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The role of the Scribble Protein Complex in the regulation of Dendrite Formation

A Thesis presented

by

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Abstract of the Thesis

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During neuronal development, the neuronal cell polarizes to specify the structures of axons and dendrites. This polarized architecture regulates the input/output functions of the neuronal cell. In my thesis, I studied the molecular mechanisms of dendrite formation. My main hypothesis was that dendrite specification results from local elevation of the cGMP signal. Soluble guanylyl cyclase (sGC) is the major enzyme for the synthesis of cGMP in the embryonic brain. I study the molecular mechanism that activates sGC and leads to dendrite specification during neuronal polarization.Scribble is a scaffold protein, part of a polarity complex that establish apical-basal polarity in epithelial cells. In neurons, Scribble has been shown to associate with neuronal nitric oxide synthase (nNOS) that produces the molecule NO. NO activates sGC and can induce cGMP elevation. I hypothesize that dendrite specification is regulated by a polarity protein complex consisting of Scribble, sGC, and nNOS. In my thesis I found the following: First, I show that Scribble and sGC associate biochemically in the rat embryonic brain. Second, I further characterized the interaction domain between Scribble and sGC and found a protein domain named IMR to be significant. I found that this single domain mutant of Scribble is a dominant negative inhibitor of the function of wild-type Scribble. Last, using shRNA-mediated knockdown of Scribble and sGC in dissociated hippocampal neuron culture, I found severe effects

on dendrite development compared to control. Thus, based on my biochemistry studies and functional analysis in cultured neurons, I conclude that the protein complex involving Scribble and sGC regulates dendrite development through the cGMP pathway.

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List of Abbreviations

sGC Soluble guanylyl cyclase
SCRIB scribble planar cell polarity protein [Human]
Scrib scribble homolog[mouse]
scrib scribbled [drosophila]
cAMP Cyclic adenosine monophosphate
cGMP Cyclic guanosine monophosphate
PLL poly-lysine
DIV day in vitro
PFA paraformaldehyde
PBS phosphate-buffered saline
NGS normal goat serum
nNOS neuronal nitric oxide synthase

Acknowledgements

First, I would like to thank my advisor, Dr. Maya Shelly, who have spent tremendous time working with me and guiding me in graduate school. We worked together for many hours on the image analysis of the dissociated hippocampal neuron culture.

This project is established on Dr. Seongil Lee's current project on studying neuronal polarization. He contributed to the rat brain lysate experiment and testified the complex in vivo. He did the cloning of the Scribble mutants, shRNAs, and flag tag sGC. He also trained me to do the biochemistry analysis and helped to grow dissociated hippocampal neurons and do nucleofection. I appreciate his great patience, detailed guidance and precious time in helping me in the experiments.

I also want to thank Sneha Rao, who provided warm conversations, generous help and good company for my time in the lab. I also want to thank lab members in Dr. Shaoyu Ge lab who help me and share some reagents with me.My thank also goes to the people in the Genetics program who are the great support of me including Dr. Martha Furie, Kate Bell and many others.

Chapter 1

Introduction

Cell polarity is the asymmetric organization of cell structures including both the cell membrane and the intracellular structures [1]. In neurons, one cell can be divided into three regions, the axon, the dendrites and the soma (cell body). Axons and dendrites are different from each other biochemically and structurally. They have distinct functions in neurons with dendrites receiving and integrating signal from other cells, and axons sends signals to other cells. During neuronal development, the specification of axons and dendrites is a primary event in neuronal polarization.

Here I hypothesized that in neuronal polarization, dendrite development is regulated by a protein complex involving Scribble, sGC and nNOS, that activates the signaling for the production of cGMP.

Scribble is a large scaffold protein that contains different domains. It belongs to the LAP (LRR And PDZ) protein family and contains 16 LRR sequence and 4 PDZ domains[2].Here, we named the other regions as intermediate region-IMR domain (the region between LRR and PDZs) and C-terminal tail (the region after PDZs, at the C terminus). Scribble can interact with lethal giant larvae (Lgl)/discs large (Dlg) proteins to establish the apical-basal polarity and asymmetry in epithelial cells[3]. In drosophila, Scribble, Lgl and Dlg1 has also been found to regulate cell migration, proliferation and tumor suppression[4, 5]. However, the function of Scribble in neurons is still unclear. In a proteomic screening analysis, Scribble has been found to associate with neuronal protein including nNOS, a neuronal specific NO synthase. In neurons, NO is known to activate sGC, the major cGMP synthase in cells[6].

My main hypothesis is that dendrite specification is triggered by cGMP signal elevation. In cultured embryonic hippocampal neurons, axon initiation is induced with an elevation of cAMP level in a single neurite while the cAMP level is suppressed in other neurites. cGMP level arises in these neurites that develop into dendrites in later time point. I therefore propose that signal transduction to activate local cGMP is essential to dendrite development [7]. I suggest here that Scribble is the scaffold protein that associate with sGC that can activate cGMP signal specify dendrite development.

To test my hypothesis, first, I need to confirm the existence of the protein complex in the nervous system. If the protein complex exists, next, I will study what is the interaction domain of Scribble between Scribble and sGC. By solving the interaction domains between Scribble and sGC, I can confirm the specificity of the interaction; Also, I can isolate the interaction domain of Scribble and construct Scribble mutants. This mutant can be used as a dominant negative inhibitor to inhibit the association of wild-type Scribble and sGC and becomes a powerful tool for functional studies in dendrite development.

To study the actual function of Scribble complex in neurons, I will use short hairpin RNAs against Scribble to knock down the expression of the Scribble protein and investigate how it affects dendrite development. After confirming the function of Scribble in neuronal development, I will continue to inquire into the downstream molecular mechanism involving sGC.Thus, I will include sGC knockdown into functional studies.

I will use dissociated hippocampal neuron culture as a primary system to study dendrite development. The major cell type in the hippocampus is pyramidal neurons. From dissected rat hippocampus, pyramidal neurons can be dissociated and grow in culture with well-defined axons and dendrites development. The easiness to manipulate and maintain the primary culture can also facilitate my study on dendrite development[8].

