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THE ROLE OF G PROTEINS IN THE POLARITY OF DROSOPHILA NEURONS

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ABSTRACT

It is well-established that axons and dendrites are specified to have different functions in the neuron, but the origin of this specification is still largely unknown. Microtubules play a vital role in the transportation of cellular components in these structures, and behave differentially between axons and dendrites. Microtubules create polarized tracks in the cell, and a more-developed understanding of the mechanism controlling microtubule polarity may give insight into various neurodegenerative diseases. It has been shown that certain cellular components are involved in the maintenance of microtubule polarity, such a component is adenomatous polyposis coli 2 (APC2). From the foundational knowledge that already exists on microtubule polarity, an experimental course was designed to examine the role of guanine nucleotide binding proteins (G proteins) in the mechanism of polarity control. Through RNAi knockdown and fluorescent microscopy experiments a G protein subunit, Gsα, was found to be a vital component in maintaining microtubule polarity in the dendrite. From this initial discovery, a broader picture of the mechanism was elucidated through further experimentation. RNAi knockdowns of a G protein coupled receptor (GPCR), Fz, and an adenylate cyclase, Rut, were found to also disrupt microtubule polarity in the dendrite. These findings suggest that Fz signals through Gsα and that Gsα signals through Rut. This signaling module used in learning and memory also seems to play a role in controlling microtubule organization.
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Introduction

The Neuron: A Fundamental Review

The nervous system is a fascinating component of invertebrates and vertebrates that performs the vital task of controlling voluntary and involuntary actions. It plays a key role in sensation and motility by sending signals from one cell of the nervous system to enact changes on the next. The highly specialized cells that are the foundation of the nervous system are called neurons.

Structurally, neurons are composed of three main compartments: axons, cell bodies, and dendrites. The functions of each of these structures are also highly specialized. Axons send signals over long distances within the organism. This function is possible because the axons are very long projections that reach distant signaling targets. Near the cell body, the axon appears simple as a single branch, but at larger distances the axon branches with more complexity in order to signal more cells. In contrast to the axon, the function of the dendrites is to receive and process information. The dendrite is a much shorter structure than the axon, and it is more highly branched proximally to the cell body in order to increase the area of which it can receive signals. In order to pass on signals or to enact cellular changes within the neuron, the signal must propagate through the cell body, also known as the soma. The main role of the cell body is protein synthesis (Lodish, Berk, & Zipursky, 2000). The neuronal structure described fits a widely-used study model containing one axon and a number of dendrites. This model is convenient for study; however, it is noted that the structure of neurons can vary but function remains fairly constant.
**Drosophila as a Model System**

*Drosophila melanogaster* development is composed of several different morphological stages. Over the course of about ten days, the organism develops from an embryo into an adult fly (Figure 1).

After fertilization, the adult female lays an egg, which hatches into a first instar larva in about one day. The larva then undergoes two additional molt giving rise to second and third instar larvae in about two days. The third instar larva is a great neurological model because it has a developed nervous system that is easily accessible to experimenters. The third instar larvae of *Drosophila* are so accessible because they are transparent, which enables microscopy experiments to be performed on them. The third instar larva stage is followed by pupation and metamorphosis into adult organisms (Alberts, 2008).

![Life Cycle of Drosophila melanogaster](image.png)

**Figure 1. Life Cycle of Drosophila melanogaster**
(Alberts, 2008)

A convenient model that is used to study neuronal function and character is a group of cells in the peripheral nervous system known as dendritic arborization sensory neurons. These
cells are divided into four main categories, Class I – Class IV, of increasing complexity based on the intricacy and size of their dendrites (Figure 2) (Jan & Jan, 2010).

![Figure 2. The Four Classes of Dendritic Arborization Neurons](Grueber, Jan, & Jan, 2002)

Class I neurons of particular interest for research are dorsal dendritic arborization neurons E, commonly referred to as ddaE (Figure 3). Each cell has a simple dendritic arbors composed of a long primary dendrite with branching secondary dendrites. These neurons are located beneath the epidermis in the lateral area of segments in D. melanogaster larvae with axons extending to the ventral nerve cord (Jan & Jan, 2010).

