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TRiC enhances retrograde axonal transport by modulating tau phosphorylation

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Abstract

The cytosolic chaperonin T-complex protein (TCP) 1-ring complex (TRiC) has been shown to exert neuroprotective effects on axonal transport through clearance of mutant Huntingtin (mHTT) in Huntington's disease. However, it is presently unknown if TRiC also has any effect on axonal transport in wild type neurons. Here we examined how TRiC impacted the retrograde axonal transport of brain-derived neurotrophic factor (BDNF), which was significantly enhanced by TRiC subunit, leading to an increase in instantaneous velocity with a concomitant decrease in pauses for retrograde BDNF transport. The transport enhancing effect by TRiC was dependent on endogenous tau expression since no effect was seen in neurons from tau knockout mice. We showed that TRiC regulated the level of cyclin-dependent kinase 5 (CDK5)/p35 positively, contributing to TRiC-mediated tau phosphorylation (ptau). Expression of a single TRiC subunit increased the level of ptau while downregulation of the TRiC complex decreased ptau. We further demonstrated that TRiC-mediated increase in ptau induced detachment of tau from microtubules. Our study has thus revealed that TRiC-mediated increase in tau phosphorylation impacts retrograde axonal transport.

Graphical Abstract

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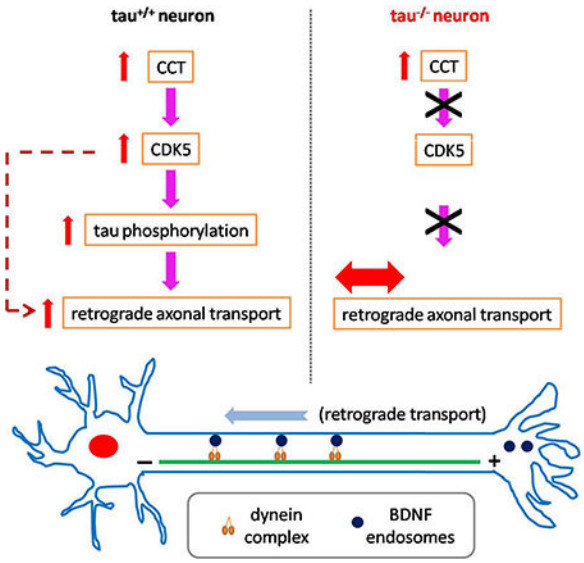
Conflict of interest

The authors declare no conflict of interest.



Keywords

TRiC/CCT chaperonin; tau; hyperphosphorylation; axonal transport

Introduction

The cytosolic chaperonin T-complex protein (TCP) 1-ring complex (TRiC) or chaperonin containing TCP-1(CCT) binds to approximately 10% of the eukaryotic newly translated polypeptides and promotes their folding (1, 2). CCT also binds to misfolded proteins and regulate their oligomerization/aggregation (1, 3, 4). Recent studies suggest that CCT exerts strong neuroprotective effects in Huntington's disease (HD) (5–9). Expression of either a single or all eight subunits (CCT1–8) or application of the 20 kDa substrate-binding apical domain of yeast CCT1 (ApiCCT1) prevented aggregation of mutant Huntingtin (mHTT) protein, leading to improved cellular or neuronal functions (5–8, 10). In additon, CCT inhibited amyloid fiber assembly of α-synuclein mutant A53T, associated with Parkinson disease (PD), pointing out a similar neuroprotective role for CCT in PD (11). Furthermore, CCT has also been also implicated to play a role in Alzheimer's disease (AD) susceptibility (12).

Importantly, we previously found that either CCT3 or CCT5 delivery could normalize the deficit in axonal transport in neurons from the BACHD mouse model of HD (8). However, it is unclear if CCT subunit has any role in the axonal transport in wild type neurons. Tau is a microtubule-associated protein that regulates microtubule dynamics and stability (13–20). Tau thus plays an important role in facilitating axonal transport that is critical for neuronal survival and maintenance. Aberrant function in tau is associated with a class of neurodegenerative diseases called tauopathies. These diseases are characterized by tau hyperphosphorylation and/or expression of mutant tau protein, leading to aggregation of tau and formation of tau-containing deposits in the brain (21–23). The best-known tauopathy is AD, whose pathological hallmarks include both β-amyloid peptide-containing amyloid

plaques and tau-containing neurofibrillary tangles (NFTs) (24). In addition, missense mutations in tau have been identified in patients with frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) tauopathy (21, 25).

