G PROTEIN-MEDIATED REGULATION OF NEURONAL MIGRATION

by

Angela Michelle Horgan

A DISSERTATION

Presented to the Department of Cell and Developmental Biology,

the Department of Neuroscience,

and the Oregon Health Sciences University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

December 1997

School of Medicine
Oregon Health Sciences University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. Thesis of

Angela M. Horgan

has been approved

[redacted]
Professor in charge of thesis

[redacted]
Member

[redacted]
Member

[redacted]
Member

Member

[redacted]
Associate Dean for Graduate Studies

TABLE OF CONTENTS

Acknowledgments

Special thanks to Philip Copenhaver;
his guidance and patience, excitement and cheerleading, confidence and support
have helped me greatly during this work.

Thanks to my Dad who encouraged this since my first science class
and never let me settle for less.

Thank you to Amy,
your love, understanding and pride have made these the best years of my life
...with many more to come.

Thanks go to everyone in the Copenhaver lab who have helped me through the years;
with special thanks to Marissa Lagrange for her participation in this project.

Also, thanks to Gillian Bunker for her friendship since that cold night of wrecked cars.

Abstract

During the formation of the nervous system, many neurons transiently acquire the ability to migrate from their point of origin to sometimes distant locations. However, little is known of the mechanisms controlling migratory behavior. This thesis investigates the role of one signal transduction molecule, the heterotrimeric G protein, G_o, and a putative downstream molecule, Ca^{2+}, in the regulation of this process. To study neuronal migration, I have used an insect preparation, the moth *Manduca sexta*. During its embryonic development, a population of ~300 post-mitotic neurons (the EP cells) migrates in a stereotyped pattern on the surface of the gut to form the enteric plexus. Since the neurons and their pathways are accessible during migration, this system allowed an *in vivo* investigation of the signal transduction mechanisms involved in EP cell migration.

Initially I characterized the expression pattern of various members of the heterotrimeric family of G proteins during embryonic development, using immunoblot and immunocytochemical techniques. While expression of $G_{s\alpha}$, $G_{i\alpha}$, and $G_{f\alpha}$ was detected in other regions of *Manduca*, only $G_{o\alpha}$ was found to be expressed in the developing EP cells. The onset of $G_{o\alpha}$ immunoreactivity in the EP cells coincided precisely with the beginning of their migration. Levels of expression then increased during the course of migration. Finally, after both EP cell migration and axon outgrowth were complete, $G_{o\alpha}$ protein expression was down regulated in a subset of the neurons. Upon cloning the *Manduca* form of $G_{o\alpha}$, we found that $MG_{o\alpha}$ mRNA was similarly expressed in the EP cells, using both developmental Northern blot and *in situ* hybridization analyses.

The timecourse of $G_{o\alpha}$ expression in the EP cells suggested that this protein might participate in the regulation of EP cell migration. Using an embryonic culture preparation which allows direct access to the individual neurons, I examined the effects of compounds known to alter G protein activity on neuronal motility. These experiments have demonstrated that stimulation of G_o in these cells resulted in the termination of migration.

In contrast, inhibition of G_o had no obvious effect. Similarly, injection of activated $G_{o\alpha}$ subunits also caused a reduction in the extent of EP cell migration. These results suggest that the function of G_o might be to transduce an inhibitory signal, either to direct migration away from inappropriate tissues or to signal the termination of migration when the cells have reached their target destination.

Since G_o has been shown to modulate Ca^{2+} in other systems, we investigated whether the effects of $G_{o\alpha}$ on EP cell migration involves a Ca^{2+} dependent process. While EP cell migration proceeded normally in 0 mM Ca^{2+} saline, elevating intracellular Ca^{2+} caused a decrease in neuronal migration. The inhibitory effects of G_o stimulation were, however, completely abolished in 0 mM Ca^{2+} saline. In addition, when G proteins were activated during Ca^{2+} imaging, large increases in intracellular Ca^{2+} were seen in the majority of EP cells examined. These results support a Ca^{2+}-mediated mechanism of action for the inhibitory effects of G_o on EP cell migration.

Introduction

Cellular Migration

Cellular migration is the ability of a cell body to translocate from one environment to another. The change in environments can be subtle, such as the rearrangement of neighboring cells during gastrulation (Hardin, 1989) and neural tube formation (Jessell et al., 1989), or dramatic, as demonstrated by the immune response which stimulates the infiltration of leukocytes from the bloodstream into any tissue of the body (Thelen et al., 1993). The specific features of each type of motile behavior is inherently dependent upon the cells' internal repertoire of signal-transduction systems, the architectural dynamics of its plasma membrane, and the regulation of its cytoskeletal apparatus. The variability in the motile phenotypes observed between different cell types illustrates the complexity of the underlying mechanisms guiding the cell's movement, necessitating an investigation of multiple systems to elucidate basic or conserved properties of motility.

Despite differences in the modes of cell movements observed between specific cell types, studies of chemotaxis and developmental motility have uncovered some fundamental strategies for the regulation of migration (Stossel, 1993; Cooper, 1991). In broad terms, motile cells receive stimulation and directional information from external sources. Cells are often stimulated to migrate by external cues in the form of diffusible molecules. Little is known about the signals that control the cessation of migration, although recent work from the field of neuronal process outgrowth has identified some putative signaling molecules that may regulate this event (see below). The majority of receptors so far identified that recognize stimulatory cues belong to two distinct types: the serpentine receptors, typically associated with heterotrimeric G proteins (Devreotes, 1994; Dillon et al., 1988), and the receptor tyrosine-kinases (Wehrle-Haller and Weston, 1997; Abedi and Zachary, 1995). This thesis will be dedicated to the discussion of G protein-mediated regulation of migration; however,

preliminary evidence from other members of the laboratory have implicated tyrosine kinase activity as an additional modulatory pathway in our system.

Some cells can also be guided, in part, by cell surface adhesion molecules that recognize ligands in the extracellular matrix or on neighboring cells. The distinction between receptor-mediated signal transduction and cell adhesion has become vague in recent years, as it is now well accepted that adhesion molecules such as integrins, cadherins, and possibly the immunoglobulin-containing superfamily, can also transduce external signals across the membrane (Schaller et al., 1995; Klymlowsky and Parr, 1995; Brummer et al., 1995). Additionally, adhesion serves a more fundamental role in the guidance of motility. For a cell to move forward, connections between the cell membrane and the underlying substrate must be made in order to produce traction (Ruoslahti and Obrink, 1996). However, these connections are not static, since during migration, membrane extensions continue to form new attachments at the leading edge while breaking contacts as the trailing edge retracts (Gumbiner, 1996). The transient nature of this adhesion implies that membrane adhesion molecules must be regulated coordinately in a cyclical pattern of high and low affinity states, or that adhesion molecules are inserted into the membrane at the front of the cell and removed at the rear, or both. It has been shown that the ligand-binding capabilities of integrins can be modulated in keratinocytes and some neurons (Adams and Watt, 1993; Neugebauer and Reichardt, 1991) by G proteins and protein kinases (Smyth et al., 1993). Their adhesive strength can also be regulated by clustering at specific sites termed focal adhesion contacts, a process that is mediated by cytoplasmic proteins that are themselves regulated by phosphorylation levels (Burridge et al., 1992). The dynamic regulation of adhesive contacts is a likely target of receptor-mediated signaling during motility, a phenomenon that is only beginning to be understood at the molecular level.

Underlying the sensory and adhesive structures of the plasma membrane is the cytoskeleton. Although there is some evidence that microtubules participate in cell motility of some cell types (Rivas and Hatten, 1995), the actin-based cytoskeleton is known to play a

primary role in most forms of cellular migration (Stossel, 1993). During cell movement, the cytoskeleton undergoes a dynamic cycling of actin assembly, disassembly, and stabilization (Mitchison and Cramer, 1996). G-actin is a monomeric protein which forms helical filaments (F-actin) by polymerization. That polymerization and depolymerization are important for motility has been shown by marking and following lamellipodial actin during fibroblast motility: actin is assembled at the distal, membrane-associated end, moves centripetally away from the leading edge, and is disassembled proximal to the cell body (Wang et al., 1985). Toxins such as cytochalasin which prevent actin polymerization also inhibit motility (Forscher and Smith, 1988; Horgan and Copenhaver, unpublished observations). Actin filaments can be bundled by proteins like α-actinin (Condeelis and Vahey, 1982), cross linked at high-branch angles by ABP (Niederman et al., 1983) and bound to proteins that associate with the plasma membrane, including the β subunit of integrins, talin, vinculin, and MARCKS (Hanks and Polte, 1997; Aderem, 1992). These interactions serve to create a dense actin network at the leading edge of the motile cell which can be rapidly altered in response to signal transduction events. To accomplish this, many actin-binding proteins are regulated by changes in levels of Ca^{2+}, kinases, and/or phosphoinositols. For example, at the level of polymerization, the actin monomer-sequestering protein, profilin, as well the F-actin capping protein, gelsolin, are both inhibited by phosphoinositol binding (Lassing and Lindberg, 1985; Janmey et al., 1987). The inhibitory regulation of polymerization can be accomplished by both gelsolin and another capping protein, gCap 39 (MCP), which are stimulated by Ca^{2+} (Lamb et al., 1993; Dabiri et al., 1992). In addition, phosphorylation of actin monomers themselves inhibits their assembly into filaments (Sonobe et al., 1986). At the level of the three-dimensional actin structure, the cross-linking of actin filaments by α-actinin might be regulated by intracellular Ca^{2+} (Condeelis and Vahey, 1982), while phosphorylation or binding by Ca^{2+}/calmodulin inhibits the ability of MARCKS to link actin to the membrane (Greengard et al., 1993).

Our present knowledge of the intracellular mechanisms used for the transduction of external signals into changes in adhesive and cytoskeletal dynamics is limited. To illustrate this current understanding, several examples of motility will be described, with particular emphasis being placed on the intracellular messengers that regulate movement. I will then describe the novel system that we are studying, and explain the experimental rationale for the identification of one signal transduction pathway utilized in the regulation of neuronal migration within our system.

Chemotaxis

The directed locomotion of cells towards or away from a chemical gradient is defined as chemotaxis. During this process, an individual cell is able to recognize an external signal, integrate this information to determine the direction of its source, and respond to changes in the concentration of the chemotactic agent with movement. Distinct from chemokinesis, in which a chemical stimulates an increase in random motion (as in bacteria; Koshland, 1988; Kleene, 1986], chemotaxis alters the direction of the cell's motion. Upon stimulation by the appropriate chemical, chemotactic cells rapidly orient their polarity in the direction of increasing (or decreasing) concentrations of the substance in the form of shape changes such as the extension of pseudopods and lamellae. Subsequent regulation of cell/substratum adhesion and cytoskeletal dynamics are then necessary to produce the traction and force required to move the cell body. The two best studied eukaryotic model systems for chemotaxis are *Dictyostelium discoideum* and mammalian leukocytes. Work on these two cell types have enabled the dissection of some of the molecular pathways by which an external chemical induces and regulates these intracellular changes.

Dictyostelium

The amoeboid *Dictyostelium discoideum* is an excellent system with which to examine intracellular mechanisms governing chemotaxis, largely due to its amicability to

genetic manipulation. During the life cycle of this amoeba, it remains unicellular in conditions of plentiful food. But upon starvation, the individual cells initiate a program of aggregation and differentiation in order to distribute spores. It is the period of aggregation which is of interest to the discussion of chemotaxis. Groups of "founder cells" begin to release high levels of cAMP, towards which more distant cells move. Individual cells respond to gradients of cAMP by extending pseudopods, filopodia-like extensions of the membrane, which orient to the direction of higher cAMP concentrations. Their response to this stimulant is the result of the direct binding of cAMP to cell surface receptors, as has been shown by thorough examination of cAMP receptor knockout strains (Sun et al., 1991; Insall et al., 1994). The cAMP receptors are a part of a family of proteins that have seven membrane-spanning domains, stereotypical of the G protein-linked receptors (Saxe et al., 1991). Dictyostelium produces eight distinct G protein alpha subunit isoforms which share considerable homology with mammalian G proteins (Gudermann et al., 1997). Mutants lacking the Gα2 protein no longer orient or aggregate to cAMP (Kumagai et al., 1991). In contrast, deletion of Gα4 prevents chemotaxis to another stimulant, folic acid, while preserving the response to cAMP (Hadwiger et al., 1994). The six other G protein α isoforms do not seem to be involved in this aspect of development, since null mutants retain wild-type abilities to aggregate (Chen et al., 1996). G proteins were initially believed to act solely through the activation of the α subunit; however, in vertebrate cells, it is now evident that the βγ dimer can also modulate effector systems (Dunlap, 1997; DeWaard et al., 1997). To date, only one β subunit has been identified in Dictyostelium. It also plays a critical role in chemotaxis, since its expression is required for migration towards either cAMP or folic acid (Wu et al., 1995). The γ subunit has not yet been identified in *Dictyostelium.*

Receptor binding of cAMP initiates a number of intracellular changes, the order and effects of which are only partly understood. Many second messenger systems appear to be activated in responses to cAMP; however, results gathered from genetic deletion of individual proteins suggests that there is no one pathway that is critical for chemotaxis. The

immediate target of Gα2 is believed to be phospholipase C (PLC), which hydrolyzes PIP_2 into IP_3 and DAG, thereby causing the release of Ca^{2+} from intracellular stores and the activation of protein kinase C (Berridge, 1993). Chemotactic responses in *Dictyostelium* are mimicked by IP_3 [Nicholas and Newell, 1985) and the activity of PLC can be stimulated by nonhydrolyzable forms of GTP (Van Haastert 1995). Besides PLC activation, there is a rapid elevation of intracellular cGMP levels as well as an influx of external Ca^{2+}. The direct effects of these changes in *Dictyostelium* are not yet known, although many cytoskeletal components have been shown to be regulated by phosphoinositides, Ca^{2+}, and kinases (as summarized above). For example, these include the increase in actin polymerization, phosphorylation of myosin heavy and light chains, and the induction, translocation and regulation of multiple actin-binding proteins (Noegel and Luna, 1995).

Leukocytes

Chemotaxis in the white blood cell population has been the focus of intense investigation, since this process has considerable significance for the functioning of the immune system. Leukocyte chemotaxis is the body's main defense mechanism after tissue injury or microbial infection in higher vertebrates. The classification of leukocytes encompasses four different cell types, all of which require the ability to translocate from the blood into the target tissue in order to eliminate the pathogen. Most research has concentrated on the polymorphonuclear leukocytes (neutrophils) because they exist in greater numbers, are easier to purify, and seem to constitute a homogenous population, as opposed to the other immune response cell types. By using a number of chemotactic assays both *in vitro* (Bottazzi et al., 1985) and, more recently, *in vivo* (Steinberg et al., 1997), a large variety of compounds have been identified which stimulate the migration of neutrophils. These chemoattractants are released from sites of injury, thus forming a natural gradient which is sensed by circulating leukocytes. This recognition induces the activation of many cellular responses, including motility, exocytosis, and a burst of respiration.

Elucidation of the transduction systems mediating this response to chemoattractants has demonstrated mechanisms involving G protein and PLC activation. Receptors to chemotactic stimuli on neutrophils are almost exclusively coupled to G proteins (Dillon et al., 1988). For example, the complement protein C5a, the bacterial-derived formylated peptides, platelet aggregation factor (PAF), leukotriene B_4, and a family of at least 16 chemotactic molecules called the chemokines all are recognized by membrane receptors with a seven transmembrane region motif (Imhof and Dunon, 1995). G protein involvement has been demonstrated by the ability of pertussis toxin (a specific inhibitor of the Go/i subclass of G proteins; West et al., 1985) to inhibit all neutrophil motility (Sprangrude et al., 1985). In addition, both AlF_4 and GTPγS, stimulators of all heterotrimeric G proteins (Bigay et al., 1985; Andrade, 1994), mimic the responses normally seen during chemotaxis (Newell et al., 1995; Saito et al., 1996).

The exact intracellular pathways regulated by the activation of G proteins are less well characterized. There is evidence that several G proteins can support neutrophil activation (Hu et al., 1992; Al-Aoukaty et al., 1996), some in a receptor-specific manner (Amatruda et al., 1993), suggesting the involvement of multiple signal cascades or cross talk between the pathways. What has been established is that, similar to *Dictyostelium*, agonist binding to G protein-coupled receptors results in the stimulation of PLC, followed by a rapid production of IP3 and DAG (Smith et al., 85). Intracellular levels of Ca^{2+} then rise and PKC is activated (Smith et al., 85; Berridge, 1993). There is an influx of Ca^{2+}, as well as the opening of a non-specific cation channel (von Tscharner et al., 1986). Many aspects of leukocyte adhesion are altered downstream of these second messengers: a class of adhesion proteins, called the selectins, are phosphorylated at multiple residues and become translocated to the membrane (Fujimoto and McEver, 1993; Weller et al., 1992), there is a change in the affinity state of some integrins (Detmers et al., 1991) and an induction of immunoglobulins (de Fougerolles and Springer, 1992; de Fougerolles et al., 1994). At the level of the cytoskeleton, agonist binding causes an increase in the number of high affinity, barbed sites

on actin, thereby mediating an increase in its polymerization (Carson et al., 1986; Omann et al., 1987). The intracellular levels of gelsolin, an actin-binding protein, are decreased (Chaponnier et al., 1987), and myosin is phosphorylated by myosin chain kinase (Fechheimer Cebra, 1983). These changes probably contribute directly to the enhanced motility induced by chemoattractants; however, it is likely that many other components are modulated to produce the coordinated changes necessary for movement. The similarities of leukocyte chemotaxis to that of *Dictyostelium* is remarkable considering their distance phylogenetically. The information provided by these two chemotactic systems will be useful to the understanding of motility in other cell types.

Nervous System Development

Axon Outgrowth

The study of the cell migration is of much importance with respect to the developing nervous system, since all neurons, at least transiently, acquire the ability to move either their cell bodies and/or cellular extensions in the form of axons and dendrites. One aspect of neuronal motility that has implications for the understanding of migration is neuronal outgrowth of an axon. The similarities in morphology between the tip of an extending axon, called a growth cone, and the leading process of a migrating neuron are striking. Both structures utilize actin-based lamellipodia and filapodia to sample the environment and integrate external signals (Bray and White, 1988; Smith et al., 1988; Singer and Kupfer, 1986). Similar pathway markers have also been characterized for both migration and axon outgrowth (Schlosshauer et al., 1988; Lin et al., 1994; Copenhaver et al., 1996) The relative ease in which axon outgrowth can be induced in culture has led to the production of a large body of literature examining the mechanisms regulating this form of motility (Chiba and Keshishian, 1996; Whitington, 1993).

With respect to G proteins, research has suggested that several of these signal transduction molecules are likely involved in the regulation of growth cone motility;

however, the precise mechanisms by which they function are not yet understood. In particular, G_o is highly expressed in growth cones within the developing nervous system (Strittmater et al., 1990; Wolfgang et al., 1991) and is ideally situated to participate in the intracellular events necessary for the control of outgrowth. When the G_o subtype of G proteins was stimulated by mastoparan or expressed in a constitutively activated form in either neuroblastoma or PC-12 cells, there was a significant increase in the number and length of the processes extended by these cells (Strittmatter et al., 1994; Xie et al., 1995). These effects could be prevented by PKC activation (Xie et al., 1995) and enhanced by a protein of unknown function, GAP-43 (Igarashi et al., 1995). In addition, the enhancement of neurite outgrowth mediated by the cell adhesion molecules L1, NCAM, and N-cadherin in a neuronal cell line was prevented by the application of the $G_{o/i}$ inhibitor pertussis toxin (Schuch et al., 1989; Doherty et al., 1991). Regulation of intracellular Ca^{2+} levels was also implicated as a downstream component of this interaction, because PTX prevented the observed increase in Ca^{2+} that accompanied neuronal outgrowth on these adhesion molecules (Schuch et al., 1989). In contrast, Ca^{2+} channel inhibitors blocked the increase of process outgrowth (Doherty et al., 1993; Williams et al., 1992; Doherty et al., 1991).

Interestingly, G proteins may also play a role in the *inhibition* of process outgrowth. For an axon to reach its correct destination, the cell likely receives information that guides its movement by both stimulating motility in one direction and inhibiting it in another (Kolodkin, 1996; Mitchell et al., 1996). Ligand-induced growth cone collapse *in vitro* is believed to represent an example of this inhibition (Fan and Raper, 1995; Kapfhammer and Raper, 1987), albeit in a more pronounced fashion than probably occurs *in vivo*. Some of the molecules responsible for repelling axons have begun to be isolated and identified (Caroni and schwab, 1988; Luo et al., 1993; Messersmith et al., 1995), but it remains to be determined how these ligands ultimately modulate components of cell adhesion and cytoskeletal structure necessary for outgrowth. However, there is recent evidence suggests that one family of "collapsing" molecules, the semaphorins, may act via G protein-mediated

pathways (Varela-Echavarria and Guthrie, 1997). For example, Igarashi et al. showed that collapse of retinal and dorsal root ganglion neurites induced by CNS myelin fractions was inhibited by PTX and stimulated by mastoparan (Igarashi et al., 1993). The purified component of this fraction, identified as collapsin, caused an increase in intracellular Ca^{2+} levels when applied to Xenopus oocytes that was similarly G protein-dependent (Goshima et al., 1995). Some neurotransmitters such as serotonin and dopamine, that act via G protein-linked receptors, also have collapsing activities which appear to be cell-type specific (Lankford et al., 1988; McCobb et al., 1988). The results of these inhibitory molecules on axon outgrowth might also be due to the induction of a rise in intracellular Ca^{2+} levels (Polak et al., 1991; Neely and Gesemann, 1994), as demonstrated in both vertebrate and invertebrate cell types (Bandtlow et al., 1993; Moorman and Hume, 1993; Snow et al., 1994). The ability of G proteins to enhance or reduce motility depending on the experimental context might be due to different intracellular pathways coupled to various G proteins, or possibly to cell-specific adaptations to the same receptor-mediated events. These issues will require a more complete elucidation of the molecular components comprising signaling mechanisms downstream of receptor activation.

Neuronal Migration

In addition to the outgrowth of axons, many neurons transiently acquire the ability to migrate away from their place of origin and to populate and innervate more distant regions of the embryo. There are many examples of neuronal migration in the central and peripheral nervous systems of vertebrates, where it is clear that the neurons follow precise pathways at specific developmental times to reach their target destination. During the development of the cerebral cortex, neurons are initially born in the ventricular layer. Once post-mitotic, neuronal precursors migrate radially through the intermediate zone to the cortical plate (Gray et al., 1990; Rakic, 1971). Cellular migration is also fundamental to the development of the peripheral nervous system. For example, the enteric nervous system of vertebrates is formed

by the migration of neurons from the neural crest into the developing walls of the gut, followed by an anterior and posterior migration along the gut (Gershon and Tennyson, 1991). However, the investigation of signal transduction mechanisms that mediate the timing and guidance of these examples of migration has proven difficult: migratory neurons are typically hard to purify; migratory behavior is hard to reproduce *in vitro*; and the neurons are relatively inaccessible to direct manipulation within the developing embryo.

One exception, however, has been the migration of cerebellar granule cells. Laboratories have begun to examine the molecular mechanisms of granule neuron migration by using both a slice preparation and a dissociated *in vitro* culture assay (Rakic and Komuro, 1994; Hatten, 1990). From these experiments, it was shown that granule neurons required a membrane molecule, astrotactin, to bind to and migrate along astroglia in culture (Fishell and Hatten, 1991). In addition, the ECM component laminin, when applied to glass fibers, supported neuronal migration via the β1 integrin receptor system (Fishman and Hatten, 1993). The intracellular events downstream of such ligand binding are still unknown, but these results indicate that cell adhesion is equally important to neuronal migration as has been seen in non-neuronal systems. Recent work by Komuro and Rakic in the cerebellum slice preparation focused on the function of the intracellular signaling molecule Ca^{2+}. They found that membrane Ca^{2+} channels were important for migration, since blockage of Ca^{2+}, but not Na^{+} or K^{+} channels, reduced the speed of granule cell motility (Komuro and Rakic, 1992). Furthermore, the rate of movement was proportional to the levels of extracellular (Komuro and Rakic, 1992) and intracellular Ca^{2+} (Komuro and Rakic, 1996). Moreover, buffering intracellular concentrations of Ca^{2+} with BAPTA-AM, reduced granule cell movement (Komuro and Rakic, 1993), demonstrating a stimulatory role for Ca^{2+} influx in the regulation of neuronal migration. Further investigation into the causes of spontaneous Ca^{2+} fluctuations *in vivo*, and the regulatory effects of Ca^{2+} with respect to both cell adhesion and cytoskeletal components, will be necessary if we are to gain an understanding of how neuronal migration is controlled. The significance of the classical G protein signaling

pathways in the migration of these, or other, migratory neurons is an area of research that has not yet been examined.

Neuronal Migration in *Manduca sexta*

The limitations of studying the migration of neurons in vertebrates has hampered the discovery of the regulatory mechanisms that control neuronal cell movements during the development of the nervous system. For this reason, I have taken advantage of an insect system, the moth *Manduca sexta.* During the formation of the enteric nervous system in *Manduca,* a small population of neurons (the EP cells) undergoes a period of migration on the surface of the gut musculature (Copenhaver and Taghert, 1989b). The migration of the EP cells is very stereotyped: it occurs at a well defined developmental time and proceeds on identifiable pathways in the embryo. The fact that these neurons, and their pathways, are easily accessible to manipulations *in vivo* has allowed me to investigate the role of various signaling molecules in migration within the context of the normally developing animal. The evidence that some signaling molecules and transduction pathways are highly conserved between diverse cell types (i.e. *Dicyostelium* and mammalian leukocytes) supports the relevance of studying neuronal migration in a relatively simple invertebrate preparation. Moreover, the results obtained by my research has indicated a role for G_o in migrating neurons that can now be tested in vertebrate preparations.

Since G proteins have been widely implicated in the regulation of motility in many cell types (see above), I proposed to investigate the possibility that this signal transduction mechanism might participate in controlling the precise migration of the EP cells observed during the development of *Manduca sexta.* In addition, I tested the role of downstream effector systems known to interact with the specific G proteins in an attempt to elucidate the mechanisms by which the G proteins act. Specifically, I carried out the following experimental investigations:

Chapter 1

Developmental Expression of G proteins in a Migratory Population of Embryonic Neurons

In order to investigate the role that G proteins might play in the regulation of motility, it was first necessary to determine the identity of the G proteins expressed in the EP cells and the time course of that expression. By using antibodies that recognize various alpha subunits in the heterotrimeric G protein family, I observed expression of only a single subtype, $G_{o\alpha}$, in the EP cells during embryogenesis. This expression coincided precisely with EP cell motility, implicating a possible role in migration. Preliminary experimental manipulation of G proteins in the migratory neurons suggested that the stimulation of Go activity was inhibitory to migration.

Chapter 2

A Developmental Role for the Heterotrimeric G Protein $G_{o\alpha}$ in a Migratory Population of Embryonic Neurons

The further characterization of $G_{o\alpha}$ and its expression was accomplished upon cloning its *Manduca* homologue. It was found that $MG_{o\alpha}$ had considerable sequence similarly to $G_{o\alpha}$ from other species, and that the expression of $MG_{o\alpha}$ mRNA in the EP cells followed a similar time course as that of the protein. In addition, to examine the function of $G_{o\alpha}$, I manipulated intracellular transduction pathways by injecting reagents known to alter normal G protein function directly into migrating EP cells. In further support of an inhibitory role for G_o, I found that its stimulation caused a reduction in migration. This effect could be prevented by coinjecting a G_o inhibitor. However, G_o inhibition alone caused no significant effect on migration.

Chapter 3

G Protein Mediated Inhibition of Neuronal Migration Requires Ca^{2+} Influx

Many of the G proteins that have been identified are classified by the effector system to which they couple. G_o has been shown in other systems to couple to the regulation of intracellular Ca^{2+} levels. I therefore examined whether manipulations of Ca^{2+} had any effect on EP cell migration. The results suggested that elevations of intracellular Ca^{2+} caused a decrease in migration, whereas buffering intracellular fluctuations of Ca^{2+} produced misguided migration. A role for Go in regulating Ca^{2+} levels was supported by evidence that the inhibitory effect of Go stimulation required Ca^{2+}, and that stimulation of G proteins caused Ca^{2+} spikes in the EP cells. My results demonstrated that the G_o could function via Ca^{2+} regulation to inhibit neuronal migration of the EP cells. However, the exact mechanism by which Ca^{2+} affects neuronal motility in this system remains to be determined.

DEVELOPMENTAL EXPRESSION OF G PROTEINS IN A MIGRATORY POPULATION OF EMBRYONIC NEURONS

A.M. Horgan, M.T. Lagrange, and P. F. Copenhaver*

Department of Cell Biology and Anatomy, Oregon Health Sciences University

Portland, Oregon 97201

Originally published in Development, 120: 729-742 April, 1994

SUMMARY

Directed neuronal migration contributes to the formation of many developing systems, but the molecular mechanisms that control the migratory process are still poorly understood. We have examined the role of heterotrimeric G proteins (guanyl nucleotide binding proteins) in regulating the migratory behavior of embryonic neurons in the enteric nervous system of the moth, *Manduca sexta*. During the formation of the enteric nervous system, a group of ~300 enteric neurons (the EP cells) participate in a precise migratory sequence, during which the undifferentiated cells populate a branching nerve plexus that lies superficially on the visceral musculature. Once migration is complete, the cells then acquire a variety of position-specific neuronal phenotypes. Using affinity-purified antisera against different G protein subtypes, we found no apparent staining for any G protein in the EP cells prior to their migration. Coincident with the onset of migration, however, the EP cells commenced the expression of one particular G protein, Go_α. The intensity of immunostaining continued to increase as migration progressed, with Go_α-immunoreactivity being detectable in the leading processes of the neurons as well as their somata. The identity of the Go_α-related proteins was confirmed by protein immunoblot analysis and by comparison with previously described forms of Go_α from *Drosophila*. When cultured embryos were treated briefly with aluminum fluoride, a compound known to stimulate the activity of heterotrimeric G proteins, both EP cell migration and process outgrowth were inhibited. The effects of aluminum fluoride were potentiated by alpha toxin, a pore-forming compound that by itself caused no significant perturbations of migration. In preliminary experiments, intracellular injections of the non-hydrolyzable nucleotide GTPγ-S also inhibited the migration of individual EP cells, supporting the hypothesis that G proteins play a key role in the control of neuronal motility in this system. In addition, once migration was complete, the expression of Go_α-related proteins in the EP cells underwent a subsequent phase of regulation, so that only certain phenotypic classes among the differentiated EP cells retained detectable levels of Go_α immunoreactivity. Thus Go may perform multiple

functions within the same population of migratory neurons in the course of embryonic development.

INTRODUCTION

The directed migration of neurons or their undifferentiated precursors is a prominent feature in the developing nervous systems of many organisms. Besides distributing the cellular components of a particular region to their appropriate locations, the process of migration exposes immature neurons to a variety of epigenetic cues, thereby influencing the morphological and biochemical phenotypes that they may subsequently express (e.g. Black et al., 1984; Teillet et al., 1987; Walsh and Cepko, 1988; Wetts et al., 1989). At the level of an individual neuron, several distinct extracellular and intracellular events must be coordinated so that migration can proceed normally. The neuron must recognize appropriate cues for orientation, presumably via specific receptor subtypes that must be expressed at the requisite stages of development. These ligand-sensitive receptors must be coupled to one or more intracellular signalling systems within the cell, which in turn must be competent to transduce appropriate stimuli to one or more effector systems that are associated with the motile apparatus of the cell. Ultimately, this sequence must result in regulated changes in the cytoskeleton (Singer and Kupfer, 1986; Smith, 1988; Theriot and Mitchison, 1992), although other cellular processes may also be affected, including modifications in the secretory apparatus, reorganization of membrane-limited receptors, altered rates of organelle transport, and specific changes in gene expression (reviewed in Singer and Kupfer, 1986; Caterina and Devreotes, 1991). Beyond a simple activation of the locomotory process, directed migration also requires that continued input from environmental cues be provided, so as to prevent the uncontrolled dispersal of neurons (or other cell types) into inappropriate domains of the developing organism (e.g. Barth, 1987; Grimstad, 1987).

Among the intracellular signalling systems that may affect motility, the heterotrimeric G proteins (guanyl-nucleotide binding proteins) have been implicated in a number of different

studies of both cell migration and process outgrowth. The G proteins comprise a conserved family of molecules that are involved in transmembrane signal transduction in virtually all eukaryotic cells (Bourne et al., 1991; Simon et al., 1991). Typically associated with specific members of the heptahelical receptor superfamily (Mollen, 1991), G proteins become activated in response to a ligand-receptor interaction: the α-subunit of the G protein exchanges bound GDP for GTP; it dissociates from the $\beta\gamma$ complex; and in most cases, the activated α-subunit then exerts a modulatory effect on one or more effector systems (Stryer and Bourne, 1986; Bourne et al., 1991; but see also Federman et al., 1992; Kleuss et al., 1992). Several different classes of G proteins have been identified in the developing nervous systems of both vertebrates (Pituello et al., 1991; Rius et al., 1991; Shinohara et al., 1992) and invertebrates (Wolfgang et al., 1990; Ray and Ganguly, 1992). In particular, members of the Go class have been associated with regions of active outgrowth and presumed synaptogenesis (Chang et al., 1988; Garibay et al., 1991; Wolfgang et al., 1991; Asano et al., 1992).

Go-related proteins have been shown to form a major component of growth cone-like processes in PC-12 cells (Strittmatter et al., 1990; Edmond et al., 1990; Garibay et al., 1991), and several different adhesion molecules stimulate process outgrowth in these cells via one or more pertussis-sensitive mechanisms (which might involve Go; Schuch et al., 1989; Doherty et al., 1991). Recently, the inhibitory response of neuronal growth cones to several different collapsing factors have also been shown to be G protein-dependent (Igarashi et al., 1993), indicating that G protein-coupled mechanisms may exert a negative regulation on neuronal motility, as well. G proteins have also been implicated in the chemotactic responses of non-neural cells, including polymorphonuclear leukocytes and cells of the slime mold Dictyostelium (Devreotes and Zigmond, 1988; Caterina and Devreotes, 1991). The mechanisms by which G proteins affect the locomotory process in these systems remain enigmatic, however. In the case of neuronal migration, the role of particular G proteins in regulating motility has not been examined.

To address this issue, we have begun an investigation of neuronal migration in a relatively simple preparation, the enteric nervous system (ENS) of the moth, *Manduca sexta*. As previously described, the ENS of *Manduca* includes a pair of small peripheral ganglia and a distributed population of ~300 enteric neurons (the EP cells) that occupy a branching nerve plexus, called the enteric plexus (Copenhaver and Taghert, 1989a; Copenhaver and Taghert, 1989b; see fig. 1A). The EP cells are noteworthy in that the entire population shares a common developmental origin, emerging from an epithelial placode in the foregut between 30-40% of embryogenesis (Copenhaver and Taghert, 1990). Immediately thereafter, the EP cells undergo two successive waves of directed migration, first spreading down both sides of the foregut-midgut boundary (from 40-55% of development) and then rapidly dispersing along a defined set of muscle bands that have recently differentiated from the visceral musculature (55-60%). It is during this second migratory phase that the mature distributions of the EP cells are established. Only after migration is complete do the neurons commence the expression of their mature phenotypes, at which time they exhibit a number of distinct characteristics in a position-specific manner. For example, several distinct morphological subtypes have been identified that correspond to the terminal locations occupied by the EP cells on either the foregut or midgut (Copenhaver and Taghert, 1989b). In addition, one particular subtype (designated "type B") has been shown to commence the expression of neuropeptides related to the molluscan peptide Phe-Met-Arg-Phe-NH_2 (FMRFamide), but only after the neurons have completed their migration onto the midgut (Copenhaver and Taghert, 1989a). Both the migration and differentiation of the EP cells have been shown to proceed in embryo culture (Copenhaver and Taghert, 1990; Copenhaver, 1993), facilitating an investigation of the migratory process in a normal developmental context.

We now report that neuronal migration in this system coincides with the expression of a particular G protein that may in turn regulate key aspects of migratory behavior. Specifically, we have found that the EP cells commence the expression of a protein related to Go_α just prior to the onset of their migratory dispersal, while the levels of expression

subsequently increase in all of the neurons throughout the period of active migration. Once migration is complete, however, the pattern of Go_α expression in the enteric plexus undergoes an additional phase of regulation, in that only certain subsets of the EP cells continue to exhibit detectable levels of Go-related protein. This subsequent pattern of expression is directly correlated with the mature phenotypes that the neurons acquire during the maturation of the ENS. In addition, stimulating G protein activity in the EP cells with a brief exposure to aluminum fluoride (AlF_4-) or by injection of the non-hydrolyzable nucleotide GTPγ-S significantly inhibits both their migratory dispersal and subsequent axonal outgrowth. Thus in this system, the developmental expression of Go_α is precisely regulated with respect to the onset of neuronal migration and may play an important role in controlling subsequent aspects of the migratory process. A preliminary account of some of these results has appeared in abstract form (Copenhaver et al., 1992).

MATERIALS AND METHODS

Animal Preparation

A laboratory colony of *Manduca sexta* was used to generate experimental animals, as previously described (Copenhaver and Taghert, 1989a). Eggs were collected in one hour intervals as needed and maintained in a 25° C incubator. At this temperature, embryogenesis is completed in ~100 hours (1 hour is equivalent to 1% of development). Established schedules of external and internal markers (Dorn et al., 1987; Copenhaver and Taghert, 1989a) were used to identify specific developmental stages. Embryos were removed from their shells and underlying membranes and dissected along the dorsal body wall to expose the ENS in the following medium (after Chen and Levi-Montalcini, 1969; and Seecof et al., 1971): 50% Schneider's *Drosophila* medium, 40% minimal essential medium (MEM) with Hanks' salts, 9.7% heat-inactivated fetal calf serum, 0.2% ecdysone, 0.1% insulin, 0.01% penicillin-streptomycin. Embryo cultures were also supplemented with *Manduca*

hemolymph (5-10%, vol:vol; Copenhaver and Taghert, 1990; Copenhaver, 1993). Under these conditions, semi-intact embryos can be cultured for up to 50% of subsequent development (Copenhaver and Taghert, 1990; Copenhaver and Taghert, 1991). For postembryonic stages, animals were dissected in a modified Weever's saline (in mM: dextrose, 160; KCl, 40; $MgCl_2$, 18; NaCl, 4; $CaCl_2$, 3; KH_2PO_4, 1.25; $KHCO_3$, 1.25; pH 6.5; after Levine and Truman, 1985). Animals to be processed for immunohistochemical staining were usually fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hour at room temperature. Alternatively, some animals were fixed in PLP (2% paraformaldehyde, 10 mM $NaIO_4$, 75 mM lysine, 37 mM phosphate buffer, pH 6.5; after McLean and Nakane, 1974) for 2 hours, which enhanced the detection of Go_α when visualized by immunofluorescence.