![Figure 3. Dorsal Dendritic Arborization Neuron E](Grueber, Jan, & Jan, 2002)
Microtubules: The Underlying Determinate of Polarity

From the fundamental review of neurons, it can be inferred that these specialized cells must be highly organized in order to maintain the signaling and receiving functions of the axons and dendrites, respectively. The support for this inference derives from microtubules, which make up a transportation network through both axons and dendrites (Baas, 2002). Microtubules are manufactured in a manner that makes them inherently directional, or polar. This inherent polarity is established during the assembly of α and β tubulin monomers that come together in a head-to-tail orientation to form linear protofilaments. The protofilaments combine to ultimately form a hollow tube composed of 13 protofilaments, a microtubule.

Microtubules consist of a “plus end”, where assembly and disassembly are favored to occur, and “minus end” that is more static. The process of assembly and disassembly at the plus end is known as dynamic instability (Figure 4). Dynamic instability is composed of the two main conversions: growth to shrinkage (catastrophe) and shrinkage to growth (rescue). Dynamic instability enables the cell to adapt to the changing environment, to generate pushing and pulling forces, and to find specific targets throughout the volume of the cell (Alberts, 2008).

Figure 4. Schematic of Microtubule Structure and Dynamics
(a) Diagram of microtubule composition highlighting the structural characteristics of directionality. (b) Cyclic representation of dynamic instability. (Conde & Cáceres, 2009)
In proliferating cells, nucleation of microtubules occurs at the centrosome, and the structures extend from this central location into the remainder of the cellular space. The minus end of the microtubule, which begins with α tubulin, is located at the point of nucleation; whereas, the plus end with exposed β tubulin is jutted out into cellular space (Bartolini & Gundersen, 2006). Although this is the predominant manner in which microtubules organize, this type of microtubule organization is not possible in neurons because of the vast distances that would need to be traversed by single microtubules originating from the centrosome. Rather, microtubules are nucleated forming overlapping arrangements throughout axons and dendrites (Figure 5) (Baas & Lin, 2010).

Figure 5. Overlapping Arrays of Microtubules
This diagram displays the uncharacteristic organization of microtubules in the extremities of the neuron. (Stone et al., 2008)

**Polarity: Axons and Dendrites**

The functions of axons and dendrites have been established, but some scientists have delved deeper into the underlying structural fundamentals. Studies on rat hippocampal neurons, rat sympathetic neurons, and chick dorsal root ganglion neurons observed uniform plus-end-distal microtubule orientation of almost all microtubules in vertebrate axons. Additional studies on hippocampal neurons and frog mitral dendrites suggested a mixed (50:50) orientation of microtubule orientation in vertebrate dendrites (Black & Baas, 1989). When this distinction was observed, some scientists believed that axon and dendrite function was secondary to
establishment of this network of microtubules. More recently scientists have described a transportation-based model, which focuses on the transport of organelles by motor proteins to either the plus or minus end of microtubules. In this model, one motor protein could in theory travel in both directions in dendrites, but not in axons. This characteristic may be responsible for the presence of cellular components, such as golgi or ribosomes that are only carried by certain motor proteins, to be present in dendrites but not in axons, which could dictate function (Baas & Lin, 2010). Studies in invertebrates paint a slightly different picture. A study using D. melanogaster as the model system showed that microtubule polarity in the axon is conserved. Nearly all microtubules aligned with their plus ends distal to the soma in the axon, but this study also showed that about 90% of microtubules in the proximal dendrites of all classes of neurons were organized with their minus ends distal to the soma (Stone, Roegiers, & Rolls, 2008). The differences between the dendrites in the vertebrate and invertebrate studies have been hypothesized to be affected by structural differences, such as shorter and narrower dendrites in invertebrates (Baas & Lin, 2010).

The differences in microtubule polarity between axons and dendrites justify the claim that microtubules can be the underlying cause for the differences in function and morphology. Just what orients these microtubules in this manner is an interesting topic for further investigation, and this investigation has lead to guanine nucleotide binding proteins.
Guanine Nucleotide Binding Proteins

Guanine nucleotide binding proteins (G proteins) are composed of three distinct subunits: α, β, and γ. The name of these proteins derives from the fundamental characteristic that they bind guanine nucleotides. This binding is carried out by the α subunit. Additionally, all three subunits of G proteins remain associated with the plasma membrane (Neer, 1995).

