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Cooperative effect of p150Glued and microtubule stabilization to suppress excitotoxicity-induced axon degeneration

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ABSTRACT

Glutamate excitotoxicity is implicated in chronic neurological disorders and acute CNS insults and causes neuronal degeneration including axons. The molecular mechanism underlying excitotoxicity-induced axon degeneration is poorly understood. Recently, we found that components of the dynein–dynactin complex that governs microtubule-dependent retrograde transport play important roles in modulating the process of excitotoxicity-induced neurodegeneration. Here we used hippocampal cultures and searched for pathways that function in concert with the components of the dynein–dynactin complex and identified microtubule stabilization as a cooperative pathway to suppress axon degeneration. We find that overexpression of p150Glued, a major component of the dynactin complex, and microtubule stabilization cooperatively suppress axon degeneration. The protective effect of p150Glued is dependent on the C-terminal region as excitotoxicity-induced C-terminal truncated form of p150Glued was unable to interact with APP cargo and altered the localization of APP in neurites when overexpressed. C-terminal truncation of p150Glued is not rescued by microtubule stabilization suggesting that the downstream effects of p150Glued and microtubule stabilization to protect axon degeneration are mutually exclusive.

1. Introduction

Glutamate is an excitatory neurotransmitter in the CNS that plays a pivotal role in long-term potentiation and cognitive functions such as learning and memory. However, prolonged exposure to excessive glutamate hyperactivates glutamate receptors and triggers neurodegenerative processes (excitotoxicity), including morphological changes in the axon [1]. Excitotoxicity is linked to chronic neurological disorders, including Alzheimer's disease and amyotrophic lateral sclerosis, and acute CNS insults. The mechanism underlying excitotoxicity is complex. Hyperactivated NMDA receptors trigger calcium influx [2], and lead to numerous events that are detrimental to normal neuronal function, including acute mitochondrial dysfunction and free radical production [3]. Given the complexity and the growing need for effective pharmacological modulators of excitotoxicity, it is important to identify converging mechanisms that could modulate excitotoxicity-induced neurodegenerative processes.

Neurodegeneration includes axon degeneration and cell body death. These two degenerative processes of distinct compartments are morphologically illustrated by swelling (beading) and/or transection of axons and swelling or shrinkage of the cell body [1,4]. Whether these two events are interconnected or distinct as well as the causal relationship is still unclear [5]. However, previous reports have shown that genetically inhibited apoptosis protects cell body death, but not the axon in a mouse model of progressive motor neuropathy and in superoxide dismutase 1 transgenic mice supporting the idea that axon degeneration and cell body death are distinct events [6,7]. Thus, degeneration of axons could be triggered by modulation in the molecular pathways that are distinct from those that trigger cell body death.

A key molecular feature of axon degeneration is deficits in axonal transport. Kinesins anterogradely transport cargos from the cell body to the distal axon, and the dynein–dynactin complex retrogradely transports cargos from the distal axon to the cell body. These microtubule-based motors supply newly synthesized materials to distal synapses, clear away misfolded/aggregated proteins from the axon, and deliver signals initiated by environmental changes from the distal axon to the cell body [8–10]. Recently, we identified p150Glued, a major component of the dynactin complex,

Abbreviations: a.a., amino acid; APP, amyloid precursor protein; CNS, central nervous system; DIC, dynein intermediate chain; DIV, days in vitro; GFP, green fluorescent protein; NMDA, *N*-methyl-D-aspartate; WT, wild type.

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and DIC, a major component of the dynein complex that interacts with p150Glued and links dynein and dynactin complexes, as novel modulators of excitotoxicity-induced neurodegeneration [11]. Excitotoxicity generates a C-terminal truncated form of p150Glued that exacerbates excitotoxicity-induced axon degeneration. Moreover, overexpression of WT p150Glued and WT DIC suppress excitotoxicity-induced axon degeneration. However, how these modulating effects are exerted and whether there are other degeneration modulating pathways that function in concert with p150Glued and DIC are unknown.