Immunohistochemistry

Fixed preparations were rinsed in PBS and incubated overnight at 4° C in an appropriate dilution of primary antibody. An affinity-purified antiserum generated against the conserved carboxyl-terminal decapentapeptide of the α-subunit of Go (Goldsmith et al., 1987; gift of Drs. M. Forte and W. Wolfgang) was used at a concentration of 1:10,000 in PBS with 0.1% Triton-X 100, 0.1% NaN_3, and 10% normal serum. The specificity of this antiserum has previously been characterized in *Drosophila* (Thambi et al., 1989), in which it recognizes a single protein species (Mr ~40 X 10^3) that has been identified as Go_α. The antiserum is completely inhibited when preadsorbed against the carboxyl terminal decapentapeptide of Go_α, but it shows no cross-reactivity with other known Gα subunits (Thambi et al., 1989; Wolfgang et al., 1990). An antiserum specific for the α-subunit of Gs (designated "RM"; Wolfgang et al., 1990) was also used at a dilution of 1:6000. The monoclonal antibody TN-1 was used as a histological marker for the EP cells at a concentration of 1:20,000. TN-1 recognizes a cell surface molecule that is related to fasciclin II (Nardi, 1990; Nardi, 1992) and that is expressed by specific subsets of neural and

nonneural cells in *Manduca* (Taghert et al., 1986; Carr and Taghert, 1988). As previously reported, TN-1 provides an unambiguous marker for the EP cells and their processes throughout embryonic development (Copenhaver and Taghert, 1989b; Copenhaver and Taghert, 1990). To characterize the expression of peptidergic phenotypes in the ENS, a polyclonal antiserum generated against synthetic FMRFamide was used at concentrations of 1:1000-1:2000 (Copenhaver and Taghert, 1989a). For double-labelling experiments, the anti-Go_α antiserum was applied in conjunction with a monoclonal antibody that was generated against the molluscan Small Cardiac Peptide (SCP; diluted 1:20; gift of Drs. B. Masinovsky and A.O.D. Willows), which also stains all FMRFamide-immunoreactive neurons in the ENS of *Manduca*. As the specific identity of the neuropeptides produced by the EP cells is still in question, these anti-peptide antisera were simply used to distinguish the type B neurons that express this particular peptidergic phenotype.

Following incubation in primary antisera, the tissues were routinely processed using a biotinylated secondary antiserum, followed by avidin-HRP (Vector Laboratories, Inc; Burlingame, CA), and reacted with 1.4 mM diaminobenzidine in 0.001% H_2O_2. Stained preparations were then mounted in 90% glycerol in PBS plus 0.1% n-propyl gallate as an antioxidant. For co-localization experiments, the anti-Go_α antiserum was detected with a biotinylated anti-rabbit antiserum followed by avidin-fluorescein, while the anti-SCP antibody was detected with an anti-mouse-rhodamine conjugate (all secondary antisera were obtained from Vector Laboratories). Fluorochrome-labelled tissues were mounted in in 90% glycerol in HEPES-buffered saline (pH 8.5) with n-propyl gallate. Preparations were subsequently analyzed and photographed at 650X using Nomarski and fluorescence optics or drawn by camera lucida techniques. Specificity controls for the anti-Go_α antiserum included omission of the primary antiserum; incubation of embryos with antisera that are specific for other classes of G proteins (Gi_α, Gs_α, Gt_α, Gf_α; Quan, et al., 1993); and preadsorption of the anti-Go_α antiserum with the Go-specific peptide against which the antiserum was generated (Goldsmith et al., 1987; Wolfgang et al., 1990) .

Immunoblot Analysis

Pooled embryonic and postembryonic tissues were collected on dry ice and then rapidly homogenized in sample buffer at 100° C. Approximately 100 μg protein from each sample was separated on 10% SDS-polyacrylamide gels. The protein was subsequently blot-transferred to nitrocellulose, which was then incubated with the same anti-Go_α antiserum that was used for immunohistochemistry (after Otter et al., 1987). Bound antibodies were then visualized by incubation with an anti-rabbit antiserum coupled to alkaline phosphatase, followed by reaction with the appropriate enzymatic substrates (Promega; Madison, WI). The specificity of the reaction was monitored by pre-incubation of the anti-Go_α antiserum with an excess quantity of the peptide used to generate the antiserum (Goldsmith et al., 1987) and by incubation of identical blots with antisera specific for other G protein α-subunits; no cross-reactivity among these antisera has been detected in insect tissues (see also Wolfgang et al., 1990; Wolfgang et al., 1991).

AlF_4- Experiments

Embryos at several different developmental stages around the time of migration were removed from their protective membranes and restrained in a Sylgard (Dow Corning; NY) chamber under culture medium. A small opening was made in the dorsal epithelium to expose the premigratory EP cells. In our initial experiments, the medium was then replaced with medium containing 25-100 uM AlF_4- (prepared from fresh stocks of 1 mM $AlCl_3$ and 500 mM NaF in culture medium, combined in a final ratio of 1:2000; after Jones et al., 1990). The embryos were then allowed to develop for an additional 6-8 hours at 28°C, followed by fixation and staining with TN-1. In subsequent experiments, the time of exposure to AlF_4- was reduced by application of bacterial alpha toxin (α-hemolysin from S. aureus; List Biologicals; Campbell, CA). This compound has been shown to form transient pores in most plasma membranes, rendering cells permeable to small molecules (M_r<4 X

10^3) without inducing cell lysis (McEwen and Arion, 1985; Hohman, 1988). Alpha toxin (0.02-0.01 mg/ml) was applied to dissected embryos in the presence of 25 uM AlF_4- for 20-30 min, followed by extensive rinsing in normal medium. The preparations were then allowed to continue to develop for an additional 8-10 hours before fixation. Standard controls for these experiments included both dissected and undissected embryos that were incubated in normal medium, or animals that were incubated for 20-30 min in either AlF_4- or alpha toxin alone. As an additional control, one group of animals was incubated first with AlF_4-, rinsed, and then incubated with alpha toxin over the course of 30 min. All preparations were subsequently labelled with TN-1 (as described above), and the extent of EP cell migration and process outgrowth was analyzed with respect to matched controls.

Intracellular Pressure Injection

Individual EP cells were injected at specific times during their migration using a WPI pneumatic ("Picopump") pressure injector. Staged embryos were dissected to expose the developing ENS and positioned under a water immersion lens on a modified Nikon UM-2 microscope equipped with an orbital stage from Meridian Instruments, Inc. (Kent, WA). Electrodes were pulled from filament glass on a Sutter P-80/PC puller to a resistance of 10-30 MΩ (when filled with 3 M KCl). Electrodes were filled at the tip with a buffered injection solution containing 1-10 mg/ml lysinated tetramethylrhodamine dextran amine (LRD; from Molecular Probes, Eugene, OR) and backfilled with 1.2 M LiCl or 2 M KAC. For some experiments, the injection solution also contained 20 mM of GTPγ–S, a non-hydrolyzable form of GTP (Stryer and Bourne, 1986). Electrodes were positioned using a Nikon/Narishige "joystick" hydraulic manipulator. Penetration of individual cells was monitored using a Getting Model 5A intracellular amplifier mounted in parallel with the pressure injector. Solutions were injected using a brief series of 100 msec pulses delivered with an ejection port pressure of ~5 psi; the ejection pressure was adjusted so that no detectable swelling of the target cells accompanied an individual pulse. Following each

injection, the preparations were briefly examined using a heavily filtered UV light source to verify the number and position of labelled cells. Embryos were then allowed to continue to develop in culture at 28° C for an additional 8-12 hr before fixation and counterstaining with TN-1. Preparations were photographed at 650X, and the images scanned and analyzed using a Microtek ScanMaker and Adobe Photoshop software.

RESULTS

Go_α expression during EP cell migration

The postembryonic ENS of *Manduca* extends the length of the alimentary tract (foregut, midgut, and hindgut) and innervates the superficial layers of the visceral musculature. In the vicinity of the foregut-midgut boundary, this innervation is supplied by the enteric plexus (fig. 1A), a branching network of nerves projecting along discrete sets of muscle fibers on the foregut and along the eight major longitudinal muscle bands of the midgut (L1-L4 and R1-R4). Anteriorly, the enteric plexus is connected with the enteric ganglia of the foregut by the recurrent nerve (RN; Copenhaver and Taghert, 1991), while posteriorly, its nerves extend most of the length of the midgut and supply a diffuse innervation to the midgut musculature (Copenhaver and Taghert, 1989a). Within the enteric plexus is distributed a population of about 300 neurons (the EP cells). Unlike most neurons of the insect CNS, the EP cells do not occupy cell-specific positions but are distributed variably among the different branches of the plexus. As previously illustrated (Copenhaver and Taghert, 1989b), these distributions are achieved by the directed migration of the EP cells into the different domains of the enteric plexus between 55-60% of embryonic development (fig. 1B).

To investigate whether G proteins may be involved in regulating the motile behavior of the EP cells, the developmental expression of different G proteins in the embryonic ENS was examined using affinity-purified antisera against various G_α protein subtypes (including Gs_α, Gi_α, Go_α, and Gf_α; Quan et al., 1993). Initially, during the slow, circumferential

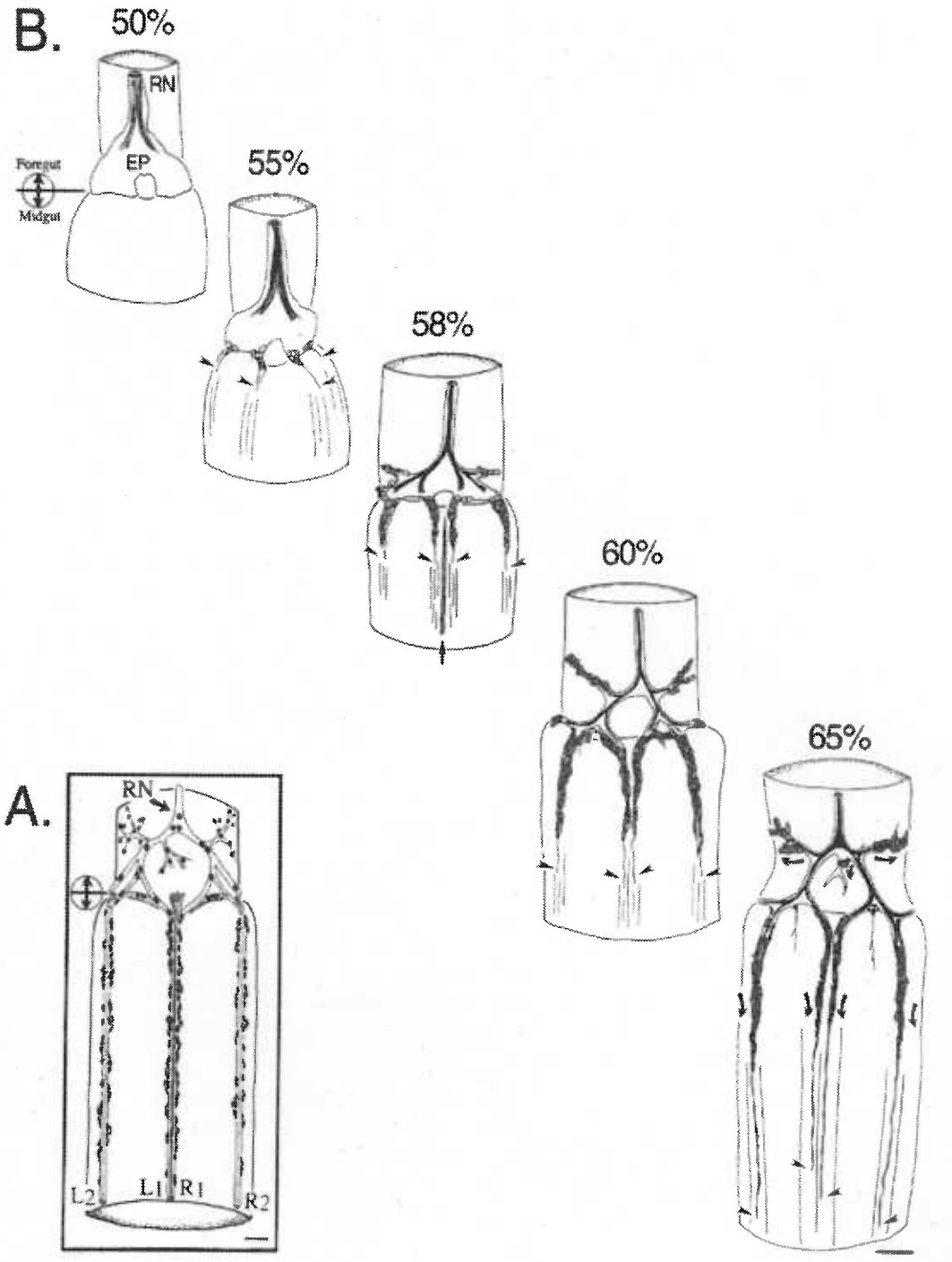
B.
50%
RN
EP
Foregut
Midgut
55%
58%
60%
65%
A.
RN
L2
L1
R1
R2

Figure 1. Onset of Go_α expression during cell migration in the ENS. **A.** Schematic representation of the mature distribution of neurons (EP cells) in the ENS. Boxed panel shows a dorsal view of the enteric plexus on either side of the foregut-midgut boundary (circled arrow). The enteric plexus is connected with the anterior enteric ganglia of the foregut by the recurrent nerve (RN); branches of the plexus extend onto specific sets of muscle fibers on the foregut and along the eight major longitudinal muscle bands of the midgut (L1-L4 and R1-R4). **B.** *Camera lucida* drawings of the developing enteric plexus at successive stages of embryogenesis (shown in percent development) to illustrate the onset and enhancement of Go_α immunoreactivity during EP cell migration. Each panel shows a dorsal view of the embryonic gut in the vicinity of the foregut-midgut boundary (anterior is to the top of the page). At 50%, the EP cells form a discrete packet of premigratory cells (labelled **EP**; compare with fig. 2) that sits adjacent to the foregut-midgut boundary; no detectable Go_α immunoreactivity is present in the EP cells, although there is staining in processes descending through the recurrent nerve (**RN**) from the anterior enteric ganglia (ganglia are not shown). At 55%, faint levels of staining can be seen in the leading groups of EP cells (stippled cell groups) and in their processes that have extended onto the midgut muscle bands (arrowheads). From 55-60%, as the EP cells migrate onto the visceral musculature of both the foregut and midgut, there is a gradual enhancement of staining in all of the migratory neurons and their processes (arrowheads indicate the growing tips of EP cell processes). Transient staining also appears within the epithelium of the midgut at the dorsal midline at ~58% (arrow), during the final phase of midgut closure. Following the completion of migration (65%), the level of staining continues to increase both in the somata of the EP cells and in their processes as they continue to elongate along the gut musculature (arrowheads). Curved arrows depict the general directions of neuronal migration. Scale: 0.05 mm.

phase of migration (40-50%), no G protein expression was seen in the EP cells, which during this stage formed a coherent packet of cells adjacent to the foregut-midgut boundary (structure labelled "EP" in fig. 1B; see also fig. 2A). Go_α immunoreactivity could be detected within processes of the recurrent nerve, descending from the more anterior ganglia (fig. 1B, 2E, arrow). Then just prior to the dispersive phase of migration (55-60%), faint levels of Go_α immunoreactivity could first be detected in the EP cells that had become aligned with the newly formed muscle bands on the adjacent midgut (fig. 1B, stippled clusters; 2E, upper arrowheads). As migration proceeded (58-60%), the intensity of Go_α staining gradually increased in the EP cells that had commenced migration. Immunoreactive material could also be traced into the leading neuronal processes that extended along the muscle bands of the midgut (compare TN-1 staining with Go_α immunoreactivity; fig 2). EP cells moving laterally onto the foregut musculature also began to stain positively for Go_α, concurrent with the onset of their migration (fig. 1B, 58-60%). By ~60% of development, the dispersive phase of migration was largely complete (Copenhaver and Taghert, 1989b); however, the neurons continued to exhibit motile behavior in the form of axonal outgrowth along the gut musculature until 75-80%. During this period, the intensity of Go_α immunoreactivity also continued to increase within the EP cell bodies and their processes (fig 2H; fig. 3, arrowheads). Preadsorption of the Go_α antiserum with the Go-specific peptide used to generate the antiserum completely blocked all immunoreactive staining (fig. 3C). Antisera against a number of other G_α subunits, including Gs_α (fig. 3D), produced no detectable staining in either the EP cells or their processes throughout the period of migration.

While Go_α-related proteins were first detectable in the EP cells just before the onset of their migration, Go_α immunoreactivity could be seen in a number of other tissues at substantially earlier times in development. In the embryonic CNS, Go_α immunoreactivity was detectable in the tracts and commissures of the developing ganglia by 35% of development and subsequently could be traced within the axonal processes of all the major nerve roots (Copenhaver and Nichols, in preparation). Neuropilar staining was also apparent

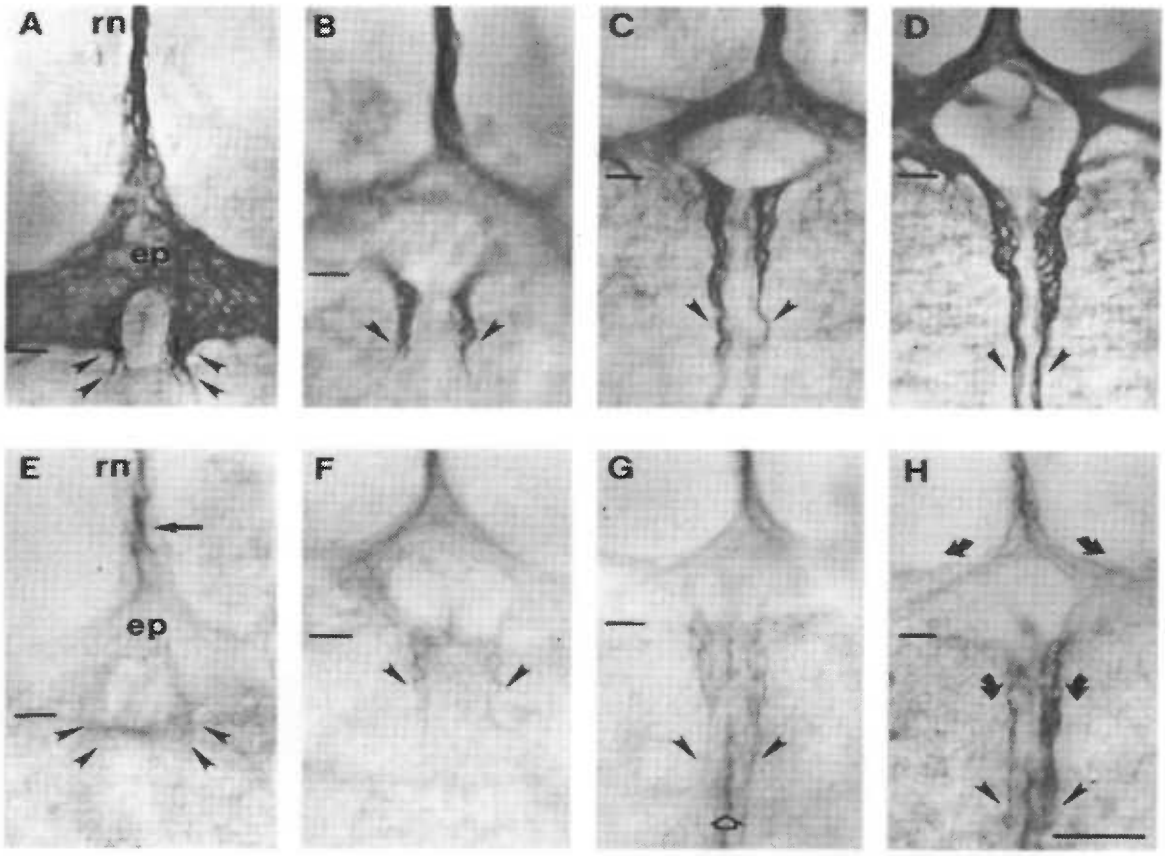

Figure 2. Photomicrographs of $G_{o\alpha}$ immunoreactivity in the developing enteric plexus. Panels A-D show a progression of developmental ages stained with TN-1 to show the overall distributions of EP cells during migration; panels E-H show similar ages stained with anti-$G_{o\alpha}$ antisera. At 54% (panel A), the EP cells still form a discrete packet on the foregut (labelled ep), although subsets of the premigratory neurons have begun to align with the developing muscle bands near the foregut-midgut boundary (indicated by the horizontal black line in each panel). At this time, faint $G_{o\alpha}$-immunoreactivity can first be detected in these leading cell groups and their processes (panel E, arrowheads). Stained processes descending from the anterior enteric ganglia through the recurrent nerve (RN) are also apparent (arrow). At 56% (B and F), just after migration onset, anti-$G_{o\alpha}$ immunoreactivity is clearly present in the advancing groups of EP cells and their processes (arrowheads). By 58% (C and G), groups of EP cells have moved onto the musculature of both the foregut and midgut and show an enhanced level of immunoreactive staining; transient staining can also be seen in a narrow strip of epithelium in the dorsal midgut (open arrow), below and between the muscle bands on which the EP cells are migrating. By 60% (D and H), most of the EP cells have migrated onto the foregut or midgut (curved arrows), but they continue to elaborate processes along the visceral musculature, and the level of $G_{o\alpha}$ immunoreactivity continues to increase throughout the neurons and their processes. Scale: 50 mm.

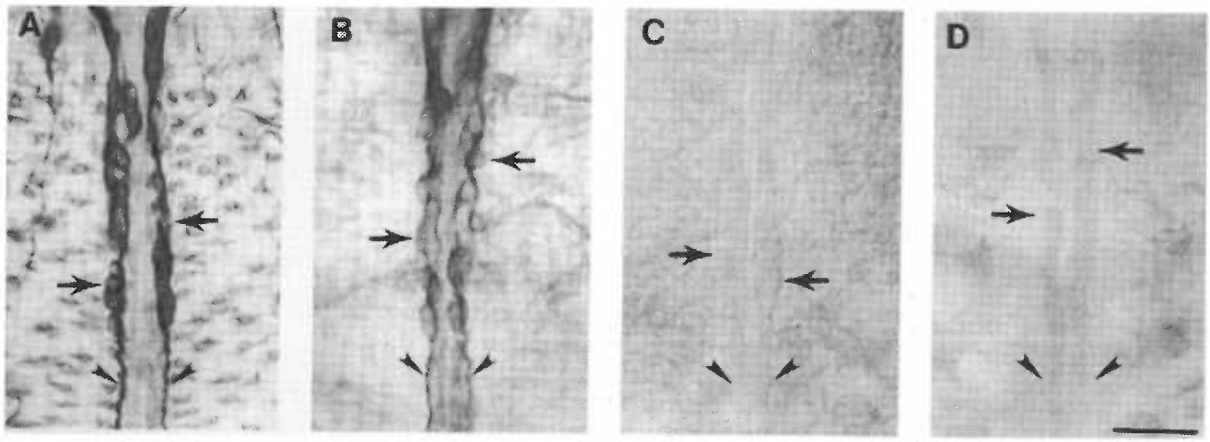

Figure 3. $G_{o\alpha}$ immunoreactivity is eliminated by preadsorption with $G_{o\alpha}$-specific peptides. (A) TN-1 stained embryo at 65% of development shows the distribution of EP cells on the mid-dorsal muscle bands of the midgut, following the completion of migration. (b) A different embryo of the same age, stained with the anti-$G_{o\alpha}$ antiserum; intense immunoreactivity is present in both the cell bodies (arrows) and in their processes that extend posteriorly along the midgut muscle bands (arrowheads). (C) $G_{o\alpha}$ immunoreactivity is completely blocked when the antibody is pre-incubated with the $G_{o\alpha}$ specific peptide used to generate the $G_{o\alpha}$ antiserum. Unstained cell bodies (arrows) and the approximate positions of their processes (arrowheads) can be faintly seen using Nomarski optics. Scale: 50 μm.

in the frontal ganglion of the foregut (not shown), and as already noted, Go_α immunoreactivity could be detected within neuronal processes that descended from the enteric ganglia through the recurrent nerve of the foregut (fig. 1B, 2E). Several non-neural tissues also exhibited positive staining for Go_α during embryonic and postembryonic development, including the gonads and prothoracic glands (not shown). Transient staining of the midline epithelium of the midgut was also apparent between 55-60% of development, coincident with the final closure of the midgut and overgrowth of the visceral musculature (see arrow at 58% in fig. 1B; arrow in fig. 2G). This staining was clearly distinguishable from the immunoreactive material within the migratory EP cells. It should be noted that several other classes of G proteins could also be detected by immunohistochemistry in the developing CNS of *Manduca*, including Gi_α, Gs_α, and Gf_α (Copenhaver and Lagrange, unpublished observations). However, none of these additional antisera resulted in positive staining of the EP cells, supporting our conclusion that these neurons selectively express Go_α-related proteins around the time of their migration.

Post-migratory regulation of Go_α expression

While the initial appearance of Go_α in the EP cells coincided with the onset of their migratory behavior, the levels of Go_α immunoreactivity continued to increase in the enteric plexus even after migration was complete (fig. 2H, 3B). When we examined the distribution of Go-related proteins in the postembryonic ENS, however, we found that the pattern of Go_α expression had changed significantly. Whereas all of the EP cells initially stained positively for Go_α during migration, only certain subsets of the neurons remained immunoreactive in postembryonic stages (fig. 4A). Specifically, all of the EP cells that had migrated onto the foregut musculature continued to exhibit Go_α immunoreactivity (fig. 4B), although the level of staining in these cells appeared more diffuse and was less clearly associated with the plasma membrane than in embryonic stages. Within the neuronal populations of the midgut, we found robust levels of Go_α immunoreactivity, but only in a

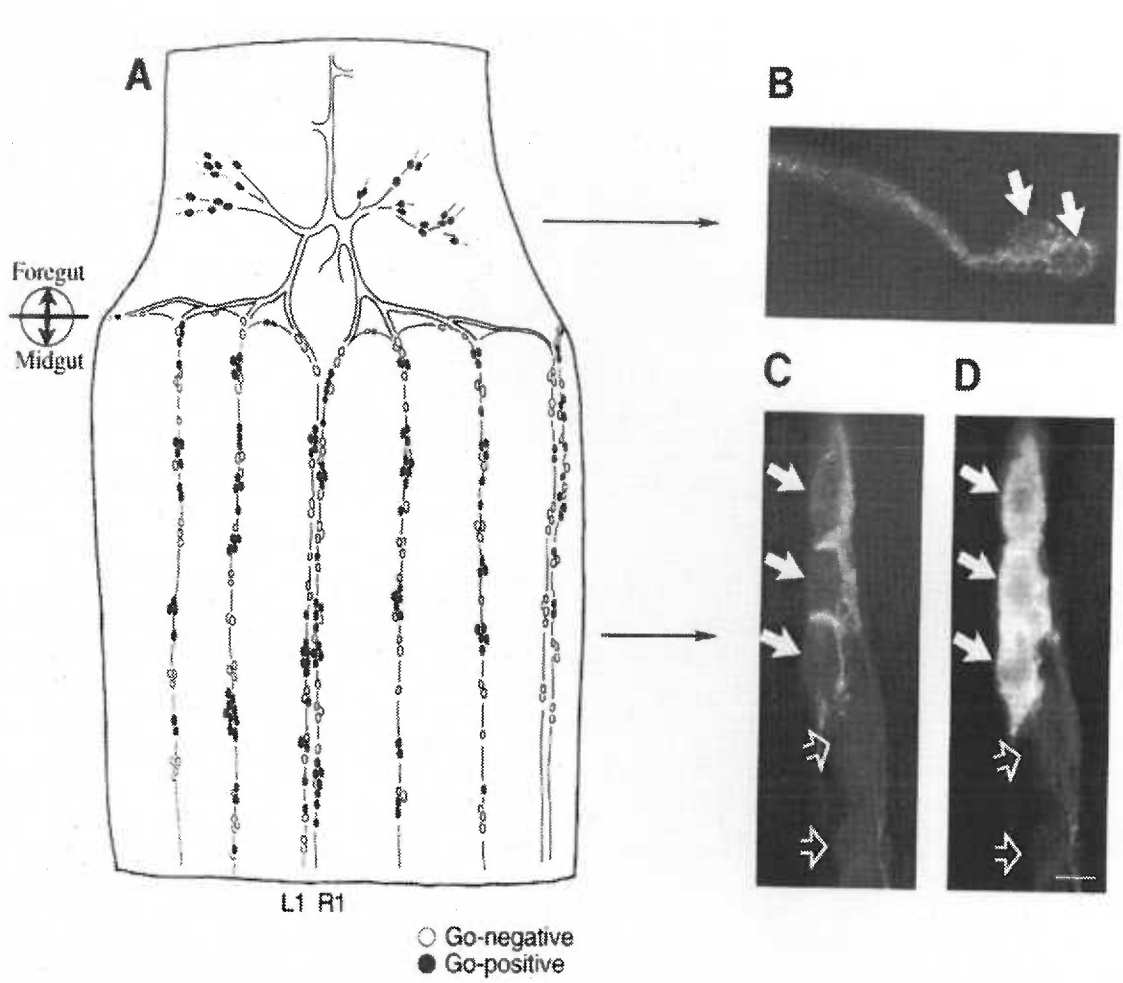

Figure 4. Postembryonic expression of $G_{o\alpha}$ in the enteric plexus is restricted to specific subsets of the differentiated EP cells. (A) Cameral lucida image of the enteric plexus in a third instar larva, stained with the anti-$G_{o\alpha}$ antiserum. All of the EP cells that migrated onto the foregut retain detectable levels of $G_{o\alpha}$-related protein, although the distribution of immunoreactive material in these neurons appears more diffuse than during their migratory stage of embryonic development. (B) A photomicrograph of EP cells (arrows) on the larval foregut, stained with the anti-$G_{o\alpha}$ antiserum and visualized by immunofluorescence with a fluorescein-conjugated marker. In contrast, only a subset of the EP cells that have migrated onto the midgut still contain detectable levels of $G_{o\alpha}$-related protein, so that immunopositive and immunonegative neurons are intermingled along the midgut muscle bands (filled and clear cells in panel A). (C) One cluster of EP cells on a midgut muscle band, stained with the anti-$G_{o\alpha}$ antiserum and a fluorescein-conjugated secondary antiserum; a subset of the neurons and their processes are clearly stained (solid arrows), with immunoreactive material primarily in the vicinity of the plasma membrane. The other EP cells show no evidence of $G_{o\alpha}$ immunoreactivity (open arrows). (D) The same preparation stained with a monoclonal antibody that recognizes FMRFamide-related peptides in the ENS, visualized with a rhodamine-conjugated secondary antibody. Only those neurons that exhibit the peptidergic phenotype still express detectable levels of $G_{o\alpha}$ immunoreactivity (compare with C).

subset of the EP cells that had migrated onto the muscle bands (fig. 4C). When preparations were also labelled with an antiserum that recognized FMRFamide-related peptides in the ENS (fig. 4D), we found that only the EP cells that exhibited this peptidergic phenotype (the "type B" cells; Copenhaver and Taghert, 1989a) also continued to stain positively for Go_α. Thus the expression of Go_α within the enteric plexus showed an additional phase of regulation once migration was complete, corresponding with the differential expression of mature phenotypes by distinct subsets of the EP cells. These observations suggest that Go may serve an additional function within the ENS that is specific to the peptidergic class of post-migratory neurons.

Immunoblot analysis of Go_α expression

To characterize the proteins being recognized by the anti-Go_α antiserum in *Manduca*, we also performed an immunoblot analysis of extracts from a variety of embryonic and postembryonic tissues. As shown in figure 5, a single moiety of M_r ~40 X 10^3 was recognized by the anti-Go_α antiserum when applied to nitrocellulose blots of protein separated in a polyacrylamide gel. The tissue distribution of labelled proteins in these blots corresponded well with the patterns of immunoreactivity that were revealed by the anti-Go_α antiserum in our whole-mount preparations. In postembryonic animals, substantial amounts of the M_r ~40 X 10^3 protein were present in extracts of larval CNS (lane B), larval midgut muscle bands (which included the EP cells and their processes; lane C), and the enteric plexus regions of hatchling midguts (lane F). In contrast, no proteins were labelled in extracts from larval fat body (lane A) or from interband regions of the midgut (regions that contains visceral muscle but were devoid of EP cells; lanes D, E), nor was any positive signal seen in extracts from skeletal muscle or malpighian tubules (not shown). In pooled tissues from embryos dissected at 75% of development, a protein band of similar molecular weight was detected in extracts from both embryonic midgut (containing the post-migratory EP cells; lane G) and in the embryonic body (which included the developing CNS; lane H).

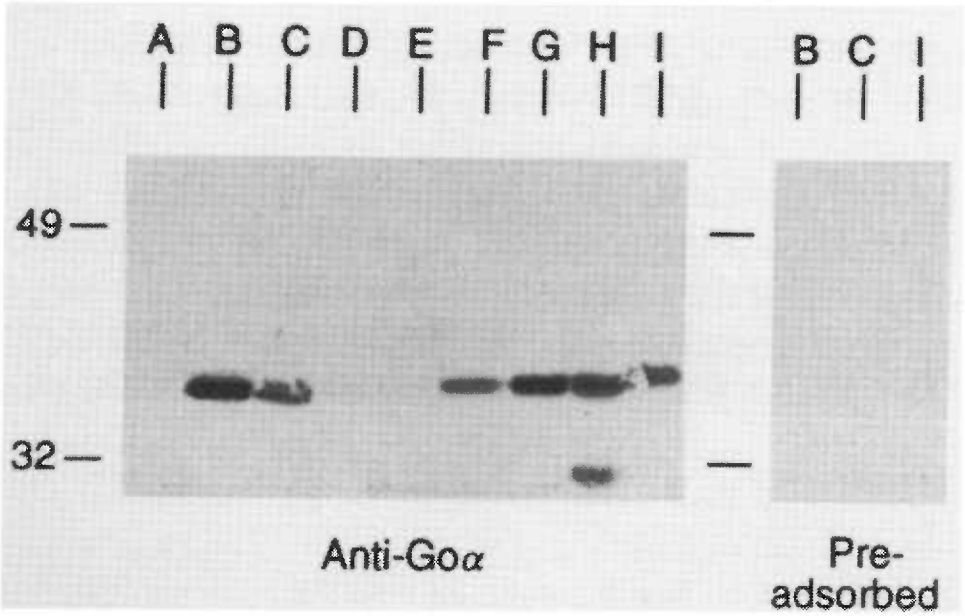

Figure 5. Immunoblot analysis of $G_{o\alpha}$-related proteins in embryonic and postembryonic tissues of *Manduca*. Blots were probed with the anti-$G_{o\alpha}$ antibody at 1 μg/ml or with antibody that had been preadsorbed with $G_{o\alpha}$-specific peptides (10 μg/ml) overnight at 4° C. Individual lanes contain extracts prepared from the following tissues (50 μg loaded per lane). (A) Larval fat body. (B) Larval CNS. (C) Larval midgut muscle bands containing the EP cells. (D) Larval interband muscles from the anterior regions of the midgut (interband regions between the EP cell groups). (E) Larval interband muscles from the posterior midgut. (F) Hatchling midgut dissected at the level of the enteric plexus. (G) Pooled embryonic midguts taken at 75% of development. (H) Pooled embryonic bodies (following removal of the midguts) at 75% of development. (I) Extract from *Drosphila* CNS (see Thambi et al., 1989). High levels of a single $G_{o\alpha}$-like protein (M_r~40X10^3) were detected in the CNS of both *Manduca* and *Drosophila* (lanes B, I), and in tissue samples that included the EP cells from embryonic (lane G) or postembryonic stages of development (lanes C, F). An additional band was present in extracts of the embryonic bodies (M_r~30X10^3). All positive signals were absent when identical tissue extracts were probed with preadsorbed antibody.

A second labelled band of M_r ~30 X 10^3 was also seen in the latter sample. The apparent size of the major protein band closely matched the molecular weight of the Go_α subunit in extracts from *Drosophila* brain (lane I; see also Thambi et al., 1989). As with our immunohistochemical staining, all positive signals were completely abolished when the anti-Go_α antiserum was preadsorbed with the synthetic Go_α peptide, prior to the application of the antiserum to the blots.

Effects of AlF_4- and GTPγ-S on EP cell migration

The appearance of Go_α-like proteins in the EP cells coincident with the onset of their migration suggested that G proteins might play a role in regulating one or more aspects of the migratory process. To investigate this possibility, we exposed the EP cells in semi-intact embryos to varying concentrations of AlF_4-, a compound that has been shown to activate all heterotrimeric G proteins in an unregulated manner (Sternweiss and Gilman, 1982; Anderson et al., 1991) but does not affect the small-molecular weight family of *ras*-related proteins (e.g. Donaldson et al., 1991). Embryos were opened in culture starting at about 52% of development (± 0.5%), at which time Go_α immunoreactivity could be faintly detected in the EP cells but the migratory dispersal of the neurons had not yet commenced (fig. 6A). When control embryos were permitted to develop in culture for 6-8 hours and then fixed and stained with TN-1, we observed that all aspects of EP cell migration and process outgrowth proceeded normally (fig. 6B). In contrast, when identically staged embryos were cultured in the presence of AlF_4-, we subsequently found a dramatic inhibition of both EP cell migration and process outgrowth (fig. 6C-E), although the overall appearance of the neurons and their expression of the surface epitope recognized by TN-1 remained relatively normal. The examples shown in figure 6 were chosen from six separate experiments (employing a total of 53 experimental preparations) to illustrate the range of effects that we observed following exposure to AlF_4-. In most of these preparations, however, we saw little or no evidence of migratory dispersal onto the musculature of the foregut and midgut.