Studies have shown that molecular chaperones such as heat shock proteins (Hsps) are involved in tau-related neurodegenerative diseases through recognizing abnormal tau and reducing its level by facilitating tau dephosphorylation or degradation (26–30). For instance, Hsp90 was shown to maintain the stability of mutant tau^{P301L} (28), one of the most prevalent FTDP-17 tau mutations (31, 32).

Given the potential role of CCT chaperonin on axonal transport in HD, we investigated here whether CCT could modulate the axonal transport in wild type neurons and if tau is involved in the regulation. We found that expression of a single CCT subunit CCT5 improved axonal transport function as measured by an increase in instantaneous velocity with a concomitant decrease in pauses for retrograde transport of brain-derived neurotrophic factor (BDNF). Mechanistically, CCT5 acted through the cyclin-dependent kinase 5 (CDK5) pathway to increase the level of ptau which resulted in detachment of tau from microtubules. Our study provides evidence that upregulation of TRiC/CCT subunit increases tau phosphorylation that impacts retrograde axonal transport.

Results

CCT modulates axonal transport of BDNF via a tau-dependent mechanism

Our previous study has shown that either CCT3 or CCT5 delivery rescued the deficit in axonal BDNF transport in cultured cortical neurons from the BACHD mouse model of Huntington's disease (8). To test if CCT had any effect on axonal transport in wild type neurons, we used lentivirus to express an Avi-tagged human CCT5 construct in rat E18 cortical neurons. To mark the neurons for lenti-infection, GFP was also expressed in the same vector but under a promoter different from hCCT5-Avi. 4 days post transduction, lenti-mediated expression routinely achieved an infection efficiency of ~95% as revealed by the GFP signal (Figure 1A).

Primary rat E18 cortical neurons were transduced with either CCT5-lenti or control-lenti were at *days in vitro* (DIV)5 (Figure 1B). At DIV9, we then used a well-established system to measure retrograde axonal transport of quantum dot-labeled BDNF (QD-BDNF) by live cell imaging (8, 20, 33–35). Interestingly, CCT5-lenti treatment enhanced retrograde axonal transport of BDNF in comparison to control lenti as revealed by kymograph analysis (Figure 1C-H); it resulted in an increase in instantaneous velocity (the absolute velocity from moving time without pause time included) from 1.33 ± 0.08 μm/s to 1.62 ± 0.08 μm/s (Figure 1C, D) and a decrease in the percentage of pause events (from 19 ± 3% to 12 ± 2%) (Figure 1C, E) and average pause duration (from 7.47 ± 1.17 s to 4.16 ± 0.74 s) (Figure 1C, F). As a result, the average retrograde velocity (the relative velocity from moving time with pause time included) in CCT5-expressing neurons was at 1.41 ± 0.09 μm/s, significantly faster than that in control neurons (1.04 ± 0.08 μm/s) (Figure 1C, G; Video S1–2). CCT5 overexpression also increased the percentage of non-pause (motile) puncta within the

observation time window (Figure 1H). These results indicate CCT5 overexpression promotes the retrograde axonal BDNF transport.

We next investigated whether not CCT5-induced enhancement of axonal transport function was tau-dependent, as tau has been demonstrated to play an important role in modulating axonal transport both under physiological and pathological conditions (22). To establish a role of tau in CCT5-mediated enhancement of axonal transport of BDNF, we elect to examine E18 cortical neurons from $tau^{-/-}$ mouse (Figure 2). The experimental design was shown in Figure 2A. To investigate the role of either wt tau (tau^{WT}) or mutant tau (tau^{P301L}), tau^{WT} or tau^{P301L} was re-introduced in tau−/− neurons through lenti-transduction. The level of tau^{WT} or tau^{P301L} was revealed by immunoblotting (Figure 2B). Interestingly, in contrast to wild type E18 cortical neurons (Figure 1), CCT5-induced enhancement of axonal transport of BDNF was not seen in $tau^{-/-}$ cortical neurons (Figure 2C-G; Video S3–4). Re-expression of tau^{WT} in $tau^{-/-}$ neurons (Figure 2C) resulted in an average transport velocity of 1.05 ± 0.12 μm/s, which is comparable to wild type $tau^{+/+}$ control neurons (1.04 ± 0.08 μm/s) (Figure 2C, G; Figure 1G). Co-expression of CCT5 together with tau^{WT} in $tau^{-/-}$ neurons induced a ~40% increase in the average retrograde velocity to 1.40 ± 0.13 μm/s (Figure 2C, G). In addition, the percentage of pause events was decreased from 40 ± 4% to 21 ± 4% and average pause duration was reduced from 17.25 ± 2.66 s to 8.77 ± 1.68 s (Figure 2C, E, F; Video S5–6). Our results have thus demonstrated that reintroducing tau^{WT} into $tau^{-/-}$ neurons restores the beneficial effect of CCT5 on retrograde axonal transport.