We developed an excitotoxicity-induced axon degeneration system in primary hippocampal cultures using a microfluidic device and show that stabilization of axonal microtubules by taxol suppresses axon degeneration. We find that axon degeneration is significantly suppressed by both overexpression of WT p150Glued and taxol treatment compared with overexpression of WT p150Glued or taxol treatment alone. Excitotoxicity-induced C-terminal truncation of p150Glued is not rescued by taxol treatment. We find that APP co-immunoprecipitates with WT p150Glued but not with the C-terminal truncated p150Glued. As a result, APP localization in neurites is altered in neurons overexpressing the C-terminal truncated p150Glued. Thus, our findings identify stabilization of microtubules as a pathway that cooperatively function with p150Glued and that the downstream effects of p150Glued and microtubule stabilization could be mutually exclusive in attenuating excitotoxicity-induced axon degeneration.

2. Materials and methods

2.1. cDNA cloning and expression vectors

Methods for cloning rat p150Glued cDNA and the generation of GFP–p150Glued WT (1-1,280 a.a.) and the GFP–p150Glued C-terminal truncated form (1-931 a.a.) in pEGFP-C1 vector (Clontech) are previously described [11].

2.2. Antibodies

Primary antibodies used are rat monoclonal anti-GFP (Nacalai Tesque), rabbit polyclonal anti-GFP (MBL), mouse monoclonal anti-p150Glued (BD Biosciences, N-terminal recognition), mouse monoclonal anti-p50 (BD Biosciences), rabbit polyclonal anti-α-tubulin (Abcam), mouse monoclonal anti-βIII-tubulin (Abcam), and rabbit polyclonal anti-APP (SIGMA). Secondary antibodies are mouse IgG TrueBlot Ultra or rabbit IgG TrueBlot (eBioscience), horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling), and Alexa Fluor® 488, 568, or 647 fluorescents (Invitrogen).

2.3. Neuron culture, immunofluorescence, and transfection

Preparation of primary Wistar rat hippocampal neurons and culture conditions for indirect immunofluorescence labeling of transfected neurons were performed as previously described [11]. Procedures were approved by the Osaka University Institutional Guidelines for the Care and Use of Laboratory Animals. Transfection was performed using AMAXA Nucleofector transfection system (Lonza) with 4 μg of plasmid DNA and 3×10^6 of cells. Images were captured by confocal laser microscopy FV1000 system (Olympus) with 20× and 40× oil-immersion objective lenses.

2.4. Compartmentalized neuron culture

Treatments with 100 nM Paclitaxel (taxol, SIGMA), 50 μM glutamate (SIGMA), or DMSO (SIGMA) on axons were achieved by AXIS™ device, 450 μm (Millipore) placed on poly-L-lysine-coated cover glasses (Matsunami). Culturing conditions were performed mainly under manufacture's instruction [12,13]. To quantify beading axons, 7–10 of 630 μm width grid aligned with 50 μm interval was overlayed to images of axons in the channel connected to wells III and IV. βIII-tubulin-labeled axons crossing each line were counted, summed, and the ratio of beading axons was quantified for each condition.

2.5. Western blot detection and immunoprecipitation

Cell lysates were obtained and quantified as previously described [11]. Samples for SDS–PAGE were prepared in sample buffer, boiled, and loaded onto 10 % polyacrylamide gel, transferred to nitrocellulose membranes, further blotted and detected [11]. Blotted membranes were subjected to new rounds of probing using WB Stripping Solution (Nacalai Tesque). Immunoprecipitation of p50 dynamitin was performed with 20–25 μg of lysates from 8 DIV hippocampal cultures added with 10 μg of either normal mouse IgG (Millipore) or p50 mouse monoclonal antibody, incubated for 2 h at 4 °C, and the antigen–antibody complex was captured by Protein G Sepharose 4 Fast Flow Beads (GE Healthcare) by further incubation for 1 h at 4 °C. For detection of APP, GFP–p150Glued constructs were immunoprecipitated using 80–220 μg of lysates from transfected and untransfected 293 cells added with 4 μg of GFP rabbit polyclonal antibody. Following 2 h incubation at 4 °C, GFP–p150Glued construct-antibody complex was captured by Protein G Sepharose 4 Fast Flow Beads by 1 h incubation at 4 °C, and the captured complexes were further incubated with 130 μg of hippocampal culture lysates for 2 h at 4 °C.

