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Synaptic Organization of Thalamocortical Axon Collaterals in the Perigeniculate Nucleus and Dorsal Lateral Geniculate Nucleus

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Abstract

We examined the synaptic targets of large non- γ -aminobutyric acid (GABA)-ergic profiles that contain round vesicles and dark mitochondria (RLD profiles) in the perigeniculate nucleus (PGN) and the dorsal lateral geniculate nucleus (dLGN). RLD profiles can provisionally be identified as the collaterals of thalamocortical axons, because their ultrastructure is distinct from all other previously described dLGN inputs. We also found that RLD profiles are larger than cholinergic terminals and contain the type 2 vesicular glutamate transporter. RLD profiles are distributed throughout the PGN and are concentrated within the interlaminar zones (IZs) of the dLGN, regions distinguished by dense binding of *Wisteria floribunda* agglutinin (WFA). To determine the synaptic targets of thalamocortical axon collaterals, we examined RLD profiles in the PGN and dLGN in tissue stained for GABA. For the PGN, we found that all RLD profiles make synaptic contacts with GABAergic PGN somata, dendrites, and spines. In the dLGN, RLD profiles primarily synapse with GABAergic dendrites that contain vesicles (F2 profiles) and non-GABAergic dendrites in glomerular arrangements that include triads. Occasional synapses on GABAergic somata and proximal dendrites were also observed in the dLGN. These results suggest that correlated dLGN activity may be enhanced via direct synaptic contacts between thalamocortical cells, whereas noncorrelated activity (such as that occurring during binocular rivalry) could be suppressed via thalamocortical collateral input to PGN cells and dLGN interneurons.

Indexing terms

RLD; GABA; synapse; ultrastructure; perigeniculate; lateral geniculate; interlaminar zone

The existence of subcortical collaterals of thalamocortical axons has been well established via a number of in vivo and in vitro physiological studies (Ahlsén et al., 1978, 1984; Ahlsén and Lindström, 1982; Lindström, 1982; Kim and McCormick, 1998). However, anatomical descriptions of these collaterals are relatively sparse, and very few studies have examined their synaptic arrangements. Ide (1982) first described large profiles with round vesicles and dark mitochondria (RLD profiles) in the cat perigeniculate nucleus (PGN) and suggested that they originate from collaterals of geniculocortical axons. RLD profiles were described as the most abundant source of input to PGN cells, which correlates with in vitro studies indicating that glutamatergic dorsal lateral geniculate nucleus (dLGN) inputs strongly affect the activity of PGN cells (Kim and McCormick, 1998). RLD profiles were also observed by Montero (1989a), who found that these terminals could be labeled via tracer injections in the optic radiations dorsal to the PGN. Additionally, Montero (1989b) observed RLD profiles within

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the interlaminar zone (IZ) between the A and A1 laminae of the dLGN as well as within lamina A (Montero, 1991); this distribution correlates with an in vitro study, which found that dLGN interneurons are innervated by geniculocortical cells (Cox et al., 2003).

The origin of RLD profiles can be shown by comparing their ultrastructure with that of all other known sources of input to the dLGN and PGN. RLD profiles can be distinguished from retinal terminals based on the appearance of the mitochondria contained within them. Retinal terminals are large profiles with round vesicles but contain distinctive mitochondria with dilated cristae; this feature gives the mitochondria a pale appearance compared with other mitochondria. Therefore, retinal terminals are characterized as RLP profiles (to indicate round vesicles, large profile size, and pale mitochondria; Guillery, 1969; Robson and Mason, 1979; Hamos et al., 1987). In contrast, the mitochondria within RLD profiles have more compact cristae and thus have a darker appearance. Most importantly, RLD profiles can be distinguished from corticogeniculate terminals based on size. Corticogeniculate terminals have consistently been described as small profiles with round vesicles and dark mitochondria (RSD profiles; Jones and Powell, 1969; Weber et al., 1989; Vidnyànszky and Hámori, 1994; Erisir et al., 1997). These terminals arise from corticogeniculate cells located exclusively in layer 6 (Gilbert and Kelly, 1975). Other thalamic nuclei, such as the pulvinar nucleus and lateral posterior nucleus, receive input from corticothalamic cells located in both layers 5 and 6, and these cells give rise to large and small terminals (Abramson and Chalupa, 1985; Vidnyànszky et al., 1996; Feig and Harting, 1998; Baldauf et al., 2005; Huppé-Gourgues et al., 2006). These two types of corticothalamic terminals are easily distinguished at the light microscopic level as type II and type I terminals, respectively (Guillery et al., 2001; Baldauf et al., 2005; Huppé-Gourgues et al., 2006). If large corticothalamic terminals innervated the dLGN, they should easily be distinguished at the light microscopic level. However, all accounts of corticogeniculate fibers have described them as exceedingly fine axons (type I; Guillery, 1968) that give rise to small boutons (Robson, 1984; McCart and Henry, 1994; Murphy and Sillito 1996; Murphy et al., 2000). Thus, it can be concluded that all corticogeniculate terminals are smaller than RLD profiles. Furthermore, all other inputs to the dLGN and PGN either are smaller than RLD profiles or contain distinct neurotransmitters (Fitzpatrick et al., 1984, 1989; Montero, 1986; Raczkowski and Fitzpatrick, 1989; Bickford et al., 1993, 1994; Erisir et al., 1997; Wang et al., 2001, 2002a). Therefore, all available evidence indicates that RLD profiles originate from the collaterals of geniculocortical axons.

Given the potential importance of geniculocortical collaterals in the circuitry of the dLGN and PGN, a more complete characterization of RLD profiles is warranted. The purpose of this study was to compare the synaptic targets of RLD profiles in the PGN and dLGN and to determine the precise distribution of RLD profiles within the dLGN laminae. Tissue was prepared for electron microscopy and stained for γ -aminobutyric acid (GABA), using postembedding immunocytochemistry, to distinguish GABAergic PGN cells and dLGN interneurons from non-GABAergic geniculocortical cells (Houser et al., 1980; Oertel et al., 1983; Fitzpatrick et al., 1984; Montero and Singer, 1984, 1985; de Biasi et al., 1986; Rinvik et al., 1987; Rinvik and Ottersen, 1988). Our results indicate that RLD profiles are abundant in the PGN and interlaminar zones of the dLGN and that they exhibit distinct synaptic arrangements in each zone.