Interestingly, reintroducing tau^{P301L} (Figure 2B) did not rescue the effect of CCT5 (Figure 2C-G; Video S7–8), suggesting that CCT5 differentially impacts tau^{WT} and tau^{P301L} to affect axonal function. Consistently, CCT5 overexpression increased the percentage of non-pause (motile) puncta within the observation time window only in tau^{WT}-introduced $tau^{-/-}$ neurons (Figure 2H). Taken together, we conclude that the enhancing effect of CCT5 on retrograde axonal transport of BDNF is dependent on tau^{WT} expression. The effect of tau^{WT} cannot be substituted with the pathogenic tau^{P301L} mutant.

CCT regulates the level of CDK5/p35/p25

Tau binds to microtubule to impact axonal cargo transport (13–20). Previous studies have shown that tau overexpression compromised vesicle transport in a phosphorylation-dependent manner in *Drosophila* (36). Therefore, we predicted that CCT subunit may affect axonal cargo transport through mediating tau phosphorylation. We firstly evaluated the level and activity of glycogen synthase kinase 3 beta (GSK3β) and cyclin dependent kinase 5 (CDK5), two prominent kinases that play important roles in tau phosphorylation in AD (37). CDK5 could be activated by neuron-specific activator p35 as well as its truncated form p25 (38). Interestingly, CCT5 overexpression significantly increased the level of CDK5, p35 and p25 (Figure 3A-D). This effect appeared to be dependent on the level of CCT5 since overexpression of CCT5 did not change the endogenous level of CCT subunits such as CCT2, CCT4 and CCT5 in rat primary E18 cortical neurons (Figure S1A). We also observed that CCT5 upregulation also increased Hsp70 level (Figure S1A).

To further define the role of CCT complex in CDK5/p35, we used a lenti-short hairpin RNA (shRNA) specific for rat CCT2 (rCCT2-shRNA-lenti) to reduce the level of CCT complex in

rat primary E18 cortical neurons. Knocking down CCT2 has been shown to effectively reduce the levels of all eight CCT subunits (39). Both control lenti and rCCT2-shRNA-lenti also expressed GFP. The rCCT2-shRNA-lenti resulted in a ~40% reduction in the level of CCT2, 4, 5 (Figure S1B). In transfected human embryonic kidney 293T (HEK293T) cells, expression of CCT5 also significantly increased the level of endogenous CDK5, while depletion of CCT complex decreased that (Figure S2). This effect also appears to be dependent on the level of CCT5 since overexpression of CCT5 did not change the endogenous level of other CCT subunits in HEK293T cells (Figure S1C). Interestingly, CCT5 upregulation or attenuation of CCT complex had no obvious effect on the activity of GSK3β (p-Ser9 and p-Tyr216) as well as its total level in either primary cultured neurons (Figure 3E, F) or HEK293T cells (Figure S2).

However, unlike GSK3β, CCT5-induced upregulation of p35 and CDK5 was abolished in $tau^{-/-}$ neurons and recovered when tau^{WT} was reintroduced into $tau^{-/-}$ neurons (Figure 3G). These results showed that endogenous tau expression is also required for the CCT5-mediated increase in CDK5 activity. Taken together, these results indicate CCT5 could modulate the activity of CDK5, but not GSK3β, in a tau-dependent manner. Furthermore, as tau was required for the effect of CCT5 on the retrograde axonal transport of BDNF (Figure 2), CDK5, but not GSK3β, may mediate the effect of CCT5 on retrograde axonal transport of BDNF.