G proteins undergo an activation cycle that relies on the two major conformations of the proteins, as a heterotrimer or as an independent α subunit and βγ dimer (Figure 6). When all three subunits of the protein are associated, the protein is inactive and bound to guanosine diphosphate (GDP). When G proteins are stimulated by sensory, hormonal, or neurotransmitter signals, they are able to regulate a variety of cellular processes. When the three subunits of a G protein are associated in a heterotrimer, the protein is inactive and does not have the ability to perform signal transduction. In this conformation, GDP is bound to the α subunit. When a ligand binds to a G protein coupled receptor (GPCR), the receptor enacts a conformational change that enables it to become a guanine nucleotide exchange factor, which exchanges GDP for guanine triphosphate (GTP). This GTP-bound protein is now able to become active. In order for the protein to enact cellular changes, the α subunit dissociates from the βγ dimer and escapes the heterotrimer. Both the GTP-bound α subunit and βγ dimer are able to activate signaling pathways. When signaling is complete, the α subunit acts as a guanosine triphosphatase and cleaves a phosphate from GTP converting it to GDP. The GDP-bound α subunit returns to an inactive conformation by associating with a βγ dimer (Neer, 1995).
There are many different types of G protein α subunits that carry out diverse functions. One particular α subunit, Gα₅ or Gsα, is of particular interest for this study. The Gα₅ subunit is expressed in nervous system development in Drosophila embryos, and its absence during development is lethal (Deng et al., 2011). The typical method of action for Gα₅ proteins is to activate adenylyl cyclases to produce cAMP, a second messenger in a number of biological processes. (Deng et al., 2011) Additionally, a recent study has found an association between a family of GPCRs, known as Frizzled receptors, and Gα₅. These Frizzled (Fz) proteins serve as receptors for secreted ligand known as Wnts. Wnt signaling is vital for a number of processes throughout development, such as cell specification and morphogenesis. The results of this study
indicate that the Gα₅ protein receives stronger signals through these Frizzled receptors than other G proteins (Nichols, Floyd, Bruinsma, Narzinski, & Baranski, 2013). Additionally, unpublished data from the Rolls Lab at the Pennsylvania State University has shown that certain Gα protein subunits are involved in an adenomatous polyposis coli-dependent microtubule organization pathway.

**Adenomatous Polyposis Coli**

A key tumor suppressor gene found to be linked to colon, liver, and other cancers is known as adenomatous polyposis coli (APC). There are two APC genes conserved across humans and D. melanogaster, APC and APC2. Although the conserved genes between species are not identical, they code for similar proteins. In mammals, APC is expressed in fetal tissue as well as most epithelial tissue in adults; whereas, APC2 is expressed throughout the organism with highest levels in the brain. In Drosophila, APC is highly expressed in the central nervous system, but APC2 is expressed ubiquitously (Aoki & Taketo, 2007).

The APC gene product is a protein with multiple domains that can bind to various intracellular components, such as proteins in the Wnt signaling pathway, cytoskeletal regulators, or cytoskeletal structures such as microtubules (Aoki & Taketo, 2007). Among the binding partners of the APC protein is an end binding (EB) protein, EB1. EB proteins are small proteins that contain specialized domains that allow them to bind to microtubule ends. EB1 has the ability to interact with the growing, plus-end of microtubules as well as with the carboxy-terminal residues of the APC gene product, providing an association between APC and microtubules that has been found to stabilize the microtubules (Galjart, 2010).
APC2 is a C-terminal truncated version of APC that does not have the binding domains that enable APC to interact with both EB1 and microtubules (Figure 7). Although there is a difference in structure between these two proteins, a recent study has found evidence that suggests APC and APC2 may work in conjunction at dendritic branch points to direct microtubule growth in a way that maintains microtubule polarity. In this study, researchers found that almost all growing microtubule plus ends turn toward the cell body at dendritic branch points. They showed that EB1 and APC are required for maintaining this polarity, and that APC2 recruits APC to the dendritic branch points (Mattie et al., 2010).