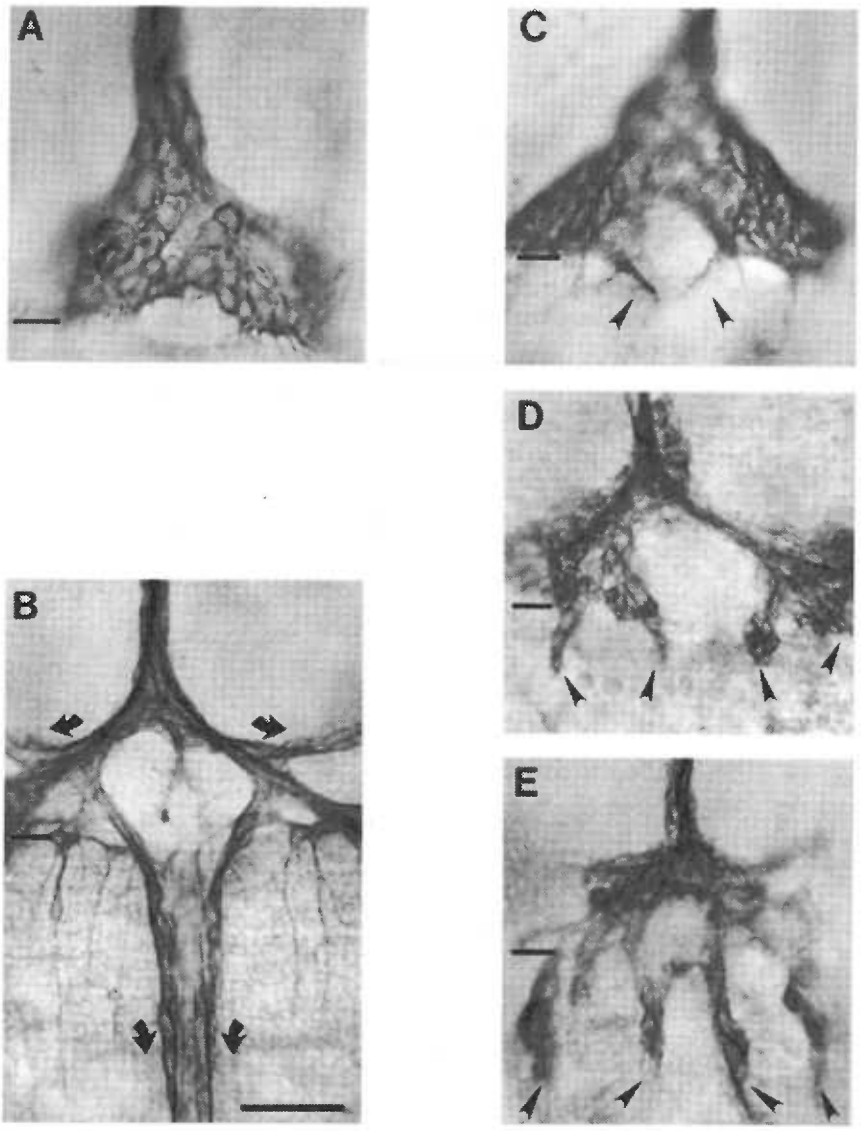

Figure 6. Effects of AlF_4^- treatment on EP cell migration in embryonic culture. (A) Staged embryo (52% of development) that was fixed and stained with TN-1 to illustrate the position of the EP cells just prior to AlF_4^- treatment; at this stage, the neurons have not begun the dispersive phase of their migration (foregut-midgut boundary is indicated by the horizontal black line in each panel). (B) Embryo that was placed in culture at 52% (same age as panel A) and allowed to develop in normal medium for 8 hours before fixation and staining with TN-1. Both cell migration and process outgrowth proceeded normally under these conditions. (C-E) Embryos that were placed in culture at 52% in the presence of 20-40 mg/ml AlF_4^- and allowed to develop for 8 hours. Arrowheads indicate the most dispersed groups of EP cells in these preparations. In contrast to cultured control animals (panel B), there was a substantial inhibition of both cell migration and process outgrowth in the presence of AlF_4^-, although the EP cells remained relatively healthy in appearance and TN-1 staining. Preparations in panels C-E were chosen to illustrate the range of migration that was observed under these conditions. Scale: 50 μm..

We also examined the effects of AlF_4- exposure at several different stages around the onset of EP cell migration (table 1). When cultured embryos were exposed to AlF_4- for various periods between 45-50% of development, we observed only minor effects on the timing and number of EP cells that subsequently migrated onto the midgut, although AlF_4- did impede the normal closure of the gut epithelium. In contrast, treatment with AlF_4- between 50-55% (just prior to migration onset) substantially inhibited both the migration and outgrowth of the EP cells, as also shown in figure 6. At still later stages, when the EP cells were actively dispersing along the muscle bands (55-60%), treatments with AlF_4- continued to have an inhibitory effect on both cell migration and process outgrowth when compared to matched controls. Thus the onset of sensitivity to AlF_4- treatment coincided reasonably well with the appearance of Go_α immunoreactivity in the EP cells (first detectable at ~52% of development; fig. 3) and persisted throughout the period of their active migration. Similar treatments with AlF_4- were also found to inhibit process outgrowth from the CNS of *Manduca* (Nichols and Copenhaver, unpublished observations), coincident with the expression of Go_α immunoreactivity in the growing axons.

To control for non-specific effects that might result from a continuous exposure to aluminum salts, we performed an additional set of experiments in which alpha toxin was used to enhance the permeability of the EP cells to an abbreviated pulse of AlF_4-. When embryos at 52% of development were exposed to alpha toxin alone for 20-30 minutes and then allowed to develop for an additional 8 hours in normal medium, no significant changes were detected in either the number of EP cells that migrated or in the extent of neuronal migration and process outgrowth (fig. 7, slashed histograms). Similarly, when embryos were exposed first to AlF_4-, rinsed, and then exposed to alpha toxin over the course of 30 minutes, no diminution in the rate or extent of EP cell motility were subsequently observed (chequered histograms). In contrast, both cell migration and process outgrowth were subsequently reduced when embryos were exposed to a combination of alpha toxin and

TABLE 1.

Developmental Stage of AlF_4- treatment:	% Inhibition of Cell Migration (vs. controls):	% Inhibition of Process Outgrowth (vs. controls)
45-50% (pre-migration):	5%	(n.m.)
50-55% (migration onset):	85%	83%
55-60% (mid-migration):	97%	90%

Effects of AlF_4- treatment on EP cell motility at various stages of development. Exposure of cultured embryos to AlF_4- at times between 45-50% of development (prior to the appearance of Go_α immunoreactivity in the EP cells) caused only minor effects on the subsequent migration of the EP cells when compared to controls; process outgrowth in these preparations was not measured (n.m.). Treatments applied between 50-55% of development (around the onset of Go_α expression in the EP cells) resulted in a substantial inhibition in both neuronal migration and process outgrowth (compare with fig. 7). Treatments applied at still later times during migration (between 55-60%) continued to have a strong inhibitory effect on both aspects of EP cell motility. Data represents the pooled averages from several different experiments involving a minimum of five animals per experiment.

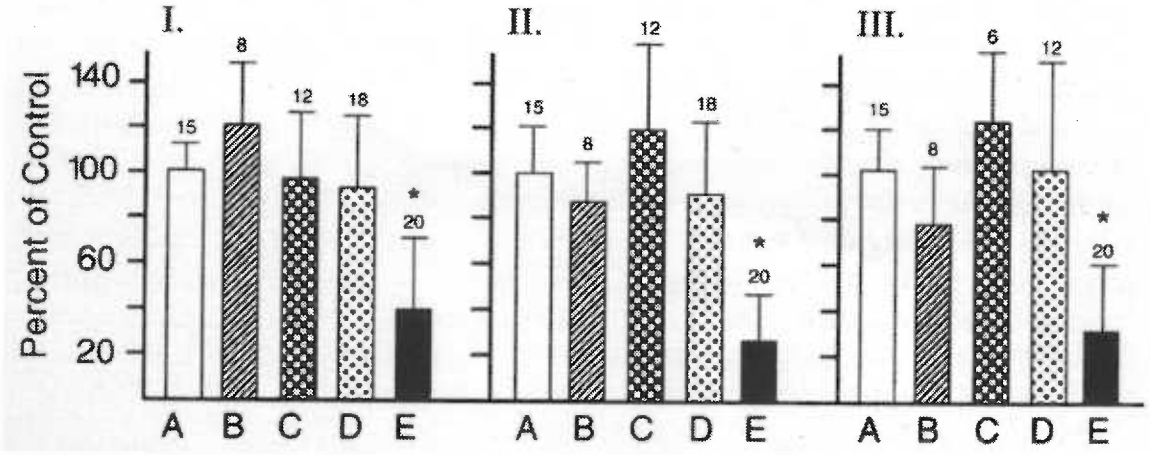

Figure 7. Effects of a brief exposure to AlF_4- on EP cell migration in the presence of the pore-forming compound, alpha toxin. Embryos were placed in culture at 52% of development (as in figure 6), allowed to develop for 6 hours at 28° C, and then stained with TN-1. Camera lucida images of these preparations were then used to calculate (I) the number of migratory EP cells, (II) the greatest distance travelled by an EP cell, and (III) the greatest distance of process outgrowth beyond the EP cells on each of the dorsal muscle bands (L1-L2 and R1-R2; see figure 1A). Results were then normalized for each band with respect to values taken from control animals; the average values and standard deviations for each treatment group are shown in the histograms (numbers indicate the number of preparations used for each histogram). Error bars indicate standard deviations. Statistical values were calculated with respect to a smaller number of matched control animals in each experiment, using a 1-tailed Student's *t*-test. A (open histograms) represent control values; error bars on these histograms were derived from pooled controls from several different experiments to illustrate the normal variation in migration and outgrowth. B (diagonal slashed histograms): a brief (20-30 min) exposure to 0.02-0.01 mg/ ml-1 alpha toxin alone followed by extensive rinsing produced no significant changes in the number of EP cells that migrated or in the distance that they migrated along the muscle bands. C (chequered histograms): sequential treatments with 25 µg/ ml-1 AlF_4- alone (for 10 min), rinsing in normal medium (for 10 min) and then alpha toxin alone (for 10 min) similarly caused no diminution in either cell migration or process outgrowth. D (stippled histograms): exposure to 25 µg/ ml-1 AlF_4- alone for (20-30 min) produced no significant changes in the number of EP cells that migrated or in the distance that they migrated along the muscle bands, although in some of these preparations there was a substantial inhibition in process outgrowth (note the large standard deviation for histogram III-C). E (solid histograms): exposure of the EP cells to AlF_4- in the presence of alpha toxin caused a significant inhibition of all three aspects of motility: *$p<0.05$ for the number of migrating EP cells (I) and the distance process outgrowth (III), and *$p<0.01$ for the distance of of the greatest distance of EP cell migration (II).

AlF_4- for 20-30 min, followed by extensive rinsing with normal medium (solid histograms). Exposure to AlF_4- by itself during this same period had only modest effects on the number or extent of migratory neurons (stippled histograms); in some of these preparations, however, there was a substantial inhibition of process outgrowth, resulting in the large standard deviation shown in histogram III-D. As with longer exposures to AlF_4- alone (fig. 6), the presence of alpha toxin in combination with AlF_4- produced no obvious deleterious effects on the level of TN-1 staining in the enteric plexus or in the general morphology of the EP cells, despite their diminished motility.

These experiments showed that a brief exposure of the EP cells to AlF_4-, a compound that has been shown to stimulate the activation of G proteins in other systems, caused a dramatic inhibition of neuronal motility in the developing ENS. However, while AlF_4- does not activate the *ras*-related family of GTP-binding proteins (Donaldson et al., 1991), it has been shown to stimulate a number of other metabolic enzymes that are unrelated to the heterotrimeric G proteins (e.g. Lange et al. 1986, Robinson et al., 1986). As a means of corroborating our results with AlF_4-, we also initiated a series of experiments using intracellular pressure injections of individual EP cells with other compounds that affect G protein activity. When individual EP cells were injected prior to the onset of migration with a control solution containing the fluorescent marker LRD (fig. 8a), we subsequently observed that most of the injected cells continued to exhibit both normal migration and process outgrowth (fig. 8b). In contrast, when individual cells were injected with GTPγ–S, a non-hydrolyzable form of GTP that has been shown to activate all heterotrimeric G proteins (Stryer and Bourne, 1986; Gilman, 1987), we observed a substantial reduction in the extent of both migration and outgrowth in approximately 50% of the injected cells. These results, though preliminary, support our experiments with AlF_4- and suggest the extent of EP cell migration may be regulated via the activation of one or more G protein-mediated events in the course of neuronal development.

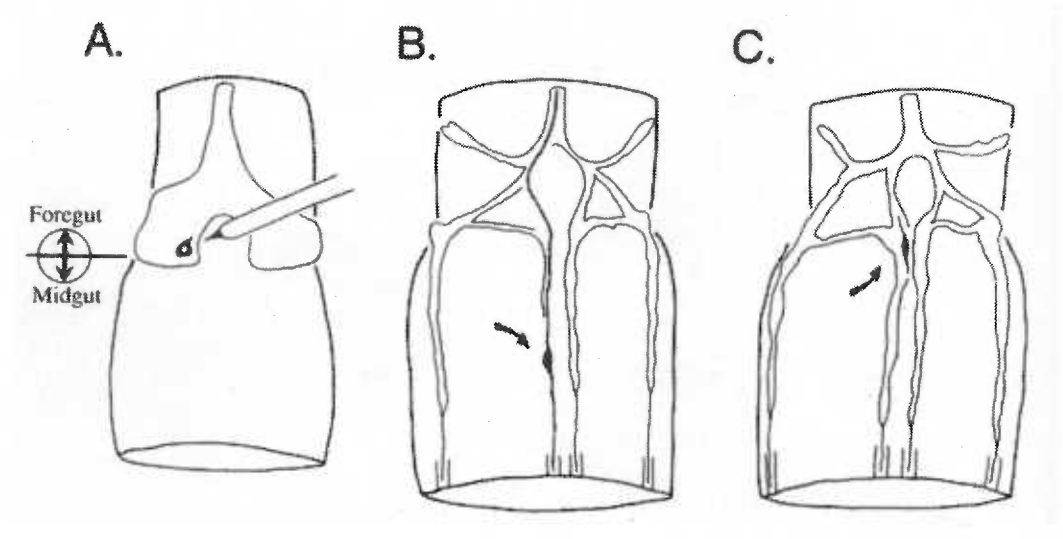

Figure 8. Intracellular injection of GTPγS inhibits EP cell migration. (A) Individual EP cell injected before the onset of migrtion with a control solution containing the fluorescent marker LRD. (B) Example of a preparation in which an individual EP cell was injected prior to migration (at a stage similar to that in A) and then allowed to develop for an additional 10 hours at 28°C. Both the migration and the process outgrowth of the injected cell were indistinguishable from that of the surrounding uninjected cells. (C) Example of an individual EP cell that was injected with a solution containing 20 mM GTPγS. In this preparation, both the migration and outgrowth of the injected cell were substantially reduced. Similar effects were seen in approximately 50% of the cells treated in this manner; images were drawn from scanned photographs of the preparations following fixation and counterstaining with TN-1.

DISCUSSION

Developmental Expression of G Proteins in the ENS

The results of this paper show that a protein related to the Go class of heterotrimeric G proteins is selectively expressed by a defined population of migratory neurons, the EP cells, and that the appearance of this protein coincides with their transition from a premigratory to a migratory stage of development. An important consideration in evaluating these results is the sensitivity of our methods for detecting G proteins in the developing ENS. Several lines of evidence support our conclusion that the EP cells express only one type of G protein, Go, coincident with the onset of their migration. As noted above, we screened embryos with a number of antisera against other G protein subtypes (including Gs, Gi, Gt, and Gf; Quan et al., 1993), all of which produced distinctive patterns of immunoreactive staining in the CNS and other regions of the embryo (Copenhaver and Lagrange, unpublished observations). We also could detect Go-related material in the CNS and in peripheral nerve branches (including the recurrent nerve of the foregut; fig. 2) at significantly younger ages than in the EP cells, indicating that our failure to detect G proteins in the premigratory EP cells was not simply due to stage-specific levels of expression in the embryo as a whole. G proteins have also been revealed by these same methods in the developing fly embryo (Wolfgang et al., 1991), in which a number of G protein subtypes (including Go) have been detected from the earliest stages of embryonic development; the presence of G proteins in the ENS of *Drosophila* has not been examined, however.

With respect to the identity of the molecules that were recognized by the anti-Go_α antiserum, several lines of evidence support our conclusion that the EP cells express an authentic form of Go_α during their migration. As noted in figure 3, Go_α immunoreactivity in the EP cells was completely suppressed when the antiserum was preadsorbed with the Go_α-specific peptide that was initially used to generate the antiserum (Goldsmith et al., 1987). In addition, Wolfgang et al. (1990) have shown that immunoreactive staining with

this antiserum in *Drosophila* is not inhibited when the antiserum is preadsorbed with peptides specific to other G proteins. Thus in the insect nervous system, the anti-Go_α antiserum appears to recognize members of the Go class of proteins selectively. Further support for this conclusion was found in our immunoblot analysis of protein extracts, in which this same antiserum recognized a single band of appropriate molecular weight (M_r ~40 X 10^3) in a tissue-specific manner, corresponding to the distribution of immunoreactive staining in whole-mount embryonic preparations. A single protein band of similar molecular weight has been found in tissue extracts from *Drosophila*, and this protein has been shown to correspond to authentic Go (Thambi et al., 1989; fig. 5, lane I). The appearance of an additional band of lower molecular weight in extracts of embryonic body (fig. 5, lane H) might be due to a degradation product of authentic Go_α or might reflect cross-reactivity of the antiserum with another protein; however, this additional band was never seen in extracts that included the EP cells. Again, all positive signals in the immunoblots were completely blocked by preadsorption of this antiserum with Go-specific peptides.

While we believe that the immunohistochemical procedures used in this paper provide a sensitive means of detecting Go-related proteins in the ENS, these results will require confirmation with an analysis of Go_α gene expression in this system. We have recently characterized a clone from a *Manduca* cDNA library that contains all the major features of Go_α in other systems (Horgan, Lagrange, and Copenhaver, in preparation), including the conserved peptide sequence (ANNLRGCGLY) that was used to generate the anti-Go_α antiserum. Using probes derived from this clone for in situ hybridization histochemistry, we have now been able to detect the presence of Go_α-specific mRNAs in the EP cells just prior to their migration. This approach can now be used to examine the developmental expression of Go_α during neuronal migration in more detail.

Role of G proteins in EP Cell Migration

The initial appearance of Go_α-related proteins in the EP cells just prior to the onset of migration suggests a number of possible functions for Go in the migratory process. One obvious possibility is that the expression of functional G proteins in the neurons is required for the initiation of migratory behavior to occur. For example, Go might be coupled to a membrane-bound receptor that recognizes extracellular cues for migration, and in turn might cause a stimulation of the intracellular machinery that underlies cellular motility. Alternatively, the initial expression of G proteins might simply be coincident with the onset of migration, in advance of some later aspect of migratory behavior in which G protein-mediated events are required. As previously described, the migration of the EP cells is tightly regulated, both in terms of directionality and duration: EP cell migration lasts from 55-60% of development and then abruptly stops (Copenhaver and Taghert, 1989b), and the EP cells will migrate on the eight muscle bands of the midgut but not the adjacent visceral musculature (Copenhaver and Taghert, 1988). Lastly, Go might serve no significant function at all during migration, being required instead for some later aspect of EP cell differentiation and appearing at the time of migration by coincidence alone.

As an initial means of distinguishing among these possibilities, we have used AlF_4- to stimulate all G protein activity in the EP cells just prior to migration onset. As shown in figures 6 and 7, we found that *stimulation* of G protein activity caused a marked *inhibition* of neuronal motility. These results support a role for Go in regulating the extent or perhaps directionality of EP cell migration, as opposed to providing the initial trigger for migration (in which case stimulation of Go should cause precocious or exuberant migration). For example, the motile behavior of the EP cells might be limited in part by a variety of membrane- or matrix-associated cues associated with their migratory environment, as has been demonstrated for migratory cells in a number of other preparations (Heagsman et al., 1987; Bronner-Fraser, 1990; Baird et al., 1992; Le Douarin and Dupin, 1993). By this scenario, detection of these inhibitory cues would involve Go-coupled membrane receptors in

the EP cells, which when stimulated would cause a termination of migratory behavior and a transition to a subsequent phase of differentiation (including the expression of mature neuronal phenotypes; Copenhaver and Taghert, 1989a; 1989b). Moreover, we found that treatment with AlF_4- cause an inhibition of neuronal motility throughout the period of EP cell migration (table 1), whereas treatments applied before the onset of Go_α expression did not cause a significant disruption in migratory behavior.

Our results support the hypothesis that a Go-mediated pathway plays an important role in regulating the motile behavior of the EP cells: both cell migration and process outgrowth were markedly inhibited in the presence of AlF_4-, a compound that is known to induce the activation of all heterotrimeric G proteins (Sternweiss and Gilman, 1982; Anderson et al., 1991), but not members of the *ras*-related family of GTP-binding proteins (Donaldson et al., 1991; Ktistakis et al., 1992). Several important caveats must be included in the interpretation of these experiments, however. While we have failed to detect other G proteins in the EP cells during migration, the possibility that unidentified members of this family are present at low levels or are unrecognized by our antisera cannot be excluded. It is also conceivable that the effects of AlF_4- were indirect, resulting from a disruption of the migratory pathways of the enteric plexus. In most preparations, however, all of the morphological features of the gut (including the differentiated midgut bands of visceral musculature) could still be distinguished, indicating that the normal pathway structures were nominally present. Similarly, it might be argued that the effects of AlF_4- were simply due to the general toxicity of aluminum salts or to an increase in the osmolarity of the culture medium. The fact that the pore-forming compound alpha toxin potentiated the effects of AlF_4- under conditions in which neither AlF_4- nor alpha toxin alone inhibited migration argues against this possibility. Moreover, increasing the osmolarity of our culture medium with mannitol by as much as 60 mOsm has previously been found to have no major effects on either EP cell migration or process outgrowth (Copenhaver and Lagrange, unpublished observations).

Beyond the limitation that AlF_4- acts as a non-selective stimulating agent for all G proteins, the use of AlF_4- is further complicated by its reported effects on a number of other metabolically important molecules, including glucose-6-phosphatase (Lange et al., 1986), mitogen-activated protein kinases (Anderson et al., 1991), and Na^+,K^+-dependent ATPase (Robinson et al., 1986). A number of more selective reagents have been described that can be used to manipulate G protein activity, including non-hydrolyzable forms of GDP and GTP (Stryer and Bourne, 1986; Gilman, 1987). Our preliminary results using intracellular pressure injections with GTPγ–S (fig. 8) provides support for the data obtained with AlF_4-, in that the introduction of a compound that should *stimulate* all heterotrimeric G proteins also caused an *inhibition* of migration. Unlike AlF_4-, GTPγ–S can also stimulate members of the *ras*-related family of guanyl nucleotide-binding proteins (Donaldson et al., 1991; Ktistakis et al., 1992), so these results must similarly be viewed with caution. Several toxins that perturb certain subsets of heterotrimeric G proteins have also been described, including pertussis toxin (e.g. Tamura et al., 1982) and mastoparin (Higashijima et al., 1988) that have been shown to affect Go activity in other systems. Neither of these compounds are specific for Go, however. Proper characterization of the role of Go during neuronal migration will require the application of G protein-selective probes to individual EP cells, such as the injection of affinity purified antisera (Harris-Warrick et al., 1988; Bergamaschi et al., 1992) or anti-sense oligonucleotide probes against the products of particular G protein genes (Kleuss et al., 1991; Wang et al., 1992).

G Proteins and the Regulation of Directed Cellular Motility

Support for the regulation of EP cell migration by Go can be drawn from work on another form of neuronal motility, the outgrowth of processes during axogenesis. Several different classes of G proteins have been localized to regions of active outgrowth and synaptogenesis in the CNS of both vertebrates and invertebrates (Chang et al., 1988; Pituello et al., 1991; Wolfgang et al., 1991; Asano et al., 1992), and high concentrations of both Gi

and Go have been found in association with growth cone membranes (Edmond et al., 1990; Strittmatter et al., 1990; Garibay et al., 1991). Strittmatter et al. (1990) have also shown that Go_{α} subunits can be activated by another growth cone protein, GAP-43, whose expression is correlated with axonal extension and may stimulate filopodial extension (Goslin et al., 1988; Zuber et al., 1989); the functional consequences of this interaction are as yet unknown.

Other studies have indicated that G proteins may serve a variety of functions in motile growth cones, depending on the environmental context in which the cells are growing. In PC-12 cells that have been treated with nerve growth factor, process outgrowth in response to several different cell adhesion molecules is mediated in part by pertussis-sensitive G proteins (Doherty et al., 1991; Doherty et al., 1992), which appear to stimulate growth cone motility via the activation of one or more calcium channels (Schuch et al., 1989; Doherty et al., 1991). In other preparations, pertussis-sensitive G proteins have been implicated in the *inhibition* of calcium currents in growth cones (Man-Son-Hing and Haydon, 1992). As demonstrated in a number of recent studies, the regulation of intracellular calcium may in turn affect growth cone motility in a variety of ways, depending on the developmental context and the particular cell type involved (e.g. Goldberg, 1988; Mattson et al., 1988; Silver et al., 1989; Rehder and Kater, 1992). Recently, pertussis-sensitive G proteins have been strongly implicated in the inhibition of cones in response to several different collapsing factors from embryonic and postembryonic brain tissue (Igarashi et al., 1993). These results suggest that G proteins may play a critical signalling function during the guidance of axons through the substrates of the developing brain and peripheral nervous system. While the mechanisms by which G proteins regulate growth cone motility are unknown, G proteins have been shown to affect the polymerization of several major cytoskeletal proteins, either directly or indirectly (Berlot et al., 1987; Wang et al., 1990; Cooper, 1991), which ultimately must underlie the motile behavior of neurons and their growing processes (Singer and Kupfer, 1986; Smith, 1988; Cooper, 1991).

G proteins have been implicated in the directed migration of non-neural cell types, as well. Among the most extensively examined, the migratory behavior of both polymorphonuclear leukocytes and the slime mold Dictyostelium in response to diffusible chemotactic factors have been shown to require the activation of membrane-associated, G protein-coupled receptors (Devreotes and Zigmond, 1988; Caterina and Devreotes, 1991). These in turn may stimulate a variety of intracellular messenger systems (Firtel et al., 1989; Hall et al., 1989; Newell, 1990) and result in specific changes in locomotory behavior . The mechanisms by which these intracellular signalling pathways induce chemotaxis are still enigmatic, however; and in the case of neuronal migration, the role of G proteins in governing particular aspects of cellular motility remains largely unexplored.

Differential Regulation of Go Expression

A final point that deserves consideration is the differential expression of Go in the EP cells once their mature neuronal phenotypes have been established. As already noted, all of the EP cells began to show positive immunoreactivity for Go_α around the time of their migration, indicating that the initial expression of the Go_α gene was uniformly regulated within this cell population. In contrast, by the time the EP cells completed their terminal differentiation, only certain subsets of the neurons still contained detectable levels of Go_α-related proteins (fig. 4). Among the EP cells that had migrated onto the midgut, only those neurons that had acquired a specific peptidergic phenotype (related to the FMRFamide class of neuropeptides) still stained positively with the Go_α-specific antiserum. As previously demonstrated, the expression of this peptidergic phenotype occurs in a position-specific manner and is delayed until after migration is complete: FMRFamide immunoreactivity appears only in the "type B" subset of EP cells that occupy the midgut domains of the enteric plexus, and the timing of expression of this phenotype is regulated in part by interactions with the post-migratory environment of the midgut (Copenhaver and Taghert, 1988; and unpublished observations). Thus epigenetic interactions may similarly contribute to the

differential regulation of the Go_α gene within the EP cells during the establishment of mature neuronal phenotypes in the ENS. While Go-like proteins have previously been associated with regions of active neuronal migration or process outgrowth (as noted above), the subsequent expression of G proteins with respect to mature neuronal phenotypes has not been characterized. The accessibility of the ENS throughout the period of neuronal migration and differentiation should now permit us to explore these issues in the context of a normally developing embryo.

ACKNOWLEDGEMENTS:

This research was supported by an NSF grant to PFC (#BNS9010538) and by a Grant-in-Aid from the American Heart Association. The authors are indebted to Dr. Michael Forte, Dr. William Wolfgang, and other members of the Forte laboratory for their support during the completion of this work, and for the generous donation of the G protein-specific antisera used in this paper. We also wish to thank Drs.Michael Forte, Karla Kent, and William Wolfgang for critical evaluations of this manuscript.

A DEVELOPMENTAL ROLE FOR THE HETEROTRIMERIC G PROTEIN Go_α IN A MIGRATORY POPULATION OF EMBRYONIC NEURONS

A.M. Horgan, M.T. Lagrange, and P.F. Copenhaver*

Dept. of Cell Biology and Anatomy L-215

Oregon Health Sciences University; Portland, OR 97201 USA

Originally published in Developmental Biology, 172: 640-653 December, 1995

SUMMARY

The heterotrimeric G proteins are an extended family of guanyl nucleotide binding proteins that serve essential functions in the mature nervous system but whose contributions to neuronal development remain poorly understood. We have investigated the potential role of one specific G protein, Go_α, in the control of neuronal migration. During embryogenesis of the moth, *Manduca sexta*, an identified population of undifferentiated neurons (the EP cells) migrate along sets of visceral muscle bands to form part of the enteric nervous system. Previously, immunohistochemical studies indicated the presence of Go_α-related proteins in the EP cells during migration. We have now verified this result, using probes derived from the Go_α gene in *Manduca*. A clone containing the full-length coding domain for Go_α was sequenced from a *Manduca* cDNA library; digoxigenin-labeled probes were then made from this clone and used to examine the developmental expression of the Go_α gene during embryogenesis. Go_α-specific transcripts could first be detected in the EP cells several hours before the onset of their migration. The level of Go_α expression in all of the EP cells continued to increase during migration, but subsequently was down-regulated in a subset of the post-migratory neurons at the time of their terminal differentiation. This pattern of regulated expression is consistent with the distribution of Go_α-related protein in the EP cells. We also used a semi-intact culture preparation of staged embryos to investigate the effects of G protein-specific toxins on the migratory process. Intracellular injections of the wasp toxin mastoparan, a specific activator of Go_α- and Gi_α-related proteins, inhibited the migration of individual EP cells. Injections of pertussis toxin (an inhibitor of Go_α and Gi_α) or cholera toxin (a selective activator of Gs_α) had no effect on migration, although pertussis toxin treatments did cause a measurable increase in the subsequent outgrowth of axonal processes. However, co-injection of mastoparan with pertussis toxin blocked the inhibitory effects of mastoparan alone. These results suggest that Go_α-coupled signaling

events within the EP cells may down-regulate their migratory behavior, possibly in response to inhibitory cues that normally guide migration in the developing embryo.

INTRODUCTION

During the formation of the nervous system, the directed migration of undifferentiated cells to their mature locations plays a central role in morphogenesis and in the expression of position-specific phenotypes by both neurons and glia. Faced with a diversity of potential guidance cues within its local environment, a migratory cell must respond first by selecting an appropriate pathway and then by sustaining its motile behavior for an appropriate duration before completing its differentiation. In the vertebrate central nervous system, for example, migratory neurons have been shown to respond to membrane-associated cues associated with adjacent radial glia (Bartsch et al., 1992; Fishman and Hatten, 1993) and to neurotransmitters such as glutamate and GABA, which may act via specific receptor subtypes to regulate motility (Behar et al., 1994; Komuro and Rakic, 1993). By contrast, the migration of neural crest cells during the formation of the vertebrate peripheral nervous system may be affected by a variety of molecules associated with the surrounding extracellular matrix (Bronner-Fraser, 1990), as well as potentially diffusible compounds, such as steel factor (Wehrle-Haller & Weston, 1995) and members of the transforming growth factor-β family (Delannet and Duband, 1992) . Ultimately, the stimulatory and inhibitory effects of these guidance cues must be integrated via intracellular signaling cascades to induce coherent changes in cytoskeletal assembly, thereby regulating both the traction and force generation needed to produce a motile response (Stossel, 1993). Examples of neuronal migration have now been documented in many systems (e.g. Jacob, 1984; Salser and Kenyon, 1992), but the molecular events that underlie this fundamental process have remained enigmatic.

We are investigating the intracellular signaling mechanisms that regulate neuronal motility in the enteric nervous system (ENS) of the moth, *Manduca sexta*. As previously described (Copenhaver and Taghert, 1989b), the formation of the ENS requires an extended phase of directed migration, during which a population of ~300 neurons (named the EP cells) become distributed along a branching nerve plexus on the visceral musculature (the enteric plexus; Fig. 1A). Following their initial generation from an ectodermal placode in the embryonic foregut (Copenhaver and Taghert, 1990), the EP cells spread bilaterally around gut, adjacent to the foregut-midgut boundary. Then, starting at about 55% of development, the EP cells participate in a rapid phase of migration (Fig. 1B), dispersing along a set of eight longitudinal muscle bands on the midgut and along radial muscle fibers on the foregut. Manipulations done in semi-intact embryos have shown that these muscle band pathways are necessary for EP cell migration: surgically isolated subsets of EP cells will continue to migrate along individual muscle bands (but not onto the interband domains of the visceral musculature), while ablation of one or more of the muscle bands prevents neuronal migration in the vicinity of the ablated pathway (unpublished data). The accessibility of the EP cells in embryonic culture has allowed us to examine the molecular mechanisms that control migration in the context of normal development.

Recently, we showed by immunohistochemical analysis that the onset of EP cell migration coincides with the expression of one specific member of the heterotrimeric G protein family, Go_{α} (Horgan et al., 1994, and Fig. 1). The G proteins (guanyl nucleotide-binding proteins) comprise an extended family of intracellular signaling molecules, each consisting of an α, β, and γ subunit (Gilman, 1987; Simon et al., 1991). These proteins are typically associated with members of the moderately conserved, heptahelical class of receptors (Mollen, 1991), although they may also interact with a variety of non-receptor proteins, as well (Nishimoto et al., 1993; Strittmatter et al., 1994). A number of G proteins exhibit regional patterns of expression within the developing nervous system (e.g. Rius et al, 1991; Shinohara et al., 1992), but the functional significance of these patterns is

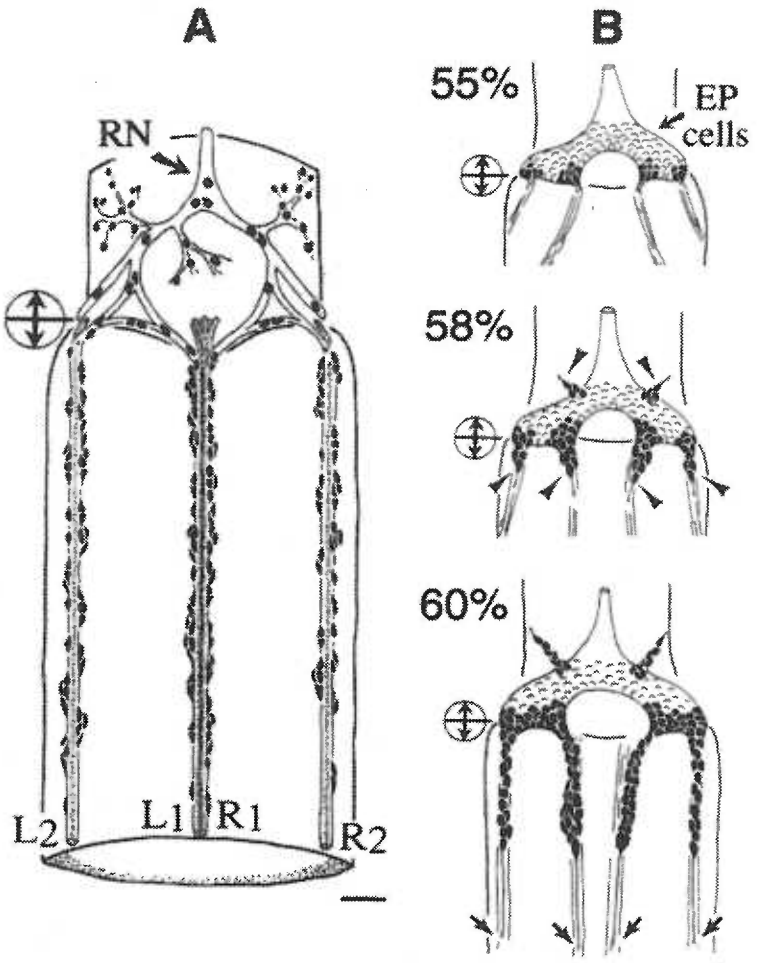

Figure 1. Migration of the EP cells into the enteric plexus coincides with their expression of $G_{o\alpha}$ immunoreactivity. (A) The mature enteric plexus (dorsal view); EP cells are distributed along the eight muscle bands on the midgut (L1-L4 and R1-R4) and in radial nerve branches on the foregut. (B) From 55-60% of embryogenesis, EP cells migrate into the enteric plexus; $G_{o\alpha}$ immunoreactivity first appears in neurons around migration onset (arrowheads), and then later is seen in axonal processes (small arrows). Double arrow = foregut-midgut boundary. RN, recurrent nerve. Scale = 0.05 mm.

poorly understood. In particular, members of the Go_α subtype are often highly expressed in regions of neuronal outgrowth and synaptogenesis in the developing CNS (Copenhaver et al., 1995; Wolfgang et al., 1990; Chang et al., 1988); they also comprise a major fraction of total protein in growth cone membranes (Edmond et al., 1990). G proteins have been implicated in the regulation of growth cone motility (Igarashi et al., 1993; Strittmatter et al., 1994) and in the chemotactic behavior of both polymorphonuclear leukocytes and the slime mold Dictyostelium (Devreotes and Zigmond, 1988). With respect to the migration of neurons in a developing embryo, however, the role of specific G proteins has remained undefined.

To determine the identity of the G protein being expressed by the migratory EP cells, we have now cloned and sequenced a full-length cDNA encoding an authentic form of Go_α in *Manduca.* Using probes derived from this clone, we have shown that the expression of the Go_α gene commences in the EP cells just prior to the onset of their migration. The levels of expression in all of the neurons increase throughout the migratory period, but are subsequently down-regulated in a subset of EP cells, coincident with the differentiation of their mature phenotypes. In addition, we have employed an embryonic culture preparation to inject G protein-specific toxins (mastoparan, pertussis toxin, and cholera toxin) within individual EP cells during their migration. The results of these experiments support a role for Go_α in regulating the extent or duration of neuronal migration within the developing ENS.