Proper axon and dendrite development is essential for forming functional neural circuits. Dendrite malformation is reported in many neurological disorders including mental retardation, autism spectrum disorders, schizophrenia and Alzheimer's disease with altered dendrite length, number and spine structures[9, 10]. My research focuses on studying a novel candidate molecule, Scribble, that regulates dendrite development. The study can provide a new insight on the intrinsic developmental programs in neuronal polarization that uses a scaffold protein complex, which may be targeted for therapeutic purposes in the future.

Chapter 2

Material and methods

HEK293T cell growth and transfection

HEK293T cells are cultured in DMEM media with 10% FBS and 1X Pen-Strep. The cells are split every 3 days to ensure cell quality. Cells are plated 24 hours before transfection and grow to 50-60% confluency during transfection. We do calcium phosphate transfection in HEK293T cells for biochemistry analysis. For each 60mm dish, we use 40ul 2.5M CaCl2, 2ug of plasmid DNA and 200ul 2x HEPES-buffered saline. Each dish will be added 400ul of Calcium-DNA-HEPES mix. Transfected cells are allowed to grow for 24 hours before lysis.

Immunoprecipitation assay

HEK293T cells are plated one day before transfection and grow to 50-60% confluency during transfection. Flag-sGC is coexpressed with HA tag Scribble mutants in HEK293T cells for 24 hours and lysed in lysis buffer. Lysis buffer is made as 1%CHAPS buffer with 0.15M NaCl and 20mM Sodium Phosphate, cOmplete, Mini Protease Inhibitors and 1mM EDTA. The lysate is incubated with mouse protein G agarose beads at 4 degree for 3 hours with Sigma Flag M2 antibody. The beads and the total lysate is run on 7% Tris-HCl SDS gel and transferred in Tris-Glycine buffer containing 20%methanol to PVDF membrane. The membrane is blocked in 5% non-fat dry milk for one hour and incubated with Flag HRP conjugated antibody at 1:20000 for one hour an a half. The membrane is then developed with Pierce ECL2 substrate onto a film.

Hippocampal neuron culture and nucleofection

The tissue and protocol are obtained from BrainBits(R) complete culturing kit (hippocampus). We chose E18 Sprague Dawley Rat. One pair of E18 rat hippocampus is stored in 2 ml Hibernate(R) EB (Hibernate E(R)/B27(R)/GlutaMAXtm;BrainBits(R) HEB 500ml)). Tissue is dissociated in 2 mg/ml Papain in HE-Ca with 10 minutes incubation at 30 degree water bath. Cells are resuspended in the growth medium, Neural Basal Medium with 2% B27 supplement, 0.5nM GluMax, and 25uM/ml Glutamine. Count cells on the glass cytometer and suspend cells in growth medium.

200ul of the cell suspension is then used for one nucleofection reaction. A final number of 2X10$\hat{4}$ nucleofected cells are plated in a 35mm cell culture dish that contains 5 PLL treated coverslips with 2.5 ml neural basal medium for future growth in a 37degree incubator with 5% CO2.

Immunohistochemistry

Cultured rat hippocampal neurons are fixed at 3 DIV of growth. The cells are washed in PBS for one time and fixed in 4% PFA for 20 minutes. We rinse the cells in PBS for three times and permeabilize the cells in PBS-0.05% Triton. After 5 minutes of treatment in PBS-0.05% Triton for three times. We block the cells in 10 NGS in PBS-0.05% Triton for one hour in the dark. After blocking, the cells are put one primary antibody incubation for 24 hours. We use NOVUS CHICKEN MAP II antibody at a concentration of 1:5000, and Mouse SMI-312 antibody at a concentration of 1:500.

Cells are washed in PBS-0.05% Triton and transferred to secondary antibody incubation in the dark for 2 hours. We use Invitrogen Alexa Fluor CHICKEN 633, Alexa Fluor MS 488 conjugated antibody at a concentration of 1:1000 when the cells are coexpressed with GFP. The cells are mounted onto slides using Fluoromount.

Data analysis

We did quantitive analysis on the number and length of the neurites of cultured hippocampal neurons. Dendrites are specified by MapII signal and Axons are specified by Smi312 signal. The cells are imaged at Olympus confocal microscope. The images are then analyzed using Fiji software. We measure neurite length from soma to the tip of a single neurite. We counted any crossing that lengths over 5um from soma as a branch and we measure the longest branch as the length of that neurite. Axons are measured and counted under Smi312 channel; Dendrites are measured and counted under Map2 channel.

Chapter 3

Results

IMR region is the interaction domain between Scribble and sGC

In figure 1, Dr. Lee has illustrated that in a developing rodent brain (E20) Scribble can associate with sGC β1 subunit. sGC is a heterodimer of one αsubunit and one βsubunit. Each subunit has two types, α1, α2, β1, and β2. This identifies a functional complex in dendrite development that consists of nNOS, sGC β1 subunit, and Scribble. I characterized the domains in Scribble that participate in these interactions. First, I have generated a Scribble mutant variant that has single domain deletion, and cloned each single domain mutant of Scribble into the mammalian expression vector pCAG fused to HA-tag, as shown in figure 2.

Next, I conducted a coimmunoprecipitation experiment to study the association between Scribble and sGC. By coexpressing different scribble mutant proteins fused to HA tag together with wild-type sGC tagged with protein flag in HEK293T cells, I investigated the differences in the stability of the complex among mutant Scirbble. As shown in figure 3, Flag immunoprecipitates Flag-sGC along with WT Scribble, LRR, LRR, and IMR mutants, but not PDZ+CTERM, CTERM, and PDZ scribble mutants.This observation suggests that LRR and IMR domains can both associate with sGC. However, the deletion of LRR domain in LRR mutants does not abolish the interaction. This further shows that the presence of IMR domain is essential for scribble and sGC interaction.

Inhibition of Scribble/sGC interaction interrupts dendrite development

Next I have examined the functional importance of the IMR region in Scribble and sGC interaction. I expected that the IMR mutant of Scribble will act as a dominant negative inhibitor of this interaction, which means that the expression of IMR mutant protein can interrupt the specific association of the Scribble/sGC complex without interfering with the expression of scribble. Thus, next I conducted a functional study to examine neuronal development using over-expression of the IMR mutant of Scribble.