**Figure 7. APC and APC2**

The truncated version of the APC gene known as APC2 does not have the microtubule binding domain or EB1 protein binding domain; therefore, APC2 does not have the same ability as APC to stabilize microtubules.
Materials and Methods

Fly Stocks

Fly Stocks were obtained from the Vienna Drosophila Resource Center and the Bloomington Drosophila Stock Center at Indiana University.

Genetic Crosses and Mounting

All Drosophila were maintained at 25°C in suitable media. Virgin female flies from experimental fly lines were mated with male flies containing a fluorescent marker expressed in the peripheral nervous system. In order to obtain virgin female flies, vials of flies were examined within 8 or 16 hours at 25°C or 18°C, respectively. After the flies were mated, embryos were collected and allowed to mature into third instar larvae over a course of 3 days at 25°C. In order to visualize the larvae under a microscope, they were mounted on glass slides. The mounting process consisted of a brief bath in 0.1% phosphate buffer solution followed by a transfer to glass slides with dried 3% agarose solution. The larvae were held in place using cover slips and clear tape after they naturally stretched into a fully elongated orientation.

Microtubule Polarity Assay

Larvae with EB1-GFP expressed in the peripheral nervous system were generated by crossing males from UAS-Dicer 2;221Gal4, UAS-EB1-GFP/TM6 with virgin females from the following lines: Rtnl 2, Gsa 60A, G-alpha 73β, Gsa on II, G-Beta 76C, CTX on II, Gsa on III, Gsa Q215L, AC3, Rut, Smo, and Fz on II. The ddaE neurons of the third, fourth, or fifth hemisegment of the mounted larvae were imaged at 63x using a Zeiss Axio Imager M2 with Zeiss Immersol Immersion Oil. The GFP
chromophore was excited by a 470nm light source from the microscope. Image series were collected with an exposure time of 750nm over 200 cycles of 1 second. Images were only collected for 10 minutes after mounting to ensure larvae were healthy.

Movies were compiled and analyzed in the Image J software. Microtubule polarity was determined by counting EB1 comets and noting the direction of movement either toward or away from the soma. In order for an EB1 comet to count as a data point, it had to remain in focus for at least three consecutive images. Additionally, EB1 comets located between the soma and the first branch point were not included in quantification. Chi Square analysis was performed on the experimental fly lines using rtnl 2 as a control to determine statistical significance. Rtnl 2 was used as a control because the phenotypes of cells containing rtnl 2 RNAi and cells with no RNAi hairpin have been consistently similar.

**APC 2 Localization Assay**

UAS-dicer2, UAS-MCD8-RFP; 221Gal4, UAS-APC2-GFP/TM6 larvae were crossed with the following RNAi knockdown lines: Rtnl 2, Gsa 60A, Gsa Q215L. The MCD8-RFP served as a membrane marker to visualize the dendritic network background. Again, ddaE neurons in the third, fourth, or fifth hemisegment were examined. The larvae were imaged using the Olympus BX61 under the 60x objective with immersion oil. The excitation wavelengths used in this study were 488nm for GFP and 543nm for RFP.

A Z-stack was created using the Image J software from time series images that captured the depth of the structure. APC2 localization was determined by counting the number of branch points within the dendrite that contained appreciable amounts of the GFP chromophore. Chi Square analysis was also used to determine significance in the APC2 localization assay.
Results

G Protein Screen

In order to determine if significant polarity phenotypes were observed for knockdowns of various G proteins, the examination of the microtubule polarity of a negative control (Rtnl 2) was the first step. The results of this microtubule polarity assay yielded a 94.8 percentage of plus-end-out microtubules in the axon and a 10.1 percentage of plus-end-out microtubules in the dendrite. These results are very similar to the accepted values for a negative control in this assay (95% in the axon and 10% in the dendrite); therefore, they can be reliable points of comparison for statistical analysis.

Next, a general screen was performed of the following G protein candidates: G-alpha 73β, Gsα on II, G-Beta 76C, and Gsα 60A. In the axon, none of the candidates presented a significant inversion of microtubules from the expected values, as determined by chi square analysis. The results of this screen yielded percentages of 92.1%, 94.9%, 98.1%, and 94.7% for G-alpha 73β, Gsα on II, G-Beta 76C, and Gsα 60A, respectively (Table 1 and Figure 8).

