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Activated Mutants of the α Subunit of G_o Promote an Increased Number of Neurites per Cell

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The high concentration of the GTP-binding protein G_o in the neuronal growth cone suggests that G_o activation state may alter neurite outgrowth. We find that activation of pertussis toxin–sensitive G-proteins by mastoparan increases neurite outgrowth from neuroblastoma cells. To examine G_o activation specifically, point mutations homologous to activating, oncogenic mutations in α_{i2} and α_s were introduced into the α subunit of G_o. The stability of the α_o mutants to tryptic digestion confirms that they are activated. When expressed in PC12 or N1E-115 cells, activated α_o doubles total neurite length per cell, primarily by increasing the number of neurites per cell. The growth cones of cells expressing activated α_o are narrower than control growth cones. Expression of wild-type α_o or the activated α subunits of other G-proteins did not affect total neurite length per cell. Thus, factors that lead to activation of G_o can modulate neurite number per cell.

[Key words: GTP-binding proteins, neuronal growth cone, G_o, neurite outgrowth, signal transduction, oncogenic mutations, axonal growth, PC12 cells, neuroblastoma cells]

The distal tip of neuritic processes, the growth cone, distinguishes between multiple pathways and synaptic targets during development and regeneration (Lockerbie, 1987; Strittmatter and Fishman, 1991). The high sensitivity of the growth cone to small gradients of attractive and repulsive signals in the environment, and the ability of a single filopodial contact to rearrange rapidly growth cone and axonal morphology suggest that it contains a powerful signal amplification system. Growth cone membranes can be enriched by a subcellular fractionation method (Pfenninger et al., 1983), and contain very high levels of the heterotrimeric GTP-binding protein G_o (Strittmatter et al., 1990). This implies that the activation state of G_o may be an important determinant of growth cone motility (Strittmatter and Fishman, 1991; Strittmatter, 1992).

As yet, no data directly confirm a role for G_o in growth cone function. However, several studies demonstrate that, as a class, heterotrimeric G-proteins participate in the regulation of growth cone physiology for specific cell types. 5-HT (Haydon et al., 1984), dopamine (Lankford et al., 1988; Rodrigues and Dowling, 1990), and thrombin (Suidan et al., 1992) all interact with G-protein–coupled receptors, causing growth cone collapse and the cessation of neurite extension. Growth cone collapse (Raper and Kapfhammer, 1990) induced by membrane-associated components of embryonic brain and of CNS myelin is blocked by pertussis toxin, implicating a signal-transducing pertussis toxin–sensitive G-protein in growth cone collapse (Igarashi et al., 1992). Some of the actions of the cell adhesion molecules NCAM, L1, and N-cadherin may also be mediated by G-proteins. Antibodies to NCAM and L1 decrease phosphoinositide hydrolysis and increase intracellular calcium levels in PC12 cells by a pertussis toxin–sensitive mechanism (Schuch et al., 1989). NCAM- and N-cadherin-induced neurite outgrowth from PC12 cells can be blocked by pertussis toxin (Doherty et al., 1991). G-protein stimulation, initiated by the introduction of GTPγS into embryonic chick sympathetic neurons, decreases neurite extension (Strittmatter et al., 1992). Taken together, these studies indicate that G-protein activation state can alter growth cone motility in certain circumstances, but specifically which G-proteins are involved and whether they are inhibitory or stimulatory in a particular cell type remain unclear.

Heterotrimeric G-proteins are composed of α, β, and γ subunits, and it is the α subunit that binds GTP and transduces signals to various second messenger systems (Gilman, 1987). Pharmacologic and mutational methods can be employed to alter G-protein activity. G_o and G_i can be activated by a wasp venom peptide, mastoparan (Higashijima et al., 1990). Stimulation of G_o and G_i by either receptor or mastoparan is blocked by pretreatment with pertussis toxin (Gilman, 1987; Higashijima et al., 1990). Several mutant α subunits of heterotrimeric G-proteins are constitutively activated and are oncogenic for specific cells. A Q227L mutant of α_s was first identified in human growth hormone–secreting pituitary tumors (Landis et al., 1989). This mutant is activated by virtue of decreased GTPase activity, which locks the protein in an α_s–GTP conformation and prevents the normal "turn-off" reaction that yields inactive α_s–GDP (Graziano and Gilman, 1989; Masters et al., 1989). This glutamine^{227} residue is homologous to one of the frequent GTPase-inhibiting oncogenic mutations of p21^{ras}. A second set of oncogenic, activating point mutations of α_s occurs at Arg^{201}, the residue that can be ADP-ribosylated by cholera toxin (Landis et al., 1989). In addition to these *gsp* mutations in α_s, *gip* mutations have been described for α_{i2}, and these are associated with adrenal cortical tumors and endocrine tumors of the ovary (Lyons et al., 1990; Wong et al., 1991). Analysis of adenylate cyclase activity and cAMP levels in extracts or cells containing

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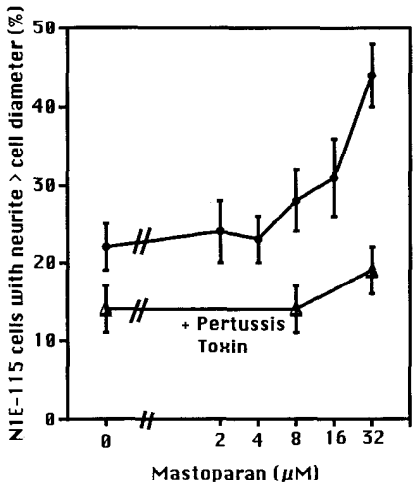


Figure 1. G-protein activation increases neurite outgrowth. Differentiated N1E-115 were plated on PLL/laminin-coated glass for 4 hr. The percentage of cells with a neurite longer than one cell diameter was measured in the presence of the indicated concentrations of mastoparan (●). Pertussis toxin at 600 ng/ml was added to some cultures (△). Note that neurite extension is increased by mastoparan in a pertussis toxin–sensitive fashion. The mean ± SEM from six experiments is shown.

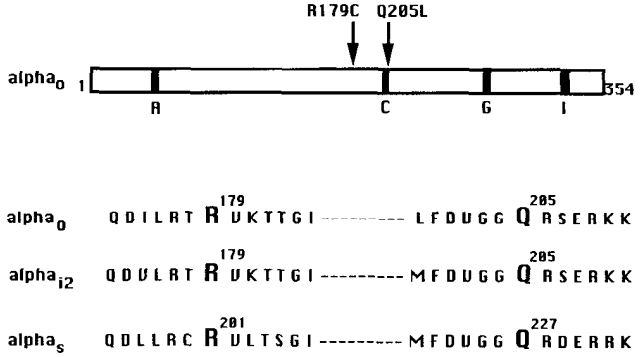


Figure 2. Activating mutations of G_α subunits. A schematic diagram of the α_oA protein and the residues surrounding the R179C and Q205L mutations is illustrated. The amino acid sequences of rat α_s, α_{i2}, and α_oA are aligned to demonstrate the high degree of sequence conservation around the sites of these point mutations that are known to activate α_s and α_{i2} (Jones and Reed, 1987; Landis et al., 1989; Lyons et al., 1990). The domains thought to be involved in GTP binding and hydrolysis are indicated *A, C, G,* and *I.*

these α_s and α_{i2} mutants demonstrates that the activated proteins maintain their specificity for cyclase activation or inhibition (Graziano and Gilman, 1989; Masters et al., 1989; Lyons et al., 1990; Wong et al., 1991).

In order to assess whether G_o has a regulatory role in neurite outgrowth, we used G-protein–specific reagents and the expression of mutant, constitutively activated α_o proteins. We find that when activated α_o is expressed in PC12 or N1E-115 cells, neurite outgrowth is increased.

Materials and Methods

Construction of α_o expression vectors. The XhoI and SphI sites were deleted from the cytomegalovirus (CMV)-based eukaryotic expression vector pcDNA1 (Invitrogen) by digestion with PstI and NsiI, and religation. A 2 kilobase EcoRI DNA fragment containing the entire rat α_oA coding sequence (Jones and Reed, 1987) was subcloned into this vector. To mutate arginine^{179} to cysteine, two restriction enzyme-digested, polymerase chain reaction (PCR)–derived DNA fragments were ligated between the XhoI site at nucleotide 37 and the SphI site at position 726 of the α_o coding region in pcDNA1–α_o. The first fragment extended from the XhoI site to position 549, and was generated with the oligonucleotides 5′-AGAGCCGCCCTCGAGCGGAGCAAGGCG-3′ and 5′-GACGCAGGTCCGGAGGATGTCCTGCTCGGT-3′. The oligonucleotide at 3′ end of this fragment encodes the R179C mutation, and a conservative base pair change at position 531, which introduces a new BspEI site. The second PCR fragment extended from position 322 to the SphI site, and was synthesized with the oligonucleotides 5′-ATCCTCCGGACCTGCGTCAAAACAACTGGC-3′ (encoding the new BspEI site and the R179C mutation) and 5′-GAGAGACT-CGTGCATGCGGTTCGTGGT-3′. The α_o Q205L mutation was introduced in a similar fashion. One PCR-derived DNA fragment extended from the XhoI site to include the Q205L substitution, and was created using the oligonucleotides 5′-AGAGCCGCCCTCGAGCGGA-GCAAGGCG-3′ and 5′-AGATCTTAAGCCCCCAACGTCAAACA-GCCT-3′. A new AflII site was introduced at the site of the substitution. The second fragment extended from the Q205L mutation to the SphI site of the α_o sequence, and was synthesized with the oligonucleotides 5′-GGGGGCTTAAGATCTGAACGTAAGAAGTGG-3′ and 5′-GA-GAGACTCGTGCATGCGGTTCGTGGT-3′. The G204A mutant was created by exactly the same method as for the Q205L mutant except that the oligonucleotides were 5′-GACGTTGGCGCCCAGCGATCT-GAACGT-3′, and 5′-TCGCTGGGCGCCAACGTCAAACAGCCT-3′, and the new restriction site was NarI. The DNA sequence of the entire PCR-derived sequence from the XhoI site to the SphI site, including the ligation sites, was confirmed by the dideoxy chain termination method.