IMR mutant Scribble was coexpressed with the wild-type protein in the

developing hippocampal neurons in culture. 3DIV hippocampal neurons were fixed and immunostained with MAP2 and Smi312 antibodies. MapII is a protein marker for dendrites and Smi312 as a protein marker for axons. Compared to the control cells that expressed the empty vector and wild type scribble, the IMR expressing neurons exhibit shorter dendrites and less number of dendrites, as shown in figure 4 and 5. I also performed a quantitive analysis of neurite length and number, using MAP2 specific labeling to identify dendrites. In figure 6, I show that the total dendrite length per cell decrease for 50% from control to IMR mutants; the number of dendrites also decreases for 40% from control to IMR mutant; each dendrite also has 40-50% decrease length by comparing the control with the IMR mutants. The effect of IMR mutant does not affect the axon length and numbers. In all, this functional study has shown that expression of IMR mutants can inhibit dendrite development.

However, we can not conclude that IMR has a dominant negative effect on Scribble in regulating dendrite development. The next step is to knock down Scribble protein in cultured hippocampal neurons and to compare the effect of both manipulations on dendrite development. Therefore, we constructed shRNA against the rat Scrib gene. We introduced either the shScribble or empty shRNA vector into cultured dissociated rat hippocampal neurons. In this experiment, we observed similar effect as IMR mutant inhibition. The shScribble decreases dendrite numbers and length in dissociated hippocampal neurons as shown in figure 7. In figure 8, I compare the number and length of axons, dendrites, and neurites among the control and shScribble introduced cells. The shScribble has decreased the number of dendrites per cell by 10% while the length of dendrites per cell has decreased by 35%. The length of the average longest axon in each cell increases as well. The average length of the dendrites from longest to shortest all decrease from control to shScribble cells and the longest dendrites from each cell show an aberrant decrease for 40% .

The effect of Scribble shRNA knock down is not as clear as the effect of IMR mutant on dendrite development. A possible explanation might be that the dominant negative effect can affect the function of a scaffold protein and shRNA knockdown solely changes the protein expression level. The inhibition of function of a protein complex is more severe than its expression knockdown. We conclude that the interruption of the Scribble affect dendrite development. The next question will be whether Scribble functions through a protein complex with sGC to act as a regulator of dendrite development. So

far, I observed that IMR is the interaction domain between Scribble and sGC and IMR mutant affects dendrite development in a similar way to Scribble knockdown. Next, it is important to examine whether the knockdown of the downstream factor sGC will have a similar effect on dendrite development.

sGC knockdown inhibits dendrite development in culture hippocampal neurons

We constructed two shRNAs against rat sGC, the shsGC 5 and shsGC 15. We introduce each shsGC and the control shRNA vector into culture hippocampal neurons. I analyze the images in the same way as previous described experiments of IMR mutants and shScribble. Interestingly, I observed that shsGC 5 had a stronger effect on dendrite development with a 50% decrease in dendrite number per cell and 70% decrease in dendrite length compared to control cells. The shsGC 15 construct has a milder effect with 30% decrease in the number of dendrite per cell and 50% decrease in the length of dendrite per cell. The combination of both shRNA also has an effect of 40% decrease in the number of dendrite per cell and 64% decrease in the length of dendrite per cell. The strong effect of sGC knockdown has confirmed the hypothesis that sGC signaling and Scribble both regulate dendrite formation. Together with my finding that that IMR mutant interrupts the Scribble association with sGC, resulting in inhibition of dendrite formation, I conclude that Scribble regulates dendrite development via a protein complex formation with sGC.

Chapter 4

Discussion

In this study, I examined a protein complex consisting of Scribble and sGC in the regulation of dendrite development. I found that Scribble interacts with sGC through its IMR region and this association is essential for proper dendrite development. I observe that Scribble knockdown, sGC knockdown and the dominant negative inhibition of Scribble/sGC all decrease dendrite outgrowth or elongation during early neuronal development from cultured hippocampal neurons.

However, to further confirm the specificity of the function of Scribble in dendrite development, it would be necessary to perform overexpression experiments and rescue experiments, where I will overexpress rat scribble in dissociated hippocampal neurons and test if this will enhance dendrite outgrowth. Also, for the shScribble experiment, I can try to rescue the dendrite phenotype by activation of the sGC function. From this experiment, I can confirm that Scribble indeed regulates dendrite development by sGC downstream signaling

Now that I have tested the necessity of Scribble protein in dendrite development in dissociated neuronal culture, it would be important to examine its role in dendrite development in vivo using Scribble mutant expression. The dendrite morphology in vivo is more complex than that in dissociated culture. Scribble mutant mice exhibit neural tube defects and die before birth[11]. It is not applicable to study Scribble in a knockout animal model, but only in conditional knockout animals. With further observation from animal studies, I will understand more about the function of Scribble in regulating dendrites in early neuronal development.

The molecular mechanism that regulate dendrite development remain unclear. Here we reported that a scaffold protein Scribble shows regulation of dendrite development. Other molecules have also been reported in this regulation including extrinsic factors: Semaphorin 3A, neurotrophins, Notch and intrinsic factors such as regulators of cytoskeletal components used to promote dendrite[12, 13]. As Scribble is a scaffolding protein, it is very likely that it can also associate with other factors in dendrite development regulation. Moreover, it can be a bridge from extrinsic factors to the intrinsic program of dendrite development induction. In all, I expect more function of Scribble will be discovered in neuron development.

Figure Legends

Figure 1 Scribble associate with sGC and nNOS in rate brain lysate : E20 rat brain lysate are immunoprecipitated with different antibody. 1 total brain lysate; 2 immunoprecipitate with mouse IgG alone; 3 immunoprecipitate with sGC α1 subunit antibody;4 immunoprecipitate with sGC α2 subunit antibody;5 immunoprecipitate with sGC β1 subunit antibody;6 immunoprecipitation with nNOS antibody;7 immunoprecipitation with STRAD antibodyA) western blotting using scribble antibody, the total lysate, coimmunoprecipitation with sGC β1 subunit and coimmunoprecipitation with nNOS all show Scribble protein signal. B) western blotting using sGC α1 subunit antibody. the total lysate, sGC α1 subunit, sGC β1 subunit antibody all show the indicated sGC α1 signal. C) western blotting using sGC β1 subunit. The total lysate, sGC α1 subunit, sGC β1 subunit antibody all show the indicated sGC β1 signal. D) western blotting using sGC β1 subunit antibody and nNOS antibody. Both sGC α1 subunit and sGCβ1 subunit pull down nNOS. This experiment data is provided by Dr. Seongil Lee.