MATERIALS AND METHODS

In total, seven cats were used for these experiments (96–01, 96–12, 99–03, 99–10, 99–11, 03–06, and 03–08). All procedures were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the University of Louisville Animal Care and Use Committee. Tissue from some of these cats was used for

previous studies (Patel and Bickford, 1997; Patel et al., 1999; Wang et al., 2002b; Kelly et al., 2003).

Perfusion and sectioning

Cats were deeply anesthetized with an intravenous injection of sodium pentobarbital (30 mg/lb) and perfused with saline or Tyrode solution, followed by a fixative solution of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4; cases 99-03 and 99-10) for preembedding immunocytochemical staining for the type 2 vesicular glutamate transporter (vGLUT2), postembedding immunocytochemical staining for GABA, and electron microscopy. A fixative of 4% paraformaldehyde and 0.5% glutaraldehyde in PB (96-01, 96-12, 99-11) was used for preembedding immunocytochemical staining for choline acetyltransferase (ChAT), postembedding immunocytochemical staining for GABA, and electron microscopy. For light-level histochemical staining, cats were perfused with 2% paraformaldehyde and 0.1% glutaraldehyde, or 4% paraformaldehyde, in PB (03-06 and 03-08). Brains were removed from the skulls, and blocks containing the thalamus were sectioned in the coronal or sagittal plane to a thickness of 50 μ m with a vibratome.

Antibody characterization

The antibodies used in this study are listed in Table 1. The ChAT antibody specifically binds a protein with a molecular weight of 68 kDa that forms 153 μ mol acetylcholine/minute/mg (Eckenstein and Thoenen, 1982). In cat tissue, this antibody stains the same pattern of thalamic terminals, and basal forebrain and mesopontine tegmentum cells, as has been demonstrated with other cholinergic markers in a variety of species (Fitzpatrick et al., 1989; Bickford et al., 1993, 1994).

The GABA antibody shows positive binding with GABA and GABA-keyhole limpet hemocyanin, but not BSA, in dot blot assays (manufacturer's product information). In cat tissue, this antibody has been shown to stain axon thalamic terminals originating from the basal forebrain, thalamic reticular nucleus (TRN)/PGN, and pretectum (Bickford et al., 1994; Wang et al., 2001, 2002a) as well as the dendritic terminals of dLGN interneurons (Bickford et al., 1999; Datskovskaia et al., 2001; Dankowski and Bickford, 2003).

The glutamic acid decarboxylase (GAD) antibody recognizes a single band at 67 kDa on Western blot analysis of rat brain (manufacturer's communication). In cat tissue, this antibody stains most neurons in the TRN and PGN and a subset of neurons in the dorsal thalamus (current study).

The SMI-32 antibody recognizes a nonphosphorylated epitope of high-molecular-weight neurofilaments (Sternberger and Sternberger, 1983). SMI-32 visualizes two bands (200 and 180 kDa), which merge into a single line on two-dimensional blots. This antibody has previously been used to stain presumed Y cells in the cat dLGN (Bickford et al., 1998).

Preabsorption of the type 1 vesicular glutamate transporter (vGLUT1) antiserum with immunogen peptide eliminates all immunostaining (manufacturer's product information). Western blot reveals a single band at approximately 60 kDa (Melone et al., 2005). With the vGLUT1 antibody, the thalamic staining pattern in the cat brain is similar to that obtained in rat by Kaneko and Fujiyama (2002). Preabsorption of the vGLUT2 antiserum with immunogen peptide eliminates all immunostaining (manufacturer's product information). Western blot reveals a single band at approximately 62 kDa (Montana et al., 2004). The vGLUT2 antibody has been shown to stain geniculocortical terminals in the ferret (Nahmani and Erisir, 2005). In cat striate cortex, the vGLUT2 antibody labels a dense band of terminals in layer 4.

ChAT and vGLUT2 immunohistochemistry for electron microscopy

Sections for ChAT or vGLUT2 immunocytochemistry were preincubated in a solution of 10% NGS in PBS for 30 minutes and then incubated for 12–48 hours at 4°C in a solution of 1% NGS in PBS containing either a 1:50 dilution of a monoclonal anti-ChAT antibody or a 1:15,000 dilution of a polyclonal anti-vGLUT2 antibody (Table 1). Sections incubated with these antibodies were then rinsed in PB and placed in 1% NGS in PBS solution containing a 1:100 dilution of a biotinylated goat anti-rat or biotinylated goat anti-guinea pig antibody (Vector, Burlingame, CA) for 1 hour at room temperature. After being rinsed in PB, sections were placed in a complex of avidin and biotinylated horseradish peroxidase (ABC solution; Vector) for 1 hour at room temperature. After PB washes, sections were reacted either with nickel-intensified diaminobenzidine (DAB) for 5 minutes or with a VIP kit (Vector) for 15 min. Sections were either mounted on slides for light microscopic examination or prepared for electron microscopy as described below.

Wisteria floribunda agglutinin histochemistry

Sections for *Wisteria floribunda* agglutinin (WFA) histochemistry were incubated for 12 hours at 4°C in 1:200 dilution of biotinylated-WFA in PB (Sigma, St. Louis, MO; catalogue No. L-1766). After rinsing in PB, sections were placed in ABC for 1 hour at room temperature. After PB washes, sections were reacted with nickel-intensified DAB for 5 minutes, rinsed in PB, and mounted on slides for light microscopic examination.

Double labeling with WFA + GAD, SMI-32, vGLUT1, or vGLUT2

Sections for double labeling were incubated for 12 hours at 4°C in 1:200 dilution of biotinylated-WFA and a 1:2,000 dilution of a mouse monoclonal anti-GAD 67-kD antibody, a 1:10,000 dilution of the mouse monoclonal SMI-32 antibody, or a 1:15,000 dilution of guinea pig polyclonal antibodies against vGLUT1 or vGLUT2 (Table 1). Sections incubated with these antibodies were then rinsed in PB and placed in 1% NGS in PBS solution containing a 1:100 dilution of streptavidin-Alexa 546 with goat anti-rabbit, goat anti-mouse, or goat anti-guinea pig antibodies conjugated to Alexa 488 (Invitrogen/Molecular Probes, Carlsbad, CA) for 1 hour at room temperature. After being rinsed in PB, sections were mounted on slides for confocal microscopic examination.

