



Amyloid Precursor Protein family as unconventional Go-coupled receptors and the control of neuronal motility

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ABSTRACT

Cleavage of the Amyloid Precursor Protein (APP) generates amyloid peptides that accumulate in Alzheimer Disease (AD), but APP is also upregulated by developing and injured neurons, suggesting that it regulates neuronal motility. APP can also function as a G protein-coupled receptor that signals via the heterotrimeric G protein $G\alpha_o$, but evidence for APP- $G\alpha_o$ signaling *in vivo* has been lacking. Using *Manduca* as a model system, we showed that insect APP (APPL) regulates neuronal migration in a $G\alpha_o$ -dependent manner. Recently, we also demonstrated that *Manduca* Contactin (expressed by glial cells) induces APPL- $G\alpha_o$ retraction responses in migratory neurons, consistent with evidence that mammalian Contactins also interact with APP family members. Preliminary studies using cultured hippocampal neurons suggest that APP- $G\alpha_o$ signaling can similarly regulate growth cone motility. Whether Contactins (or other APP ligands) induce this response within the developing nervous system, and how this pathway is disrupted in AD, remains to be explored.

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Amyloid precursor protein and the control of neuronal migration

The Amyloid Precursor Protein (APP) has been the focus of intense scrutiny for many years, but its normal functions remain controversial. Originally identified as the source of β -amyloid ($A\beta$) peptides that accumulate in Alzheimer Disease (AD),¹ APP is a type-1 transmembrane glycoprotein that may play a variety of roles in both the embryonic and adult nervous system.^{2,3} Like many proteins, APP can be rapidly cleaved by membrane-associated proteases (secretases) that help clear the holoprotein from the cell surface.⁴ During “non-amyloidogenic” processing, sequential cleavage of APP by α - and γ -secretases liberates soluble ectodomain fragments (sAPP α) and cytoplasmic AICDs (APP intracellular domains). By comparison, during “amyloidogenic” processing, cleavage by β - and γ -secretases generates a smaller ectodomain fragment (sAPP β), an identical AICD, and the infamous $A\beta$ peptides that accumulate in the brains of AD patients and that can induce neurotoxic responses in model systems. Frustratingly, drugs targeting $A\beta$ have been largely unsuccessful

in treating human subjects,^{5,6} bolstering the argument that the loss of normal APP-dependent functions might also contribute to neurodegeneration in AD.^{3,7}

Although several cleavage products derived from APP exhibit biologic activities in different assays, increasing evidence suggests that holo-APP can function as a transmembrane receptor, capable of regulating neuronal responses in both the developing and mature brain.^{2,8} Notably, APP family proteins are upregulated in regions of active neuronal motility, where they may function as both cell adhesion molecules and guidance receptors.^{2,7} For example, studies have suggested that APP regulates neuroblast migration and growth cone navigation;^{9,10} synaptic growth and remodeling;^{11,12} and retraction/regrowth responses following injury.^{13,14} Conversely, disrupting these functions might contribute to diseases linked with abnormal APP levels, including Down Syndrome and AD.^{15,16}

However, establishing authentic functions for APP has been complicated by 2 close orthologs in mammalian systems (APP-Like Proteins; APLP1 and 2) that are co-expressed with APP and exhibit overlapping

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biologic activities.¹⁷ Moreover, APP can either restrict^{10,18} or promote^{9,19} motile responses under different conditions, akin to other guidance receptors whose functions can be modulated by alternative ligands and co-receptors. Indeed, APP can potentially bind a wide variety of membrane-associated proteins, extracellular matrix components, and cytoplasmic adaptor proteins,^{7,20} although many of these interactions have yet to be validated *in vivo*.

Particularly noteworthy are studies implicating APP in the control of neuronal migration within the developing mammalian cortex, although different experimental strategies have yielded contradictory results. By genetically deleting all 3 APP family members (APP, APLP1 & ALP2) in mice, Herms and colleagues induced a striking pattern of ectopic migration, whereby cortical neurons traveled beyond the pial membrane to form cortical heterotopias (resembling cobblestone lissencephaly).¹⁰ These results suggest that signaling by APP family proteins normally *restricts* the extent or directionality of migration, potentially in response to guidance cues encountered in the developing brain. In contrast, inhibiting APP expression using RNA interference methods resulted in premature stalling and arrest of neuronal migration,⁹ arguing that APP normally *promotes* (rather than restricts) migratory behavior. A potential advantage of this method is that acutely knocking down APP expression might circumvent potential compensatory responses mediated by other proteins (including APLP1 and 2). However, since APP family members also regulate the mitotic behavior of cortical progenitors,^{21,22} these manipulations might have altered cell fate decisions during neurogenesis rather than directly affecting their subsequent migratory behavior. Other studies have suggested that molecular redundancy with additional guidance receptors might mask the normal functions of APP within the complex environment of the developing cortex.²³ Ultimately, to understand how APP family proteins can elicit different types of neuronal behavior in a context-dependent manner, we first need to define the signal transduction mechanisms by which they regulate motile responses under physiologic conditions.

