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Immunocytochemical Localization of the Guanine Nucleotide-Binding Protein G_o in Primary Cultures of Neuronal and Glial Cells

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We have localized the guanine nucleotide-binding protein, G_o, in primary cultures of pure neuronal and glial cells prepared from different mouse brain areas. Immunoblotting experiments with selective affinity-purified polyclonal rabbit antibodies to the 39 kDa alpha subunit of G_o (G_o alpha) indicated that G_o is distributed in both neurons and glial cells. G_o alpha accounts for 0.3% of total membrane proteins in striatal neurons. High specific G_o immunoreactivity was also detected in cortical neurons and cerebellar granule cells. Similarly, striatal glial cells contain large amounts of G_o (0.2% of total membrane proteins), as do glial cells from cerebral cortex and colliculi. Surprisingly, G_o was barely detectable in cerebellar glial cells. ^{32}P-ADP-ribosylation of the same neuronal and glial cell membranes with pertussis toxin indicated the presence of at least 3 substrates related to G_o alpha, G_i alpha (41 kDa), and a 40 kDa protein. This 40 kDa protein is the major pertussis toxin substrate in glial cells, while G_o alpha is predominant in neuronal membranes. Confirming immunoblotting, no labeled band was detected at 39 kDa in cerebellar glial cells with pertussis toxin. Indirect immunofluorescence staining of cerebellar granule cells and striatal neurons with purified G_o alpha antibodies was pronounced at the plasma membrane level, particularly at cell-cell contact areas, and in neurite arborization. More discrete staining was also apparent in the cytoplasm, whereas nuclei remained unstained. In striatal glial cells, specific immunolabeling was more diffused over the whole cell, and dense around the nucleus. The localization of G_o suggests that this protein must perform important functions in both the neuronal and glial cells that are discussed.

The contribution of guanine nucleotide-binding proteins (G proteins) in signal-transducing mechanisms is now firmly established (Stryer and Bourne, 1986; Dolphin, 1987). G_s, G_i, and G_T (transducin) are the functionally identified members belonging to the G protein family. G_s and G_i, respectively, mediate the stimulation and inhibition of adenylate cyclase activity (Hildebrandt et al., 1983; Gilman, 1984; Neer et al., 1984). G_T is the retinal rod outer-segment transducer that couples rhodopsin to cyclic GMP phosphodiesterase (Kühn, 1980; Fung et al., 1981). Another protein, termed G_o, was initially isolated from bovine brain (Neer et al., 1984; Sternweis and Robishaw, 1984). This protein exhibits structural and biochemical properties similar to the previously purified G ones that justify its designation as a member of the family (Huff et al., 1985; Pines et al., 1985). Its precise function has not yet been defined, although recent evidence suggests the involvement of G proteins and perhaps the G_o protein in the neurotransmitter coupling to neuronal calcium channels (Holz et al., 1986; Lewis et al., 1986; Scott and Dolphin, 1986; Hescheler et al., 1987).

Like G_s and G_i, G_o is a membrane-associated heterotrimer consisting of alpha, beta, and gamma subunits. The beta and gamma subunits are common components, which constitute a tightly associated and hydrophobic dimer that could have a regulatory function (Manning and Gilman, 1983; Sternweis, 1986). In SDS-PAGE analysis, the beta subunit can be resolved into a doublet of 36 and 35 kDa, while the gamma subunit migrates at about 8 kDa. The alpha subunits differ for each G protein; they possess the guanine nucleotide-binding site, a GTPase activity, and can be ADP-ribosylated with bacterial toxins (Roof et al., 1985; Itoh et al., 1986). G_s, alpha subunits (2 forms at 45-52 kDa) are substrates for cholera toxin, while both G_o alpha (39 kDa) and G_i alpha (41 kDa) subunits are susceptible to covalent modification by ADP-ribosylating Bordetella pertussis toxin (Bokoch et al., 1983; Kahn and Gilman, 1984; Katada et al., 1986).

Several studies evidenced that G_o is a highly conserved protein that is predominantly localized in neuronal tissues (Neer et al., 1984; Sternweis and Robishaw, 1984; Gierschik et al., 1986a; Homburger et al., 1987). Immunohistochemical observations in rat brain indicated a selective enrichment of G_o in neuropil areas (Worley et al., 1986). However, nothing is known about the cellular localization of G_o in the CNS cells.

We have produced polyclonal rabbit antibodies against the purified bovine brain G_o alpha subunit and obtained very selective probes towards the G_o alpha subunit. Furthermore, culture methods were developed in our laboratory to obtain highly differentiated neurons and glial cells in separated primary cultures from mouse brain (Ebersolt et al., 1981; Weiss et al., 1986). These primary cultures provide ideal models for examining the cellular physiology of nerve cells (Bockaert et al., 1986).

In the present study, we first used these antibodies to investigate the G_o alpha immunoreactivity in nerve cells and then described intracellular localization of G_o by light immunocytochemistry. Our results indicated that G_o is distributed in neurons, as well as in some glial cells, and mainly, but not exclusively, associated with the cytoplasmic surface of the plasma membrane.

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Materials and Methods

Materials. Lubrol PX, ATP, GTP, and L-myristyl phosphatidylcholine were obtained from Sigma. Lubrol PX was deionized prior to use. α-^{32}P-NAD and ^{125}I-protein A were purchased from New England Nuclear. Pertussis toxin was provided from List Laboratories. All other chemicals were of high purity from commercial sources.

Cell cultures

Neuronal cell cultures. Neuronal cultures generated from the striatum and cerebral cortex of 14–15-d-old Swiss albino mouse fetuses were grown for about 7 d under conditions previously described by Weiss et al. (1986). These cultures were grown in the absence of serum and contained less than 10% of glial cells (Bockaert et al., 1986). Cerebellar granule cells were cultured from cerebella of 7-d-old mice, as originally described by Messer (1977), with minor modification. Briefly, cells were isolated by mechanical dissociation with a fire-narrowed Pasteur pipette after 5 min incubation in 1 mM EDTA buffer, pH 7.4. Granule cells (1.5×10^6 cells/ml) were grown for 8–14 d in Dulbecco's modified Eagle's medium plus Ham F-12 nutrient (1:1) containing 5% fetal calf serum and 5% horse serum. After 48 hr in culture, 40 μM cytosine arabinofuranoside (ara-C) was added, and after 6 d this medium was changed. Mitotic inhibitors were present throughout the culture.