2.6. Neurite beading and APP dot analyses

Quantification of bead-containing neurites was performed as previously described [11]. Neurons at 8 DIV treated with 50 μM glutamate, 100 nM taxol, and vehicles for 3 h were subjected for quantification. For APP-labeled dot analysis, (1) APP dot was defined by using FV10-ASW1.7 software (Olympus), images were captured with the same exposure and the signal intensity of a dot labeled with the APP antibody was "more than 1500." When the signal intensity of the neurite shaft labeled with the APP antibody adjacent to the dot was "0", the dot was defined as "an APP dot," (2) 50 μm GFP and βIII-tubulin-positive neurite was defined as one segment, (3) more than 5 APP dots per segment was defined as APP dot-containing neurite, and (4) two segments were analyzed per neuron. Neurons with both segments as APP dot-containing neurite were regarded as APP dots-containing neurons.

2.7. Statistics

All statistical analyses were done by one-way ANOVA with Tukey–Kramer post test. In all instances, a value of $p < 0.05$ was considered significant.

3. Results

3.1. Microtubule stabilization suppresses axon degeneration

A focal bead-like swelling phenotype in neurites is an early neurodegenerative feature in acute and chronic neurological disorders [14]. In order to elucidate the significance of microtubule-based functions on excitotoxicity-induced axon degeneration, we asked whether microtubule stabilization in axons affects the process of axon degeneration by analyzing the axonal beading phenotype. For this purpose, we used a microfluidic device to

compartmentalize axons [12,13]. The device allows testing of treatments specifically on axons that extend into the channel III–IV that connects wells III and IV (Fig. 1A). Dissected rat hippocampal preparation of 5×10^4 cells was seeded in the channel I–II that connects wells I and II (Fig. 1A). To facilitate the extension of axons through the microgrooves located between the two channels, 200 μl of culture medium was added to wells I and II, and 100 μl to wells III and IV to create hydrostatic pressure (Fig. 1A). At 8 DIV, axons sufficiently occupied the channel III–IV suitable for further analyses. Taxol is a well-established compound that selectively stabilizes microtubules and has been extensively used in studies of microtubule-based functions [15]. We administered 100 nM taxol or DMSO to the channel III–IV and 50 μM glutamate or vehicle to the channel I–II simultaneously for 3 h (Fig. 1B). We found that glutamate treatment on the cell body/dendrite compartment (channel I–II) induced axonal beading and that simultaneous treatment with taxol on the axonal compartment (channel III–IV) suppressed axonal beading compared with DMSO treatment (Fig. 1B). To quantify the axonal beading phenotype, 7–10 of 630 μm width lines aligned with 50 μm interval was overlayed to images of axons in the channel III–IV and axons crossing each line were analyzed (Fig. 1C, right). Approximately 4.7 of 10 axons showed a beading phenotype with simultaneous glutamate (channel I–II) and DMSO (channel III–IV) treatments, whereas glutamate (channel I–II) and taxol (channel III–IV) treatments showed approximately 2.6 of 10 axons, a more than 40% suppression by taxol compared with DMSO (Fig. 1C, left). Treatments with 50 μM glutamate or 100 nM taxol (channel III–IV) and vehicle (channel I–II) for 3 h had subtle effects showing approximately 0.7 of 10 or 0.2 of 10 axons with a beading phenotype, respectively (Fig. 1C, left). This result indicates that stabilization of axonal microtubules suppresses excitotoxicity-induced axon degeneration.

