

# Localization of mRNAs encoding the $\alpha$ -subunits of signal-transducing G-proteins within rat brain and among peripheral tissues

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The sequence of the mRNAs which encode the  $\alpha$ -subunits of the signal-transducing G-proteins  $G_s$ ,  $G_o$  and two forms of  $G_i$  (termed  $G_{i1}$  and  $G_{i2}$ ) have recently been reported. Based on rat sequences we prepared oligodeoxynucleotide probes for measurement of these mRNAs in rat brain and peripheral tissues. The relative abundance of these mRNA species in brain was  $G_s > G_o \sim G_{i2} > G_{i1}$ . The  $G_s$  and  $G_{i2}$  mRNAs had somewhat lower levels in heart, kidney and liver than in brain, and  $G_o$  and  $G_{i1}$  mRNAs were not detected in the peripheral tissues. Using in situ hybridization we localized each of these mRNAs within slices of the rat brain. The patterns of distribution of  $G_s$  and  $G_{i2}$  mRNA were very similar, but very different from that of  $G_o$  and  $G_{i1}$  mRNA. These data illustrate that receptor-effector coupling G-proteins are regionally specialized in their expression. This regional specialization may reflect a selective coupling of individual G-proteins with the various neurotransmitter receptors and effector pathways.

G-protein; Signal transduction; Neurotransmitter receptor; Hybridization; mRNA

## 1. INTRODUCTION

Signal transduction by many neurotransmitter receptors is mediated by a family of structurally related GTP-binding proteins (G-proteins). The best characterized G-proteins regulate adenylate cyclase activity.  $\beta$ -Adrenergic and various neuropeptide receptors stimulate adenylate cyclase by coupling with the G-protein  $G_s$ . Muscarinic,

somatostatin,  $\alpha_2$ -adrenergic and opiate receptors inhibit adenylate cyclase by coupling with the G-protein  $G_i$ .  $G_s$  and  $G_i$  each consist of an  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunit. The  $\beta$ -subunits are identical for  $G_s$  and  $G_i$  while the  $\alpha$ -subunits are similar but structurally distinct. A third G-protein, named  $G_o$ , has recently been purified from brain, it has an identical  $\beta$ -subunit to  $G_s$  and  $G_i$  and an  $\alpha$ -subunit which is more similar to  $G_i$  than to  $G_s$  [1–3].

A number of other receptor-mediated events have been attributed to G-proteins. Examples include: (i) stimulation of phospholipase C activity by serotonin [4] and bradykinin receptors [5]; (ii) opening of  $K^+$  channels by  $\gamma$ -aminobutyric acid and serotonin receptors [6]; and (iii) inhibition of voltage-dependent  $Ca^{2+}$  channels by noradrenalin,  $\gamma$ -aminobutyric acid [7] and somatostatin [8]. Many of these responses to receptor activation are

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**Abbreviations:**  $G_s$  and  $G_i$ , the G-proteins that stimulate and inhibit adenylate cyclase, respectively;  $G_o$ , an abundant G-protein of brain

blocked by pertussis toxin and mimicked by guanine nucleotides. Since both  $G_i$  and  $G_o$ , but not  $G_s$ , are sensitive to pertussis toxin the precise identity of the G-protein or proteins which mediate these events is unknown [1–3].

Two events mediated by receptors have recently been examined in detail. In pacemaker cells of the heart, muscarinic receptors open  $K^+$  channels via a G-protein [9], and in pertussis toxin-treated cells  $G_i$  is more potent than  $G_o$  in reconstituting the ability of the receptor to couple to the channel [10]. Similarly, in neuroblastoma  $\times$  glioma hybrid cells opiates inhibit  $Ca^{2+}$  channels via a G-protein. After the cells are treated with pertussis toxin, however,  $G_o$  is more potent than  $G_i$  in reconstituting receptor coupling [11].

While G-proteins are essential for signal transduction by neurotransmitter receptors, little is known about the distribution of G-proteins within the nervous system. Antibodies to  $G_o$  have recently been used to map its distribution in the brain using immunohistochemistry [12]. Similar antibodies have not been available for use in the mapping of  $G_s$  and  $G_i$ . An alternative method for determining the regional specialization of G-protein expression is the use of in situ hybridization histochemistry to map the distribution of mRNAs which encode these proteins. Since the  $\alpha$ -subunits of the individual G-proteins are unique, localization studies can focus on these structures.