Non-neuronal cell cultures. Glial cells generated from cerebral cortex of newborn Swiss mice were grown for 18–21 d in the same medium used for neuronal cultures, but containing 10% fetal calf serum, according to the methods described by Ebersolt et al. (1981). More than 95% of the cells were identified as glial cells since they were stained with anti-GFA (glial fibrillary acidic protein) antibodies. Meningeal layers were prepared under the same conditions described by Ebersolt et al. (1981). Cultures of glial cells from the striatum and of 14–15-d-old Swiss mouse fetuses were grown for 18–21 d in the serum medium described above at a concentration of 8×10^5 cells/ml. This medium was changed 5 d after seeding and subsequently twice a week. Cultures of cerebellar glial cells were prepared according to the technique described for cerebellar granule cells, except that mitotic inhibitors were omitted and the medium was changed after 6 d of seeding and twice a week thereafter.

All the neuronal and non-neuronal cells were grown either in Falcon dishes (10 cm diameter) for the preparation of membranes or on glass coverslips incubated in culture dishes for immunofluorescent detection.

Preparation of particulate fractions from cells

Cells were gently washed 3 times at room temperature (RT) with 0.15 M NaCl, scraped off with a rubber spatula in 0.15 M NaCl (10 ml/dish), and centrifuged for 10 min at 300 × *g*. The cell pellets were then swollen for 10 min in 1 ml ice-cold lysing buffer containing 50 mM Tris-HCl, pH 7.5, 3 mM EDTA, 5 μg/ml soybean trypsin inhibitor, and 0.1 mM fresh phenylmethylsulfonyl fluoride (PMSF). Each suspension of lysed cells was homogenized at 0°C in a Potter-Elvehjem and centrifuged for 15 min at 40,000 × *g* in a Beckman J2-21 centrifuge at 2°C. Each homogenate was resuspended in the same buffer at a final protein concentration of about 1–2 mg/ml and stored at −180°C until use.

ADP-ribosylation with pertussis toxin

For ADP-ribosylation with pertussis toxin, 100 μg of proteins from particulate fractions were pelleted for 10 min in an Eppendorf centrifuge and resuspended in 20 mM Tris-HCl, pH 8, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.05% (vol/vol) of Lubrol PX. Samples were then incubated for 60 min at 30°C in 70 mM Tris-HCl, pH 8, containing 0.5 μM nicotinamide adenine dinucleotide (NAD), 0.5–1 μCi (α-^{32}P)-NAD, 1 mM ATP, 100 μM GTP, 10 mM thymidine, 1 mM EDTA, 0.1 mM $MgCl_2$, 1 mg/ml L-myristyl phosphatidylcholine, 10 mM nicotinamide, 25 mM DTT, and 100 ng activated pertussis toxin in a 60 μl assay volume. The reaction was stopped by the addition of 2% SDS plus 100 μg/ml bovine serum albumin and proteins were precipitated overnight at 4°C with 10% trichloracetic acid. After a 10 min centrifugation at 10,000 × *g*, the pellets were washed twice with ethyl ether and dried. The dried pellets were solubilized in 20 μl of 50 mM Tris-HCl, pH 6.8, 10% SDS, and 0.5 mM DTT by incubation for 2 hr at 30°C. The samples were cooled, mixed with 10 μl of 9 mM N-ethylmaleimide (NEM), and allowed to stand for 15 min at RT. Then 35 μl of 100 mM Tris-HCl, pH 6.8, 10% SDS, 10% beta-mercaptoethanol and 20% glycerol were added and an aliquot was loaded onto the gel. Analysis in SDS–PAGE was performed on 1.5-mm-thick slab gels with a 10% acrylamide, 0.13% bis-separating gel containing 0.1% SDS in 0.375 M Tris-HCl, pH 8.8. Electrophoresis was carried out at 4°C for about 90 min at 100 V, followed by 5 hr at 35 mA constant. Gels were then stained with Coomassie blue and dried. Autoradiography was performed using Kodak-X Omat AR5 film without intensifying screens for 24–96 hr.

Immunoblotting

Membrane proteins (100 μg) were pelleted for 10 min in an Eppendorf centrifuge and solubilized in 10 μl of 50 mM Tris-HCl, pH 6.8, 10% SDS, and 0.5 mM DTT by incubation for 2 hr at 30°C. The samples were then alkylated with NEM, as described above, prior to electrophoresis. Analysis of the molecular weight of the proteins was performed on 10% polyacrylamide gels prepared according to Laemmli (1970). Proteins were then transferred onto nitrocellulose sheets, as previously described (Audigier et al., 1985). The nitrocellulose sheets were dried and stained with red Ponceau S in order to assess the quality of the transfer. After blocking nonspecific protein binding with 3% gelatin, blots were incubated overnight at RT in 10 mM Tris-HCl, pH 7.5, 500 mM NaCl containing 0.3% gelatin, and affinity-purified anti-G_o alpha antibodies (6 μg/ml). The blots were then washed and incubated at RT with ^{125}I-protein A (100,000 cpm/ml) for 60 min. They were then thoroughly washed, dried, and exposed to Kodak X-AR5 with Dupont Cronex image-intensifying screens at −80°C for 16–48 hr.

Affinity purification of antibodies

Characteristics of antisera against anti-G_o alpha have been described previously by Homburger et al. (1987). For purification of antisera, 2 mg of a purified mixture of G_o and G_i (heptylamine Sepharose elution fraction) from bovine brain was coupled to activated CH-Sepharose (Pharmacia) to a final concentration of 1 mg protein/ml gel. Thirty-three percent $(NH_4)_2SO_4$-precipitated IgG fractions were applied to the G_o–G_i-immobilized Sepharose gel and recycled through the column overnight at 4°C. After extensive washing with 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, specifically bound antibodies were eluted with 3 M sodium isothiocyanate in 10 mM phosphate buffer, pH 7.4. The eluate was then thoroughly dialyzed against phosphate buffer, pH 7.4, 150 mM NaCl, concentrated at about 1 mg IgG/ml, aliquoted, and stored at −80°C.

Immunofluorescence detection of G_o alpha

Cell cultures generated on Thermanox glass coverslips (Miles, Norjerville, IL) were rinsed in PBS at 37°C and immediately fixed with 4% paraformaldehyde in 120 mM phosphate buffer containing 120 mM glucose for 30 min at RT. Coverslips were then rinsed in 0.1 M glycine, pH 7.4, for 30 min in order to quench free aldehyde groups. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. After washing with PBS, coverslips were incubated with affinity-purified anti-G_o alpha (10–20 μg/ml) in 20 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.2% gelatin, for 3 hr at 37°C. Coverslips were washed 3 times for 30 min with the same buffer and exposed to goat anti-rabbit second antibody conjugated to rhodamine, 1:50, in phosphate buffer for 60 min at RT. Coverslips were washed for 30 min with phosphate buffer, rinsed in 5 mM phosphate buffer, pH 7.4, mounted in Moviol 4.88 (Hoechst) mounting medium, and visualized with a Leitz Dialux 20 equipped with interferential filters. To demonstrate the specificity of the immunostaining, absorption of affinity-purified anti-G_o alpha was performed with a 10-fold molar excess of G_o alpha covalently linked to activated Sepharose beads. Bovine serum albumin coupled to the same gel was used as control. The beads were then pelleted for 2 min in an Eppendorf centrifuge and the supernatants were applied to the coverslips.