Electron microscopy and GABA immunocytochemistry

Sections were prepared for electron microscopy by post-fixing in osmium, dehydrating in an alcohol series, and embedding in Durcupan resin (Ted Pella, Redding, CA). Blocks containing portions of the PGN, A, A1, IZ, and magnocellular or parvocellular C laminae of the dLGN were cut into ultrathin sections in serial order (silver-gray interference color). To avoid counting the same synapse in multiple sections, every fifth section was collected on Formvar-coated nickel slot grids, and every fourth collected section was examined (a spacing of approximately 1.5 μ m between examined sections).

These sections were stained for the presence of GABA by using previously reported postembedding immunocytochemical techniques (Patel and Bickford, 1997). For this study, we used a rabbit polyclonal anti-GABA antibody at a dilution of 1:2,000 (cases 99-03 and 99-10) or 1:500–1:1,000 (cases 96-01, 96-12, and 99-11). As in our previous studies, the GABA antibody was tagged with a goat anti-rabbit antibody conjugated to 15-nm gold particles (Amersham, Arlington Heights, IL). Selected GABA-stained sections within the series were examined with an electron microscope, and RLD profiles were photographed when sectioned through a synaptic contact. As illustrated in Figure 1, the thin sections lightened as they were scanned in the electron microscope. Thus, by examining sections at low magnification when an RLD profile was encountered, the location of the RLDs contained within the section could

be pinpointed fairly easily. In this way, the location of the RLD profiles in relation to laminar borders could be ascertained.

GABA labeling was quantified by comparing the density of gold particles overlying RLD profiles with the density overlying their postsynaptic partners. The density for each profile was calculated in Image J software (<http://rsb.info.nih.gov/ij/>). For quantification of synapses, each postsynaptic partner was counted only once. In other words, if an RLD profiles formed several synapses with one postsynaptic profile in a single section, this was counted as one synaptic contact.

For sections previously stained with a ChAT antibody, ChAT-stained terminals and adjacent RLD profiles were photographed when sectioned through a synaptic contact. RLD profiles were included in the analysis only when located within zones that contained ChAT-stained profiles (within the zone of ChAT antibody penetration).

Computer-generated figures

Light-level photographs were taken with a digitizing camera (Spot RT; Diagnostic Instruments, Sterling Heights, MI). Confocal images were taken with an Olympus Fluoview laser scanning microscope (BX61W1). Electron microscopic images were taken with a digitizing camera (SIA-7C; SIA, Duluth, GA) or negatives, which were subsequently scanned and digitized (SprintScan 45i; Polaroid, Waltham, MA). In Adobe Photoshop (Adobe Systems, San Jose, CA), the brightness and contrast were adjusted to optimize the images.

RESULTS

Identification of RLD profiles

In the PGN and dLGN, RLD profiles were easily identified as very large (PGN $4.09 \pm 2.7 \mu\text{m}^2$, $n = 50$; dLGN $4.1 \pm 2.2 \mu\text{m}^2$, $n = 101$, case 99-10), non-GABAergic profiles that contain loosely packed, round synaptic vesicles and dark mitochondria (Figs. 2–11). RLD synaptic contacts were identified by a collection of vesicles adjacent to a synaptic cleft. When cut perpendicular to the center of the synapse, the postsynaptic density was slightly thicker than the presynaptic density (asymmetric synapse). Figure 4 illustrates the appearance of RLD synaptic contacts in comparison with other identified contacts within the dLGN. RLD profiles and their postsynaptic profiles were also occasionally attached via puncta adherentia (see Fig. 7). Puncta adherentia are characterized by short segments of dense material extending from the membranes of adjacent profiles; no vesicles are associated with these contacts (Figs. 4, 7, arrowheads). Neurofilaments were often present within RLD profiles, and dense-core vesicles were also occasionally observed, as previously described (Fig. 4I; Ide, 1982; Rinovik and Ottersen, 1988). We examined several RLD profiles in serial sections in an attempt to confirm their axonal origin. However, in all cases, the terminal profile abruptly transitioned to a profile of fine caliber that could not be confidently identified in subsequent sections. Occasional fortuitous sections revealed that RLD profiles are connected to small, unmyelinated processes that contain microtubules and some vesicles (Figs. 3, 5, 7).

As illustrated in Figure 6, RLD profiles are easily distinguished from RLP profiles, named for their round vesicles, large size, and pale mitochondria (Guillery, 1969). It is well established that RLP profiles represent the synaptic endings of retinal axons (Robson and Mason, 1979; Hamos et al., 1987). Although the size of RLD profiles is close to that of RLP profiles, they are easily distinguished based on the ultrastructure of the mitochondria contained within them. In addition, the vesicle density within RLP profiles is higher than that within RLD profiles (Figs. 4, 6).

RLD profiles were also clearly distinct from RSD profiles, named for their round vesicles, small size, and dark mitochondria (Guillery, 1969; Fig. 4G). Terminals of cortical and brainstem origin exhibit RSD morphology, although cortical terminals in the dLGN are smaller ($0.36 \pm 0.14 \mu\text{m}^2$) than cholinergic terminals ($0.52 \pm 0.32 \mu\text{m}^2$), and some cholinergic terminals have previously been identified as RLD profiles (Erisir et al., 1997). To determine whether RLD profiles identified in the present study could be cholinergic, we examined PGN tissue stained for ChAT and GABA (tissue generated for previous studies of the pulvinar and lateral posterior nucleus; Patel and Bickford, 1997; Patel et al., 1999). As illustrated in Figure 10, when we examined tissue specifically within zones that contained ChAT-labeled terminals (within the zone of ChAT antibody penetration), unlabeled RLD terminals were frequently observed. In the ChAT-stained tissue, we photographed RLD profiles engaged in synaptic contacts and found that their sizes were similar to those observed in the tissue that was not additionally stained for ChAT ($3.18 \pm 1.38 \mu\text{m}^2$, $n = 80$). We also observed that ChAT-stained terminals engaged in synaptic contacts were of medium size ($0.81 \pm 0.11 \mu\text{m}^2$, $n = 53$), i.e., larger than RSD profiles (as previously described; Raczkowski and Fitzpatrick, 1989; Erisir et al., 1997) but smaller than RLD profiles. Thus, we conclude that RLD profiles are distinct from cholinergic terminals and can be classified as non-GABAergic, noncholinergic profiles with loosely packed vesicles and sizes in the range of $2\text{--}4 \mu\text{m}^2$. We did not examine the IZs of the dLGN in ChAT-stained tissue because the cholinergic innervation of the IZs is quite sparse (Fitzpatrick et al., 1989).

