Cell Surface Molecules Containing *N*-Acetylgalactosamine Are Associated with Basket Cells and Neurogliaform Cells in Cat Visual Cortex

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In the cerebral cortex, the plant lectin *Vicia villosa* (VVA) selectively stains the surfaces of nonpyramidal neurons. This lectin binds specifically to α - and β -linked *N*-acetylgalactosamine (GalNac). VVA-reactive carbohydrate is highly concentrated in layer 4 of the primary visual cortex of the cat, where it is associated exclusively with GABAergic local circuit neurons. We have studied this neuronal subset with intracellular electrophysiological recording and dye marking to identify the particular cell types expressing surface GalNac. Five different types of local circuit neurons were stained intracellularly ($N = 45$), but only 2 types, the columnar basket and large neurogliaform cells, were also labeled by the lectin ($N = 19/45$). Lectin negative types included small basket, chandelier, and large bitufted cells (26/45). Spiny stellate and pyramidal neurons were also lectin negative. Electrophysiological recordings revealed differences in the duration of action potentials in smooth versus spiny stellates but no differences between lectin-positive or -negative types. A biochemical analysis of cortical glycoproteins by SDS-PAGE and lectin blotting revealed multiple bands containing GalNac enriched in membrane fractions. These carbohydrate-containing molecules may be part of a biochemical mechanism for linking together cells with common functional properties.

The primary visual cortex of cats and primates synthesizes a number of fundamental physiological response properties, including selectivity for stimulus orientation, direction, and velocity (Hubel and Wiesel, 1977). Despite intensive investigations of cortical microcircuitry with both anatomical and electrophysiological approaches, the specific cell types and synaptic connections involved in generating even these basic cortical properties remain unclear. In the vertebrate retina, detailed information about the axonal and dendritic arbors of distinct cell types, combined with knowledge about the number and spatial distribution of a specific class, has provided considerable

insight into the processing of light-induced signals in the retina (Wassle et al., 1981; Dowling, 1987). A similar approach in the visual cortex could help reveal some basic organizational principles underlying the generation of receptive field properties.

Electrophysiological response properties of cerebral cortical neurons are known to involve synaptic contributions from inhibitory smooth-dendritic or sparsely spiny local circuit neurons and excitatory pyramidal and spiny stellate neurons. Most of the smooth-dendritic cortical neurons contain GABA and its biosynthetic enzyme, GAD. These neuronal types are of special interest because several models for the generation of orientation selectivity, end-stop inhibition, and directional specificity postulate specific contributions by these cells (Sillito, 1977; McGuire et al., 1984; Boltz and Gilbert, 1986; Ferster and Koch, 1987).

The morphology, synaptic targets, and distributions of specific types of GABAergic neurons are not as well studied as those of pyramidal cells, chiefly for a number of technical reasons. While specific classes of pyramidal cells can be identified by combining retrograde labeling and intracellular staining (Katz, 1987), GABAergic cells cannot be retrogradely labeled since their axons do not project into the white matter. They are encountered rarely in studies employing electrophysiological recording and intracellular filling *in vivo*. Immunocytochemical staining for GABA and GAD reveals the distribution of most local circuit neurons but not the distribution of each morphological type (Jones, 1975; Lund et al., 1979; Hendrickson et al., 1981; Gabbott and Somogyi, 1986; Somogyi, 1986; Hendry et al., 1987; Lund, 1987; Meinecke and Peters, 1987). Immunocytochemical staining for neuropeptides or calcium-binding proteins identifies subpopulations of local circuit neurons (Hendry et al., 1984; Schmechel et al., 1984; Somogyi et al., 1984; Celio, 1986; Demeulemeester et al., 1988; Jones et al., 1988), but the majority of local circuit neurons do not exhibit immunoreactivity for any of the known neuropeptides (Jones and Hendry, 1986). Finally, although intracellular dye injections or Golgi stains identify different local circuit neuron types, these approaches provide little information about either the distribution or relative abundance of different cell types.

Because the above approaches provide limited information about the number and spatial distributions of many types of local circuit neurons, probes of greater cellular specificity are needed. There is now evidence that certain plant lectins and monoclonal antibodies identify subsets of cortical local circuit neurons. Lectins from *Vicia villosa* (VVA) label *N*-acetylgalactosamine (GalNac)-containing molecules which are selectively enriched on the surfaces of many GABAergic neurons (Naka-

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gawa et al., 1986a, b; Naegele et al., 1987; Mulligan et al., 1989). Monoclonal antibodies VC1.1 and VC5.1 identify integral membrane or extracellular proteins on approximately 35% of GABAergic neurons with multipolar morphology in the cat visual cortex (Arimatsu et al., 1987; Naegele et al., 1988). Monoclonal antibody Cat-301 labels a large proteoglycan restricted to cell bodies and dendrites of approximately 80% of the GABAergic neurons in cat area 17, as well as some pyramidal neurons (Hockfield and McKay, 1983; Hendry et al., 1988; Zarembo et al., 1989a). Additional probes for subsets of cortical local circuit neurons include monoclonal antibodies generated against epidermal growth factor receptor, hyaluronectin, *Drosophila* heads, or *Torpedo* electric organs (reviewed in Naegele and Barnstable, 1989).

Previous studies had demonstrated that GABAergic neurons with either bitufted or multipolar dendrites were stained preferentially by monoclonal antibodies VC1.1 and VC5.1 or the lectin VVA (Naegele et al., 1988; Mulligan et al., 1989). These findings suggested that each marker identified more than one fundamental cell type. With none of these markers was the identity of the stained neurons known, since the labeling included cell bodies and dendrites, but not axons.

As a first step towards defining molecular and structural relationships for cortical local circuit neurons, we have studied the morphology of cells exhibiting surface GalNac-containing molecules. Unambiguous identification of these local circuit neurons required visualization of axonal and dendritic patterns; therefore, we developed a double-labeling technique combining VVA staining with intracellular recording and dye injections of Lucifer yellow in living slices of cat visual cortex. Our investigations focused on layer 4 of area 17, where many of the basic cortical receptive field properties are first observed. Some of this work was reported in abstract form (Naegele et al., 1987).

Materials and Methods

Intracellular dye injections in vitro. Neurons in layer 4 of the cat visual cortex were stained with Lucifer yellow by intracellular injections. They were subsequently labeled with fluorescent VVA lectin as described in the following section and double-labeled neurons were identified. Nine cats were used for intracellular experiments. Preliminary experiments indicated that optimal intracellular filling and lectin staining were obtained with cortical slices prepared from animals 12–18 weeks old. Animals were anesthetized with Nembutal (40 mg/kg i.p.), and a large craniotomy was performed to expose area 17. Five to 10 min prior to removal of area 17, ketamine was injected (20 mg/kg i.m.), to block cortical NMDA receptors and thus minimize excitotoxicity. After removal of the occipital cortices, the animals were killed by an overdose of Nembutal (60 mg/kg, i.p.). Coronal slices from area 17 were prepared, maintained, intracellularly stained, and processed according to methods previously described (Katz, 1987) with the following modifications. Addition of 1 mM kynurenic acid (Sigma) improved the viability of GABAergic neurons in the slices. The artificial cerebral spinal fluid (ACSF) contained 1 mM GalNac and 1 mM *N*-acetylglucosamine (GlcNac; Sigma). Addition of the sugars enhanced subsequent lectin staining.

During intracellular recording and staining, action potentials were recorded onto a video cassette recorder and later photographed from a digital oscilloscope. We restricted our intracellular investigations to layer 4, which was clearly visible in living slices when they were viewed with transmitted light. Under these conditions, this layer appeared as a dark band due to the presence of myelin.

Fluorescent lectin staining of slices. Intracellular recording and staining with Lucifer yellow was followed by fixing individual slices by immersion in freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 12–24 hr. After individual neurons were filled by intracellular staining the slices were placed in fixative as soon as possible. The time interval between intracellular staining and fixation varied for each neuron and slice, depending on the number of neurons filled in

the slice, but it was typically less than 10 min. Slices were rinsed, cryoprotected in 30% sucrose in 0.1 M sodium phosphate, and 40- μ m-thick frozen sections were cut on a sliding microtome. Sections were rinsed in two 30 min washes in 0.1 M Tris-Cl, pH 7.4, and incubated overnight in 10 μ g/ml VVA-Texas red (E. Y. Labs) in 0.1 M Tris-Cl at 4°C, with agitation. Sections were rinsed in three 20 min washes in Tris-Cl, mounted onto gelatin-coated slides, air-dried, dipped in xylene for 1 min, and coverslipped in Krystalon. Fluorescent sections were stored at –20°C in the dark.