The rat α_oB Q205L mutant was a generous gift of T. Okamoto and I. Nishimoto (Massachusetts General Hospital, Charlestown, MA). It was derived from α_oA Q205L by removing the 3′ portion of α_oA, from the SphI site to the EcoRI site, and substituting the corresponding fragment from α_oB.

Other expression vectors. The activated mutants of rat α_{i1}, mouse α_{i2}, rat α_{i3}, rat α_s, mouse α_q, and rat α_z are all contained in the pcDNA1 vector and were generously provided by H. Bourne and colleagues (Pace et al., 1991; Wong et al., 1991; Conklin et al., 1992).

The pCMV–βgal vector was created by ligating the CMV promoter contained in the SpeI–HindIII fragment of pCDM8 (Invitrogen), and the *lacZ* gene in the HindIII–BamHI fragment of pCH110 (Pharmacia) to the XbaI and BamHI sites of pSP72 (Promega).

Tryptic sensitivity of α_o mutants. RNA encoding the wild-type α_o and mutant α_o sequences was synthesized from linearized plasmids using T7 RNA polymerase. ^{35}S-labeled α_o proteins were translated from this RNA using a rabbit reticulocyte lysate system (Promega). To examine tryptic sensitivity, material from the translation reactions was incubated in 40 μl of 10 μg/ml trypsin-TPCK in 10 mM Na-HEPES pH 7.8, 100 mM NaCl, 100 μM $MgCl_2$ for 15 min at 30°C, in the presence of 200 μM GTP or 200 μM GTPγS. The reaction was terminated by the addition of soybean trypsin inhibitor to 20 μg/ml. The samples were boiled in 2% SDS, separated by SDS-PAGE, and analyzed by fluorography with enHance (New England Nuclear).

Cell culture and transfection. COS-7 cells were transfected with equal amounts of different DNAs by electroporation and 40 hr later cells were scraped from the plate. One hundred micrograms of total cellular protein were analyzed for α_o reactivity on immunoblots using the α_o-specific antibody GC2 (New England Nuclear). Bound antibody was detected by an avidin-biotin complex method (Vectastain) with tetramethylbenzidine as a peroxidase substrate.

PC12K cells were maintained in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, penicillin, and streptomycin. For studies of differentiated cells, NGF (100 ng/ml) was added to the cultures for 4–7 d prior to analysis. N1E-115 cells were maintained in F12 medium with 10% fetal bovine serum, penicillin, and streptomycin. To induce differentiation, the cells were cultured in the presence of 2% dimethyl sulfoxide (DMSO) for 7 d prior to analysis.

For transient transfections of PC12 cells, cultures were differentiated in the presence of NGF for 7 d, and then transfected with 3 μg of a pcDNA1-derived vector containing sequences for various α subunits, together with 0.4 μg of pCMV–βgal in a 60 mm dish by the lipofectamine method (GIBCO). In general, neurite outgrowth from transfected cultures was analyzed 40 hr later.

To isolate the stably transfected clones, a neomycin resistance gene expression vector, pDOJ (Bloch et al., 1989), and a sevenfold molar excess of pcDNA1 or one of the α_o expression vectors derived from

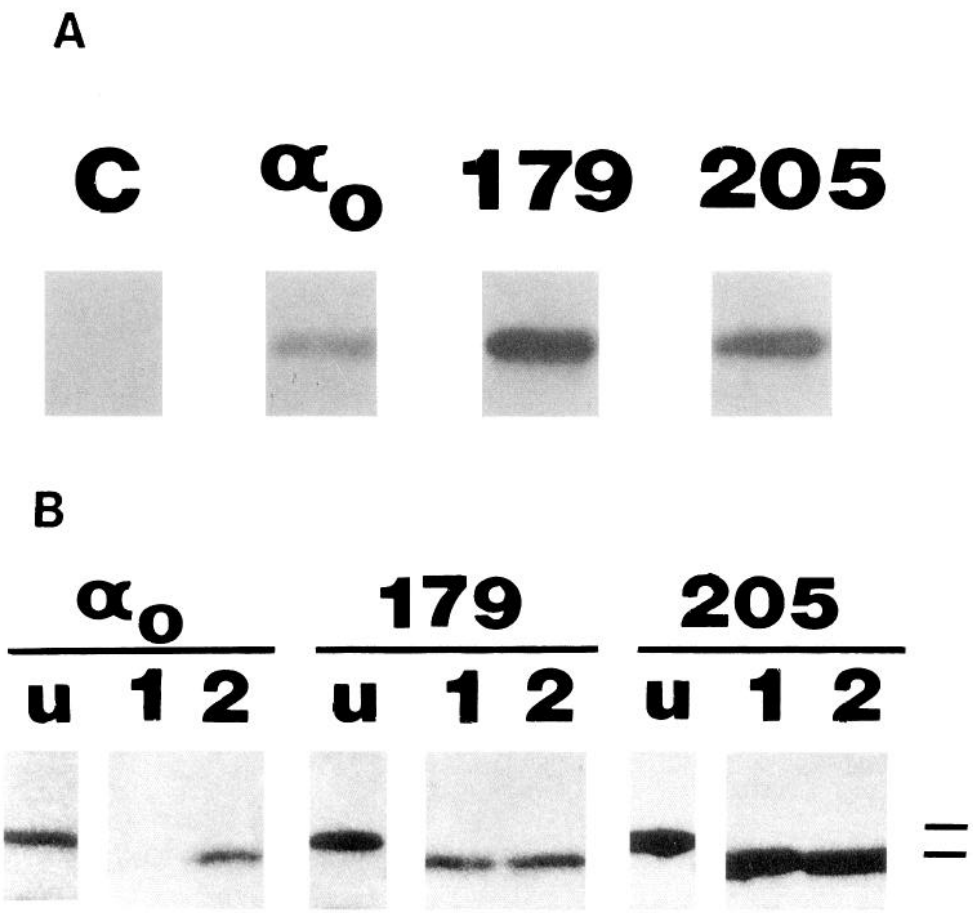


Figure 3. Expression of activated α_oA protein. *A*, COS-7 cells were transfected with the control plasmid pcDNA1 (*C*), with pcDNA1–α_oA (α_o), with pcDNA1–α_oA R179C (*179*), or with pcDNA1–α_oA Q205L (*205*) and analyzed for α_o immunoreactivity. Control cells have no detectable α_o, but all of the expression vectors are capable of inducing detectable levels of α_o immunoreactivity of the appropriate molecular weight, 39 kDa. *B*, A fluorogram demonstrates the stability of wild-type α_oA and the two mutant α_oA proteins to trypsin. For each protein, *in vitro* transcribed/translated material was incubated without trypsin (*u*), with trypsin and GTP (*1*), or with trypsin plus GTPγS (*2*). The wild-type protein has a molecular weight of 39 kDa (*upper marker at right*), and in the presence of trypsin and GTP the protein is completely degraded to much smaller fragments, as reported previously (Hurley et al., 1984; Winslow et al., 1986). In the presence of GTPγS, a 37 kDa form (*lower marker at right*), is stabilized to further tryptic digestion. In contrast to the wild-type protein, both the R179C and Q205L mutants are as effectively stabilized by GTP as by GTPγS to tryptic degradation.

pcDNA1 were cotransfected by the calcium phosphate procedure. Stable transfectants were selected in the presence of 300 μg/ml G418. After clones were isolated, G418 was not present in the culture medium. All experiments were completed with fewer than 10 passages from the initial isolation of the clones.

Neurite outgrowth assay. For stably transfected clones, 30–70% confluent cultures of differentiated or undifferentiated PC12 or N1E-115 cells were trypsinized with 0.25% trypsin, 1 mM EDTA at room temperature for 5 min. A 50-fold volume excess of complete medium was added to the cells, and then appropriate dilutions were replated onto glass slides precoated with poly-L-lysine (PLL), 0.1 mg/ml for 1 hr, and with laminin, 0 or 10 μg/ml for 1 hr. After a 3–8 hr incubation at 37°C, the cells were fixed in PBS, 2% glutaraldehyde, and then stained with Coomassie blue.

In transient expression experiments, cells were trypsinized 40 hr after transfection, and then incubated for 3–24 hr in six-well plastic dishes before fixation. The presence of β-galactosidase (βgal) in 2–20% of the cells was detected by incubation with Xgal (5-bromo-4-chloro-3-indolyl-β-D-galatopyranoside), and only those cells staining blue were analyzed morphologically. The percentage of cells stained was not altered by any of the α subunit expression vectors (not shown).