Results

Qualitative and quantitative analysis of G_o content in primary cultures of striatal neurons and glial cells

Immunoblotting experiments were carried out to identify the G_o alpha subunit on particulate fractions of cultured striatal neurons and glial cells. These fractions, subjected to electrophoresis, were either silver-stained (Fig. 1*A*) or blotted onto nitrocellulose sheets and analyzed with affinity-purified anti-G_o

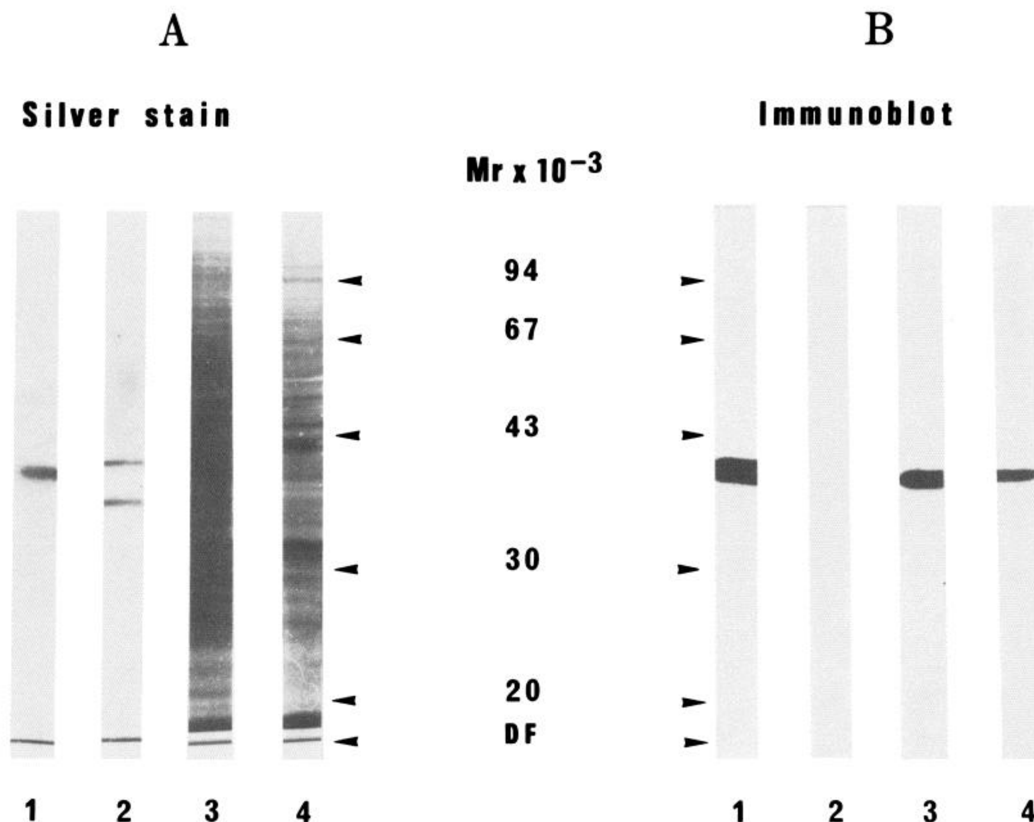

Figure 1. Detection of G_o alpha in striatal neurons and glial cells. One hundred micrograms of particulate fraction proteins from striatal neurons and glial cells, 1 μg of purified G_o alpha, and 1 μg of purified G_i were subjected to electrophoresis through 10% polyacrylamide gels and either stained with silver (*A*) or blotted onto nitrocellulose sheets and analyzed with affinity-purified antibodies against the alpha subunit of the G_o protein (*B*). Lane *1*, G_o alpha; lane *2*, G_i; lanes *3* and *4*, particulate fraction from striatal neurons and glial cells, respectively.

alpha antibodies (Fig. 1*B*). Figure 1*B* shows that both striatal neurons and glial cells display a positive immunoreactivity towards these antibodies (lanes 3 and 4). Comparison of silver-staining and immunoblot indicated that a single band at 39 kDa was specifically labeled. Electrophoresis of purified bovine brain G_o alpha subunit and G_i protein run in parallel confirmed the presence of a 39 kDa protein immunologically identical to G_o alpha in striatal neurons and glial cells (lanes 1 and 2, respectively).

To assess the relative G_o and total G protein concentrations in the same cells, we performed quantitative immunoblotting with anti-G_o alpha and anti-G beta antisera, respectively. We have previously described this quantitative method and the characteristics of these antisera (Homburger et al., 1987). Table 1 summarizes the results of our measurements. The data show that G_o accounts for 72 ± 5 pmol/mg protein in particulate fractions of striatal neurons and 44 ± 3 pmol/mg protein in glial cells. These values correspond to 0.3 and 0.2% of total membrane proteins, respectively. This quantitative method also indicated lower concentrations of the beta subunit than of G_o alpha in these cells. Thus, we detected only 54 ± 3 pmol/mg protein and 23 ± 6 pmol/mg protein in particulate fractions of striatal neurons and glial cells, respectively.

Immunoblotting distribution of G_o in neuronal and glial cells from various regions of mouse brain

We used affinity-purified anti-G_o alpha antibodies to probe immunoblots of electrophoretically separated proteins of particulate fractions from neuronal and glial cells in culture prepared from various mouse nervous tissues. The autoradiogram of a typical immunoblot is shown in Figure 2. The results pointed out the existence of a positive immunoreactivity at 39 kDa, corresponding to G_o alpha in cortical and striatal neurons and cerebellar granule cells (lanes 2, 3, and 4, respectively). In contrast, the particulate fractions of a nonconfluent neuroblastoma cell line, N1E-115, were not labeled (lane 5). However, additional experiments with the same cell line at confluency indicated clearly positive G_o alpha immunoreactivity (data not shown). Similarly, glial cells from cerebral cortex, striatum, and colliculi exhibited high G_o alpha immunoreactivity (lanes 6, 7,

Table 1. Quantitative estimation of G_o alpha and G beta subunit content in striatal neurons and glial cells

	Membrane protein (pmol/mg)	
Subunit	Striatal neurons	Striatal glial cells
$Alpha_o$	72 ± 5	44 ± 3
Beta	54 ± 3	23 ± 6

The presence of immunoreactivity was assessed on autoradiograms after electrotransfer of the proteins from SDS-PAGE onto nitrocellulose sheets, successive incubation with anti-G_o alpha or anti-G beta sera, and then with radioiodinated protein A. Standard curves were made for the amount of immunoreactivity corresponding to standards of G_o alpha and G beta-gamma by cutting out the radioactive bands and counting by gamma spectroscopy. The amounts of G_o alpha and beta in particulate fractions of striatal neurons and glial cells were determined from the standard curves. Results are expressed as the mean ± SEM of 3 independent experiments.