Reconstructions of double-labeled neurons. All observations were done with a Zeiss Standard microscope equipped with epifluorescence. Texas red and fluorescein filters were used to visualize the Texas red- and Lucifer yellow-stained cells. Photography was done with either Ektachrome ASA 800 or TMAX ASA 100 or 400. Reconstructions of Lucifer yellow-stained neurons were made with a 63 $\times$ Plan Neofluor objective (N.A. 1.3) and a camera lucida, using a wide-band fluorescein filter set. Cells were selected for reconstruction based on extensive filling of axon collaterals, and cells with evident truncation of axons were not used. However, some truncation of particularly long axons in a 400- μ m-thick slice is unavoidable. Thus, the maximum horizontal spread of the axons of our reconstructed cells is somewhat less than that described using *in vivo* intracellular staining (e.g., Martin, 1984). After reconstructing the cells, the coverslip was removed and laminar boundaries were determined by fluorescent counterstaining for 3 min in 0.0001% Nuclear yellow in distilled water (Schmued et al., 1982). The laminar boundaries in sections containing the cell body were determined using an ultraviolet filter and the cytoarchitectonic criteria of Otsuka and Hassler (1962).

Lectin cytochemistry in perfusion-fixed brain sections. Two adult cats were used to study VVA lectin staining following transcardiac perfusion with fixative containing 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brains were removed, postfixed for 1–12 hr, blocked, and cryoprotected according to the method of Eldred et al. (1983). Blocks through area 17 were embedded in TissueTek (Miles), frozen on dry ice, and stored at –80°C. For free-floating staining, 40- μ m-thick sections were cut on either a sliding freezing microtome or on a Vibratome in ice-cold 10 mM Tris-HCl buffer, pH 8.0. The sections were washed in the same buffer containing 150 mM NaCl, pH 8.0, for 1–2 hr before an overnight incubation in the same buffer containing 10–20 μ g/ml of B4 isolectin from VVA conjugated to peroxidase (VVA-POD, E. Y. Labs). Control sections were incubated in VVA-POD mixed 12 hr earlier with 1 mg/ml GalNac (Sigma). The VVA-POD sections were rinsed 4 $\times$ 30 min in 10 mM Tris-HCl and reacted in the same buffer containing 25 mg/100 ml of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.0002% hydrogen peroxide for 10–20 min. Following two 30 min rinses, the sections were mounted onto slides, air-dried, cleared in xylene, and coverslipped.

Effects of in vitro incubation on lectin staining. To determine whether reductions in surface GalNac occurred during the experimental procedures, we compared the number of GalNac⁺ neurons in a pair of slices maintained *in vitro* for different periods of time. To control for variations in the number of GalNac⁺ cells in different parts of area 17 or in different animals, the slices were taken from the same part of area 17, in the same cat. Both slices were prepared according to standard procedures described in Katz (1987). One slice was incubated for less than 1 hr *in vitro* and then placed in fresh fixative. The second slice was incubated for 10 hr *in vitro* before fixation. Subsequently, 40- μ m-thick frozen sections were prepared from the 2 slices and stained with VVA-POD, as described above. Camera lucida drawings were made of 2 of the stained sections (one from each slice) showing the positions of all cell bodies outlined by peroxidase reaction product and the boundaries between cortical layers. Counts were then made of the number of GalNac⁺ neurons in layers 2+3, 4, or 5+6, in three 1-mm-wide strips along the medial bank of area 17.

Size measurements of VVA-stained neurons. The sizes of the GalNac⁺ neurons also stained intracellularly with Lucifer yellow were compared with the size distribution of all GalNac⁺ cells in middle cortical layers in order to determine whether our sample of double-labeled neurons was biased toward a particular cell type. The measurements were made in 40- μ m-thick sections cut from a brain slice fixed by immersion immediately after slicing procedures and stained for GalNac using VVA-POD as described above. Only GalNac⁺ neurons with an obvious nucleus were selected for measurement ($n = 118$). To aid visualization of nuclei and cortical layers, the sections were counterstained with cresyl violet and measurements were made with an eyepiece micrometer while viewing the section with a 100 $\times$ oil-immersion objective under No-

Figure 1. Electrophysiological differences between spiny and smooth-dendritic neurons *in vitro*. *A* and *B*, Action potentials of spiny stellate and pyramidal neurons had average durations of 1.5–2 msec at half-height, small afterhyperpolarizations, and rapid accommodation to prolonged current pulses. *C–E*, Smooth-dendritic neurons had fast spikes, 0.5 msec at half-amplitude, little or no accommodation to longer current pulses, and lacked a prolonged afterhyperpolarization. Calibration bars in *A–C*, 10 mV, 5 msec; *D* and *E*, 10 mV, 20 msec.

marški optics. The maximum and minimum diameters of 118 VVA-stained cells were measured from 5 different sections and the values for each cell were averaged.

SDS-PAGE and lectin blots. SDS-PAGE and Western blotting were used to identify the GalNac containing molecules responsible for cell surface staining of GABAergic neurons. Tissue fractions were prepared from cat visual cortex and various membrane or cytosolic fractions were subjected to electrophoresis and transferred electrophoretically as described previously in Arimatsu et al. (1987). Approximately 500 μ g of protein was loaded per lane. In pilot experiments it was determined that this was the minimum amount of protein required for detection of the major GalNac-containing bands, following 2 hr of electrophoretic transfer onto nitrocellulose (7 mA, Hoeffer TE Transphor Unit). Subsequent to transfer, filters were washed in blocking buffer (10 mM Tris HCl, pH 8.2, 0.05% Tween-20, 0.5 M NaCl, 0.2% sodium azide) and incubated overnight with VVA lectin conjugated to HRP (10 μ g/ml) diluted in TBS (10 mM Tris HCl, pH 8.2, 0.05 M NaCl), washed twice in TBS, rinsed briefly in distilled water and reacted with 0.05% 4-chloro-1-naphthol (BioRad) and 0.01% H_2O_2 in 40% ethanol. The relative mobility of reactive bands (M_r) was calculated by comparison with the mobilities of molecular weight standards (Bio-Rad), using the log molecular weights. The M_r 's of VVA-reactive polypeptides were averaged from 3 separate experiments.

Results

Our double-labeling experiments focused on identifying the axonal and dendritic morphologies of GalNac⁺ cells within layer 4. We restricted our analysis to layer 4 for 2 reasons. First, it allowed us to obtain a larger sample size of morphological types

and, second, because, as we describe below, surface GalNac was more stable or present at higher levels on layer 4 cells.

Electrophysiological identification of smooth stellate cells in vitro

Impaled neurons exhibited 2 distinct forms of action potentials (Fig. 1, *A*, *C*). The most common form had a duration of 1–2 msec at one-half amplitude, small afterhyperpolarization, and accommodated rapidly in response to longer current pulses (Fig. 1*B*). A rarer type had a fast spike with a duration of 0.5 msec at one-half amplitude and little or no accommodation to longer current pulses (Fig. 1, *C–E*). In initial experiments, neurons exhibiting both forms of action potentials were stained intracellularly with Lucifer yellow and the morphologies were compared.

These morphological comparisons indicated that it was possible to identify unambiguously the smooth dendritic local circuit neurons by the shorter time course of their action potentials. These findings are in agreement with a previous study in guinea pig sensorimotor and anterior cingulate cortex in which GAD immunoreactive, smooth dendritic neurons exhibited similar properties (McCormick et al., 1985). Less than 5% of the intracellularly stained neurons were smooth dendritic types when fills were made randomly; however, our later experiments yielded nearly 80% smooth dendritic cells when only cells with fast spikes were injected.

The final sample of 156 Lucifer yellow-stained cells included 45 nonpyramidal neurons with smooth dendrites and 111 other cells including spiny stellates, spiny pyramidal neurons, or astrocytes. We further categorized the smooth dendritic neurons on the basis of dendritic form, preponderance of dendritic spines, and laminar patterns of axonal arborization (Peters and Regidor, 1981; Fairen et al., 1984). Our sample of smooth or sparsely spiny types included basket, chandelier, neurogliaform, and large bitufted neurons.

