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ORIGINAL PAPER

Co-localization of Glutamic Acid Decarboxylase and Phosphate-activated Glutaminase in Neurons of Lateral Reticular Nucleus in Feline Thalamus

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Abstract Immunohistochemical methods were used to label singly and/or in combination glutamic acid decarboxylase (GAD, the sole synthesizing enzyme for the inhibitory neurotransmitter γ-aminobutyric acid) and phosphate-activated glutaminase (GLN, a synthesizing enzyme for glutamate) in neurons of lateral reticular nucleus (LRN) of thalamus of adult cats. (1) GAD- and GLN-immunoreactivity (IR) exhibited matching regional patterns of organization within LRN. (2) GAD- and GLN-IR co-localized within most if not all LRN neuronal cell bodies as shown by light microscopy. (3) GAD- and GLN-IR had distinct subcellular localizations in LRN neurons as shown by correlative light/electron microscopy. LRN neurons are important conceptual models where strongly inhibitory cells receive predominant excitatory glutamatergic afferents (from neocortex). Consistent with known actions of intermediary astrocytes, LRN neurons demonstrate GLN enrichment synergistically coupled with glutamatergic innervation to supplement the glutamate pool for GABA synthesis (via GAD) and for metabolic utilization (via the GABA shunt/tricarboxylic acid cycle) but not, apparently, for excitatory neurotransmission.

Keywords GAD · GABA · Glutaminase · Glutamate · Co-localization · Thalamus

Special issue dedicated to Anthony Campagnoni.

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Introduction

The metabolic pathways of glutamate and γ-aminobutyric acid (GABA) are intimately entwined in the central nervous system (CNS) of mammals. Unable to cross the blood-brain barrier, they must be synthesized anew within the organ [1–4]. Glutamate derives principally from glucose, can be exchanged by cells via both uptake and re-uptake mechanisms and forms with α-ketoglutarate and glutamine a metabolic pool spanning neurons and astrocytes [1, 3, 5–7]. In neurons, glutamate functions mainly as the predominant excitatory neurotransmitter in brain, a metabolic participant in the tricarboxylic acid (TCA) cycle and a structural component of proteins [1, 3]. It is also the immediate precursor for GABA. In neurons, GABA functions mainly as the predominant inhibitory neurotransmitter in forebrain and a metabolic participant in the TCA cycle via the GABA shunt [2, 4].

Two synthesizing enzymes play key roles in this arrangement. Glutamate decarboxylase (GAD) synthesizes GABA from glutamate in a well-characterized fashion [2, 4]. Because of its exclusive and limited neuronal localization, GAD expression clearly defines the GABAergic neurons of the CNS [8–12]. In contrast, phosphate-activated glutaminase (GLN) synthesizes glutamate from glutamine in a less straight–forward fashion that may occur at low levels in astrocytes as well as at higher levels in neurons [1, 13, 14]. GLN expression would permit neurons to re-cycle glutamate obtained by direct uptake or indirect uptake from astrocytic glutamine metabolism to supplement de novo glutamate synthesis from glucose. Unlike GAD, GLN localization defines neurons that could employ enriched glutamate production to support

glutamatergic and/or GABAergic neurotransmission and to contribute to the TCA cycle when high metabolic demand occurs, perhaps as a consequence of sustained synaptic excitation [15–18].

Earlier reports of GLN localization focused on the glutamatergic enrichment in corticothalamic and thalamocortical projection neurons in rats consistent with the functional excitation produced and received by these cells [15, 19–23]. While inhibitory neurons were not strictly excluded in these observations, neither were they clearly delineated. Several of these studies suggested that considerable levels of both amino acids were produced by infrequent neurons in neocortex and retina . A possible explanation of these findings is that enriched GLN expression supplements GABA synthesis particularly in neurons receiving predominant synaptic excitation. This situation may occur in a well-characterized and anatomically advantageous brain site, the lateral reticular nucleus (LRN) of the thalamus, where essentially all neurons show strong GAD immunoreactivity (IR), inhibit their thalamic targets and receive strong synaptic excitation via glutamatergic corticothalamic fibers [10, 20, 24, 25]. The present investigation was designed to test this hypothesis by immunohistochemical means assessing precisely the co-expression of GAD and GLN (as GAD and GLN–IR) within feline LRN neurons.