To classify the RLD profiles further, we examined tissue stained for vGLUT2, which has been established as a marker for thalamocortical terminals (Nahmani and Erisir, 2005). To examine terminals labeled with the vGLUT2 antibody at the ultrastructural level, we used a Vector VIP kit to reveal the antibody distribution. The VIP kit gives a punctate reaction product that allows examination of ultrastructural details when relatively sparse reaction product is present (Figs. 5, 11). The penetration of the vGLUT2 antibody was quite limited. In profiles labeled immediately adjacent to the tissue surface, the VIP reaction product obscured ultrastructure, but profiles labeled approximately $1\text{--}5 \mu\text{m}$ from the surface were more lightly labeled, and the ultrastructure could be distinguished. We found that the vGLUT2 antibody stained RLD profiles in the PGN and both RLD and RLP profiles in the dLGN. Labeling could also be found within non-GABAergic dendrites, but not within GABAergic profiles or RSD profiles. We measured the size of vGLUT2-labeled terminals that did not contain pale mitochondria and found that their size was similar to that of RLD profiles identified in unstained tissue ($5.5 \pm 2.7 \mu\text{m}^2$, $n = 50$, case 99-03).

Finally, it should be noted that, with fixation that does not result in the flattening of vesicles, the ultrastructure of RLD profiles is quite similar to that of F2 profiles, which are the dendritic terminals of GABAergic interneurons (Hamos et al., 1985; Montero, 1986). However, in tissue stained for GABA, RLD profiles were clearly distinguished from F2 profiles based on the density of gold particles overlying each profile (Figs. 2–7). In addition, unlike F2 profiles, RLD profiles were never observed to be postsynaptic to other terminals.

Distribution of RLD profiles in the dLGN and PGN

To determine the distribution of RLD profiles within the PGN and dLGN, we examined the location of RLD profiles in sections from two cases (99-03 and 99-10) that contained 1) the PGN, lamina A, and IZ; 2) the IZ, lamina A1, and magnocellular C lamina (C_m); or 3) the C_m and parvocellular C (C_p) laminae and optic tract. For each section, we carefully examined the entire section and identified the location of RLD profiles (regardless of whether they were engaged in synaptic contacts). Figure 12 shows a low-magnification micrograph of an examined thin section; the locations of RLD profiles observed within the section are indicated with dots. By identifying the location of each identified RLD at low magnification and

comparing its location with blood vessel patterns in drawings of the 50- μ m-thick mounted section, we found that RLD profiles were easily identified within 1) the PGN and the adjacent regions of the dorsal lamina A, 2) the IZ between lamina A and A1 and adjacent regions of the ventral lamina A and dorsal lamina A1, and 3) the IZ between lamina A1 and C_m and adjacent regions of the ventral A1 and C_m. No RLD profiles were observed in the C_p.

In blocks containing the PGN, it was clear that some RLD profiles were distributed in adjacent regions of the dorsal lamina A. The transition from the PGN to the dorsal A lamina is marked by a relatively abrupt change in the neuropil; no RLP profiles were observed in the PGN, whereas RLP profiles are abundant within lamina A. It was more difficult to distinguish precisely the transition between the laminae of the dLGN. However, RLP profiles were relatively sparse within the IZs, whereas the A and C laminae contained prominent RLP profiles within glomeruli. RLD profiles were distributed within each IZ, but their distribution also clearly extended into adjacent regions of the dLGN laminae, where RLD profiles were observed adjacent to RLP profiles (Fig. 6).

Synaptic targets of RLD profiles in the dLGN

As described above, RLD profiles in the dLGN were concentrated near the transition zones between PGN and lamina A, between lamina A and A1, between lamina A1 and C_m, and within the C_m. In all of these regions, the synaptic arrangements of the RLD profiles were similar. RLD profiles within the dLGN primarily participated in glomerular arrangements (Figs. 2, 4–7). That is, RLD profiles were often centrally located within complex synaptic arrangements in which the RLD profile made multiple synaptic contacts with F2 profiles and dendrites. The dendrites and F2 profiles postsynaptic to single RLD profiles were often connected by a synapse (triadic arrangements). Examples of profiles participating in triads with RLD profiles are indicated in Figures 2 and 6 with subscripts (F2_T and D_T), and the three synapses that make up a triad are illustrated in Figure 6B. Although RLD profiles were found adjacent to RLP profiles, RLD and RLP glomerular arrangements were segregated. We did not observe any profiles that were contacted by both an RLD and an RLP profile.

More rarely, we observed RLD profiles that made synaptic contacts with somata and proximal dendrites in the dLGN (Fig. 8). To characterize these cells, we used postembedding immunocytochemical staining for GABA, a neurotransmitter used by the interneurons but not thalamocortical cells (Fitzpatrick et al., 1984; Montero and Singer, 1984). Since staining intensity can vary from section to section as a result of slight variations in their thickness, we used the ratio of gold particle density overlying postsynaptic profiles to the gold particle density overlying their presynaptic partners to determine whether RLD profiles synapse on GABAergic or non-GABAergic profiles. The gold particle density overlying cells and proximal dendrites was found to be higher than that within their presynaptic RLD profiles (postsynaptic/presynaptic ratio mean and standard deviation = 17.27 ± 11.66 , $n = 5$). Thus, RLD profiles in the dLGN directly synapse on the somata and proximal dendrites of GABAergic interneurons. We found that, within glomerular RLD arrangements, the density of gold particles overlying postsynaptic dendrites that contained vesicles (F2 profiles; Figs. 2–7) was higher than that overlying their presynaptic RLD profiles (postsynaptic/presynaptic ratio 17.23 ± 12.17 , $n = 61$). Thus we can identify these profiles as the dendritic terminals of GABAergic interneurons. A smaller population of RLD targets was identified as GABAergic (postsynaptic/presynaptic ratio 7.76 ± 6.26 , $n = 25$) but did contain abundant vesicles. These dendrites were located both inside and outside glomeruli. RLD targets identified as thalamocortical cell dendrites (D; Figs. 2–7) contained a low density of gold particles (postsynaptic/presynaptic ratio 1.74 ± 0.87 , $n = 17$). These dendrites were located within glomeruli and were often involved in triadic arrangements (Figs. 2, 6). Finally, small numbers of postsynaptic dendrites were deemed too

small to detect GABA accurately. The proportions of RLD synaptic contacts in the dLGN are summarized in Figure 13.