Double-labeled neurons visualized with Lucifer yellow and VVA-Texas red fluorescence

Neurons double-labeled with Lucifer yellow and VVA-Texas red were identified by viewing sections alternately under FITC and Texas red filters. Typically 3–5 Lucifer yellow-stained neurons were present in each slice. These neurons were subsequently viewed with Texas red filters to determine whether they were lectin positive. An example of a double-labeled neuron is shown in Figure 2, *A*, *B*. Lucifer yellow staining revealed the cell body, dendrites, and axons (Fig. 2, *A*, *C*, *D*). When this same neuron was viewed with Texas red excitation wavelengths, VVA reactive puncta clearly surrounded the cell body and extended onto the proximal dendrites (Fig. 2*B*). Of the 45 intracellularly stained neurons with smooth dendrites, 19 had distinct pericellular VVA-Texas red staining.

Double-labeled neurons had 1 of 2 distinct dendritic forms. Twelve of the 19 double-labeled neurons were multipolar, with long, aspinous, varicose dendrites (Fig. 2*C*). The axonal features of these cells corresponded to subtypes of basket cells as described in greater detail below. The second type of VVA-reactive neurons (3/19) had medium-sized somata and short, recurving dendrites (Fig. 2*D*). These resembled one of several types of neurogliaform cells described in previous studies in cats in which they have also been termed “smooth spherical multipolar neurons” (Peters and Regidor, 1981) or “multipolar neurons of layers 2–5 with neurogliaform axonal plexus” (Meyer, 1983). A

Figure 2. Basket and neurogliaform cell types exhibiting surface GalNac. *A*, High-magnification view of Lucifer yellow-injected basket cell in layer 4, viewed with FITC filter set. Note the pronounced dendritic varicosities. Cell body diameter was 19 μm . The axon initial segment emerged from an apical dendrite out of plane of focus. *B*, Same neuron viewed with Texas red filters to visualize VVA-Texas red staining of surface GalNac. *C*, Lower-magnification view of a GalNac⁺ basket cell showing multipolar morphology, varicose dendrites, and portions of the pericellular axonal terminations, characteristic of basket cells. *D*, GalNac⁺ neurogliaform cell which had a superficial tuft of recurving dendrites and a prominent basal dendrite giving rise to multiple fine recurving axonal branchlets, heavily beaded with small *en passant* swellings. A serial section reconstruction of this neurogliaform cell is shown in Figure 7. The cell bodies in *A*, *B*, and *D* photographed with 63 $\times$ Plan Neofluar objective; *C*, with 25 $\times$ Plan Neofluar objective.

similar cell type in primate visual cortex has also been termed "type 5 cells" (Jones, 1975), "neurogliaform cells with dense dendritic arborisation" (Szentagothai, 1973), and "spine-free stellate neurons type d" (Lund et al., 1981) based on the axonal appearance in Golgi-impregnated material.

In some cases (4/19) the axons of double-labeled cells were

too weakly stained to allow classification. However, their dendritic shapes were morphologically similar to either basket or neurogliaform neurons. These findings indicate that VVA-reactive glycoconjugates are restricted to 2 specific types of local circuit neurons in layer 4, the basket and neurogliaform cells.

All of the double-labeled neurons had medium or large cell

Figure 3. Frequency histogram showing the diameters of GalNac⁺ neurons in layer 4 of area 17. Measurements were made using a 100 $\times$ oil-immersion objective and Nomarski optics. One hundred and eighteen neurons were measured from a series of five 40- μ m-thick sections stained with VVA-POD. Standard deviations for each group are shown as error bars. The mean cell body diameter was 22 μ m.

bodies that ranged in diameter from 14 to 29 μ m. To determine whether our sample was representative of all GalNac⁺ neurons in layer 4, we measured the somatic diameters of layer 4 GalNac⁺ neurons. These data are shown in Figure 3. The soma diameters of 118 GalNac⁺ neurons in layer 4 ranged from 14 to 32 μ m, with an average diameter of 21 μ m. Thus, the sizes of the double-labeled neurons were similar to the overall population; however, we cannot exclude the possibility that some very large GalNac⁺ neurons were not intracellularly stained. Previous studies have shown that basket cells range in cell body sizes, with some basket cell soma sizes comparable to the large pyramidal cells of layers 5 and 6. Thus, the large GalNac⁺ neurons in our material may correspond to these large basket cells.

The double-labeled cells were categorized under the broad classification scheme in which basket cells were identified on the basis of characteristic pericellular or *en passant* synaptic endings which looped around other cell bodies in the same layer (Fig. 4, *A, B*; Somogyi et al., 1983; Kisvarday et al., 1985). In many instances, the endings looped around other GalNac⁺ neurons. An example of this is given in Figure 4*A*, which shows a double-exposure of a VVA-stained neuron receiving pericellular contacts from a Lucifer yellow-injected axon.

Not all basket cells intracellularly stained exhibited surface GalNac. In fact, 10 out of 26 GalNac⁻ interneurons had axonal arbors that clearly identified them as basket cells. However, as the analysis below indicates, GalNac⁺ basket cells had distinc-

Figure 4. Basket-like pericellular endings formed by dye-injected neurons on other GalNac⁺ (*A*) or GalNac⁻ somata (*B*) in layer 4.

Figure 5. Serial section, 2-dimensional reconstructions of 2 layer 4ab GalNac⁺ basket cells shown in the coronal plane. In these reconstructions, the cell bodies and dendrites are separated from the axonal arbors for clarity. *A*, Cell body diameter was 20 μm; *B*, cell body diameter was 18 μm. Both basket cells had recurrent axons originating from the pial side of the cell body (arrows). Each axon formed a dense columnar plexus throughout layers 2 + 3, 4ab, 5, and 6. The terminal plexi in each layer were in register with the main plexus in the upper half of layer 4ab, forming a columnar arbor. Descending branches were unbranched and myelinated through layer 4c but then formed multiple collateral branches in the infragranular layers. Shaded areas indicate other GalNac⁺ layer 4 neurons which were contacted by the axons in layer 4. *Inset*, Low-power drawing of the sections containing the cell bodies. The position of both the cells on the medial bank of area 17 is indicated by the asterisk on the low-power reconstruction.

Figure 6. Serial section reconstructions of 2 large GalNac⁺ basket cells in layer 4ab. *A*, GalNac⁺ basket cell in layer 4ab with columnar arbor. The cell body had an average diameter of 29 µm and varicose multipolar dendrites which formed few secondary branchlets. The initial axon segment formed from an apical dendrite (*arrow*) and gave rise to descending and ascending portions of a highly asymmetrical arbor. Extensive portions of the arbor terminated in layer 4ab, as well as supra- and infragranular layers. The projection to layer 4c was sparse. *B*, Reconstruction of a basket cell with a large arbor in layer 4ab. The average soma diameter was 28 µm. This cell was located at the apex of the lateral gyrus. The axon formed only sparse projections to layers 2+3, 5, and 6. Within layer 4ab, the arbor extended 650 µm in the coronal plane.

4AB

Figure 7. Serial section reconstruction of a GalNac⁺ neurogliaform cell in layer 4c. The soma diameter was 13 μ m on average. The cell's dendrites and axon were highly intertwined; however, the axon extended further into layers 5 and 6 and into sublaminae 4ab.

tive laminar patterns of axonal projections that differed markedly from the axonal arbors of GalNac⁻ cells.

Axonal morphologies of GalNac⁺ basket cells

The axonal arbors of all 12 GalNac⁺ basket cells shared a number of distinctive features. When viewed in the coronal plane, the axons of all cells formed a 400–700- μ m-diameter axonal arbor with distinct, although not identical patterns of laminar arborizations. Figures 5 and 6 show 4 GalNac⁺ basket neurons, located in layer 4ab, with morphologically similar axonal arbors. The neurons differed in the amount of axonal arbor in layers 3

and 4ab and shared in common the lack of an arbor in layer 4c. In addition, 3 of the 4 neurons formed sizable projections to layers 5 and 6. For example, the layer 4ab basket cell shown in Figure 5A had an ascending axon which gave rise to a dense plexus of collaterals within layer 4ab. Some collaterals traveled horizontally within this layer or layer 3 for 100–200 μ m. Other collaterals descended through layer 4c and subsequently arborized in layers 5 and 6. Within layer 4c, this neuron did not branch, nor did it form pericellular endings or *en passant* varicosities.