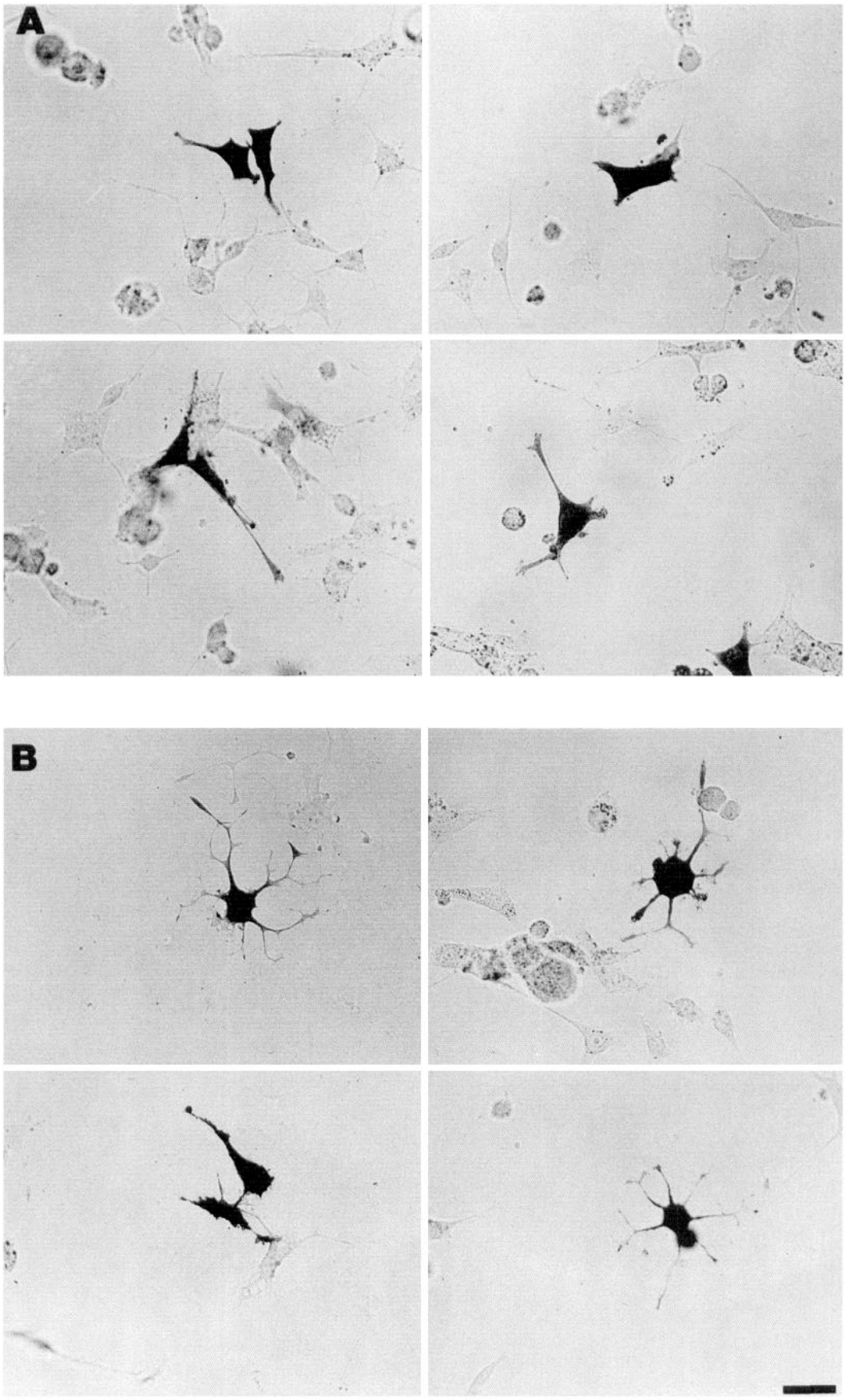
Aspects of cellular morphology were quantitated for 75–300 consecutive cells for each experiment by an observer unaware of the protocol. The number of primary neurites per cell was defined as the number of separate protrusions from the cell body of greater than 5 μm. For each neurite the length of the longest branch was measured. Minor branches were not included in the measurements. Thus, neurite length per neurite was defined as the distance from the cell body to the tip of the longest branch derived from that neurite. Neurite length per cell was defined as the sum of the distances from the cell body to the most distal tip of each primary neurite derived from that cell. Branching behavior was not qualitatively different between groups and was not quantitated separately. In a number of cases, cells were scored positive or negative for any neurite with a length from cell body to distal tip that exceeded the cell diameter. This simple measure of neurite outgrowth does not distinguish between the rate of individual components of neurite outgrowth such as neurite extension, retraction, formation, and dissolution. The statistical significance of differences between groups of clones was analyzed by a two-tailed Student's *t* test.

Cell adhesion assay. PC12 clones were differentiated with NGF for 4 d and then cultured overnight in the presence of 3 μCi/ml ^{35}S-methionine in NGF-containing PC12 medium. Adhesion was then assayed by the method of Lotz et al. (1989) and of Murray and Jensen (1992). In brief, the cells were trypsinized, washed in PBS, and then resuspended in PBS with 2 mg/ml BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$. Cells were plated into 96-well plates precoated with laminin or BSA. After incubating for 15 min at 0°C or 37°C, the plates were covered with a second medium-filled plate, sealed with carpet tape, inverted, and centrifuged at 80 × *g* for 10 min. The top and bottom wells were cut from the plates and radioactivity was quantitated by liquid scintillation spectrophotometry. The percentage of adherent cells was defined as the fraction of the total cpm that remained attached to the top well during centrifugation.

Results

Activation of pertussis toxin–sensitive G-proteins increases neurite outgrowth from neuroblastoma cells

The initial rate of neurite outgrowth from differentiated N1E-115 neuroblastoma cells was assayed by counting the percentage of cells with a neurite longer than one cell diameter after a 4 hr incubation on a PLL/laminin surface. Mastoparan nearly doubles neurite outgrowth at a concentration of 30 μM (Fig. 1). Pertussis toxin blocks the effect of mastoparan and decreases basal outgrowth slightly. Thus, activation of a pertussis toxin–sensitive G-protein in these cells increases neurite outgrowth. The decrease caused by pertussis toxin suggests that there is endogenous receptor activation of G-proteins in this culture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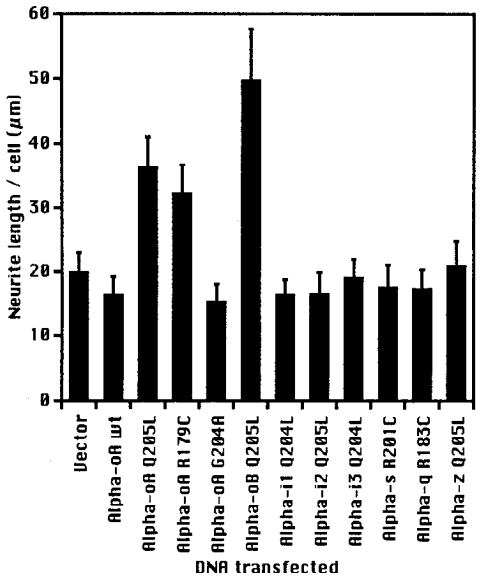

Figure 5. The effect of different α subunits on neurite outgrowth. Differentiated PC12 were transfected with different α subunit vectors as described in Figure 4. The neurite length per cell was determined for those cells expressing βgal. Note that activated mutants of α_oA (Q205L, R179C) increase neurite outgrowth significantly ($p < 0.01$). Activated α_oB (Q205L) also stimulates neurite outgrowth ($p < 0.001$). Wild-type α_oA and activated mutants of other α subunits have no effect. The values are means ± SEM for 76 cells per transfection. This is one of three experiments with similar results.

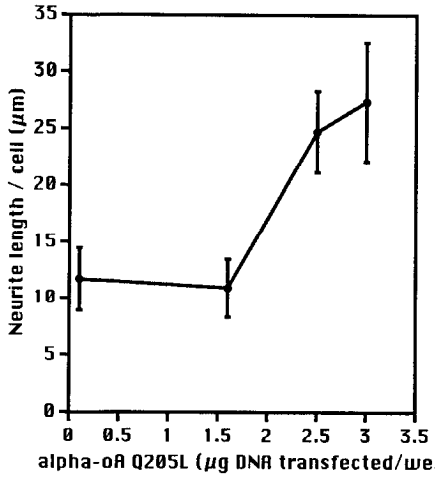

Figure 6. The stimulation of neurite outgrowth by α_oA Q205L is dependent on the amount of DNA transfected. Differentiated PC12 cells were transfected with 0.4 μg of pCMV–βgal and 3 μg of a mixture of pcDNA1 and α_oA Q205L vector. The amount of α_oA Q205L vector transfected is indicated. Cells were incubated and neurite outgrowth was determined as for Figures 4 and 5. The values are means ± SEM for 76 cells per transfection. Neurite outgrowth is significantly increased ($p < 0.01$) at 2.4 and 3.0 μg of α_oA Q205L vector transfected.

system that can be prevented by the toxin. The data do not indicate which toxin-sensitive G-protein(s) mediates these changes.