Nucleotide sequences of cDNAs coding for the  $\alpha$ -subunits of many G-proteins have recently been determined. The sequences of rat [13], mouse [14], human [15,16], and bovine  $G_s$  [17,18]; rat [13] and bovine  $G_o$  [19]; bovine rod [20–22] and cone [23] transducins; and bovine [24], human [25], rat [13] and mouse [14]  $G_i$  have been reported. For  $G_o$  and  $G_s$  the protein sequences predicted from cDNAs are almost completely conserved across species. In the case of  $G_o$ , the predicted sequences agree with sequences derived from purified proteins [13,20]. The amino acid sequence of  $G_s$  has not been chemically determined, but the identity of  $G_s$  clones has been verified by indirect methods [18] and by transfection of  $G_s$ -deficient  $cyc^-$  cells with  $G_s$  cDNA [26].

The identification of cDNAs encoding  $G_i$   $\alpha$ -subunits is more problematic. Several distinct cDNA clones have been obtained from rat [27] and human [25,28] libraries. While all of the isolated

$G_i$  cDNAs predict proteins with strong homology (they are more similar to each other than to  $G_o$ , the transducins and  $G_s$ ), not all of them agree in sequence with proteins already purified. Finally, none of the clones have yet been shown to encode proteins which inhibit adenylate cyclase.

The anomalies in  $G_i$  sequences can be reconciled if one postulates the existence of multiple forms of  $G_i$ . We call the bovine [24] and human [28]  $G_i$  sequences  $G_{i1}$ . These sequences, obtained from brain cDNA libraries, agree precisely with the chemically determined sequence of  $G_i$  from rat [13] and bovine [24] brain. We term the  $G_i$  cloned from rat C6 glioma [13], mouse macrophage [14] and human monocyte [25] libraries  $G_{i2}$ .  $G_{i1}$ ,  $G_{i2}$  and a third  $G_i$ -like sequence have been cloned from a rat olfactory mucosa library [27]. Based on the rat cDNA sequences, we prepared oligodeoxynucleotide probes to identify mRNA encoding  $G_s$ ,  $G_o$ ,  $G_{i1}$  and  $G_{i2}$ . We used these probes to map the distribution of mRNA coding for these proteins within brain and among peripheral tissues.

## 2. MATERIALS AND METHODS

### 2.1. Probes

The synthetic oligodeoxynucleotide probes were complementary to bases encoding amino acids 379–394 of  $G_s$  (5'-GAGCAGCTCGTATTGGCGAAGATGCATGCCCTGGATGATGTCACGGCA-3'), 118–133 of  $G_{i1}$  (5'-GTCCTTCCACAGTCTCTTTATGACGCCGCGAGCTCCGCGGT-CATAAA-3') and 109–124 of  $G_{i2}$  (5'-AGGTCTTCCGGAAGCATGCCTTGCTCCTCGGCAGC-ACAGGACAGTGCG-3') and 108–123 of  $G_o$  (5'-GTTCTGCAGAGAATGGTTTCAGTGTCCTCCATGCGACTCACCACGTCAC-3') mRNA [13,27]. These regions were selected because of the lack of homology among the mRNAs within these regions. The oligodeoxynucleotides were made on an Applied Biosystems DNA synthesizer and purified on an 8% polyacrylamide/8 M urea preparative sequencing gel. The probes were labeled by tailing the 3'-end with terminal transferase (Bethesda Research Laboratories) and deoxyadenosine triphosphate labeled in the  $\alpha$ -position with  $^{35}S$  for in situ hybridizations and  $^{32}P$  for Northern blots ( $^{35}S > 1000$  Ci/mmol,  $^{32}P > 3000$  Ci/mmol, New England Nuclear). Using a sequencing gel we have observed that the most com-

mon length of our oligonucleotide 3'-tails was 10 bases.