Experimental procedure

Animals and tissue preparation

Tissue was obtained from six adult cats of known age [1–2 years; three males, three females). Cats were born and reared under standard conditions in the kitten breeding colony at the UCLA Mental Retardation Research Center. All procedures were conducted in accordance with the ''NIH Guide for the Care and Use of Laboratory Animals''.

Many of the methods were detailed in previous reports [11, 12]. Briefly, cats were terminated by barbiturate overdose (100 mg/kg b.wt. Nembutal®) and perfused transcardially with 1 l of phosphate-buffered saline rinse (PBS; 19 mM $NaH_2PO_4H_2O$, 81 mM Na_2HPO_4, 150 mM NaCl; pH = 7.4) followed by 2 l of fixative (1.33 mM paraformaldehyde, 200 μM glutaraldehyde, 89 mM acrolein in PBS; pH = 7.4; 4°C; delivered over 20 min). Brains were then extracted and immersed in fixative for 60 min.

After blocking and gelatin embedment, brain sections through the thalamus were sliced with a Vibratome into chilled Tris–buffered saline (TBS; 77 mM Trizma–HCl, 150 mM NaCl; pH = 7.4). At least 24 serial sections including the LRN bilaterally were obtained from each cat. This series was divided into adjacent alternating sections immunolabeled for GAD, GLN or both antigens.

GAD single labeling method

In all six cases, GAD was labeled first by the indirect peroxidase-antiperoxidase (PAP) immunohistochemical method [26]. Sections were rinsed in TBS (diluent and rinse for all subsequent steps; diluent for immunohistochemical steps also contained 1% serum from the species providing secondary antibodies), treated to quench residual peroxidase activity (3.1 M methanol, 882 mM H_2O_2; 5 min) and soaked in detergent (0.25% Triton X–100; 5 min) for partial membrane solubilization. Non-specific IgG binding was blocked with 100 mM D,L-lysine and 10% normal rabbit serum (60 min). The first immunohistochemical incubation was in polyclonal sheep antirat GAD IgGs (Kopin-Oertel 1440 antiplasma lysate; 1:2000 dilution from 75 mg solid/ml reconstituted plasma; 16 h; 4°C; see Acknowledgement). The specificity of these antibodies has been characterized extensively and they reliably identify neuronal GAD without colchicine pretreatment [8–12]. The second immunohistochemical incubation was in polyclonal rabbit antisheep IgGs presorbed with acetone-extracted cat brain powder (1 h). These antibodies were cross-reactive with goat IgGs. The third immunohistochemical incubation was in polyclonal goat PAP IgG complex (1 h). After PBS rinses, GAD was labeled histochemically with DAB chromagen (1.4 mM 3,3'-diaminobenzidine, 11 mMβ D(+)-glucose, 7.4 mM NH_4Cl, 10 mg glucose oxidase/liter of PBS diluent; 45 min, 35°C) and rinsed in PBS [27]. For simple light microscopy, thick specimens were mounted on gelatin-coated slides, post-fixed in 4 mM OsO_4 (5 min), dehydrated in ethanols, cleared in xylenes and cover-slipped with DePeX®. Selected specimens were counterstained with cresyl violet to aid regional identification and mapping of labeled neurons.

GLN single labeling method

In all six cases, GLN was labeled by comparable methods with immunoreagents revised to accommodate the species providing the primary antibodies (Curthoys polyclonal rabbit antirat IgGs directed against renal phosphate-activated GLN; 1:2000 optimal dilution; see Acknowledgement). Thus, the blocking

serum was from mouse, the second immunohistochemical incubation was in polyclonal mouse antirabbit IgGs and the third immunohistochemical incubation was in polyclonal rabbit PAP IgG complex. These primary antibodies have been used infrequently in brain, but label specifically the expected renal mitochondrial site of GLN [14, 15].

In five cases, the morphological distributions of GAD and GLN-IR neurons as observed by transmitted light microscopy were compared in the LRN of singly labeled specimens in adjacent serial sections to assess the degree of overlap in the regional patterns of the cell bodies. For each tested case, specific labeling was represented by the differential accumulation of brown DAB reaction product in ''experimental'' specimens incubated in primary antibodies directed against GAD and GLN versus adjacent ''control'' specimens incubated in non-immune sera from the species providing the primary antibodies. Like omission of primary immunohistochemical incubations, use of non-immune sera in primary incubations resulted in no clearly apparent labeling except from the endogenous peroxidase-like activity of erythrocytes. In one case, the GAD series of sections was completed as described above, but the GLN series of sections was treated with serial dilutions of primary antibodies to establish optimal conditions for specific immunolabeling similar to previous tests for the GAD antibodies [11].