MATERIALS AND METHODS

Tissue preparation and immunoanalysis

Timed egg collections were taken from a laboratory colony of *Manduca sexta* and incubated at 25°C; at this temperature, embryonic development is complete in 100 hr (1 hr is equivalent to 1% of development). Embryos were staged according to a schedule of internal and external markers (Copenhaver and Taghert, 1989a). Animals were dissected

to expose the ENS as described in Horgan et al. (1994). For immunohistochemical staining, animals were fixed in 4% paraformaldehyde for 1 hr at room temperature. For immunoblot and Northern blot analyses, tissues from identically staged animals were dissected in culture medium and immediately frozen on dry ice. To visualize the EP cells, embryos were incubated in whole-mount with the monoclonal antibody TN-1 (1:20,000; gift of P. Taghert), which recognizes a molecule related to fasciclin II (Nardi, 1992, and P. Copenhaver, unpublished data) that is expressed by the neurons throughout their migration (Copenhaver and Taghert, 1989b). Go_α-related proteins were detected using an affinity-purified antiserum against Go_α at 1:10,000 (Goldsmith et al., 1987, gift of M. Forte). The specificity of this antibody for Go_α has previously been described in detail (Horgan et al., 1994; Wolfgang et al., 1990). Bound antibodies were visualized using the avidin-biotin-HRP protocol of Vector Laboratories (Burlingame, CA). For immunoblotting, protein was extracted from pooled tissue samples and quantified using the BCA protein assay method (Pierce; Rockford, IL). 100 μg of each protein sample was then separated on 10% acrylamide gels, blot-transferred to nitrocellulose, and reacted with the same anti-Go_α antiserum used for immunohistochemistry (at 1-5 μg/ml). Bound antibody was visualized with an alkaline phosphatase-conjugated secondary antiserum, followed by reaction with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega; Madison, WI).

Isolation and sequencing of Go_α-related cDNAs

A cDNA library from *Manduca* brain (in Lambda ZAP; gift of F. Horodyski) was screened with a ^{32}P-labeled probe, prepared by nick-translation from a 1.2 kb *Bam*HI-*Eco*RI fragment of a clone for *Drosophila* Go_α (Thambi et al., 1989, gift of M. Forte). Hybridizations were performed in 5X SSC, 5X Denhart's solution, 1% SDS, 50 mM NaP_i, and 1% dry milk for 48 hr at 37°C. Approximately 4 X 10^5 plaques were screened. The filters were then washed at low stringency in 1X SSC, 0.2% SDS, at 50°C. Positive clones were further characterized by restriction digests, and selected fragments were

subcloned into Bluescript for sequencing by the dideoxy nucleotide chain termination method (Sanger et al., 1977).

Northern blot analysis

RNA was prepared from embryonic and post-embryonic tissues using the Tri-Reagent extraction method (MRC; Cincinnati, OH). Approximately 10 μg total RNA of each sample was separated on 1% agarose-formaldehyde gels and transferred onto nylon membranes. Gels were stained with methylene blue to monitor for equal loading. The blots were then destained and hybridized for 72 hr at 62°C in 0.9% NaCl and 1% SDS, using the High Efficiency Hybridization System of MRC (Cincinnati, OH). Random hexamer ^{32}P-labeled probes were prepared from an 800 bp *Eco* RI/*Eco*RV fragment from the 5' end of the *Manduca* cDNA clone for Go_α. A control probe was also made against a 600 bp *Eco*RI/*Bam*HI fragment from a *Drosophila* cDNA clone for the ribosomal 49 kDa protein (gift of M. Forte). Washes were done in 1% SDS, 1X SSC, at room temperature and at 55°C. Blots were then exposed to x-ray film for 24-72 hr.

Whole-mount *In Situ* Hybridization

Animals were dissected to expose the ENS and fixed with 4% paraformaldehyde for 1-2 hr, dehydrated in ethanol, and then re-hydrated into PBS plus 0.1% Tween-20. The tissue was then re-fixed for 20 min and digested for 5 min with 50 μg/ml Proteinase K (Boehringer, Indianapolis, IN). After rinsing in 2 mg/ml glycine, the tissue was fixed again for 20 min and incubated in hybridization solution (50% deionized formamide, 5X SSC, 10 μg/ml herring sperm DNA, 100 μg/ml tRNA, 50 μg/ml heparin, 0.1% Tween 20, in DEPC-treated dH_20). The tissue was then hybridized with digoxigenin-labeled probes made from the same *Eco*RI/*Eco*RV fragment of the Go_α clone described above, using digoxigenin-11-UTP (Boehringer) after the method of (Tautz and Pfeifle, 1989). Probes were boiled for 5 min and then added to the hybridization solution at 20% (vol/vol),

for hybridization against the tissue for 48 hr at 48°C. Alternatively, single-strand probes were generated in both sense and antisense configurations by linearizing the Go_α clone and generating runoff probes using T7/T3 primers with *Taq* polymerase (Promega) in a PCR reaction, after the method of Patel and Goodman (1992). Probes prepared in this manner were then fragmented by boiling for 40-60 min in hybridization buffer before being added to tissue samples. After rinsing, the tissue was incubated in an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer; diluted 1:2000) that had been pre-adsorbed against *Manduca* skeletal muscle. Detection of bound antibody was performed in 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris, 100 M levamisol, 0.1% Tween-20, pH 9.5 and the appropriate enzymatic substrates.

Embryonic culture and intracellular injections

Staged embryos were isolated in a modified culture medium and minimally dissected to expose the developing ENS (Horgan et al., 1994). G protein-specific toxins (suspended in culture medium, with 0.1% DMSO) were then introduced by bath application directly onto the developing ENS. Alternatively, individual EP cells were injected at specific times during migration with a WPI pressure injector, while viewing the preparation with a modified Nikon UM-2 compound microscope equipped with Nomarski optics and a 40X water immersion lens. Electrodes were filled at the tip either with a buffered control solution (containing mixtures of 10-20 mg/ml tetramethylrhodamine dextran and biotin dextran; from Molecular Probes, Eugene, OR), or with the same control solution mixed with G protein-specific toxins (from Biomol; Plymouth Meeting, PA). Mastoparan was prepared at an injection concentration at 100-500 μM; pertussis toxin (activated A protomer) was prepared at an injection concentration of 50-100 μg/ml; cholera toxin was pre-activated by incubation with dithiothreotol (50 mM for 20 min at 37° C) and injected at 100 μg/ml. Actual intracellular concentrations of these toxins were approximately 100 fold lower, based on our estimates of injection volumes. For bath

application, pertussis toxin (intact molecule; Gibco/BRL) was pre-activated with 1 M dithiothreotol and used at concentrations of 10-100 ng/ml.

Following these treatments, the embryos were then allowed to develop for 12 hr at 28°C, after which they were fixed, incubated in avidin-HRP (Vector Laboratories), and reacted with 1.4 mM diaminobenzidine (DAB), 0.001% H_2O_2, and 1% NiCl, which resulted in a black reaction product in the injected cells. The preparations were then counterstained with the antibody TN-1 as described above, using DAB-H_2O_2 without NiCl (to produce a brown reaction product). Using photomicrography and *camera lucida* techniques, the distance of migration and axonal outgrowth was then calculated for all EP cell groups on the dorsal midgut pathways. Migratory distances in both experimental and control preparations were measured with respect to the foregut-midgut boundary, pooled by group, and subjected to statistical analysis using Student's T test. For neurons that had been intracellularly injected, the distance of migration was calculated as a percentage of the distance traveled by the leading neuron on the same pathway, to normalize for intrinsic variability between different EP cell groups. The relative distributions of injected and uninjected EP cells were then photographed and documented by camera lucida techniques for statistical analysis. In some preparations, individual EP cells were also labeled with a solution of 25 mg/ml DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes).

RESULTS

Isolation of a cDNA clone for Go_α

To show that the migratory EP cells express authentic Go_α during their migration, we used probes derived from a clone of *Drosophila* Go_α (Thambi et al., 1989) to screen a *Manduca* cDNA library made from developing adult brain mRNA. Two distinct inserts (approximately 1.8 kb and 3.6 kb) were isolated and characterized by restriction analysis. Based on similarities with the restriction digest pattern for *Drosophila* Go_α, the 1.8 kb

insert was selected for further analysis; the identity of the 3.6 kb insert is still under investigation. The complete nucleotide and deduced amino acid sequences of the 1.8 kb clone are shown in Fig. 2. The insert contains one long open reading frame encoding a 354-residue protein with a calculated mass (*Mr*) of ~40.6 X 10^3. The initiator methionine was selected by alignment with the conserved Met-Gly-Cys sequence found at the amino termini of other G_α subunits. The nucleotide sequence around the proposed initiator ATG (CTGCCATGG) is also a good match for the Kozak consensus sequence for translation initiation (CC(A/G)CCATGG; Kozak, 1986). The predicted protein contains many of the hallmark features found in other G proteins, including several conserved sequence domains (regions A, C, E, G in Figs. 2 and 3) that are thought to be important for GTP binding and hydrolysis (Halliday, 1984). The clone did not include an obvious polyadenylation site, however, suggesting that it does not contain a complete 3' untranslated region.

Fig. 3 shows the alignment of the deduced amino acid sequence with sequences for G proteins from other species. There is strongest conservation between the *Manduca* protein and other members of the Go_α family, including the two reported isoforms of *Drosophila* Go_α (both 93% identical; Thambi et al., 1989) and rat Go_α (82%; Jones and Reed, 1987), with substantially less similarity to *Drosophila* Gi_α and Gs_α (64% and 43% respectively; Provost et al., 1988; Quan et al., 1989). Sequence conservation is particularly strong in regions that distinguish Go_α-related proteins from other G proteins (Masters et al., 1986). For example, residues 116-148 are 94% identical to fly Go_α and 78% identical to rat Go_α, but only 50% and 34% identical to fly Gi_α and Gs_α, respectively. In addition, the carboxy-terminal decapentapeptide is 100% conserved with rat Go_α, which encodes the peptide sequence that was used to generate the Go_α-specific antiserum described above (Goldsmith et al., 1987). This region also contains a cysteine four residues from the carboxy terminus (asterisk in Fig. 2) that represents the predicted site for ADP-ribosylation by pertussis toxin (West et al., 1985). Together, these similarities indicate that the 1.8 kb insert encodes a full-length sequence for *Manduca* Go_α.

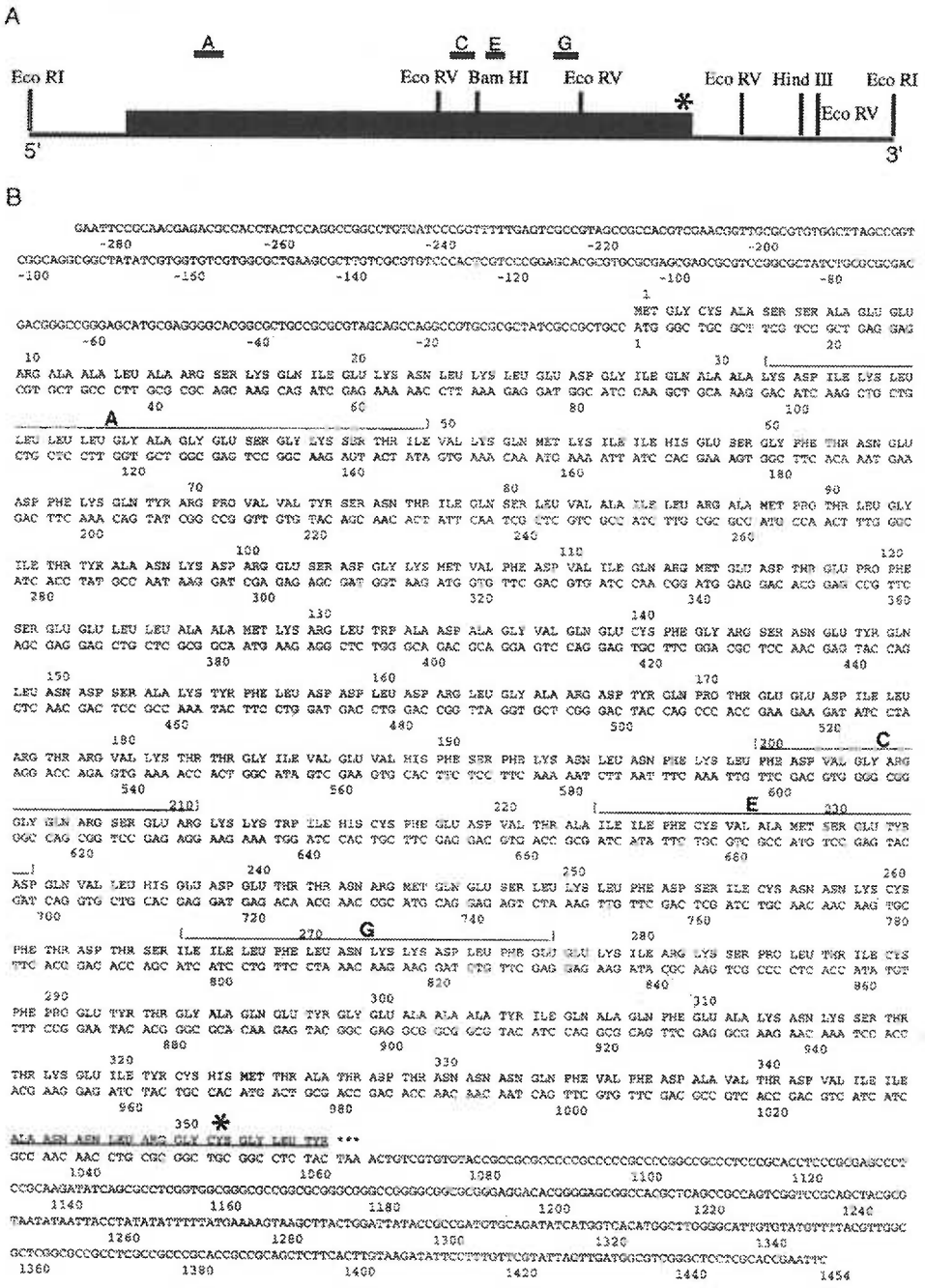

Figure 2. A cDNA from *Manduca* encodes an authentic form of $G_{o\alpha}$. (A) Restriction map of the 1.8 kb clone. (B) Nucleotide and deduced amino acid sequence of the single large open reading frame. Nucleotide #1 indicates the ATG translation start codon. Putative regions of guanyl nucleotide binding and hydrolysis (A, C, E, and G) are overlined. The C-terminal decapentapeptide (underlined) shows 100% conservation with rat $G_{o\alpha}$. Asterisk shows the putative binding site for pertussis toxin.

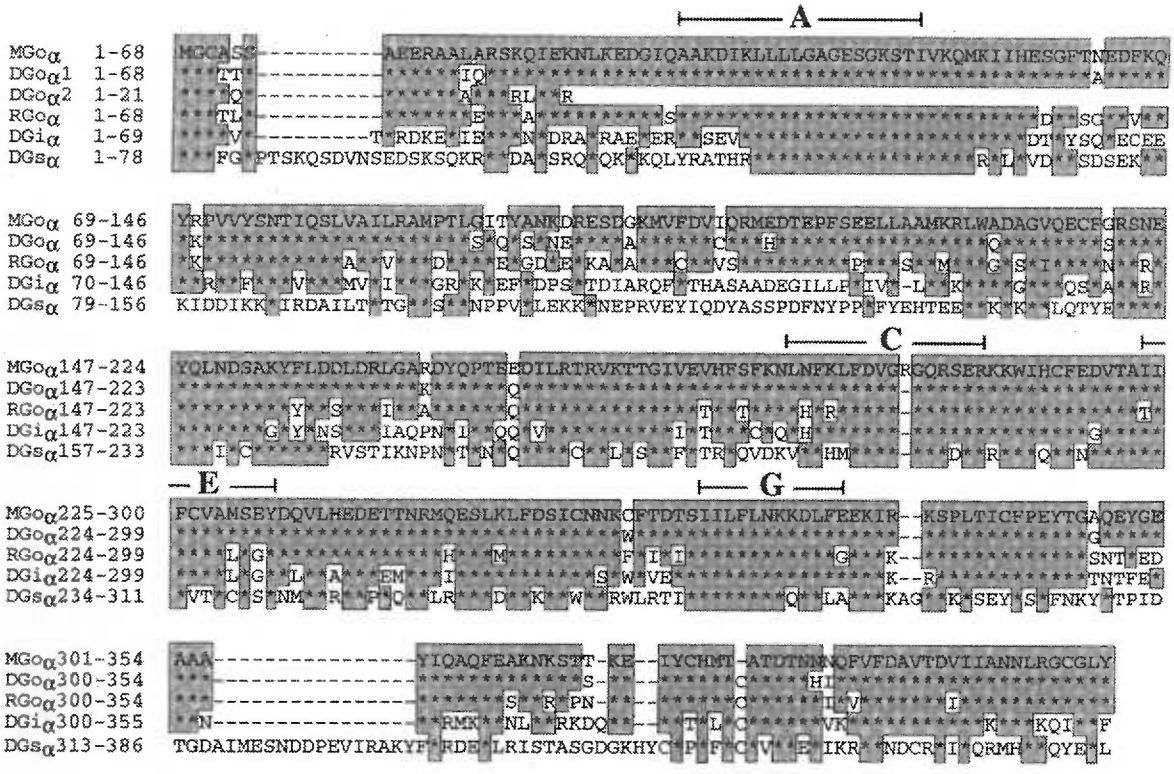

Figure 3. Alignment of amino acid sequence for *Manduca* $G_{o\alpha}$ with other G_{α} subunits. The deduced sequence from the 1.8 kb clone ($MG_{o\alpha}$) has been aligned with the two known isoforms of *Drosophila* $G_{o\alpha}$ ($DG_{o\alpha1}$ and $DG_{o\alpha2}$), rat $G_{o\alpha}$ ($RG_{o\alpha}$), *Drosophila* $G_{i\alpha}$ ($DG_{i\alpha}$) and *Drosophila* $G_{s\alpha}$ ($G_{s\alpha}$). Residues identical to *Manduca* $G_{o\alpha}$ are shown as shaded asterisks. Putative regions of guanyl nucleotide binding and hydrolysis (A, C, E, and G) in the *Manduca* sequence are overlined (see fig. 2).

Tissue distribution of Go_α gene expression

When immunoblots of *Manduca* protein were stained with an antibody specific for Go_α (Fig. 4A), a single prominent protein species was detected in extracts from prothoracic gland (lane PT), nervous system (lane NS), and the midgut muscle bands (lane B; which included the post-migratory EP cells), but not in extracts of the adjacent interband musculature (lane IB; lacking the EP cells) or in several control tissues (including skeletal muscle and fat body; see also Horgan et al., 1994). The immunoreactive protein had an apparent molecular mass of ~40 X 10^3, which coincided with the predicted mass of the protein encoded by the 1.8 kb clone for *Manduca* Go_α, and with authentic Go_α from *Drosophila* (Wolfgang et al., 1991). The larger protein species (~47 X 10^3) in nervous system extract was only rarely observed and may be due to incomplete denaturation. When probes against the 5' end of the cloned cDNA for *Manduca* Go_α were used in a Northern blot analysis of the same tissues, a similar expression pattern for Go_α-related transcripts was found (Fig. 4B). An intensely hybridized mRNA of about 6 kb was seen in prothoracic gland, nervous system, and bands, with only trace amounts being detectable in the other tissues. A second transcript (approx. 3.5 kb), though much less strongly hybridized, showed a similar distribution pattern. In Northern blots that were substantially overexposed, a third transcript at about 2 kb was also detected in all tissues examined (not shown). Methylene blue staining of the 1.8 kb ribosomal RNA band (fig. 4B, lower panel) indicated that equivalent amounts of RNA were loaded in each lane. These results showed that the distribution of transcripts corresponding to our cDNA clone for Go_α coincided with the predicted patterns of Go_α gene expression, based on the distribution of Go_α-related proteins in post-embryonic tissues.

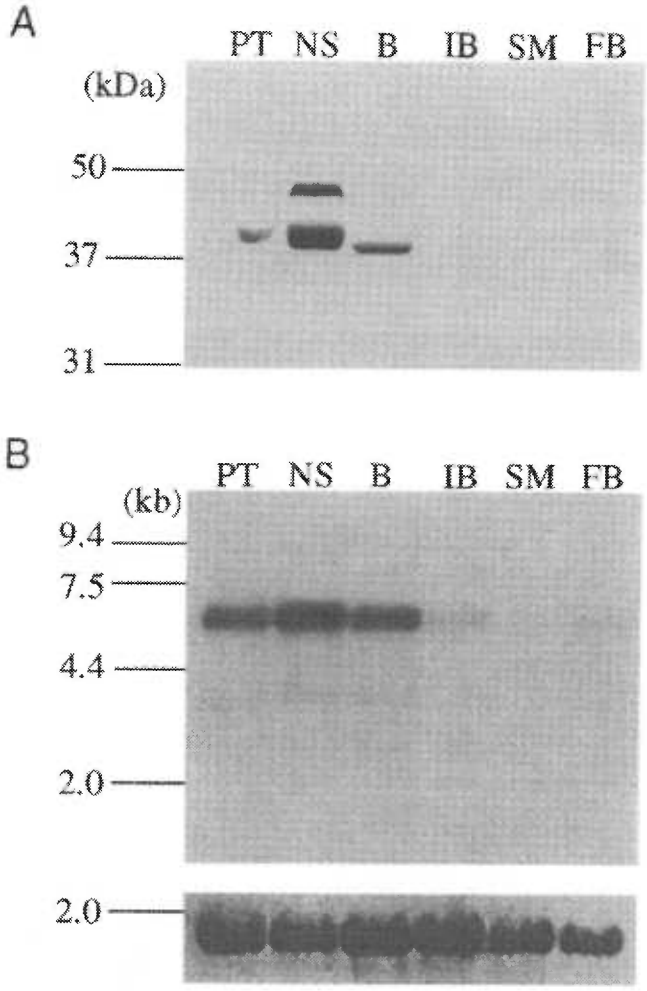

Figure 4. Distribution of $G_{o\alpha}$-specific protein and mRNA in post-embryonic tissues. (A) Immunoblot of extracted proteins stained with an affinity-purified anti-$G_{o\alpha}$ antiserum. The protein band at ~40 X 10^3 coincides with the predicted mass of $G_{o\alpha}$; the larger protein band (~47 X 10^3) may be due to incomplete denaturation. (B) Northern blot of mRNA extracted from the same tissues and reacted with a ^{32}P-labeled probe made from the 1.8 kb clone for Manduca $G_{o\alpha}$; transcripts of ~6 kb and ~3.5 kb were predominantly found in the same tissues that contained substantial amounts of $G_{o\alpha}$-related protein. Bottom panel: methylene blue staining of the 1.8 kb ribosomal RNA band in the original gel showed that equal amounts of RNA were loaded per lane. PT, prothoracic gland; NS, central nervous system; B, midgut bands containing the EP cells; IB, interband midgut muscles without EP cells; SM, skeletal muscle; FB, fat body.

Developmental expression of Go_α during neuronal migration

Using digoxigenin-labeled probes made from the cloned insert for *Manduca* Go_α, we next examined the developmental expression of Go_α-specific transcripts within the EP cells throughout their migratory period. Figure 5 shows a series of developmental stages stained either with the cell surface marker TN-1 (top panels) or by whole-mount *in situ* hybridization for Go_α. Prior to about 52% of development (Fig. 5A), no detectable expression of the Go_α gene could be seen within the pre-migratory EP cells (Fig. 5E), although there were substantial levels of Go_α expression within the CNS at this time (Fig. 6). Then, starting at about 54% of development (Fig. 5B), faint levels of staining could first be seen within the EP cells that were closest to the foregut-midgut boundary (Fig. 5F). By 56%, after the onset of migration (Fig. 5C), all of the actively migrating neurons showed detectable levels of cytoplasmic staining (Fig. 5G). The levels of Go_α expression appeared to increase in the EP cells as they migrated throughout the ENS, both along the muscle bands of the midgut (Fig. 5H; 58%) and onto the radial muscle fibers of the foregut (not shown).

Following the completion of migration, the EP cells continue to extend axonal processes along the visceral musculature (from ~60-75% of development) and acquire their mature transmitter phenotypes (Copenhaver & Taghert, 1989a, b). Although individual EP cells are not uniquely identifiable, several distinct neuronal subtypes become apparent within the population at this time, including one subtype that expresses peptides related to the molluscan peptide Phe-Met-Arg-Phe-amide (FMRFamide; Copenhaver & Taghert, 1989a). Previously, we showed that this peptidergic subtype continues to be immunoreactive for Go_α but that other EP cells on the midgut do not (Horgan et al., 1994), suggesting that the Go_α gene is differentially regulated in the post-migratory neurons. When we compared the distribution of Go_α-specific mRNA and protein in the mature ENS, we found that robust levels of expression persisted in approximately half of the midgut EP cells (Fig. 6A, B), while no detectable expression was seen in their interspersed

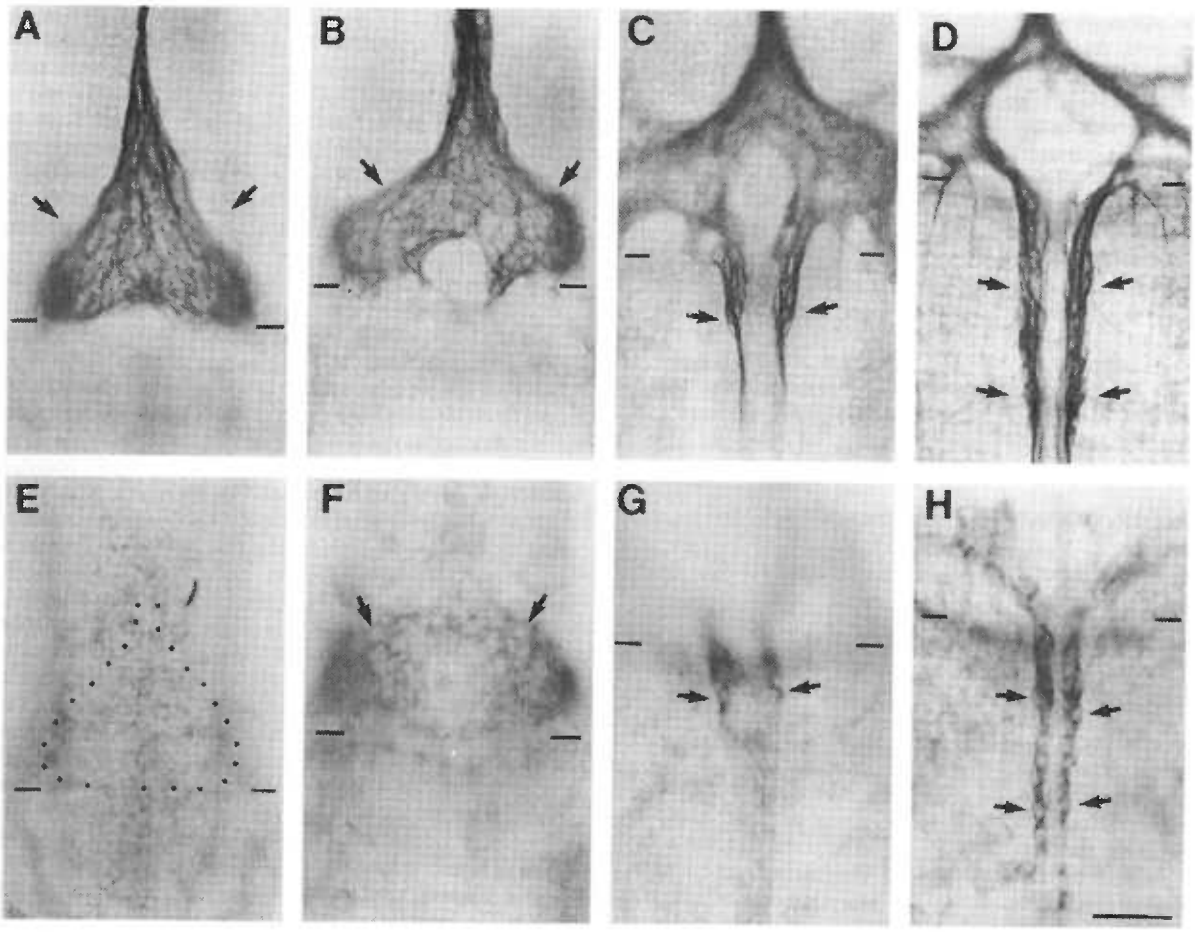

Figure 5. Developmental expression of $G_{o\alpha}$ in the migratory EP cells. A-D, embryos stained with the monoclonal antibody TN-1, a cell-surface marker to indicate EP cell positions. E-H, identically staged embryos stained by whole mount in situ hybridization histochemistry with digoxigenin-labeled probes for Manduca $G_{o\alpha}$. Arrows indicate equivalent EP cell positions in the matched photographs. A and E: at 52%, the premigratory EP cells show no $G_{o\alpha}$ transcript expression (dotted outline indicates EP cell position in E). B and F: at 54%, the EP cells show a faint hybridization signal, just prior to migration onset. C and G: at 56%, increased levels of staining can be seen in the EP cells as they migrate onto the midgut muscle bands. D and H: at 58%, all of the EP cells on the midgut still show strong levels of $G_{o\alpha}$ expression; staining is restricted to the cytoplasm surrounding the large nuclei of the migratory neurons. Hatchmarks indicate the foregut-midgut boundary. Scale = 50 μm.

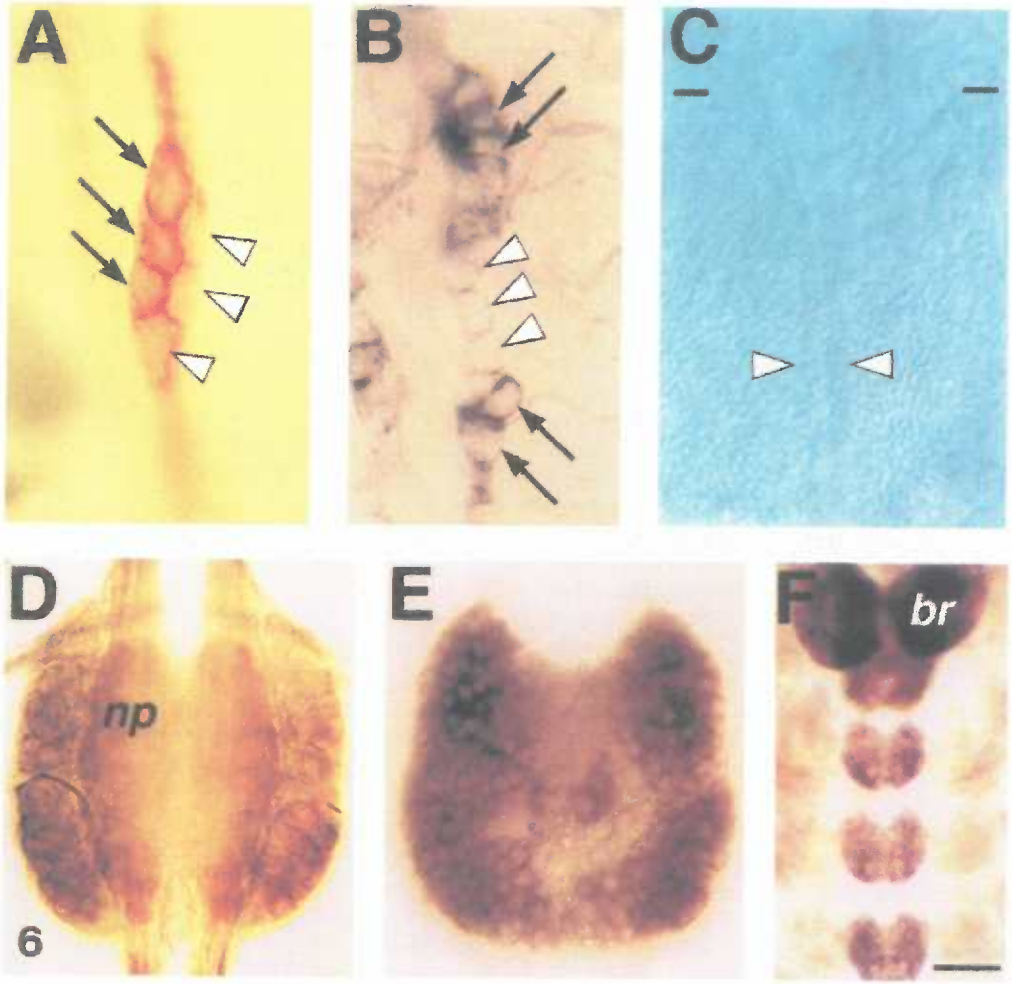

Figure 6. Co-localization of $G_{o\alpha}$-related protein and transcript expression in the nervous system following EP cell migration. Black arrows indicate positively stained cells; white arrowheads indicate EP cells that no longer show detectable levels of expression. (A) $G_{o\alpha}$ immunoreactivity in the EP cells on the midgut of a mature embryo (at the time of hatching); robust staining is still present in a subset of the EP cells, interspersed with other neurons that are no longer expressing detectable levels of the protein. (B) same developmental age stained by in situ hybridization with $G_{o\alpha}$-specific probes; a similar pattern of differential expression is seen in a subset of the mature EP cells. (C) Sense-oriented probes showed no positive staining in either neural or non-neural tissue. White arrowheads indicate the position of the mid-dorsal EP cells and muscle bands on the embryonic midgut at 60% of development. Black hatchmarks indicate the foregut-midgut boundary (same as in Fig. 5). (D) $G_{o\alpha}$ immunoreactivity in an abdominal ganglion of a 55% embryo; positive staining can be seen in both the cortical cell bodies and centrally located neuropilar regions (np). (E) $G_{o\alpha}$-specific in situ hybridization staining in an identically staged ganglion; positive signal is restricted to the neuronal somata. (F) Lower magnification of in situ hybridization staining for $G_{o\alpha}$ in the brain (br) and segmental ganglia at 55% of development. Scale = 50 μm for A-E and 150 μm for F.

neighboring cells. Co-localization of Go_α-specific mRNA and protein was also demonstrated in the embryonic CNS. Strong levels of Go_α immunoreactivity were present in both the cortical regions (containing the neuronal cell bodies) and central neuropil (containing their processes) of the segmental ganglia (Fig. 6D; Copenhaver et al., 1995), while Go_α-specific transcripts were confined to the neuronal somata, as expected (Fig. 6E-F). No hybridization staining was detected in a variety of control reactions, including the omission of digoxigenin probe or the application of sense-oriented probes (Fig. 6C).

We also examined the developmental pattern of Go_α gene expression by Northern blot analysis of mRNA from the embryonic gut (containing the EP cells) and body (including the CNS) at selected stages of development (Fig. 7). At 30-35% of embryogenesis, shortly after the generation of the EP cells (Copenhaver and Taghert, 1990), little or no Go_α expression was seen in the gut-derived mRNA (lane A), although there were detectable levels of expression elsewhere in the body (lane E). By 50-55%, however, a robust signal was seen in gut-associated mRNA (lanes B), coincident with the onset of Go_α expression by the EP cells (fig. 5). The relative levels of expression continued to increase in both the gut (lanes C-D) and body (lanes G-H) throughout the remainder of embryogenesis. In most embryonic stages examined, only the larger transcript (approx. 6 kb) could be detected, although faint levels of the 3.5 and 2 kb transcripts could be seen by the completion of development (100%; lanes D and H).

Effects of G protein activity on EP cell migration

The onset of Go_α expression in the EP cells coincident with their migration suggested that this intracellular signaling molecule might be involved in regulating one or more aspects of migratory behavior. Using an embryonic culture preparation that provides access to the developing ENS (Horgan et al., 1994), we exposed the EP cells to one of several G protein-specific toxins and then monitored the effects of these treatments on their migratory behavior. Individual EP cells were injected with either a control solution

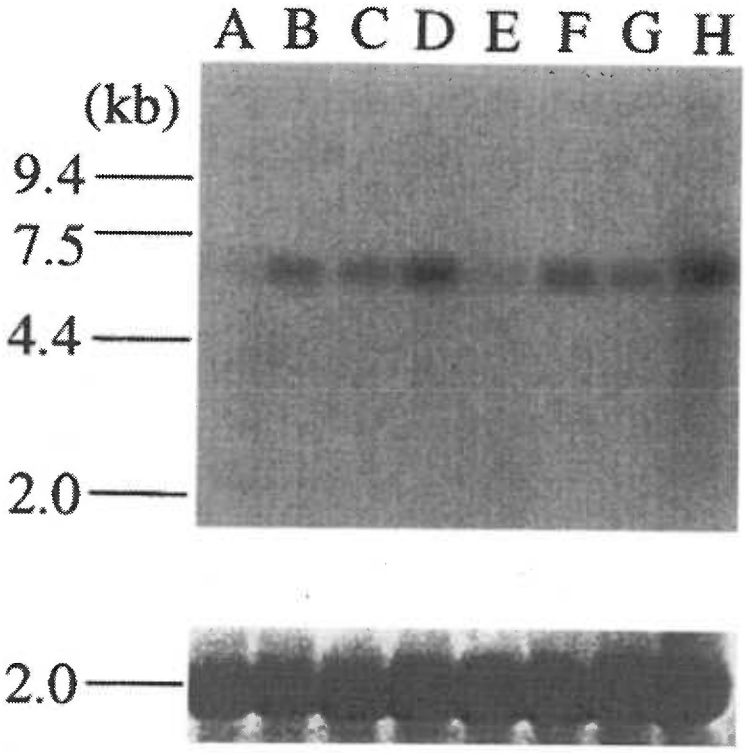

Figure 7. Developmental expression of $G_{o\alpha}$-specific transcripts during embryogenesis. A ^{32}P-labeled probe made from the 1.8 kb clone for Manduca $G_{o\alpha}$ was used for hybridization to a Northern blot of mRNA isolated from the embryonic gut regions containing the EP cells at the following stages of development: lane A, 30-35% embryos; lane B, 50-55% embryos; lane C, 70-75% embryos; lane D, 100% (hatchling). Lanes E-H contain mRNA extracted from the bodies (including the CNS) of the same ordered stages. Bottom panel: methylene blue staining of the 1.8 kb ribosomal RNA band in the original gel showed that ~equal amounts of RNA were loaded per lane.

containing dextran-coupled marker dyes (Dx; Fig. 8A) or with solutions that also contained a variety of G protein-specific toxins (Dx + toxin). Following the completion of the migratory period (8-12 hr in culture), the preparations were then fixed and counterstained so that the positions of the injected neurons could be compared with that of their uninjected neighboring cells (Fig. 8B, open and filled arrows). For consistency, we always injected EP cells within the leading group of migratory neurons (Fig. 9A) and subsequently measured the distance of their migration with respect to the leading neuron on the same muscle band (Fig. 9B-C). Control neurons that were injected with dextran-conjugated dyes exhibited some variability in the extent of their migration, consistent with our earlier observations that EP cells in the mature ENS are stochastically distributed along the muscle bands (Copenhaver and Taghert, 1989a). On average, however, the injected control neurons migrated approximately 54±5% (SEM) as far as the leading neuron on the same pathway (Fig. 8C). In contrast, when cells were injected with mastoparan, a wasp venom that selectively activates Go_α- and Gi_α-related G proteins (Higashijima et al., 1990), there was a pronounced reduction in the extent of their migration (32±5%). There was no detectable increase in EP cell death as a result of these injections, and the overall morphology of the injected neurons appeared normal at the termination of these experiments (fig. 9).