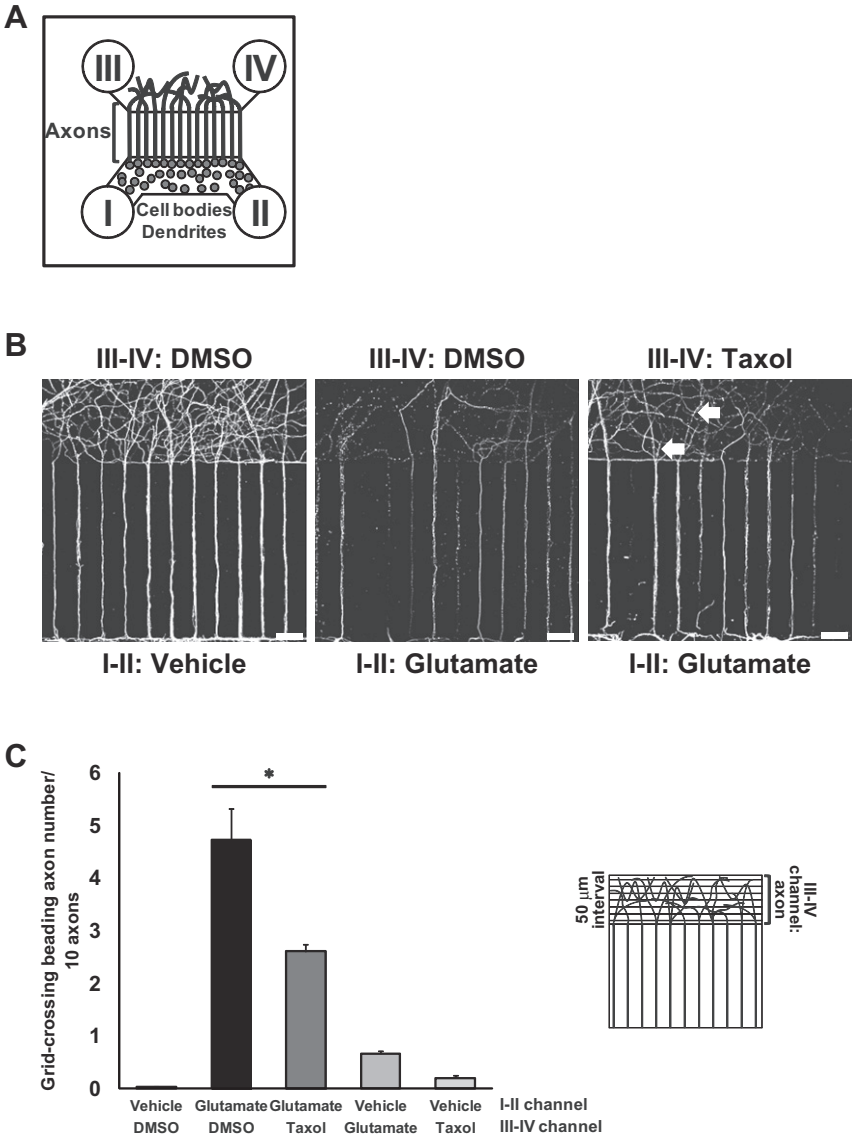

Fig. 1. Axonal microtubule stabilization suppresses axon degeneration. (A) Compartmentalized axons using a microfluidic device. Neurons seeded in the channel, connecting wells I–II, extend axons through microgrooves toward the channel connecting wells III–IV. (B) Images of representative compartmentalized axons treated with vehicle, DMSO, 50 μM glutamate, and 100 nM taxol, indicated within the images. Taxol treatment on axons suppressed degeneration (arrows) compared with DMSO treatment. Bars: 50 μm. (C) Quantification of beading axons in the channel III-IV (left). Grids of 50 μm interval were overlayed to each image and beading axons crossing each grid were quantified (right). Axonal treatment of taxol suppressed glutamate-induced degeneration (*$p < 0.01$) compared to DMSO. Statistics represent mean ± SEM of 3–6 independent images and total of >692 axons counted for each condition.