Table 1. G Protein Screen: Axon Microtubule Orientation

<table>
<thead>
<tr>
<th>Stock Line</th>
<th>% Plus-End-Out Microtubules</th>
<th>Number of EB1 Comets (n)</th>
<th>Chi Square P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rtnl 2</td>
<td>94.8</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td>G-alpha 73β</td>
<td>92.1</td>
<td>253</td>
<td>0.562</td>
</tr>
<tr>
<td>Gsα on II</td>
<td>94.9</td>
<td>138</td>
<td>0.633</td>
</tr>
<tr>
<td>G-Beta 76C</td>
<td>98.1</td>
<td>54</td>
<td>0.198</td>
</tr>
<tr>
<td>Gsα 60A</td>
<td>94.7</td>
<td>171</td>
<td>0.668</td>
</tr>
</tbody>
</table>
The same screen was performed in the dendrite of ddaE neurons of these lines (Figure 9). Similarly negative data was obtained in the dendrite for G-alpha 73β (14.0%), Gsα on II (11.3%), and G-Beta 76C (11.4%) when statistically analyzed against Rtnl 2 (Table 2). However, a very significant phenotype was discovered in the dendrite of the Gsα 60A experimental cross. The percentage of plus-end-out microtubules in the Gsα 60A experimental cross was found to be 19.2%. This data was statistically analyzed using chi square analysis to give a p value of 0.003, which is very statistically significant (p<0.01).

**Table 2. G Protein Screen: Dendrite Microtubule Orientation**

<table>
<thead>
<tr>
<th>Stock Line</th>
<th>% Plus-End-Out Microtubules</th>
<th>Number of EB1 Comets (n)</th>
<th>Chi Square P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rtnl 2</td>
<td>10.1</td>
<td>341</td>
<td></td>
</tr>
<tr>
<td>G-alpha 73β</td>
<td>14.0</td>
<td>385</td>
<td>0.197</td>
</tr>
<tr>
<td>Gsα on II</td>
<td>11.3</td>
<td>212</td>
<td>0.864</td>
</tr>
<tr>
<td>G-Beta 76C</td>
<td>11.4</td>
<td>144</td>
<td>0.918</td>
</tr>
<tr>
<td>Gsα 60A</td>
<td>19.2</td>
<td>261</td>
<td>0.003</td>
</tr>
</tbody>
</table>
After discovering the very significantly altered microtubule polarity phenotype in the dendrite of the Gsα 60A experimental cross, confirmation of this phenotype in the dendrite was performed using an alternate RNAi construct (Gsα on III), a GTP-bound mutant (Gsα Q215L), and a cholera toxin RNAi (CTX on II). A cholera toxin RNAi was chosen because the cholera toxin is an ADP-ribosyltransferase that activates Gsα. The results of Gsα on III and Gsα Q215L mutant with were found to have plus-end-out microtubule percentages of 15.9% and 20.3%, respectively (Figure 10). After statistical analysis, Gsα on III and Gsα Q215L confirmed the previously observed phenotype with p values of 0.030 and 0.009 (Table 3). Although the alternate Gsα RNAi and the GTP-bound did confirm the phenotype, the cholera toxin line showed statistical significance of a different sort with a very low percentage of plus-end-out microtubules at 5.7% (Table 3). This differing phenotype does not negate the confirmation of the previously
determined phenotype because other cellular components may be affected by the knockdown of the function of an ADP-ribosyltransferase, but the phenotype should be noted.

### Table 3. Gsα: Dendrite Microtubule Orientation

<table>
<thead>
<tr>
<th>Stock Line</th>
<th>% Plus-End-Out Microtubules</th>
<th>Number of EB1 Comets (n)</th>
<th>Chi Square P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rtnl 2</td>
<td>10.1</td>
<td>341</td>
<td></td>
</tr>
<tr>
<td>Gsα 60A</td>
<td>19.2</td>
<td>261</td>
<td>0.003</td>
</tr>
<tr>
<td>Gsα on III</td>
<td>15.9</td>
<td>253</td>
<td>0.030</td>
</tr>
<tr>
<td>Gsα Q215L</td>
<td>20.3</td>
<td>118</td>
<td>0.009</td>
</tr>
<tr>
<td>CTX on II</td>
<td>5.7</td>
<td>123</td>
<td>8.17 E-12</td>
</tr>
</tbody>
</table>