CCT subunit modulates tau phosphorylation

To validate the effect of CCT on tau phosphorylation, we analyzed the AT8 site of ptau in our analysis using primary neurons, as AT8 is one of the best well-known pathological epitopes (40). Expression of hCCT5-Avi induced a significant increase in the level of ptau at the AT8 site, but had no effect on the total tau level (Figure 4A). In addition, other CCT subunits such as CCT3, CCT6 also increased the level of ptau (Figure 4B). Furthermore, CCT2 shRNA-mediated knockdown against CCT complex induced a decrease of AT8 signal, while the total level of tau was not affected (Figure 4C). Additionally, CCT5 upregulation promoted ptau-PHF signal while reduction of the CCT complex attenuated PHF signal in neurons (Figure 4D, E).

To further investigate the role of CDK5 and GSK3β in mediating the effect of CCT5 on AT8, we tested the effect of roscovitine, a CDK5 inhibitor and LiCl, a GSK3β inhibitor in CCT-mediated tau phosphorylation. Roscovitine treatment blocked CCT5-induced increase in ptau-AT8 signal in primary cortical neurons, albeit without any effect on the basal AT8 level (Figure 4F). To rule out the possible contribution of GSK3β, we compared AT8 levels from samples from the following conditions: 1) control neuron without virus infection; 2) neuron with lenti-control infection; 3) neuron with lenti-CCT5 infection; 4) neuron with lenti-control infection and LiCl treatment; 5) neuron with lenti-CCT5 infection and LiCl treatment. Our results showed that: 1) consistent with our previous finding (Figure 4A), CCT5 increased AT8 signal while control-lenti did not seem to change the basal level of AT8 (Figure 4G); 2) interestingly, LiCl treatment alone reduced the basal level of AT8 signal (Figure 4G), a finding consistent with our previous report (20); 3) importantly, LiCl treatment did not prevent an increase in AT8 signal by CCT5-lenti (Figure 4G). Based on

these findings, we conclude that GSK3β may not contribute importantly to CCT5-induced increase in AT8 in our studies. We thus conclude that CCT5 expression selectively modulated the activity of CDK5, but not GSK3β.

We then confirm the effect of CCT on tau phosphorylation in transfected HEK293T cells, a cell line that does not express endogenous tau. CCT5 overexpression significantly increased the level of phosphorylated EGFP-tau^{WT} at multiple pathological epitopes (AT8, PHF-1, pT181, pT231) without impacting the total level of EGFP-tau^{WT} (Figure S3A, B). Expression of CCT5 increased the total level of EGFP-tau^{P301L} with a concomitant increase in ptau^{P301L} species, albeit at a much lower level than CCT5-induced increase in ptau^{WT} (Figure S3A, B). The ratio of ptau^{P301L} over total tau^{P301L} is not altered by CCT5 (Figure S3A, B). In addition, other CCT subunits such as CCT3, CCT6 and CCT8 also significantly increased the level of EGFP-ptau^{WT} (Figure S3C, D). The level of EGFP-ptau^{WT} was correlated with the expression level of CCT subunit(s) (Figure S3E). These results have demonstrated that upregulation of a single CCT subunit promotes phosphorylation of EGFP-tau^{WT}. Knockdown of the endogenous CCT complex with lentivirus expressing hCCT2-shRNA induced a reduction in the level of CCT2 and other CCT subunits by ~40% in HEK293T cells as well as ptau (AT8) level (Figure S1D; Figure S3F).

Consistently, in HEK293T cells roscovitine, but not LiCl, prevented the increase in ptau by CCT5 (Figure S4A). Furthermore, CCT5-mediated increase in ptau^{WT} was blocked by the expression of a dominant negative CDK5^{D145N} not the wild type CDK5^{WT}construct (Figure S4B). Therefore, CDK5, not GSK3β, mediates CCT-induced increase in ptau.

The substrate-binding apical domain of CCT1 (ApiCCT1) was shown to enter cells and inhibit toxicity of mHTT (7, 8). We wondered whether exogenous delivery of ApiCCT1 could mimic the effect of full-length CCT subunit on ptau^{WT} as well. Recombinant ApiCCT1 proteins were added at indicated concentrations into the culture medium of tau^{WT}-transfected HEK293T cell for 2 days. Our results showed that ApiCCT1 did not show any impact on the level of AT8 phosphorylation of tau^{WT} (Figure S4C). We also tested the mobility retardation profile of tau and CCT5 using native gel electrophoresis. We found overexpressed CCT5 formed a complex with a molecular weight (MW) between 720~1048 kD, from which tau was absent (Figure S4D). It has been reported that tau phosphorylation promotes formation of tau oligomer (41). Our results showed that CCT5, although increased ptau, had no effect on tau^{WT} oligomerization (Figure S4D); as a control, tau^{P301L} has been shown to form various oligomers in native gel (42)(Figure S4D).