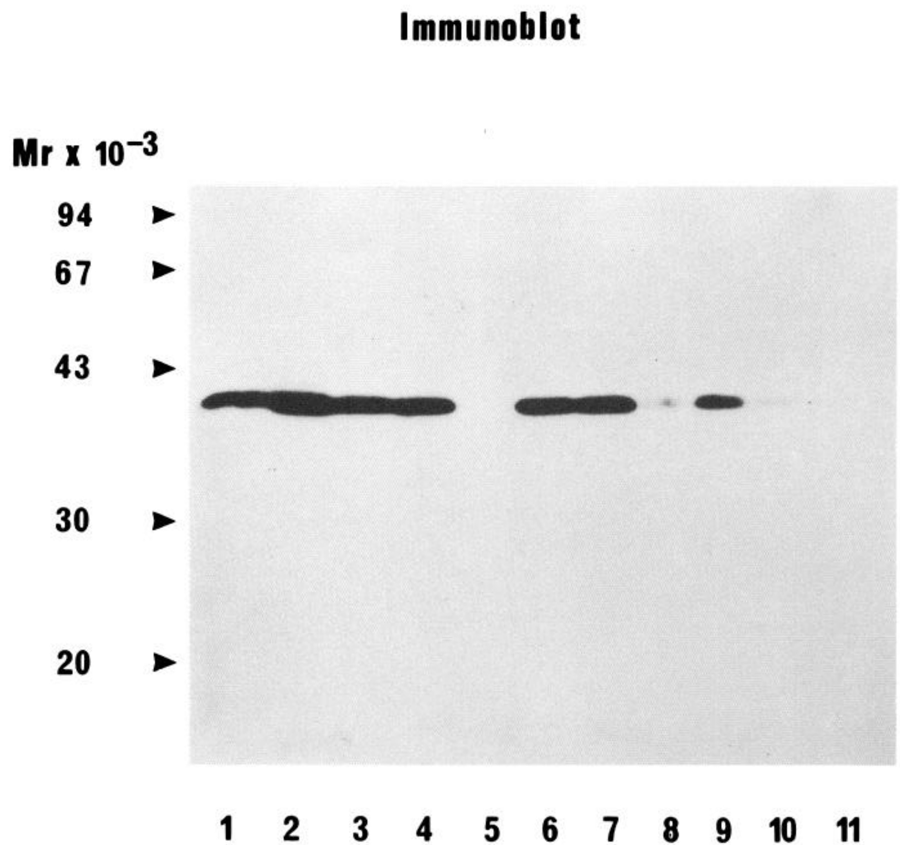

Figure 2. Immunoblot analysis of particulate fractions of neuronal, glial, and meningeal cells with affinity-purified anti-G_o alpha antibodies. One hundred micrograms of particulate fractions from cells and 0.2 μg of G_o alpha subunit were subjected to electrophoresis through 10% polyacrylamide gels, transferred onto nitrocellulose sheets, and analyzed with affinity-purified anti-G_o alpha antibodies, as described in Materials and Methods. An autoradiogram of the immunoblot of G_o alpha (lane *1*), cortical neurons (lane *2*), striatal neurons (lane *3*), cerebellar granule cells (lane *4*), neuroblastoma N1E-115 cells (lane *5*), cortical glial cells (lane *6*), striatal glial cells (lane *7*), cerebellar glial cells (lane *8*), colliculi glial cells (lane *9*), C_6 glioma cells (lane *10*), and meningeal cells (lane *11*) is shown. The autoradiogram was exposed for 48 hr.

and 9), while cerebellar glial cells appeared to contain a very low amount of G_o (lane 8). Specific immunolabeling was barely detected in rat C_6 glioma and was absent in meningeal cells (lanes 10 and 11). The immunoblot method was sensitive to as little as 0.1 pmol of protein, reproducibly detected with affinity-purified antibodies.

ADP-ribosylation of proteins by pertussis toxin in neurons and glial cells from various brain tissues

Particulate fraction proteins from neuronal, glial, and meningeal cells were ADP-ribosylated with pertussis toxin, solubilized, treated with NEM, and separated by SDS–PAGE. Several ADP-ribosylated proteins were observed in a molecular-mass range of 39–41 kDa (Fig. 3). No labeling occurred when pertussis toxin was omitted (not shown). In order to investigate the nature of these proteins, we compared their migrations to those of ADP-ribosylated alpha subunits of partially purified G proteins (heptylamine Sepharose elution fraction) and enriched G_o and G_i fractions (lanes 1, 2, and 3, respectively), all obtained from bovine brain. As shown in Figure 3 (lane 1), bovine brain contains 3 major ADP-ribosylated proteins: 2 of them migrate in the same manner as G_i alpha (41 kDa) and G_o alpha (39 kDa) subunits, whereas the third is an intermediate band (40 kDa) detected between G_i and G_o alpha subunits.

Similarly, particulate fractions of cortical and striatal neurons and cerebellar granule cells contain these 3 pertussis toxin substrates (lanes 4, 5, and 6). Note that the most greatly stained band corresponds to the 39 kDa G_o alpha subunit. No G_o alpha subunit was detected by pertussis toxin-catalyzed ADP-ribosylation in nonconfluent N1E-115 cells (lane 10). This result agrees with the immunoblotting experiments (Fig. 2, lane 5). In particulate fractions from glial cells, the major pertussis toxin substrate was the 40 kDa protein (lanes 7–9). More discrete ADP-ribosylation of G_i alpha and G_o alpha subunits also took place, except in cerebellar glial cells, where the 39 kDa band was not detectable (lane 9). Rat C_6 glioma and meningeal cells displayed the same pattern of pertussis toxin substrates as cerebellar glial cells (lanes 11 and 12).

Immunofluorescent localization of G_o in cerebellar granule cells, striatal neurons, and glial cells

The intracellular localization of G_o alpha recognized by affinity-purified antibodies was determined by light immunocytochemistry using prefixed and permeabilized nerve cells. Figure 4, *c, d,* illustrates the immunofluorescence staining pattern in striatal neurons and cerebellar granule cells. The fluorescence in cerebellar granule cells was strongly pronounced at the plasma membrane level in the perikarya and seemed to be reinforced at cell–cell and cell–neurite contact areas. A low level of labeling was also apparent in the cytoplasm and neurite arborization, while nuclei remained unstained. A similar pattern of immunofluorescence was obtained with striatal neurons, but the labeling of the cytoplasm was more intense than in cerebellar granule cells. In striatal glial cells, specific immunolabeling was relatively diffused within the cell and more pronounced around the unstained nucleus (Fig. 4*e*). However, no fluorescence staining was particularly observed at the cell–cell contact. Figure 4, *a, f,* shows additional specificity controls. Immunofluorescent labeling in striatal neurons was completely abolished after preincubation of anti-G_o alpha antibodies with an excess of purified bovine