**Figure 10. Gsα: Dendrite Microtubule Orientation**

This figure is a graphical representation of the data obtained from the microtubule polarity assay in the dendrites of the ddaE neurons of Gsα confirmation candidates shown in Table 3. As displayed above, the percentage of plus-end-out microtubules for the following *Drosophila* lines confirm the Gsα 60A phenotype: Gsα on III and Gsα Q215L. The CTX on II experiment shows a significant phenotype of the opposite sort with a very small percentage of plus-end-out microtubules.
APC 2 Screen

In order to determine if the function of APC2, recruitment of APC to the branch points, is involved in the $\text{Gs} \alpha$ mechanism of inverting the polarity of microtubules in the dendrite, an APC2 branch point localization assay was performed.

Figure 11. APC2 Assay Images
The images above were taken with the Olympus in order to visualize the presence of APC2 at the branch points of ddaE neurons. The GFP tagged APC2 is signified by the green color in the images; whereas, the red color indicates the presence of the MCD-RFP membrane marker.

Again, Rtnl 2 was used as a negative control yielding a fraction of 0.898 branch point localization with APC2. The $\text{Gs} \alpha$ 60A and Gsα Q215L experimental lines were found to have a similar fraction of branch points with APC2, 0.938 and 0.881, respectively (Figure 12). After chi square analysis, the similar fractions of branch points in the experimental crosses were determined to be insignificant (Table 4).
Table 4. Gsα: Dendrite APC2 Localization

<table>
<thead>
<tr>
<th>Stock Line</th>
<th>Fractional Branch Points with APC2</th>
<th>Total Branch Points</th>
<th>Chi Square P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rtnl 2</td>
<td>0.898</td>
<td>215</td>
<td></td>
</tr>
<tr>
<td>Gsα 60A</td>
<td>0.938</td>
<td>78</td>
<td>0.174</td>
</tr>
<tr>
<td>Gsα Q215L</td>
<td>0.881</td>
<td>269</td>
<td>0.564</td>
</tr>
</tbody>
</table>

This figure is a graphical representation of the data obtained from the APC2 localization assay in the dendrites of the ddaE neurons of Gsα 60A and Gsα Q215L shown in Table 4. As displayed above, the fraction of branch points with APC2 for all Drosophila lines tested was found to be similar.

Adenylate Cyclases

In order to determine if the Gsα polarity control mechanism involves the activation of an adenylate cyclase, two adenylate cyclase RNAi lines, AC3 and Rut, were examined. These adenylate cyclase lines were chosen because they have known neuronal expression and function. The AC3 RNAi experimental cross was found to have a microtubule plus-end-out percentage of 12.6%, and the Rut RNAi experimental cross was found to have a higher microtubule plus-end-out percentage of 17.5% (Figure 13). Chi square analysis of these adenylate cyclase...
Experimental crosses determined that the phenotype of Rut was significant with a p value of 0.021, but the phenotype of AC3 was not significant with a p value of 0.556 (Table 5). This result suggests that Rut may be involved in the Gsα pathway for regulating microtubule polarity.

Table 5. Adenylate Cyclases: Dendrite Microtubule Orientation

<table>
<thead>
<tr>
<th>Stock Line</th>
<th>% Plus-End-Out Microtubules</th>
<th>Number of EB1 Comets (n)</th>
<th>Chi Square P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rtnl 2</td>
<td>10.1</td>
<td>341</td>
<td></td>
</tr>
<tr>
<td>AC3</td>
<td>12.6</td>
<td>183</td>
<td>0.556</td>
</tr>
<tr>
<td>Rut</td>
<td>17.5</td>
<td>252</td>
<td>0.021</td>
</tr>
</tbody>
</table>

![Graph showing microtubule orientation](image)

**Figure 13. Adenylate Cyclases: Dendrite Microtubule Orientation**

This figure is a graphical representation of the data obtained from the microtubule polarity assay in the dendrites of the ddaE neurons of adenylate cyclase candidates shown in Table 5. As displayed above, the percentage of plus-end-out microtubules for AC3 is not very different than the control; whereas, the percentage for Rut is significantly higher than the control.
G Protein Coupled Receptors