Point mutations activate α_o

The domains of α_s and α_{i2} in which point mutations produce activated, oncogenic proteins are highly conserved. The mutated residues and their sequence similarity with α_o are shown in Figure 2. We introduced a substitution of cysteine for arginine at position 179 and also leucine for glutamine at position 205 in the α_o sequence. These mutations were made in the α_oA alternate splice form, which is the most prominent species in brain tissue (Strathmann et al., 1990). Mutated and wild-type α_oA constructs were expressed in COS cells using a CMV-based eukaryotic expression vector. Immunoblot analysis of transiently transfected cells demonstrates that protein of the appropriate molecular weight is produced from all three vectors (Fig. 3*A*).

The activation state of G-proteins can be monitored by their tryptic sensitivity (Hurley et al., 1984; Graziano and Gilman, 1989; Masters et al., 1989; Denker et al., 1992). In the absence of guanine nucleotides or in the presence of GTP, wild-type α_oA is rapidly degraded by trypsin. However, in the presence of GTPγS, α_oA assumes an activated confirmation that is cleaved by trypsin from 39 kDa to 37 kDa, but then is stable upon further incubation with trypsin (Hurley et al., 1984; Winslow et al., 1986; Denker et al., 1992). Because activated oncogenic α_s mutants have impaired GTPase function, GTP is as effective as GTPγS at stabilizing these mutants (Graziano and Gilman, 1989; Masters et al., 1989). To test the tryptic sensitivity of mutated α_o proteins, we synthesized ^{35}S-labeled protein by *in vitro* transcription/translation. As expected, the wild-type α_oA protein is rapidly degraded in the presence of GTP, but is stabilized in a slightly smaller form by the presence of GTPγS (Fig. 3*B*). In contrast, both of the point-mutated α_oA proteins can be stabilized against tryptic digestion by the presence of GTP as effectively as GTPγS (Fig. 3*B*). Most likely this is because they have impaired GTP hydrolysis and, after binding GTP, remain in an activated confirmation. The same conclusion has been reached for the α_o Q205L mutant produced in *Escherichia coli* by Slepak et al. (1993).

Neurite number is increased by activated α_oA

To examine the effect of activated α_oA on neurite extension, we transfected these wild-type and mutant α_oA expression vectors into PC12 pheochromocytoma cells. Transiently transfected cells were identified by staining for the presence of βgal expressed from a cotransfected *lacZ* vector, which utilizes the same CMV promoter as do the G-protein expression vectors. This enzyme marker for transfected cells was reliably detected in 2–20% of transfected cells examined 36–72 hr after transfection (Fig. 4). The extension of neurites from cells replated 40 hr posttransfection was compared. The expression of the Q205L or the R179C α_oA mutant approximately doubles the degree of neurite outgrowth per cell, as compared to vector or wild-type α_oA (Figs.

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Figure 4. Transfection of α_oA Q205L increases neurite outgrowth. Differentiated PC12 cells were transfected with pcDNA1 and pCMV–βgal (*A*), or with α_oA Q205L vector and pCMV–βgal (*B*). After 40 hr, the transfected cells were replated on plastic dishes, incubated for 24 hr, and then stained for βgal expression. Note that about 10% of the cells are expressing the *lacZ* gene in both cases. Transfection of α_oA Q205L vector increases neurite outgrowth from the stained cells primarily by increasing the number of neurites. Also note that several of the growth cones in the control transfection are broadened, whereas the growth cones of the α_o Q205L cells are of lesser width. Scale bar, 20 μm.

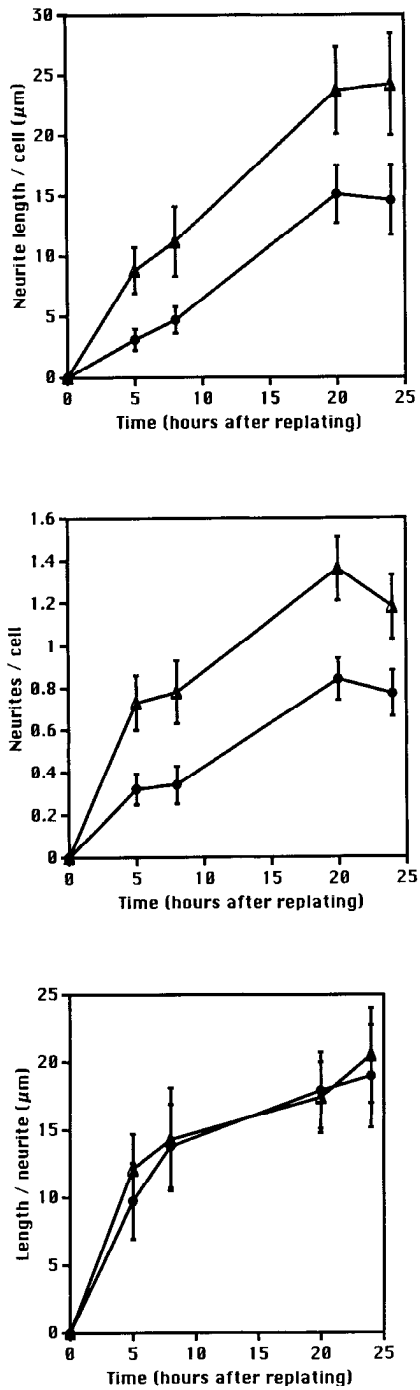

Figure 7. The expression of α_oA Q205L increases neurite number shortly after plating PC12 cells. Differentiated PC12 cells were transfected with pcDNA1 and pCMV–βgal (●), or with α_oA Q205L vector and pCMV–βgal (△). Forty hours later the cells were trypsinized, replated, and incubated for the indicated times before fixation. The morphology of cells staining for βgal was analyzed for neurite length per cell (*A*), number of neurites per cell (*B*), and neurite length per neurite (*C*). The α_oA Q205L vector significantly increases neurite length/cell ($p < 0.05$) and neurite number/cell ($p < 0.02$) at all time points. The values are means ± SEM for 76 cells from one experiment. Similar results were obtained in three separate experiments.

4, 5). The effect of the Q205L mutant is dose dependent for the amount of DNA added to the transfection (Fig. 6). Because the transfection includes a sevenfold molar excess of the G-protein expression vector over the CMV–βgal vector, most stained cells are expected to express the transfected G-protein. If some stained cells do not express the G-protein of interest, then the effects of G-protein expression would be greater than measured here.

To better characterize the stimulatory effect of the Q205L α_oA mutant on neurite outgrowth, we measured the length and number of neurites at various times after plating. The cells transfected with activated α_o exhibit more neurite length per cell during a period 5–24 hr after plating on plastic and the effect is greatest at the earliest time points, just as neurites are forming (Fig. 7*A*). An increase in the number of neurites per cell accounts for nearly all of the Q205L mutant effect (Fig. 7*B*); there is no significant change in the length of the average neurite (Fig. 7*C*). Thus, activated α_o is likely to enhance neuritogenesis or stabilization under these conditions.

To confirm and extend these results, we stably transfected PC12 and N1E-115 neuroblastoma cells with the α_oA expression vectors and a neomycin resistance gene. Multiple individual clones were isolated after selection in G418 (Fig. 8). Immunoblot analysis of these clones for α_o demonstrates that the mock-transfected cells contain easily detectable amounts of α_o, and that the total level of α_o is not dramatically altered by the transfections (not shown). Thus, although we could not directly measure the proportion of activated α_oA, the R179C and Q205L clones are likely to contain an increased amount of activated α_oA without a major alteration in the total amount of α_o.

In the absence of agents that induce neuronal differentiation, both the PC12 and N1E-115 clones transfected with control plasmids or the activated α_oA constructs exhibit few or no neuritic processes (Fig. 9*C*). Treatment with NGF is known to induce neuronal differentiation in PC12 cells, and DMSO causes neurite outgrowth from N1E-115 cells. After differentiation with NGF (PC12) or DMSO (N1E-115), cells were trypsinized and replated, and neurite outgrowth was observed after 3–6 hr on a laminin substratum. There is a high degree of variability in neurite length, even among the control clones expressing the neomycin resistance gene alone (Figs. 8, 9*A*). This variability is largely attributable to differences between individual clones; repeated assays of single clones show much less variation in the data (Fig. 9*A*). The range of results is not unexpected given the known phenotypic instability of both neuroblastoma and PC12 clones. Due to the need to measure a large number of clones, we utilized a simplified measure of neurite outgrowth, the percentage of cells with a neurite longer than the cell body diameter.