We also treated the EP cells with pertussis toxin, which in other systems has been shown to inhibit Go_α- and Gi_α-related subtypes by ADP-ribosylating a cysteine residue near their carboxyl termini (West et al., 1985). In contrast to mastoparan, intracellular injections of the active A-protomer of pertussis toxin caused no significant changes in the migratory behavior of treated cells when compared with controls (Fig. 8C). Similarly, when pre-activated pertussis toxin was used to treat the entire EP cell population by bath application, there was no significant effect seen on EP cell migration, although these treatments did cause a measurable increase in the subsequent outgrowth of their axonal processes (measured in terms of axonal length from the foregut-midgut boundary; Table 1).

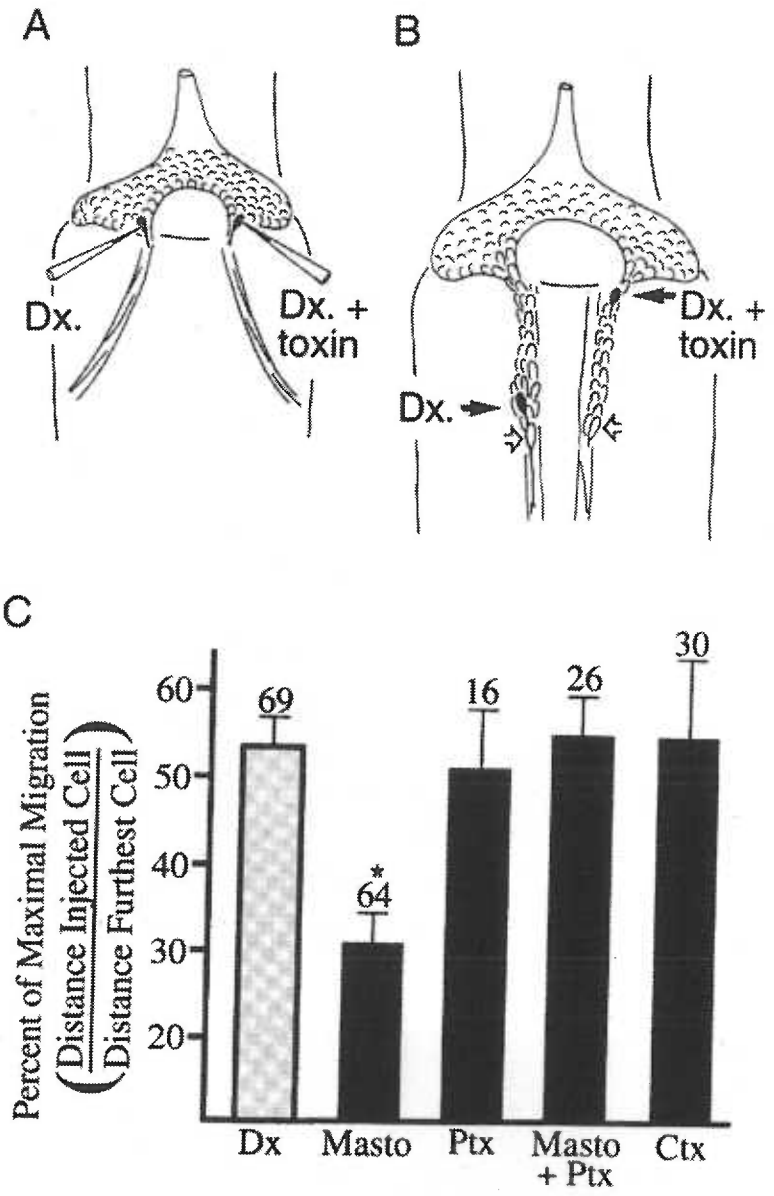

Figure 8. Intracellular injection of mastoparan causes a pertussis-sensitive inhibition of EP cell migration. (A) Individual EP cells were injected at ~55% of development with either dextran-conjugated markers (Dx) or with a solution that also contained G protein-specific toxins (Dx + toxin). (B) After 8-12 hr in culture, the preparations were fixed and stained to show the relative position of the dye-injected cells (filled arrows) with respect to the leading uninjected cell on the same pathway (open arrows). (C) The distances of migration for dye-injected cells were calculated as a percentage of the distance traveled by the furthest cell on the same pathway. Dx: control cells injected with dextran markers. Masto: cells injected with 100 μM mastoparan. Ptx: cells injected with 10 ng/ml pertussis toxin. Masto + Ptx: cells injected with 100 μM mastoparan and 10 ng/ml pertussis toxin. Ctx: cells injected with 100 μg/ml cholera toxin. Final intracellular concentrations were estimated to be ~100 fold less than the electrode concentrations. Numbers = N cells injected. *Migration of mastoparan-injected cells was significantly less than controls ($p < 0.01$; Student's T test). All other treatments were not significantly different.

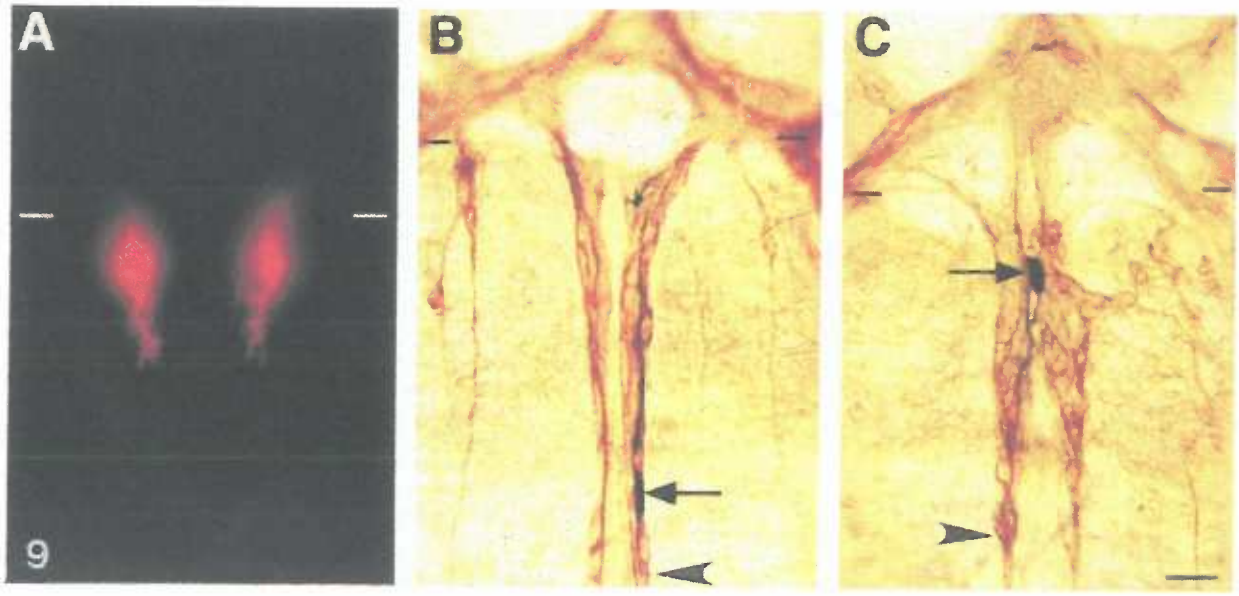

Figure 9. Dye-labeled EP cells before and after migration. (A) Two leading EP cells at the foregut-midgut boundary, labeled at the onset of migration with DiI. (B) Position of an EP cell (arrow) that was injected at migration onset with dextran-coupled markers, and then fixed and stained after 8 hr in culture. The surrounding EP cells were counterstained with TN-1. (C) Position of an EP cell (arrow) after 8 hr in culture that was injected at migration onset with both dextran-coupled markers and mastoparan. Arrowheads in B and C indicate the leading uninjected neurons on the same pathways as the injected cells. Hatchmarks indicate the foregut-midgut boundary (as shown in Fig. 5). Scale = 50 μm.

TABLE 1.

EFFECTS OF PERTUSSIS TOXIN ON EP CELL MOTILITY

Cell Migration:	% of controls:	N	P
Controls:	100% ± 22%	16	
PTX (10 ng/ml):	95% ± 14%	16	ns
PTX (100 ng/ml):	86% ± 22%	8	ns
Process Outgrowth:			
Controls:	100% ± 11%	8	
PTX (10 ng/ml):	125% ± 21%	8	<.02
PTX (100 ng/ml):	140% ±10%	8	<.001

Note: Distances are shown as percent of controls (±SD). N, number of preparations measured. P, statistical significance of experimental values when compared with controls; ns, not significantly different from controls.

However, when pertussis toxin was co-injected with mastoparan into individual EP cells, it eliminated the inhibitory effects of mastoparan alone (fig. 8C). Injections of cholera toxin, which acts selectively on Gs_α-related proteins (Freissmuth & Gilman, 1989), also had no effect on migration. These results indicate that the effects of mastoparan on EP cell migration involve a pertussis-sensitive G protein. They are also consistent with previous data that Go_α is the only G protein that we have detected in the EP cells during migration (Horgan et al., 1994), suggesting that stimulation of Go_α in the EP cells induces a down-regulation of their motile behavior. A model for the role of Go_α during migration is discussed below.

DISCUSSION

Go_α gene expression in the developing nervous system

Several lines of evidence indicate that the 1.8 kb cDNA clone described in this paper encodes an authentic form of Go_α. Both the primary sequence and the deduced amino acid sequence of this clone contain a strong degree of conservation with Go_α proteins from other species, whereas a comparison with other insect G proteins (including Gi_α and Gs_α) showed more modest levels of sequence identity. The similarity of *Manduca* Go_α with fly Go_α was particularly strong in regions that have previously been used to distinguish Go_α-related molecules from other heterotrimeric G proteins (Masters et al., 1986), including the C-terminal peptide that was used to generate the Go_α-specific antiserum used in this study (Goldsmith et al., 1987). The predicted size of the encoded protein (~41 kD) coincides with the single prominent band detected by immunoblot analysis of *Manduca* tissues (fig. 4) and closely matches the size of Go_α from *Drosophila* (Thambi et al., 1989). The *Manduca* protein also contains a putative binding site for pertussis toxin (fig. 2) in an identical position to that seen in fly and rat Go_α (Jones and Reed, 1987; Thambi et al., 1989). Lastly, the expression patterns of mRNA corresponding to this clone are identical to the distribution of Go_α-related proteins in both tissue extracts and whole-

mount embryos. On the basis of these results, we conclude that the 1.8 kb clone from *Manduca* encodes an authentic form of Go_α.

Whereas in our immunoblot analysis, we typically detected only a single protein band (at ~40 kD), we could detect as many as three distinct transcript sizes (with apparent sizes of 6 kb, 3.5 kb, and 2 kb) in Northern blots of the same tissues. In *Drosophila*, a single gene for Go_α has been identified that produces at least two distinct transcripts by alternative splicing at the 5' end of the coding region (de Sousa et al., 1989; Thambi et al., 1989; Yoon et al., 1989). Multiple transcripts have also been found in Northern blots of fly mRNA (ranging in size from 6.0 to 3.4 kb), which show some tissue-specific differences in expression (Thambi et al., 1989; Yoon et al., 1989). In the present study, we only characterized a single cDNA clone for Go_α from *Manduca*; whether alternative processing of Go_α mRNA contributes to transcript heterogeneity in this species remains to be determined. Similarly, in both invertebrates and vertebrates, there is also evidence for multiple isoforms of Go_α proteins with closely similar molecular weights and antigenicities (de Sousa et al., 1989; Goldsmith et al., 1987; Thambi et al., 1989). Since the affinity-purified antiserum used in this study was directed against the conserved C-terminus of Go_α, it would probably not distinguish among putative Go_α isoforms.

Nevertheless, the combined application of our probes against Go_α-specific mRNA and protein revealed a consistent pattern of Go_α gene expression in both embryonic and post-embryonic tissues. In particular, we found a good correlation between Go_α-specific transcription and translation in the migratory EP cells: Go_α mRNA could first be detected at ~52% of development, while Go_α-immunoreactivity appeared shortly thereafter (at ~54-55%; Horgan et al., 1994). Similar results were obtained by Northern blot analysis (fig. 7), which showed barely detectable levels of Go_α transcript expression in the gut prior to migration (at ~30-35% of development), but a substantial increase in expression levels by 50-55%, coinciding with migration onset. Some diminution in signal intensity was seen in extracts of 75% embryonic gut (Fig. 7, compare lanes B and C), which may simply be due

to variability in our extraction technique. Alternatively, we also observed that some of the EP cells stopped expressing Go_α-related mRNA, once migration was complete (fig. 6). As previously noted by immunohistochemical staining (Horgan et al., 1994), all of the EP cells contain Go_α-related proteins during migration, but only a subset shows persistent Go_α expression once they have completed their differentiation. This same subset of neurons also commences the expression of a peptidergic phenotype at this time (Copenhaver and Taghert, 1989b; Horgan et al., 1994). Thus, the same G protein may serve distinct functions during the migration of immature EP cells and in the mature ENS.

Go_α in the control of neuronal migration

The developmental expression of Go_α during EP cell migration suggests that this intracellular signaling molecule might modulate specific aspects of the migratory process. Since the first appearance of detectable levels of Go_α-specific transcripts and protein coincided with the onset of migration, we initially thought that Go_α might be involved in triggering the migratory process. For example, activation of Go-coupled receptors in response to local environmental stimuli might lead to the initiation of migration. Our manipulations of G protein activity in the EP cells failed to support this hypothesis, however. Exposing the EP cells to pertussis toxin (an inhibitor of mammalian Go and Gi) produced no discernible inhibition of EP cell migration. These results indicate that Go_α-mediated events are not required for the initiation of migration.

Alternatively, the role of Go_α in the EP cells might be to terminate the migratory process. As previously described (Copenhaver and Taghert, 1989a, 1989b), the period of active migration is relatively brief (lasting from 55-60% of development), after which the EP cells extend axonal processes and acquire their mature neuronal phenotypes. Conceivably, Go_α-coupled receptors within the EP cells might respond to localized cues along the muscle bands, triggering a cessation of migratory behavior and initiating their subsequent differentiation. In support of this model, intracellular injections of the wasp

toxin mastoparan (a specific activator of Go and Gi) caused a marked inhibition of migration in individual EP cells, without affecting the behavior of the surrounding neurons or inducing any measurable increase in cell death. Significantly, the effects of mastoparan were abrogated by co-injection of pertussis toxin, indicating that the effects of mastoparan involve a pertussis toxin-sensitive G protein. To date, the only G protein that has been shown to be modified by pertussis toxin in insects is Go_α (Provost et al., 1988; Thambi et al., 1989); and as already noted, we have found no detectable Gi_α expression within the EP cells at any stage of their development (Horgan et al., 1994; and unpublished observations). Cholera toxin, which selectively activates Gs_α by ADP ribosylation (Freissmuth & Gilman, 1989), also had no effect on migration. These results indicate that in the developing EP cells, the normal role of Go_α may be to provide a means of down-regulating their migratory behavior.

However, our data also indicate that Go_α-coupled signaling mechanisms are not the only means of limiting EP cell migration. Specifically, we found that injections of pertussis toxin alone did not result in exuberant or inappropriate migration, as might be expected if Go_α activation was explicitly necessary for terminating the migratory process. Similarly, while bath applications of pertussis toxin did cause a measurable *increase* in process outgrowth (Table 1), there was no evidence of aberrant or misdirected axonal sprouting, as might be expected if Go_α-mediated events were uniquely involved in down-regulating neuronal motility. These results indicate that within the developing embryo, there may exist both Go_α-dependent and independent processes that act in concert to restrict the extent of neuronal migration. A similar conclusion may be inferred from the effects of deletion mutations for Go_α in the nematode, which caused a number of abnormalities in adult behavior but no gross defects in neuronal development (Segalat et al., 1995; Mendel et al., 1995). An appealing model suggested by our data is that Go_α-mediated signaling events within the EP cells may underlie their response to inhibitory cues on non-permissive domains of the midgut. By this model, the activation of Go_α-coupled

receptors might induce local steering responses of the EP cells away from inappropriate domains, such as the interband musculature, thereby confining migration to the longitudinal muscle bands. The robust inhibitory action of injected mastoparan would therefore reflect an extreme example of the normal modulatory effect of Go_α on migratory behavior. A similar model has recently been proposed for the effects of collapsin, a secreted protein in the developing vertebrate nervous system that *in vitro* can cause a complete inhibition of growth cone motility, but *in vivo* may provide a more graded effect on growth cone guidance (Fan and Raper, 1995). We are currently testing for putative inhibitory cues of this type that might be associated with the visceral musculature in *Manduca.*

G protein-coupled signaling mechanisms have been shown to modulate cellular motility in a variety of other contexts. In the slime mold *Dictyostelium*, for example, the chemotactic response of individual cells to diffusible cAMP requires receptor-mediated activation of one specific G_α subunit ($G\alpha 2$; Kumagai et al., 1991). Directed migratory behavior in a variety of hematopoeitic cell types (responding to specific growth factors or other chemoattractants) has also been shown to be a G protein-coupled event, although the specific G proteins involved vary with respect to particular stimuli (Devreotes and Zigmond, 1988; Amatruda et al., 1993). In another form of neuronal motility, the extension of growth cones and growth cone-like processes, G proteins have been shown to mediate cell-specific responses to diffusible neurotransmitters (e.g. Behar et al., 1994; Haydon et al., 1984) and cell adhesion molecules (Doherty and Walsh, 1994). In particular, the inhibitory effects of proteins isolated from CNS myelin that curtail neurite outgrowth are mimicked by mastoparan and blocked by pertussis toxin (Igarashi et al., 1993). Recently, Go_α has also been shown to interact with another prominent protein in growth cones, GAP 43, possibly contributing to the regulation of growth cone motility or shape (Strittmatter et al., 1994). The role of G proteins in migratory neurons, however, and the potential mechanisms by which G proteins may regulate their motile behavior, have remained poorly defined. Recently, we have found that transient increases in intracellular

calcium will also inhibit EP cell migration (Copenhaver and Horgan, unpublished observations). We are now investigating whether the modulatory effects of Go_α on the migratory process may involve regulated changes in intracellular calcium within individual EP cells.

Our current results provide evidence that Go_α contributes to the regulation of migratory behavior of developing enteric neurons in *Manduca*. Both Go_α-specific mRNA and protein are expressed within the EP cells coincident with their migratory phase of development, in contrast to other G proteins that we have examined. The inhibitory effects of G protein activity on EP cell migration, while not completely characterized, support a role for Go_α in regulating specific aspects of the migratory process. With the cloning and characterization of Go_α from *Manduca*, it will now be possible to conduct more specific manipulations of Go_α expression in the EP cells, using intracellular injections of antisense oligonucleotides and constitutively activated forms of different G proteins to determine their effects on migratory behavior. By applying these techniques to individual neurons within the developing ENS, we should also gain insight into the intracellular processes by which G proteins may regulate neuronal motility in the context of a developing embryo.

ACKNOWLEDGMENTS

We wish to thank Drs. Michael Forte and William Wolfgang for their continued input and help during the completion of this work. We also thank Drs. Rae Nishi, Michael Forte, and William Wolfgang for their critiques of this manuscript. The sequence for *Manduca* Go_α has been recorded with EMBL (acquisition #Z49080). This research was supported by NIH #MH51833-01 and a grant from the Medical Research Foundation of Oregon.

G Protein-Mediated Inhibition of Neuronal Migration Requires Ca^{2+} Influx

Angela M. Horgan and Philip F. Copenhaver*

Dept. of Cell and Developmental Biology, L215

Oregon Health Sciences University; Portland, OR 97201 USA

Submitted for publication in Neuron

SUMMARY

Neuronal migration is an essential feature of the developing nervous system, but the intracellular signaling mechanisms that regulate this process are poorly understood. In the insect enteric nervous system, the migration of identified neurons is regulated in part by $G_{o\alpha}$. Using an *in vivo* culture preparation, we have shown that migration is similarly regulated by intracellular Ca^{2+}: actively migrating neurons exhibited only small fluctuations in intracellular Ca^{2+}, while elevating internal Ca^{2+} inhibited the migratory process. Activated $G_{o\alpha}$ also inhibited migration, while G protein stimulation induced multiple Ca^{2+} spikes within the migratory neurons; both effects were reversed by removal of external Ca^{2+}. These results indicate that the G protein-mediated regulation of migration involves a Ca^{2+}-dependent process requiring Ca^{2+} influx.

INTRODUCTION

During the formation of the nervous system, many neurons or their precursors undergo a transient period of migration, translocating their cell bodies to unique and sometimes distant locations. The ability of a cell to migrate requires not only the expression of a motile phenotype but also depends upon information from the surrounding environment to guide the selection of appropriate pathways and destinations. Some of the repulsive and attractive molecules which may provide this information have been identified (Fishman and Hatten, 1993; Muller and Kypta, 1995). However, the orchestrated intracellular events by which these cues are transduced into an appropriate migratory response are unknown.

Previously, we demonstrated that $G_{o\alpha}$, a member of the heterotrimeric class of guanyl-nucleotide binding proteins (G proteins), is expressed by a population of embryonic neurons at the time of their migration and may participate in the regulation of their motility (Horgan et al., 1994; Horgan et al., 1995). Using the enteric nervous system (ENS) of the moth *Manduca sexta*, in which a defined population of neurons (the EP cells) undergoes a

stereotypic pattern of migration on the gut musculature (Copenhaver and Taghert, 1989), we showed that the onset of $G_{o\alpha}$ expression within the EP cells coincided with their migratory period (Horgan et al., 1994). Furthermore, the accessibility of the ENS has permitted direct manipulations of these neurons within the embryo: injections of mastoparan (which stimulates $G_{o\alpha}$ and $G_{i\alpha}$) into individual EP cells caused a marked inhibition of migration, an effect that was reversed by pertussis toxin (Horgan et al., 1995). Because $G_{o\alpha}$ is the only G protein in insects known to be sensitive to pertussis toxin (Quan et al., 1989; Thambi et al., 1989), these results suggested that activation of G_o-mediated events within the EP cells might serve to inhibit or terminate their migratory behavior. However, the precise role of Go in these developing neurons and the mechanism by which G proteins down-regulate migration remained undefined.

Other work on the function of G proteins has provided much insight into their potential modes of action. G proteins, consisting of an α, β and γ subunit, typically bind to heptahelical membrane receptors (Rens-Domiano and Hamm, 1995). Upon activation of the receptor by an appropriate ligand, the trimeric complex dissociates, and both the α and $\beta\gamma$ subunits may then impinge upon a variety of other intracellular proteins. In some cells, G_o has been shown to modulate voltage-dependent Ca^{2+} channels within the plasma membrane (Dunlap, 1997; Wickman and Clapham, 1995). Other evidence, however, suggests that G_o may also act via the regulation of a phospholipase C- (PLC) or protein kinase C- (PKC) dependent pathway. For example, constitutively active $G_{o\alpha}$ mutants enhanced neurite outgrowth in PC-12 cells by a PKC-dependent mechanism (Xie et al., 1995), while in myenteric neurons, Pan et al. (1997) found that antibodies to $G_{o\alpha}$ inhibited a PKC-mediated response to serotonin, possibly by preventing G_o activation of phosphatidylcholine-specific PLC. As PLC activity has been shown to modulate Ca^{2+} release from intracellular stores (Berridge, 1993), the involvement of this signal transduction pathway may also provide an alternative mechanism for the regulation of intracellular Ca^{2+} levels by G_o.

With respect to neuronal motility, the regulation of intracellular Ca^{2+} is known to play an important role both during cell migration and process outgrowth (Kater and Mills, 1991; Rakic and Komuro, 1994). Elucidation of the precise function of Ca^{2+}, however, has been complicated by evidence that Ca^{2+} can have both inhibitory and stimulatory effects on motility, depending on both cell type and experimental context. For example, Letourneau et al. (1994) showed that in dorsal root ganglion cells, contact with an inhibitory proteoglycan caused an influx of Ca^{2+}, resulting in the depolymerization of actin filaments and collapse of their growth cones. In contrast, neurite outgrowth from PC-12 cells in response to a variety of adhesion molecules was stimulated by Ca^{2+} influx, most likely via a G protein-coupled mechanism (Doherty and Walsh, 1994; Williams et al., 1992). In studies of neuronal migration, contradictory effects of Ca^{2+} have been similarly documented. Neural crest cells *in vitro* were found to migrate precociously when exposed to Ca^{2+} channel antagonists (Newgreen and Gooday, 1985), while Komuro and Rakic (1996) reported that Ca^{2+} influx through ligand- and voltage-gated ion channels stimulated the rate of migration of cerebellar granule cells. With respect to G_o, the potential role of Ca^{2+} in mediating its inhibitory effect on migration have not been explored.

In this paper, we have examined the interaction of these two intracellular signaling molecules during neuronal migration by examining their respective effects on the EP cells within an *in vivo* culture preparation. Specifically, we have tested the role that Ca^{2+} plays in regulating EP cell migration by manipulating extracellular and intracellular levels of Ca^{2+} during the migratory period. We have also used activated α-subunits to demonstrate the specific effects of $G_{o\alpha}$ on migration, and we have tested whether the inhibition of the EP cells by $G_{o\alpha}$ is Ca^{2+}-dependent. Lastly, we have examined the effects of G protein activation on spontaneous changes in Ca^{2+} within individual EP cells as they migrate in the developing embryo.

MATERIALS AND METHODS

Animal Preparation and Culture

Animal dissection and culture were performed as described previously (Horgan et al., 1994; Horgan et al., 1995). Briefly, timed egg collections from a *Manduca sexta* colony were maintained at 25°C, at which temperature embryonic development lasts ~100 hours (1 hour = 1% development). Embryos were dissected from the egg shell and underlying membranes and restrained in a Sylgard-coated chamber in either culture medium (50% Schneider's *Drosophila* medium, 40% MEM with Hank's salts, 9.7% heat-inactivated fetal calf serum, 0.2% ecdysone, 0.1% insulin, and 0.01% penicillin-streptomycin) or a defined saline (140 mM NaCl, 5 mM KCl, 28 mM glucose, 5 mM HEPES, 0.2% ecdysone, 0.1% insulin, and 0.01% penicillin-streptomycin; pH 7.4). In experiments where external Ca^{2+} concentrations were manipulated, the solutions were osmotically balanced with mannitol. A small incision was made in the dorsal epidermis above the foregut/midgut boundary to expose the premigratory EP cell population. The animals were then allowed to develop in culture for 8 to 16 hours in a humidified chamber at 28°C.

Bath application of water-soluble compounds, including caffeine (MCB) and BAPTA (Molecular Probes), were diluted into culture medium or defined saline. A23187 (Sigma), BAPTA-AM (Molecular Probes), IBMX (Sigma), and ionomycin (Calbiochem) were dissolved into 100% DMSO and then diluted into culture medium or defined saline with a final concentration of 0.5-1% DMSO. AlF_4^- was made by diluting premixed solutions of 0.6 M NaF and 0.5 mM $AlCl_3$ at 1:40 into the culturing solution (Horgan et al., 1994). Equivalent concentrations of NaCl premixed with $AlCl_3$ served as a control.

After culturing, animals were dissected completely and fixed with 4% paraformaldehyde in PBS. To visualize the ENS, the preparations were immunohistochemically stained with a monoclonal antibody against *Manduca* Fasciclin II (MFasII, at 1/20,000; gift of Dr. Paul Taghert), followed by the avidin-biotin-HRP protocol from Vector Laboratories. The distribution of post-migratory EP cells was then analyzed

using camera lucida techniques. The extent of migration in each experiment was calculated by measuring the distance of the furthest cell from the foregut/midgut boundary (normalized to matched control cultures). Statistical analyses were performed using a two-tailed Student's t-test.

Intracellular Injection

Myristoylated rat recombinant $G_{o\alpha}$ subunits (10 μM; Calbiochem) were activated by a 30 min incubation with 10 μM GTPγS (in 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, and 20 mM HEPES, pH 8.0) at 20°C. Control injections were performed with an identical solution lacking $G_{o\alpha}$. Heat inactivation of activated $G_{o\alpha}$ subunits was performed by boiling for 30 min prior to injection. Mastoparan (100 μM; Calbiochem) was initially dissolved in dH_20 and stored at -80°C. Just prior to use, injection solutions were diluted 1:10 with an intracellular buffer containing 132 mM K-Asp, 64 mM mannitol, 5 mM HEPES, 2 mM EGTA, 2 mM MgATP, and 1 mM $CaCl_2$ plus rhodamine-dextran and biotin-dextran (both at 20 μg/μl; Molecular Probes). Glass electrodes (30-50 MΩ) were used for pressure injection of these solutions into individual EP cells (Horgan et al., 1995). Injected cells were then briefly visualized with a heavily filtered UV lamp source and the intensity and position of the cell noted. In neurons injected with control solutions, this brief UV exposure was not found to disturb normal migration. We estimate that the actual concentration delivered into cells was 1:100 of the pipette concentration. For fluorescent labeling of single cells, 10 mg/ml DiI (1,1'-dioleyl-3,3,3',3'-tetramethylindocarbocyanine methanesulfonate; from Molecular Probes) diluted in methanol was injected as described previously (Copenhaver et al., 1996).

Intracellular Calcium Imaging

Individual EP cells were injected with the calcium indicator Calcium Green-1 Dextran (10,000 MW; at 10 mg/ml, from Molecular Probes). Embryos were then inverted and restrained in chambers made from Sylgard-coated glass coverslips. Cells were observed

through a 60X Plan Apochromat water-immersion lens attached to a Nikon Diaphot 200 inverted microscope that was mounted on a vibration isolation table. A heavily filtered UV-light source from a 100W mercury bulb was passed through an FITC (450 nm excitation/535 nm emission) filter (Omega). The duration and timing of excitation was controlled with a digital IO board (National Instruments) connected to a Lambda-10 filter wheel (Sutter Instruments). A Cohn 12V CCD camera and Hamamatsu Intensifier mounted to the microscope was used to capture and enhance the emitted signal. Data was processed using a Power PC 7100 (Macintosh) interfaced with a PixelPipeline (Perceptics) data acquisition imaging board. The image analysis software used was Cytos III (Applied Scientific Instrumentation Inc.). Twelve frames were captured every 5-10 seconds and averaged to produce individual images. The images were then analyzed for changes in relative fluorescent intensity. Average pixel intensities within a rectangular box surrounding each EP cell (32 X 32 pixels/box), as well as in similarly sized boxes to measure background levels, were monitored simultaneously. Focus and box placement were adjusted approximately every 2-3 minutes to correct for cell movements. Although this procedure did result in small changes in apparent cell intensity, the fluctuations were not significantly above background noise. The duration of most experiments was 30 minutes; however, a few neurons were observed for up to 2 hours. During experiments involving AlF_4^-, baseline images were recorded for 10 minutes before application. Immediately after AlF_4^- was added to the bath, cells were refocused and then imaged over the next 20 minutes.

RESULTS

Neuronal Migration of EP cells During Embryogenesis

During the formation of the ENS, a population of ~300 post-mitotic neurons (the EP cells) arise from a neurogenic placode in the foregut epithelium (Copenhaver and Taghert, 1990) and then migrate into a branched nerve plexus on the surface of the gut musculature

(Fig. 1; Copenhaver and Taghert, 1989). By immunostaining embryos with antibodies to the adhesion molecule fasciclin II (designated in *Manduca* as MFas II), the sequence of EP cell development could be readily visualized. Before migration commences (at 55% of embryogenesis), the EP cells reside in a packet encircling the gut at the foregut and midgut boundary (fg/mg; Fig. 1A). As migration begins, individual neurons extend leading processes across the fg/mg boundary and align themselves with one of eight specific longitudinal muscle bands on the midgut (L1-L4 & R1-R4; Figs. 1B-D). The EP cell bodies migrate posteriorly for a period of 5-7 hours (Fig. 1B-D arrows; Fig. 1E), traveling up to 200 μm and distributing themselves along the muscle bands. Anteriorly, a small number of EP neurons also migrate laterally onto the radial muscle bands of the foregut (open arrows; Figs. 1B, C). At the end of migration (60%-62% of development), the neurons then extend axonal processes posteriorly and laterally to innervate the gut musculature. Of note is the specificity by which the EP cells follow their migratory pathways on the midgut: once aligned with one of the muscle bands, each neuron invariably remains closely apposed to the fibers of that band, never wandering onto the adjacent interband musculature.

Effects of Calcium Manipulations on Migration

To determine the effect of altering extracellular levels of Ca^{2+} on EP cell migration, an *in vivo* culture preparation was used that permitted access to the migrating neurons and their surrounding environment. Staged embryos were cultured during the period of EP cell migration in a serum-free, defined saline which supported embryonic development for up to 24 hours. During the period of EP migration, experimental embryos were incubated for 10-12 hours in saline containing 4 mM $CaCl_2$ (a concentration similar to that estimated in complete culture medium containing serum). In these conditions, the overall extent of migration, as measured by the distance traveled by the leading neuron, was slightly reduced but appeared essentially normal when compared with dissected and undissected controls (compare Figs. 1C and F). When the external concentration of Ca^{2+} was elevated, the

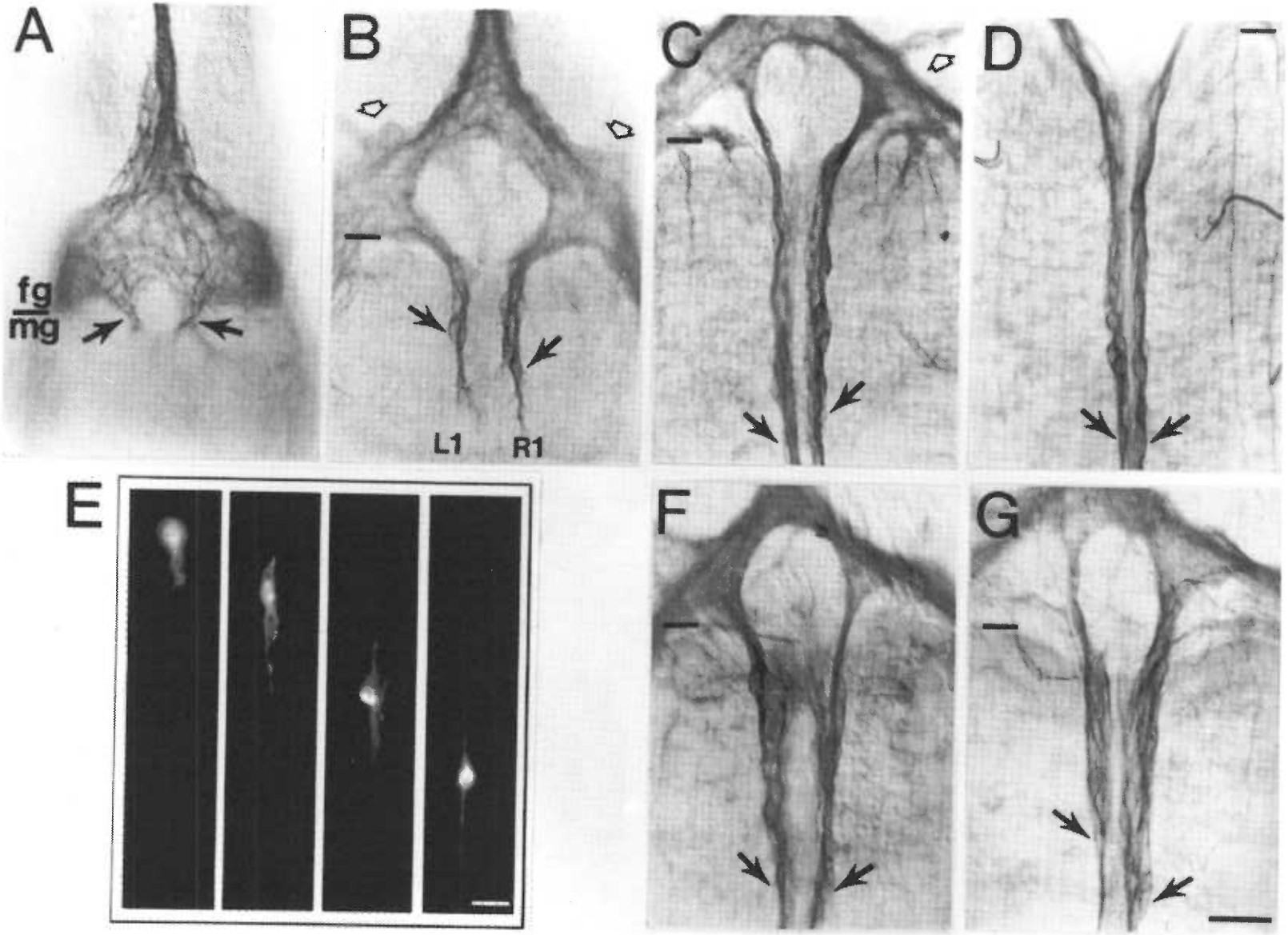
A
fg
mg
B
L1
R1
C
D
E
F
G

Figure 1. Neuronal Migration is Essential to the Formation of the ENS
(A-D) Developmental sequence of EP cell migration (visualized by anti-MFas II immunostaining) to show the stereotyped pattern of migration on pre-formed muscle band pathways; only the mid-dorsal muscle bands of the midgut (L1 and R1) are shown. (A), at 55% of embryonic development; (B), at 57%; (C), at 59%; (D), 61%. One percent of development is equivalent to one hour; "fg/mg" indicates the boundary between the foregut and midgut. Solid arrows indicate the leading cells on L1 and R1; open arrows show the lateral pathways on the foregut (out of focus).
(E) Individual EP cells labeled with DiI during migration onto the midgut; panels show a series of neurons at times and positions indicated by the solid arrows in (A-D). Note that the filopodia and subsequent axonal extensions remain primarily restricted to the pathway on which the neuron is migrating.
(F-G) Normal migration of EP cells when embryos were cultured for 10 hours in serum-free defined saline containing either 4 mM Ca^{2+} (F) or 0 mM Ca^{2+} (G). The removal of external Ca^{2+} caused no apparent deleterious effect on neuronal development. Scale bar = 20 μm (A-D) and (F-G), 50 μm in (E).

development of the EP cells proceeded normally; however, there was a statistically significant decrease in the extent of migration when compared with 4 mM $CaCl_2$ saline controls (Fig. 2A). In 12 mM $CaCl_2$, the extent of migration was 85% of that measured in 4 mM $CaCl_2$ ($p<0.05$), while in 20 mM $CaCl_2$, migration was only 53% of controls ($p<0.001$). In contrast, when no $CaCl_2$ was added to the saline (0 mM), the formation of the ENS proceeded normally (Fig. 1G) and the extent of EP cell migration was not significantly different from control cultures incubated in 4 mM $CaCl_2$ (Fig. 2A). Besides measuring the distance of the leading neuron on each of the migratory pathways (as indicated in Fig. 2A), we also quantified the extent of migration in these experiments by (a) measuring the average distance traveled by each neuron on a given pathway, and (b) measuring the total number of neurons that had migrated on each pathway (not shown). However, because these alternative methods yielded histograms that were essentially identical to those shown in figure 2A, we used this first form of quantitation for all subsequent manipulations. These results demonstrated that the defined saline used in these studies could support embryonic development and normal EP cell migration, thereby allowing us to vary external levels of Ca^{2+} in a controlled manner. In addition, increasing Ca^{2+} levels above 4 mM caused a dose-dependent decrease in migration, suggesting that proportional elevations in intracellular Ca^{2+} within the EP cells may inhibit their motility.