A second layer 4ab GalNac⁺ basket cell shown in Figure 5B

had a very similar pattern of axon collaterals. In both cases, these GalNac⁺ basket cells formed pericellular endings around 6–10 other GalNac⁺ cell bodies within layer 4ab and a smaller number of GalNac⁺ neurons within layer 6.

In most cases, the GalNac⁺ basket cells had axonal arbors extending over an area of <500 μm in the coronal plane, but on occasion a cell would extend its axon over a larger area, as seen in the 2 basket cells shown in Figure 6. The axon collaterals of these cells traveled tangentially over 700 μm within layer 4, considerably further than the arbors of the previous 2 basket cells. The arbor of the basket cell shown in Figure 6A was asymmetrically arranged, with most branches displaced to one side of the cell body in layer 4ab. This neuron, unlike the previous 2 cells shown, formed a few sparse collaterals within layer 4c. Finally, the GalNac⁺ basket cell shown in Figure 6B, formed a large tangential projection in layer 4ab but lacked extensive projections to other layers, in contrast to the 3 previous examples.

Within the population of GalNac⁺ basket cells, we did not observe any features of the dendritic arbors that correlated with these variants of the axonal arbors. While the dendrites of some cells were restricted to layer 4, others reached into the supragranular layers. The 2 neurons with dendrites extending the most superficially into supragranular layers also had the most markedly different axonal arbors (compare Figs. 5B and 6B) and the 2 types with the most similar axonal arbors (Fig. 5, A, B) formed dendritic arbors whose laminar patterns differed.

GalNac⁺ neurogliaform neurons

A second distinct type of GalNac⁺ cell in layer 4 corresponded to the neurogliaform cell. A reconstruction of the complete dendritic and axonal morphologies of one of these cells is shown in Figure 7. As this reconstruction indicates, the axon of this cell type is strikingly different from the GalNac⁺ basket cell axons shown above; however, both types formed descending interlaminar connections between layer 4 and layers 5 and 6. This neurogliaform cell had strong surface VVA reactivity, a soma diameter of about 15 μm , and irregular dendrites which radiated from one pole of the soma and branched close to the cell body. The branches were recurrent and intertwined with the axon. A single prominent process emerged from the cell body and divided into a fine axonal plexus (arrow).

GalNac⁻ basket neurons

Among the 45 intracellularly stained local circuit neurons, 26 did not show any detectable VVA labeling. Although these types were morphologically different from the GalNac⁺ cells, we cannot be certain whether they would exhibit detectable levels of GalNac under different experimental conditions. These 26 GalNac⁻ neurons were representatives of several well-described types of interneurons, including small basket cells (3), chandelier cells (2), bitufted cells (2), small neurogliaform cells (14), and a variety of multipolar cells that were not conclusively identified (5). The dendritic morphologies of 3 GalNac⁻ types are shown in Figure 8.

Since the principal goal of these investigations was to determine whether a specific cell surface molecule could be correlated with specific cell types, we were especially interested in comparing the laminar pattern of arborization of GalNac⁻ and GalNac⁺ basket cells. As we show below, the patterns of interlaminar connections are distinctly different in populations lacking surface GalNac.

These GalNac⁻ basket cells shared several morphological features which were not observed in GalNac⁺ cells. The most distinctive feature was the extent of axonal arborization within layer 4c. The neurons shown in Figure 9 (these are the same cells shown in Fig. 8, A, B) both formed a dense plexus in layer 4c. The soma and dendrites of the basket cell shown in Figure 9A were completely restricted to layer 4ab. This laminar position was similar to the GalNac⁺ cells shown in Figures 5 and 6. In contrast to the previous basket cells shown, this neuron formed a 2-tiered arbor that was equally dense within layer 4ab and 4c. In addition, few collaterals were observed in either supra- or infragranular layers; these were layers receiving considerable input from the GalNac⁺ basket cells. The pattern of interlaminar connections was similar for the basket cell shown in Figure 9B. This neuron also formed a narrow, columnar arbor throughout 4ab and 4c, with additional collaterals extending into either layers 2+3 or 5 and 6.

Within layer 4ab, the axons of both GalNac⁻ and GalNac⁺ neurons appeared to form looping pericellular contacts around other GalNac⁺ neurons with about the same frequency. Within layer 4c, however, the GalNac⁻ basket cells contacted GalNac⁺ somata, a feature not observed in the intracellularly stained GalNac⁺ cells.

One GalNac⁻ basket cell had projections to layer 2+3 and 4ab which were similar to the other GalNac⁺ basket cells (Fig. 10). Unlike most GalNac⁺ cells, however, this neuron had no projections to layers 5 and 6 and formed relatively sparse collaterals within layer 4c. Several other features of this axonal arbor were also distinct from the GalNac⁺ cells. Most notably, the initial axon segment emerged from the basal pole of the soma, rather than the apical pole. Furthermore, the axon made a distinctive U-turn at the lower boundary of layer 4c, then returned to layer 4ab, where it branched extensively to form a dense arbor. The branches of this axonal arbor were densely covered by *en passant* swellings which looped around other cell bodies.

The dendritic features of the GalNac⁻ basket cells had little in common with each other. For example, the cell in Figure 9A had a sparse covering of spines, while the cell in Figure 10 had the completely smooth dendrites characteristic of GalNac⁺ basket neurons.

Other GalNac⁻ neurons

Among the other 26 GalNac⁻ neurons were representatives of a well-defined type of cortical local circuit neuron, the chandelier cells. An example of an intracellularly injected chandelier cell is presented in Figure 8, C, D. The axon formed distinct, vertically ascending synaptic cartridges, characteristic of this cell type. The postsynaptic targets of this cell type have been identified as the initial axon segments of pyramidal cells (Somogyi et al., 1982). As described above, we believe that the postsynaptic targets of the GalNac⁺ cells include other GalNac⁺ neurogliaform and basket cells. Thus, there is a marked difference in the postsynaptic targets of the GalNac⁺ basket cells and the GalNac⁻ chandelier cells.

Two GalNac⁻ neurons were bitufted cell types with large vertically arranged dendrites extending through all cortical layers, except layer 1. One of these is shown in Figure 11. Most of the dendrites remained unbranched, except near the distal tips. Each cell formed a radially oriented axon tightly confined to a narrow column coextensive with the apical and basal dendrites. These

Figure 8. Immunofluorescence photomicrographs of 3 types of GalNac⁻ local circuit neurons. *A* and *B*, Types of small basket cells with sparsely spiny (*A*) or smooth dendrites (*B*). These 2 cells are shown fully reconstructed in Figure 9. *C* and *D*, Photomicrographs of 2 serial sections through a GalNac⁻ chandelier cell. In *C*, the cell body and portions of the axon are visible; in *D*, short portions of the distal dendrites are shown, which were not contained within the previous section (shown in *C*). *D* also shows the "vertical synaptic cartridges," typical of chandelier cell axons (arrows).

narrow arbors measured less than 100 μm in diameter, in contrast with the 500- to 700- μm -wide arbors of GalNac⁺ basket cells. The bitufted neuron shown in Figure 11 formed an axon from the apical pole of the soma which was located at the layer 4c/5 border. The axon ascended through layer 4c before forming fine recurrent collateral branches. Some of these descended through layer 5 before becoming too faint to detect. Additional fine branches ascended toward the pia in a narrow bunch. Some of the branches formed a looping series of *en passant* varicos-

ities. The second GalNac⁻ bitufted cell was reconstructed partially (not shown). This neuron also formed a narrow cylindrical axon arbor with looping pericellular axonal endings, which surrounded some GalNac⁺ neurons. Although more complete filling of the axonal arbors of these cell types is necessary for exact identification, these neurons strongly resembled some of the large CCK-immunoreactive bitufted cells of layer 2+3 with basket-like endings (Somogyi and Cowey, 1984; Meyer and Wahle, 1988).