Although there is considerable overlap in the results for clones from the different transfections, the α_oA Q205L mutant clones exhibit a significantly increased ($p < 0.001$) degree of neurite outgrowth as compared to control clones (Figs. 8–10). On the other hand, transfection with wild-type α_oA has no effect on the percentage of cells with the longest neurite greater than one cell diameter (Figs. 8–10). The Q205L mutant has a larger effect than the R179C mutant. The greater effect of the Q205L mutation, as compared to the R179C mutation, is similar to the

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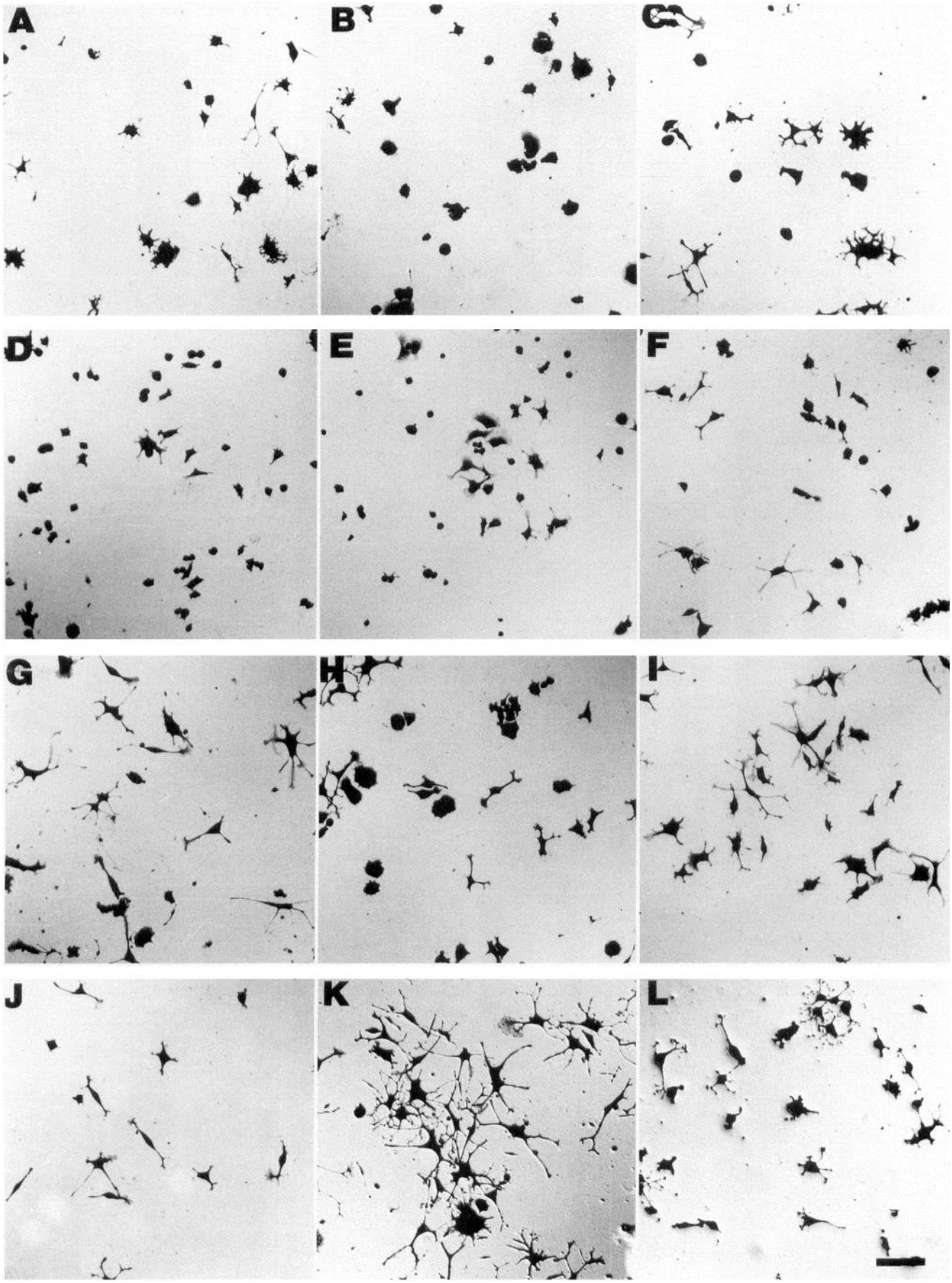
Figure 8. Increased neurite outgrowth from PC12 clones stably transfected with activated α_o. Independent PC12 clones stably transfected with control plasmid, with wild-type α_oA, with R179C α_oA, or with Q205L α_oA were differentiated in the presence of NGF and then replated on PLL/laminin for 4 hr. Note that the control cells (*A–C*) and the wild-type α_o cells (*D–F*) exhibit less neurite outgrowth than the α_oA R179C–transfected clones (*G–I*) or the α_oA Q205L–transfected clones (*J–L*). Scale bar, 30 μm.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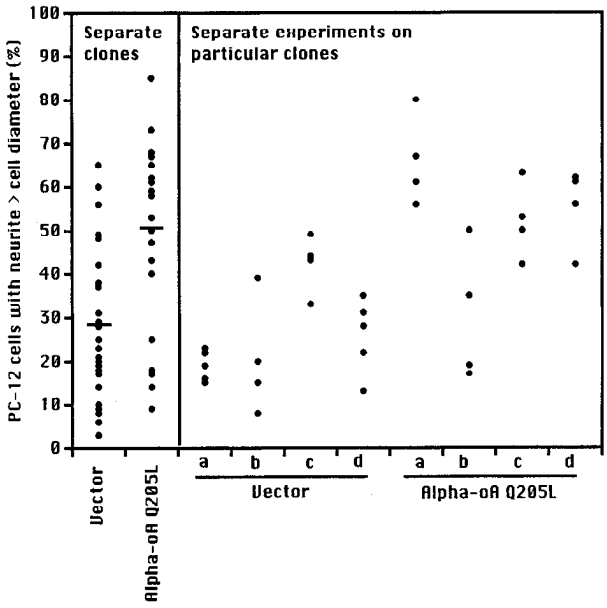


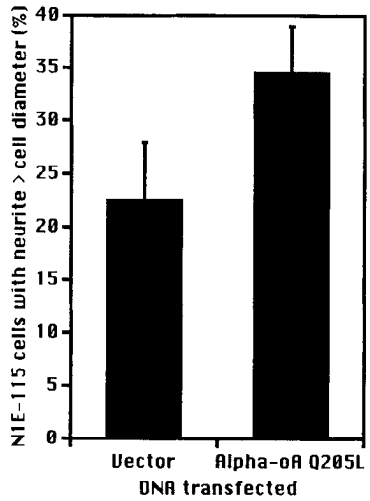
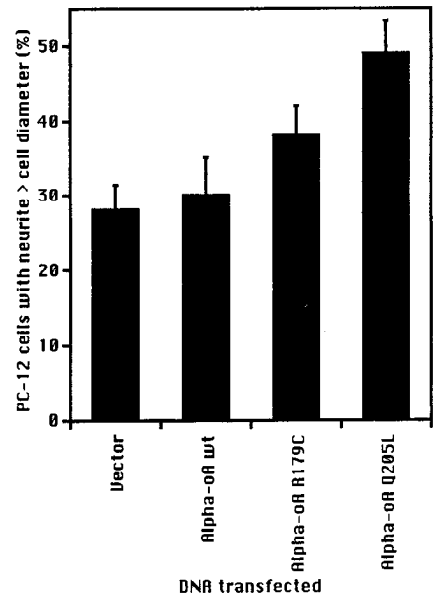
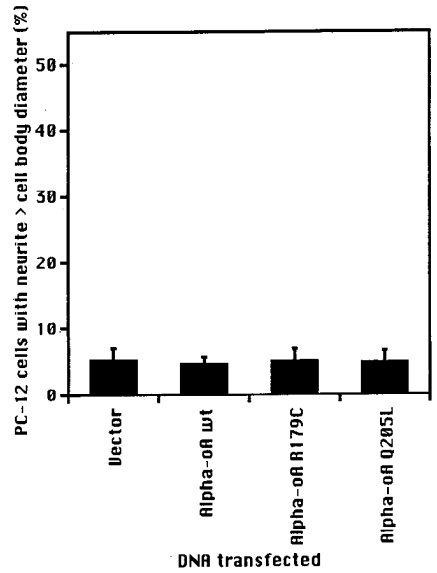


Figure 10. N1E-115 cells exhibit increased neurite outgrowth when transfected with activated α_oA. The percentage of cells with a neurite greater than one cell diameter after a 4 hr incubation on a PLL/laminin-coated surface is shown for independent clones transacted with the indicated expression vectors. All clones were differentiated in 2% DMSO for 7 d prior to plating. The means ± SEM are shown for 10–12 clones from each transfection, and each clone was analyzed in three separate experiments. The α_oA Q205L clones ($p < 0.05$) had significantly increased neurite outgrowth as compared to control clones.





effect of homologous mutations in α_{i2} on cAMP levels (Wong et al., 1991). The stably transfected clones may have lower levels of transfected α_oA than do the transiently transfected cells, in which α_oA R179C is as efficacious as α_oA Q205L. For this reason, the stably transfected clones may be more sensitive to a small difference in the degree of activation achieved by the two mutations.

To confirm that the major effect of activated α_oA is on neurite number, we counted the number of neurites per cell in different groups of PC12 clones (Fig. 11). Wild-type α_oA has no effect on neurite number, but the Q205L α_oA mutant nearly doubles the number of neurites per cell. Thus, as for the transiently transfected cells, stable transfection with α_oA Q205L augments neurite outgrowth primarily by increasing the number of neurites per cell.

