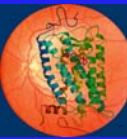


# Role of G protein $\beta\gamma$ subunit and dynein light chain Tctex-1 in neuronal differentiation

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## Introduction

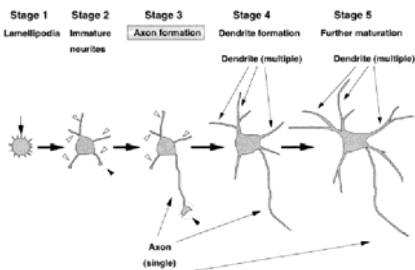
Signaling pathways that lead to neurite outgrowth and the establishment of neuronal polarity remain poorly understood. Dynein light chain component Tctex-1 was recently demonstrated to regulate multiple steps in neurogenesis in a dynein-independent manner [1]. However how dynein-associated and dynein-free pools of Tctex-1 are maintained in the cell remains unclear. Tctex-1 has also been identified as a receptor-independent activator of G protein signaling and was shown to bind G protein  $\beta\gamma$  subunit ( $G\beta\gamma$ ) [2,3]. We propose a novel role for the  $G\beta\gamma$ -Tctex-1 interaction in neurite outgrowth and neuronal differentiation.

## Project Goals

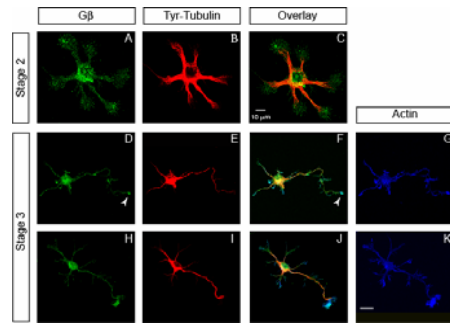
- To demonstrate a role for the  $G\beta\gamma$ -Tctex-1 complex in regulating neuronal differentiation.
- To establish a cell line model to explore the cellular signaling pathways regulated by the  $G\beta\gamma$ -Tctex-1 complex in neuronal differentiation.

## Neuronal Models

**Primary Hippocampal Neurons:** In culture, primary neurons differentiate to a polarized state in five distinct morphological stages [5].



**Neuro 2A Neuroblastoma (N2A) cell line:** N2A cells show clear signs of differentiation within 24 h of serum withdrawal whereas they remain undifferentiated and proliferative in the presence of serum (see Figure 3) [5].



**Figure 1. Expression pattern of  $G\beta$  in various stages of cultured primary hippocampal neuron differentiation**  
 $G\beta$  distributes diffusely throughout the cell body and the minor processes in stage 2 neurons (A-C). As the neurons develop through stage 2-3 and reach stage 3 and adopt the well differentiated neuronal phenotype,  $G\beta$  shows two distinct labeling patterns. Approximately 40% of stage 3 neurons ( $n > 50$ ) showed  $G\beta$  labeling in the central region of the axonal growth cone (D-K). An example of stage 3 neuron that failed to show any detectable enhancement of  $G\beta$  in the growth cones is represented in L-N. Neurons were co-labeled for  $G\beta$  (green in A, D, H and L), Tyr-Tubulin (red in B, E, I and M), and actin (blue in G and K). Overlayed images are shown in C, F, J and N. Scale bars equal 10 mm in A-C, and 20 mm in D-N.

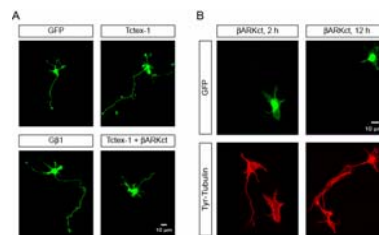


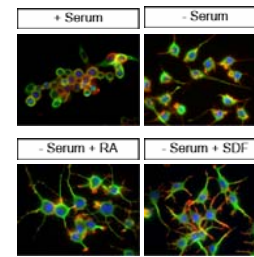
Table 1. Quantitative Analyses of Morphological Changes of Transfected Neurons

Overexpressed Protein	% Cells Stage 1	% Cells Stage 2	% Cells with a Single Neurite Stage 3	% Cells with Multiple Neurites $\geq 70-80 \mu m$
GFP alone	0 ± 4	52 ± 8	40 ± 6	0.6 ± 0.1
βARKct	78 ± 6*	17 ± 3*	4 ± 2*	ND
FLAG-Gβ1	2 ± 1	36 ± 9*	42 ± 14	22 ± 6*
FLAG-Tctex1	2 ± 1	24 ± 8*	28 ± 6	38 ± 8*
FLAG-Tctex1 + βARKct	10 ± 4	38 ± 4*	40 ± 12	12 ± 6*

Cells were transfected at 2 h after plating and fixed 24 h later. Each transfection received 1 μg of GFP expressing vector for visualizing the transfected cells. For all other constructs 2 μg of plasmid were typically used. The total amount of DNA added was kept constant by adding appropriate amount of control vector. A neurite longer than 70-80 μm was considered to be an axon in these analyses. Each value represents the mean ± S.E.M of at least 50-75 cells for each experimental condition. \* Asterisk represents value significantly different from that of the GFP-transfected group ( $p < 0.01$ ). ND, not detected