G proteins receive signals from GPCRs, so the next step was to determine if there was a GPCR in the Gsα polarity control mechanism. Fz receptors were chosen as likely candidates due to the recent studies that linked the Gsα subunit to the Fz receptors. Two receptors from the Fz family, Fz and Smo, were examined using the microtubule polarity assay. The Fz experimental cross yielded a high percentage of plus-end-out microtubules in the dendrite at 20.1%; whereas, the Smo experimental cross yielded low a percentage of 8.1% (Figure 14). Statistical analysis determined the Fz phenotype to be very significant with a p value of 0.006 and the Smo phenotype to be insignificant with a p value of 0.299 (Table 6).

<table>
<thead>
<tr>
<th>Stock Line</th>
<th>% Plus-End-Out Microtubules</th>
<th>Number of EB1 Comets (n)</th>
<th>Chi Square P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rtnl 2</td>
<td>10.1</td>
<td>341</td>
<td></td>
</tr>
<tr>
<td>Fz</td>
<td>20.1</td>
<td>149</td>
<td>0.006</td>
</tr>
<tr>
<td>Smo</td>
<td>8.1</td>
<td>209</td>
<td>0.299</td>
</tr>
</tbody>
</table>

**Figure 14. GPCR: Dendrite Microtubule Orientation**

This figure is a graphical representation of the data obtained from the microtubule polarity assay in the dendrites of the ddaE neurons of GPCR candidates shown in Table 6. As displayed above, the percentage of plus-end-out microtubules for Smo is not very different than the control; whereas, the percentage for Fz is drastically higher than the control.
Discussion

G proteins have shown signal transduction significance in sensory neurons in previous studies, and subunits have even been found to differentially signal in various locations (Boto, Gomez-Diaz, & Alcorta, 2010). The microtubule polarity assay of various G proteins was carried out based on the hypothesis that certain G proteins may be active components in the mechanism of polarity control. Although many of the G proteins that were chosen as candidates did not show any significant phenotype, there was one line that exhibited substantial inversion of polarity in the dendrite – Gsα. Due to the lack of significance in axons of any genetic construct, axon examination was abandoned after the initial screen to focus efforts on the significance in dendrites.

The confirmation of the Gsα phenotype was carried out in order to ensure that it was not an artifact of the genetic alteration made to create the RNAi knockdown. An additional RNAi line was chosen that contained a very different genetic construct that targeted a different location on the Gsα gene (Figure 15).

**Figure 15. Gsα RNAi Constructs**
The diagram above displays the RNAi constructs of Gsα 60A (KK107742) and Gsα on III (HMC03106). Both constructs knockdown the expression of Gsα, but target different sequences of the gene.
The GTP-bound Gsα mutant was tested as well to ensure that the faulty Gsα protein was truly the cause of the phenotype. A mutant enables the organism to translate without targeted destruction of mRNA. The mutant therefore serves as a reassurance that the RNAi process is not causing the altered phenotype. Although the GTP-bound mutant did show a significant phenotype, the same was not true of the cholera toxin line. This was a bit peculiar because the cholera toxin ADP-ribosylates Gsα at an arginine residue, which is required for GTP hydrolysis (Offermanns & Rosenthal, 2008). In turn, the cholera toxin holds Gsα in the GTP-bound state because it cannot cleave the GTP to GDP. Therefore, it was expected to have a similar phenotype to the mutant, but it did not have similar results. A possible explanation for this disparity between the GTP-bound mutant and the cholera toxin is that the cholera toxin may not effectively ADP-ribosylate Gsα in neurons, which would allow close to normal function of Gsα.

The APC2 localization assay results showed no significant changes when Gsα expression was altered. This lack of significance suggests that the APC2 pathway that controls microtubule polarity at dendritic branch points is independent of the newly discovered Gsα pathway. Although the results of this assay were negative, the positive conclusion that the pathways may be separate can be extrapolated.

It is interesting to note that both adenylate cyclase RNAi constructs did increase the percentage of plus-end-out microtubules, although only Rut was found to be significant. A possible explanation for this phenomenon arises in a study performed on mushroom body neurons in drosophila.