## 2.2. *In situ* hybridization

Our methods for *in situ* hybridization were similar to those developed for measurement of neuropeptide mRNA expression [29]. We have previously applied these methods to measurement of mRNAs encoding the  $\alpha$ -subunit of transducin, the G-protein of photoreceptor rods [30,31]. Male Sprague-Dawley rats (200–300 g, NIH) were killed by decapitation and the brains were removed, frozen on dry ice and stored at  $-80^{\circ}\text{C}$  until sectioned. 12- $\mu\text{m}$  sections were cut and thaw-mounted onto gelatin-coated slides. The sections were then warmed for 2 min at  $40^{\circ}\text{C}$  and stored at  $-80^{\circ}\text{C}$  until use. Before hybridizations, sections were warmed to room temperature and then fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 5 min. Sections were rinsed in PBS and incubated in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl (pH 8) for 10 min at room temperature and then transferred through 70 (1 min), 80 (1 min), 95 (2 min), and 100% ethanol (1 min), 100% chloroform (5 min), 100 and 95% ethanol and air-dried. Hybridizations were performed in  $4 \times \text{SSC}$  ( $1 \times \text{SSC} = 0.15 \text{ M NaCl}$ , 0.015 M sodium citrate, pH 7.2), 50% formamide,  $1 \times \text{Denhardt's}$  (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 250  $\mu\text{g/ml}$  sheared single-stranded salmon sperm DNA, 100 mM dithiothreitol, and 10% dextran sulfate.  $1 \times 10^6$  dpm oligodeoxynucleotide probe in 50  $\mu\text{l}$  buffer were applied to each section. Sections were covered with a parafilm coverslip and incubated overnight at  $37^{\circ}\text{C}$  in a humid chamber. The coverslips were removed and the sections rinsed in four 15-min changes of  $1 \times \text{SSC}$  at  $55^{\circ}\text{C}$ , then twice in the same buffer for 30 min at room temperature, dipped in water and air-dried. Autoradiographic localization of bound probe was performed by exposure of the sections to X-ray film for 1–5 weeks. For higher resolution, the sections were then dipped into NTB3 nuclear emulsion (Kodak) and exposed for 3–6 weeks at  $4^{\circ}\text{C}$ . Tissue was stained with cresyl violet.

## 2.3. RNA extraction and Northern blots

For extraction of RNA, the whole brain was

homogenized in guanidinium thiocyanate (329) and total RNA separated on a cesium trifluoroacetate (Pharmacia) gradient. Approx. 5  $\mu\text{g}$  denatured total RNA was loaded on an agarose gel containing formaldehyde. After elec-