CCT modulates binding of tau to microtubules

Phosphorylated tau is believed to reduce its capacity to bind to microtubules (43, 44). We then assessed the effect of CCT5 expression on tau binding to microtubules by measuring free tau and microtubule-bound tau in primary rat cortical neurons. Expression of CCT5 induced a decrease in the level of tau bound to the Taxol-stabilized microtubules as detected in the pellet fraction (P) (Figure 5A). The ratio of tau in the pellet to the supernatant fractions (P/S) was markedly decreased in CCT5-overexpressed neurons, as compared with that in controls (Figure 5A). In addition, roscovitine treatment reversed the change of the distribution of tau induced by CCT5 (Figure 5A). These results provide evidence that CCT5

acts through CDK5 to promote tau phosphorylation to induce detachment of tau from microtubules.

One of the most striking subcellular phenotypes of tau overexpression is the formation of microtubule bundling, likely due to hyper-stabilization of microtubules by excessive tau (45). We next tested the effect of CCT on tau-induced microtubule bundling in Chinese Hamster Ovary cells (CHO). CHO cells have been used to induce microtubule bundling due to their specific pattern of microtubule characteristics (46) that can be detected by immunostaining with an anti-α-tubulin antibody. CHO cells transiently expressing tau displayed microtubule bundling at the cell periphery in 80% of the cells, in contrast to 20% of the cells expressing GFP/mCherry or GFP/CCT5-mCherry. CCT5 co-expression with tau dramatically reduced the percentage of cells containing microtubule bundles to ~50% (Figure 5B). These results indicate that CCT5-mediated increase in ptau reduces microtubule bundling, a finding consistent with a recent report that tau-induced microtubule bundling could be reversed by a tau phosphor-mimic mutant (46).

We further examined if polymerization of microtubules was impacted by CCT5 expression. We found that CCT5 had no effect on microtubule polymerization in primary neurons (Figure 5C). In addition, expression of CCT5 did not affect tubulin acetylation (a marker for stable microtubules) in primary cortical neurons (Figure 5D). These results indicate that CCT-mediated tau phosphorylation weakens tau binding to microtubules and reduces tau-mediated microtubule bundling without affecting the stability of microtubules.

Hypophosphorylated tau, not pseudophosphorylated tau, was found to impair axonal transport likely due to enhanced binding of tau to microtubule in *Drosophila* (36). Consistently, treatment with the CDK5 inhibitor roscovitine abolished the effect of CCT5 on axonal retrograde transport (Figure 6; Video S9–10). Taken together, it is likely that reduced tau binding to the microtubule, due to CCT5 overexpression, makes the microtubule network more favorable for conducting axonal transport as observed (13, 14, 36).

Discussion

Recent studies have established a strong link between the TRiC/CCT cytosolic chaperonin and neurodegenerative diseases. A H147R mutation in CCT5 has been identified to cause autosomal recessive mutilating sensory neuropathy with spastic paraplegia in patients (47) and a C450Y mutation in CCT4 was found to be associated with an early onset sensory neuropathy in Sprague-Dawley rat strain (48). Conversely, increasing TRiC/CCT has been shown to be neuroprotective. Expression of all eight or a single CCT subunit inhibits aggregation of mHTT and prevents its cellular toxicity (5–8, 10). Enhanced expression of CCT subunits or exougenously added ApiCCT1-mediated clearance of mHTT is believed to be the principal mechanism underlying the neuroprotective effect of TRiC/CCT (7, 8). We thus postulated a similar mechansim that might exist for CCT in preventing neurotoxicity by pathogenic tau species.

Surprinsingly, our current study has demonstrated that CCT appeared to impact tau species through mechanims very different from mHTT. The key difference is overexpression of a

single CCT subunit increased, but not reduced, the level of ptau. Nevertheless, CCT expression appeared to enhance axonal function under these settings. CCT thus plays an important role through modulation of “misfolding” protein tau. These results have shed new light into the complex roles of CCT in protein misfolding diseases.