APP may function as a non-canonical G protein-coupled receptor

Although many intracellular proteins can interact with APP under certain conditions,⁷ considerable evidence

has shown that holo-APP directly binds and regulates the heterotrimeric G protein Go,²⁴⁻²⁶ suggesting that APP functions as an unconventional G protein-coupled receptor (GPCR). Whereas canonical GPCRs assume a heptahelical configuration, several type-1 (single pass) receptors also regulate heterotrimeric G protein signaling.²⁷ Based on structural analyses of Insulin-like Growth Factor II (which activates Gi), Nishimoto and colleagues identified a 20-amino acid motif within the cytoplasmic region of APP as a candidate G protein-interacting domain, and they showed that this “peptide 20” domain bound the α -subunit of Go ($G\alpha o$) but not other G proteins *in vitro*.^{24,28} Notably, $G\alpha o$ is the most abundant heterotrimeric G protein in the brain and is highly enriched in developing neurons.²⁹ Also like APP, $G\alpha o$ -dependent signaling can both promote and inhibit neuronal motility under different circumstances,^{29,30} supporting the concept that APP- $G\alpha o$ interactions might serve important functions in both the developing and adult brain.

Lacking authentic ligands for APP, several groups subsequently showed that crosslinked antibodies against APP could induce Go activation, while hyperactivating this response in cultured neurons provoked calcium (Ca^{2+}) overload, increased reactive oxygen species (ROS) levels, and induced apoptosis.³¹⁻³³ Moreover, mutant forms of APP that cause familial forms of AD were found to hyperactivate $G\alpha o$ and induce neurotoxic responses in cell culture, arguing that disease-associated isoforms of APP function as constitutively active Go-coupled receptors.³⁴ Notably, these neurotoxic effects were *independent* of $A\beta$,³¹ although subsequent work showed that $A\beta$ could also provoke APP-dependent cytotoxic responses that were blocked by pertussis toxin (PTX), a specific inhibitor of Gi/Go proteins.^{35,36} Intriguingly, studies using human brain samples revealed a decline in APP- $G\alpha o$ interactions in patients with elevated levels of amyloid pathology.³⁶ In combination, these experiments support the model that APP normally functions as a Go-coupled receptor that when chronically activated (by $A\beta$ or other age-related factors) provokes neurodegenerative responses.

Unfortunately, a variety of issues have hindered efforts to develop this model more fully. Many early studies relied on overexpression assays in transfected cells that generated contradictory results. For example, some experiments suggested that APP-dependent responses were mediated by $G\beta\gamma$ dimers rather than

$G\alpha o$, or that APP stimulation might inhibit (rather than activate) Go.^{25,37} By comparison, another group recently demonstrated that CTFs and membrane-tethered AICD fragments derived from APP could interact with a different G protein ($G\alpha s$) and promote $G\alpha s$ -dependent outgrowth.^{38,39} Whether APP or its fragments endogenously interact with $G\alpha s$ (as well as $G\alpha o$) in developing neurons remains to be determined.

An alternative perspective has recently been developed by Kögel and colleagues, who have focused on the neuroprotective role of the APP-Go pathway. Based on reports that soluble sAPP α ectodomain fragments of APP (produced by α -secretase cleavage) can directly bind the transmembrane holoprotein,⁴⁰ they showed that acute treatment with sAPP α induced pro-survival responses mediated by the PI3K/Akt pathway, a response that was both APP- and PTX-sensitive (implicating $G\alpha o/i$ proteins).⁴¹ These results integrate previous evidence that both holo-APP and sAPP α fragments can elicit neuroprotective responses, providing support for the concept that APP- $G\alpha o$ signaling serves important physiologic functions in the adult brain.

A role for APP- $G\alpha o$ signaling in neuronal migration: *Manduca* as a model system

Besides its association with AD in humans, APP is actually a member of an evolutionarily ancient class of transmembrane proteins found in most animals. In contrast to vertebrate systems, however, invertebrates typically express only one APP ortholog, facilitating experiments designed to explore their normal functions. In particular, insects express APPL (APP-like), which has been shown to play important roles in both the developing nervous system and in genetic models of AD.⁴² Structurally, APPL contains the same motifs found in human APP₆₉₅ (the most prominent neuronal isoform), including an A β -like domain that induces neurodegenerative responses when overexpressed in the fly brain,⁴³ and a well-conserved Go domain that specifically binds $G\alpha o$ but not other G proteins.^{19,26} Moreover, genetic manipulations in *Drosophila* have shown that human APP₆₉₅ can rescue deficits caused by the loss of APPL, demonstrating that these proteins are both functionally and structurally homologous.⁴⁴ Unlike human APP, however, insect APPL is only expressed by neurons,^{45,46} further simplifying an analysis of its normal functions *in vivo*.