GAD and GLN double labeling methods

Based on consistent and replicable observations of matched regional distributions for specific GAD-IR and GLN-IR neurons in the LRN, co-localization of GAD and GLN was performed in two cases. As expected from previous reports and as described below, GAD-IR had a disseminated cytoplasmic distribution associated with a wide variety of organelles including synaptic vesicles while GLN-IR had a much more limited, punctuate distribution associated with mitochondria.

In one case, co-localization at the cellular level was performed by sequential immunolabeling using two different peroxidase chromagens. GAD was first labeled using DAB chromagen as described above. After peroxidase blocking, GLN was then labeled as described above using *o*-dianisidine chromagen in place of DAB [12, 28]. In contrast to the diffuse brown peroxidase reaction product labeling GAD, transmitted light microscopy revealed a punctate dark blue reaction product labeling GLN in a pattern clearly associated with mitochondria. Label specificities were maintained as defined above when one or both primary antibodies were replaced with non-immune serum. Additionally, the *o*-dianisidine label was recognized by weak birefringence in polarized light, a property not found for the DAB label.

In one case, co-localization at the cellular and subcellular levels was performed by sequential immunolabeling using two different markers. GAD was first labeled using DAB chromagen as described above. After extensive peroxidase exhaustion and IgG blocking with mouse serum, GLN was labeled with protein A colloidal gold conjugate (1–2 nm nominal particle diameter) [12, 29]. In contrast to the diffuse brown peroxidase reaction product labeling GAD, transmitted light microscopy revealed a punctate dark red reaction product labeling GLN in a pattern again clearly associated with mitochondria. These labels were specific as defined previously by the results replacement, singly or in combination, of primary antibodies.

This form of combined pre-embedment labeling proved to be amenable to correlative light/electron microscopy [11, 12]. LRN samples were dissected from immunoreactive specimen faces (approximately $1 \times 2 \times 0.1$ mm), postfixed in 40 mM OsO_4 (1 h), dehydrated in ethanols, infiltrated with propylene oxide-resin mixtures and flat-embedded in Epon-Araldite®. Semithin (1–2 μm thickness; toluidine blue counterstain) and thin specimens (silver-gold interference colors; lead citrate poststain) were obtained by ultramicrotomy. Semithin specimens were mounted on glass slides for transmitted light microscopy and adjacent thin sections were mounted on Formvar®-coated copper grids for transmission electron microscopy. In thin sections, the DAB label for GAD-IR was ligand-bound to electron-dense OsO_4 while the colloidal gold label for GLN-IR was electron-opaque. In semithin sections, the DAB label was a diffuse dark brown while the colloidal gold label was a punctuate blue-black. In thick, semi-thin and thin specimens, these labels were specific in results of non-immune sera replacements for primary antibodies.

Results

Methodologically specific and replicable immunolabeling was seen by transmitted light microscopy and/or transmission electron microscopy in all tested cases for all tested methods for both GAD-IR and GLN-IR. The observations reported in this section will detail these labeling patterns obtained from ''experimental'' sections.

GAD- and GLN-IR neurons are morphologically identical in LRN

As demonstrated by light microscopy, single labeling of GAD-IR was contained in three morphological components of the LRN: cell bodies, processes, and punctae (Fig. 1). The labeling was clearly within the profiles as determined by examination with a 100X oil objective, a condition yielding a depth of field of less than 2 μm and resolution of approximately 0.2 μm. For present purposes, the cell bodies were most significant.

The GAD-IR cells consisted entirely of neurons as shown by their medium to large size (somatic diameters approximately 15–30 μm) and characteristic dense patches of neuronal counterstaining with cresyl violet and toluidine blue. GAD-IR was excluded from their nuclear profiles which tended to be centrally or somewhat eccentrically placed within the perikaryal cytoplasm. Dense GAD-IR was typical in these neurons and usually extended into several proximal dendrites. GAD-IR was diffusely arranged throughout the perikaryal cytoplasm with denser, scattered accumulations indicative of localization in the Golgi apparatus and rough endoplasmic reticulum arranged in proximity to nuclear profiles. These dense accumulations of GAD-IR were greater than 2 μm in diameter and thus tended to be larger than typical perikaryal mitochondria.