Figure 2: An illustration of the Scribble mutant constructs and Scribble functional domains: The Human Scribble mutant genes are clone in to the PCAG vectors along with an HA protein tag. The mutant clones are provided by Dr. Seongil Lee.

Figure 3: Scribble associates with sGC through the IMR domain: HA tag scribble mutants are transfected into HEK293T cells along with Flag-sGC. Two blots are shown in this figure; the blot on the left is immunoblotted with Flag antibody and detects the level of sGC in total cell lysate and immunoprecipitation samples. The blot on the right is immunoblotted with HA antibody and detects the level of Scribble mutants in both total cell lysate and immunoprecipitation samples. Each lane is one transfection of different scribble mutants with Flag-sGC. The signal of PDZ-CTERM mutant and CTERM mutant is completely lost after immunoprecipitation with Flag which means that they can not associate with sGC. The loss of LRR domain does not affect the assocaition of IMR to sGC.

Figure 4: IMR mutant of Scribble interferes with dendrite development. A-C) 3DIV E18 rat hippocampal neurons cultured with control vectors with Venus and show normal neuronal development. A GFP channel show the

complete morphology of the cell; B MAPII signal shown in red color specifies dendrites and proximal axon; C SMI312 signal specifies axonal structure in blue color.

D-E) 3DIV E18 rat hippocampal neurons cultured with IMR mutant Scribble construct with Venus and show decreased number of dendrite structures with shorter length. A GFP channel show the complete morphology of the cell; B MAPII signal shown in red color specifies dendrites and proximal axon; C SMI312 signal specifies axonal structure in blue color.

Figure 5: Soma and dendrite structures in control and IMR mutants. The MAPII channel of 3DIV E18 rat hippocampal neurons cultured with control vectors or IMR mutants. Dendrite number and length are decreased from control to IMR mutant expressed cells.

Figure 6: The Quantitive analysis of the effects of IMR mutants on dendrite development. A) the total length measurement of axons, dendrites and neurites is divided by cell number. Total length of the axons per cell has no difference between control cells and IMR cells. However, total dendrite length per cell decrease by 50% and total neurite length per cell decrease by 20%.

B) The average primary axon length

C) The number of axons, dendrite and neurites averaged by cell number.

D) The individual dendrite length comparison between control cells and IMR mutant introduced cells. The dendrites are number according to their length. The error bar is obtained for the difference in two repetition experiments.

Figure 7: Scribble protein knockdown affects dendrite development in culture hippocampal neurons. A-C) 3DIV E18 rat hippocampal neurons cultured with control vectors with Venus and show normal neuronal development. A GFP channel show the complete morphology of the cell; B MAPII signal shown in red color specifies dendrites and proximal axon; C SMI312 signal specifies axonal structure in blue color.

D-E) 3DIV E18 rat hippocampal neurons cultured with shScribble construct with Venus targeted to Scribble and show decreased number of dendrite structures with shorter length. A GFP channel show the complete morphology of the cell; B MAPII signal shown in red color specifies dendrites and

proximal axon; C SMI312 signal specifies axonal structure in blue color.

Figure 8: The Quantitive analysis of the effects of Scribble knock-down on dendrite development. A) the total length measurement of axons, dendrites and neurites is divided by cell number.

B) The number of axons, dendrite and neurites averaged by cell number.

C) The average primary axon length

D) The individual dendrite length comparison between control cells and shScribble introduced cells. The dendrites are number according to their length.

Figure 9: sGC knockdown disturbs dendrite development in culture hippocampal neurons.First lane: 3DIV E18 rat hippocampal neurons cultured with control vectors with Venus and show normal neuronal development. A GFP channel show the complete morphology of the cell; B MAPII signal shown in red color specifies dendrites and proximal axon; C SMI312 signal specifies axonal structure in blue color.

Second lane: 3DIV E18 rat hippocampal neurons cultured with shsGC construct no.5 with venus targeted to sGC and show decreased number of dendrite structures with shorter length. A GFP channel show the complete morphologuy of the cell; B MAPII signal shown in red color specifies dendrites and proximal axon; C SMI312 signal specifies axonal structure in blue color.

Third lane: 3DIV E18 rat hippocampal neurons cultured with shsGC construct no.15

Fourth lane: 3DIV E18 rat hippocampal neurons cultured with shsGC construct no.15 and shsGC construct no.5.

Figure 10: The Quantitive analysis of the effects of sGC knockdown on dendrite development. A) The number of axons, dendrite and neurites averaged by cell number.

B) the total length measurement of axons, dendrites and neurites is divided by cell number.

C) The average primary axon length

D) The individual dendrite length comparison between control cells and shsGC introduced cells. The dendrites are number according to their length.

Figure 1

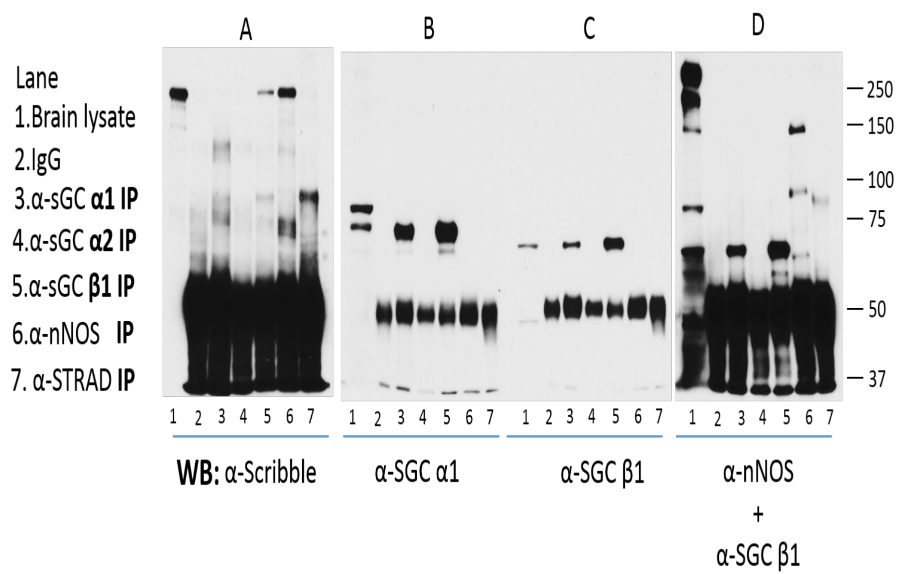
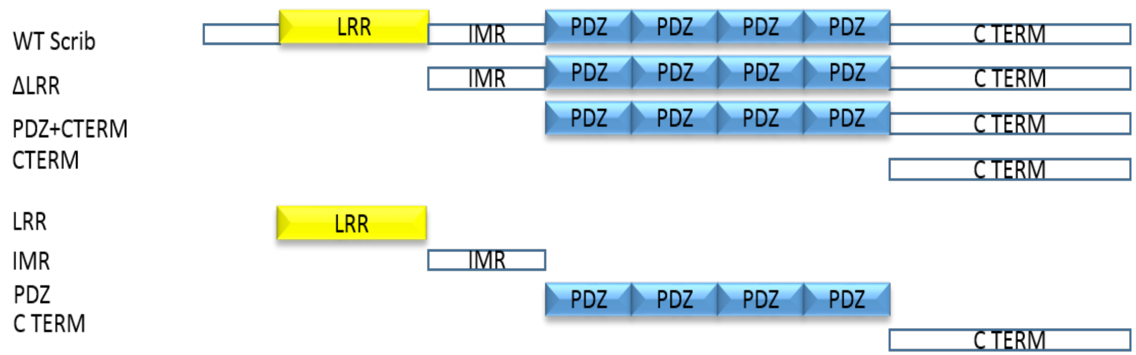