In previous work, we established the enteric nervous system (ENS) of *Manduca sexta* (hawkmoth) as a unique preparation for testing the role of APPL- $G\alpha o$ signaling in neuronal migration.^{26,47} As in vertebrates, the insect ENS is organized into small peripheral ganglia and interconnected nerve plexuses that are formed via sequential waves of neuronal and glial migration (Fig. 1A). In particular, an identified set of ~300 neurons (EP cells; magenta) delaminates from a neurogenic placode in the foregut and then disperse to form an Enteric Plexus that spans the foregut-midgut boundary.⁴⁷ After encircling the foregut (Fig. 1A₁), subsets of EP cells migrate and extend axons along 8 equivalent muscle bands (“b”) on the midgut (Fig. 1A₂). As they migrate, the neurons become rapidly ensheathed by a trailing population of proliferating glial cells (green) that also help regulate their trajectories (as noted below). Throughout this period, the EP cells extend exploratory processes onto the adjacent interband regions (“ib”) but normally remain on their band pathways until after migration and axon outgrowth are complete, whereupon they branch laterally to provide a diffuse innervation to the visceral musculature. Of note is that the superficial location of the EP cells allows them to be directly imaged and manipulated in cultured embryos,^{47,48} while subsequent immunostaining with antibodies against the cell adhesion receptor Fasciclin II (Fas II) labels the neurons and their processes, permitting unambiguous quantification of their migration and outgrowth.²⁶

In published studies, we showed that the EP cells robustly express APPL as they migrate along their band pathways, and that APPL colocalizes with $G\alpha o$ in their leading processes.⁴⁶ Using co-immunoprecipitation protocols, we demonstrated that APPL endogenously interacts with $G\alpha o$ (but not $G\alpha s$ or $G\alpha i$) in lysates from both *Manduca* and *Drosophila*, and we showed that this interaction was downregulated by $G\alpha o$ activation (similar to conventional GPCRs). In parallel, we showed that APP endogenously interacts with $G\alpha o$ (but not $G\alpha s$) in mouse and human brain lysates.²⁶ Using bimolecular fluorescence complementation (BiFC) methods, we then demonstrated that APPL specifically binds $G\alpha o$ (but not $G\alpha s$ or $G\alpha i$) via its Go domain in transfected COS7 cells. More importantly, by co-expressing our BiFC constructs in developing photoreceptors of transgenic *Drosophila*, we demonstrated for the first time that APP family proteins *directly* bind $G\alpha o$ within synaptic-terminals of the CNS.