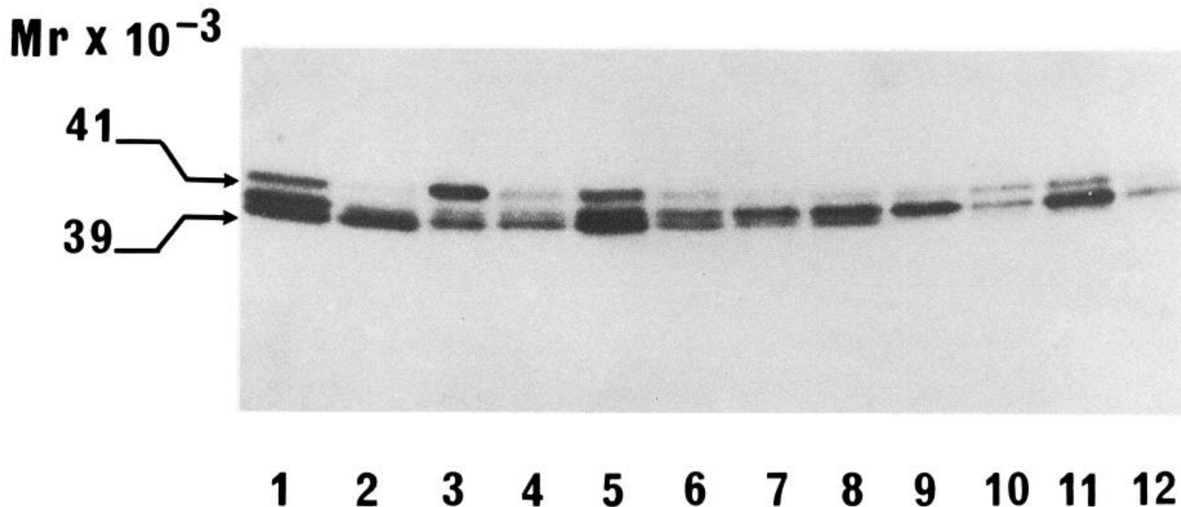

Figure 3. ADP-ribosylated substrates of Bordetella pertussis toxin in particulate fractions of neurons and glial cells from various mouse brain regions. One hundred micrograms of particulate fractions from cells and about 1 μg of purified G proteins were α-^{32}P-ADP-ribosylated by Bordetella pertussis toxin. Aliquots of α-^{32}P-ADP-ribosylated material were loaded onto a 10% polyacrylamide gel, as described in Materials and Methods. An autoradiogram of labeled proteins of heptylamine Sepharose elution fraction (lane *1*), enriched G_o fraction (lane *2*), enriched G_i fraction (lane *3*), cortical neurons (lane *4*), striatal neurons (lane *5*), cerebellar granule cells (lane *6*), cortical glial cells (lane *7*), striatal glial cells (lane *8*), cerebellar glial cells (lane *9*), neuroblastoma N1E-115 cells (lane *10*), meningeal cells (lane *11*), and C_6 glioma cells (lane *12*) is shown. The autoradiogram was exposed for 96 hr without intensifying screens.

brain G_o protein (Fig. 4*a*). Similarly, a negative result was obtained with meningeal cells (Fig. 4*f*), a primary culture devoid of G_o alpha, as shown in Figures 2 and 3, lane 11.

Discussion

The present study was undertaken to clearly localize the guanine nucleotide-binding protein, G_o, in CNS cells. The main finding that emerged was that cultured neurons and glial cells contained high amounts of this protein. Since both cells are known to possess similar neuropharmacological and physiological characteristics, such as neurotransmitter-sensitive adenylate cyclase (Ebersolt et al., 1981; Bockaert et al., 1986), receptors linked to inositol phosphate metabolism (Pearce et al., 1985; Sladeczek et al., 1985), as well as many voltage-dependent ion channels (Gray and Ritchie, 1985; Miller, 1987), the ubiquitous distribution of G_o among these nerve cells can help clarify its function.

Previous studies have shown that G_o is distributed throughout the CNS, with particularly high concentrations in forebrain (Sternweis and Robishaw, 1984; Huff et al., 1985; Gierschik et al., 1986a). However, an important question has been, In which cell types can this protein be found? A recent report (Milligan et al., 1986) suggested that the major G (or N) protein of neuroblastoma × glioma hybrid cell (NG 108-15) membranes is G_o, whereas that of C_6 glioma cells differs from G_i, G_o, and G_T and is a 40 kDa protein. This novel protein (40 kDa) is the predominant pertussis toxin substrate in C_6 cell membranes. In agreement with these observations, we describe here our finding that C_6 glioma cells contain at least 2 major pertussis toxin substrates, distinctly separated on SDS–PAGE by the experimental procedures used in Materials and Methods (Fig. 3, lane 12). The 41 kDa component is believed to be G_i alpha, while the major labeled band appeared to be the 40 kDa protein. We did not find any G_o in nonconfluent N1E-115 neuroblastoma cells (Fig. 3, lane 10). However, preliminary experiments indicated that at confluency, G_o could be expressed (data not shown). The possible identity between the 40 kDa protein and the bovine brain G_o alpha subunit is excluded, since affinity-purified anti-G_o alpha antibodies did not recognize any proteins in cells containing only the 41 and 40 kDa proteins, like meningeal cells (Figs. 2, lane 11; 4*f*). These data demonstrated, likewise, the high selectivity of these antibodies for the G_o alpha subunit.

Nevertheless, these established cell lines are transformed nerve cells in which many specific properties may have been lost, attenuated, or replaced. In contrast, primary cultures of neurons and glial cells prepared from dissociated normal brain tissues have provided a way to obtain functionally and morphologically well-differentiated and homogeneous nerve cells. We were therefore able to investigate the presence of G_o in primary cultures of neuronal and glial cells dissociated from different brain structures (Figs. 1, 2). We observed that G_o is largely distributed in both cultured neurons and glial cells. However, all neuronal cells contained high amounts of G_o, while glial cells displayed more differences in G_o content. Thus, immunoblotting as well as pertussis toxin-stimulated ADP-ribosylation clearly showed that cerebellar glial cells contain barely detectable amounts of G_o. This apparent heterogeneity can be explained either by developmental changes in the G_o level in glial cells during onto-