Figure 2



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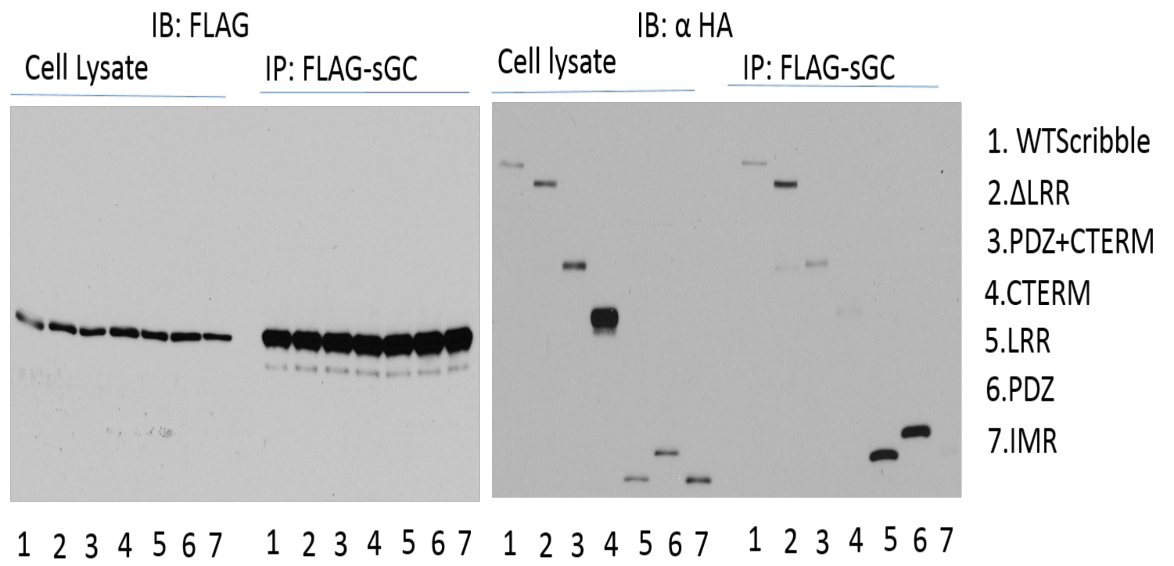

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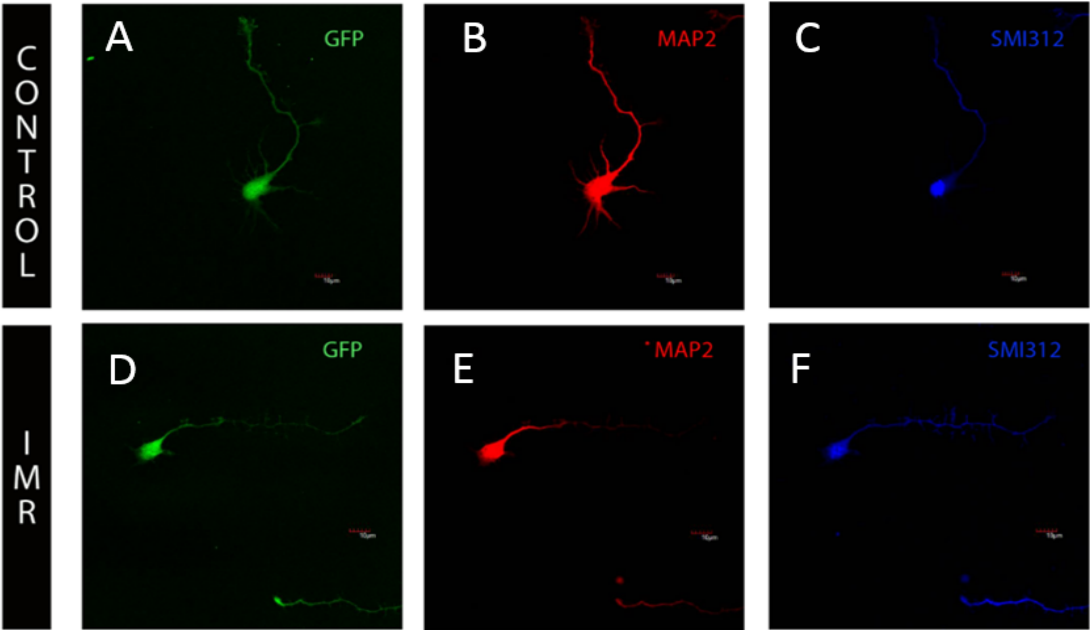