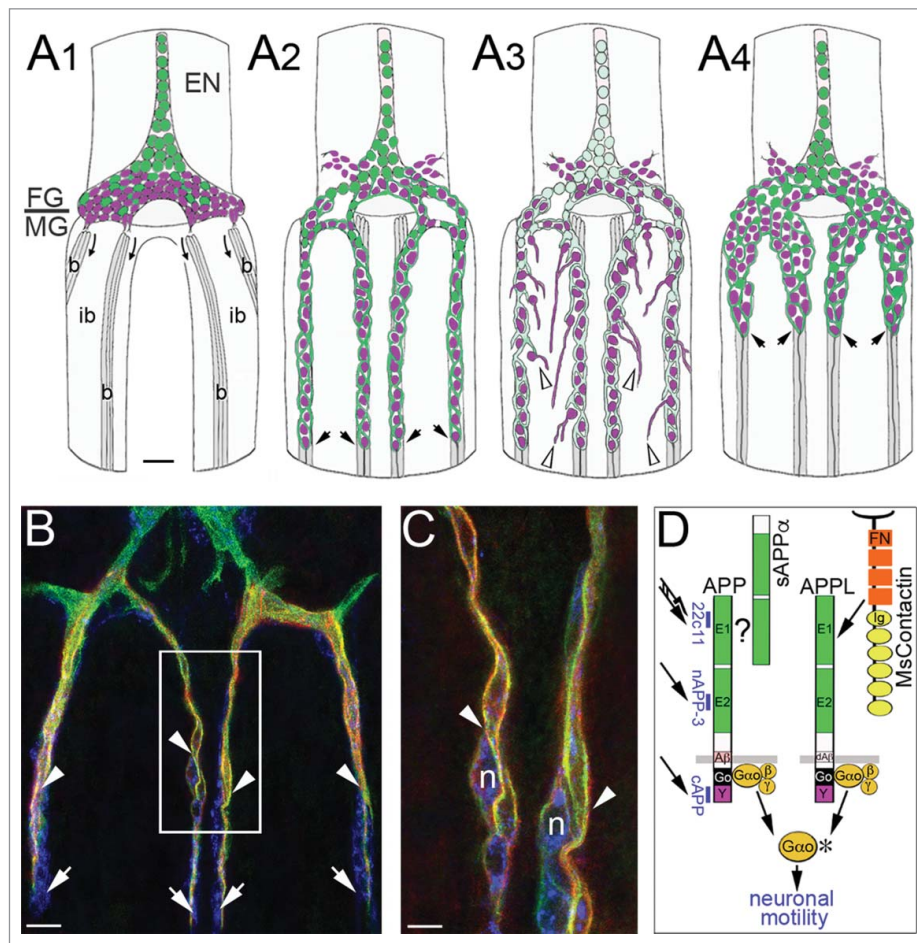


Figure 1. Neuronal migration in *Manduca* is regulated by MsContactin-dependent activation of APPL-G α signaling. (A), Schematic representation of neuronal and glial cell migration during the formation of the Enteric Plexus in *Manduca*. Each panel shows a dorsal view of the embryonic ENS near the foregut-midgut boundary (FG/MG); embryos raised at 25°C complete their development in 100 hr post-fertilization (HPF). (A₁), Embryo at 55 HPF: Enteric Plexus neurons (EP cells; magenta) have delaminated from a neurogenic placode in the foregut epithelium and spread bilaterally to encircle the foregut. Subsets of EP cells align with one of eight longitudinal muscle bands (“b;” curved arrows) on the midgut surface; only the 4 dorsal bands are shown. Homophilic interactions mediated by the Ig-CAM Fas II (Fasciclin II; expressed by both the neurons and the bands) subsequently promote EP cell migration and outgrowth specifically along the bands, during which the neurons typically avoid the adjacent interband regions (“ib”). Concurrently, proliferating glial cells (green) closely follow the migratory neurons, rapidly surrounding their somata and processes. (A₂), By 70 HPF, the EP cells have transitioned from migration to a prolonged period of axon outgrowth, during which they extend processes posteriorly along the bands (beyond the field of view). Glial ensheathment of the neurons is typically complete by this stage (arrows). Only once axon elongation is complete (80 HPF) will the neurons extend terminal synaptic processes onto the interband regions (not shown). (A₃), Manipulations that inhibit APPL expression (by the EP cells) or MsContactin expression (by the glial cells; illustrated by fainter green shading) permit the neurons and their processes to travel inappropriately onto the adjacent interband regions (open arrowheads).⁴⁹ Inhibiting G α activity in the EP cells also induces the same distinctive pattern of ectopic migration and outgrowth.²⁶ By comparison, glial migration is unaffected by reduced MsContactin expression. (A₄), Overstimulating APPL-G α signaling in the EP cells (with MsContactin-Fc fusion proteins) induces a dramatic collapse/stall response in the neurons, resulting in the premature termination of migration (arrows) and reduced axonal outgrowth. (B), Enteric Plexus in a filleted *Manduca* embryo (at 60 HPF) immunostained with anti-APPL (blue), anti-GPI-Fas II (green), and anti-MsContactin (red). Anti-APPL specifically labels the migratory EP cells (arrows), while anti-MsContactin and anti-GPI-Fas II colabel adjacent glial cells (arrowheads). (C), Higher magnification of the boxed region in panel B. Arrowheads indicate areas of colocalization of MsContactin (red) with GPI-Fas II (green) in glial processes ensheathing the migratory neurons (“n”) expressing APPL (blue). Scale bar = 45 μ m in (A); 30 μ m in (B); 10 μ m in (C). (D), Model of how G α -dependent signaling might be regulated by APP family proteins. In the migratory EP cells of *Manduca*, glial MsContactin functions as a ligand for neuronal APPL, inducing the local activation of G α in their leading processes; in turn, activated G α can regulate their motile behavior (potentially via both Ca²⁺-dependent and independent effectors), resulting in local retraction responses that prevent ectopic migration and outgrowth. In cultured hippocampal neurons, activation of APP signaling by antibodies against its extracellular domain, including crosslinked 22C11 (double arrows) or non-crosslinked nAPP-3 (single arrow), also induces collapse/stall responses in a G α -dependent manner, whereas antibodies against the cytodomain (cAPP) do not. Whether physiologic activation of APP-G α signaling regulates the behavior of developing neurons in the mammalian nervous system remains to be explored. Different candidate ligands (including other Contactins and sAPP α ectodomain fragments) might also promote or restrict motile responses in a context-specific manner, depending on the presence of additional co-receptors or G α effectors.

Using our *Manduca* embryo culture assay, we subsequently showed that inhibiting either APPL expression or G α o activity in the EP cells induced a distinctive pattern of ectopic migration and outgrowth, whereby the neurons and their processes traveled into the interband regions (illustrated schematically in Fig. 1A₃). This effect was remarkably similar to the pattern of ectopic growth caused by blocking G protein-dependent Ca²⁺ currents in the migratory neurons.⁴⁸ Together, these results support the model that activation of APPL-G α o signaling in the EP cells mediates their inhibitory response to guidance cues encountered as they migrate within the developing ENS, thereby preventing ectopic growth into inappropriate regions.²⁶ In general terms, our results in *Manduca* are also analogous to the cortical dysplasias caused by deleting all 3 APP family proteins in mice, wherein subsets of developing neurons migrated ectopically beyond their normal locations.¹⁰