GAD-IR processes consisted of dendrites (> 1 μm diameter) and axons (< 1 μm diameter) arising at least in part from GAD-IR LRN neurons. GAD-IR punctae also exhibited dense labeling and represented accumulations of GAD-IR synaptic vesicles clustered in both en bouton and en passage terminals as demonstrated by the occurrence of labeled punctae along the lengths and ends of labeled processes. In some cases, these punctae were located along the lengths of thicker processes likely to be dendrites. Taken together, these GAD-IR labeling patterns in the LRN entirely replicated with previous reports from other investigators with particular regard to terminal arrangements consistent with the occurrence of GABAergic dendritic terminals in feline as well as rodent and primate LRN [10, 20, 24, 25].

As demonstrated by light microscopy, single labeling of GLN-IR was contained in two morphological components of the LRN: cell bodies of two types with distinct size differences and widely diffused punctae (Fig. 2). Once more, examination with a 100X oil objective demonstrated that the labeling was contained within at least the somatic profiles. For present purposes, the larger type of cell body was most significant.

The larger GLN-IR cells consisted entirely of neurons as shown by their medium to large size (somatic diameters approximately 15–30 μm) and characteristic dense patches of neuronal counterstaining with cresyl violet and toluidine blue. GLN-IR was excluded from their nuclear profiles which tended to be centrally or somewhat eccentrically placed within the perikaryal cytoplasm. These features matched the GAD-IR neurons in the LRN as described above. These observations supported the co-localization of GAD- and GLN-IR in LRN neurons because the morphological features for such cells were essentially identical in singly labeled specimens. However, GLN-IR within the perikaryal cytoplasm of LRN neurons was diffuse and punctate, extending only slightly into proximal

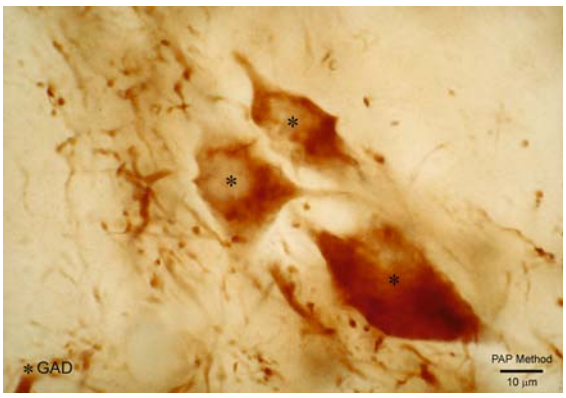

Fig. 1 Light photomicrograph of typical GAD-IR observed in neuronal cell bodies (asterisks), processes and punctae in the LRN of an adult cat (singly labeled specimen). Peroxidase-antiperoxidase labeling method using DAB chromagen

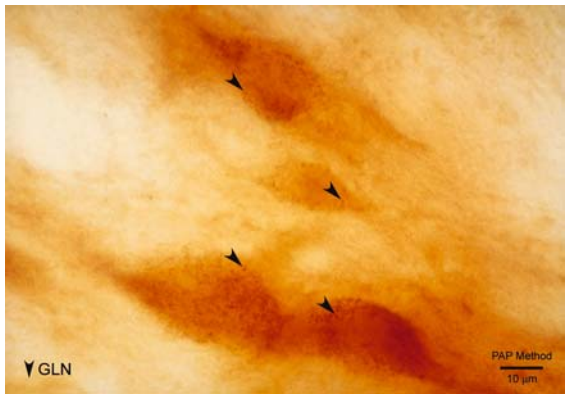

Fig. 2 Light photomicrograph of typical GLN-IR observed in cell bodies and the neuropil in the LRN of an adult cat (singly labeled specimen). Arrows point to labeled components that represent mitochondria. Note the clear size difference between the large neurons at the top and bottom of the panel versus the smaller, more lightly labeled astrocyte at the center of the panel. Peroxidase-antiperoxidase labeling method using DAB chromagen

dendrites. The punctae of GLN-IR were almost always greater than 1 μm in diameter in accord with the size of typical perikaryal mitochondria.

The smaller GLN-IR cells (somatic diameters greater than 15 μm) always contained many fewer labeled punctae than the LRN neurons and their perikaryal space was dominated by a centrally placed nuclear profile. Based on their smaller size and very sparse Nissl counterstaining, these cells were astrocytes, a cell lineage thought to express GLN at lower levels than neurons [6, 7, 13]. It was difficult to ascertain the morphological location(s) of the widely diffused GLN-IR punctae within the LRN neuropil by light microscopy alone. By transmission electron microscopy, they were subsequently found to represent labeled mitochondria mainly in neuronal processes and terminal regions, but unlike GAD-IR, the GLN-IR mitochondria were associated with Gray Type 1 asymmetrical synaptic specializations most likely to represent excitatory corticothalamic projections as well as Gray Type 2 symmetrical synaptic specializations most likely to represent local GABAergic connections within the LRN [25, 30].