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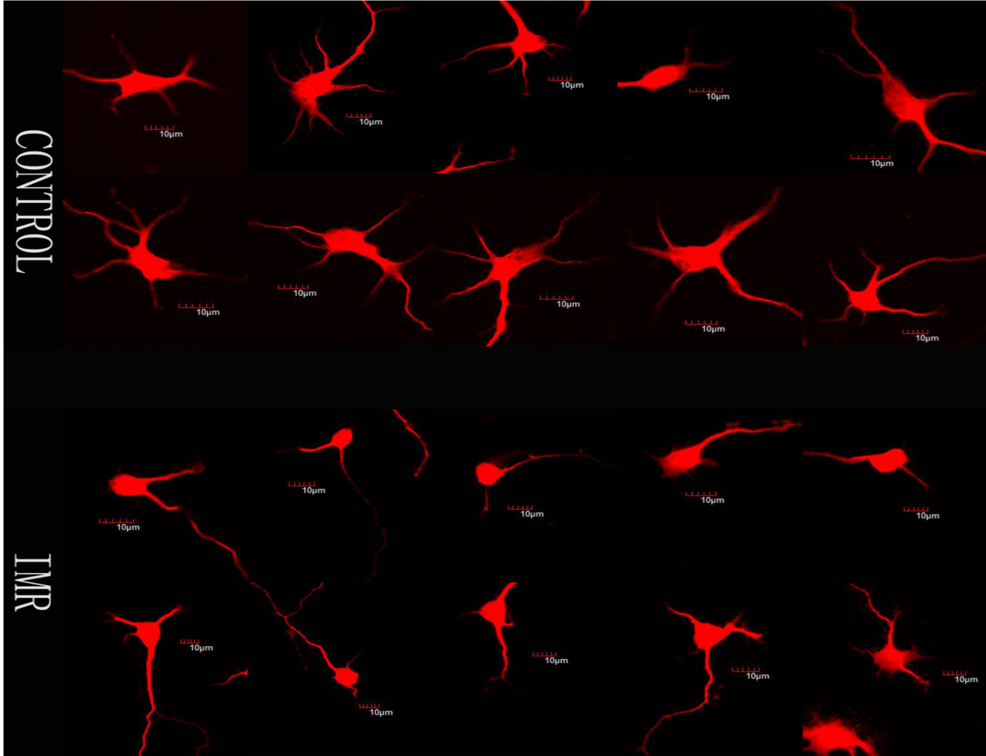

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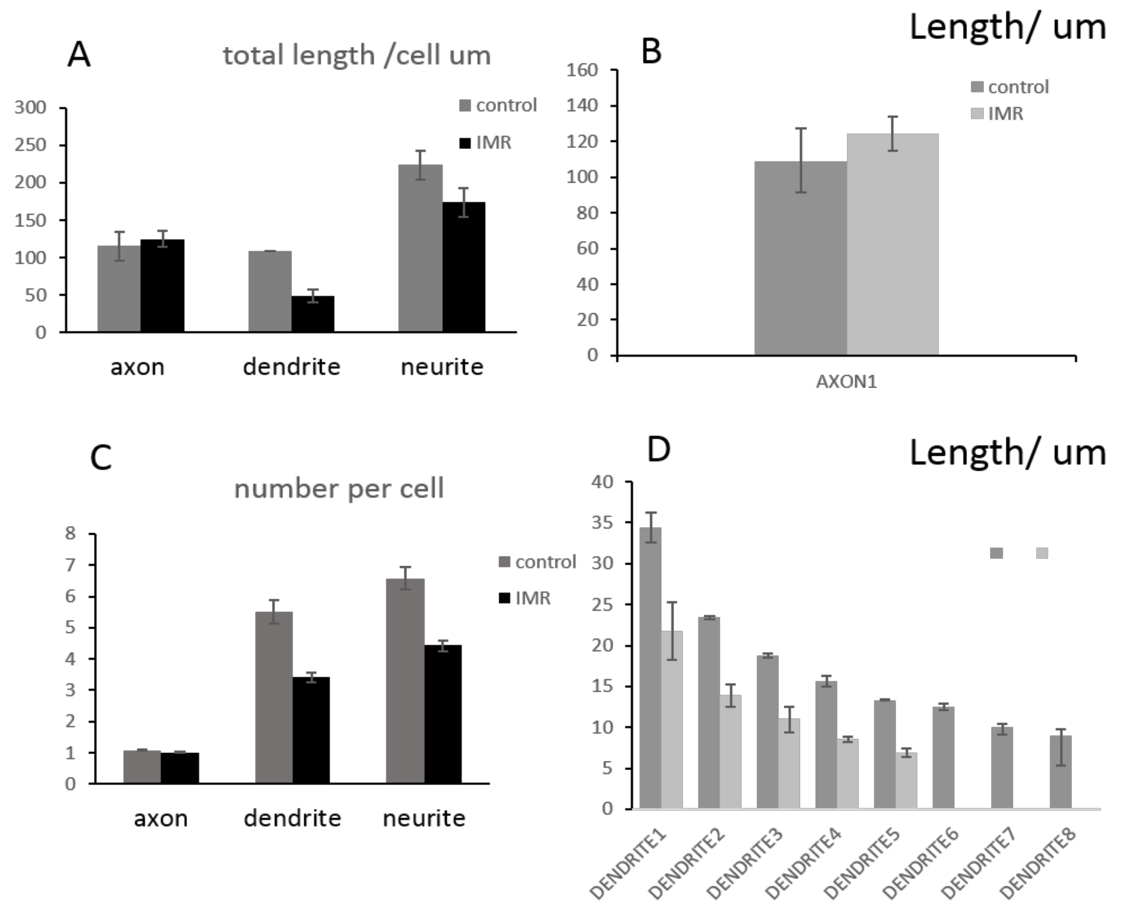