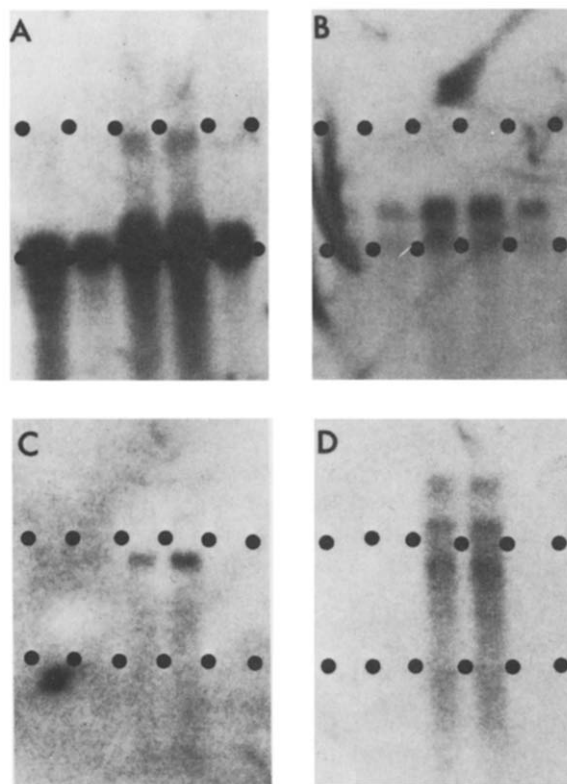


Fig.1. Northern blots of RNA hybridized with oligonucleotide probes directed to the  $\alpha$ -subunits of (A)  $G_s$ , (B)  $G_{i2}$ , (C)  $G_{i1}$  and (D)  $G_o$  mRNA. 5  $\mu\text{g}$  total RNA extracted from (left to right) heart, kidney, cerebral cortex, cerebellum, and liver and separated on a denaturing agarose gel. Identical RNA preparations were used for each blot. To confirm RNA transfer and integrity, each gel was stained with ethidium bromide and the transferred ribosomal bands photographed (the positions of the ribosomal bands are indicated by circles). Slightly more ribosomal RNA ( $\sim 15\%$ ) was observed on the liver than the other blots.  $1-2 \times 10^6$  dpm/ml probe was hybridized with each blot overnight at  $37^{\circ}\text{C}$ . The blots were washed at  $\sim 20^{\circ}\text{C}$  below the theoretical melting temperature ( $55^{\circ}\text{C}$ ,  $1 \times \text{SSPE}$ ) of the cDNA-mRNA hybrids. The blots were exposed to X-ray film for  $\sim 7$  days at  $-70^{\circ}\text{C}$  with two intensification screens.

trophoresis, RNA was transferred to Genescreen (NEN) and baked for 2 h at 80°C. The blots were prehybridized in 4 ml hybridization buffer [50% formamide, 1 × Denhardt's, 1 × SSPE (1 × SSPE = 0.5 M NaCl, 10 mM sodium phosphate, 1 mM EDTA), 250 µg yeast tRNA, 500 µg single-stranded salmon sperm DNA] for 8 h and then hybridized with 3 ml probe at a concentration of  $1-2 \times 10^6$  dpm/ml for 20 h. Blots were then washed in 1 × SSPE, 0.2% SDS five times at 55°C for 15 min, and then twice in the same buffer at room temperature for 30 min. Wash protocols for both in situ and blot hybridizations used conditions ~20°C below the theoretical melting temperature of the oligodeoxynucleotide-mRNA hybrids [33]. Autoradiographic localization of bound probes was performed by exposure of the blot to X-ray film.

### 3. RESULTS

Northern blot analysis of several tissues with each of our oligodeoxynucleotide probes is illustrated in fig.1. Each probe was hybridized with 5 µg total RNA extracted from heart, kidney, cerebral cortex, cerebellum, and liver. The  $G_s$  probe labeled two mRNA species: an abundant species of ~1900 bases and a much less abundant one of ~3900 bases. The  $G_{i2}$  probe labeled mRNA with a size of ~2300 bases.  $G_s$  and  $G_{i2}$  mRNAs were most abundant in brain, and their concentrations were similar in the cerebellum relative to the cerebral cortex.  $G_s$  and  $G_{i2}$  mRNA were also abundant in the heart, kidney and liver. The  $G_{i1}$  probe labeled a single mRNA species of ~4000 bases. The  $G_o$  probe labeled three mRNA species, the most abundant had a size of ~3500 bases. Unlike  $G_s$  and  $G_{i2}$  mRNA,  $G_{i1}$  and  $G_o$  mRNA concentrations

were higher in the cerebellum than the cerebral cortex, and were not detectable in the peripheral tissues. The relative abundance of mRNA detected with the probes in brain was  $G_s > G_o \sim G_{i2} > G_{i1}$ .

The distribution of cells labeled with the  $G_s$ ,  $G_o$ ,  $G_{i2}$ , and  $G_{i1}$  probes in sagittal and coronal sections of rat brain is illustrated in fig.2. As was the case in the septal nucleus, the  $G_s$  and  $G_{i2}$  mRNAs had the same regional distribution in all brain regions examined, and as in the case of Northern blots,  $G_s$  mRNA was more abundant than  $G_{i2}$  mRNA. These mRNAs were abundant in the ependymal lining of the ventricles and choroid plexus, hippocampus (fields CA 1-4 being higher than the dentate gyrus), cerebellum (cortical layers and lateral nuclei) and cerebral cortex. Within the cerebral cortex these mRNAs were least abundant within a central layer. As was the case in Northern blots, the levels of  $G_s$  and  $G_{i2}$  mRNA were similar in the cerebellum relative to the cerebral cortex. High concentrations of these mRNAs were also observed in the globus pallidus, primary olfactory cortex, various nuclei of the thalamus, subthalamic nuclei and brain stem. A low density of mRNA was observed in the regions rich in white matter such as the corpus callosum and anterior commissure and portions of the cerebellum. The caudate putamen and olfactory tubercle also contained a relatively low concentration of  $G_s$  and  $G_{i2}$  mRNA.