As a more direct means of manipulating intracellular Ca^{2+}, we used the ionophores A23187 (Reed and Lardy, 1972) and ionomycin (Liu and Hermann, 1978) to permeabilize the neurons to Ca^{2+}. The ionophores were applied externally to the EP cells by their addition to the culture medium at the beginning of the migratory period. When applied in normal culture medium, both of these compounds caused a dose-dependent decrease in EP cell migration (Fig. 2B). At low doses (1 μM), the distance traveled by the EP cells was reduced by 20% ($p<0.01$) but the neurons proceeded along their normal pathways. At higher concentrations, even a brief exposure to the ionophores completely inhibited the migration of most EP cells, with only a few neurons travelling for short distances onto the midgut. The

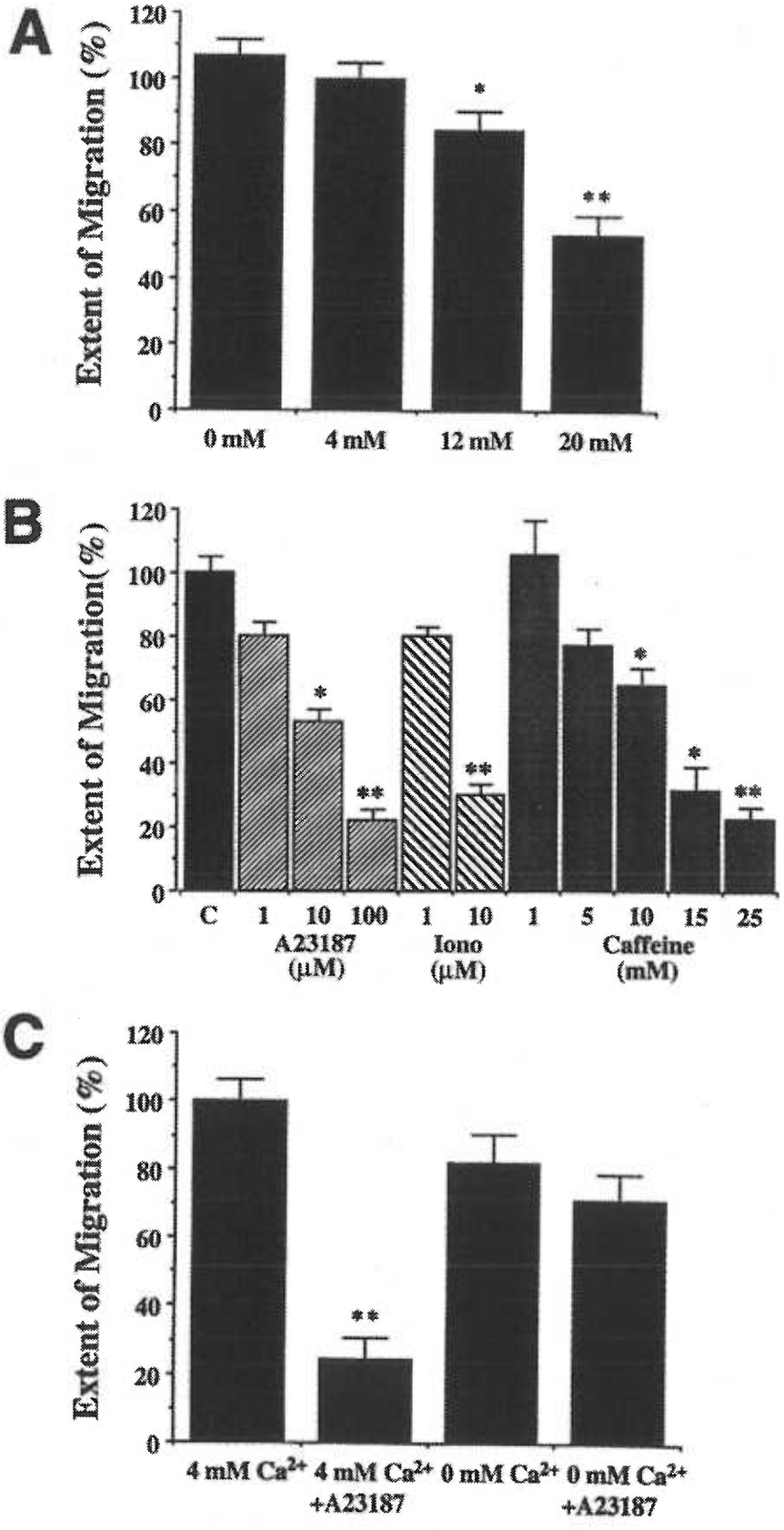
A
Extent of Migration (%)
120
100
80
60
40
20
0
*
**
0 mM
4 mM
12 mM
20 mM
B
Extent of Migration(%)
120
100
80
60
40
20
0
*
**
*
**
*
**
C
1
10
100
1
10
1
5
10
15
25
A23187 (μM)
Iono (μM)
Caffeine (mM)
C
Extent of Migration (%)
120
100
80
60
40
20
0
**
4 mM Ca2+
4 mM Ca2+ +A23187
0 mM Ca2+
0 mM Ca2+ +A23187

Figure 2. Increased Intracellular Ca^{2+} Causes a Concentration-Dependent Inhibition of Migration

(A). Effects of increasing external levels of Ca^{2+}. Embryos were cultured in defined saline containing 0, 4, 12, or 20 mM Ca^{2+} during the normal period of EP cell migration. Extent of migration was calculated by measuring the distance traveled by the leading cell on each pathway from the fg/mg boundary as a proportion of control measurements taken from cultures incubated in complete medium. Averaged data are presented as $\pm$ SEM. Histograms were normalized to values in 4 mM Ca^{2+}. $N \geq 16$ for each condition. *, $p<0.05$; **, $p<0.001$ (two-tail Student's t test).

(B). Effects of increasing internal levels of Ca^{2+}. Embryos were cultured in complete culture medium containing A23187, ionomycin, or caffeine at indicated concentrations during the migratory period. Preparations cultured in 100 μM A23187 were rinsed after 2 hr and incubated in normal culture medium until the completion of the experiment. The distance of migration was normalized to values taken from matched cultures. Averaged data are presented as $\pm$ SEM; N = 8-20. *, $p<0.05$; **, $p<0.001$.

(C). The inhibitory effect of A23187 is markedly attenuated in 0 mM Ca^{2+} Saline. Preparations were cultured in serum-free defined saline containing 4 mM or 0 mM Ca^{2+} plus or minus 3 μM A23187. The distance of migration was normalized to that of control embryos cultured in saline containing 4 mM Ca^{2+}. Averaged data are presented as $\pm$ SEM; $N \geq 12$. **, $p<0.001$.

majority of cells remained within the packet on the foregut, although they continued to express MFasII. As an additional means of increasing intracellular levels of Ca^{2+} in the EP cells, we also applied caffeine (Mironov and Usachev, 1991; Palade et al., 1989) to cultured embryos at the onset of migration. Similar to the ionophores, caffeine caused a statistically significant and dose-dependent inhibition of migration (Fig. 2B). Besides inducing the release of internal Ca^{2+} stores, caffeine has also been reported to inhibit phosphodiesterase activity (Nehlig et al., 1992). However, when we applied IBMX (100 μM) to control for this effect, we observed no change in the extent of EP cell migration (not shown). Because all of our experiments were conducted within the developing embryo, we were unable to use UV-activated compounds such as caged Ca^{2+} to induce selective changes in intracellular Ca^{2+} within individual neurons. Nevertheless, these results showed that raising intracellular concentrations of Ca^{2+}, either by increasing the cell permeability to Ca^{2+} or releasing Ca^{2+} from intracellular stores, consistently caused a dose-dependent decrease in migration.

To show that the effect of the ionophores was dependent on external Ca^{2+}, we performed an additional experiment with A23187 applied in defined saline with and without added Ca^{2+} (Fig. 2C). When an intermediate concentration of A23187 (3 μM) was applied to cultured embryos in defined saline containing 4 mM $CaCl_2$, there was a robust inhibition of migration. The more dramatic effect of A23187 in defined saline compared with A23187 in normal culture medium (Fig. 2B) was due to the absence of serum, and might reflect either non-specific binding of the ionophore by serum proteins or stabilization of the neuronal membranes. In this experiment, control preparations cultured in saline without Ca^{2+} showed a slight reduction in migration but were not significantly different from controls. In contrast, when A23187 was applied in 0 mM Ca^{2+}, its inhibitory effect was greatly attenuated (Fig. 2C). The reduction in migration caused by the ionophore changed from an 86% inhibition in 4 mM Ca^{2+} to only a 26% inhibition in 0 mM Ca^{2+}, which was not significantly different from the 0 mM Ca^{2+} controls. These results indicated that the inhibitory effect of the ionophore was dependent on extracellular Ca^{2+}, supporting our conclusion that

manipulations which increase cytoplasmic Ca^{2+} in the EP cells cause a dose-dependent inhibition of neuronal migration.

To examine the effects of reducing intracellular levels of Ca^{2+} within the EP cells, we applied BAPTA-AM, a membrane-permeable form of the Ca^{2+} chelator BAPTA (Dickens et al., 1990; Tsien, 1980), which becomes active inside the cell after hydrolysis by cytosolic esterases. BAPTA-AM was applied in 0 mM Ca^{2+} saline to the EP cells at the onset of migration, and the embryos were subsequently cultured for 8 hours. Both embryonic development and EP cell migration proceeded in these experimental conditions; however, in many of the embryos, the normal guidance of the EP cells was severely disrupted. In contrast to control preparations cultured in 0 mM Ca^{2+} saline (Fig. 3A), EP cells exposed to BAPTA-AM underwent extensive misrouting onto inappropriate musculature. Figure 3B shows an example of a plexus treated with BAPTA-AM, in which a large number of neurons wandered off their longitudinal muscle band pathways (arrows) and migrated onto the interband musculature. The severity of this effect was decreased if BAPTA-AM was applied in saline containing 4 mM $CaCl_2$ (Fig. 3C), although under these conditions, some signs of aberrant pathfinding were still seen on the foregut (arrowheads). In contrast, when a cell-impermeable form of BAPTA (100 μM) was applied in 0 mM Ca^{2+} saline as a control, no defects in migration were observed (Fig. 3D). These results suggest that buffering intracellular Ca^{2+} in the EP cells as they migrate may alter a Ca^{2+}-dependent signaling mechanism involved in restricting the neurons to their normal pathways.

Inhibition of Migration Caused by G protein Stimulation Requires Calcium

Previously, we presented evidence that activation of G proteins within the EP cells regulates their migratory behavior (Horgan et al., 1994; 1995). In particular, intracellular injections of the wasp toxin mastoparan (which is specific for G_o/G_i family subtypes; Higashijima et al., 1990) inhibited neuronal migration in a pertussis toxin-sensitive manner (Horgan et al., 1995). To verify the specificity of this effect, we injected constitutively

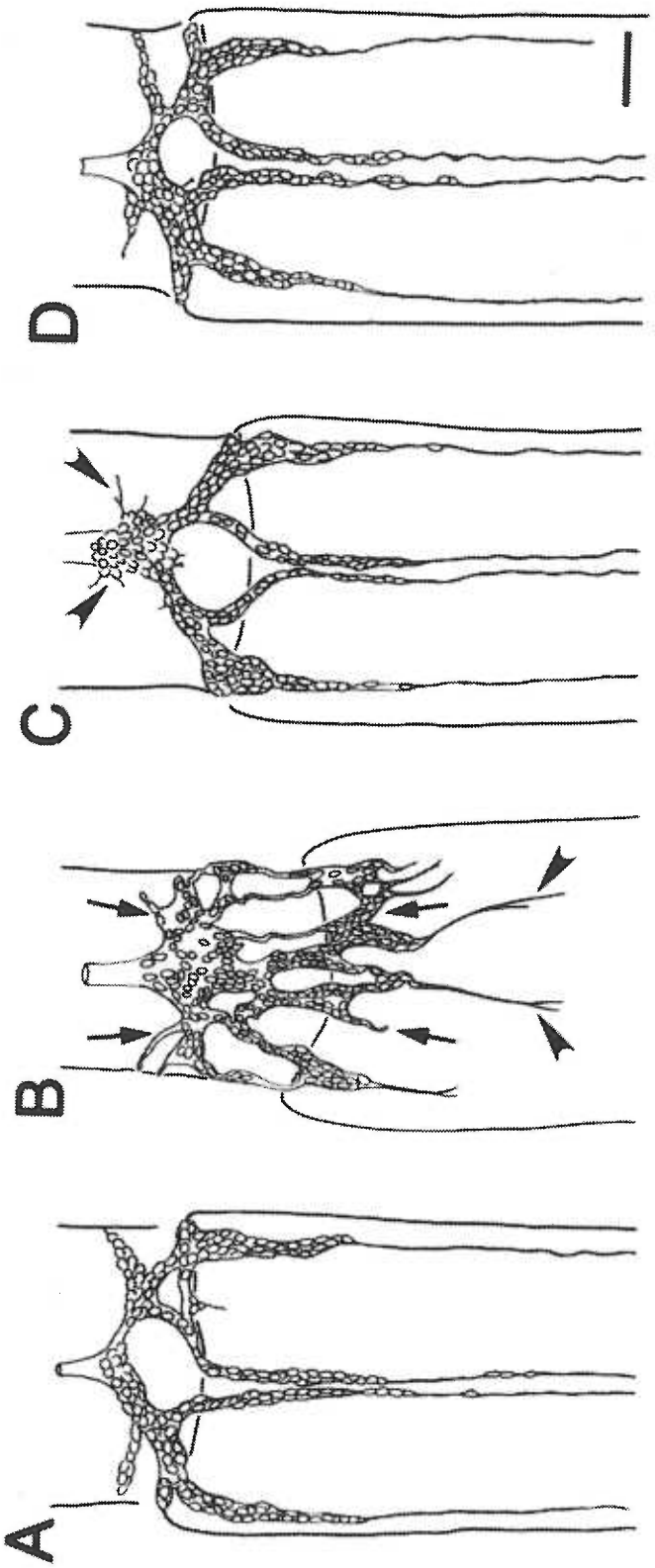
D
C
B
A

Figure 3. Buffering Intracellular Ca^{2+} Disrupts Normal Pathfinding in Migration Camera lucida drawings of individual preparations representative of experiments in which embryos were cultured during the migratory period in the following conditions: (A) 0.5% DMSO control in defined saline with 0 mM Ca^{2+} (n= 24); (B) 100 μM BAPTA-AM in 0 mM Ca^{2+} saline (n= 15); (C) 100 μM BAPTA-AM in 4 mM Ca^{2+} saline (n= 8); (D) 100 μM BAPTA (non-cell permeable) in 4 mM Ca^{2+} saline (n=8). Solid arrows indicate disrupted migration of the EP cells; open arrows indicate misdirected process outgrowth. Scale bar = 75 μm.

activated $G_{o\alpha}$ subunits ($G_{o\alpha}^*$) into individual neurons at the onset of their migration. As shown in figure 4A, injections of $G_{o\alpha}^*$ caused a -42% decrease in the extent of EP cell migration when compared with injected control neurons, an effect that was highly significant ($p<0.001$). In contrast, injections of heat-inactivated $G_{o\alpha}^*$ had no significant effect on migration. These results show that activation of $G_{o\alpha}$ within the EP cells, at a stage when only $G_{o\alpha}$ can be detected in these neurons, regulates their motile behavior in an inhibitory manner.

Because G_o has been shown to act via the modulation of intracellular Ca^{2+} concentrations in other systems, we examined whether its effect on neuronal migration might similarly be mediated by Ca^{2+} in the developing ENS. When mastoparan was injected into migrating EP cells cultured in 4 mM Ca^{2+} saline, we observed a significant decrease (-52% of controls) in their migration when compared with injected control neurons ($p<0.001$); these results were similar to the effects of mastoparan injections in the presence of complete culture medium containing serum (Horgan et al., 1995). However, when embryos were cultured in 0 mM Ca^{2+} saline, the inhibitory effect of mastoparan was completely eliminated (Fig. 4). Therefore, the inhibitory effect of mastoparan is dependent upon the presence of extracellular Ca^{2+}, indicating that Ca^{2+} influx is required for the G protein-mediated inhibition of EP cell migration.

Next, to examine spontaneous changes in intracellular Ca^{2+} levels within the EP cells, individual neurons were injected as they migrated with the Ca^{2+}-indicator dye Calcium Green-1 Dextran. The embryos were then maintained in saline with or without Ca^{2+}, and the cells were imaged *in vivo* every 5 seconds for 30-120 minutes to monitor changes in fluorescent intensity. Throughout this period of development, we observed small fluctuations in intracellular Ca^{2+} levels within most of the EP cells (Figs. 5A & 6A), but no large transients were observed in any of the neurons examined (Fig. 6D; see discussion). For technical reasons, we could not simultaneously inject compounds that stimulate G_o and image intracellular Ca^{2+} within the migrating neurons. However, when AlF_4^- (which also

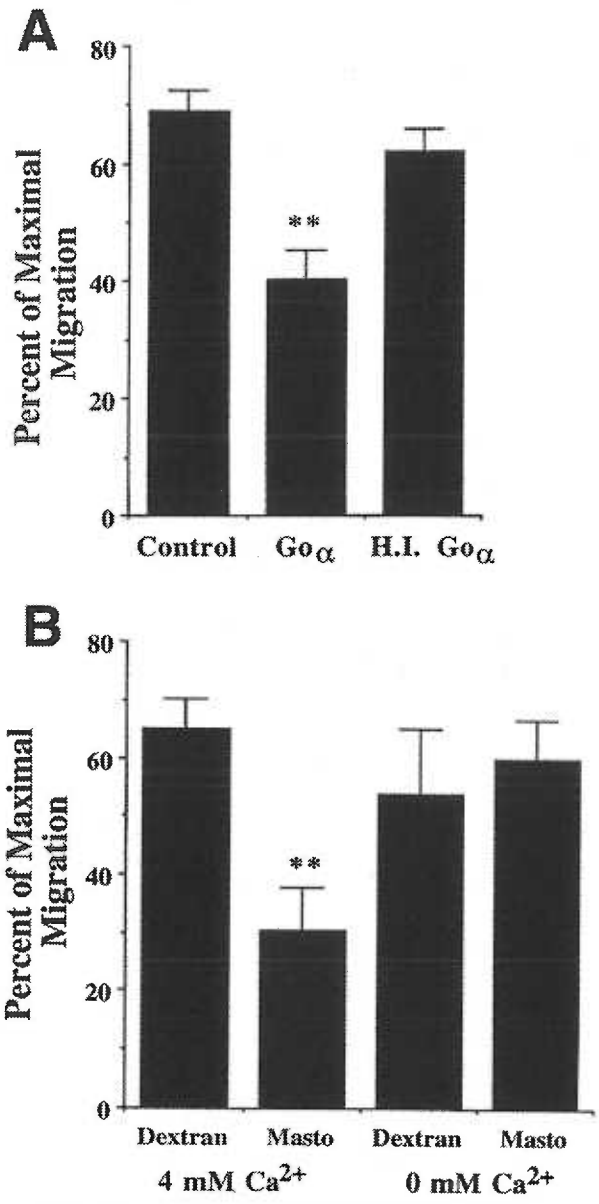

Figure 4. $G_{o\alpha}$ Stimulation Inhibits Migration and Requires the Presence of External Ca^{2+}.

(A). Individual EP cells were injected at the onset of migration with a solution of dextran-conjugated dyes plus or minus GTPγS-activated $G_{o\alpha}$ subunits (1 mM). Control injections were performed using the activation solution without $G_{o\alpha}$. Heat-inactivated solutions of $G_{o\alpha}$ were boiled for 30 min prior to injection. After culturing the embryos for 12-16 hours, the percent of maximal migration was calculated by measuring the distance that the injected neuron had migrated as a proportion of the distance traveled by the leading neuron on the same pathway. N > 30 for each histogram. Averaged data are presented + SEM; **, $p<0.001$.

(B). Embryos were cultured in defined saline containing 4 mM or 0 mM Ca^{2+}, and individual EP cells were injected at the onset of migration with a solution of dextran-conjugated dyes plus or minus 100 mM mastoparan. After 12-16 hours in culture, the percent of maximal migration was calculated as described in 4A. N > 15 for each histogram.

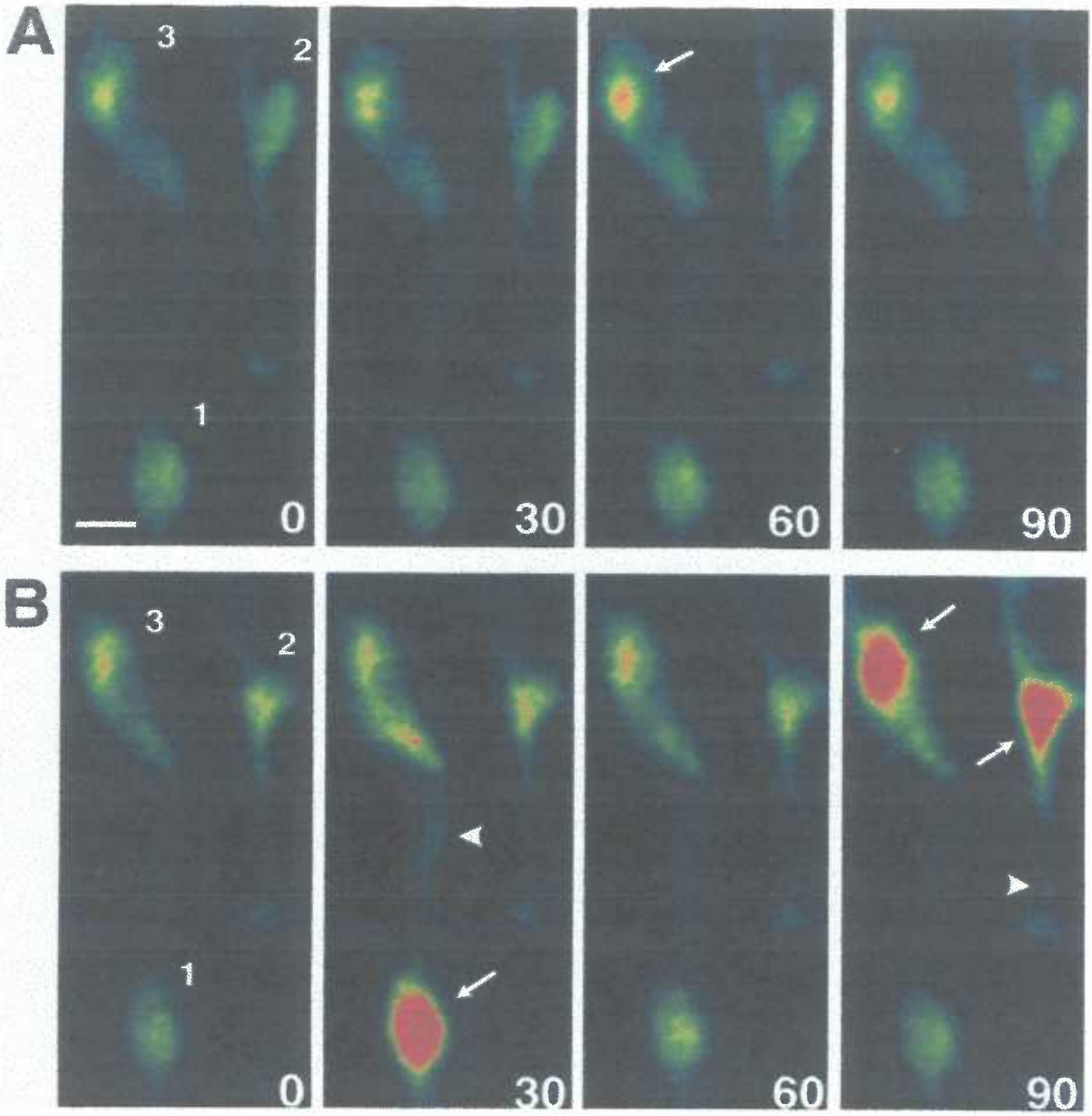

Figure 5. Intracellular Ca^{2+} Activity Imaged in Migratory Neurons Before and After AlF_4- Exposure.

Embryo cultured in defined saline containing 4 mM Ca^{2+} in which three EP cells were injected with Calcium Green Dextran-1. The cells were then imaged for 20 minutes before (A) and after (B) bath application of AlF_4-. Images were generated by averaging 12 individual frames collected over ~2 seconds. Pseudocolor representation shows relative changes in fluorescent intensity; Blue = low, red = high. Each row of time points represents consecutive 30 second intervals during the imaging period. Arrows highlight increased Ca^{2+} concentrations in the cell bodies; arrowheads, increased Ca^{2+} concentrations in their leading processes. Scale bar = 10 μM

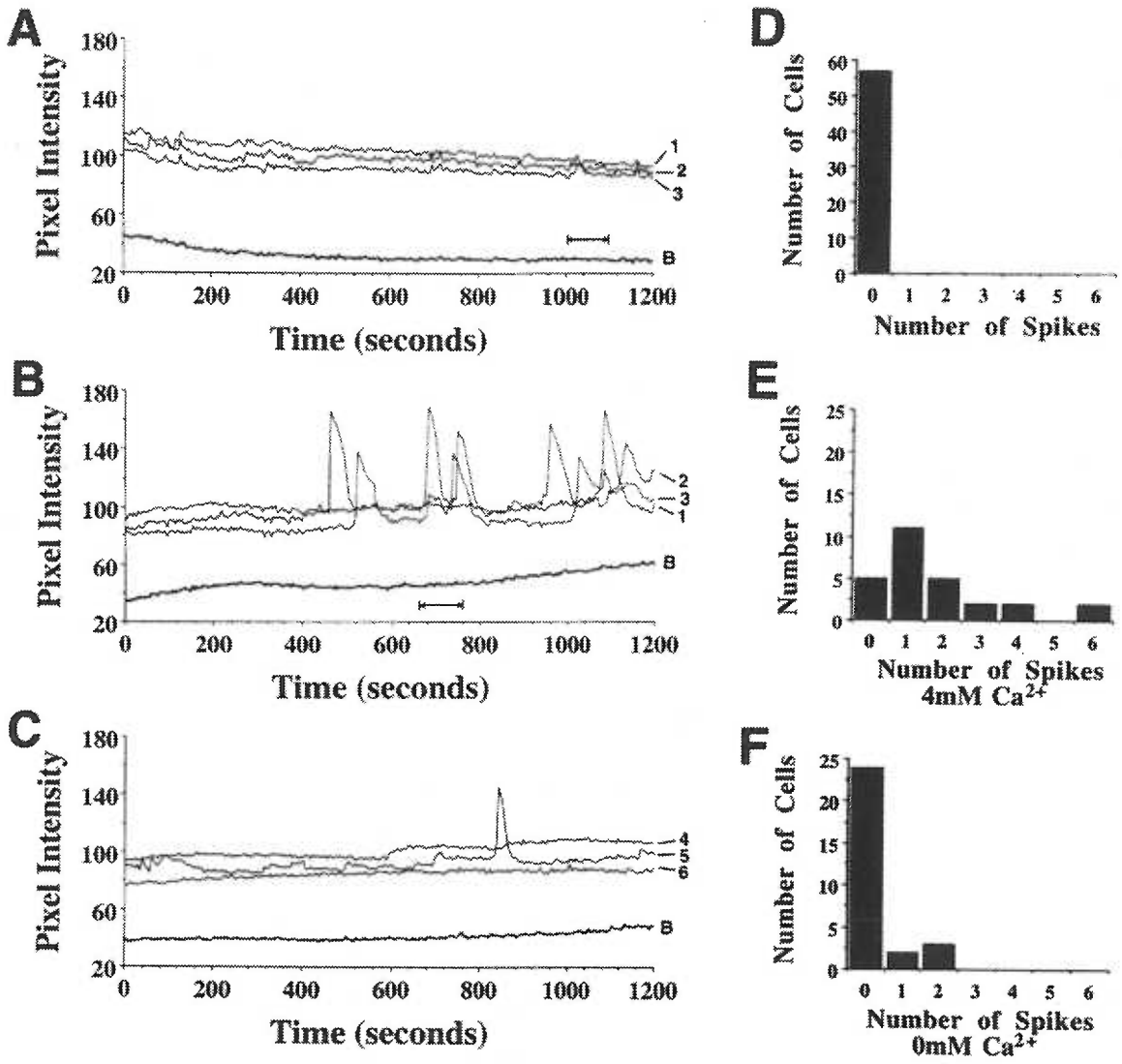

Figure 6. AlF_4-induced Ca^{2+} Spiking Activity is Greatly Diminished in the Absence of External Ca^{2+}

(A-C). Each graph shows the time course of changes in fluorescent intensity measured in three separate neurons (numbered traces). Trace labeled "B" in each panel represents simultaneous measurements of the background intensity. (A) Imaging during the period of migration in 4 mM Ca^{2+}. (B) Imaging after the addition of AlF_4- in saline containing 4 mM Ca^{2+}. (C) Imaging after the addition of AlF_4- in saline containing 0 mM Ca^{2+}. Panels A and B show the time course of Ca^{2+} activity in the same three cells shown in Figure 5 (double-headed arrows indicate times when images were captured for generating Figs. 5A and B); traces in panel C represent cells from a separate preparation.

(D-F). Histograms of the number of spikes displayed per injected cell during 20 minutes of continuous imaging: (D), before AlF_4- application; (E), after AlF_4 in 4 mM Ca^{2+} saline; and (F), after AlF_4- in 0 mM Ca^{2+} saline. A spike was defined as a transient increase in fluorescence greater than 50% of baseline intensity in the cell. The total # of cells in each condition was 56, 27, and 29, respectively.

stimulates heterotrimeric G proteins; Kahn, 1991; Northrup et al., 1983) was applied to the cultured embryos, large, transient increases in Ca^{2+} occurred in the majority of cells imaged (Fig. 5B). Figure 6A and 6B show the changes in the average pixel intensities of the same three cells injected in figure 5, before and after AlF_4^- administration in the presence of external Ca^{2+}. After a short delay, all three cells began to spike intermittently. In 81% of the EP cells examined (Fig. 6E), AlF_4^- caused at least one Ca^{2+} spike which transiently elevated the fluorescent intensity of the cells by an average of 75% above baseline. The spikes were characterized by a relatively steep average rise time of 12.8 seconds, followed by a slower recovery of 54.5 seconds before returning to baseline. The number of spikes displayed by an individual cell within the recording period was variable: 11/22 cells displayed only a single transient, while the rest exhibited multiple spikes (Fig. 6E). To control for non-specific effects of aluminum on the cells, we substituted AlF_4^- with $AlCl_3$, a compound that does not stimulate G protein activity (Northrup et al., 1983) and has no effect on EP cell migration (Horgan et al., 1994). This treatment did not induce changes in intracellular Ca^{2+} within any of the migrating neurons examined (0/14 cells; data not shown). These results provide a correlation between the inhibitory effects of AlF_4^- on migration (Horgan et al., 1994) and that of elevated Ca^{2+}, suggesting that G protein activation in the migratory EP cells regulates their motility in a Ca^{2+}-dependent manner.

To determine if the AlF_4^--induced spikes were dependent upon extracellular Ca^{2+}, we imaged neurons in embryos that were cultured in saline containing 0 mM added Ca^{2+}. Under these conditions, AlF_4^- no longer induced spiking activity in most of cells observed (Fig. 6C & F). Both the total number and frequency of Ca^{2+} transients observed in 0 mM Ca^{2+} were decreased: only 17% of cells responded to AlF_4^- with any Ca^{2+} transient activity, and most of those displayed only a single spike over the duration of the experiment. In addition, the overall size of the residual spikes was somewhat reduced (only a 47% increase in fluorescent intensity in 0 mM Ca^{2+} versus a 75% increase in 4 mM Ca^{2+}). These observations indicate that Ca^{2+} influx from extracellular sources is required for initiating the periodic spiking

activity stimulated by AlF_4^- and contributes to the observed increase in intracellular Ca^{2+}. In combination with the results of our mastoparan treatments in defined saline, they support a model for the Ca^{2+}-dependent regulation of neuronal migration by G_o.

DISCUSSION

The experiments described in this paper demonstrate a functional relationship between intracellular levels of Ca^{2+} and the activity of G proteins in the control of neuronal migration. By using an embryonic preparation of *Manduca*, we have been able to investigate the role of these two signaling molecules within individual migratory neurons *in vivo*. Manipulations designed to increase intracellular concentrations of Ca^{2+} consistently caused dose-dependent decreases in migration. In contrast, lowering Ca^{2+} levels within the neurons promoted nonselective or misdirected migration and outgrowth onto inappropriate musculature. As previously shown, the stimulation of G protein activity in the EP cells negatively regulates their migratory behavior (Horgan et al., 1994; 1995). In this paper we have expanded these findings by showing that $G_{o\alpha}$, the one G protein that we have detected in the undifferentiated EP cells (Horgan et al., 1994), inhibited migration. Moreover, G protein stimulation caused large, transient increases in Ca^{2+} within the EP cells, while the inhibitory effect of G protein activity on neuronal migration required the presence of external Ca^{2+}. These results suggest a model whereby receptor-mediated G protein activation transduces an external avoidance or termination signal for migration. This signal results in the activation of $G_{o\alpha}$, either locally within the leading filopodia or globally throughout the migratory neuron. G_o activation subsequently leads to an increase in intracellular Ca^{2+}, thereby down regulating neuronal motility by one of a variety of Ca^{2+}-dependent mechanisms (discussed below).

Calcium as a Regulator of Neuronal Motility

As a preliminary investigation into the role of Ca^{2+} in EP cell migration, we used a number of means to alter Ca^{2+} levels within the EP cells. In many of these experiments, embryos were cultured in a serum-free defined saline. Although incubating embryos with defined saline did reduce the final average distance of EP cell migration by 10-15% when compared with embryos grown in normal culture medium, this was likely due to the slight overall reduction in the rate of embryogenesis seen in the absence of serum (unpublished observations). Using defined saline, we were able to manipulate the relative levels of Ca^{2+} surrounding the EP cells. Elevations of external Ca^{2+} caused a dose-dependent inhibition in the distance of EP cell migration, as did both the application of Ca^{2+} ionophores and the induction of intracellular Ca^{2+} release. As noted above, the use of an *in vivo* preparation precluded the use of UV-activated compounds such as caged Ca^{2+} to affect Ca^{2+} levels within individual neurons. However, none of the treatments that we employed to manipulate intracellular Ca^{2+} altered the morphology of the EP cells or their expression of MFas II, indicating that these manipulations did not damage the neurons. Rather, the elevation of intracellular Ca^{2+} appeared to inhibit or down-regulate the migratory process selectively.

Conversely, experiments designed to lower intracellular concentrations of Ca^{2+} in the EP cells resulted in different consequences. BAPTA-AM, a cell-permeable Ca^{2+} chelator, caused a marked increase in misdirected migration, whereas lowering external Ca^{2+} levels, either by excluding Ca^{2+} from the culture medium or by applying the non-cell permeable form of BAPTA, had no measurable effect on migration. The effect of BAPTA-AM could be due to a reduction in the overall cytoplasmic level of Ca^{2+} (Dickens et al., 1990) or to the ability of this compound to suppress fluctuations of Ca^{2+} within the cell (Gu and Spitzer, 1995; Kuijpers et al., 1992). Our results support the latter, since lowering external Ca^{2+} (which causes a proportional reduction in intracellular Ca^{2+}; Gomez et al., 1995; Komuro and Rakic, 1992; Rehder et al., 1991) did not by itself alter migration. However, the fact that neither 0 mM Ca^{2+} saline nor external BAPTA disrupted migration could be due either to

the incomplete removal of Ca^{2+} by these treatments or to the release of Ca^{2+} into the medium from the embryo itself. Complete removal of trace levels of external Ca^{2+} with EGTA caused a general dissociation of the migratory neurons and embryonic death (not shown). Residual levels of Ca^{2+} in our 0 mM Ca^{2+} saline might therefore be sufficient to support migration, although in the presence of this saline, both the effects of the Ca^{2+} ionophore A23187 (Fig. 2C) and the inhibitory effects of mastoparan (Fig. 4B) were eliminated. Alternatively, perhaps the neurons are able to compensate for low external Ca^{2+} by drawing upon intracellular sources in order to maintain their migratory behavior.