Figure 7

Dissociated embryonic rodent Hippocampal neurons

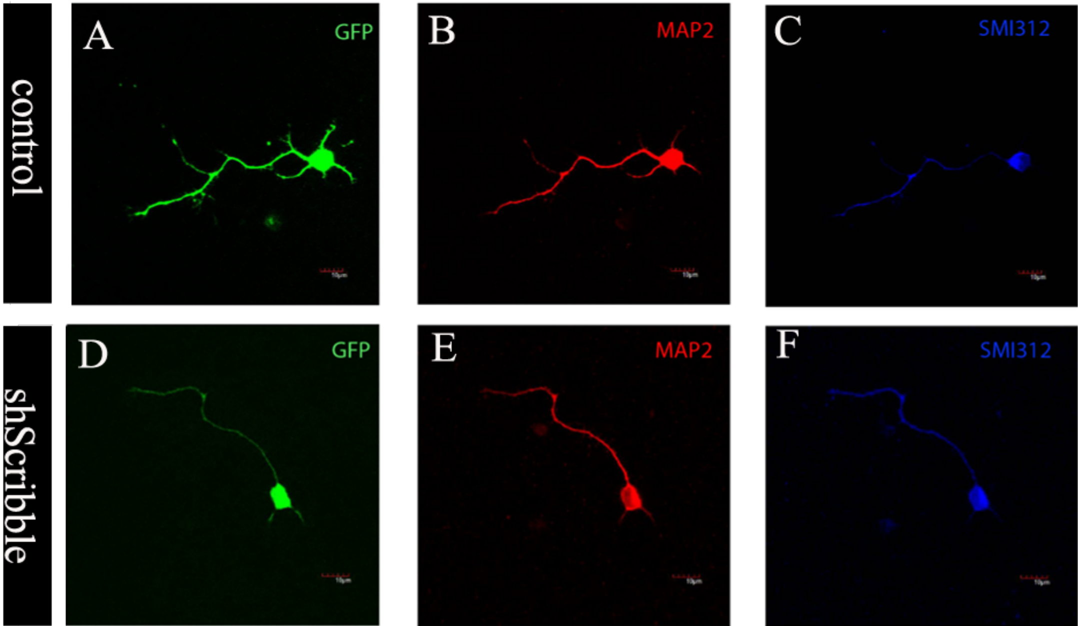


Figure 8

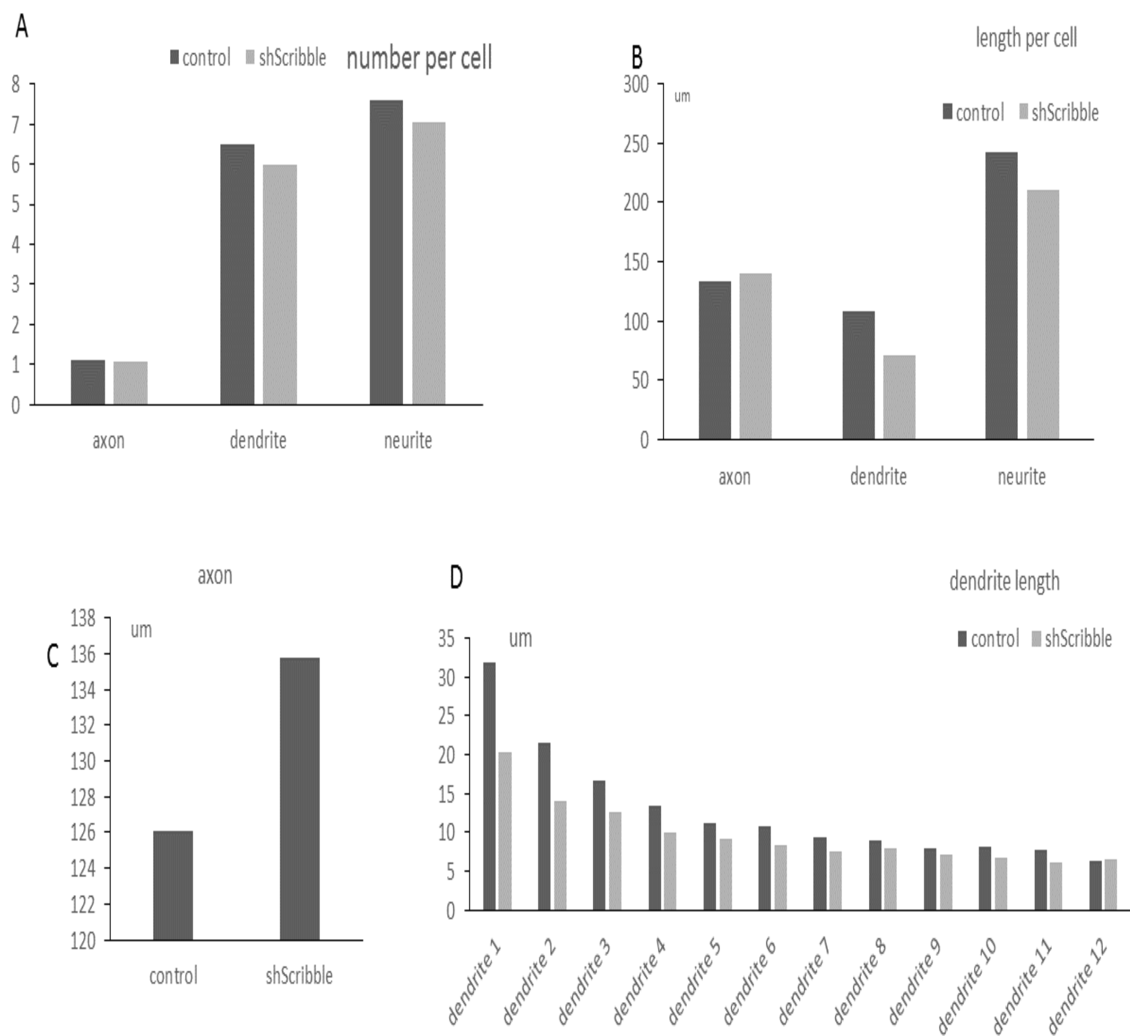