The distributions of  $G_{i1}$  and  $G_o$  mRNA in similar sagittal and coronal sections of rat brain are also illustrated in fig.2. Like  $G_s$  and  $G_{i2}$  mRNAs,  $G_{i1}$  and  $G_o$  mRNAs were abundant in the cerebral and cerebellar cortices, hippocampus, and primary olfactory cortex. Unlike  $G_s$  and  $G_{i2}$  mRNAs,  $G_{i1}$  and  $G_o$  mRNAs were much more abundant in the cerebellar cortex than the cerebral cortex and had low abundance in the central and

Fig.2. Sections of rat brain were hybridized with probes directed to mRNAs coding for G-protein  $\alpha$ -subunits. 12-µm sagittal (A,C,E,G) and coronal (B,D,F,H) sections were hybridized with the  $G_s$  (A,B),  $G_{i2}$  (C,D),  $G_{i1}$  (E,F) and  $G_o$  (G,H) probes. A central nucleus of the cerebellum is indicated by a horizontal arrow in A, C, E and G. A central layer of the cerebral cortex (cx) is illustrated by a vertical arrow in all sections. The ependymal lining of the ventricles and choroid plexus is illustrated by a triangle in all sections. The caudate putamen (cp) and globus pallidus (gp) are illustrated by vertical arrows in D, F and H. The hippocampus (h) is labeled in E and F. The  $^{35}$ S-labeled probes were incubated with the sections overnight. The sections were washed at ~20°C below the theoretical melting temperatures of the probe-mRNA hybridizations, dried and exposed to X-ray film for 4 weeks. The sagittal and coronal sections approximate brain levels (lateral 3.4 mm and interaural 6.2 mm) as indicated [41].