In primary embryonic cultured neurons, we found the antibodies that we tested (PHF, AT8 and tau) all detected a major band with a molecular weight (MW) of ~50 kD and a second band with a slightly higher MW of ~54 kD. Tau has six spliced isoforms: 0N3R, 1N3R, 2N3R, 0N4R, 1N4R, 2N4R(49, 50). While expression of the 4R forms (0N4R, 1N4R, 2N4R) is only detected starting at 2 months of age in mice(51), the 0N3R form of tau is the predominant form that is expressed in neurons at embryonic stages(51). Low level of 1N3R form is also detected (51). Therefore, the 50 kD and 54 kD bands that we observed in our studies likely represents the 0N3R, 1N3R form of tau, respectively(51). The two forms of tau, 0N3R and 1N3R, differ only in the presence of an N-terminal insert (29 residues) in 1N3R, but with exact same microtubule binding domains in the C-terminus. Our results showed that phosphorylated forms of both 0N3R and 1N3R were present in rat E18 cortical neurons since LiCl treatment induced a reduction in the intensity of both the 50 kD and 54 kD bands. Of note, the difference in the relative intensity of the 54 kD band varied among different sets of experiments in our study. We believe this is likely due to slight variations in the experimental conditions such as exposure times. Nevertheless, results from samples within the same set of experiments (i.e. Figure 3A left panel or right panel) were consistent.

The role of classical chaperone proteins such as heat shock proteins (Hsp70, Hsp90) in regulating tau processing in tauopathies have been extensively studied (52–54). Induction of Hsp70 (53, 54) or induction of heat shock response through inhibition of Hsp90 leads to downregulation of ptau through various mechanisms (55, 56). Interestingly, Hsp90 inhibition reduces the stability of tau^{P301L} (28) and tau^{WT} (57). In the present study, we found CCT5 overexpression did increase Hsp70 level in primary neurons. Although consistent with a previous report (58), the increase in Hsp70 may not explain the CCT5-induced increase in ptau, since Hsp70 and its cochaperone have been reported to facilitate the clearance, but not increase of ptau(52, 54). In addition, other CCT subunits such as CCT3, CCT6 and CCT8 also increased the level of ptau, pointing the effect on ptau by CCT is not subunit specific. However, this effect appears to be dependent on the level of overexpressed individual subunit only as upregulation of CCT5 did not alter the level of other CCT subunits.

Tau^{WT} and tau^{P301L} may possess different biochemical, structural and conformational properties that impact their aggregation tendency and microtubule binding/stabilization capacity (59, 60). It is possible that these different properties of tau^{WT} and tau^{P301L} dictate the differential effects of CCT on tau^{WT} and mutant tau, as CCT5 promoted the phosphorylation of tau^{WT} while had little effect on that of tau^{P301L}.

Furthermore, almost all the transfected CCT5 subunits formed a complex in which tau was apparently absent. Therefore, the effect of CCT5 on tau phosphorylation requires oligomeric complex either self-assembled by transfected CCT5 alone or assembled by CCT5 with other endogenous CCT subunits. Previous studies suggest that hyperphosphorylated tau tends to oligomerize to form toxic species (41) that inhibit axonal transport (61, 62). Interestingly,

our findings suggest that CCT5 upregulation somehow prevents tau oligomerization despite of an increase in ptau. This may explain why CCT5 introduction promotes the retrograde axonal transport.

Tau, as a microtubule associated protein, binds to and stabilizes microtubule, plays an important role in axonal transport of vesicles, organelles as well as other cargoes (22), as evident by acute reduction of hippocampal tau in adult mice resulting learning and memory deficits(63). However, overexpression of tau, as observed early in some forms of tauopathies (64), also impaired axonal transport before the breakdown of microtubules (13, 14, 36, 46, 65). Interestingly, this inhibitory effect of excessive tau could be rescued by phosphorylation-driven tau off microtubules (65). On the other hand, hyperphosphorylation drives tau to detach from microtubules, thus destabilizing microtubules that could also interrupt axonal transport (22, 66). Furthermore, detached soluble and hyperphosphorylated tau tends to aggregate into paired helical filaments (67), which causes spatial clogging within axon (66). Therefore, cellular tau, presented either in excess amount or in hyperphosphorylated forms, shows deleterious effect on axonal function.