3.2. p150Glued C-terminal truncation alters cargo localization in axons

Excitotoxicity generates a C-terminal truncated form of p150Glued, a major component of the dynactin complex, that exacerbates axon degeneration [11]. Since taxol suppresses axonal beading (Fig. 1B and C), we addressed whether microtubule stabilization could rescue p150Glued from truncation. Simultaneous treatment with 50 μM glutamate and 100 nM taxol did not rescue p150Glued from truncation as detected by an antibody that recognizes the N-terminal region of p150Glued (Fig. 2A). This result indicates that taxol-mediated microtubule stabilization does not function upstream of p150Glued. The dynactin complex has previously been shown to facilitate dynein-mediated retrograde transport of cargos [16–19]. p150Glued binds microtubules at the N-terminal and p50, a component of the dynactin complex, at the C-terminal region which links to cargos [20]. To elucidate the significance of the C-terminal truncated p150Glued and cargo interaction, we tested the binding ability of the endogenous C-terminal truncated p150Glued with endogenous p50. Neurons were treated with 50 μM glutamate or vehicle for 3 h and immunoprecipitation of endogenous p50 was performed. We found co-immunoprecipitation of endogenous WT p150Glued with p50 but not the C-terminal truncated p150Glued (Fig. 2B). This result suggests that the C-terminal truncated p150Glued is unable to interact with cargos. Amyloid precursor protein (APP) is a known cargo for fast axonal transport and impaired transport shows accumulated APP vesicular staining (APP dots) in neurites which is rarely observed in normal neurites [1,21,22]. We recently cloned rat p150Glued and generated a C-terminal truncated mutant of p150Glued that resembles the molecular weight of the endogenous

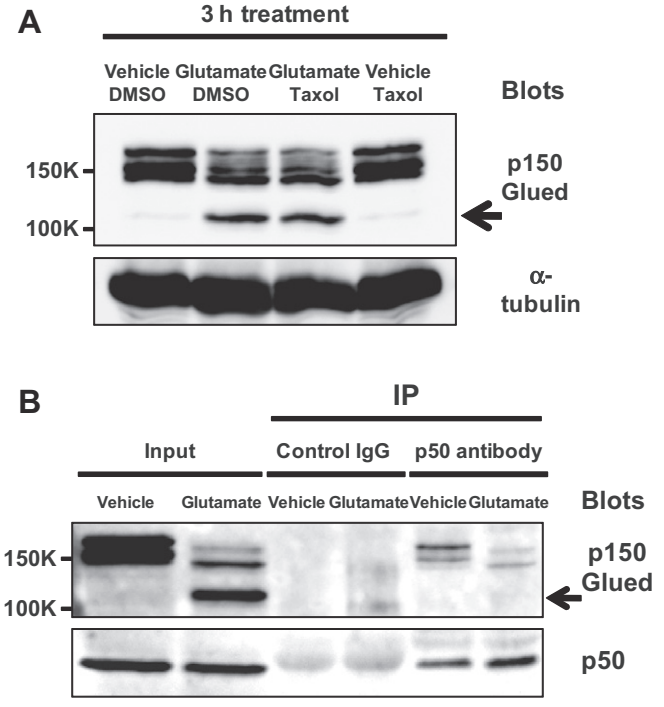

Fig. 2. Microtubule stabilization cannot suppress p150Glued truncation. (A) Western blot detections of p150Glued and α-tubulin on a single sheet using lysates from hippocampal cultures treated as indicated for 3 h. (B) Immunoprecipitation (IP) of endogenous p50 from hippocampal cultures treated with 50 μM glutamate or vehicle for 3 h. Co-immunoprecipitation of endogenous WT p150Glued was observed but not with the truncated form (arrow). Western blot detections were performed on a single sheet. Detection of p150Glued was performed with the N-terminal-recognizing antibody.

C-terminal truncated p150Glued [11]. We used GFP-fused p150Glued WT (1-1,280 a.a.) and the C-terminal truncated form (1-931 a.a.) in this study (Fig. 3A), and assessed whether APP co-immunoprecipitates with these GFP–p150Glued constructs (Fig. 3B). We found that APP co-immunoprecipitates with GFP–p150Glued WT but not with the C-terminal truncated form (Fig. 3B). The impact of GFP–p150Glued C-terminal truncated form overexpression on the localization of endogenous APP was further assessed. For this purpose, transfected neurons had to be visually isolated. Transfection efficiency was approximately 70% as evaluated by indirect immunofluorescence using anti-GFP antibody (unpublished observation, T.F. and K.M.), therefore analyses observing the transfected population should represent the majority of the neuronal cultures. Overexpression of the GFP–p150Glued C-terminal truncated form increased approximately 2.5-fold the number of neurons with endogenous APP dots in neurites compared with neurons overexpressing control GFP or GFP–p150Glued WT (Fig. 3C and D). These results indicate that the C-terminal truncated p150Glued cannot interact with cargos and alters the localization of cargos in neurites.