A

Figure 9. Serial-section reconstructions of GalNac⁺ local basket cells in layer 4. Each formed a focused arbor in layer 4. One type was a basket cell in layer 4ab, with sparsely spiny, bitufted dendrites. The axon formed from an ascending dendrite (arrow) and issued recurving collateral branches which terminated in distinct horizontal tiers spaced approximately 200 μm apart. The most superficial tier formed at the border between layers 2+3 and 4ab. A second tier of horizontally oriented processes formed in the middle of layer 4ab. The third and fourth tiers formed at the border of layer 4ab and within layer 4c. Between these distinct horizontal plexuses, the branches were oriented radially. **B**, Smooth-dendritic, bitufted basket cell located in layer 4c which formed a narrow, arcade-type axonal plexus through layers 4 and 2+3. One weakly filled descending axonal branch was followed to the white matter. The cell body diameters were 18 μm (**A**) and 16 μm (**B**).

B

Figure 10. Serial-section reconstruction of a "modified" GalNac⁻ basket cell in layer 4ab. The axonal branches were restricted to layers 2+3 and 4ab and lacked a descending component to layers 5 and 6. The fan-shaped arbor had a diameter of 500 μm within layer 4ab. Unlike the other basket cells, the axon formed at the basal pole of the soma and descended to the layer 4c border before forming a U-turn and returning to the vicinity of the cell body. Within layers 4ab and 2+3, the axon formed numerous asymmetrical collateral branches, covered with *en passant* swellings. The cell body diameter was 13 μm.

Effects of slice incubation on the reactivity of surface GalNac glycoconjugates

In interpreting our results, we were aware that the absence of staining of certain cells could result from loss of lectin staining as a result of the slicing procedures and *in vitro* incubations. We assessed this possibility by comparing the distribution and intensity of VVA staining obtained in perfusion fixed material from an intact animal to that obtained from slices immersion-fixed after various incubation times.

In the first condition, free-floating microtome sections through area 17 were stained with VVA-POD, following normal perfusion-fixation (Fig. 12). In agreement with previous studies in the human, monkey, and mouse, VVA lectin binding is strongest along the surfaces of multipolar and large bitufted neurons in cortical layers 2–6 (Nakagawa et al., 1986a; Mulligan et al., 1989). In highly magnified views, VVA formed a fenestrated pattern covering perikarya and some proximal dendrites (Fig.

12, B, C). The most intensely stained neurons were multipolar types within layer 4ab. In addition to the neuronal staining, diffuse VVA reactivity was associated with the neuropil in layers 3 and 4 (Fig. 12D).

In a second series of experiments, GalNac⁺ neurons were compared in sections prepared from brain slices maintained *in vitro* for 1–10 hr before immersion-fixation. The extent of cellular staining given by VVA-POD in slices with short *in vitro* incubations was similar to the staining observed after aldehyde perfusion. With longer *in vitro* incubations, pericellular staining was well preserved; however, there were reductions in the extent of dendritic staining and in the number of reactive neurons outside of layer 4. We therefore compared directly sections of cat visual cortex from brain slices maintained *in vitro* for periods of 1–10 hr prior to fixation. Figure 13A shows GalNac⁺ neurons plotted from a section of area 17 taken from a slice maintained *in vitro* for 1 hr. After 10 hr *in vitro*, the number of GalNac⁺ neurons was somewhat diminished (Fig. 13B). To obtain an

Figure 11. A large smooth-dendritic bitufted neuron which was GalNac⁺. The cell body was located in layer 5, near the border with layer 4c. This neuron had long, unbranched and smooth dendrites which extended through cortical layers 2+3–6 in a narrow column. The axon was not filled completely enough to determine whether this was a double-bouquet type or another type of bitufted cell. The ascending axon (arrow) was extremely fine and formed short side collaterals at 90° angles with the main ascending axon. These collateral branches formed symmetrical secondary branches which were radially oriented. The descending portions could not be followed past the layer 5/6 border. The cell body diameter was 11 μm on average.

estimate of this effect we counted the number of neurons stained by VVA after short- and long-term incubations. This comparison reveals a 35% decrease in the number of VVA reactive neurons between 1 and 10 hr in culture (358 vs. 232). This loss occurs predominantly outside of layer 4 since no significant reductions were found within layer 4 (145 vs. 144).

Biochemical analysis of VVA-binding glycoproteins

Initial studies of lectin binding in blots revealed a prominent doublet of GalNac⁺ glycoproteins present in crude homogenates of cerebral cortex (Fig. 14). Based on calculated relative mobilities from 8 separate experiments, this doublet had an average M_r 77,000 and M_r 70,000 (± 3000). A number of additional,

faintly stained bands were also present in crude homogenates. These included bands of M_r 95,000, M_r 45,000, and M_r 26,000, as well as a band which traveled at the dye front (Fig. 14, arrows). Further subcellular fractionation revealed a selective enrichment of the M_r 45,000 and dye-front bands in membrane fraction P3, and the M_r 76,000 and M_r 72,000 bands in the P2' fractions but not in the cytosolic fraction. These observations indicate that GalNac⁺ glycoproteins from cerebral cortex are heterogeneous in size and are probably membrane associated. In order to resolve whether some or all of these GalNac-reactive bands correspond to the lectin-binding molecules outlining neuronal populations requires further studies with antibody probes specific for each of the separate GalNac-reactive bands.

Figure 12. Fluorescence and transmitted-light photomicrographs of VVA staining in cat area 17. *A*, Examples of layer 4 basket cells exhibiting strong VVA-Texas red surface reactivity. *B* and *C*, Successively higher-power views of meshwork pattern of surface staining. *A–C*, Cryostat sections, 12 μ m thick. *D*, Low-power Nomarski optics photomontage showing laminar positions of GalNac⁺ neurons stained with VVA-POD in a 40- μ m-thick Vibratome section cut from area 17 in the coronal plane. Perfusion fixation.

Figure 13. Plots of GalNac⁺ neurons in cat area 17 following 1 hr (A) and 10 hr (B) of *in vitro* incubation before fixation by immersion. Plots made from immersion-fixed, 40- μ m-thick sections cut on a freezing microtome and stained with VVA-peroxidase.

Discussion

In some brain areas such as the cerebellum and hippocampus, there are a relatively restricted number of cell types with stereotyped synaptic relationships. In the neocortex, however, there appears to be a tremendous diversity of cell types and a range of possible synaptic interactions. The criteria that define a functionally related class of cortical cells remain elusive. In this study, we attempted to uncover whether the presence of specific cell surface molecules, those containing GalNac, identified a specific cell type, or subset of cell types. By combining cytochemistry with intracellular staining, we were able to correlate the presence of GalNac-containing surface molecules with 2 distinct cortical cell types: the basket cells with columnar axons and the neurogliaform cells. We further observed that other specific neuronal cell types, including chandelier and large bitufted neurons, lacked detectable levels of these molecules.

Our findings also show that differences in the interlaminar projections of layer 4 basket cells correlate with surface GalNac staining. Types of basket cells consistently showing undetectable levels of surface GalNac tended to have smaller arbors confined to both sublamina of layer 4. The GalNac⁺ basket cell types had extensive projections to supra- and infragranular layers but lacked a sizable projection to layer 4c. Several lines of evidence indicate that it is unlikely that technical artifacts alone can account for these findings. First, quantitative and qualitative comparisons showed that the numbers of neurons with pericellular GalNac

Figure 14. Lectin blot showing GalNac-containing polypeptides enriched in various subcellular fractions of cat cortical tissue. Each aliquot of a different cortical subcellular fraction contained 500 μ g protein. Fractions were electrophoresed, blotted, and stained with VVA-peroxidase, as described in the text. Based on mobilities calculated from 3 separate experiments, crude homogenates of cortex contained multiple bands with M_r 93,000, M_r 83,000, M_r 77,000, M_r 70,000, M_r 72,000, and an additional smaller weight band which traveled at the dye front. After further fractionation, bands corresponding to M_r 40,000 and M_r 33,000 were enriched in the P3 fraction. The higher-molecular-weight bands were selectively enriched in the washed membrane pellet (P2). No staining was detected in a lane containing the cytosol fraction (C). (Note that the same amount of protein was loaded in all lanes.) The supernatant of synaptosomal lysate (LS1) contained weak immuno-reactivity for some of the higher-molecular-weight bands. Reference proteins indicated on the right are myosin (M_r 200,000), β -galactosidase (M_r 116,000), phosphorylase B (M_r 97,000), BSA (M_r 66,000) and ovalbumin (M_r 42,669). H, crude homogenate; P3, crude membrane pellet.

were not reduced in layer 4 as a result of slicing and incubating the living tissue (see Fig. 13). Thus, it would be unlikely that one set of layer 4 basket cells (those with small layer 4 arbors) would lose surface GalNac selectively. Second, other types of local circuit neurons were consistently GalNac⁺ besides the basket neurons with small arbors restricted to layer 4. These included chandelier, large bitufted, and small neurogliaform cells. If random degradation of GalNac were occurring, we would expect similar numbers from each morphological category to be GalNac⁺ and GalNac⁻. The observed differences are more likely due to molecular and functional distinctions present *in vivo*.