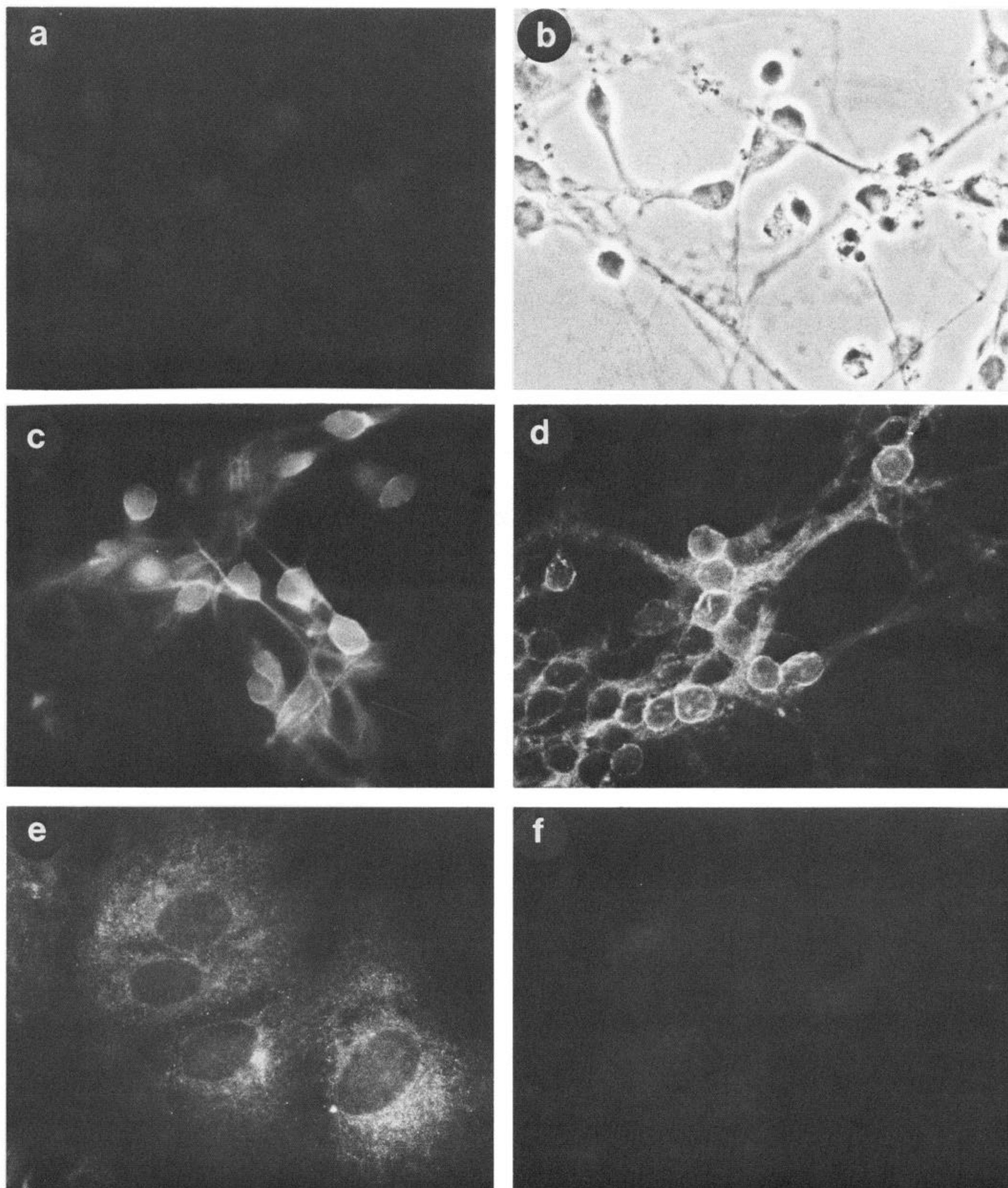

Figure 4. Indirect immunofluorescent localization of G_o alpha in cerebellar granule cells, striatal neurons, and glial cells. Cells cultured on glass coverslips were fixed and permeabilized with Triton X-100. Absorption of affinity-purified anti-G_o alpha antibodies was carried out with a 10-fold molar excess of G_o alpha, as described in Materials and Methods. Secondary antibodies were rhodamine-conjugated goat anti-rabbit IgG. Magnification, ×620. *a,* Prior incubation of affinity-purified anti-G_o alpha antibodies with a 10-fold molar excess of G_o alpha prevents specific staining of striatal neurons. *b,* The same field as shown in *a,* viewed by phase-contrast optics. *c–e,* Striatal neurons (*c*), cerebellar granule cells (*d*), and striatal glial cells (*e*) stained with unabsorbed affinity-purified anti-G_o alpha antibodies. Note the marked immunolabeling at neuronal cell–cell contact areas. *f,* Absence of staining of meningeal cells with affinity-purified anti-G_o alpha antibodies.

genesis or by the existence of heterogeneous populations of glial cells, according to their topographical localization in CNS (Denis-Donini et al., 1984). Recent data have showed that G_o protein expression soon changes during maturation of the embryonic chick heart (Liang et al., 1986), as well as during differentiation of 3T3-L1 cells (Gierschik et al., 1986b). Embryonic development has been reported to be associated with an increase of $alpha_{39}$ levels (related to the G_o alpha subunit), while 3T3-L1 cell differentiation was accompanied by an increased level of G_o alpha at day 2, followed by a substantial decrease. Thus, further investigation into whether the G_o alpha subunit is present in mature glial cells will be required. Indeed, differentiation of cultured glial cells depends greatly on external factors (Shapiro, 1973; Lim et al., 1977).

We have investigated the G_o cellular localization by indirect immunofluorescence on striatal neurons and cerebellar granule cells from 7–14-d-old cultures (Fig. 4, *c, d*). The data showed that immunolabeling was strongly delineated at the periphery of perikarya and was particularly reinforced at cell–cell contacts, mainly in cerebellar granule cells (Fig. 4*d*). Neurite arborizations and cytoplasm were also immunolabeled, while nuclei remained unstained. No preferential labeling was observed at the synaptic level, as revealed by specific anti-synapsin antibodies (Weiss et al., 1986). Striatal glial cells (Fig. 4*e*), as well as cortical glial cells (data not shown), also exhibited a positive specific immunofluorescence. However, the G_o localization was more difficult to elucidate without comparison with other specific markers because immunolabeling was relatively diffuse over the cytoplasm. The absence of increased fluorescence at cell–cell contact points may be due to a different localization than that in neurons. The presence of G_o immunoreactivity at the cytoplasmic level suggested that its function may not be limited to the plasma membrane (Toutant et al., 1987) and is compatible with the existence of free G_o alpha in the cytosolic compartment, as is expected for a hydrophilic molecule (Sternweis, 1986). However, our results differ from those of Worley et al. (1986) obtained on adult rat brain, as these authors reported that G_o was specifically localized in neuropil areas and absent in neuronal cell bodies. This discrepancy could be explained by the fact that our experiments were carried out on primary cultures generated from fetal and neonatal mouse brain tissues. This suggests that alterations in G_o alpha subunit turnover would occur in both neurons and glial cells during brain maturation.