As with other instances of cellular motility, the actions of Ca^{2+} during neuronal migration are likely to be both complex and cell type-specific (Caterina and Devreotes, 1991). During axon outgrowth, for example, normal growth cone motility requires the maintenance of intracellular Ca^{2+} levels within a specific range, above or below which motility is inhibited (Kater and Mills, 1991). However, the functions of Ca^{2+} are undoubtedly more than just permissive: many cell surface receptors known to affect motility have been shown to be linked to the regulation of Ca^{2+} (Cohan et al., 1987; Dillon et al., 1988; Schuch et al., 1989; Snow et al., 1994), while a variety of cytoskeletal components (including several actin-binding proteins and microtubule-associated proteins) are known to be modulated by Ca^{2+} in turn (Matus, 1991; McLaughlin et al., 1993; Sun et al., 1995).

In contrast to axon outgrowth, the molecular mechanisms of neuronal migration are less well understood. The evidence to date refutes a solitary or generalized role for Ca^{2+}. In avian neural crest cells, for example, Ca^{2+} seems to act in an inhibitory manner, since blocking voltage-sensitive Ca^{2+} channels stimulated their migration (Newgreen and Gooday, 1985). In addition, measurements of intracellular Ca^{2+} during migration showed that migrating crest cells had lower levels of Ca^{2+} than did their non-motile counterparts (Dickens et al., 1990). However, in cerebellar granule cells of the CNS (Fishman and Hatten, 1993), elevations in Ca^{2+} have been suggested to play a stimulatory role, possibly in response to the neurotransmitter glutamate (Rakic and Komuro, 1994). Using a cerebellar brain slice

preparation, Komuro and Rakic (1992; 1993) found that a variety of treatments intended to lower Ca^{2+} levels (including application of N-type Ca^{2+} channel blockers, NMDA receptor antagonists, and reduced Ca^{2+} saline) caused a decrease in granule cell migration. In support of this model, they subsequently showed that fluctuating changes in Ca^{2+} within granule cells *in vitro* correlated with their saltatory movement (Komuro and Rakic, 1996). Using a fluorescent Ca^{2+}-indicator, they observed oscillating increases and decreases in Ca^{2+}-dependent fluorescence of 5-25% that last ~1-2 minutes in the somata of migrating cells. In contrast, we did not observe any regular, periodic oscillations in the relative levels of Ca^{2+} within the EP cells during their normal migration *in vivo*, although brief, spontaneous changes on the order of 5-10% were seen at irregular intervals throughout the recording period (Figs. 5A & 6A). It is possible that these relatively small Ca^{2+} fluctuations are similar to those observed by Komuro & Rakic, or they may represent Ca^{2+} oscillations within the filopodia of the EP cells that were beyond the resolution of our detection system. Conversely, the much larger Ca^{2+} spikes that we observed following G protein stimulation appear to play an inhibitory role, curtailing migration. Thus, Ca^{2+} oscillations may serve both stimulatory and inhibitory functions during neuronal migration, depending on the size and possibly the location of the event within the cell. However, it remains to be determined in our system whether the oscillation of intracellular Ca^{2+} (Gu and Spitzer, 1995) or a concomitant elevation in the baseline levels of Ca^{2+} (Kater and Mills, 1991) is ultimately responsible for regulating neuronal migratory behavior.

G Protein Regulation of Migration

Unlike the well-studied mechanisms of G_O-dependent processes in the mature nervous system, the roles that G_O serves during neuronal development are only partially understood. However, information from other cell types has implicated G proteins, and specifically G_O, in the control of cellular motility. For example, in the immune system, the chemotactic responses of polymorphonuclear leukocytes towards both N-formylated peptides

and platelet activating factor are mediated by a pertussis toxin-sensitive G protein (Caterina and Devreotes, 1991; Dillon et al., 1988; Ptasznik et al., 1995), implicating members of the $G_{o/i}$ family. In addition, a variety of chemokines (including RANTES and interleukin-8) are able to stimulate the migration of leukocytes by activating $G_{o/i}$ mediated pathways (Bacon et al., 1995; Wu et al., 1993). Studies of the migratory behavior of *Dictyostelium* have similarly shown that multiple G proteins mediate the response to specific chemoattractants (Caterina and Devreotes, 1991; Devreotes and Zigmond, 1988).

G proteins have also been implicated in the control of process outgrowth from neurons and neuron-like cells, although their specific functions appear to vary with respect to cell type. In PC-12 cells, for example, neurite outgrowth was prevented by pertussis toxin (Doherty et al., 1991; Schuch et al., 1989; Williams et al., 1992), while the activation of G_o within both PC-12 cells and N1E-115 neuroblastoma cells caused a marked increase in neurite outgrowth (Strittmatter et al., 1994; Xie et al., 1995). Conversely, in cultures of both DRG and retinal neurons, a pertussis-sensitive G protein has been shown to be required for the induction of growth cone collapse by brain membrane extracts (Igarashi et al., 1993), the active component of which has been identified as collapsin (Luo et al., 1993). Furthermore, dopamine and serotonin, both of which cause neuron-specific growth cone collapse in cultured neurons (Haydon et al., 1984; Lankford et al., 1988; McCobb et al., 1988), exert their effects via G protein-coupled receptors (Jackson and Westlind-Danielsson, 1994; Sandou and Hen, 1994). G_o is a highly abundant protein in the nervous system (Brabet et al., 1988; Strittmatter et al., 1990); its onset of expression coincides with the initial differentiation of embryonic neurons (Otte et al., 1992; Pituello et al., 1991) and continues throughout the periods of their migration and outgrowth (Garibay et al., 1991; Schmidt et al., 1994; Wolfgang et al., 1991), making G_o a likely participant in the control of these processes. However, the specific functions of G_o during the formation of the nervous system have remained elusive.

In the migratory EP cells of *Manduca*, onset of $G_{o\alpha}$ expression precisely corresponds with the initiation of their migration and continues until the end of their outgrowth (Horgan et al., 1994; 1995), suggesting that it might participate in the regulation of motility. In the present work, we have confirmed the inhibitory role of G proteins with respect to EP cell migration and, in conjunction with our previous work, demonstrated that $G_{o\alpha}$ can itself down-regulate the migratory process (Fig. 4A). We have also begun to investigate the downstream signals by which G_o might induce this inhibition. The demonstration that AlF_4^- leads to large, global increases in Ca^{2+} within the EP cells explains the inhibitory effect of AlF_4^- on migratory behavior Horgan et al., 1994), since increases in intracellular Ca^{2+} levels also reduced migration (see above). Although AlF_4^- is known to stimulate all heterotrimeric G proteins (but not Ras-related small G proteins; Kahn, 1991), our previous investigations into the developmental expression of G proteins in the EP cells indicated that only G_o is present during their migration (Horgan et al., 1994). Similarly, intracellular injections of mastoparan (a specific activator of G_o and G_i) also inhibited EP cell migration, an effect that was prevented when extracellular Ca^{2+} was removed (Fig. 4B). Because all of the present studies were performed *in vivo*, it was not possible to image changes in intracellular Ca^{2+} simultaneously with the injection of individual neurons; nevertheless, these results suggest that the activation of G_o requires an influx of Ca^{2+} to mediate its inhibitory effect, and that activated $G_{o\alpha}$ induces this influx either directly or indirectly. In combination, our data favor a role for G_o in raising the intracellular concentration of Ca^{2+}, which in turn results in a regulated inhibition of EP cell migration.

Calcium-Mediated Effects of G proteins

While a variety of interactions between G proteins and Ca^{2+}-dependent processes have been described in other systems, the mechanism by which G_o might regulate Ca^{2+} in the EP cells is still unclear. One obvious explanation would involve a G_o-mediated enhancement of voltage-sensitive Ca^{2+} currents (Hille, 1994). However, based on

preliminary data, this is not likely to be the case within the migratory EP cells. In unpublished observations, we have found that 1) a number of specific Ca^{2+} channel blockers applied in embryonic culture had no effect on migration; 2) the application of high K^+ saline solutions to the embryos, which should cause the depolarization of neurons and lead to the opening of voltage-sensitive Ca^{2+} channels, also had no effect; 3) a preliminary analysis of the electrophysiological characteristics of the EP cells has suggested that the neurons do not begin to acquire any voltage-gated inward currents until the end of migration; and 4) G_O activity is normally associated with the *inhibition* of voltage-activated Ca^{2+} currents in other preparations (Hille, 1994). Moreover, a large variety of neurotransmitters and peptides that might act to depolarize the neurons via the activation of ligand-gated currents neither stimulated nor inhibited EP cell motility (unpublished observations).

Alternatively, it is possible that G_O might exert its effects via the modulation of non-voltage gated ion channels. For example, in mast cells, a ~50 pS cation conductance has been identified whose activation and inhibition could be mimicked by non-hydrolyzable analogs of GTP/GDP (Fasolato et al., 1994; Penner et al., 1988). A similar inward Ca^{2+} current is activated by purinergic-receptor agonists in PC-12 cells (Reber et al., 1992), and it may also underlie the influx of Ca^{2+} following muscarinic receptor activation in fibroblasts (Felder et al., 1992; 1993). More recently, a long-lasting, non-selective cation current has been identified in hippocampal pyramidal neurons that is stimulated by a G protein-mediated process (Congar et al., 1997; Crepel et al., 1994). G protein-mediated inhibition of migration might also involve the calcium release-activated current (Berridge, 1995), which opens in response to the depletion of Ca^{2+} from intracellular stores and has been shown to be regulated indirectly by a variety of intracellular signaling molecules (Berridge, 1993). Currents of this type, and their possible regulation by G_O, warrant further investigation with respect to the control of neuronal migration.

Cytosolic oscillations in Ca^{2+} are a widespread phenomenon resulting from the temporal coordination of Ca^{2+} influx and Ca^{2+} release from intracellular stores (Berridge,

1992). These events require a complex interaction between multiple Ca^{2+} channels that display both calcium-induced calcium release and calcium-induced current inactivation (Ehrlich, 1995; Murphy et al., 1995). It is possible that multiple sources of Ca^{2+} are responsible for the transient elevations we observed upon G protein stimulation in the EP cells. For example, the few AlF_4-induced spikes that we observed in 0 mM Ca^{2+} saline (Fig. 6C) displayed a similar time course and shape as the spikes recorded in the presence of Ca^{2+}, although the peak magnitude was decreased by 38%. This result suggests that intracellular pools of Ca^{2+} might act to amplify an initial Ca^{2+} influx (Berridge, 1997), which occasionally may be sufficient to initiate a spike even when external Ca^{2+} levels have been substantially reduced.

Alternatively, internal Ca^{2+} stores require external Ca^{2+} for their replenishment (Hoth and Penner, 1993; Putney and Bird, 1993). Therefore, in a cell undergoing spontaneous Ca^{2+} fluctuations, low levels of external Ca^{2+} might be necessary to maintain such activity for any significant length of time (Berridge, 1992). Thus, it is possible that during EP cell migration, the function of G_o involves the cyclic regulation of intracellular Ca^{2+} release, but that transmembrane Ca^{2+} influx is indirectly required in the refilling of these same Ca^{2+} stores. Evidence from other systems has shown that both the $G_{o\alpha}$ subunit (Moriarty et al., 1990) and $G_{\beta\gamma}$ (Blank et al., 1992; Katz et al., 1992) can induce phosphoinositol hydrolysis, resulting in the production of inositol phosphate (IP_3) and diacylglycerol (DAG). IP_3 can then induce the release Ca^{2+} from IP_3-sensitive stores, while DAG is known to stimulate protein kinase C (PKC), which can also raise intracellular levels of Ca^{2+} (Evans et al., 1995; Strittmatter et al., 1994). Although members of the G_q subtype of heterotrimeric G proteins have been most strongly implicated in stimulating the PLC pathway (Smrcka et al., 1991; Taylor et al., 1991), G_o has also been shown to act via regulation of PKC (Pan et al., 1997; Strittmatter et al., 1994; van Biesen et al., 1996; Xie et al., 1995) and therefore may participate in the control of Ca^{2+} modulation by way of a PLC- or PKC-mediated effect. By

using selective agonists and antagonists of the PLC/PKC pathway, we are currently investigating whether such a mechanism might be involved in controlling EP cell migration.

A Model for the Inhibitory Actions of G_o and Calcium on Neuronal Migration

Taken together, our results support the following hypothesis by which G_o may regulate the migratory behavior of EP cells. During the course of active EP cell migration, the neurons come in contact with an inhibitory signal in the local environment. This molecule (or molecules), once recognized by its G protein-coupled receptor, would cause the stimulation of G_o and then lead to an increase in intracellular Ca^{2+} levels via the regulation of Ca^{2+} influx and/or intracellular Ca^{2+} release. The results of this Ca^{2+} increase would include the down-regulation of the ongoing cytoskeletal dynamics that underlie migratory behavior.

One candidate family of proteins which is known to have this type of inhibitory effect on motile growth cones are members of the semaphorin/collapsin family. These proteins, either secreted or attached to the cell membrane, have been shown to induce local growth cone collapse and cause turning by the axon (Fan and Raper, 1995; Goodman, 1994). In addition, it has been suggested that these inhibitory molecules are recognized by G protein-coupled receptors (Goshima et al., 1995; Varela-Echavarria and Guthrie, 1997). In the developing enteric nervous system of *Manduca*, a semaphorin-like protein might be located on the non-pathway regions of the gut or possibly be expressed at the normal termination site for migration. This ligand would then be recognized by the appropriate G protein-coupled receptor expressed in the migrating EP cells (as yet unidentified), which in this case would be associated with G_o. If an inhibitory molecule of this type were located on the nonsupportive, interband musculature, it might induce a local collapse of an exploring filopodium that had extended off the pathway, thereby steering the leading process away from the inappropriate substrate. This more subtle inhibition could contribute to the small Ca^{2+} fluctuations we observed during normal migration (Fig. 6A). Moreover, this model is supported by the

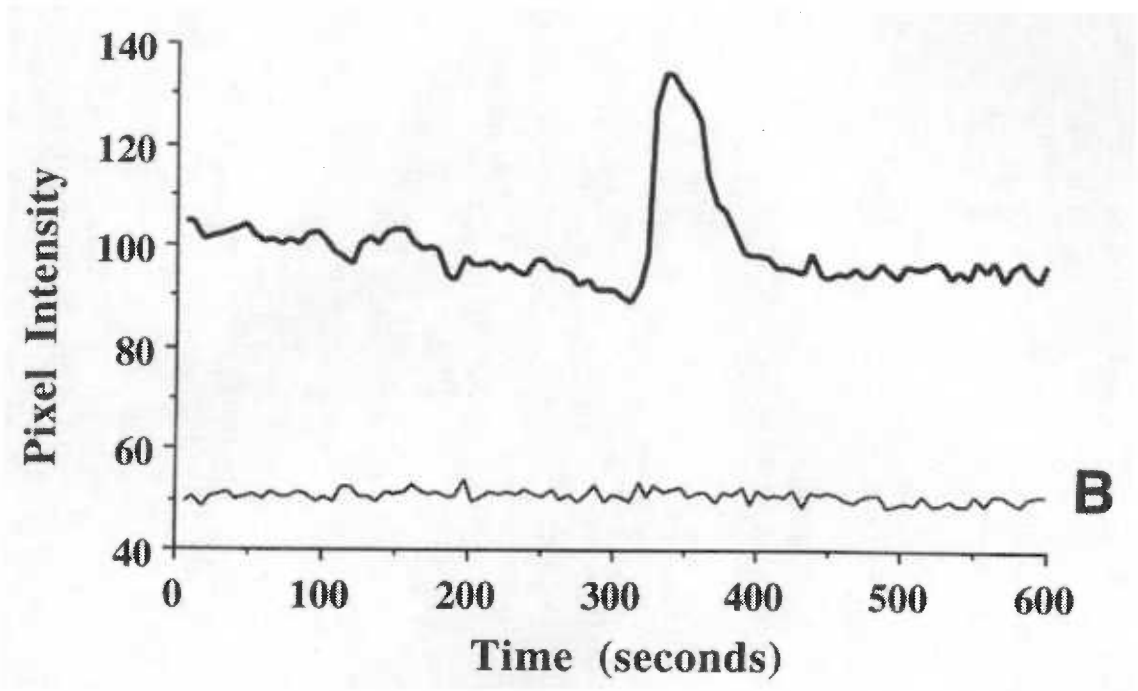

Figure 7. Spontaneous Ca^{2+} Spike Observed Towards the End of EP cell Migration
Graph shows the time course of changes in fluorescent intensity measured in an EP cell at the end of its migration. Lower trace (labeled "B") represents changes in background fluorescence. Embryo at ~65% of embryogenesis was imaged in 4 mM Ca^{2+} saline over a period of one hour. Spikes of this magnitude have been observed in 2/14 cells imaged.

effects of BAPTA-AM, which should dampen any transient changes in intracellular Ca^{2+} and was found to induce the misdirected migration of EP cells onto inappropriate regions of the gut (Fig. 3).

Alternatively, inhibitory molecules might be expressed only at the termination sites for migration along the muscle band pathways, serving to end the migratory phase of EP cell development and possibly induce the transition from cell migration to axon outgrowth (Copenhaver and Taghert, 1989). While most of the recordings described in this paper did not extend into the normal termination period for EP cell migration, in preliminary studies, we have now observed examples of an EP cell undergoing a large Ca^{2+} spike at the end of its migration (Fig. 7), similar to those induced by our treatments with AlF_4^- (Fig. 6). Our results suggest that the regulation of intracellular Ca^{2+} levels might contribute to the control of multiple aspects of neuronal migration. Elucidation of the mechanisms by which G_o regulates EP cell migration in this *in vivo* preparation should further our understanding of the signal transduction pathways that control neuronal motility and the role of particular G proteins in the developing nervous system.

ACKNOWLEDGMENTS

We are indebted to Drs. Steven Matsumoto and Marc Carey for their assistance and generosity during the calcium imaging studies. We thank Drs. Michael Forte, Edwin McClesky, and Steven Matsumoto for critical reviews of this manuscript, and other members of our laboratory for their support and helpful discussions. This work was funded by NIH grant NS-35369.

Summary and Future Directions

For a neuron to reach its correct destination, multiple aspects of migration must be regulated. For example, the onset, direction, route selection, and cessation of motility are critical parameters which are mediated in part by external cues in the cell's environment (Lauffenburger, 1996). Little is known of the intracellular signaling events that coordinate these behaviors in migratory neurons. By studying the enteric nervous system of *Manduca sexta*, I have investigated one member of a family of signaling molecules, the heterotrimeric G proteins, and demonstrated that it may participate in the regulation of EP cell migration *in vivo*. Specifically, I characterized the appearance of $G_{o\alpha}$ in *Manduca* embryos by monitoring the developmental expression of both mRNA and protein levels. From these studies, I found that both could be detected in the EP cells, and the onset of this expression coincided with the period of EP cell migration and process outgrowth.

In order to test whether G_o plays a functional role in the migrating neurons, I used a variety of reagents known to stimulate or inhibit G proteins. Application of AlF_4 (an activator of all heterotrimeric G proteins) directly onto the developing ENS blocked EP cell migration. As a more selective test of G_o function, I injected the stimulatory toxin mastoparan (which activates G_i and G_o) directly into migrating cells. This treatment reduced the distance that the injected cells migrated by ~50%. These effects were specific, since the inhibitory effect of mastoparan could be eliminated by the coinjection of pertussis toxin (an inhibitor of G_o and G_i), while no effect was observed when cholera toxin (specific for G_s) was injected. The strongest evidence that $G_{o\alpha}$ can itself mediate the down regulation of motility was produced by my injections of the activated $G_{o\alpha}$ subunit, which also reduced the distance of migration by ~40%. However, inhibition of G_o by the injection of pertussis toxin or the guanyl nucleotide analog GDPβS caused no observable enhancement of migration, suggesting the involvement of additional regulatory mechanisms in the control of EP cell migration.

Since G_o has been shown to modulate Ca^{2+} in many cell types (Dunlap, 1997), and since intracellular Ca^{2+} levels are critical in other forms of neuronal motility (Kater & Mills, 1991), I hypothesized that G_o mediated the inhibition of EP cell migration by regulating Ca^{2+} within the motile cells. Manipulations designed to alter the intracellular levels of Ca^{2+} in migrating EP cells demonstrated that Ca^{2+} could cause a down-regulation of their motility, since elevations of Ca^{2+} by a variety of means caused dose-dependent decreases in migration. In contrast, the buffering of intracellular Ca^{2+} resulted in aberrant migration of neurons off their correct pathways. To determine whether G_o activation might inhibit migration via a Ca^{2+}-dependent process, I imaged intracellular fluctuations of Ca^{2+} in individual EP cells while stimulating G_o. Application of AlF_4 caused large transient, elevations of Ca^{2+} which could be prevented by the removal of external Ca^{2+}. In addition, the lowering of Ca^{2+} in the culture medium eliminated the inhibitory effect of mastoparan on EP cell migration. These results support the conclusion that G_o activation reduces migration by increasing the intracellular levels of Ca^{2+}.

A number of additional experiments could be performed to test more conclusively the role of this signaling pathway. Imaging Ca^{2+} within migratory neurons while injecting specific modulatory reagents of G_o would allow us to eliminate the possible involvement of other, as yet unidentified G proteins which might contribute to the rise of Ca^{2+} in the EP cells. The question of whether G_o stimulation elevates Ca^{2+} by intracellular release or Ca^{2+} influx could be tested by combining G_o activation and/or Ca^{2+} imaging with specific inhibitors of particular Ca^{2+} sources. The effects of these inhibitors could then be tested on the normal migration of the EP cells. In addition, electrophysiological observations of Ca^{2+} channel properties within migrating EP cells might clarify why the effects of stimulating G_o are dependent on external Ca^{2+}, thereby lending further insight into the mechanism of G_o action.

The question of whether G_o functions in the guidance of EP cells by inhibiting their migration onto inappropriate substrates or by down regulating migration at the termination

of the normal motile period is critical for our understanding of neuronal migration. Future experiments to examine the first of these possibilities will require a better resolution of Ca^{2+} dynamics within individual cells. Since pathfinding molecules are likely to regulate the behavior of the leading process of a migrating neuron, an examination of more subtle changes in Ca^{2+} levels within the filopodia and/or lamellipodia might provide insight into this question. Manipulations could also be designed that elevate or lower Ca^{2+} selectively within the leading process by the use of caged Ca^{2+} or Ca^{2+} buffers. Furthermore, the local activation of G proteins with caged guanyl nucleotide analogs would allow us to test directly the role of G proteins on pathfinding.

The alternative hypothesis, that G_o functions to terminate EP cell migration during the transition from migration to process outgrowth, is supported by our observation of occasional, large Ca^{2+} transients in EP cell bodies at the completion of their migration. Imaging Ca^{2+} within individual EP cells for extended durations would be necessary to determine if events of this type are a regular feature of EP cell development. Additionally, this transition occurs at the time when the EP cells are undergoing differentiation of their neurotransmitter phenotype (Copenhaver & Taghert, 1989a), a stage when the expression of $G_{o\alpha}$ is selectively maintained in the same subpopulation of EP cells that develops FMRFamide-like immunoreactivity (Horgan et al 1994). It would therefore be interesting to test the effects of G_o manipulations on this aspect of differentiation.

The experiments and results described in this thesis indicate that G_o is a signaling molecule in the EP cells that transduces inhibitory information from the extracellular environment to the motile machinery of the migrating cell. However, the external ligands and receptors that cause the activation of this pathway are still unknown. Preliminary evidence from other systems suggest that a family of molecules called semaphorins, which have been shown to regulate growth cone motility (Kolodkin et al., 1993), may act via G protein coupled receptors (Goshima et al., 1995). The identification of the putative

inhibitory molecules that couple to G_o in the developing CNS might have broad implications for the understanding of neuronal migration in the developing nervous system.

References

Abedi, H. and Zachary, I. (1995). Signalling mechanisms in the regulation of vascular cell migration. Cardiovascular Research 30, 544-56.

Adams, J.C. and Watt, F.M. (1993). Regulation of development and differentiation by the extracellular matrix. Development 117, 1183-1198.

Aderem, A. (1992). Signal transduction and the actin cytoskeleton: the roles of MARCKS and profilin. Tr. Biochemi. Sci. 17, 438-443.

Al-Aoukaty, A., Schall, T.J., and Maghazachi, A.A. (1996). Differential coupling of CC chemokine receptors to multiple heterotrimeric G proteins in human interleukin-2-activated natural killer cells. Blood 87, 4255-4260.

Amatruda III, T. T., Gerard, N. P., Gerard, C., and Simon, M. I. (1993). Specific interactions of chemoattractant factor receptors with G proteins. J. Biol. Chem. 268, 10139 10144.

Anderson, N. G., Kilgaar, E., and Sturgill, T. W. (1991). Activation of mitogen-activated protein kinase in BC3H1 myocytes by fluoroaluminate. J. Biol. Chem. 266, 10131-35.

Andrade, R. (1994). Infusion of guanine nucleotides through recording electrodes for studies on G-protein regulation of ion currents and channels. Methods in Enzymology 238, 348-356.

Asano, T., Morishita, R., and Kato, K. (1992). Two forms of Go type proteins: identification and distribution in various rat tissues and cloned cells. J. Neurochem. 58, 2176-81.

Bacon, K.B., Premack, B.A., Gardner, P., and Schall, T.J. (1995). Activation of dual T cell signaling pathways by the chemokine RANTES. Science 269, 1727-1730.

Baird, D. H., Hatten, M. E., and Mason, C. A. (1992). Cerebellar target neurons provide a stop signal for afferent neurite extension *in vitro*. J. Neurosci. 12, 619-634.

Bandtlow, C.E., Schmidt, M.F., Hassinger, T.D., Schwab, M.E., and Kater, S.B. (1993). Role of intracellular calcium in NI-35-evoked collapse of neuronal growth cones. Science 259, 80-83.

Barth, P. G. (1987). Disorders of neuronal migration. Can. J. Neurol. Sci. 14, 1-16.

Bartsch, S., Bartsch, U., Dorries, U., Faissner, A., Weller, A., Ekblom, P., and Schachner, M. (1992). Expression of tenascin in the developing and adult cerebellar cortex. J. Neurosci. 12, 736-749.

Behar, T. N., Shaffner, A. E., Colton, C. A., Somogyi, R., Olah, Z., Lehel, C., and Barker, J. L. (1994). GABA-induced chemokinesis and NGF-induced chemotaxis of embryonic spinal cord neurons. J. Neurosci. 14, 29-38.

Bergamaschi, S., Govoni, S., Battaini, F., Trabuichi, M., Del Monaco, S., and Parenti, M. (1992). G protein modulation of ω-conotoxin binding sites in neuroblastoma x glioma NG 108-15 hybrid cells. J. Neurochem. 59, 536-543.

Berlot, C. H., Devreotes, P. N., and Spudich, J. A. (1987). Chemoattractant-elicited increases in *Dictyostelium* myosin phosphorylation are due to changes in myosin localization and increases in kinase activity. J. Biol. Chem. 262, 3918-3926.

Berridge, M.J. (1992). Inositol trisphosphate and calcium oscillations. Adv. in Second Messenger and Phosphoprotein Res. 26, 211-223.

Berridge, M.J. (1993). Inositol trisphosphate and calcium signaling. Nature 361, 315-325.

Berridge, M.J. (1995). Capacitative calcium entry. Biochem. J. 312, 1-11.

Bronner-Fraser, M. (1990). Role of extracellular environments in neural crest migration. In "Cytoplasmic Organization Systems" (G. M. Malacinski, Ed.), pp. 375-402. McGraw-Hill, N.Y.

Berridge, M.J. (1997). Annual Review Prize Lecture: Elementary and global aspects of calcium signalling. J. Physiol. (London) 499.2, 291-306.

Bigay, J., Deterre, P., Pfister, C., and Chabre, M. (1985). Fluoroaluminates activate transducin-GDP by mimicking the γ-phosphate of GTP in its binding site. FEBS 191, 181-185.

Black, I. B., Adler, J. E., Dreyfus, C. F., Jonakait, G. M., Katz, D. M., La Gamma, E. F., and Markey, K. M. (1984). Neurotransmitter plasticity at the molecular level. Science 225, 1266-1270.

Blank, J.L., Brattain, K.A., and Exton, J.H. (1992). Activation of cytosolic phosphoinositide phospholipase C by G-protein βγ subunits. J. Biol. Chem. 267, 23069-23075.

Bornfeldt, K.E., Graves, L.M., Raines, E.W., Igarashi, Y., Wayman, G., Yamamura, S., Yatomi, Y., Sidhu, J.S., Krebs, E.G., Hakomori, S.-i., and Ross, R. (1995). Sphingosine-1-Phosphate Inhibits PDGF-induced Chemotaxis of Arterial Smooth Muscle Cells: Spatial and Temporal Modulation of PDGF Chemotactic Signal Transduction. J. Cell Biol. 130, 193-206.

Bottazzi, B., Introna, M., Allavena, P., Villa, A., and Mantovani, A. (1985). In vito migration of human large granular lymphocytes. J. Immunology 134, 2316-2321.

Bourne, H. R., Sanders, D. A., and McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanisms. Nature 349, 117-121.

Brabet, P., Dumuis, A., Sebben, M., Pantaloni, C., Bockaert, J., and Homburger, V. (1988). Immunocytochemical localization of the guanine nucleotide-binding Protein G_o in primary cultures of neuronal and glial cells. J. Neurosci. 8, 701-708.

Bray, D. and White, J.G. (1988). Cortical flow in animal cells. Science 239, 883-888.

Bronner-Fraser, M. (1990.) Role of extracellular environments in neural crest migration. In "Cytoplasmic Organization Systems" Vol. (ed. G.M. Malacinski), pp. 375-402. McGraw-Hill, N.Y.

Brummer, J., Neumaier, M., Gopfert, C., and Wagener, C. (1995). Association of $pp60^{c\text{-}src}$ with biliary glycoprotein (CD66a), an adhesion molecule of the carcinoembryonic antigen family downregulated in colorectal carcinomas. Oncogene 11, 1649-1655.

Burridge, K., Turner, C.E., and romer, L.H. (1992). Tyrosine phosphorylation of paxillin and $pp125^{FAK}$ accompanies cell adhesion to extracellular matrix: A role in cytoskeletal assembly. J. Cell Biol. 119, 893-903.

Caroni, P. and Schwab, M.E. (1988). Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. J. Cell Biol. 106, 1281-1288.

Carr, J. N. and Taghert, P. H. (1988). Formation of the transverse nerve in moth embryos 1. A scaffold of non-neuronal cells prefigures the nerve. Dev. Biol. 130, 487-499.

Carson, M., Weber, A., and Zigmond, S.H. (1986). An actin-nucleating activity in polymorphonuclear leukocytes is modulated by chemotactic peptides. J. Cell Biol. 103, 2707-2714.

Caterina, M. J. and Devreotes, P. N. (1991). Molecular insights into eukaryotic chemotaxis. FASEB J. 5, 3078-3085.

Chang, K. J., Pugh, W., Blanchard, S. G., McDermed, J., and Tam, J. P. (1988). Antibody specific to the α subunit of the guanine nucleotide-binding regulatory protein Go: developmental appearance and immunocytochemical localization in the brain. Proc. Nat. Acad. Sci. (USA) 85, 4929-4933.

Chaponnier, C., Yin, H.L., and Stossel, T.P. (1987). Reversibility of gelsolin/actin interaction in macrophges. J. Exp. Med. 165, 97-106.

Chen, J. S. and Levi-Montalcini, R. (1969). Axonal outgrowth and cell migration *in vitro* from nervous systems of cockroach embryos. Science 166, 631-632.

Chen, M.-Y., Insall, R.H., and Devreotes, P.N. (1996). Signaling through chemoattractant receptors in *Dictyostelium*. Trends Gen. 12, 52-57.

Chiba, A. and Keshishian, H. (1996). Neuronal pathfinding and recognition: Roles of cell adhesion molecules. Dev. Biol. 180, 424-432.

Cohan, C.S., Connor, J.A., and Kater, S.B. (1987). Electrically and chemically mediated increases in intracellular calcium in neuronal growth cones. J. Neurosci. 7(11), 3588-3599.

Condeelis, J. and Vahey, M. (1982). A calcium- and pH-regulated protein from *Dictyostelium discoideum* that cross-links actin filaments. J. Cell Biol. 94, 466-471.

Congar, P., Leinekugal, X., Ben-Ari, Y., and Crepel, V. (1997). A long-lasting calcium-activated nonselective cationic current is generated by synaptic stimulation or exogenous activation of group I metabotropic glutamate receptors in CA1 pyramidal neurons. J. Neurosci. 17, 5366-5379.

Cooper, J. A. (1991). The role of actin polymerization in cell motility. Ann. Rev. Physiol. 53, 585-605.

Copenhaver, P. F. (1993). Origins, migration, and differentiation of glial cells in the insect nervous system from a discrete set of glial precursors. Development 117, 59-74.

Copenhaver, P.F., Horgan, A.M., and Combes, S. (1996). An identified set of visceral muscle bands is essential for the guidance of migratory neurons in the enteric nervous system of *Manduca sexta*. Dev. Biol. 179, 412-426.

Copenhaver, P. F., Horgan, A. M., and Rasmussen, M. A. (1995). Developmental expression of heterotrimeric G proteins in the nervous system of *Manduca sexta*. J. Neurobiol. 26, 461-484.

Copenhaver, P. F., Lagrange, M., and Horgan, A. (1992). Developmental expression of G proteins in migratory neurons of the insect enteric nervous system. Soc. Neurosci. Abs. 18, 1469.

Copenhaver, P. F. and Taghert, P. H. (1991). Origins of the insect enteric nervous system: differentiation of the enteric ganglia from a neurogenic epithelium. Development 113, 1115-1132.

Copenhaver, P.F. and Taghert, P. (1990). Neurogenesis in the insect enteric nervous system: generation of premigratory neurons from an epithelial placode. Development 109, 17-28.

Copenhaver, P. F., and Taghert, P. H. (1989a). Development of the enteric nervous system in the moth I. Diversity of cell types and the embryonic expression of FMRFamide-related neuropeptides. Dev. Biol. 131, 70-84.

Copenhaver, P. F., and Taghert, P. H. (1989b). Development of the enteric nervous system in the moth II. Stereotyped cell migration precedes the differentiation of embryonic neurons. Dev. Biol. 131, 85-101.

Copenhaver, P. F. and Taghert, P. H. (1988). Position-dependent expression of peptidergic phenotype in post-migratory neurons of the insect enteric system. Soc. Neurosci. Abs. 14, 770.

Copenhaver, P.F., Wright, J., Snyder, M., Combes, C. (1996). Developmental expression and possible role of fasciclin II in the guidance of migratory enteric neurons. Soc. Neurosci Abs. 22, 531.

Crepel, V., Aniksztejn, L., Ben-Ari, Y., and Hammond, C. (1994). Glutamate metabotropic receptors increase a Ca^{2+}-activated nonspecific cation current in CA1 hippocampal neurons. J. Neurophysiol. 72, 1561-1569.

Dabiri, G.A., Young, C.L., Rosenbloom, J., and Southwick, F.S. (1992). Molecular cloning of human macrophage capping protein cDNA. J. Biol. Chem. 267, 16545-16552.

de Fougerolles, A.R., Qin, X., and Springer, T. (1994). Characterization of the function of intercellular adhesion molecule (ICAM)-3 and comparison with ICAM-1 and ICAM-2 in immune responses. J. Exp. Med. 179, 619-629.

de Fougerolles, A.R. and Springer, T.A. (1992). Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. J. Exp. Med. 175, 185-190.

de Sousa, S. M., Hoveland, L. L., Yarfitz, S., and Hurley, J. B. (1989). The *Drosophila* Goα-like protein gene produces multiple transcripts and is expressed in the nervous system and its ovaries. J. Biol. Chem. 264, 18544-18551.

Delannet, M., and Duband, J.-L. (1992). Transforming growth factor-β control of cell-substratum adhesion during avian neural crest cell emigration *in vitro*. Development 116, 275-287.

Detmers, P.A., Powell, D.E., Walz, A., Clark-Lewis, I., Baggliolini, M., and Cohn, Z. (1991). Differential effects of neutrophil-activating peptide 1/IL-8 and its homologues on leukocyte adhesion and phagocytosis. J. Immunology 147, 4211-4217.

Devreotes, P.N. (1994). G protein-linked signaling pathways control the developmental program of *Dictyostelium*. Neuron 12, 235-41.

Devreotes, P. N. and Zigmond, S. H. (1988). Chemotaxis in eukaryotic cells: a focus on leukocytes and *Dictyostelium*. Ann. Rev. Cell Biol. 4, 649-686.

DeWaard, M., Liu, H., Walker, D., Scott, V.E.S., Gurnett, C.A., and Campbell, K.P. (1997). Direct binding of G-protein βγ complex to voltage-dependent calcium channels. Nature 385, 446-450.

Dickens, C.J., Fillespie, J.I., and Greenwell, J.R. (1990). Measurement of intracellular calcium and pH in avian neural crest cells. J. Physiol. 428, 531-544.

Dillon, S.B., Verghese, M.W., and Snyderman, R. (1988). Signal transduction in cells following binding of chemoattractants to membrane receptors. Vir. Archiv. Cell Pathol. 55, 65-80.

Doherty, P., Ashton, S. V., Moore, S. E., and Walsh, F. S. (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, 21-23.

Doherty, P., Singh, A., Rimon, G., Bolsover, S.R., and Walsh, F.S. (1993). Thy-1 antibody-triggered neurite outgrowth requires an influx of calcium into neurons via N- and L-type calcium channels. J. Cell Biol. 122, 181-189.

Doherty, P., Skaper, S. D., Moore, S. E., Leon, A., and Walsh, F. S. (1992). A developmentally regulated switch in neuronal responsiveness to NCAM and N-Cadherin in the rat hippocampus. Development 115, 885-892.

Doherty, P., and Walsh, F. S. (1994). Signal transduction events underlying neurite outgrowth stimulated by cell adhesion molecules. Curr. Opin. Neurobiol. 4, 49-55.

Donaldson, J. G., Kahn, R. A., Lippincott-Schwartz, J., and Klausner, R. D. (1991). Binding of ARF and *B*-COP to Golgi membranes: Possible regulation by a trimeric G protein. Science 254, 1197-1199.

Dorn, A., Bishoff, S. T., and Gilbert, L. I. (1987). An incremental analysis of the embryonic development of the tobacco hornworm, *Manduca sexta*. Intern. J. Invert. Repro. and Devt. 11, 137-158.