GAD- and GLN-IR neurons are organized identically in LRN

In singly labeled serial sections, light microscopy demonstrated GAD- or GLN-IR in essentially all of the LRN neurons located in the immunoreactive section faces (approximately 10–12 μm). As schematically mapped in Fig. 3, the complements of GAD- and GLN-IR neurons in the LRN occupied the entire crescent-shaped nucleus bilaterally to form a shell between the more medial thalamic nuclei and the more lateral white matter of the internal capsule and cerebral peduncle. While showing slight variation between sections from single cases and between cases, these observations supported the co-localization of GAD and GLN-IR in LRN neurons because the complements and patterns of regional organization for such cells were essentially identical in singly labeled specimens.

GAD and GLN-IR are co-localized in LRN neurons by double labeling

The correspondence of GAD and GLN-IR found in LRN neurons in adjacent singly labeled serial sections strongly supported the inference of GAD and GLN co-localization therein. However, double labeling of GAD and GLN-IR within LRN neurons provided a direct, empirical proof for their co-localization. Both double labeling methods(two chromagen PAP and PAP/Protein A-colloidal gold) yielded specific, replicable and comparable results. As shown in Fig. 4 for a PAP/Protein A colloidal gold specimen, the doubly labeled GAD and GLN-IR neurons in the LRN were identical to the singly labeled GAD and GLN-IR neurons described above. Interestingly, double labeling tended to reduce the apparent efficiencies of labeling for both markers as determined by light microscopy such that GAD-IR processes and punctae were much less evident while GLN-IR astrocytes and punctae in the

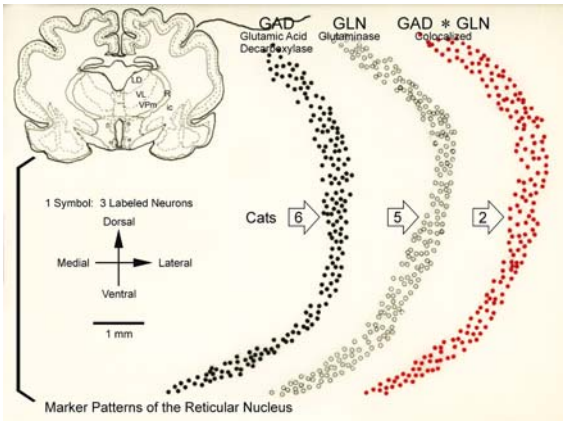

Fig. 3 Maps demonstrating the location of the LRN (R) within the thalamus in a coronal section of feline brain (inset) and representative distributions obtained from cases where adjacent sections were singly labeled for GAD (left arc, closed dark circles, six cases) and GLN (center arc, open circles, five cases) or doubly labeled for GAD and GLN within the same sections (right arc, closed light circles, two cases). Scaling, directions and dimensions for the maps are provided to the left of the panel

Fig. 4 Light photomicrograph of typical GAD-IR (diffuse cytoplasmic labeling denoted by asterisk) and GLN-IR (punctuate mitochondrial labeling denoted by arrow) observed in neuronal cell bodies in the LRN of an adult cat (doubly labeled specimen from a thick section of brain). GAD-IR from peroxidase-antiperoxidase labeling method using DAB chromagen and GLN-IR from protein A colloidal gold

neuropil were completely unrecognizable. However, the complement of doubly labeled GAD and GLN-IR cells included practically all of the LRN neurons situated in the immunoreactive section faces as found for singly labeled GAD and GLN-IR neurons. The regional pattern of these doubly labeled neurons within the LRN also matched the patterns found for singly labeled GAD and GLN-IR neurons (Fig. 3). These findings cross-validated the results obtained and conclusions drawn by single and double labeling methods.

GAD and GLN-IR have distinct subcellular localizations in LRN neurons

Correlative light and electron microscopy allowed relatively precise subcellular localization of GAD and GLN-IR in doubly labeled LRN neurons. Semithin specimens revealed the same forms of diffuse GAD-IR and punctuate GLN-IR within the perikaryal cytoplasm of neurons having the same size and Nissl counterstaining properties described above for both singly and doubly labeled cells (Fig. 5). Transmission electron microscopy confirmed the neuronal type of these cells by showing their synaptic junctions (Fig. 6).