3.3. Overexpression of p150Glued and microtubule stabilization cooperatively suppress axon degeneration

Microtubule stabilization cannot rescue p150Glued from truncation and yet overexpression of WT p150Glued protects axons from degeneration. This generates a hypothesis that p150Glued function and microtubule stabilization pathway could act cooperatively for protection of axons against degeneration. We examined this hypothesis by quantifying the neurite beading phenotype of visually isolated neurons overexpressing GFP–p150Glued WT and control GFP simultaneously treated with 50 μM glutamate and 100 nM taxol. Overexpression of GFP–p150Glued WT suppressed approximately 30% the number of neurons with beading neurites compared with that of control GFP-overexpressing neurons in 3 h glutamate incubation (Fig. 4). Control GFP-overexpressing neurons simultaneously treated with glutamate and taxol for 3 h suppressed approximately 30% the number of neurons with beading neurites compared with that of control GFP-overexpressing neurons treated with glutamate for 3 h (Fig. 4). GFP–p150Glued WT-overexpressing neurons simultaneously treated with glutamate and taxol for 3 h suppressed more than 55% the number of neurons with beading neurites compared with that of control GFP-overexpressing neurons treated with glutamate for 3 h (Fig. 4). In addition, GFP–p150Glued WT-overexpressing neurons simultaneously treated with glutamate and taxol for 3 h suppressed approximately 40% the number of neurons with beading neurites compared with that of GFP–p150Glued WT-overexpressing neurons treated with glutamate, and control GFP-overexpressing neurons simultaneously treated with glutamate and taxol (Fig. 4). This result indicates that overexpression of p150Glued and microtubule stabilization functions cooperatively to protect axons from excitotoxicity-induced degeneration.

4. Discussion

Our results demonstrate for the first time that increased axonal microtubule stability protects axons from degeneration induced by glutamate excitotoxicity. Importantly, overexpression of WT p150Glued further protects axons from excitotoxicity-induced degeneration by a cooperative effect with the microtubule stabilization. Excitotoxicity-induced C-terminal truncated p150Glued is dysfunctional as it is unable to interact with APP cargo and alters the localization of APP in neurites when overexpressed. Thus our results support the idea that both the stability of microtubules