In a screen for APPL ligands, we identified *Manduca* Contactin (MsContactin),⁴⁹ a glycosylphosphatidylinositol (GPI)-linked member of the immunoglobulin superfamily of cell adhesion molecules.⁵⁰ Whereas mammals express 6 Contactins, several of which may interact with APP family proteins either in *cis* or *trans*,⁵¹⁻⁵³ insects only express a single Contactin ortholog that is typically restricted to epithelial cells and glial cells.⁵⁴ Using *in situ* hybridization histochemistry and whole-mount immunostaining protocols, we found that MsContactin is expressed by the glial cells that ensheath the migratory EP cells (Fig. 1B–C, red), co-localizing with the glial marker GPI-Fas II (green), whereas APPL is exclusively expressed by the neurons (blue). We also showed that MsContactin fusion proteins bound to the EP cells in an APPL-specific manner. Again using our embryo culture assay, we then demonstrated that knocking down MsContactin expression in the glial cells produced the same distinctive pattern of ectopic migration and outgrowth caused by inhibiting APPL or G α o (Fig. 1A₃). In contrast, hyperstimulating APPL signaling with Contactin fusion proteins (Cont-Fc) induced a dramatic collapse/stall response (Fig. 1A₄) that was both APPL- and G α o-dependent.⁴⁹ Notably, this response was also induced by hyperstimulating G α o in the EP cells.⁴⁸

In combination, these results support the model that APP and its orthologs function as unconventional G α o-coupled receptors in the nervous system. In

addition, our analysis of neuronal migration in *Manduca* offers new evidence that Contactins can serve as authentic ligands for APP family proteins (shown schematically in Fig. 1D). Within the developing ENS, we propose that glial MsContactin stimulates APPL-dependent activation of G α o in the EP cells, which in turn induces local retraction responses that prevent their ectopic outgrowth into inappropriate regions. More generally, our experiments provide the framework for investigating how APP-G α o signaling might regulate neuronal responses to a variety of guidance cues (including mammalian Contactins and sAPP α), and how APP signaling might be modulated to promote or restrict neuronal growth in a context-dependent manner.

Does APP-G α o signaling regulate neuronal motility in developing hippocampal neurons?

Based on our results in *Manduca*, we recently investigated whether APP-G α o might also regulate similar types of motile responses in mammalian neurons. For these studies, we used cultured rat hippocampal neurons prepared from both male and female embryos, following the protocols developed by Kaech and Banker.⁵⁵ Briefly, hippocampi were dissected from E18 pups, digested with Trypsin, and triturated into single-cell suspensions. Neurons were plated on 18 mm glass coverslips coated with poly-L-lysine at a density of 20,000–25,000 cells per coverslip, and then maintained above astroglial feeder layers in Neurobasal Medium supplemented with B27 and glutaMAX (Thermo Fisher). After 24 hr, the neurons were fixed in 4% buffered paraformaldehyde, permeabilized for 10 min with 0.1% Triton-X 100 in phosphate-buffered saline, and pre-blocked with 5% fish skin gelatin for 30 min.

Replicate cultures were then immunolabeled with different combinations of the following antibodies: mouse anti-APP 22C11 (Millipore #MAB348; 1:25), targeting AA 66–81 of human APP₆₉₅; rabbit anti-APP-Y₇₅₆ (Abgent #AP9053a; 1:200), targeting AA 735–763 of human APP₇₇₀; chick anti-APP-3 (Aves Labs # nAPP-3), targeting AA 433–452 of human APP₇₇₀; chick anti-APP-4 (Aves Labs # nAPP-4), targeting AA 501–522 of human APP₇₇₀; chick anti-cAPP (Aves Labs # cAPP); targeting AA 749–770 within the cytoplasmic domain of APP₇₇₀; and affinity-purified anti-G α o (1:50); generated against a

conserved sequence shared by *Manduca* and mammalian Gαo isoforms.^{26,56} Primary antibodies were detected with secondary antibodies conjugated to Alexa Fluor 488, 568, or 647 (Life Technologies/Thermo Fisher; 1:1000). Coverslips containing the neurons were mounted in Elvanol and imaged on a Nikon compound microscope (Optiphot2-UD), using a DXM1200F digital camera and Nikon ACT-1 software; subsequent cropping and adjustments of brightness and contrast were performed with Adobe Photoshop software. Similar images of APP-Gαo colocalization were obtained with each of the foregoing antibody combinations.

To examine whether APP and Gαo colocalize within axonal growth cones, we focused on neurons

that had initiated axon elongation, accompanied by active growth cone exploration. As shown in Fig. 2A₁, we found that Gαo (magenta) was readily detectable throughout the axons and dendrites of cultured hippocampal neurons and was noticeably more concentrated in the leading growth cones, where it colocalized with APP (green). This colocalization was particularly apparent at the lamellipodial margins and filopodial protrusions associated with active motility (Fig. 2A₂, arrowheads). Similar results were obtained using antibodies against both the N- and C-terminal domains of APP, indicating that Gαo colocalizes with the transmembrane holoprotein in growth cones. This result is also supported by our co-immunoprecipitation studies showing that Gαo interacts with full-