As in previous reports, electron-dense GAD-IR in doubly labeled neurons was diffusely associated with most membranous, fibrous, and granular organelles within the cytoplasm, a subcellular distribution suggesting promiscuous aldehyde precipitation of GAD onto adjacent infrastructure as well as sites of native localization that would necessarily include Golgi apparatus, rough endoplasmic reticulum and synaptic vesicles [11, 12]. It was interesting to note that GAD-IR tended to be confined to the external surface of mitochondrial membrane but always extended into the interior of synaptic vesicles while also studding their external surface.

In contrast, the electron–opaque colloidal gold conjugates signifying GLN-IR in doubly labeled neurons were confined to the interior of mitochondria (Fig. 6). In these cases, internal mitochondrial membranes were often disrupted by less than optimal ultra structural preservation, a common difficulty in pre-embedment labeling methods. However, the intramitochondrial localization of GLN-IR was consistent with its expected sites based on previous studies of kidney and the close association of GLN with the TCA cycle[1, 14]. It was also distinctly different than the sub-cellular labeling of GAD-IR. The subcellular associations of both GAD and GLN-IR corresponded closely to the arrangements expected from light microscopic observations of singly and doubly labeled specimens.

While GAD-IR was often encountered in dendritic, axonal, and terminal profiles in the LRN, GLN-IR was infrequently observed. This outcome was consistent with the inefficient production of GLN-IR noted previously for doubly labeled specimens examined by light microscopy. Perhaps the accessibility and preservation of GLN antigen were less robust in the processes and terminals than in the perikaryal cytoplasm in these highly processed specimens. Another possibility worth consideration was that levels of GLN antigen might be lower in the transported mitochondria of axonal and dendritic arbors than in the more settled mitochondria of cell bodies.

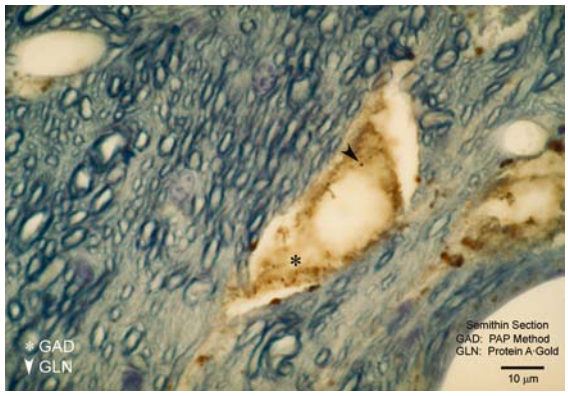

Fig. 5 Light photomicrograph of typical GAD-IR (diffuse cytoplasmic labeling denoted by asterisk) and GLN-IR (punctuate mitochondrial labeling denoted by arrow) observed in neuronal cell bodies in the LRN of an adult cat (doubly labeled specimen from a semi-thin section of brain). GAD-IR from peroxidase-antiperoxidase labeling method using DAB chromagen and GLN-IR from protein A-colloidal gold

Discussion

In summary, the present investigation obtained three principal results: (1) GAD and GLN-IR had identical neuronal and regional patterns of organization within LRN. (2) GAD and GLN-IR co-localized within most if not all LRN neuronal cell bodies as shown by light microscopy. (3) GAD and GLN-IR were clearly recognized with distinct subcellular localizations within the limits of single LRN neurons as shown by correlative light or electron microscopy. Consistent in combination, all of these observations demonstrated that GAD and GLN are co-expressed in feline LRN neurons. This outcome supports the hypothesis that enriched GLN expression supplements GABA synthesis at least for the population of inhibitory LRN neurons receiving predominant synaptic excitation from corticothalamic projections.