Synaptic targets of RLD profiles in the PGN

We sampled PGN tissue from regions immediately dorsal to the A lamina of the dLGN. We avoided sampling tissue from PGN regions adjacent to the rostral or lateral surfaces of the dLGN, where the PGN is difficult to distinguish from the TRN or ventral lateral geniculate nucleus, respectively (Ahlsén et al., 1982; Ide, 1982; Fitzgibbon, 2002). Because the PGN receives only a very sparse direct retinal input (Laties and Sprague, 1966; Sanderson and Pearson, 1977; Schmielau, 1979), we distinguished the PGN as a region in which we observed no RLP profiles.

As previously described by Ide (1982), RLD profiles in the PGN synapsed primarily on the somata and proximal dendrites of PGN cells (Figs. 9–11). In addition, RLD profiles synapsed on small spines that could in many instances be identified as originating from PGN somata or dendrites (Fig. 9D,F).

To distinguish PGN cells from the dendrites of thalamocortical cells located within lamina A that can extend into the PGN (Ferster and LeVay, 1978; Friedlander et al., 1981), we used postembedding immunocytochemical staining for GABA, a neurotransmitter used by PGN neurons but not by dLGN thalamocortical cells (Fitzpatrick et al., 1984; Montero and Singer, 1984; Rinovik et al., 1987). With the exception of spines, all profiles in the PGN postsynaptic to RLD profiles contained a density of gold particles that was higher than the density of gold particles overlying the RLD profiles that contacted them (postsynaptic/presynaptic ratio mean and SD = 8.15 ± 6.87 , $n = 48$). The density of gold particles overlying postsynaptic spines was not consistently greater than that overlying their presynaptic RLD profiles. This likely is due to the limited area of the spines ($0.209 \mu\text{m}^2 \pm 0.163$, $n = 41$). Because we observed spines emerging from somata and proximal of GABAergic dendrites within the PGN, we assume that the spines are too small to detect the presence of GABA accurately. However, because we never observed spines emerging from non-GABAergic dendrites, we conclude that geniculocortical axon collaterals in the PGN exclusively target GABAergic cells and do not synapse on thalamocortical cell dendrites that extend into the PGN. The proportions of RLD targets in the PGN of case 99-10 are summarized in Figure 13.

WFA binding defines zones of thalamocortical axon collaterals

We found that the distribution of RLD profiles is correlated with regions of the PGN and dLGN that show strong binding of WFA, a plant lectin with affinity for N-acetyl-D-galactosamine within the extracellular matrix of subsets of neurons. Cells within the PGN are densely stained, as are cells between the A and C laminae of the dLGN. The staining between the A and A1 laminae defines the IZ. The staining between the A1 and C_p laminae appears to include the C_m and adjacent IZs.

Because the WFA staining pattern is similar to that obtained with the Cat-301 antibody, a putative Y cell marker which recognizes a protein epitope of aggrecan (Sur et al., 1988; Hockfield and Sur, 1990; Mathews et al., 2002), we examined tissue stained with WFA and the SMI-32 antibody, another putative Y cell marker that recognizes nonphosphorylated high-molecular-weight neurofilament proteins (Bickford et al., 1998). As illustrated in Figure 15F,G, some colocalization of WFA and SMI-32 was observed near IZs, but many SMI-32-stained cells within the A and C laminae did not bind WFA, and many WFA-stained cells did not stain for SMI-32. There was no colocalization in the PGN; as previously illustrated, SMI-32 does not stain PGN cells (Bickford et al., 1998), although Cat-301 staining is strong within the

PGN (Sur et al., 1988; Hockfield and Sur, 1990). WFA binds to GABAergic PGN cells (14A) and a small subset of dLGN interneurons located within IZs or close to laminar borders (14E).

Furthermore, in tissue stained for vGLUT2 and WFA, vGLUT2 terminals are associated with the somata and proximal dendrites of PGN cells (Fig. 15C,D). This correlates with the location of most RLD synapses within the PGN. In contrast, terminals stained for vGLUT1 are associated with the more distal dendrites of PGN cells (Fig. 15B). Because the cortex expresses high levels of vGLUT1 mRNA, but not vGLUT2 mRNA, the vGLUT1 antibody is a presumed marker for corticothalamic terminals (Freneau et al., 2001; Kaneko and Fujiyama, 2002), although lower levels of vGLUT1 mRNA have been detected in the thalamus (Barroso-Chinea et al., 2007).

We attempted to examine the ultrastructural location of WFA binding, but we found WFA bound to cells in the interlaminar zones only when the tissue was lightly fixed. WFA binding in the PGN was more robust, but, as the quality of tissue fixation increased, WFA binding decreased. In other words, WFA localization and the preservation of ultrastructure were not compatible.

DISCUSSION

RLD profiles have been previously identified in the rat, cat, and monkey (Ide, 1982; Ohara, 1988; Rinvik and Ottersen, 1988; Montero, 1989a, 1991; Liu and Jones, 1999), but the present study represents the first detailed comparison of their synaptic arrangements and distribution within the PGN and dLGN. As discussed below, we found in the PGN that all RLD profiles make synaptic contacts with GABAergic somata, dendrites, and spines, supporting previous studies. In contrast, we found in the dLGN that RLD profiles form complex glomerular arrangements with both thalamocortical cells and interneurons. Because these arrangements are located near laminar borders and within interlaminar zones, the contribution of this novel circuitry to dLGN function is unrecognized; most previous studies of dLGN ultrastructure have focused on the A laminae. Our anatomical observations call attention to circuits that may be potentially important in enhancing correlated dLGN activity and suppressing noncorrelated activity.

Identification of RLD profiles as thalamocortical axon collaterals

RLD profiles have been shown to be labeled via tracer injections in the optic radiations (Montero, 1989a) and to degenerate following dLGN but not cortical lesions (Ohara, 1988). It has also been demonstrated that all terminals labeled via striate cortex tracer injections exhibit RSD morphology in the dLGN (Jones and Powell, 1969; Vidnyánszky and Hámori, 1994; Erisir et al., 1997). Brief examinations of collaterals of thalamocortical cells labeled by intracellular injection were carried out by Wilson et al. (1984) and Hamos (1990). Although the labeled terminals were described as RSD-like, one illustrated terminal was over $5 \mu\text{m}^2$ (Fig. 4 in Hamos, 1990). Finally, we found that RLD profiles are not cholinergic and contain vGLUT2, a marker for thalamocortical terminals (Nahmani and Erisir, 2005). Thus, all available data support the identification of RLD profiles as originating from the collaterals of geniculocortical axons.