We found that the addition of the sugars GalNac and GlcNac to the slice incubation media ameliorated the loss of VVA staining during *in vitro* incubations. Even with this modification, a comparison of lectin reactivity in perfusion-fixed material and in slices maintained for prolonged periods *in vitro* indicated that a diminution of lectin binding sites occurred outside of layer 4 during the *in vitro* incubations. The comparison of surface GalNac

in slices after short or longer *in vitro* incubations indicated that this effect was minimal within layer 4. The reductions in GalNac in other layers may reflect a higher turnover of membrane glycoconjugates which are normally replenished *in vivo*. Because adding exogenous GalNac to the slice incubation media partially prevented this loss, endogenous glycosidases might be responsible for the decrease in staining. Although we have not investigated alternative methods for retaining GalNac *in vitro*, addition of glycosidase inhibitors might prevent this loss altogether.

All the neurons which formed looping pericellular contacts were broadly classified as various types of basket cells (Somogyi et al., 1983). Some of these cell types correspond morphologically to some cell types described in the literature. However, we found greater morphological variability than was reported previously. The GalNac⁺ basket cells in Figures 5 and 6 resemble the "clutch" cells described previously by Kisvarday et al. (1985). In contrast to the published examples of these cells, we found stronger projections to layers 2+3, 5, and 6 (Fig. 5). The GalNac⁺ basket cell shown in Figure 6B resembles the large basket cells with clustered projections described previously by Gilbert and Wiesel (1979; Fig. 2B) and Martin et al. (1983; Fig. 1), except that it has a shorter horizontal spread of the axon. This difference is probably a technical artifact, due to the inevitable truncation of some long axons which may be cut off at the surface of the brain slice. Three other GalNac⁻ types, also with looping pericellular axons, probably represent additional morphological varieties of basket cells (Fig. 9). One (Fig. 9A) differed from the clutch cells and the large basket cells in that the axon terminated nearly exclusively within layer 4, primarily on nonpyramidal cell somata. This neuron and a second GalNac⁻ basket cell (Fig. 9B) may correspond to the bitufted neurons with "arcade" axons, described in Golgi-impregnated material (see figure 15 in Meyer, 1983). Alternatively, they may each belong to separate morphological categories, although a larger number of examples of each type would be required to determine this.

Several of our stained cells corresponded closely to previous descriptions of neurogliaform cells. These are probably the first intracellularly stained examples of such cells. Although we did not analyze them in detail electrophysiologically, their spike shape was indistinguishable from that of other smooth stellate cells in layer 4. These results, in combination with the previous demonstration that nearly all GalNac⁺ cortical cells are GABA immunoreactive (Nakagawa et al., 1986b), indicate that neurogliaform cells are probably GABAergic. In contrast to the large tangential spread of GalNac⁺ basket cell axons in layer 4, the neurogliaform cells in this layer form very local projections, which can include portions of layers 5 and 6. The short-range axon, together with the extremely dense axonal plexus, suggests that the neurogliaform cells exert a strong, local inhibitory effect.

We did not observe any clear terminations of the axons from these cells onto other layer 4 GalNac⁺ somata, although the intricate nature of the arborization would make visualization of such contacts difficult. Previous ultrastructural studies, however, indicate that the postsynaptic targets of neurogliaform cells are spines and small dendritic shafts (LeVay, 1973; Somogyi, 1986). Thus, the GalNac⁺ layer 4 neurogliaform cells that we have described could form inhibitory connections onto layer 4 spiny stellates, as well as onto pyramidal neurons in layers 5 and 6.

The connection between the expression of the GalNac⁺ cell surface molecules and functional properties are not yet known, although it should now be possible to begin to dissect out the

functions of the layer 4 basket cells and neurogliaform cells from other local circuit neurons by using VVA in combination with electrophysiology. Differences in the postsynaptic targets of GalNac⁻ and GalNac⁺ cell types suggests that the expression of the GalNac-containing molecules may be related to specific functional differences. The chandelier cells were the most distinctive GABAergic cell type which did not express GalNac. The synaptic projections of chandelier cells are highly specific for the initial segments of pyramidal cell axons (Somogyi, 1979; Fairen and Valverde, 1980; Somogyi et al., 1982). Thus, the chandelier cells' highly specific projections would allow them to control the output of groups of nearby pyramidal cells. The postsynaptic targets of the chandelier cells are very different from the basket or neurogliaform cells, which terminate on cell bodies, dendritic shafts, or spines of both pyramidal and nonpyramidal cortical neurons (Somogyi, 1986).

Several roles have been hypothesized for the basket and neurogliaform cells. Both have been implicated in sharpening receptive field properties, although the basket cells are thought to act over longer distances (Jones, 1984; Koch, 1987; Hata et al., 1988). Inhibition between basket cells may enhance orientation selectivity; in a recent cross-correlation analysis, it was shown that horizontal inhibitory interactions exist between pairs of visual cortical neurons with somewhat different orientation preferences, but not between neurons with orthogonal orientation preferences (Hata et al., 1988). The cell pairs forming these inhibitory connections were situated less than 1 mm away from each other, in layers 4, 5, and 6. Hata et al. were probably recording from pairs of basket cells similar to those in Figure 6 which extended collaterals 300–700 μ m through layer 4 (see also Somogyi et al., 1983). These results indicate that GalNac⁺ basket cells may be involved in enhancing orientation selectivity. It would be interesting to determine whether the GalNac-containing molecules on the surfaces of basket cells provide a molecular mechanism for stabilizing synaptic connections between pairs of basket cells with slightly different orientations.

The neurogliaform cells are less well known, but they have also been implicated in sharpening receptive field properties. Jones has speculated that the vertical axons of basket cells might control the flow of visual signals from layer 4 to the supra- and infragranular layers within a single orientation column, while the local connections of neurogliaform cells would sharpen the boundaries between columns (Jones, 1984). 2-Deoxyglucose studies in the visual cortex have shown that orientation columns are mapped in a system of parallel bands (Lowel et al., 1987). Variations in the thickness of these bands gives them a beaded appearance, when viewed tangentially in flat mounts of the visual cortex. It is not yet clear how these bands are formed during ontogeny or what leads to this beaded organization. One possibility is that neurogliaform cells may have a nonuniform distribution across the visual cortex and that clusters of this cell type lead to variations in orientation column thicknesses.

It is also possible that the expression of GalNac-containing cell surface molecules is related to a particular extrinsic afferent fiber system. Previous work has shown that separate sets of local circuit neurons in layer 4 are contacted by either the X- or Y-geniculocortical fibers. GABAergic neurons in layer 4 that received contacts from X-type geniculocortical afferents were considerably smaller (average diameter, 15 μ m) than those that received Y-type inputs (average diameter, 24 μ m) (Freund et al., 1985b). Our findings indicate that GalNac is not expressed selectively on neurons that are postsynaptic to only one of these

pathways since GalNac⁺ neurons include both small and large cells. Furthermore, neither the laminar distribution of the GalNac⁺ cells nor their axons would suggest any correspondence with either the X- or the Y-pathways. Geniculocortical fibers subserving the Y-pathway terminate in layer 6 and the upper portion of layer 4, avoiding layer 4c (Freund et al., 1985a; Humphrey et al., 1985). The X-afferents were also found to terminate in layer 6, but in layer 4, they exhibited a more heterogeneous pattern of termination, ending in either layer 4ab or 4c. In contrast, GalNac⁺ cells are located throughout layers 3–6. Finally, although GalNac⁺ basket cells terminated in a pattern overlapping with the Y-afferents, the projections of GalNac⁺ neurogliaform cells did not (see Fig. 7). Taken together, these comparisons indicate that the expression of surface GalNac is probably not related directly to either the X- or the Y-geniculocortical afferents. It seems more likely that the GalNac⁺ neurons in the visual cortex are subserving a physiological role common to both X- and Y-pathways.