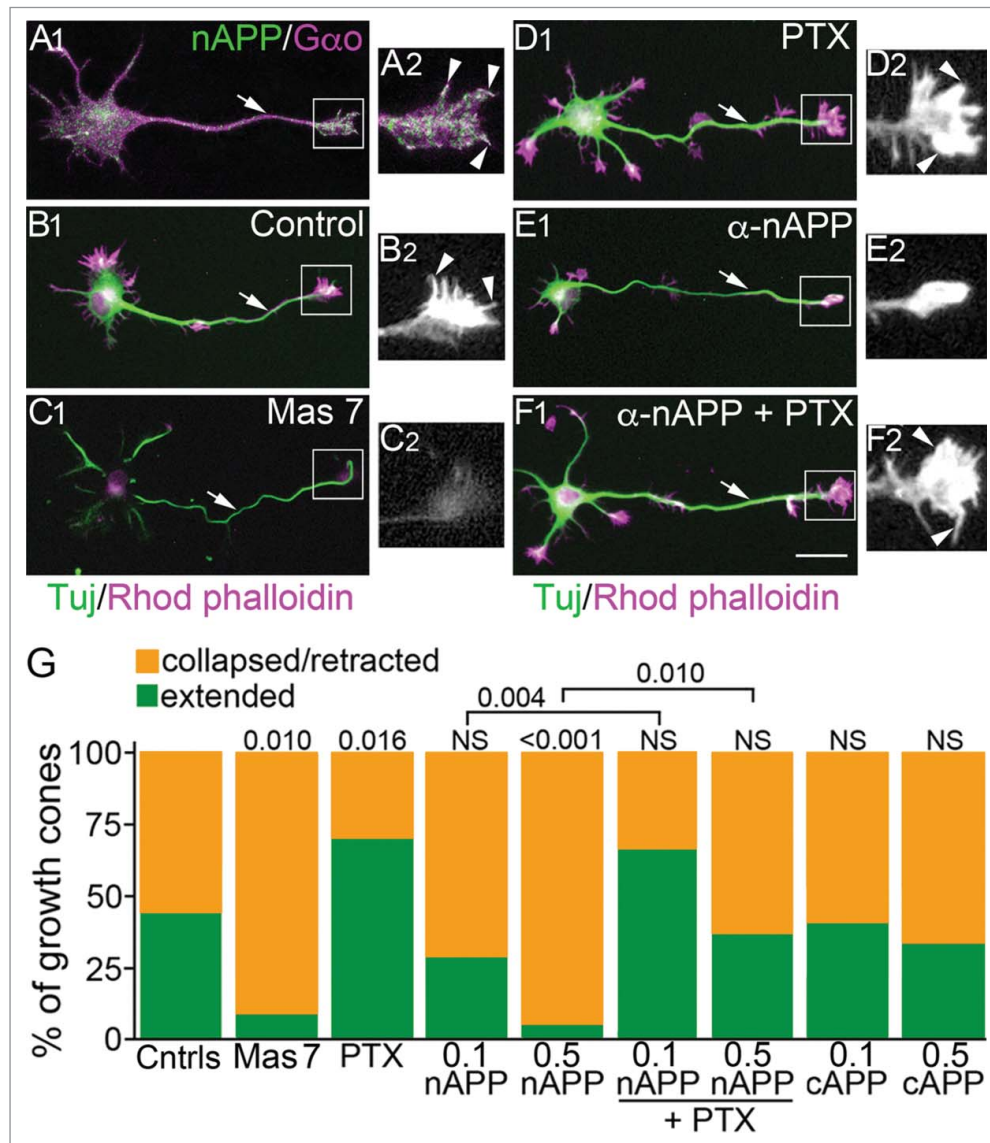


Figure 2. (For figure legend, see page 7.)

length APP in both mouse and human brain lysates, whereas we were unable to detect interactions between $G\alpha_o$ and the smaller C-terminal cleavage fragments derived by secretase processing of the holoprotein (including CTFs and AICDs).²⁶

To explore whether APP- $G\alpha_o$ signaling regulates the motile behavior of developing hippocampal neurons, we treated replicate cultures of neurons (grown for 24 hr *in vitro*) with reagents targeting APP or $G\alpha_o$, then fixed and immunolabeled the neurons with anti- β -III tubulin (Tuj-1), followed by counterstaining with Phalloidin-Tetramethylrhodamine B isothiocyanate (Rhod-Phalloidin) to label polymerized actin filaments. Figure 2B shows an example of a neuron treated with control medium: anti- β -III tubulin antibodies robustly labeled microtubules throughout the axon (arrow) and primary dendrites, while the growth cone was enriched in polymerized actin (as previously reported).⁵⁷ Figure 2B₂ shows an enlarged view of the growth cone labeled with Rhod-Phalloidin (boxed region in Fig. 2B₁; shown in gray scale); arrowheads indicate strongly labeled filopodia.

Cultured hippocampal neurons exhibit a dynamic pattern of motility as they extend processes, during which their growth cones undergo cyclic patterns of expansion and retraction.^{57,58} To assess how manipulating the APP- $G\alpha_o$ pathway affected their overall behavior, we quantified the morphologies of axonal growth cones in our different treatment groups, focusing on neurons that did not contact other somata or processes. Growth cones were classified as “extended” if they widened at their tips and had lamellipodial veils between filopodial protrusions; “collapsed” if they had truncated filopodial protrusions and minimal lamellipodial veils between filopodia; and “retracted” if they lacked an actin-rich growth cone and exhibited thinning of the axonal process, associated with retraction debris.