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Figure 9. Quantitation of neurite outgrowth from PC12 clones expressing α_oA mutants. *A,* The percentage of cells with at least one neurite longer than the cell diameter was determined for individual clones transfected with control plasmid or with the α_oA Q205L vector. The cells were differentiated for 5 d with NGF and then plated on a PLL/laminin surface for 4 hr. Each point is a separate clone in the *left panel,* and the mean value is indicated by a *horizontal line.* The scatter of points reveals the degree of variability between clones isolated from the same transfection. In the *right panel,* each point is the value from separate experiments on four particular control and four particular α_oA Q205L clones. Note the lower level of variability in the assay procedure itself. *B,* The percentage of cells with at least one neurite longer than the cell diameter is shown for a group of independent clones transfected with each of the indicated DNAs. Cells were differentiated for 5 d with NGF and then plated on a PLL/laminin surface for 4 hr. The mean percentages with SEM for 22–27 clones in each group are shown. The α_oA Q205L clones had significantly increased neurite outgrowth as compared to control clones ($p < 0.001$). *C,* PC12 clones were analyzed in the same fashion as in *B,* except that NGF was not present. Note that there is very little neurite outgrowth, even in the α_oA Q205L–transfected clones.

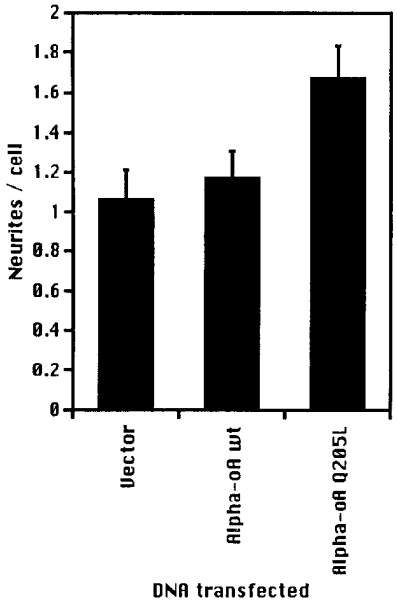


Figure 11. Activated α_oA Q205L increases neurite number in PC12 cells. The number of neurites per cell was determined for NGF-differentiated PC12 clones transfected with each of the indicated DNAs and incubated for 4 hr on a PLL/laminin-coated substrate. Note the increase in neurite number for the α_oA Q205L–transfected clones ($p < 0.001$ as compared to control clones). The data are shown as means ± SEM for 22–27 independent clones derived from each transfection, and each clone was analyzed in two separate experiments.

Growth cone morphology and substratum adhesion in mutant α_oA-transfected cells

The strength of adhesive interactions with the substratum has a major impact on the rate of neurite outgrowth from many cells. However, the action of the activated α_oA subunit is not restricted to laminin-dependent outgrowth. The assays for transiently transfected cells utilized a plastic substratum, whereas the stably transfected clones were assayed on a substratum promoting rapid neurite outgrowth, composed of both PLL and laminin. When the substratum is coated with PLL alone, a threefold longer incubation is required for PC12 clones to achieve the degree of neurite outgrowth as observed on a PLL/laminin-coated surface. However, clones transfected with α_oA Q205L show the same twofold increase in neurite outgrowth on the PLL substrate (Fig. 12*A*).

We also assessed the effect of activated α_oA on adhesion per se. Adhesive interactions of metabolically labeled PC12 cells that could withstand 80 × *g* centrifugal force were quantitated (Lotz et al., 1989; Murray and Jensen, 1992). Less than 5% of cells adhere to BSA at 37°C, or to laminin at 0°C. However, 30–40% of cells became tightly adherent to a laminin-coated surface after a 15 min incubation at 37°C (Fig. 12*B*). There was no detectable difference in adhesion between the control and α_oA Q205L–transfected clones. Activated α_oA therefore has no detectable effect on laminin-dependent adhesive mechanisms.

Because growth cone morphology has been associated with alterations in the rate of growth cone advance (Argiro et al., 1984; Tosney and Landmesser, 1985; Raper and Kapfhammer, 1990; Kaethner and Struermer, 1992), we measured the length and width of growth cones in α_oA mutant–transfected cells as compared to control cells. In transiently transfected cells, the Q205L α_oA mutant decreases growth cone width by 40%, without altering growth cone length (Fig. 13*A*). In the stably transfected clones, we determine the percentage of neuritic tips that had a diameter greater than twice the shaft of their neurite, as a simple quantitative measure of growth cone shape (Fig. 13*B*). In control and in wild-type α_oA-transfected clones, this is about 40%. Half as many of the neuritic tips from the activated α_oA Q205L mutant–transfected clones exhibit such a broad and widened morphology. Thus, expression of activated α_oA results in narrower growth cones and more numerous neurites.

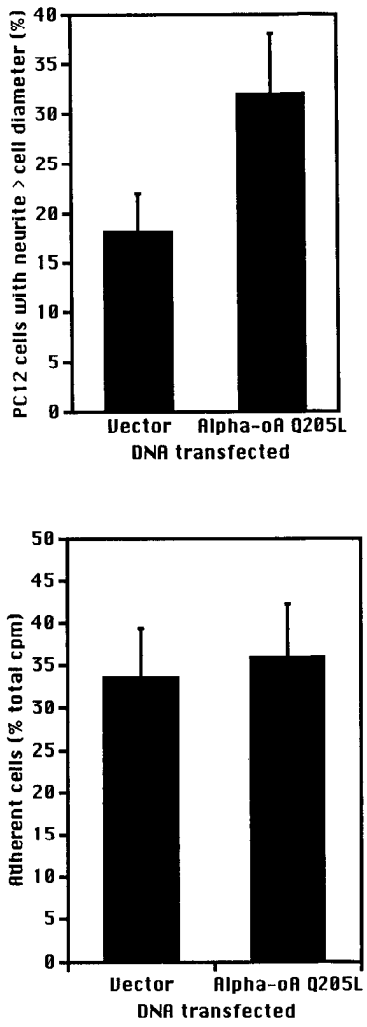
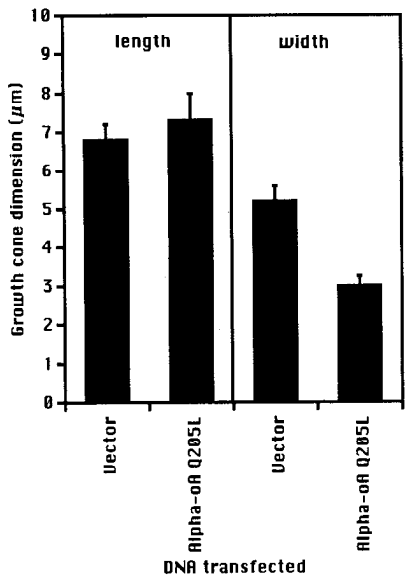


Figure 12. The effect of α_oA Q205L on neurite outgrowth is not dependent on laminin-based adhesion. *A*, Neurite outgrowth from NGF-differentiated PC12 clones transfected with the indicated DNAs was examined as for Figure 9*A*, except that the surface was coated with PLL alone and no laminin was added. The incubation time was 8 hr instead of 3–4 hr as for Figure 9*A*, because the rate of outgrowth was significantly slower without the addition of laminin. Note that the clones transfected with Q205L α_oA exhibit a significantly ($p < 0.05$) greater percentage of cells with a neurite longer than one cell diameter. The data are the mean ± SEM for 16–22 clones in each group. *B*, The adhesion of metabolically labeled NGF-differentiated PC12 clones transfected with the indicated DNAs was measured. The data are reported for adhesion after a 15 min, 37°C incubation on a laminin-coated surface. The level of adhesion on BSA-coated surfaces was less than 5% for both control and α_oA Q205L–transfected clones. The data are the mean ± SEM for eight clones in each group.

Other activated α subunits do not alter neurite outgrowth

To test the specificity of the α_oA effects, activated mutants of other α subunits were transiently transfected into PC12 cells



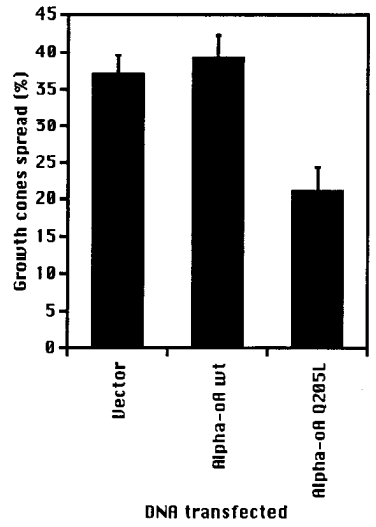


Figure 13. PC12 growth cone morphology is altered by the expression of activated α_oA. *A,* Differentiated PC12 were transiently transfected with pcDNA1 and PCMV–βgal (*Vector*), or with α_oA Q205L vector and pCMV–βgal (*Alpha-oA Q205L*). Cells were plated and stained as for Figure 4. The length and width of neuritic growth cones from βgal-expressing cells are reported as the mean ± SEM for 76 cells. The expression of α_o Q205L significantly decreased ($p < 0.001$) growth cone width. *B,* PC12 cells were transfected with the indicated expression vectors and independent stably transfected clones were analyzed after NGF-induced differentiation and incubation for 4 hr on a PLL/laminin-coated surface. The percentage of neuritic tips with a diameter greater than twice the shaft of the neurite is presented as mean ± SEM for 22–27 independent clones from each transfection. The α_oA Q205L clones have a significantly decreased ($p < 0.001$) percentage of spread growth cones as compared to control clones.