Immunocytochemical studies comparing the distribution of surface molecules detected by monoclonal antibodies VC1.1, VC5.1, and Cat-301 or the lectin VVA suggest that GalNac⁺ cells may be part of several functionally or spatially overlapping circuits in area 17. In these experiments, a subset of cortical neurons was stained by monoclonal antibodies VC1.1 or VC5.1 and the lectin VVA. Another subset, however, was positive for only VVA (Naegele et al., 1987, and unpublished observations). Additional double-labeling studies were done in cat area 17 comparing the distributions of neurons stained by monoclonal antibody Cat-301 and VC1.1. The results of these studies indicated 3 partially overlapping neuronal subsets, some cells expressing only the Cat-301 epitope or the VC1.1 epitopes and a third subset which expressed both epitopes (Zaremba et al., 1989b).

Our biochemical findings suggest that the GalNac-containing molecules include a number of distinct membrane-associated polypeptides ranging from small glycoproteins of about 20,000 Da to larger glycoproteins of over 100,000 Da. It is not known what function these molecules serve or whether any correspond to known cell surface molecules. From our results, however, it appears that the GalNac⁺ bands have different molecular weights from those stained on Western blots by monoclonal antibody markers for GABAergic neurons. Monoclonal antibody VC1.1 and a similar antibody, HNK-1, recognize a sulfate-containing glucuronic acid found on the cell-adhesion molecules N-CAM and myelin-associated glycoprotein (MAG) (Noronha et al., 1986; Arimatsu et al., 1987; Schwarting and Yamamoto, 1988; Barnstable and Naegele, 1989b). Recent studies also showed that the VC1.1 carbohydrate is found on several larger molecules with molecular weights of 680–700,000 (Zaremba et al., 1989b). The antibody Cat-301 recognizes a band of 680,000 Da which corresponds to a chondroitin sulfate proteoglycan (Zaremba et al., 1989a). Although detailed comparisons between these molecules and the molecules stained by VVA have not yet been done, these data suggest distinct differences.

It is not yet known whether the heterogeneity in the number of GalNac-reactive bands is due to the selective expression of some of the molecules on the basket cells and others on the neurogliaform cells or whether both cell types express the same molecules. If molecular heterogeneity exists between these cell types, then it should be possible to generate more selective probes which react with one but not the other cell type. More selective

markers would allow the separate distributions of the basket cells or the neurogliaform cells to be visualized.

A general conclusion to be drawn from this study is that the detection of cell surface carbohydrates is a powerful tool for identifying groups of neurons with similar functional and morphological properties. We believe our approach of combining labeling with molecular markers and intracellular staining can be widely applied to study the individual components of complex neural circuits and the assembly of these circuits during ontogeny.

References

- Arimatsu, Y., J. R. Naegele, and C. J. Barnstable (1987) Molecular markers of neuronal subpopulations in layers 4, 5 and 6 of cat primary visual cortex. *J. Neurosci.* 7: 1250–1263.
- Barnstable, C. J., and J. R. Naegele (1989) Biochemical analysis of cortical cell surface molecules recognized by antibodies VC1.1 and VC5.1. *Soc. Neurosci. Abstr.* 15: 892.
- Boltz, J., and C. D. Gilbert (1986) Generation of end-inhibition in the visual cortex via interlaminar connections. *Nature* 320: 362–365.
- Celio, M. R. (1986) Parvalbumin in most γ -aminobutyric acid-containing neurons of the rat cerebral cortex. *Science* 231: 995–997.
- Demeulemeester, H., F. Vandesande, G. A. Orban, C. Brandon, and J. J. Vanderhaeghen (1988) Heterogeneity of GABAergic cells in cat visual cortex. *J. Neurosci.* 8: 988–1000.
- Dowling, J. E. (1987) *The Retina*, Harvard Univ. Press, Boston.
- Eldred, W. D., C. Zucker, H. J. Karten, and S. Yazulla (1983) Comparison of fixation and penetration enhancement techniques for use in ultrastructural immunocytochemistry. *J. Histochem. Cytochem.* 31: 285–292.
- Fairen, A., and F. Valverde (1980) A specialized type of neuron in the visual cortex of cat: A Golgi and electron microscope study of chandelier cells. *J. Comp. Neurol.* 194: 761–779.
- Fairen, A., J. DeFelipe, and J. Regidor (1984) Nonpyramidal neurons: General account. In *Cerebral Cortex. Cellular Components of the Cerebral Cortex*, Vol. 1, A. Peters and E. G. Jones, eds., pp. 201–253. Plenum, New York.
- Ferster, D., and C. Koch (1987) Neuronal connections underlying orientation selectivity in cat visual cortex. *Trends Neurosci.* 10: 487–492.
- Freund, T. F., K. A. C. Martin, and D. Whitteridge (1985a) Innervation of cat visual areas 17 and 18 by physiologically identified X- and Y-type thalamic afferents. I. Arborization patterns and quantitative distribution of postsynaptic elements. *J. Comp. Neurol.* 242: 263–274.
- Freund, T. F., K. A. C. Martin, P. Somogyi, and D. Whitteridge (1985b) Innervation of cat visual areas 17 and 18 by physiologically identified X- and Y-type thalamic afferents. II. Identification of postsynaptic targets by GABA immunocytochemistry and Golgi impregnation. *J. Comp. Neurol.* 242: 275–291.
- Gabbott, P. L. A., and P. Somogyi (1986) Quantitative distribution of GABA-immunoreactive neurons in the visual cortex (area 17) of the cat. *Exp. Brain Res.* 61: 323–331.
- Gilbert, C. D., and T. N. Wiesel (1979) Morphology and intracortical projections of functionally identified neurons in cat visual cortex. *Nature* 280: 120–125.
- Hata, Y., T. Tsumoto, H. Sata, K. Hagihara, and H. Tamura (1988) Inhibition contributes to orientation selectivity in visual cortex of cat. *Nature* 335: 815–817.
- Hendrickson, A. E., S. P. Hunt, and J.-Y. Wu (1981) Immunocytochemical localization of glutamic acid decarboxylase in monkey striate cortex. *Nature* 292: 605–607.
- Hendry, S. H. C., E. G. Jones, J. DeFelipe, D. Schmechel, C. Brandon, and P. C. Emson (1984) Neuropeptide-containing neurons of the cerebral cortex are also GABAergic. *Proc. Natl. Acad. Sci. USA* 81: 6526–6530.
- Hendry, S. H. C., H. D. Schwark, E. G. Jones, and J. Yan (1987) Numbers and proportions of GABA-immunoreactive neurons in different areas of monkey cerebral cortex. *J. Neurosci.* 7: 1503–1519.
- Hendry, S. H. C., E. G. Jones, S. Hockfield, and R. D. G. McKay (1988) Neuronal populations stained with the monoclonal antibody Cat-301