Quantitative data obtained from striatal cells showed that both G_o alpha and G beta subunits are major membrane proteins (Table 1). However, ratios of G_o alpha to beta in excess of 1 raised the possibility that G_o might also be present as the free G_o alpha subunit in membranes, as previously suggested by Gierschik et al. (1986a). In addition, we cannot exclude the possibility that certain forms of G beta subunit in mouse brain tissues are not detected with our anti-G beta antisera compared with purified bovine brain G beta subunits (Rosenthal et al., 1986; Evans et al., 1987). Moreover, we show that nerve cells contain at least 3 pertussis toxin substrates (Fig. 3). The nature of these proteins has been investigated as with cell lines (see above). They correspond to G_o alpha (39 kDa), G_i alpha (41 kDa), and a 40 kDa protein that is the major pertussis toxin substrate in glial cells, while G_o is predominant in neurons. To date, this undefined 40 kDa protein has been found in tissues as various as brain (Neer et al., 1984), adipocytes (Malbon et al., 1984), neutrophils (Gierschik et al., 1986c), monocytes (Verghese et al., 1986), anterior pituitary cells (Journot et al., 1987), and chromaffin cells (Toutant et al., 1987). However, the identity between all these 40 kDa pertussis toxin substrates has yet to be established.

In conclusion, the present data demonstrate that the G_o protein is not exclusively associated with neuronal cells, but also with glial cells. This localization may provide important clues as to its function. Indeed, the G_o function should be invovled in a mechanism common to neurons and glial cells. For instance, it has been reported that G_o possibly mediates hormonal regulation of adenylate cyclase and/or phosphatidylinositol breakdown (Liang et al., 1986; Rapiejko et al., 1986; Worley et al., 1986). However, G_i is as effective as G_o in this regard, and no biochemical data have been provided for such a role for G_o in the regulation of phosphatidylinositide turnover. Recent evidence (Hescheler et al., 1987) suggests that G_o is 10-fold more potent than G_i in the functional coupling of opiate receptors to neuronal voltage-sensitive calcium channels (VSCC). The presence of these VSCC in both neurons and glial cells is consistent with this observation (MacVicar, 1984; Newman, 1985; Nowycky et al., 1985; Pin and Bockaert, 1987). In this regard, electron immunocytochemistry will provide a way of studying the precise subcellular localization of G_o and therefore help ascribe to it a specific function.

References

Audigier, Y., C. Pantaloni, J. Bigay, P. Deterre, J. Bockaert, and V. Homburger (1985) Tissue expression and phylogenetic appearance of the beta and gamma subunits of GTP-binding protein. FEBS Lett. *189:* 1–7.

Bockaert, J., J. Gabrion, F. Sladeczek, J. P. Pin, M. Recasens, M. Sebben, D. Kemp, A. Dumuis, and S. Weiss (1986) Primary culture of striatal neurons: A model of choice for pharmacological and biochemical studies of neurotransmitter receptors. J. Physiol. (Paris) *81:* 219–227.

Bokoch, G. R., T. Katada, J. K. Northup, E. L. Hewlett, and A. G. Gilman (1983) Identification of the predominant substrate for ADP-ribosylation by Islet Activating Protein. J. Biol. Chem. *258:* 2072–2075.

Denis-Donini, S., J. Glowinsky, and A. Prochiantz (1984) Glial heterogeneity may define the three-dimensional shape of mouse mesencephalic dopaminergic neurons. Nature *307:* 641–643.

Dolphin, A. C. (1987) Nucleotide binding proteins in signal transduction and disease. Trends Neurosci. *10:* 53–57.

Ebersolt, C., M. Perez, and J. Bockaert (1981) Neuronal, glial and meningeal localizations of neurotransmitter-sensitive adenylate cyclases in cerebral cortex of mice. Brain Res. *213:* 139–150.

Evans, T., A. Fawzi, E. D. Fraser, M. L. Brown, and J. K. Northup (1987) Purification of a $beta_{35}$ form of the beta-gamma complex from human placenta membranes. J. Biol. Chem. *262:* 176–181.

Fung, B. K., J. B. Hurley, and L. Stryer (1981) Flow of information in the light-triggered cyclic nucleotide cascade of vision. Proc. Natl. Acad. Sci. USA *78:* 152–156.

Gierschik, P., G. Milligan, M. Pines, P. Golsmith, J. Codina, W. Klee, and A. Spiegel (1986a) Use of specific antibodies to quantitate the guanine nucleotide-binding protein G_o in brain. Proc. Natl. Acad. Sci. USA *83:* 2258–2262.

Gierschik, P., B. Morrow, G. Milligan, C. Rubin, and A. Spiegel (1986b) Changes in the guanine nucleotide-binding proteins, G_i and G_o, during differentiation of 3T3-L1 cells. FEBS Lett. *199:* 103–106.

Gierschik, P., J. Falloon, G. Milligan, M. Pines, J. I. Gallin, and A. Spiegel (1986c) Immunochemical evidence for a novel pertussis toxin substrate in human neutrophils. J. Biol. Chem. *261:* 8058–8062.

Gilman, A. G. (1984) G proteins and dual control of adenylate cyclase. Cell *36:* 577–579.

Gray, P. T. A., and J. M. Ritchie (1985) Ion channels in Schwann and glial cells. Trends Neurosci. *8:* 411–415.

Hescheler, J., W. Rosenthal, W. Trautwein, and G. Schultz (1987) The GTP-binding protein, G_o, regulates neuronal calcium channels. Nature *325:* 445–447.

Hildebrandt, J. D., R. D. Sekura, J. Codina, R. Iyengar, C. R. Manclark, and L. Birnbaumer (1983) Stimulation and inhibition of adenylyl

cyclase mediated by distinct regulatory proteins. Nature *302:* 706–709.

Holz IV, G. G., S. G. Rane, and K. Dunlap (1986) GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. Nature *319:* 670–672.

Homburger, V., P. Brabet, Y. Audigier, C. Pantaloni, J. Bockaert, and B. Rouot (1987) Immunological localization of the GTP-binding protein G_o in different tissues of vertebrates and invertebrates. Mol. Pharmacol. *34:* 313–319.

Huff, R. M., J. M. Axton, and E. J. Neer (1985) Physical and immunological characterization of the guanine nucleotide-binding proteins guanine nucleotide-binding protein purified from bovine cerebral cortex. J. Biol. Chem. *260:* 10864–10871.

Itoh, H., T. Kozasa, S. Nagata, S. Nakamura, T. Katada, M. Ui, S. Iwai, E. Ohtsuka, H. Kawasaki, K. Suzuki, and Y. Kaziro (1986) Molecular cloning and sequence determination of $_c$DNAs for alpha subunits of the guanine nucleotide-binding proteins G_s, G_i and G_o from rat brain. Proc. Natl. Acad. Sci. USA *83:* 3776–3780.