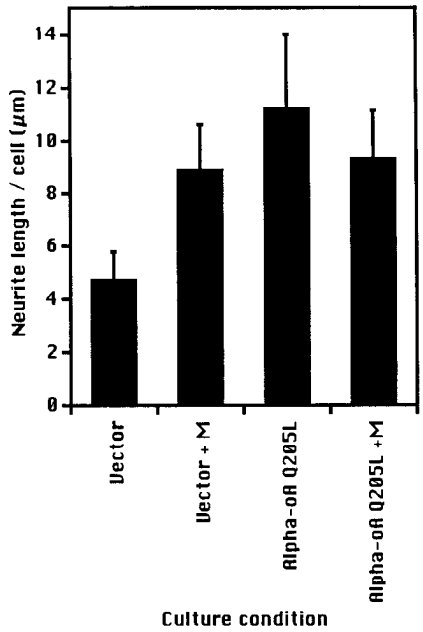


Figure 14. Mastoparan does not stimulate neurite outgrowth from α_oA Q205L–transfected cells. Differentiated PC12 cells were transiently transfected with pcDNA1 and pCMV–βgal (*Vector*), or with α_oA Q205L vector and pCMV-βgal (*Alpha-oA Q205L*). Forty hours after transfection, the cells were trypsinized and replated in the presence or absence of 30 μM mastoparan (*M*). After incubation for 8 hr the cells were fixed and the morphology of βgal-positive cells was determined. Note that mastoparan stimulates outgrowth from control cells ($p < 0.05$), but not the cells transfected with α_o Q205L. The values are the means ± SEM from 76 cells. The experiment was conducted twice with similar results.

under the same conditions (Fig. 5). A Q205L mutant of the minor splice form of α_o (α_oB) is at least as effective in increasing neurite outgrowth as the Q205L α_oA mutant. Transfection of cells with a vector encoding an inactive form of α_oA (G204A; Slepak et al., 1993) did not change neurite length (Fig. 5). Activated mutants of α_{i1}, α_{i2}, α_{i3}, α_s, α_q, and α_z have no effect on PC12 neurite length when examined in this protocol. Thus, the stimulatory effect is quite specific for α_o.

Mastoparan in α_oA Q205L–transfected cells

These data suggest that the activation of G_o specifically is responsible for an increase in neurite outgrowth. This interpretation predicts that the effect of the $G_{o/i}$ activator mastoparan should be lessened in α_oA Q205L–transfected cells since they already possess the activated form of the relevant G-protein. Indeed, whereas mastoparan significantly increases outgrowth from control PC12 cells, the agent does not alter the morphology of the α_oA Q205L–transfected cells (Fig. 14).

Discussion

Activation of pertussis toxin–sensitive G-proteins by mastoparan enhances neurite outgrowth from neuroblastoma cells. However, pharmacological agents such as mastoparan, pertussis toxin, GDPβS, and GTPγS are known to alter the activity of multiple G-proteins (Gilman, 1987; Higashijima et al., 1990). We designed mutationally activated α_o proteins to investigate G_o function in the growth cone. Mutations homologous to those that produce activated oncogenic α_{i2} and α_s appear to activate α_o. The ability of GTP to protect mutant α subunits from tryptic digestion provides evidence that these mutations inhibit GTPase function and produce constitutively activated α_o.

The effect of activated α_o is similar in PC12 cells and N1E-115 cells. It is clear that transfection of the Q205L or the R179C mutant increases neurite outgrowth as compared to transfection of control plasmids, or transfection of other activated α subunits. The enhanced neurite outgrowth is dependent on predifferen-

tiation of the cells, so the activated α_o subunit must act in concert with other cellular constituents that are induced as part of the neuronal phenotype. Although we differentiated the cells for an extended time period, it is still conceivable that activated α_o enhances outgrowth indirectly, through an influence on differentiation. One limitation of the assay is that it is not feasible to distinguish activated α_o from native α_o in neuronal cells because the electrophoretic mobility of the two proteins is identical. Therefore, we cannot correlate the level of activated α_o with neurite outgrowth in particular clones. However, the transient transfection results show a dose dependence on the amount of α_oA Q205L DNA added.

Measurements of neurite number and length at various times after plating indicate that activated α_o increases neurite number and total neurite length per cell, but does not alter the average length of neurites. The simplest interpretation is that activated α_o augments neuritogenesis with relatively little effect on the rate of extension of established neurites. Thus, the increase in neurite "outgrowth" would not be due to change in the rate of advance of individual growth cones. However, a more complicated mechanism in which activated α_o modifies neurite formation, extension, and retraction is also possible.

Several lines of evidence argue for a specific role for G_o in regulating neurite outgrowth. G_o is the major GTP-binding protein in the growth cone membrane (Strittmatter et al., 1990). Transfection of PC12 cells with other mutationally activated α subunits does not stimulate neurite outgrowth. Finally, the ability of α_oA Q205L transfection to abrogate mastoparan action on PC12 outgrowth implies that activated α_o can fully substitute for whichever G-proteins are stimulated by mastoparan.

A role for activated G-proteins in promoting neurite growth is compatible with several prior observations. For example, the ability of the cell adhesion molecules NCAM and N-cadherin to induce extended neurites in PC12 cells is blocked by pertussis toxin (Doherty et al., 1991). Experiments with purified proteins have demonstrated that the intracellular growth-associated molecule GAP-43 can stimulate G_o (Strittmatter et al., 1990, 1991, 1993; Sudo et al., 1992). In PC12 cells overexpression of GAP-43 increases neurite outgrowth caused by NGF (Yankner et al., 1990), and in neuroblastoma cells intracellular antibodies to GAP-43 can inhibit neuritogenesis (Shea et al., 1991). These studies did not carefully distinguish between GAP-43 effects on the formation of new neurites and on the rate of extension of established growth cones. Thus, the reported actions of GAP-43 could be explained by altered levels of activated α_o.

In some other neuronal cell types, G-protein activation has been associated with decreased neurite outgrowth. For example 5-HT reduces neurite outgrowth rates in certain heliosome neurons (Haydon et al., 1984), and dopamine decreases neurite outgrowth from certain retinal neurons in primary culture (Lankford et al., 1988; Rodrigues and Dowling, 1990). Thrombin acts through a G-protein–coupled receptor and causes neurite retraction from NB2a neuroblastoma (Suidan et al., 1992). In chick embryonic sympathetic neurons, activation of G-proteins inhibits neurite outgrowth (Strittmatter et al., 1992). The collapse of dorsal root ganglion cell growth cones by inhibitory membrane-bound factors is mediated by a pertussis toxin–sensitive G-protein (Igarashi et al., 1992). There are several reasons why activation of G-proteins might increase growth under some circumstances and inhibit it in others. In some cells, a pertussis toxin–sensitive G-protein other than G_o might predominate and have opposite effects on neurite extension. Alternatively, different phases of neurite outgrowth might be affected differently, such that activated G_o might enhance the formation of neurites but then increase the sensitivity of established growth cones to collapsing factors. A third possible explanation is that different cells may have different repertoires of second messenger systems to which G_o is coupled. Increased G_o activation might also have a biphasic effect on neurite extension, being stimulatory at some levels and inhibitory at others, as suggested for intracellular calcium ion concentrations (Kater and Mills, 1991).

Depending on the intracellular environment, activated G_o can couple to inhibition of calcium channels, activation of phospholipase C, inhibition of potassium channels, and stimulation of phospholipase A_2 (Bloch et al., 1989; Hille, 1992). Although still under investigation, modulation of intracellular calcium levels has been proposed as a final common pathway in growth cone regulation by many factors (Kater and Mills, 1991). Activated α_o could alter calcium levels via a direct action on plasma membrane calcium channels, via phosphoinositide-induced calcium release, or via changes in membrane potential and voltage-activated calcium channel flux initiated by variation in potassium channel openings.

Growth cone morphology as well as neurite extension is altered by activated α_o. A wide variety of growth cone shapes have been described both *in vitro* and *in vivo,* but in only a small number of cases has a correlation of growth cone appearance with function been possible (Argiro et al., 1984; Tosney and Landmesser, 1985; Patterson, 1988; Raper and Kapfhammer, 1990; Kaethner and Struermer, 1992). We have not directly correlated growth cone morphology with the rate of individual growth cone advance and retraction in our studies.

Other activated α subunits are oncogenic in certain cell types (Landis et al., 1989; Lyons et al., 1990; Pace et al., 1991), but we detected no obvious difference in the cell division rate or contact inhibition of neuroblastoma or pheochromocytoma cells transfected with activated α_o. It is possible that activated α_o would alter cell division in primary neuroblasts or neurons, but not in these transformed cell lines. Alternatively, the ability of activated α_o to alter neurite outgrowth suggests that germline or somatic mutations of the α_o sequence might lead to disorders of nervous system development, regeneration, or synaptic plasticity. Overactivity of the G-protein–coupled receptor rhodopsin has been associated with photoreceptor cell degeneration in certain forms of retinitis pigmentosa (Keen et al., 1991; Robinson et al., 1992). It will be of interest to determine whether G_o-activating mutations exist in some unexplained genetic disorders of human brain development, and also in human diseases manifesting abnormal sprouting and neuronal degeneration, such as Alzheimer's disease (Hanley and Selkoe, 1993).

