. Leica TX2 filter cubes (Excitation 560 nm, Emission 645 nm, Dichroic 595 nm) were used to visualize calretinin signal and Leica L5 filter cubes (Excitation 480 nm, Emission 527 nm, Dichroic 505 nm) were used to visualize calbindin signal. Images were captured by using Q Capture Pro software. In direct imaging, the Capturer software was used to overlay the CR and CB images to create the double-labeled images.

Neurolucida software (MBF Biosciences) was used for cell counting. For this, raw images were imported into Neurolucida for analysis. The subdivisions of the thalamus were distinguished using Nissl-stains of the same sections used for immunostaining. For approximately 40% of sections, Nissl-stains were not available due to tissue damage during processing, and a mouse brain atlas (Paxinos and Franklin, 2008), was used for these sections. Borders were traced using Neurolucida software, and for the MGBd, MGBm, SG and PP, cells within 25 μm of any border were not counted to ensure that each cell could be unambiguously assigned to a particular nucleus. For PIN, which is a large structure with a large