For this analysis, we combined the collapsed and retracted phenotypes into a single category to quantify average growth cone responses. Approximately 50 neurons per condition were selected at random by an investigator blinded to the treatment conditions. The frequency of growth cones with extended or collapsed/retracted morphologies within each experiment

Figure 2. (see previous page) Growth cone motility in cultured rat hippocampal neurons may also be regulated by APP- $G\alpha_o$ signaling. (A-F), Embryonic rat hippocampal neurons after 24 hr that had established normal polarity (Stage 3), with a single elongating axon (arrows) and a variable number of smaller dendrites extending from their somata. (A₁), Neuron that was immunolabeled with anti-nAPP (22c11; green) and anti- $G\alpha_o$ (magenta). (A₂), Enlarged view of boxed region in (A₁), showing that APP and $G\alpha_o$ colocalize within the axonal growth cone, particularly at the leading lamellipodial margin and in some of the larger filopodial extensions (arrowheads; colocalized immunolabeling appears white). (B-F), Representative examples of neurons that were treated with reagents targeting APP or $G\alpha_o$, then immunolabeled with anti- β -III tubulin (Tuj; green) and counterstained with Phalloidin-Tetramethylrhodamine B (Rhod-Phalloidin; green) to label polymerized actin. Enlarged regions (indicated by white boxes in each panel) show only Rhod-Phalloidin staining in gray scale to highlight growth cone morphologies. (B₁), Neuron treated with control medium, exhibiting anti-Tuj1 immunolabeling along the length of its axon and dendrite, and strong Rhod-Phalloidin staining in its growth cones (associated with active motility). (B₂), The axonal growth cone in this neuron exhibited a typical “extended” morphology. (C₁), Neuron treated with the $G\alpha_o/G\alpha_i$ activator Mastoparan 7 (Mas 7, 50 μ M; Enzo Life Sciences) exhibited a dramatic reduction in Rhod-Phalloidin staining and a more moderate reduction in anti-Tuj1 immunoreactivity. (C₂), The axonal growth cone in this neuron exhibited a typical “collapsed” morphology. (D₁), Neuron treated with the $G\alpha_o/G\alpha_i$ inhibitor PTX (100 ng/ml; List Biological Labs) exhibited an expansion of its axonal and dendritic growth cones. (D₂), The axonal growth cone in this neuron exhibited an “extended” morphology. (E₁), Neuron treated with non-crosslinked anti-nAPP antibodies (nAPP-3; 0.1–0.5 μ M; Aves Laboratories) exhibited a collapsed/retracted morphology (highlighted in panel E₂). (F₁), Neuron that was pre-incubated with PTX before treatment with anti-nAPP antibodies showed morphological features similar to control neurons. (F₂), The axonal growth cone in this neuron exhibited an “extended” morphology, similar to the control neuron shown in panel (B). Scale bar = 30 μ m in low-magnification images and 3 μ m in highlighted boxed regions. (G), Quantification of the proportion of neurons in each treatment group that possessed axonal growth cones with either extended (green bars) or collapsed/retracted morphologies (orange bars). P-values above each histogram are derived from pairwise comparisons with controls after applying the Bonferroni correction; NS = not significantly different. Compared to controls, treatment with Mas 7 induced a significant increase in neurons exhibiting collapsed/retracted growth cones, whereas PTX treatment had the opposite effect, increasing the number of growth cones with extended morphologies. Treatment with anti-nAPP-3 induced a concentration-dependent increase in the proportion of growth cones with collapsed/retracted morphologies, with a maximal response similar to the effects of Mas 7. Pre-incubation with PTX prevented the effects of anti-nAPP treatment. The percentage of extended growth cones in neurons treated with PTX plus anti-nAPP (0.1 μ M) was significantly greater than in cultures treated with 0.1 μ M anti-nAPP alone ($p = 0.004$), but was not significantly different from controls after applying the Bonferroni correction. Likewise the percentage of extended growth cones in neurons treated with PTX plus anti-nAPP (0.5 μ M) was significantly greater than in cultures treated with 0.5 μ M anti-nAPP alone ($p = 0.010$), and was not significantly different from controls. By comparison, growth cone morphologies were unaffected by treatment with antibodies targeting the cytoplasmic domain of APP (cAPP; see Fig. 1D).

were compared across all treatment groups, using row-by-column contingency tables. If significant differences between groups were identified, pairwise chi-square tests were subsequently performed to compare each treatment group with control neurons. For experiments using PTX pretreatment, pairwise chi-square tests were conducted to compare neurons treated with each concentration of anti-nAPP with the matched sets of neurons that were pretreated with PTX. The Bonferroni correction was applied to reported p-values (to account for 10 pairwise comparisons), and p-values were considered significant at the $\alpha = 0.05$ level.