It has been noted that, in chronically decorticated cats, thalamocortical cell dendrites contain vesicles and form synaptic contacts (Takács et al., 1991). The synapses formed by these dendrites are very similar to those of RLD profiles. Therefore, synaptic contacts formed by geniculocortical cells, either normally via axons, or by dendrites when induced by cortical lesions, may be similar throughout the dLGN.

In contrast, the RLD profiles are much larger than the cortical terminals that originate from the dLGN (Dehay et al., 1991; Ding and Casagrande, 1998; Erisir and Dreusicke, 2005), and the vesicles in geniculocortical terminals are more densely packed than those within RLD profiles. However, several previous studies indicate that terminals originating from single axons do not necessarily exhibit the same morphology in different target structures (Acsady et al., 1998; Boka et al., 2006). On the other hand, excitatory postsynaptic potentials (EPSPs) elicited by stimulation of either thalamocortical terminals or their collaterals exhibit frequency-dependent depression (Kim and McCormick, 1998; Gentet and Ulrich, 2002; Boudreau and Ferster, 2005). This suggests that glutamate release from geniculocortical terminals and their collaterals may be similarly influenced by the activity patterns of geniculocortical cells. For example, bursts of action potentials activated by low-threshold calcium spikes in thalamocortical cells are particularly effective in generating large postsynaptic potentials in the cortex and PGN, because of temporal summation and/or recovery from synaptic depression (Kim and McCormick, 1998; Swadlow and Gusev, 2001; Swadlow et al., 2002; Usrey, 2002).

RLD synaptic contacts in the PGN

For the PGN, we conclude that RLD profiles form synaptic contacts exclusively with GABAergic profiles and that they synapse primarily on somata, large dendrites, and their spines, in good agreement with Ide (1982). The location of RLD input to PGN cells suggests that the activity of geniculocortical cells can strongly influence the activity of PGN cells. Paired recordings of connected PGN and geniculocortical cells, carried out by Kim and McCormick (1998), clearly demonstrate the strong influence of geniculocortical cells on PGN cell activity. When combined with the previous finding that PGN/TRN terminals synapse primarily on geniculocortical cells (Wang et al., 2001), these anatomical results support models of thalamic circuitry that include strong reciprocal connections between thalamocortical cells and PGN cells. Such connections are thought to be critical to the generation of normal synchronized thalamocortical rhythms during sleep as well as abnormal rhythms associated with absence epilepsy (Steriade and Deschênes, 1988; Steriade et al., 1993; Bal et al., 1995a,b; Kim et al., 1995; Kim and McCormick, 1998).

RLD synaptic contacts in the dLGN

In contrast to the PGN, RLD profiles observed within the dLGN were located primarily within glomeruli, where they synaptically contacted thalamocortical cell dendrites and the dendritic terminals of interneurons, often within triadic arrangements. In fact, the synaptic arrangements of RLD profiles in the dLGN are very similar to those of retinal terminals, except that RLD synapses on interneurons are more numerous (80%) than RLP synapses on interneurons (30%; Montero, 1991; Erisir et al., 1998). This is in good agreement with the results of Montero (1991), who observed RLD profiles in glomerular arrangements in lamina A and estimated that RLD profiles contact interneurons four times more frequently than thalamocortical cell dendrites.

These anatomical results could explain the physiological results of Cox et al. (2003). Using an *in vitro* preparation of the cat dLGN, these authors found that excitation of thalamocortical cells, via the pharmacological activation of metabotropic glutamate receptors, increased spontaneous miniature EPSPs recorded from interneurons in the A and A1 laminae. Our results suggest that these potentials could arise from RLD synapses on GABAergic F2 profiles within or near the IZ. On the other hand, it is possible that the thalamocortical collaterals that do not display the distinctive RLD morphology form synaptic contacts with interneurons within the A and A1 laminae. In fact, RSD-like intralaminar collaterals have been described (Wilson et al., 1984; Hamos, 1990), so it is possible that only the largest of the thalamocortical axon collaterals can be distinguished based on ultrastructural features alone. In this case, our sample may represent only a subset of thalamocortical axon collaterals.

RLD connections within the dLGN could also contribute to the synchronous firing of thalamocortical cells that promotes rhythmic thalamic and cortical activity. In vitro activation of metabotropic glutamate receptors or stimulation of corticothalamic inputs in the cat dLGN can induce rhythmic high-threshold bursting of geniculocortical cells, which likely contributes to the generation of α and θ cortical rhythms (Hughes and Crunelli, 2007). Although gap junctions between geniculocortical cells may be primarily responsible for synchronizing these rhythms, RLD connections within the dLGN are likely to play an additional role in coordinating geniculate activity.

Another feature of the dLGN circuitry involving thalamocortical collaterals that remains to be clarified is the source of the F2 profiles contacted by RLD profiles. Physiological and anatomical evidence suggests that multiple types of GABAergic interneurons exist within the dLGN. In addition to intralaminar interneurons, Sanchez-Vives et al. (1996) identified two types of interneurons in the ferret dLGN that were located in or near IZs. PGN-like interneurons, recorded within the IZs, displayed physiological and morphological properties similar to those of PGN cells and were excited by thalamocortical axon collaterals (also identified by Lindström and Wrobel, 1990). Chirping interneurons, which fire with rhythmic bursts of high-frequency action potentials, were also recorded in or near the IZs. Anatomical studies also indicate that interneurons can vary in size, location, and presence or absence of parvalbumin, calbindin, or nitric oxide synthase (Montero and Zempel, 1985; Demeulemeester et al., 1991; Sanchez-Vives et al., 1996; Erisir et al., 1997; Bickford et al., 1999). Thus RLD profiles could target multiple types of interneurons, although to date class III intralaminar interneurons are the only known source of F2 profiles (Hamos et al., 1985; Montero, 1986). Montero (1989b) previously identified RLD synaptic contacts on the somata and proximal dendrites of two reconstructed GABAergic interneurons within the IZ, but these cells were not found to give rise to F2 profiles. We also observed RLD synapses on GABAergic somata and proximal dendrites but did not determine whether they gave rise to F2 profiles.