Journot, L., V. Homburger, C. Pantaloni, M. Priam, and J. Bockaert, and A. Enjalbert (1987) An IAP-sensitive G protein is involved in dopamine inhibition of angiotensin and TRH stimulated inositol phosphate production in anterior pituitary cells. J. Biol. Chem. (in press).

Kahn, R. A., and A. G. Gilman (1984) ADP-ribosylation of G_s promotes the dissociation of the alpha and beta subunits. J. Biol. Chem. *259:* 6235–6240.

Katada, T., M. Oinuma, and M. Ui (1986) Two guanine nucleotide-binding proteins in rat brain serving as the specific substrates of Islet-Activating Protein, pertussis toxin. J. Biol. Chem. *261:* 8182–8191.

Kühn, H. (1980) Light- and GTP-regulated interaction of GTPase and other proteins with bovine photoreceptor membranes. Nature *283:* 587–589.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227:* 680–685.

Lewis, D. L., F. F. Weight, and A. Luini (1986) A guanine nucleotide-binding protein mediates the inhibition of voltage dependent calcium current by somatostatin in a pituitary cell line. Proc. Natl. Acad. Sci. USA *83:* 9035–9039.

Liang, B. T., M. R. Hellmich, E. J. Neer, and J. B. Galper (1986) Development of muscarinic cholinergic inhibition of adenylate cyclase in embryonic chick heart. J. Biol. Chem. *261:* 9011–9021.

Lim, R., D. E. Turrif, S. Troy, B. W. Moore, and L. F. Eng (1977) Glial maturation factor: Effect on chemical differentiation of glioblasts in culture. Science *195:* 195–196.

MacVicar, B. A. (1984) Voltage-dependent calcium channels in glial cells. Science *226:* 1345–1347.

Malbon, C. C., P. J. Rapiejko, and J. A. Garcia-Sainz (1984) Pertussis toxin catalyses the ADP-ribosylation of two distinct peptides, 40 and 41 kDa, in rat fat cell membranes. FEBS Lett. *176:* 301–306.

Manning, D. R., and A. G. Gilman (1983) The regulatory components of adenylate cyclase and transducin. J. Biol. Chem. *258:* 7059–7063.

Messer, A. (1977) The maintenance and identification of mouse cerebellar granule cells in monolayer culture. Brain Res. *130:* 1–12.

Miller, R. J. (1987) Multiple calcium channels and neuronal function. Science *235:* 46–52.

Milligan, G., P. Gierschik, A. M. Spiegel, and W. A. Klee (1986) The GTP-binding regulatory proteins of neuroblastoma × glioma, NG 108-15, and glioma, C_6, cells. FEBS Lett. *195:* 225–230.

Neer, E. J., J. M. Lok, and L. G. Wolf (1984) Purification and properties of the inhibitory guanine nucleotide regulatory unit of brain adenylate cyclase. J. Biol. Chem. *259:* 14222–14229.

Newman, E. A. (1985) Voltage-dependent calcium and potassium channels in retina glial cells. Nature *317:* 809–811.

Nowycky, M. C., A. P. Fox, and R. W. Tsien (1985) Three types of neuronal calcium channel with different calcium agonist sensitivity. Nature *316:* 440–443.

Pearce, B., M. Cambray-Deakin, C. Morrow, J. Grimble, and S. Murphy (1985) Activation of muscarinic and alpha$_1$-adrenergic receptors on astrocytes results in the accumulation of inositol phosphates. J. Neurochem. *45:* 1534–1540.

Pin, J. P., and J. Bockaert (1987) Multiple voltage-sensitive calcium channels probably involved in endogenous GABA release from striatal neurons differentiated in primary culture. Naunyn Schmiedebergs Arch. Pharmacol. *336:* 190–196.

Pines, M., P. Gierschik, G. Milligan, W. Klee, and A. Spiegel (1985) Antibodies against the carboxyl-terminal 5-kDa peptide of the alpha subunit of transducin cross-react with the 40-kDa but not the 39-kDa GTP-binding proteins from brain. Proc. Natl. Acad. Sci. USA *82:* 4095–4099.

Rapiejko, P. J., J. K. Northup, T. Evans, J. E. Brown, and C. C. Malbon (1986) G proteins of fat cells. Biochem. J. *240:* 35–40.

Roof, D. J., M. L. Applebury, and P. C. Sternweis (1985) Relationships within the family of GTP-binding proteins isolated from bovine central nervous system. J. Biol. Chem. *260:* 16242–16249.

Rosenthal, W., D. Koesling, U. Rudolph, C. Kleuss, M. Pallast, M. Yajima, and G. Schultz (1986) Identification and characterization of the 35-kDa beta subunit of guanine nucleotide-binding proteins by an antiserum raised against transducin. Eur. J. Biochem. *158:* 255–263.

Scott, R. H., and A. C. Dolphin (1986) Regulation of calcium currents by a GTP analogue: Potentiation of (−)-baclofen-mediated inhibition. Neurosci. Lett. *69:* 59–64.

Shapiro, D. L. (1973) Morphological and biochemical alterations in fetal rat brain cells cultured in the presence of monobutyryl cyclic AMP. Nature *241:* 203–204.

Sladeczek, F., J. P. Pin, M. Recasens, J. Bockaert, and S. Weiss (1985) Glutamate stimulates inositol phosphate formation in striatal neurons. Nature *317:* 717–719.

Sternweis, P. C. (1986) The purified alpha subunits of G_o and G_i from bovine brain require beta-gamma for association with phospholipid vesicles. J. Biol. Chem. *261:* 631–637.

Sternweis, P. C., and J. D. Robishaw (1984) Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. J. Biol. Chem. *259:* 13806–13813.

Stryer, L., and H. R. Bourne (1986) G proteins: A family of signal transducers. Annu. Rev. Cell Biol. *2:* 391–419.

Toutant, M., D. Aunis, J. Bockaert, V. Homburger, and B. Rouot (1987) Presence moreof three pertussis toxin substrates and G_o-alpha immunoreactivity in both plasma and granule membranes of chromaffin cells. FEBS Lett. *215:* 339–344.

Verghese, M., R. J. Uhing, and R. Snyderman (1986) A pertussis/cholera toxin-sensitive N protein may mediate chemoattractant receptor signal transduction. Biochem. Biophys. Res. Commun. *138:* 887–894.

Weiss, S., J. P. Pin, M. Sebben, D. E. Kemp, F. Sladeczek, J. Gabrion, and J. Bockaert (1986) Synaptogenesis of cultured striatal neurons in serum-free medium: A morphological and biochemical study. Proc. Natl. Acad. Sci. USA *83:* 2238–2242.

Worley, P. F., J. M. Baraban, C. Van Dop, E. J. Neer, and S. H. Snyder (1986) G_o, a guanine nucleotide-binding protein: Immunohistochemical localization in rat brain resembles distribution of second messenger systems. Proc. Natl. Acad. Sci. USA *83:* 4561–4565.