Figure 9

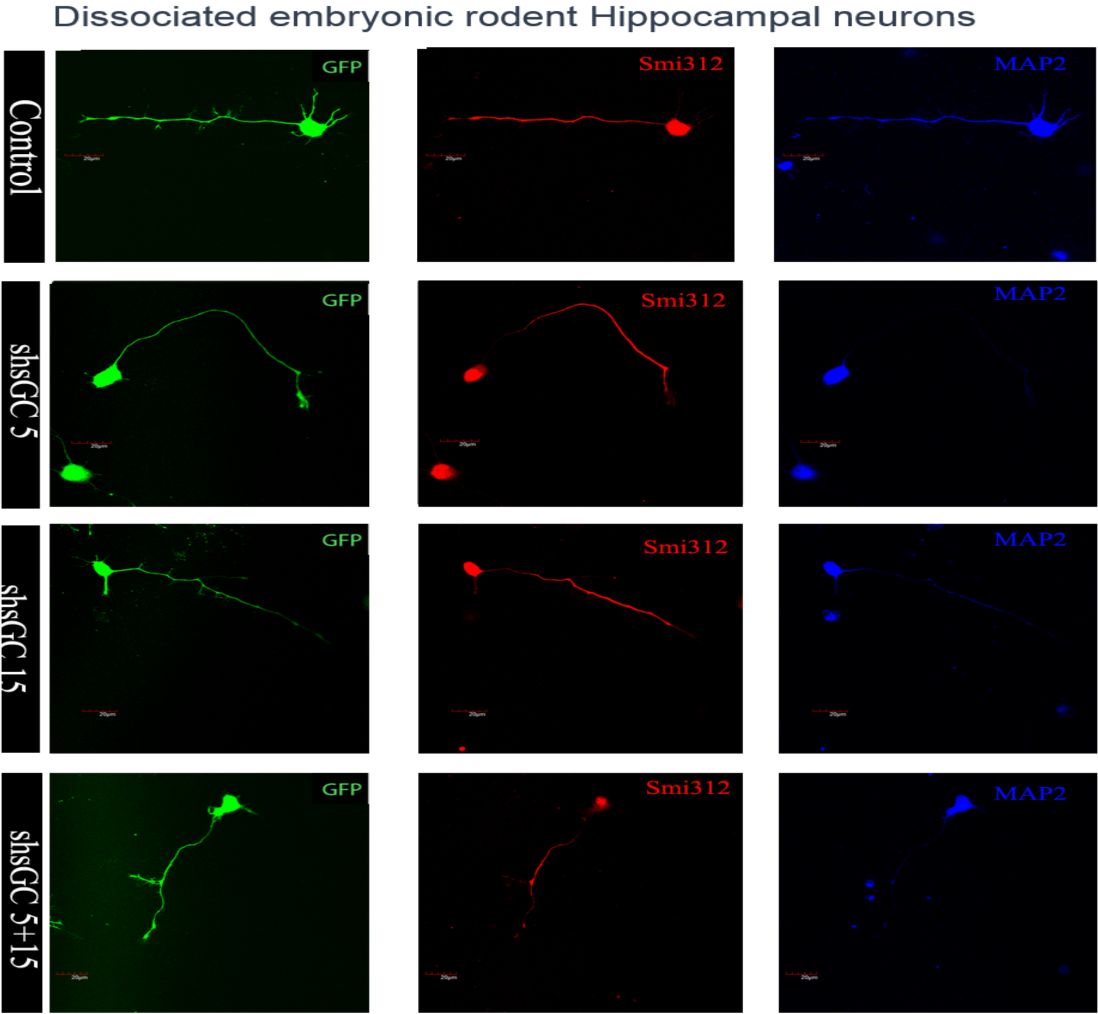
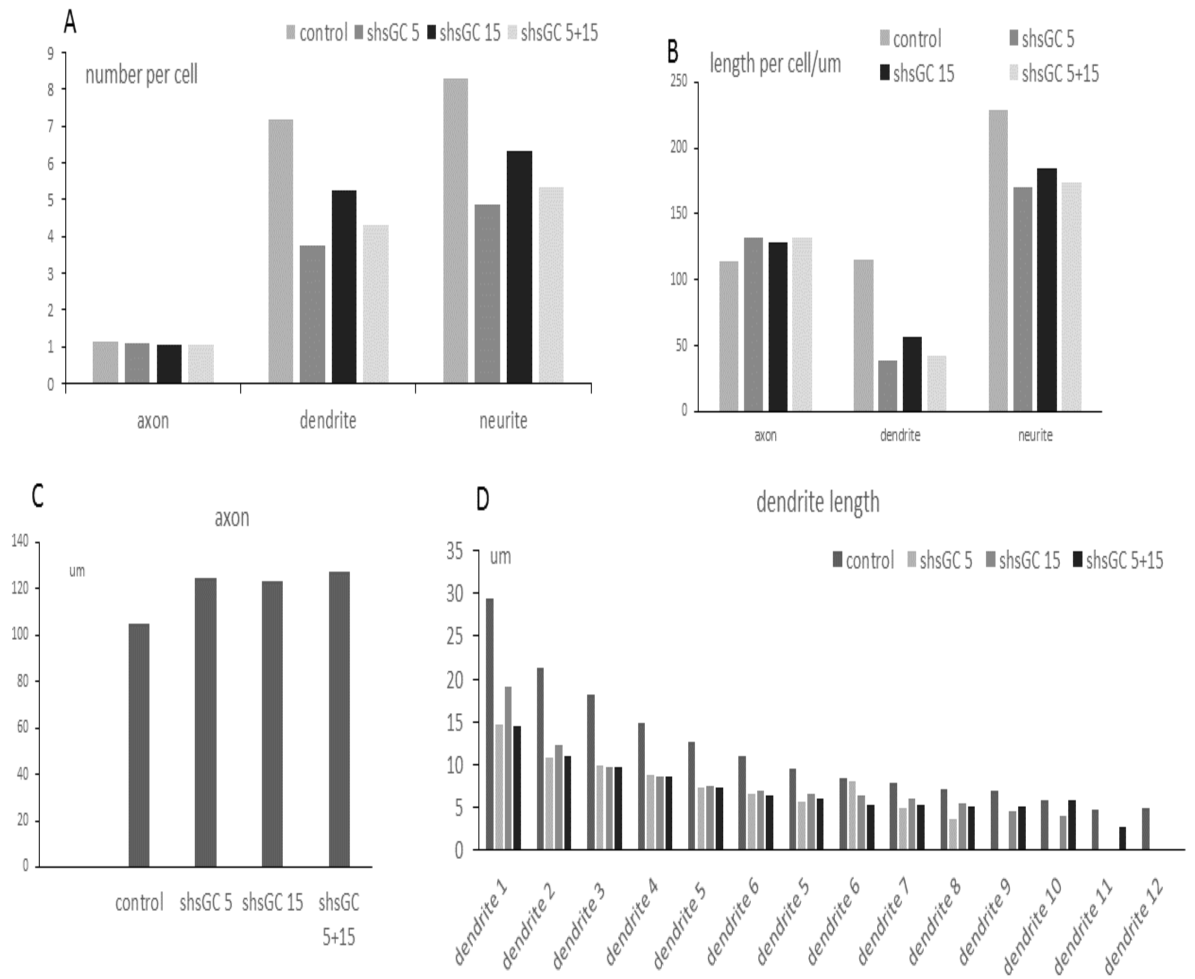


Figure 10



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