- in the mammalian cerebral cortex and thalamus. *J. Neurosci.* 8: 518–542.
- Hockfield, S., and R. D. G. McKay (1983) A surface antigen expressed by a subset of neurons in the vertebrate central nervous system. *Proc. Natl. Acad. Sci. USA* 80: 5758–5761.
- Hubel, D. H., and T. N. Wiesel (1977) Functional architecture of the macaque monkey visual cortex. Ferrier Lecture. *Proc. R. Soc. London* 198: 1–59.
- Humphrey, A. L., M. Sur, D. J. Uhrlich, and S. M. Sherman (1985) Projection patterns of individual X- and Y-cell axons from the lateral geniculate nucleus to cortical area 17 in the cat. *J. Comp. Neurol.* 233: 159–189.
- Jones, E. G. (1975) Varieties and distribution of non-pyramidal cells in the somatic sensory cortex of the squirrel monkey. *J. Comp. Neurol.* 160: 205–268.
- Jones, E. G. (1984) Neurogliaform or spiderweb cells. In *Cerebral Cortex: Cellular Components of the Cerebral Cortex*, A. Peters and E. G. Jones, eds., pp. 409–418, Plenum, New York.
- Jones, E. G., and S. H. C. Hendry (1986) Co-localization of GABA and neuropeptides in neocortical neurons. *Trends Neurosci.* 9: 71–76.
- Jones, E. G., J. DeFilipe, S. H. C. Hendry, and J. E. Maggio (1988) A study of tachykinin-immunoreactive neurons in monkey cerebral cortex. *J. Neurosci.* 8: 1206–1224.
- Katz, L. C. (1987) Local circuitry of identified projection neurons in cat visual cortex brain slices. *J. Neurosci.* 7: 1223–1249.
- Kisvarday, Z. F., K. A. C. Martin, D. Whitteridge, and P. Somogyi (1985) Synaptic connections of intracellularly-filled clutch cells: A type of small basket cell in the visual cortex of the cat. *J. Comp. Neurol.* 241: 111–137.
- Koch, C. (1987) A network model for cortical orientation selectivity in cat striate cortex. *Invest. Ophthalmol. Vis. Sci.* 28: 126.
- LeVay, S. (1973) Synaptic patterns in the visual cortex of the cat and monkey: Electron microscopy of Golgi preparations. *J. Comp. Neurol.* 150: 53–86.
- Lowel, S., B. Freeman, and W. Singer (1987) Topographic organization of the orientation column system in large flat-mounts of the cat visual cortex: A 2-deoxyglucose study. *J. Comp. Neurol.* 255: 401–415.
- Lund, J. S. (1987) Local circuit neurons of macaque monkey striate cortex: I. Neurons of laminae 4C and 5A. *J. Comp. Neurol.* 257: 60–92.
- Lund, J. S., G. H. Henry, C. L. Macqueen, and A. R. Harvey (1979) Anatomical organization of the primary visual cortex (area 17) of the cat. A comparison with area 17 of the macaque monkey. *J. Comp. Neurol.* 184: 599–618.
- Lund, J. S., A. E. Hendrickson, M. P. Ogren, and E. A. Tobin (1981) Anatomical organization of primate visual cortex area VII. *J. Comp. Neurol.* 202: 19–45.
- Martin, K. A. C. (1984) Neuronal circuits in cat striate cortex. In *Cerebral Cortex: Functional Properties of Cortical Cells*, Vol. 2, E. G. Jones and A. Peters, pp. 241–284, Plenum, New York.
- Martin, K. A. C., P. Somogyi, and D. Whitteridge (1983) Physiological and morphological properties of identified basket cells in the cat's visual cortex. *Exp. Brain Res.* 50: 193–200.
- McCormick, D. A., B. W. Connors, J. W. Lighthall, and D. A. Prince (1985) Comparative electrophysiology of pyramidal and sparsely spiny stellate neurons of the neocortex. *J. Neurophysiol.* 54: 782–806.
- McGuire, B. A., J.-P. Hornung, C. D. Gilbert, and T. N. Wiesel (1984) Patterns of synaptic input to layer 4 of cat striate cortex. *J. Neurosci.* 4: 3021–3033.
- Meinecke, D. L., and A. Peters (1987) GABA immunoreactive neurons in rat visual cortex. *J. Comp. Neurol.* 261: 388–404.
- Meyer, G. (1983) Axonal patterns and topography of short-axon neurons in visual areas 17, 18 and 19 of the cat. *J. Comp. Neurol.* 220: 405–438.
- Meyer, G., and P. Wahle (1988) Early postnatal development of cholecystokinin-immunoreactive structures in the visual cortex of the cat. *J. Comp. Neurol.* 276: 360–386.
- Mulligan, K. A., J. F. M. van Brederode, and A. E. Hendrickson (1989) The lectin *Vicia villosa* labels a distinct subset of GABAergic cells in macaque visual cortex. *Vis. Neurosci.* 2: 63–72.
- Nakagawa, F., B. A. Schulte, and S. S. Spicer (1986a) Selective cytochemical demonstration of glycoconjugate-containing terminal N-acetylgalactosamine on some brain neurons. *J. Comp. Neurol.* 243: 280–290.
- Nakagawa, F., B. A. Schulte, J.-Y. Wu, and S. S. Spicer (1986b) GABAergic neurons of rodent brain correspond partially with those staining for glycoconjugate with terminal N-acetylgalactosamine. *J. Neurocytol.* 15: 389–396.
- Naegele, J. R., and C. J. Barnstable (1989) Molecular determinants of GABAergic local circuit neurons in the visual cortex. *Trends Neurosci.* 12: 28–34.
- Naegele, J. R., L. C. Katz, and C. J. Barnstable (1987) Morphologically distinct subsets of GABAergic neurons in cat area 17 have unique patterns of glycosylated molecules on their surfaces. *Soc. Neurosci. Abstr.* 13: 359.
- Naegele, J. R., Y. Arimatsu, P. Schwartz, and C. J. Barnstable (1988) Selective staining of a subset of GABAergic neurons in cat visual cortex by monoclonal antibody VC1.1. *J. Neurosci.* 8: 79–89.
- Noronha, A. B., A. Ilyas, H. Antonicek, M. Schachner, and R. H. Quarles (1986) Molecular specificity of L2 monoclonal antibodies that bind to carbohydrate determinants of neural cell adhesion molecules and their resemblance to other monoclonal antibodies recognizing the myelin-associated glycoprotein. *Brain Res.* 385: 237–244.
- Otsuka, R., and H. Hassler (1962) Über Aufbau und Gliederung der corticalen Schizophrenie bei der Katze. *Arch. Psych. Neuvenkr.* 203: 212–234.
- Peters, A., and J. Regidor (1981) A re-assessment of the forms of nonpyramidal neurons in area 17 of cat visual cortex. *J. Comp. Neurol.* 203: 685–716.
- Schmechel, D. G., B. G. Vickrey, D. Fitzpatrick, and R. P. Elde (1984) GABAergic neurons of mammalian cerebral cortex: Widespread subclass defined by somatostatin content. *Neurosci. Lett.* 47: 227–232.
- Schmued, L. C., L. W. Swanson, and P. E. Sawchenko (1982) Some fluorescent counterstains for neuroanatomical studies. *J. Histochem. Cytochem.* 302: 123–128.
- Schwartz, G. A., and M. Yamamoto (1988) Expression of glycoconjugates during development of the vertebrate nervous system. *BioEssays* 9: 19–23.
- Sillito, A. M. (1977) Inhibitory processes underlying the directional specificity of simple, complex and hypercomplex cells in the cat's visual cortex. *J. Physiol. (Lond.)* 271: 689–720.
- Somogyi, P. (1979) An interneuron making synapses specifically on the axon initial segment (AIS) of pyramidal cells in the cerebral cortex of the cat. *J. Physiol. (Lond.)* 296: 18–19P.
- Somogyi, P. (1986) Seven distinct types of GABA-immunoreactive neuron in the visual cortex of cat. *Soc. Neurosci. Abstr.* 12: 583.
- Somogyi, P., and A. Cowey (1984) Double bouquet cells. In *The Cerebral Cortex: Cellular Components of the Cerebral Cortex*, Vol. 1, A. Peters and E. G. Jones, eds., pp. 337–360, Plenum, New York.
- Somogyi, P., T. F. Freund, and A. Cowey (1982) The axo-axonic interneuron in the cerebral cortex of the rat, cat and monkey. *Neuroscience* 7: 2577–2607.
- Somogyi, P., Z. F. Kisvarday, K. A. Martin, and D. Whitteridge (1983) Synaptic connections of morphologically identified and physiologically characterized large basket cells in the striate cortex of cat. *Neuroscience* 10: 261–294.
- Somogyi, P., A. J. Hodgson, A. D. Smith, M. G. Nunzi, A. Gorio, and J.-Y. Wu (1984) Different populations of GABAergic neurons in the visual cortex and hippocampus of cat contain somatostatin- or cholecystokinin-immunoreactive material. *J. Neurosci.* 4: 2590–2603.
- Szentagothai, J. (1973) Synaptology of the visual cortex. In *Handbook of Sensory Physiology. VII/3 Central Processing of Visual Information*. R. Jung, ed., pp. 269–324, Springer-Verlag, New York.
- Wassle, H., B. B. Boycott, and R. B. Illing (1981) Morphology and mosaic of on- and off-beta cells in cat retina and some functional considerations. *Proc. R. Soc. London [Biol.]* 212: 177–195.
- Zaremba, S., A. Guimares, R. G. Kalb, and S. Hockfield (1989a) Characterization of an activity-dependent, neuronal surface proteoglycan identified with monoclonal antibody Cat-301. *Neuron* 2: 1207–1219.
- Zaremba, S., J. Naegele, D. Geschwind, C. Barnstable, and S. Hockfield (1989b) Biochemical microheterogeneity of Cat-301 and VC1.1 high molecular weight antigens. *Soc. Neurosci. Abstr.* 15: 892.