Consistent with previous studies,^{59,60} we found ~40–50% of the growth cones in control cultures exhibited an extended profile (Fig. 2B), while the remainder exhibited collapsed/retracted morphologies (quantified in Fig. 2G). In contrast, when we treated neurons with the mastoparan analog Mastoparan 7 (Mas 7), which selectively activates members of the $G\alpha_o/G\alpha_i$ subfamily, we found that most neurons exhibited growth cone collapse and retraction (Fig. 2C), whereby only 11% retained an extended morphology (Fig. 2G). By comparison, neurons were unaffected by treatment with the inactive analog of Mastoparan 17, exhibiting ~45% extended growth cone morphologies (not shown). Conversely, treating neurons with PTX had the opposite effect, resulting in growth cone expansion (Fig. 2D) and a significant reduction in the proportion of growth cones exhibiting collapsed/retracted morphologies (Fig. 2G). Whereas PTX is specific for $G\alpha_o$ in insect systems,⁶¹ it inhibits both $G\alpha_o$ and $G\alpha_i$ in mammalian neurons;⁶² hence, this finding will need to be validated with more selective reagents that target only $G\alpha_o$. Nevertheless, these results support past experiments (including our studies in *Manduca*) showing that local $G\alpha_o$ activation in developing neurons can induce filopodial retraction, inhibit growth cone exploration, and terminate migratory behavior.^{29,30,56}

We then tested whether activating APP signaling also affected the motile behavior of developing hippocampal neurons. Initially, we treated neurons with crosslinked antibodies targeting the extracellular domain of APP (22C11) to mimic ligand activation (shown schematically in Fig. 1D), based on past reports that this method induced APP- $G\alpha_o$ responses in transfected cells.^{32,33,63} Although we consistently observed an increase in growth cone collapse/stall

responses in these cultures, a caveat is that forced multimerization with crosslinked antibodies might induce pathological changes in APP-dependent responses, including caspase-associated regulation of secretase trafficking.⁶⁴ As an alternative, we used a new generation of anti-peptide polyclonal antibodies targeting different domains in APP (Aves Laboratories), which recognize both membrane-associated and intracellular pools of APP in mammalian neurons (unpublished observations). As illustrated in Fig. 2E, we found that treating neurons with anti-nAPP-3 (targeting the E2 domain of APP) increased growth cone collapse/retraction responses, compared to controls (including neurons treated with nonspecific IgY). This response did not require antibody crosslinking and was concentration-dependent, recapitulating the inhibitory effects of Mas 7 (Fig. 2G). In contrast, treatment with antibodies against the cytoplasmic domain of APP (anti-cAPP) had no obvious effect on growth cone behavior.

Lastly, to explore whether these effects were $G\alpha_o$ -dependent, we pre-incubated cultured neurons with PTX before stimulation with anti-APP antibodies. As illustrated in Fig. 2F, we found that PTX prevented the collapse/stall responses caused by subsequent treatment with anti-nAPP antibodies, resulting in a significant increase in the proportion of growth cones that maintained extended morphologies (Fig. 2G). Given considerable evidence that APP family proteins directly interact with $G\alpha_o$ but not $G\alpha_i$,^{24,26,28} and that the effects of anti-APP antibodies require APP expression,^{24,63} these results suggest that APP induces growth cone collapse and retraction in cultured hippocampal neurons via $G\alpha_o$ -dependent pathways.

Based on these experiments, we postulate that the APPL- $G\alpha_o$ pathway identified in *Manduca* may be evolutionarily conserved (Fig. 1D). Just as MsContactin appears to function as an authentic ligand for APPL in the developing ENS, mammalian Contactins that are known to interact with APP family proteins might also induce $G\alpha_o$ -dependent responses in the developing nervous system. This hypothesis is also supported by reports that Contactin and APP family members are often expressed in similar brain regions and are associated with similar developmental functions, including axon guidance, synaptogenesis and remodeling, and neuronal migration.^{50,52,65} Moreover, since both protein families may interact in *cis* as well as *trans*, activation of APP- $G\alpha_o$ signaling by

Contactins might potentially modulate $G\alpha$ -dependent responses both positively and negatively, depending on their expression patterns. Alternatively, given strong evidence that sAPP α ectodomains can induce $G\alpha$ -dependent responses to modulate stress responses in the brain,^{8,41} it will be interesting to explore whether sAPP α -dependent activation of $G\alpha$ can also regulate motile responses in developing neurons (Fig. 1D).

Lastly, although our studies in *Manduca* focused on the role of APPL- $G\alpha$ signaling during prenatal aspects of neuronal motility (including Ca^{2+} -dependent migratory and outgrowth responses), many other studies have shown that APP family proteins are also upregulated during synaptic remodeling in the brain, as well as by neurons undergoing retraction/regrowth responses following injury.^{14,66} Hence, modulation of the APP- $G\alpha$ pathway by either physiologic or pathological factors might continue to regulate the motile responses of neurons throughout life.^{7,67} Moreover, given suggestive evidence that the misregulation of normal APP signaling can induce neurodegenerative responses (as noted above), we speculate that chronic activation of $G\alpha$ -dependent targets (including Ca^{2+} channels and stress/survival kinases) might also perturb synaptic remodeling and provoke neuronal dystrophy, thereby contributing to brain atrophy. In this context, determining the downstream effectors of the APP- $G\alpha$ pathway in our developmental assays might reveal new therapeutic targets for diseases in which APP is misregulated, including Down Syndrome and AD.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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