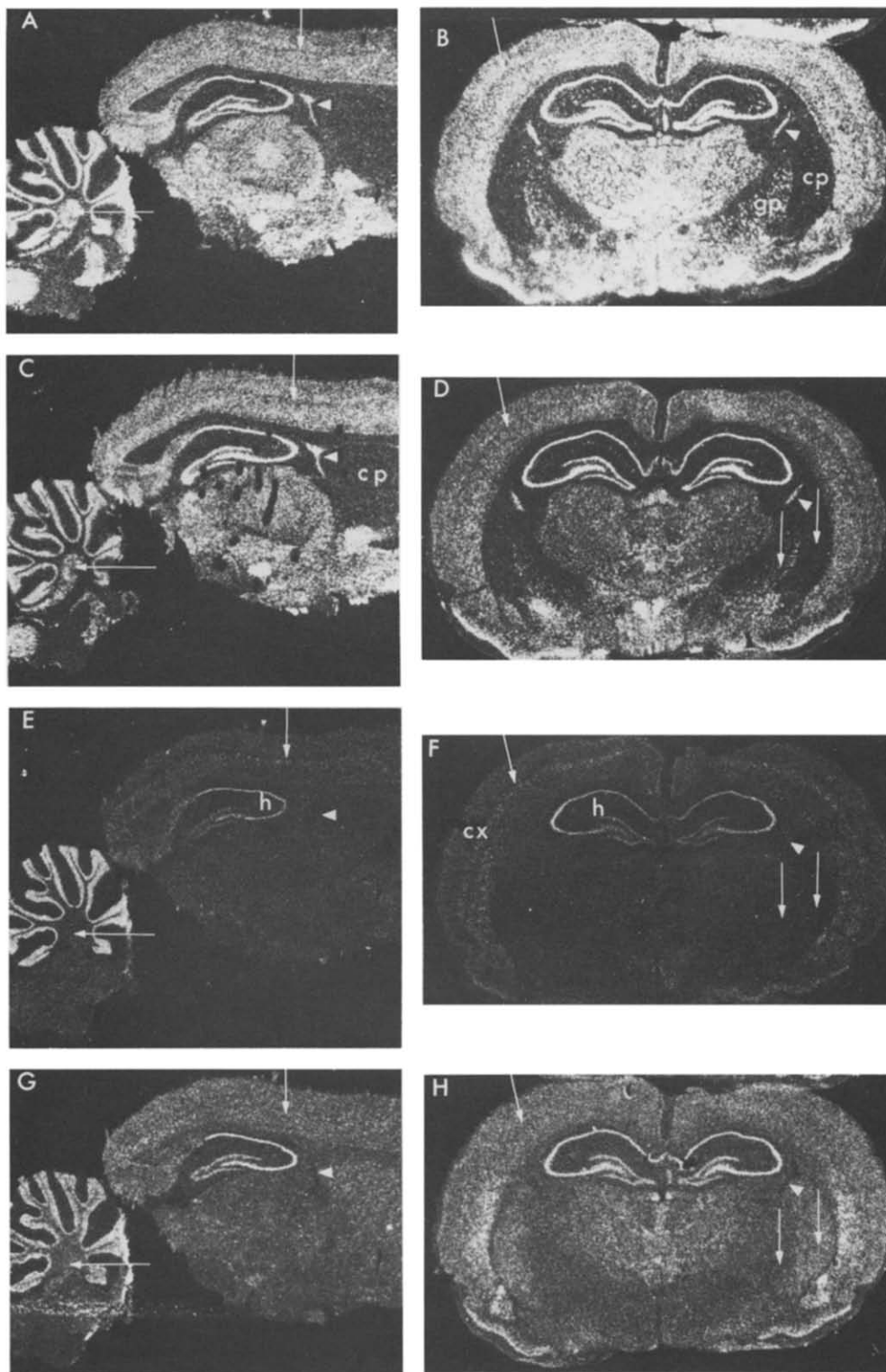


Table 1

Relative abundance of mRNAs coding for G-proteins in brain and peripheral tissues

	G <sub>s</sub>	G <sub>i2</sub>	G <sub>i1</sub>	G <sub>o</sub>
Cerebral cortex	++++	+++	++	+++
Hippocampus	++++	++++	+++	++++
Caudate putamen	+	—	—	+++
Globus pallidus	++++	+++	—	—
Cerebellum	++++	+++	+++	+++
Cortex	++++	+++	+++	+++
Lateral nuc.	++++	+++	—	—
Choroid plexus	++++	+++	—	—
Liver	+++	++	—	—
Heart	+++	++	—	—
Kidney	+++	++	—	—

Data were collected from Northern blots and in situ hybridizations. Relative amounts of the mRNAs were evaluated by visual inspection of the autoradiograms

lateral nuclei of the cerebellum, and the ependymal lining of the ventricles. Unlike the other mRNAs, G<sub>i1</sub> mRNA showed a distinct layered pattern of expression within the cerebral cortex, with a central layer having the most mRNA. Unlike the other mRNAs, G<sub>o</sub> mRNA was highly abundant in the claustrum and amygdala, relatively abundant in the caudate putamen, and had low abundance in the globus pallidus. In general, G<sub>o</sub> mRNA was more homogeneously distributed than G<sub>s</sub>, G<sub>i1</sub> and G<sub>i2</sub> mRNAs in the rat brain. A summary of the distributions of G-protein mRNAs as determined from Northern blots and in situ hybridization is presented in table 1.

#### 4. DISCUSSION

The specificity of cDNA-mRNA hybridizations was verified in several ways. (i) All of the probes were complementary to regions of the G-protein mRNAs which showed the least homology between each other [13], and the hybridizations and washes were performed under conditions which precluded hybridization of the probes to the related mRNAs. (ii) Using stringencies of hybridization identical to those used for in situ hybridizations, all of the probes identified mRNA species on Northern blots with the same size as previously reported using

long cloned probes [14–18,24,25,27,28]. We suggest that the additional, larger, mRNA species observed with the G<sub>s</sub> and G<sub>o</sub> probes may represent unprocessed RNA transcripts. (iii) The patterns of in situ hybridizations were compared with data which we have previously collected using similar methods to localize other mRNAs present in brain (i.e. neuropeptides [29]) and not present in brain (i.e. transducin and opsin [30,31]). All of the patterns reported here are unique for G-protein mRNAs, and nonspecific binding of oligonucleotides to lipids and other non-mRNA species were not detectable at the exposure times used (defined with the probes to the mRNAs not present in brain).