Distribution of RLD profiles in the dLGN

The distribution of RLD profiles within the dLGN may provide clues to their function. We found that RLD profiles are concentrated within IZs and the C_m . This distribution agrees quite well with the distribution of presumed thalamocortical collaterals labeled by the transneuronal transport of wheat germ agglutinin injected intraocularly (Fitzgibbon, 2002). We also found that regions with concentrated RLD profiles stained densely with WFA, similar to the pattern of staining observed with the Cat-301 antibody, a Y cell marker (Sur et al., 1988). Y cells are located in the C_m , and the dendrites of A laminae Y cells cross laminar borders, whereas the dendrites of X cells are confined to the lamina of origin (Friedlander et al., 1981; Stanford et al., 1983; Wilson et al., 1984). Although the triadic arrangements of RLD profiles might imply an association with X cells (which have been suggested to be the primary target of interneurons; Sherman and Friedlander, 1988), we have found that Y retinogeniculate axons contact interneurons and that glomeruli are particularly prominent in the C_m , where X cells are absent (Datskovskaia et al., 2001; Dankowski and Bickford, 2003). Thus, RLD profiles could primarily participate in circuitry involving Y cells.

A number of studies have demonstrated that thalamocortical collaterals in the PGN arise from class I, II, and IV cells as well as X, Y, and W cells (Ahlsén et al., 1978, 1984; Ferster and LeVay, 1978; Mooney et al., 1979; Friedlander et al., 1981; Stanford et al., 1983; Humphrey et al., 1985; Sanchez-Vives et al., 1996). Fewer studies have labeled intrageniculate axon collaterals, which may be particularly difficult to fill because they arise from small-caliber axons (Friedlander et al., 1981; and suggested by Figs. 3, 5, 7). However, small samples of intrageniculate collateral projections of X, Y, and W axons have been described (Friedlander et al., 1981; Stanford et al., 1983; Wilson et al., 1984; Hamos, 1990). Collaterals of X cells

have been described as being confined to the lamina of origin, whereas collaterals of Y cells are concentrated near IZs (Friedlander et al., 1981). This raises the possibility that Y cells are the source of RLD profiles that we observed near interlaminar zones in the dLGN.

The concentration of RLD profiles near zones that transition from left to right eye innervation suggests a potential involvement in binocular interactions. Geniculate neurons have been shown to be affected by stimulation of the nondominant eye, and these effects include inhibition, excitation, or a combination of inhibition and excitation (Pape and Eysel 1986; Xue et al., 1987; Guido et al., 1989; Murphy and Sillito, 1989; Tong et al., 1992; Wang et al., 1994; Zhou et al., 2003). These effects have also been recorded in the absence of cortical input, and application of a GABA antagonist reveals excitatory responses elicited from stimulation of the nondominant eye (Murphy and Sillito, 1989). The arrangement of RLD profiles in the dLGN, which includes synaptic contacts with both GABAergic F2 profiles and non-GABAergic thalamocortical cell dendrites in triadic relationships, could easily explain these physiological results. Furthermore, these arrangements appear to be ideally positioned to mediate the suppression of inputs during binocular rivalry as well as to enhance correlated binocular inputs required to achieve stereo vision.

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Fig. 1.

A–G: To determine the distribution of RLD profiles, the entire section was scanned at a magnification of $\times 3,400$ while viewing the section through the binoculars of the electron microscope. When an RLD was encountered, its identity was verified at $\times 8,700$, and the location was noted by examining blood vessel and cell patterns at low magnification. This was facilitated by the lightening of section areas that had been previously scanned. The image in A was taken before the section was scanned. The images in B–F were taken as the section was progressively scanned. The distribution of RLD profiles within the section (G, black dots) is plotted on a low-magnification image of the $300\text{-}\mu\text{m} \times 530\text{-}\mu\text{m}$ section. Each RLD observed is indicated by a single dot, regardless of whether it was involved in a synapse. Lines indicate

the approximate borders of the A and A1 laminae and the interlaminar zone (IZ) as determined by examining the flat-embedded 50- μ m-thick section prior to thin sectioning.

Fig. 2.

In the dLGN, RLD profiles form synaptic contacts (arrows) with GABAergic dendritic terminals (F2 profiles, high density of gold particles) and non-GABAergic thalamocortical cell dendrites (D, low density of gold particles), often in triadic arrangements (F2_T and D_T). The profile shown was located in the dorsal region of lamina A (just ventral to the PGN). Scale bar = 1 μ m.

Fig. 3.

In some sections, RLD profiles are seen to emerge from small unmyelinated processes (arrowheads) that contain microtubules and vesicles. The illustrated RLD profile forms synaptic contacts (arrows) with two GABAergic dendritic terminals (F2 profiles, high density of gold particles) and one non-GABAergic thalamocortical cell dendrites (D, low density of gold particles). The RLD profile was located in the A/A1 interlaminar zone. Scale bar = 1 μ m.

Fig. 4.

RLD synapses are distinct from all other identified contacts observed in the dLGN. **A:** Synaptic contact (arrow) formed by a large profile with round vesicles and pale mitochondria (RFP profile) onto a GABAergic dendritic terminal (F2 profile). **B:** Puncta adherentia (arrowheads) formed between an RFP profile and a non-GABAergic dendrite. **D:** Synaptic contact (arrow) formed by a GABAergic axon terminal (F1 profile) on a nonGABAergic dendrite. **E:** Synaptic contact (arrow) formed by an F2 profile on a nonGABAergic dendrite. **G:** Synaptic contact (arrow) formed by a small profile with round vesicles (RSD profile) on a non-GABAergic dendrite. **H:** Puncta adherentia (arrowheads) formed between two non-GABAergic dendrites. **C,F,I-M:** Synaptic contacts (arrows) formed by RLD profiles on non-GABAergic dendrites (C,J) and F2 profiles (F,I,K-M). RLD synaptic contacts are identified by a cluster of vesicles adjacent to a synaptic cleft. The postsynaptic density is slightly thicker than the presynaptic density. Occasional dense-core vesicles are observed in RLD profiles (I, arrowheads). Scale bar = 0.5 μ m.

Fig. 5.

A–C: Immunohistochemically, vGLUT2 labels RLD profiles. Shown is an RLD in the A/A1 interlaminar zone labeled with a VIP reaction. The profile synapses on (arrows, shown at higher magnification in C) two thalamocortical cell dendrites (D, low density of gold particles) and an interneuron dendritic terminal (F2, high density of gold particles). The RLD emerges from a small, unmyelinated process that contains microtubules and vesicles (outlined with white dots, shown at higher magnification in B). The VIP reaction forms small, electron-dense granules (indicated by white asterisks in C). Scale bars = 1 μ m in A; 0.25 μ m in B,C.

Fig. 6.