In this study, olfactory learning was shown to require Gsα signaling. This requirement determined because all learning ceased when Gsα function was removed; however, null alleles of
Rut and another downstream protein of Gsα were shown to partially impair learning. The authors of the article suggested that disrupting more than one adenylate cyclase at a time would produce an even greater phenotype (Connolly et al., 1996). These findings suggest an association in the function between Gsα and Rut. It can be hypothesized that Gsα signals through the adenylate cyclase Rut and produce cAMP. This signaling suggests that the Gsα microtubule polarity pathway is a cAMP-dependent pathway. On the other hand, microtubule polarity in the dendrites is not totally disrupted by the knockdown of Gsα, which suggests that it is also possible that Gsα can signal through a pathway that is not dependent on cAMP and that other pathways make significant contributions to the control of microtubule polarity, such as the APC2-dependent pathway.

The Fz receptor that was determined to show significant polarity phenotype in the dendrite using the microtubule polarity assay follows a canonical signaling pathway. The Fz receptor activates Gsα, a G protein. Then Gsα activates Rut, an adenylate cyclase that produces cAMP, and the cAMP activates downstream regulators of microtubule polarity (Figure 16).
Future studies on the Gsα pathway should target a combination of adenylate cyclases to see if the combinatorial knockdown results in an even stronger inversion of polarity. Additionally, experiments should be performed to paint an even broader picture of the pathway. Suggestions for future experiments revolve around the microtubule polarity assay and they include alteration of cAMP levels and knockdowns of specific cAMP kinases, such as Protein Kinase A (PKA).

A study in which PKA levels were altered has shown that PKA does play a role in the development of the dendritic arbor in class IV neurons. This study found that the alteration of PKA levels resulted in reduced complexity of arbors, decreased length, and fewer branch points.
in dendrites (Copf, 2014). This finding suggests that PKA has functional significance in the dendrite. Another recent study demonstrated that PKA plays a role in microtubule regulation through phosphorylation of stathmin, a microtubule-destabilizing protein that has been found to disrupt microtubule dynamics in the Rolls Lab. The study found that there is communication between PKA and c-Jun N-terminal kinases in the control of microtubule architecture (Yip, Yeap, & Ng, 2014). Taken together, these studies suggest that PKA has functionality in the control of cellular morphology and microtubule regulation in dendrites; therefore, it is a strong candidate for further investigation.


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Education
Scranton Preparatory School, Scranton, PA
The Pennsylvania State University, University Park, PA
The Schreyer Honors College
B.S., Biochemistry and Molecular Biology

Experience
Center for Cellular Dynamics, The Pennsylvania State University
Research Student

• Performed hundreds of microscopy experiments that require intense focus and fine motor skills
• Developed a standardized method for collecting relevant data points
• Analyzed trends in data in order to extrapolate pertinent conclusions

Order of Omega, Epsilon Psi Chapter
President

• Performed executive duties: recruit members, hold meetings, remain nationally recognized
• Collaborated with advisor, officers, and members to ensure the success of the organization
• Worked hand-in-hand with other organizations to impact a larger impact on the community

Community Service Chairman

• Partnered with public service organizations in order to develop community service projects
• Contributed to the executive board of the organization as a strong voice in leadership

Theta Delta Chi Fraternity, Sigma Triton Charge
VP of Philanthropy and Community Service

• Planned and oversaw philanthropy and community service events for over 75 individuals
• Assisted with fraternity Penn State Dance Marathon fundraising – $147,000 (2014)
• Organized a 5K event to raise over $100,000 for autism awareness

Pennsylvania Republican Party
Intern

• Assisted field office director in management of the Lackawanna County Field Office
• Oversaw and participated in grass root operations to reach potential voters throughout the nation

Interventional Cardiologist Extern, Lankenau Medical Center, PA

• Observed multiple catheterizations and stent placements performed by 4 cardiologists
• Witnessed logistics of an emergency operation to relieve ST-elevation Myocardial Infarction

Honors and Activities
• Alpha Epsilon Delta Pre-medical Honor Society of Penn State (2012-Present)
• Schreyer Academic Excellence Scholarship, $3,500 annually (2011-2014)
• Penn State Spanish Club (2011-2013)
  o Penn State Dance Marathon Chairman (2012)
  o Competency in Spanish
• Intramural Sports: Soccer (captain), Volleyball (captain), Basketball