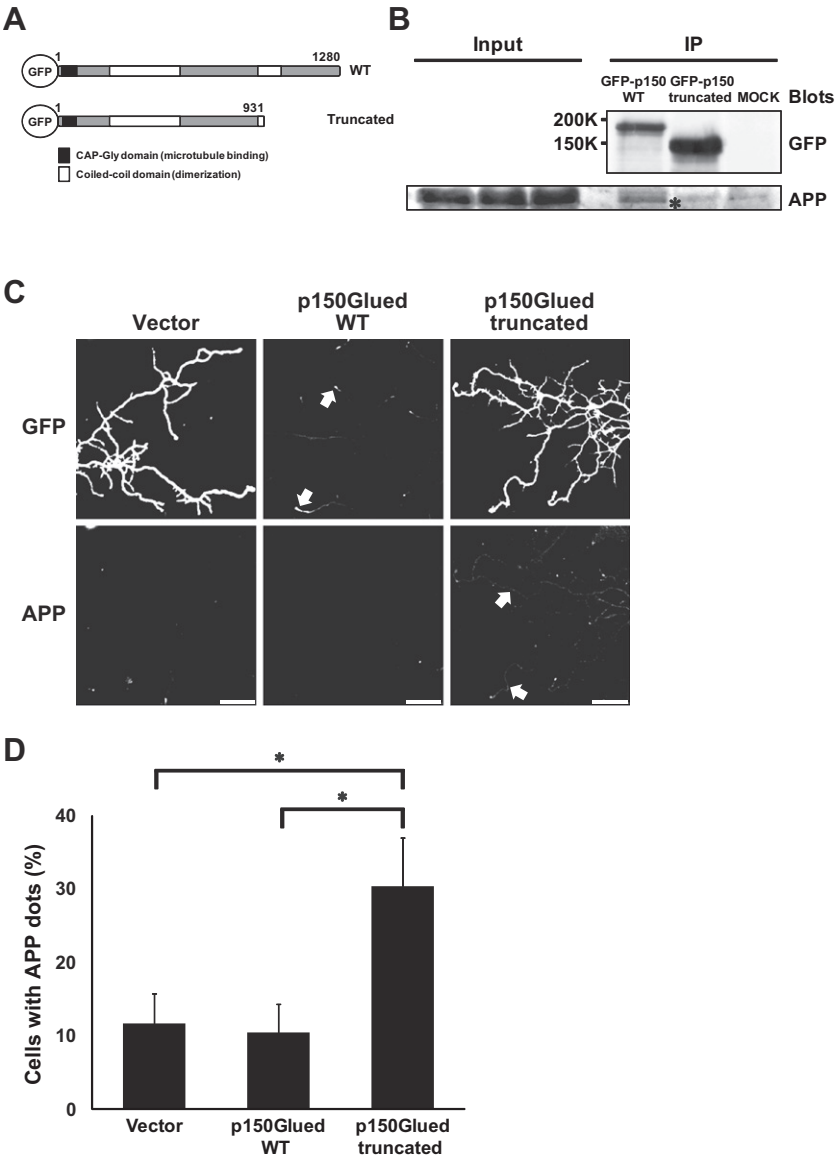

Fig. 3. C-terminal truncated p150Glued cannot interact with APP and alters APP localization. (A) Schematic representation of WT (1,280 a.a.) and the C-terminal truncated (931 a.a.) p150Glued N-terminally fused to GFP. (B) Co-immunoprecipitation of APP with GFP–p150Glued WT (asterisk). Immunoprecipitated (IP) GFP–p150Glued WT and C-terminal truncated form by polyclonal GFP antibody were further loaded with 130 μg of lysate from hippocampal cultures for each sample to detect APP (input and IP). Western blot detections were performed on a single sheet. (C) Representative images of neurons transfected with vector, GFP–p150Glued WT, and GFP–p150Glued C-terminal truncated form, labeled for GFP and APP. GFP–p150Glued WT accumulates at distal neurites (arrows). APP dots are present in neurites overexpressing the C-terminal truncated form (arrows). Bars: 40 μm. (D) Quantification of the number of neurons with APP dots in neurites (>218 cells for each condition). Neurons transfected with vector, WT, and C-terminal truncated form, showed 11.7%, 10.4%, and 30.4%, respectively (*$p < 0.01$) Statistics represent mean ± SEM of three independent experiments.

and microtubule-based retrograde transport in axons are compromised by glutamate excitotoxicity and that protection of both of these functions is required to maintain axonal integrity.

Taxol stabilizes microtubules by binding to tubulin and lowers the critical concentration required for polymerization of tubulin [23,24]. The taxol-containing microtubules are resistant to concentrations of intracellular calcium that normally depolymerize microtubules. This explains our results that taxol-induced axonal microtubule stabilization prevents degeneration of axons induced by excitotoxicity which is known to trigger excessive calcium influx [2]. Taxol has also been shown to protect cell body death of neurons induced by excitotoxicity and β-amyloid in hippocampal and cortical neuronal cultures, respectively [25,26]. Thus, stabilization of microtubules does protect both axon and the cell body from toxic effects induced by glutamate excitotoxicity.