Mechanistically, tau has been shown to compete with motor proteins for binding to microtubule (13) and reduce the number of engaged motors per cargo for transport (68). As a cargo, tau itself also probably competes with other cargoes for availability of motor (69). Further tau was also reported to regulate the release of cargo from kinesin involving PP1/GSK3β (70). Therefore, excessive non-phosphorylated tau may also pose an adverse effect on axonal transport, as revealed in a recent study in *Drosophila*, in which hypophosphorylated tau, not pseudophosphorylated tau, was found to impair axonal transport, likely due to enhanced binding of tau to microtubule (36).

The function of tau could be complemented by other microtubule-associated protein like MAP1b (71), since no obvious alternation of axonal transport in neurons that were either partially or completely depleted of tau (72, 73).

Our current study has shown that the increase in ptau by CCT attenuated binding of tau to microtubules and inhibited formation of tau-induced microtubule bundles, likely resulting in enhancement of axonal transport. It is conceivable that reduction in tau binding to microtubules by overexpression of CCT subunit makes track more favorable for motor proteins and cargoes to move along microtubules. Indeed, this tau-dependent CCT modification of axonal transport was supported by $tau^{-/-}$ experiments; in which reintroducing tau^{WT} but not tau^{P301L}, into $tau^{-/-}$ primary neurons rescued the effect of CCT on axonal transport of BDNF. Our results are consistent with that tau^{P301L} was shown to have a weaker binding capacity to microtubules than tau^{WT} (74–76).

Consistent with previous studies (77–79), we have demonstrated that CCT acted on CDK5 to modulate retrograde axonal transport. It is possible that CDK5 signals through a mechanism involving dynein regulators Ndel1/Lis1 to stimulate retrograde axonal transport (77). Interestingly, CDK5 phosphorylation of Ndel1 was reported to inhibit processive mobility of dynein-driven cargoes (78). Although the exact reasons are unknown presently, the discrepancy might be due to different system and different cargoes analyzed. The effect

of CCT5 on the retrograde axonal transport of QD-BDNF may arise from both direct tau-dependent mechanism(s) and indirect mechanism(s) including CDK5-mediated Ndel1 phosphorylation.

Although there are reports that the GSK3/ptau connection also affects axonal transport (20, 80), our results show that GSK3β is unlikely involved in CCT-induced phosphorylation of tau. Evidence supporting this notion includes that: 1) CCT5 expression had no effect on the level and activity of GSK3β as revealed by phosphorylation-specific antibodies (both pSer9 and pTyr216); 2) LiCl pretreatment of cortical neurons did not prevent CCT5-mediated increase in AT8 signal, albeit the measure reduced basal level of AT8 signal; and 3) inhibition of CDK5 effectively abolished CCT5-induced tau phosphorylation in AT8. Nevertheless, TRiC/CCT complex modulates ~10% of the eukaryotic proteins, we cannot exclude the possibility that other effectors of GSK3β, in addition to CDK5/p35, might be affected and synergistically enhance the CCT5-mediated effect on axonal transport.

It has been well established that tau regulates the activity of various protein kinases (70). Similarly, TRiC/CCT also regulates various many important kinases such as sphingosine kinase 1(81), mitogen-activated protein kinases(82) and phosphoinositide 3-kinase(83). CCT could potentially regulate the activity of these kinases through transcriptional alterations or turnover at the protein level or both. In this study, we have demonstrated that CCT acted on CDK5/p35 to impact tau phosphorylation and function. This is a novel finding in defining cellular pathway(s) by which CCT regulates neuronal function. Future studies are needed to elucidate the molecular details of regulation of CDK5/p35 activity by CCT.

In our previous study, both CCT3 and CCT5 rescued the axonal transport deficit in mHTT-expressing neurons (8). These effects are primarily due to CCT-mediated clearance of mHTT (8). In addition, ApiCCT1 delivery also rescued the axonal transport deficit in mHTT-expressing neuron (8). However, different mechanism(s) is likely responsible for CCT5 acting on tau to impact axonal transport as described herein. Currently, we do not know if there is any link between the pathways governing CCT5/mHTT and CCT5/tau in axonal transport in Huntington disease.