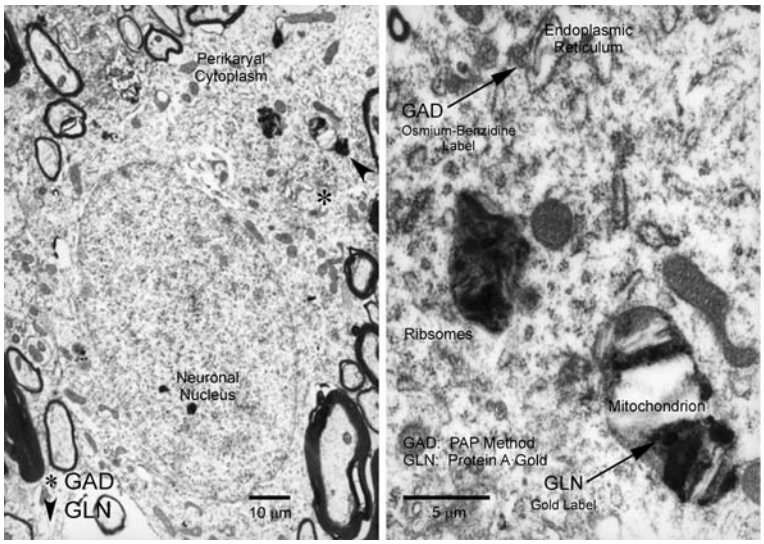

Fig. 6 Electron photomicrographs of typical GAD-IR (diffuse electron dense cytoplasmic labeling denoted by asterisks) and GLN-IR (punctuate electron-opaque labeling associated with mitochondria, for example at the arrow in the left panel) observed in neuronal cell bodies from the LRN of an adult cat (doubly labeled specimen from a thin section of brain). The left panel is a low-magnification view of the cell body. The right panel is a higher magnification view of the upper right region of the perikaryal cytoplasm. GAD-IR from peroxidase-antiperoxidase labeling method using DAB chromagen with osmication and GLN-IR from protein A colloidal gold

To best possible knowledge, this is the first demonstration of GAD and GLN co-expression in LRN neurons. While the outcome is not surprising in itself, new observations of GAD and GLN co-localization in neurons provides a clear picture of important aspects of the regional, cellular, and sub-cellular morphological patterns of organization for the two important synthesizing enzymes underlying the metabolic interactions of glutamate and GABA within a specific brain site and a readily identified type of neuron. There is little doubt that LRN neurons produce, accumulate and utilize GABA as an inhibitory neurotransmitter, and their levels of GAD are perhaps among the greatest in mammalian brain considered on a label per-cell basis in immunohistochemical investigations [10, 24]. In contrast, the arrangement of GLN and glutamate in LRN neurons is less certain as delineated in previous reports. The new observations for GLN in feline LRN neurons are consistent with descriptions provided by Kaneko and Mizuno [15] in rat, but uncover a more definitive and precise picture of sub-cellular distribution of GLN-bearing mitochondria based on both indirect and direct evidence for the morphological association of enriched glutamate and GABA synthesis. Furthermore, the rather ubiquitous cellular localization of glutamate within the CNS of rodents revealed by immunohistochemical methods suggests that LRN neurons can accumulate glutamate but the functional implications of this property remain open to question [31–36]. This expectation of glutamate accumulation was confirmed in feline LRN neurons using the Petrusz-Rustioni antisera directed against glutamate [32] (data not shown) to show that both GLN and its product are indeed evident within the same LRN neurons. The functional implications of this arrangement can now be viewed in light of the mechanisms of synaptic transmission and oxidative metabolism within a particular neuronal phenotype.

The new observations provide critical evidence for the occurrence of the following scenario of glutamate and GABA interactions in the LRN. LRN neurons driven principally by excitatory corticothalamic afferents express enriched neuronal GLN activity. This GLN can utilize glutamine released and transported from adjacent astrocytes where it is formed mainly as a supportive, protective consequence of glutamatergic neurotransmission. With the addition of glutamate synthesized by GLN, LRN neurons can produce a greater level of intraneuronal glutamate than that available from direct uptake and/or de novo synthesis from glucose. Glutamate supplementation would flow, at least in part, into the substrate pool of glutamate enabling GAD to synthesize a greater level of GABA in active LRN neurons. This may finally contribute to the replenishment of the GABA neurotransmitter pool and energy intermediates of oxidative metabolism (via the GABA shunt and the tricarboxylic acid cycle), processes with unusually high demands in the cellular

economy of LRN neurons. This scenario is congruent with our present understanding of the neurochemistry of glutamate and GABA within the mammalian CNS [1–4, 34–36]. It also illustrates the emerging co-dependency of neuronal and astrocytic functions as it depicts the important hierarchical linkage of glutamate and GABA synthesis [5, 6, 37].