Dunlap, K. (1997). Integration hot-spot gets hotter. Nature 385, 394-397.

Edmond, B. T., Moomaw, C. R., Hsu, J. T., Slaughter, C., and Ellis, L. (1990). The p38 and p34 polypeptides of growth cone particle membranes are the α and β subunits of G proteins. Dev. Brain Res. 56, 131-136.

Ehrlich, B.E. (1995). Functional properties of intracellular calcium-release channels. Curr. Opin. Neurobiol. 5, 304-309.

Evans, P.D., Robb, S., Cheek, T.R., Reale, V., Hannan, F.L., Swales, L.S., Hall, L.M., and Midgley, J.M. (1995). Agonist-specific coupling of G-protein-coupled receptors to second-messenger systems. Prog. Brain Res. 106, 259-268.

Fan, J., and Raper, J. A. (1995). Localized collapsing cues can steer growth cones without inducing their full collapse. Neuron 14, 263-274.

Fasolato, C., Innocenti, B., and Pozzan, T. (1994). Receptor-activated Ca^{2+} influx: how many mechanisms for how many channels? Trends Pharm. Sci. 15, 77-83.

Fechheimer, M. and Cebra, J.J. (1982). Phosphorylation of lymphocyte myosin catalyzed *in vitro* and in intact cells. J. Cell Biol. 93, 261-268.

Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, K. R., and Bourne, H. R. (1992). Hormonal stimulation of adenylyl cyclase through Gi protein βγ subunits. Nature 356, 159-161.

Felder, C.C., MacArthur, L., Ma, A.L., Gusovsky, F., and Kohn, E. (1993). Tumor-suppressor function of muscarinic acetylcholine receptors is associated with activation of receptor-operated calcium influx. Proc. Natl. Acad. Sci. (USA) 90, 1706-1710.

Felder, C.C., Poulter, M.O., and Wess, J. (1992). Muscarinic receptor-operated Ca^{2+} influx in transfected fibroblast cells is independent of inositol phosphates and release of intracellular Ca^{2+}. Proc. Nat. Acad. Sci. (USA) 89, 509-513.

Firtel, R. A., Van Haastert, P. J. M., Kimmel, A. R., and Devreotes, P. N. (1989). G protein linked signal transduction pathways in development: *Dictyostelium* as an experimental system. Cell 58, 235-239.

Fishell, G. and Hatten, M.E. (1991). Astrotactin provides a receptor system for CNS neuronal migration. Development 113, 755-765.

Fishman, R. B., and Hatten, M. E. (1993). Multiple receptor systems promote CNS neural migration. J. Neurosci. 13, 3485-48.

Forscher, P. and Smith, S.J. (1988). Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J. Cell Biol. 107, 1505-1516.

Freissmuth M, and Gilman A.G. (1989) Mutations of Gs_{α} designed to alter the reactivity of the protein with bacterial toxins. J. Biol. Chem. 264, p. 21907-21914.

Fujimoto, T. and McEver, R.P. (1993). The cytoplasmic domain of P-selectin is phosphorylated on serine and threonine residues. Blood 82, 1758-1766.

Garibay, J.L., Kozusa, T., Itoh, H., Tsukamoto, T., and Matsuoka, M. (1991). Analysis by mRNA levels of the expression of six G protein α-subunit genes in mammalian cells and tissues. Biochim. Biophys. Acta 1094, 193-199.

Gershon, M.D. and Tennyson, V.M. (1991). Microenvironmental factors in the normal and abnormal development of the enteric nervous system. Prog. Clin. Biol. Res. 373, 257-276.

Gilman, A. G. (1987). G-proteins: transduction of receptor-generated systems. Ann. Rev. Biochem. 56, 615-649.

Golberg, D. J. (1988). Local role of calcium on formation of veils by growth cones. J. Neurosci. 8, 2596-2605.

Goldsmith, P., Gierschik, P., Milligan, G., Unson, C., Vinitsky, R., Halech, H., and Spiegel, A. (1987). Antibodies directed against synthetic peptides distinguish between GTP-binding proteins in neutrophils and brain. J. Biol. Chem. 262, 14683-14688.

Gomez, T.M., Snow, D.M., and Letouneau, P.C. (1995). Characterization of spontaneous calcium transients in nerve growth cones and their effect on growth cone migration. Neuron 14, 1233-1246.

Goodman, C.S. (1994). The likeness of being: Phylogenetically conserved molecular mechanisms of growth cone guidance. Cell 78, 353-356.

Goshima, Y., Nakamura, F., Strittmatter, P., and Strittmatter, S.M. (1995). Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. Nature 376, 509-514.

Goslin, K., Schreger, D. J., Skene, J. H. P., and Banker, G. (1988). Development of neuronal polarity: GAP-43 distinguishes axonal from dendritic growth cones. Nature 336, 672-674.

Gray, G.E., Leber, S.M., and Sanes, J.R. (1990). Migratory patterns of clonally related cells in the developing central nervous system. Experientia 46, 929-940.

Greengard, P., Valtorta, F., Czernik, A.J., and Benfenati, F. (1993). Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259, 780-785.

Grimstad, I. A. (1987). Direct evidence that cancer cell locomotion contributes importantly to invasion. Exp. Cell Res. 173, 515-523.

Gu, X. and Spitzer, N.C. (1995). Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. Nature 375, 784-787.

Gudermann, T., Schoneberg, T., and Schultz, G. (1997). Functional and structural complexity of signal transduction via G-protein-coupled receptors. Ann. Rev. Neurosci. 20, 399-427.

Gumbiner, B.M. (1996). Cell adhesion: The molecular basis of tissue architecture and morphogenesis. Cell 84, 345-357s.

Hadwiger, J.A., Lee, S., and Firtel, R.A. (1994). The Gα subunit Gα4 couples to pterin receptors and identifies a signaling pathway that is essential for multicellular development in *Dictyostelium*. Proc. Natl. Acad. Sci. 91, 10566-10570.

Hall, A. L., Warren, V., and Condeelis, J. (1989). Transduction of the chemotactic signal to the actin cytoskeleton of *Dictyostelium discoidum*. Dev. Biol. 136, 517-525.

Halliday, K. (1984). Regional homology in GTP binding proto-oncogene products and elongation factors. J. Cyclic Nuc. Res 9, 434-448.

Hanks, S.K. and Polte, T.R. (1997). Signaling through focal adhesion kinase. Bioessays 19, 137-145.

Hardin, J. (1989). Local shifts in position and polarized motility drive cell rearrangement during sea urchin gastrulation. Dev. Biol. 136, 430-445.

Harris-Warrick, R. M., Hammond, C., Paupardin-Tritsch, D., Homburger, V., Rouot, B., Bockaert, J., and Gerschenfeld, H. M. (1988). An α40 subunit of a GTP-binding protein immunologically related to Go mediates a dopamine-induced decrease of Ca^{2+} current in snail neurons. Neuron 1, 27-32.

Hatten, M.E. (1990). Riding the glial monorail: a common mechanism for glial-guided neuronal migration in different regions of the developing mammalian brain. Tr. Neurosci. 13, 179-184.

Haydon, P.G., McCobb, D.P., and Kater, S.B. (1984). Serotonin selectively inhibits growth cone motility and synaptogenesis of specific identified neurons. Science 226, 561-564.

Heagsman, J. E. M., Middleton, C. A., and Watt, F. M. (1987). Cell behavior: shape, adhesion, and motility. J. Cell Sci. Suppl. 8, 1-449.

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

Higashijima, T., Uzu, S., Nakajima, T., and Ross, G. M. (1988). Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins. J. Biol. Chem. 263, 6491-6494.

Hille, B. (1994). Modulation of ion-channel function by G-protein-coupled receptors. Tr. Neurosci. 17, 531-536.

Hohman, R. J. (1988). Aggregation of IgE receptors induces degranulation in rat basophilic leukemia cells permeabilized with α-toxin from *Staphylococcus aureus*. Proc. Nat. Acad. Sci. (USA) 85, 1624-1628.

Horgan, A.M., Lagrange, M.T., and Copenhaver, P.F. (1994). Developmental expression of G proteins in a migratory population of embryonic neurons. Development 120, 729-742.

Horgan, A.M., Lagrange, M.T., and Copenhaver, P.F. (1995). A developmental role for the heterotrimeric G protein $G_{o\alpha}$ in a migratory population of embryonic neurons. Dev. Biol. 172, 640-653.

Hoth, M. and Penner, R. (1993). Calcium release-activated calcium current in rat mast cells. J. Physiol. 465, 359-386.

Hu, M.C.-T., Siegelman, M.H., Holzmann, B., Crowe, D.T., and Weissman, I.L. (1992). Lymphocyte homing receptors. Cold Spring Harbor Symp. Quant. Biol. 57, 291-308.

Igarashi, M., Strittmatter, S.M., Vartanian, T., and Fishman, M.C. (1993). Mediation by G proteins of signals that cause collapse of growth cones. Science 259, 77-79.

Imhof, B.A. and Dunon, D. (1995). Leukocyte migration and adhesion. Adv. Immun. 58, 345-416.

Insall, R.H., Soede, R.D.M., Schap, P., and Devreotes, P.N. (1994). Two cAMP receptors activate common signaling pathways in *Dictyostelium*. Mol. Biol. Cell 5, 703-711.

Jackson, D.M. and Westlind-Danielsson, A. (1994). Dopamine receptors: Molecular biology, biochemistry and behavioural aspects. Pharmac. & Therap. 64, 291-369.

Jacob, M. H. (1984). Neurogenesis in *Aplysia californica* resembles nervous system formation in vertebrates. J. Neurosci. 4, 1225-1239.

Janmey, P.A., Iida, K., Yin, H.L., and Stossel, T.P. (1987). Polyphosphoinositide micelles and polyphosphoinositide-containing vesicles dissociate endogenous gelsolin-actin complexes and promote actin assembly from the fast-growing end of actin filaments blocked by gelsolin. J. Biol. Chem. 262, 12228-12236.

Jessell, T.M., Bovolenta, P., Placzek, M., Tessier-Lavigne, M., and Dodd, J. (1989). Polarity and patterning in the neural tube: the origin and function of the floor plate. Ciba Foundation 144, 255-280.

Jones, D. T., Masters, S.B., Bourne, H.R., and Reed, R.R. (1990). Biochemical characterization of three stimulatory GTP-binding protiens (the large and small forms of Gs and the olfactory-specific G protein, Golf). J. Biol. Chem. 265, 2671-2676.

Jones, D. T., and Reed, R. R. (1987). Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. J. Biol. Chem. 262, 14241-14249.

Kahn, R.A. (1991). Fluoride is not an activator of the smaller (20-25 kDa) GTP-binding proteins. J. Biol. Chem. 266, 15595-15597.

Kapfhammer, J.P. and Raper, J.A. (1987). Collapse of growth cone structure on contact with specific neurites in culture. J. Neurosci. 7, 201-212.

Kater, S.B. and Mills, L.R. (1991). Regulation of growth cone behavior by calcium. J. Neurosci. 11, 891-899.

Katz, A., Wu, D., and Simon, M.I. (1992). βγ subunits of heterotrimeric G protein active β2 isoform of phospholipase C. Nature 360, 686-689.

Kleene, S.J. (1986). Bacterial chemotaxis and vertebrate olfaction. Experientia 42, 241-250.

Kleuss, C., Hescheler, J., Ewel, C., Rosenthal, W., Schultz, G., and Wittig, B. (1991). Assignment of G protein subtypes to specific receptors inducing inhibition of calcium currents. Nature 353, 43-48.

Kleuss, C., Scherubl, H., Hescheler, J., Schultz, G., and Wittig, B. (1992). Differential β-subunits determine G protein interaction with transmembrane receptors. Nature 358, 424-426.

Klymlowsky, M.W. and Parr, B. (1995). The body language of cells: The intimate connection between cell adhesion and behavior. Cell 83, 5-8s.

Kolodkin, A.L. (1996). Growth cones and the cues that repel them. Tr. Neurosci. 19, 507-513.

Komuro, H. and Rakic, P. (1992). Selective role of N-type calcium channels in neuronal migration. Science 257, 806-809.

Komuro, H. and Rakic, P. (1993). Modulation of neuronal migration by NMDA receptors. Science 260, 95-97.

Komuro, H. and Rakic, P. (1996). Intracellular Ca^{2+} fluctuations modulate the rate of neuronal migration. Neuron 17, 275-285.

Koshland, D.E.J. (1988). Chemotaxis as a model second-messenger system. Biochem. 27, 5829-5834.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44, 283-292.

Ktistakis, N. T., Linder, M. E., and Roth, M. G. (1992). Action of brefeldin: A blocked by activation of a pertussis toxin- sensitive G protein. Nature 356, 344-346.

Kuijpers, T.W., Hoogerwerf, M., and Roos, D. (1992). Neutrophil migration across monolayers of resting or cytokine-activated endothelial cells. J. Immun. 148, 72-77.

Kumagai, A., Hadwiger, J. A., Pupillo, M., and Firtel, R. A. (1991). Molecular genetic analysis of two Gα protein subunits in *Dictyostelium*. J. Biol. Chem. 266, 1220-1228.

Lamb, J.A., Allen, P.G., Tuan, B.Y., and Janmey, P.A. (1993). Modulation of gelsolin function: Activation at low pH overrides Ca^{2+} requirement. J. Biol. Chem. 268, 8999-9004.

Lange, A. J., Arion, W. J., Burchell, A., and Burchell, B. (1986). Aluminum ions are required for stabilization and inhibition of hepatic microsomal glucose-6-phosphatase by sodium fluoride. J. Biol. Chem. 261, 101-107.

Lankford, K.L., DeMello, F.G., and Klein, W.L. (1988). D1-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) 85, 2839-2843.

Lassing, I. and Lindberg, U. (1985). Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. Nature 314, 472-474.

Le Douarin, N. M. and Dupin, E. (1993). Cell lineage analysis in neural crest ontogeny. J. Neurobiol. 24, 146-162.

Letourneau, P.C., Snow, D.M., and Gomez, T.M. (1994). Growth cone motility: substratum-bound molecules, cytoplasmic [Ca^{2+}] and Ca^{2+} -regulated proteins. Prog. Brain Res. 102, 35-48.

Levine, R. B. and Truman, J. W. (1985). Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, *Manduca sexta*. J. Neurosci. 5, 2424-2431.

Lin, D.M., Fetter, R.S., Kopczynski, C., Grenningloh, G., and Goodman, C. (1994). Genetic analysis of Fasciclin II in *Drosophila*: Defasciculation, refasciculation, and altered fasciculation. Neuron 13, 1055-1063.

Liu, C.-M. and Hermann, T.E. (1978). Characterization of ionomycin as a calcium ionophore. J. Biol. Chem. 253, 5892-5894.

Luo, Y., Raible, D., and Raper, J.A. (1993). Collapsin: A protein that induces the collapse and paralysis of a neural growth cone. Cell 75, 217-227.

Man-Son-Hing, H. and Haydon, P. G. (1992). Modulation of growth cone calcium current in mediated by a PTX-sensitive G protein. Neurosci. Letts. 137, 133-136.

Masters, S. B., Stroud, R. M., and Bourne, H. R. (1986). Family of G protein a chains: amphipathic analysis and predicted structure of functional domains. Protein Eng. 1, 47-54.

Mattson, M. P., Taylor-Hunter, A., and Kater, S. B. (1988). Neurite outgrowth in individual neurons of a neuronal population is differentially regulated by calcium and cyclic AMP. J. Neurosci. 8, 1704-1711.

Matus, A. (1991). Microtubule-associated proteins and neuronal membranes. J. Cell Sci. Suppl. 15, 61-67.

McCobb, D.P., Haydon, P.G., and Kater, S.B. (1988). Dopamine and serotonin inhibition of neurite elongation of different identified neurons. J. Neurosci. Res. 19, 19-26.

McEwen, B. F. and Arion, W. J. (1985). Permeabilization of rat hepatocytes with *Staphylococcus aureus* α–toxin. J. Cell Biol. 100, 1922-1929.

McLaughlin, P.J., Gooch, J.T., Mannhertz, H.G., and Weeds, A.G. (1993). Structure of gelsolin segment 1-actin complex and the mechanisms of filament severing. Nature 364, 685-692.

McLean, I. W. and Nakane, P. K. (1974). Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. J. Histochem. Cytochem. 22, 1077-1083.

Mendel, J. E., Korswagen, H.C, Lieu, K.S., Hajdu-Cronin, Y. M, Simon, M. I., Plasterik, R. H. A, and Sternberg P. W. (1995) Participation of the protein Go in multiple aspects of behavior in *Caenorhabditis elegans*. Science 267, p. 1652-1655.

Messersmith, E.K., Leonardo, E.D., Shatz, C.J., Tesier-Lavigne, M., Goodman, C.S., and Kolodkin, A.L. (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron 14, 949-959.

Mironov, S.L. and Usachev, J.M. (1991). Caffeine affects Ca^{2+} uptake and Ca^{2+} release from intracellular stores: Fura-2 measurements in isolated snail neurons. Neurosci. Lett. 123, 200-202.

Mitchell, K.J., Doyle, J.L., Serafini, T., Kennedy, T.E., tessier-Lavigne, M., Goodman, C.S., and Dickson, B.J. (1996). Genetic analysis of netrin genes in *Drosphila*: Netrins guide CNS commissural axons and peripheral motor axons. Neuron 17, 203-215.

Mitchison, T.J. and Cramer, L.P. (1996). Actin-based cell motility and cell locomotion. Cell 84, 371-379.

Mollen, J. (1991). G protein-coupled receptors. Nature 351, 696-697.

Moorman, S.J. and Hume, R.I. (1993). ω-Conotoxin prevents myelin-evoked growth cone collapse in neonatal rat locus coeruleus neurons *in vitro*. J. Neurosci. 13, 4727-4736.

Moriarty, T.M., Padrell, E., Carty, D.J., Omri, G., Landau, E.M., and Iyengar, R. (1990). G_o protein as signal transducer in the pertussis toxin-sensitive phosphatidylinositol pathway. Nature 343, 79-82.

Muller, U. and Kypta, R. (1995). Molecular genetics of neuronal adhesion. Curr. Opin. Neurobiol. 5, 36-41.

Murphy, C.T., Poll, C.T., and Westwick, J. (1995). The whoosh and trickle of calcium signalling. Cell Calcium 18, 245-251.

Nardi, J. B. (1990). Expression of a surface epitope on cells that link branches in the tracheal network of *Manduca sexta*. Development 110, 681-688.

Nardi, J. B. (1992). Dynamic expression of a cell surface protein during rearrangement of epithelial cells in the *Manduca* wing monolayer. Dev. Biol. 152, 161-171.

Neely, M.D. and Gesemann, M. (1994). Disruption of microfilaments in growth cones following depolarization and calcium Influx. J. Neurosci. 14, 7511-7520.

Nehlig, A., Daval, J.-L., and Debry, G. (1992). Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. Brain Res. Rev. 17, 139-170.

Neugebauer, K.M. and Reichardt, L.F. (1991). Cell-surface regulation of β_1-integrin activity on developing retinal neurons. Nature 350, 68-71.

Newell, P. C. (1990). Signal transduction for chemotaxis in *Dictyostelium* amoebae. Sem. Cell Biol. 1, 105-113.

Newell, P.C., Malchow, D., and Gross, J.D. (1995). The role of calcium in aggregation and development of *Dictyostelium*. Experientia 51, 1155-1165.

Newgreen, D.F. and Gooday, D. (1985). Control of the onset of migration of neural crest cells in avian embryos. Cell Tissue Res. 239, 329-336.

Nicholas, E.-F.G. and Newell, P.C. (1985). Inositol 1.4.5-trisphosphate induces cyclic GMP formation in *Dictyostelium Discoideum*. Biochem. and Biophys. Res. Comm. 130, 1115-1122.

Niederman, R., Amrein, P.C., and Hartwig, J. (1983). Three-dimensional structure of actin filaments and of an actin gel made with actin-binding protein. J. Cell Biol. 96, 1400-1413.

Nishimoto, I., Okamoto, T., Matsaura, Y., and Takahashi, S. (1993). Alzheimer amyloid protein precursor complexes with brain GTP-binding protein Go. Nature 362, 75-79.

Noegel, A.A. and Luna, J.E. (1995). The *Dictyostelium* cytoskeleton. Experientia 51, 1135-1143.

Northrup, J.K., Sternweis, P.C., and Gilman, A.G. (1983). The subunits of the stimulatory regulatory component of adenylate cyclase. J. Biol. Chem. 258, 11361-68.

Omann, G.M., Traynor, A.E., Harris, A.L., and Sklar, L.A. (1987). LTB4-induced activation signals and responses in neutrophils are short-lived compared to formylpeptide. J. Immun. 138, 2626-2632.

Otte, A.P., McGrew, L.L., Olate, J., Nathanson, N.M., and Moon, R.T. (1992). Expression and potential functions of G-protein α subunits in embryos of *Xenopus* laevis. Development 116, 141-146.

Otter, T., King, S. M., and Witman, G. B. (1987). A two-step procedure for efficient electrotransfer of both high-molecular-weight and low-molecular-weight proteins. Anal. Biochem. 162, 370-377.

Palade, P., Dettbarn, C., Alderson, B., and Volpe, P. (1989). Pharmacologic differentiation between inositol-1,4,5-trisphosphate-induced Ca^{2+} release and Ca^{2+}- or caffeine-induced Ca^{2+} release from intracellular membrane systems. Mol. Pharmac. 36, 673-680.

Pan, H., Wang, H.-Y., Friedman, E., and Gershon, M.D. (1997). Mediation by protein kinases C and A of G_o-linked slow responses of enteric neurons to 5-HT. J. Neurosci. 17, 1011-1024.

Patel, N. H., and Goodman, C. S. (1992). DIG-labeled single-stranded DNA probes for *in situ* hybridization. In "Non-radioactive labelling and detection of biomolecules" (C. Kessler, Ed.), pp. 377-381. Springer, Heidelberg.

Penner, R., Matthews, G., and Neher, E. (1988). Regulation of calcium influx by second messengers in rat mast cells. Nature 334, 499-504.

Pituello, F., Homburger, V., Saint-Jeannet, J.-P., Audigier, Y., Bockaert, J., and Duprat, A.-M. (1991). Expression of the guanine nucleotide-binding protein G_o correlates with the state of neural competence in the amphibian embryo. Dev. Biol. 145, 311-322.

Polak, K.A., Edelman, A.M., Wasley, J.W.F., and Cohan, C.S. (1991). A novel calmodulin antagonist, CGS 9343B, modulates calcium-dependent changes in neurite outgrowth and growth cone novements. J. Neurosci. 11, 534-542.

Provost, N. M., Somers, D. E., and Hurley, J. B. (1988). A *Drosophila melanogaster* G protein α subunit gene is expressed primarily in embryos and pupae. J. Biol. Chem. 263, 12070-12076.

Ptasznik, A., Traynor-Kaplan, A., and Bokoch, G.M. (1995). G Protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase-Shc adapter protein signaling complexes. J. Biol. Chem. 270, 19969-19973.

Putney, J.W.J. and Bird, G.S.J. (1993). The signal for capacitative calcium entry. Cell 75, 199-201.

Quan, F., Wolfgang, W.J., and Forte, M.A. (1989). The *Drosophila* gene coding for the α subunit of a stimulatory G protein is preferentially expressed in the nervous system. Proc. Natl. Acad. Sci. (USA) 86, 4321-4325.

Quan, F., Wolfgang, W. J., and Forte, M. (1993). A *Drosophila* Gα subunit, Gf_{α}, expressed in a spatially and temporally restricted pattern during development. Proc. Nat. Acad. Sci. (USA) 90, 4236-4240.

Rakic, P. (1971). Neuron-glia relationship during ranule cell migration in the developing cerebellar cortex: A golgi and electron microscopic study in *Macacus rhesus*. J. Comp. Neurol. 141, 283-312.

Rakic, P., Cameron, R.S., and Komuro, H. (1994). Recognition, adhesion, transmembrane signaling and cell motility in guided neuronal migration. Curr. Opin. Neuro. 4, 63-69.

Rakic, P. and Komuro, H. (1994). The role of receptor/channel activity in neuronal cell migration. J. Neurobiol. 26, 299-315.

Ray, Y. and Ganguly, R. (1992). The *Drosophila* G protein γ subunit gene (D-$G_{\gamma 1}$) produces 3 developmentally regulated transcripts and is predominantly expressed in the central nervous system. J. Biol. Chem. 267, 6086-6092.

Reber, B., F. X., Neuhaus, R., and Reuter, H. (1992). Activation of different pathways for calcium elevation by bradykinin and ATP in rat pheochromocytoma (PC-12) cells. Pflug. Arch. 420, 213-218.

Reed, P.W. and Lardy, H.A. (1972). A23187: A divalent cation ionophore. J. Biol. Chem. 247, 6970-6977.

Rehder, V., Jenson, J.R., Dou, R., and Kater, S.B. (1991). A comparison of calcium homeostasis in isolated and attached growth cones of the snail *Helisoma*. J. Neurobiol. 225, 499-511.

Rehder, V. and Kater, S. B. (1992). Regulation of neuronal growth cone filopodia by intracellular calcium. J. Neurosci. 12, 3175-3186.

Rens-Domiano, S. and Hamm, H.E. (1995). Structural and functional relationships of heterotrimeric G-proteins. FASEB 9, 1059-1066.

Rius, R. A., Streaty, R. A., Y., P. L., and Klee, W. A. (1991). Developmental expression of G proteins that differentially modulate adenylyl cyclase activity in mouse brain. FEBS Letts. 288, 51-54.

Rivas, R.J. and Hatten, M.E. (1995). Motility and cytoskeletal organization of migrating cerebellar granule neurons. J. Neurosci. 15, 981-989.

Robinson, J. D., Davis, R. L., and Steinberg, M. (1986). Fluoride and Beryllium interact with the Na, K-dependent ATPase as analogs of phosphate. J. Bioenerg. Biomemb. 18, 521-531.

Ruoslahti, E. and Obrink, B. (1996). Common principles in cell adhesion. Exp. Cell Res. 227, 1-11.

Saito, M., Sato, R., Hisatome, I., and Narahashi, T. (1996). RANTES and platelet-activating factor open Ca^{2+}-activated K^+ channels in eosinophils. FASEB J. 10, 792-798.

Salser, S. J., and Kenyon, C. (1992). Activation of a *C. elegans* antennapedia homologue in migrating cells controls their direction of migration. Nature 355, 255-258.

Sandou, F. and R., H. (1994). 5-Hydroxytryptamine receptor subtypes: Molecular and functional diversity. Adv. Pharmac. 30, 327-80.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. Sci. (USA) 74, 5463-5467.

Saxe, C.L.I., Johnson, R.L., Devreotes, P.N., and Kimmel, A.R. (1991). Expression of a cAMP receptor gene of *Dictyostelium* and evidence for a multigene family. Genes and Development 5, 1-8.

Schaller, M.D., Otey, C.A., Hildebrand, J.D., and Parsons, J.T. (1995). Focal adhesion kinase and paxillin bind to peptides mimicking β integrin cytoplasmic domains. J. Cell Biol. 130, 1181-1187.

Schlosshauer, N., Blum, A.S., Mendez-Otero, R., Barnstable, C.J., and Constantine-Paton, M. (1988). Developmental regulation of ganglioside antigens recognized by the JONES antibody. J. Neurosci. 8, 580-592.

Schmidt, C.J., Zubiaur, M., D., V., Neer, E.J., and Drager, U.C. (1994). G_o, a guanine nucleotide binding protein, is expressed during neurite extension in the embryonic mouse. J. Neurosci. Res. 38, 182-187.

Schuch, U., Lohse, M.J., and Schachner, M. (1989). Neural cell adhesion molecules influence second messenger systems. Neuron 3, 13-20.

Seecof, R. L., Alleaume, N., Teplitz, R. L., and Gershon, I. (1971). Differentiation of neurons and myocytes in cell cultures made from *Drosophila* gastrulae. Exp. Cell Res. 69, 161-173.

Segalat, L., Elkes, D. A., and Kaplan, J. M. (1995) Modulation of serotonin-controlled behaviors by Go in *Caenorhabditis elegans*. Science 267, 1648-1651.

Shinohara, H., Asano, T., and Kato, K. (1992). Differential localization of G proteins Gi and Go in the accessory olfactory bulb of the rat. J. Neurosci. 12, 1275-1279.

Silver, R. A., Lamb, A. G., and Bolsover, S. R. (1989). Elevated cytosolic calcium in the growth cone inhibits neurite elongation in neuroblastoma cells: correlation of behavioral states with cytosolic calcium concentrations. J. Neurosci. 9, 4007-4020.

Simon, M. I., Strathmann, M. P., and Gautam, N. (1991). Diversity of G proteins in signal transduction. Science 252, 802-808.

Singer, S. J. and Kupfer, A. (1986). The directed migration of eukaryotic cells. Ann. Rev. Cell Biol. 2, 337-365.

Smith, C.D., Lane, B.C., Kusaka, I., Verghese, M.W., and Snyderman, R. (1985). Chemoattractant receptor induced hydrolysis of phosphatidylinositol 4,5-bisphosphate in human polymorphonuclear leukocyte membranes. J. Biol. Chem. 260, 5875-5878.

Smith, S. J. (1988). Neuronal cytomechanics: the actin-based motility of growth cones. Science 242, 708-715.

Smrcka, A.V., Hepler, J.R., Brown, D.O., and Sternweis, P.C. (1991). Regulation of phosphoinositide-specific phospholipase C activity by purified G_q. Science 25, 804-807.

Smyth, S.S., Joneckis, C.C., and Parise, L.V. (1993). Regulation of vascular integrins. Blood 81, 2827-2843.

Snow, D.M., Atkinson, P.B., Hassinger, T.D., Letourneau, P.C., and Kater, S.B. (1994). Chondroitin sulfate proteoglycan elevates cytoplasmic calcium in DRG neurons. Dev. Biol. 166, 87-100.

Sonobe, S., Takahashi, S., Hatano, S., and Kuroda, K. (1986). Phosphorylation of *Amoeba* G-actin and its effect on actin polymerization. J. Biol. Chem. 261, 14837-14843.

Sprangrude, G.J., Sacchi, F., Hill, H.R., Van Epps, D.E., and Daynes, R.A. (1985). Inhibition of lymphocyte and neutrophil chemotaxix by pertussis toxin. J. Immun.135, 4135-4143.

Steinberg, D., Khoo, J.C., Glass, C.K., Palinski, W., and Almazan, F. (1997). A new approach to determining the rates of recruitment of circulating leukocytes into tissues: Application to the meaurement of leukocyte recruitment into atherosclerotic lesions. Proc. Natl. Acad. Sci.(USA) 94, 4040-4044.

Sternweiss, P. C. and Gilman, A. G. (1982). Aluminum: a requirement for activation of the regulatory component of adenylate cyclase by fluoride. Proc. Nat. Acad. Sci. (USA) 79, 4888-4891.

Stossel, T. P. (1993). On the crawling of animal cells. Science 260, 1086-1094.

Strittmatter, S.M., Fishman, M.C., and Zhu, X.-P. (1994). Activated mutants of the α subunit of G_o promote an increased number of neurites per cell. J. Neurosci. 14, 2327-2338.

Strittmatter, S. M., Igarashi, M., and Fishman, M. C. (1994). GAP 43 amino terminal peptides modulate growth cone morphology and neurite outgrowth. J. Neurosci. 14, 5503-5513.

Strittmatter, S.M., Valenzuela, D., Kennedy, T.E., Neer, E.J., and Fishman, M.C. (1990). G_o is a major growth cone protein subject to regulation by GAP-43. Nature 344, 836-841.

Stryer, L. and Bourne, H. R. (1986). G proteins: a family of signal transducers. Ann. Rev. Cell Biol. 2, 391-419.

Sun, H.-Q., Kwiatkowsk, K., Wooten, D.C., and Yin, H.L. (1995). Effects of CapG overexpression on agonist-induced motility and second messenger generation. J. Cell Biol. 129, 147-156.

Sun, T.J. and Devroetes, P.N. (1991). Gene targeting of the aggregation stage cAMP receptor cAR1 in *Dictyostelium*. Genes & Devt. 5, 572-582.

Taghert, P. H., Carr, J. N., Wall, J. B., and Copenhaver, P. F. (1986.) Embryonic formation of a simple neurosecretory nerve in the moth, *Manduca sexta*. In "Insect Neurochemistry and Neurophysiology" Vol. (ed. A.B. Borkovec and D.B. Gelman), pp. 143-172. Humana Press, Clifton, New Jersey.

Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M., and Ishii, S. (1982). Subunit structure of islet-activating protein, pertussis toxin, in conforming with the A-B model. Biochem. 21, 5516-5522.

Tautz, D., and Pfeifle, C. (1989). A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of segmentation gene hunchback. Chromosoma 98, 81-85.

Taylor, S.J., Chae, H.Z., Rhee, S.G., and Exton, T.H. (1991). Activation of the β1 isozyme of phospholipase C by α subunits of the G_q class of G proteins. Nature 350, 516-518.

Teillet, M.-A., Kalcheim, C., and Le Douarin, N. M. (1987). Formation of the dorsal root ganglia in the avian embryo: Segmental origin and migratory behavior of neural crest progenitor cells. Dev. Biol. 120, 329-347.

Thambi, N.C., Quan, F., Wolfgang, W.J., Spiegel, A., and Forte, M. (1989). Immunological and molecular characterization of $G_{o\alpha}$-like proteins in the *Drosophila* central nervous system. J. Biol. Chem. 264, 18552-18560.

Thelen, M., Dewald, B., and Baggiolini, M. (1993). Neutrophil signal transduction and activation of the respiratory burst. Physiol. Rev. 73, 797-821.

Theriot, J. A. and Mitchison, T. J. (1992). Actin microfilament dynamics in locomoting cells. Nature 352, 126-131.

Tsien, R.Y. (1980). New calcium indicators and buffers with high selectivity against magnesium and protons: Design, synthesis, and properties of prototype structures. Biochem. 19, 2404--2410.

van Biesen, T., Hawes, B.E., Raymond, J.R., and Luttrell, L.M. (1996). G_o-protein α-subunits activate mitogen-activated protein kinase via a novel protein kinase C-dependent mechanism. J. Biol. Chem. 271, 1266-1269.

Van Haastert, P.J.M. (1995). Transduction of the chemotactic cAMP signal across the plasma membrane of *Dictyostelium* cells. Experientia 51, 1144-1154.

Varela-Echavarria, A. and Guthrie, S. (1997). Molecules making waves in axon guidance. Genes Dev. 11, 545-557.

von Tscharner, V., Deranleau, D.A., and Baggiolini, M. (1986). Calcium fluxes and calcium buffering in human neutrophils. J. Biol. Chem. 261, 10163-10168.

Walsh, C. and Cepko, C. L. (1988). Clonally related cortical cells show several migration patterns. Science 241, 1342-1345.

Wang, H. Y., Watkins, D. C., and Malbon, C. C. (1992). Antisense oligonucleotides to Gs protein α-subunit sequence accelerates differentiation of fibroblasts to adipocytes. Nature 358, 334-337.

Wang, N., Yan, K., and Rasenick, M. M. (1990). Tubulin binds specifically to the signal transducing protein, Gs_{α} and $Gi_{\alpha 1}$. J. Biol. Chem. 265, 1239-1242.

Wang, Y.-L. (1985). Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling. J. Cell Biol. 101, 597-602.

Wehrle-Haller, B. and Weston, J.A. (1997). Receptor tyrosine kinase-dependent neural crest migration in response to differentially localized growth factors. Bioessays 19, 337-45.

Wehrle-Haller B., and Weston, J. A. (1995) Soluble and cell-bound forms of steel factor activity play distinct roles in melanocyte precursor dispersal and survival on the lateral neural crest migration pathway. Development 121, p. 731-42.

Weller, A., Isenmann, S., and Vestweber, D. (1992). Cloning of the mouse endothelial selectins. J. Biol. Chem. 267, 15176-15183.

West, R.E., Moss, J., Liu, T., and Lie, T.-Y. (1985). Pertussis toxin-catalyzed ADP-ribosylation of transducin. J. Biol. Chem. 260, 14428-14430.

Wetts, R., Serbedzija, G. N., and Fraser, S. E. (1989). Cell lineage analysis reveals multipotent precursors in the ciliary margin of the frog retina. Dev. Biol. 136, 254-263.

Whitington, P.M. (1993). Axon guidance factors in invertebrate development. Pharm. & Therap. 58, 263-299.

Wickman, K.D. and Clapham, D.E. (1995). G-protein regulation of ion channels. Curr. Opin. Neurobiol. 5, 278-285.

Williams, E.J., Doherty, P., Turner, G., Reid, R.A., Hemperly, J.J., and Walsh, F.S. (1992). Calcium influx into neurons can solely account for cell contact-dependent neurite outgrowth stimulated by transfected L1. J. Cell Biol. 119, 883-892.

Wolfgang, W. J., Quan, F., Goldsmith, P., Unson, C., Spiegel, A., and Forte, M. (1990). Immunolocalization of G protein α subunits in the *Drosophila* central nervous system. J. Neurosci. 10, 1014-1024.

Wolfgang, W.J., Quan, F., Thambi, N., and Forte, M. (1991). Restricted spatial and temporal expression of G protein α subunits during *Drosophila* embryogenesis. Development 113, 527-538.

Wu, D., LaRosa, G.J., and Simon, M.I. (1993). G Protein-coupled signal transduction pathways for interleukin-8. Science 261, 101-104.

Wu, L., Valkema, R., Van Haastert, P.J.M., and Devreotes, P.N. (1995). The G protein β subunit Is essential for multiple responses to chemoattractants in *Dictyostelium*. J. Cell Biol. 129, 1667-1675.

Xie, R., Li, L., Goshima, Y., and Strittmatter, S.M. (1995). An activated mutant of the α subunit of Go increases neurite outgrowth via protein kinase C. Dev. Brain Res. 87, 77-86.

Yoon, J., Shortridge, R. D., Bloomquist, B. T., Schneuwly, S., Perdew, M. H., and Pak, W. L. (1989). Molecular characterization of *Drosophila* gene encoding Goα subunit homolog. J. Biol. Chem. 264, 18536-18543.

Zuber, M. X., Goodman, D. W., Karnes, L. R., and Fishman, M. L. (1989). The neuronal growth-associated protein GAP-43 induces filopodia in non-neural cells. Science 244, 1193-1195.