Clearly, the G-protein mRNAs are heterogeneously expressed in the rat brain and have widely differing concentrations. The relative abundance of these mRNAs is surprising in light of the concentrations of the proteins for which they encode, i.e. G<sub>o</sub> > G<sub>i</sub> > G<sub>s</sub> [34]. The relative abundance of G<sub>i1</sub> vs G<sub>i2</sub> protein is unknown, however, since G<sub>i1</sub> has the sequence which has been determined from protein purified from brain, it is probable that G<sub>i1</sub> is more abundant than G<sub>i2</sub>. The lack of concordance in the mRNA and protein levels implies that the proteins are turned over at different rates and/or these mRNAs have markedly different translational efficiencies.

The pattern of distribution of G<sub>s</sub> mRNA is in striking contrast to the pattern of distribution previously reported for [<sup>3</sup>H]forskolin binding sites. [<sup>3</sup>H]Forskolin binds with high affinity to a complex between G<sub>s</sub> and adenylate cyclase, and these sites are highly enriched in the caudate putamen relative to the cerebral cortex and globus pallidus. Furthermore, lesion studies indicate [<sup>3</sup>H]forskolin binding sites are synthesized by neurons intrinsic to the caudate putamen [35]. Thus, the levels of expression of G<sub>s</sub> mRNA may not be directly related to the density of complexes between G<sub>s</sub> and adenylate cyclase. It is tempting to speculate that in the cortex and globus pallidus G<sub>s</sub> may complex with some effector protein in addition to adenylate cyclase. These interpretations must be considered with caution as [<sup>3</sup>H]forskolin binding sites have not been extensively characterized in terms of a possible molecular diversity and whether they are universally associated with adenylate cyclase.

The coexpression of G<sub>s</sub> and G<sub>i2</sub> mRNAs in all of

the brain regions examined suggests that the transcription of these two mRNAs is related. The coexpression of  $G_s$  and  $G_{i2}$  mRNA, but not  $G_{i1}$  mRNA, also provides indirect evidence that of the two  $G_i$  mRNAs,  $G_{i2}$  mRNA is more likely to be functionally associated with  $G_s$ , for example as an inhibitor of adenylate cyclase. In addition to adenylate cyclase inhibition, purified  $G_i$ -like proteins have been shown to couple with  $K^+$  channels [10]. Since  $G_{i1}$  mRNA is highly enriched in neuronal tissues, and neuronal tissues would be expected to be enriched with respect to these  $K^+$  channels, it is tempting to suggest that  $G_{i1}$  may be associated with this or other effectors enriched in neurons. A similar relationship is suggested by the high abundance of  $G_o$  mRNA in the brain relative to the peripheral tissues, as  $G_o$  couples with certain calcium channels [11] and these calcium channels are associated with neuronal tissues. Of course these tentative assignments of function must be tested using transfection of G-protein cDNAs and reconstitution of expressed or highly purified G-proteins.

The regionally specialized expression of  $G_s$  and  $G_{i2}$  mRNA vs  $G_o$  and  $G_{i1}$  mRNA strongly suggests that different neuronal pathways may predominantly use distinct G-protein-mediated signal-transduction systems. This regional expression of G-protein mRNAs may be related to both the neurotransmitter and effector phenotypes of cells within the individual brain regions. For example, in the caudate putamen cells predominantly synthesize mRNA encoding  $G_o$ , suggestive of a role for  $G_o$  in signal transduction of neurotransmitter receptors in the caudate. An analogous cell-specific expression of G-proteins has recently been shown in the retina. Transducin consists of two forms, one of which is expressed exclusively in cones and the other in rods [36].

These global relationships should only be considered as first approximations as all of the brain regions examined are complex mixtures of many cell types. Perhaps the most powerful predictions from these data will only be made when individual receptors and receptor mRNAs are colocalized with G-proteins and G-protein mRNAs within cells which have been defined in terms of their neurotransmitter and effector phenotypes. Work with neuropeptide mRNAs [29], protein kinase C mRNAs [37], rat muscarinic receptor mRNAs [38],

and brain nicotinic receptors [39,40] indicates the feasibility of this approach.

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