The LRN was selected for present study because of its anatomical features and advantageously high level of GAD expression, properties which made the determination of double labeling for GAD- and GLN-IR a much less formidable task. However, it is not unreasonable to expect that the mechanism of glutamate-GABA interaction found in LRN neurons is also employed by other populations of neurons in the mammalian CNS. In the feline material available for examination, GLN-IR was apparent in nearly all thalamic, neostriatal and pallidal neurons located in the immunoreactive section faces (data not shown). Co-localization of GAD-IR and GLN-IR was ubiquitous in neostriatal and pallidal neurons consistent with their typical GABAergic neurotransmitter identities [10, 12]. Like LRN neurons, neostriatal neurons receive predominant excitatory afferents (mainly glutamatergic corticostriatal and thalamostriatal projections) [11, 15]. Yet, these excitatory afferents are not a rigid requirement for co-expression of GAD- and GLN-IR since the predominant (albeit not the exclusive) afferents of pallidal neurons are GABAergic inhibitory striatopallidal projections [12]. Within the thalamic nuclei lying deep to the LRN shell, co-localization of GAD-IR and GLN-IR was limited to approximately 10–15% of the neurons where the generally smaller size of the cells was indicative of GABAergic interneurons [20, 25]. These cells are unusual because many, if not most, lack axonal processes and instead utilize dendrites for synaptic transmission to their adjoining target thalamic output neurons [20, 25, 30]. The more frequent, larger thalamic neurons exhibited GLN-IR, but not GAD-IR, consistent with their expected role as excitatory output neurons [20, 25], an observation replicating previous reports obtained from rodents [15, 23]. Finally, the expression of GAD does not appear to be an absolute predictor of GLN expression which seems to be absent in subclasses of neurons, perhaps local circuit cells, in the red nucleus [21].

When interpreting the present observations, it is worthwhile to consider that co-localization of GAD and GLN-IR was demonstrated only in the cell bodies, not the axonal terminals, of LRN neurons. The functional implications of the perikaryal cytoplasmic site for these synthesizing enzymes are perhaps greater for metabolic than neurotransmitter replenishment. The enriched glutamate and GABA pools these enzymes produce are first present in the immediate vicinity of mitochondria where they could contribute to oxidative metabolism. However, it is not unreasonable to expect extension of GAD and GLN co-localization into the cytoplasmic organelles of axonal, and possibly dendritic, terminal synaptic regions due to centrifugal trafficking of GAD-IR in synaptic vesicles and GLN-IR in mitochondria. Such co-localization is difficult to establish by light microscopy alone given the limited resolution of this detection method and the relatively small size of the terminals. It is also difficult to accomplish by transmission electron microscopy because of the dispersion of mitochondria throughout the entire lengths of cellular processes and somewhat uncertain origins of the axonal terminals. Thus, assessment of the relative impact of GAD and GLN co-localization on neurotransmitter versus metabolic pools of amino acids remains an open question meriting attention in future investigations.

Co-localization of significant components and products of glutamate and GABA metabolism in neurons inevitably raises the question of singularity of neurotransmitter identity. Reports of co-expression of glutamate and GABA transporters for the limiting membrane and vesicles of certain classes of retina and neocortical neurons suggest the possibility that LRN neurons may also have the means necessary to utilize both glutamate and GABA as classical neurotransmitters [36, 38–40]. However, a singular, inhibitory, GABAergic means of synaptic transmission has been repeatedly attributed to LRN neurons [20, 24, 25]. Practically all thalamic neurons are likely to express and utilize postsynaptic glutamatergic receptors for the receipt of excitatory corticothalamic afferents. It is conceivable that the unitary inhibition conveyed by LRN neurons reflects a paucity of glutamatergic receptors within inhibitory postsynaptic regions of thalamic target neurons associated with Gray Type 2 synaptic junctions. Yet, it is more likely that glutamate simply never becomes available for synaptic release from LRN neurons. Whether glutamate is synthesized de novo, recycled by GLN or scavenged by uptake, the high demands of energy and GABA production in LRN neurons are always present, at least in part, in the form of GAD. In fact, the localization of GAD in the synaptic vesicles of these neurons may represent the final destination of any glutamate entering terminal regions of LRN neurons, enforcing complete conversion of glutamate substrate into the GABA neurotransmitter pool and yielding only GABA release from synaptic vesicles. A comparably fail-safe glutamate scavenging mechanism may be lacking in

non-GABAergic neurons with the possibility for synaptic co-release of glutamate and another neurotransmitter correspondingly greater.

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