**Figure 2. Role of  $G\beta\gamma$  and Tctex-1 in neuronal differentiation**

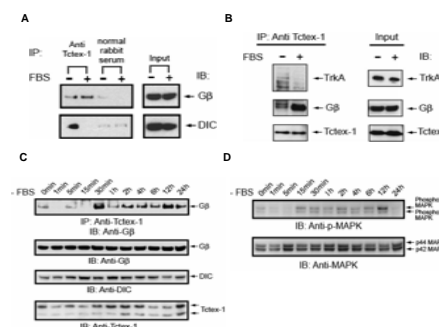
(A) Ectopic expression of  $G\beta 1$  and Tctex-1 induces multiple, long neurites in hippocampal neurons. Hippocampal neurons were transfected as indicated, with expression vectors for GFP, GFP + FLAG- $G\beta 1$ , GFP + FLAG-Tctex-1 and GFP + Tctex-1 +  $\beta$ ARKct 2 h after plating. The transfected neurons were fixed and processed for GFP fluorescence 24 h after transfection. (B) Expression of  $G\beta\gamma$ -sequestering reagent,  $\beta$ ARKct inhibits neurite outgrowth. Hippocampal neurons were transfected either 2 h or 12 h after plating, with expression vectors for GFP and  $\beta$ ARKct and analyzed 24 h after transfection for GFP expression (green) and Tyr-Tubulin labeling (red),  $\beta$ ARKct expression results in arresting the cells in stage 1 when transfected 2 h after plating and in stage 2 when transfected 12 h after plating. Note that, under both conditions, the un-transfected cells within the same field appear healthy and have reached stage 3. The images shown here are representative of three independent transfections. Quantification of the images from these transfections is shown in Table 1.



**Figure 3: Differentiation of N2A cells.** N2A cells, maintained in complete growth media, remain undifferentiated in the presence of serum. Serum withdrawal induces neurite outgrowth within 24h and retinoic acid (RA) and SDF-1 $\alpha$  treatment potentiates neurite outgrowth and neuronal differentiation of these cells implying that pathways activated downstream of both of these molecules play important roles in neuronal differentiation.



**Figure 7.  $G\beta$  overlaps with Dynein-free pool of Tctex-1 and competes with dynein for Tctex-1-binding.** (A)  $G\beta$  co-sediments with the dynein-free lighter fractions of Tctex-1. Homogenates from E15 mouse brain were sedimented in a 5%-20% linear sucrose gradient. Each fraction was analyzed by SDS-PAGE and immunoblotted with indicated antibodies. (B) Competition of dynein-Tctex-1 interaction by  $G\beta$ -peptide. Dynein intermediate chain (DIC) was immunoprecipitated (IP) from lysates generated from HEK cells transfected with Tctex-1 cDNA. The DIC IP was titrated with increasing concentration of unlabeled  $G\beta$ -peptide (0 – 1000 nM) as indicated. As the concentration of the peptide increased, a decreasing amount of Tctex-1 was detected in the immune complex as measured using an anti-Tctex-1 antibody. (C) Model of  $G\beta\gamma$ -dependent regulation of dynein-free function of Tctex-1 in inducing neurite outgrowth.



**Figure 4: Effect of N2A cell differentiation on  $G\beta\gamma$ -Tctex-1-dynein interaction and MAPK activation.** (A-B)  $G\beta\gamma$ -Tctex-1 interaction decreases in differentiated N2A cells whereas there seems to be an increase in DIC-Tctex-1 and TrkA-Tctex-1 interaction. (C) Kinetic analysis of the  $G\beta\gamma$ -Tctex-1 interaction suggests that the interaction initially increases during early stages of differentiation, peaks 12 h post-serum withdrawal and starts decreasing by 24 h when  $>80\%$  cells show signs of differentiation. (D) Kinetic analysis of MAPK activation, depicted by phospho-MAPK antibodies, demonstrates that MAPK is activated within 15 min of serum withdrawal and this activation is maintained for several hours, peaking at 12 h and then gradually subsiding by 24h when the cells are differentiated.

## References

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## Results and Conclusions

- Ectopic expression of either  $G\beta\gamma$  or Tctex-1 promotes neurite outgrowth whereas interfering with their function inhibits neurogenesis.
- Endogenous  $G\beta\gamma$ -Tctex-1 complex exists in mouse embryonic brain extracts and  $G\beta\gamma$  co-segregates with dynein-free fractions of Tctex-1.
- $G\beta\gamma$  competes with dynein for Tctex-1 binding and regulates dynein-independent functions of Tctex-1.
- Neuronal cell line Neuro 2A (N2A) serves as a good model for exploring the role of the  $G\beta\gamma$ -Tctex-1 complex in neuronal differentiation.
- $G\beta\gamma$ -Tctex-1 interaction is modulated in a differentiation-dependent manner in N2A cells: the interaction increases during initial stages of neuronal differentiation and decreases upon differentiation. Inversely, the dynein-Tctex-1 interaction increases as the N2A cells become more differentiated.
- Our results in primary hippocampal neurons and N2A cells support our model which stipulates different cellular roles for  $G\beta\gamma$ -Tctex-1 and dynein-Tctex-1 complex.