These experiments have provided a means to demonstrate that a specific G-protein, α_o, is capable of modulating neurite outgrowth, as suggested by the extremely high concentrations of G_o in the growth cone membrane (Strittmatter et al., 1990). Further studies are necessary to determine which proteins act upstream of G_o in the growth cone to determine its activation state, as well as which second messenger systems function downstream to mediate α_o action. Other G-proteins and other types of transduction systems, such as receptor tyrosine kinases, may have parallel and overlapping functions in specific cell types. However, G_o may be a focal point for the convergence of multiple signals that control neurite outgrowth, including G-protein–coupled receptors, cell adhesion molecules, and intracellular growth regulators, such as GAP-43 (Strittmatter, 1992).

References

Argiro V, Bunge MB, Johnson MI (1984) Correlation between growth form and movement and their dependence on neuronal age. J Neurosci 4:3051–3062.

Bloch DB, Bonventre JV, Neer EJ, Seidman JG (1989) The G protein α_o subunit alters morphology, growth kinetics and phospholipid metabolism of somatic cells. Mol Cell Biol 9:5434–5439.

Conklin BR, Chabre O, Wong YH, Federman AD, Bourne HR (1992) Recombinant $G_q\alpha$. J Biol Chem 267:31–34.

Denker BM, Schmidt CJ, Neer EJ (1992) Promotion of the GTP-liganded state of $G_{o\alpha}$ protein by deletion of the C terminus. J Biol Chem 267:9998–10002.

Doherty P, Ashton SV, Moore SE, Walsh FS (1991) Morphoregulatory activities of NCAM and N-cadherin can be accounted for by G protein-dependent activation of L- and N-type neuronal Ca^{2+} channels. Cell 67:21–33.

Gilman AG (1987) G proteins: transducers of receptor-generated signals. Annu Rev Biochem 56:615–649.

Graziano MP, Gilman AG (1989) Synthesis in *Escherichia coli* of GTPase-deficient mutants of $G_{s\alpha}$. J Biol Chem 264:15475–15482.

Hanley DR, Selkoe DJ (1993) Amyloid precursor on the G_o. Nature 362:14–15.

Haydon PG, McCobb DP, Kater SB (1984) Serotonin selectively inhibits growth cone motility and synaptogenesis of specific identified neurons. Science 226:561–564.

Higashijima T, Burnier J, Ross EM (1990) Regulation of G_i and G_o by mastoparan, related amphiphilic peptides, and hydrophobic amines. J Biol Chem 265:14176–14186.

Hille B (1992) G protein-coupled mechanisms and nervous signaling. Neuron 9:187–195.

Hurley JB, Simon MI, Teplow DB, Robishaw JD, Gilman AG (1984) Homologies between signal transducing G proteins and *ras* gene products. Science 226:860–862.

Igarashi M, Strittmatter SM, Vartanian T, Fishman MC (1992) G protein mediation of signals that cause growth cone collapse. Science 259:77–80.

Jones DT, Reed RR (1987) Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. J Biol Chem 262:14241–14249.

Kaethner RJ, Struermer CA (1992) Dynamics of terminal arbor formation and target approach of retinotectal axons in living zebrafish embryos: a time-lapse study of single axons. J Neurosci 12:3257–3271.

Kater SB, Mills LR (1991) Regulation of growth cone behavior by calcium. J Neurosci 11:891–899.

Keen TJ, Inglehearn CF, Lester DH, Bashir R, Jay M, Bird AC, Jay B, Bhattachayra SS (1991) Autosomal dominant retinitis pigmentosa: four new mutations in rhodopsin, one of them in the retinal attachment site. Genomics 11:199–205.

Landis CA, Masters SB, Spada A, Pace AM, Bourne HR, Vallar L (1989) GTPase inhibiting mutations activate the α chain and G_s and stimulate adenylyl cyclase in human pituitary tumors. Nature 340: 692–696.

Lankford KL, DeMello FG, Klein WL (1988) D_1-type dopamine receptors inhibit growth cone motility in cultured retina neurons: evidence that neurotransmitters act as morphogenic growth regulators in the developing central nervous system. Proc Natl Acad Sci USA 95:2839–2843.

Lockerbie RO (1987) The neuronal growth cone: a review of its locomotory, navigational and target recognition capabilities. Neuroscience 20:719–729.

Lotz MM, Burdsal CA, Erickson HP, McClay DR (1989) Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. J Cell Biol 109:1705–1805.

Lyons J, Landis CA, Harsh G, Vallar L, Grunewald K, Feichtinger H, Duh Q-Y, Clark OH, Kawasaki E, Bourne HR, McCormick F (1990) Two G protein oncogenes in human endocrine tumors. Science 249: 655–659.

Masters SB, Miller RT, Chi M-H, Chang F-H, Beiderman B, Lopez NG, Bourne HR (1989) Mutations in the GTP-binding site of $G_{s\alpha}$ alter stimulation of adenylyl cyclase. J Biol Chem 264:15467–15474.

Murray BA, Jensen JJ (1992) Evidence for heterophilic adhesion of embryonic retinal cells and neuroblastoma cells to stubstratum-adsorbed NCAM. J Cell Biol 117:1311–1320.

Pace AM, Wong YH, Bourne HR (1991) A mutant α subunit of G_{i2} induces neoplastic transformation of Rat-1 cells. Proc Natl Acad Sci USA 88:7031–7035.

Patterson PH (1988) On the importance of being inhibited, or saying no to growth cones. Neuron 1:263–267.

Pfenninger KH, Ellis L, Johnson MP, Friedman LB, Somio S (1983) Nerve growth cones isolated from fetal rat brain: subcellular fractionation and characterization. Cell 35:573–584.

Raper JA, Kapfhammer JP (1990) The enrichment of a neuronal growth cone collapsing activity from embryonic chick brain. Neuron 2:21–29.

Robinson PR, Cohen GB, Zhukovsky EA, Oprian DD (1992) Constitutively active mutants of rhodopsin. Neuron 9:719–725.

Rodrigues PS, Dowling JE (1990) Dopamine induces neurite retraction in retinal horizontal cells via diacylglycerol and protein kinase C. Proc Natl Acad Sci USA 87:9693–9697.

Schuch U, Lohse MJ, Schachner M (1989) Neural cell adhesion molecules influence second messenger systems. Neuron 3:13–20.

Shea TB, Perrone-Bizzozero NI, Beermann ML, Benowitz LI (1991) Phospholipid-mediated delivery of anti-GAP-43 antibodies into neuroblastoma cells prevents neuritogenesis. J Neurosci 11:1685–1690.

Slepak VZ, Wilkie TM, Simon MI (1993) Mutational analysis of G protein α subunit $G_o\alpha$ expressed in *Escherichia coli*. J Biol Chem 266: 1414–1423.

Strathmann M, Wilkie TM, Simon MI (1990) Alternative splicing produces transcripts encoding two forms of the α subunit of GTP-binding protein G_o. Proc Natl Acad Sci USA 87:6477–6481.

Strittmatter SM (1992) GAP-43 as a modulator of G protein transduction in the growth cone. Perspect Dev Neurobiol 1:13–19.

Strittmatter SM, Fishman MC (1991) The neuronal growth cone as a specialized transduction system. Bioessays 13:127.

Strittmatter SM, Valenzuela D, Kennedy TE, Neer EJ, Fishman MC (1990) G_o is a major growth cone protein subject to regulation by GAP-43. Nature 344:836–841.

Strittmatter SM, Valenzuela D, Sudo Y, Linder ME, Fishman MC (1991) An intracellular guanine nucleotide release protein for G_o: GAP-43 stimulates isolated α subuntis by a novel mechanism. J Biol Chem 266:22465–22471.

Strittmatter SM, Vartanian T, Fishman MC (1992) GAP-43 as a plasticity protein in neuronal form and repair. J Neurobiol 23:507–520.

Strittmatter SM, Cannon SC, Ross EM, Higashijima T, Fishman MC (1993) GAP-43 augments G protein-coupled receptor transduction in *Xenopus laevis* oocytes. Proc Natl Acad Sci USA 90:5327–5331.

Sudo Y, Valenzuela D, Beck-Sickinger AB, Fishman MC, Strittmatter SM (1992) Palmitoylation alters protein activity: blockade of G_o stimulation by GAP-43. EMBO J 11:2095–2102.

Suidan HS, Stone SR, Hemmings BA, Monrad D (1992) Thrombin causes neurite retraction in neuronal cells through activation of cell surface receptors. Neuron 8:363–375.

Tosney KW, Landmesser LT (1985) Growth cone morphology and trajectory in the lumbrosacral region of the chick embryo. J Neurosci 5:2345–2358.

Winslow JW, Van Amsterdam JKR, Neer EJ (1986) Conformations of the α 39, α 41 and $\beta\gamma$ components of brain guanine nucleotide binding proteins: analysis by limited proteolysis. J Biol Chem 261: 7571–7579.

Wong YH, Federman A, Pace AM, Zachary I, Evans T, Poyssegur J, Bourne HR (1991) Mutant α subunits of G_{i2} inhibit cyclic AMP accumulation. Nature 351:63–65.

Yankner BA, Benowitz LI, Villa-Komaroff L, Neve RL (1990) Transfection of PC12 cells with the human GAP43 gene: effects on neurite outgrowth and regeneration. Mol Brain Res 7:39–44.