A: RLD profiles can be easily distinguished from adjacent retinal terminals, identified by their round vesicles, large size, and pale mitochondria (RLP). The RLD profile illustrated was located near the interlaminar zone between lamina A and A1. The RLD profile synapses on (arrows) GABAergic dendritic terminals (F2 profiles, high density of gold particles) and non-GABAergic thalamocortical cell dendrites (D, low density of gold particles). Profiles involved in triadic arrangements are indicated (F2_T and D_T). **B:** The three synapses that constitute one of the RLD triadic arrangements are shown at higher magnification. Scale bars = 1 μ m in A; 0.5 μ m in B.

Fig. 7.

Ultrastructure of two connected RLD profiles in the A/A1 interlaminar zone. Areas shown at higher magnification in **B–E** are indicated by arrows in **A**. The left RLD profile synapses (shown at higher magnification in **B**, arrow) on a GABAergic dendritic terminal (F2 profile, high density of gold particles) and a non-GABAergic thalamocortical cell dendrite (**D**, low density of gold particles, shown at higher magnification in **C**, arrow). The RLD profiles are connected via a thin process that contains microtubules (shown at higher magnification in **E**, arrows). The RLD profiles are also connected to dendrites via puncta adherentia (arrowheads in **C**, **D**). Scale bars = 1 μ m in **A**; 0.2 μ m in **B** (applies to **B–E**).

Fig. 8. RLD profiles occasionally contact somata in the dLGN. **A:** Three RLD profiles are associated with a GABAergic (high density of gold particles) cell in the magnocellular lamina C. **B:** The RLD synaptic contacts (arrows) on the soma in A are illustrated at higher magnification. Scale bars = 1 μ m.

Fig. 9.

In the PGN, RLD profiles form synaptic contacts with somata, proximal dendrites, and spines. **A,B** illustrate the association of RLD profiles with a soma and proximal dendrites. RLDs indicated by the asterisk in **B** are shown at higher magnification in **C**. A synaptic contact formed by one of these profiles is illustrated at higher magnification in **H** (arrow). **D,E** illustrate RLD synapses with spines. Synaptic contacts are shown at higher magnification in **F,G** (arrows). The gold particle density overlying the soma and dendrites indicates the presence of GABA detected by postembedding immunocytochemistry. Spines are too small to detect significant levels of GABA but can be seen to arise from GABAergic profiles (**D,F**). Scale bars = 5 μm in **A,B**; 1 μm in **C** (applies to **C-E**); 0.25 μm in **F** (applies to **F,G**); 0.2 μm for **H**.

Fig. 10.

In PGN tissue stained for choline acetyltransferase (ChAT) with diaminobenzidine, and GABA with gold particles, RLD profiles are unlabeled and larger than ChAT-stained profiles. An RLD synapse on a GABAergic PGN dendrite is indicated with an arrow. Scale bar = 1 μ m.

Fig. 11.

A–C: Immunohistochemical labeling of vGLUT2 labels RLD profiles. Shown is an RLD profile in the PGN labeled with a VIP reaction. The profile synapses on (arrows, shown at higher magnification in B and C) three spines (S). The VIP reaction forms small, electron-dense granules (indicated by white asterisks). Scale bars = 1 μ m in A; 0.5 μ m in C (applies to B,C).

Fig. 12. Locations of observed RLD profiles (dots) plotted on a low-magnification image of the examined $400\text{-}\mu\text{m} \times 700\text{-}\mu\text{m}$ thin section that contained the PGN, lamina A, and IZ. The approximate laminar borders are indicated by arrows.

Fig. 13.

The pie charts summarize the synaptic targets of RLD profiles in the PGN and dLGN of case 99-10 (n = 203 synaptic contacts; 89 PGN, 114 dLGN). See text for details.

Fig. 14.

Wisteria floribunda agglutinin (WFA) binding densely labels cells in the perigeniculate nucleus (PGN), and in the dorsal lateral geniculate nucleus (dLGN) is distributed primarily between the A, A₁, and parvocellular C (C_p) laminae. Shown are four coronal sections through the dLGN arranged from caudal to rostral (A–D). MGN, medial geniculate nucleus; OT, optic tract; PRN, perireticular nucleus; PUL, pulvinar nucleus; TRN, thalamic reticular nucleus; VB, ventrobasal nucleus; ZI, zona incerta. Scale bar = 1 mm.

Fig. 15.

Confocal images illustrate WFA binding in the PGN (A–D) and dLGN (E–G). **A:** WFA (purple) outlines PGN cells that contain glutamic acid decarboxylase (GAD, green). A single 1-μm optical section is displayed. **B:** Terminals stained for the type 1 vesicular glutamate transporter (green) are associated with the dendrites of WFA-stained cells (purple) in the PGN. **C,D:** Terminals stained for the type 2 vesicular glutamate transporter (green) are associated with the somata and proximal dendrites of WFA-stained cells (purple) in the PGN. **E:** WFA (purple) binds to some GAD-positive cells in the dLGN (green). The double-labeled cell illustrated is located in lamina A close to the PGN border. **F,G:** WFA (purple) binds to some

putative Y cells identified by SMI-32 staining (green). Shown are cells near the A/A1 interlaminar zone (F) and the magnocellular C lamina (G). Scale bar = 20 μ m.

TABLE 1

Primary Antibodies Used in This Study

Antigen	Dilution	Host species	Source	Catalogue No.	Immunogen
ChAT	1:50	Mouse myeloma cells fused with spleen cells of immunized Wistar rats	Purchased in 1996 from Boehringer Mannheim (Indianapolis, IN)	770990	ChAT purified from pig brain (Eckenstein and Thoenen, 1982)
GABA	1:500–1:2,000	Rabbit	Sigma (St. Louis, MO)	A2052	GABA conjugated to bovine serum albumin using glutaraldehyde
GAD67	1:2,000	Mouse	Chemicon (Temecula, CA)	MAB 406	Amino acids residues 4–101 of human GAD67
SMI-32	1:10,000	Mouse	Purchased in 1997 from Sternberger Monoclonal (Baltimore, MD)	SMI-32R	Homogenized hypothalmi recovered from Fischer 344 rats
vGLUT1	1:15,000	Guinea pig	Chemicon (Temecula, CA)	AB5905	Amino acid residues 541–560 of rat vGLUT1
vGLUT2	1:15,000	Guinea pig	Chemicon (Temecula, CA)	AB5907	Amino acid residues 565–582 of rat vGLUT2