The generation of C-terminal truncated p150Glued was not suppressed by taxol treatment indicating that pathways activated by taxol to protect axons from degeneration are not upstream but are likely to lie downstream or parallel of p150Glued function. From our results, the degree of protection against axon degeneration in neurons overexpressing WT p150Glued was similar to that in taxol-treated neurons overexpressing control GFP suggesting that p150Glued function and microtubule stabilization exert their effects through a linear pathway. This idea is supported by the findings in cultured non-neuronal cells showing that overexpression of WT p150Glued bundles microtubules by promoting the nucleation step [27], placing p150Glued function upstream of microtubule stabilization. However, as taxol-treated neurons overexpressing WT p150Glued show greater protective effect against axon degeneration compared with either taxol-treated neurons

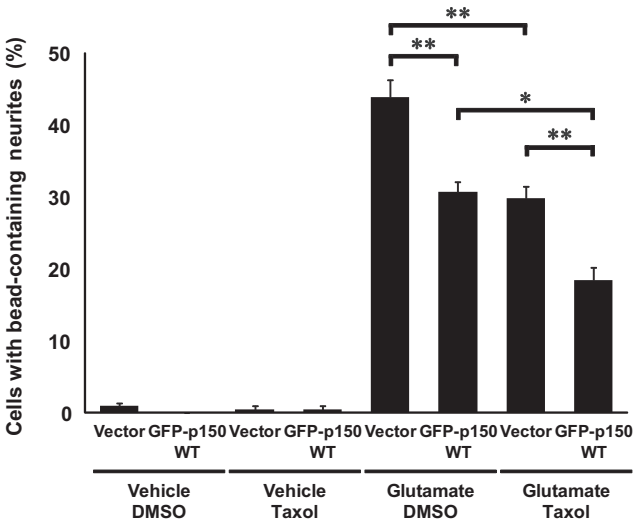

Fig. 4. Cooperative effect of WT p150Glued overexpression and microtubule stabilization on axon degeneration. Vector and GFP–p150Glued WT-transfected neurons treated with glutamate and taxol for 3 h labeled for GFP, βIII-tubulin, and DAPI. Quantification of neurons containing beading neurites (>164 cells counted for each condition). Glutamate-treated neurons transfected with vector and GFP–p150Glued WT, glutamate and taxol-treated neurons transfected with vector and GFP–p150Glued WT showed 43.9%, 30.7%, 29.8%, and 18.4%, respectively ($^{*}p < 0.01$, $^{**}p < 0.05$). Statistics represent mean ± SEM of three independent experiments.

overexpressing control GFP or neurons overexpressing WT p150Glued, p150Glued function and microtubule stabilization pathway are likely to be acting cooperatively and in parallel to each other to maintain axonal integrity.

p150Glued is a major component of the dynactin complex which facilitates retrograde axonal transport of cargos by the dynein complex [28]. Glutamate excitotoxicity-induced C-terminal truncated form of p150Glued could not interact with p50 dynamitin that enables p150Glued to link to cargos. Consistently, the C-terminal truncated p150Glued could not interact with APP cargo and resulted in altered localization of APP in neurites of neurons overexpressing the C-terminal truncated p150Glued. Thus, altered localization of p150Glued-dependent cargos per se could play a central role to activate the process of axon degeneration presumably by inducing ectopic signaling within neurites. This scenario could be argued as we have recently shown that overexpression of the C-terminal truncated p150Glued does not induce axon degeneration. An alternative idea would be that intracellular signaling pathways that exert protective/survival effects against axon degeneration require enzymes that are activated by p150Glued, possibly through its' C-terminal region. Previous studies have found activated forms of pro-survival Erk1/2 MAP kinases in retrogradely transported signaling endosomes isolated from peripheral nerves [29]. Thus, p150Glued could be activating enzymes involved in the pro-survival signaling cascades that might involve MAP kinases to propagate protective signals against excitotoxicity-induced axon degeneration. Identification of p150Glued C-terminal region-interacting enzymes or phosphorylated enzymes by p150Glued would enable us to clarify this hypothesis.

Finally, when excitotoxicity is triggered, the direct upstream event of p150Glued would be an enzymatic cleavage of the p150Glued C-terminal region. We have not identified the responsible enzyme. As excitotoxicity results in excessive intracellular calcium concentration, calcium-dependent proteases could be candidate enzymes. Future studies by setting up optimized biochemical assays and with production of stable recombinant WT p150Glued would enable the identification of the enzyme that cleaves the C-terminal region of p150Glued.

Acknowledgments

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