Recent studies have demonstrated that mRNA levels of the TRiC/CCT complex were decreased in brain samples of Alzheimer’s disease patients (84). Further CCT5 expression was found to be also decreased in Alzheimer’s disease (85). It is thus important to understand how decreased activity of CCT complex or individual CCT subunit impacts the stabilization and turnover of tau, whose pathogenic species also accumulates in Alzheimer’s disease. Although the physiological implications remain to be defined, our study has established that increased expression of CCT5 had a clear impact on tau-mediated functions such as axonal transport.

In summary, our present study has demonstrated that CCT5 expression affects tau phosphorylation and axonal transport. Although we show this effect on BDNF, it is likely microtubule-based transport of other cargoes is also affected. Further studies are needed to define this issue.

Materials and Methods

Ethic statement

This study was performed strictly according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments involving the use of animals have been approved by the University of California San Diego Institutional Animal Care and Use Committee (Protocol# S09371, S15159). The best efforts were made to minimize the suffering of animals.

Plasmids

The following plasmids were obtained from the Addgene: pRK5-EGFP-tau, pRK5-EGFP-tau^{P301L}, CDK5-HA, CDK5-DN-HA, psPAX2, pMD2.G, and pLL3.7. hCCT3, 5, 6, 8 cDNAs were cloned into pcDNA3.1 with mCherry fused to the C termini of hCCTs. Lox-Syn lentivirus vector (86) was used for hCCT5-Avi, hCCT3-Avi, hCCT6-Avi, tau^{WT} and tau^{P301L} expression in neurons. This vector contains two separate neuronal-specific synapsin promoters with one controlling the expression of indicated proteins and the other controlling the GFP expression. The corresponding control vector was made by deleting of DsRed while keeping GFP in Lox-Syn. Human and rat CCT2 shRNA knockdown constructs were generated using the following primers: 5′-TGGACTCTAGAACAGTTTATTTCAAGAGAATAAACTGTTCTAGAGTCCTTTTTTC-3′, and 5’-TGCGGAATCTTTAATTGCAATTCAAGAGATTGCAATTAAAGATTCCGCTTTTTTC-3’, targeting the sequence: 1207–1224 and 338–355 base pairs of human and rat CCT2, respectively. Annealed oligonucleotides were cloned into the lentiviral vector pLL3.7 in which the U6 promoter drives shRNA expression and GFP is expressed under a separate CMV promoter.

Cell culture, transfection and microfluidic chamber neuronal culture

HEK293T and CHO cells were from the American Type Culture Collection (ATCC, Maryland) and cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin, streptomycin). Transient expression was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Rat cortical neurons or tau knockout (tau$^{-/-}$) mouse cortical neurons were prepared from E17–18 embryos. Dissected cortices were dissociated with 0.25% trypsin and triturated. Cells were seeded on poly-D-lysine (Sigma)-coated plate or poly-L-lysine (Cultres)-coated microfluidic chamber with 450 μm microgrooves (Xona Microfluidics) and cultured in Neurobasal medium supplemented with B-27 (2%) and Glutamax (Invitrogen). Experiments were carried out using DIV (days *in vitro*) 9–10 neurons.

Lentivirus production and transduction

Lentiviral particles were generated in HEK293T cells co-transfected with the viral packaging plasmids pMD2.G and psPAX2 by calcium phosphate transfection method. Viruses were concentrated by ultracentrifugation, resuspended in PBS (Ca^{2+}-Mg^{2+}-free) and added to neurons at DIV5. To knockdown endogenous CCT2 in HEK293T cells, the cells

were infected with control or hCCT2-shRNA lentiviral particles in the presence of 8 μg/ml polybrene (Santa Cruz Biotechnology).

Live imaging of axonal transport of Quantum dot-labeled brain-derived neurotrophic factor (QD-BDNF)

Monobiotinylated BDNF conjugated to Quantum dot 655 (Inviotrgen) (QD-BDNF) was prepared and used for tracking axonal retrograde transport of BDNF in microfluidic chamber-cultured neurons by live imaging (34). Following 2-h starvation in neurobasal media, QD-BDNF (final concentration of 0.1 nM) was added to the distal chamber and allowed to incubate for another 4 h before imaging. All images were captured with a Leica DMI6000B inverted microscope using a 100x oil objective lens with a Rolera XR CCD camera (Q Imaging) in an environmental chamber (37 °C, 5% CO_2). Kymographs generation and analysis of puncta movement were carried out with ImageJ software.

Immunocytochemistry

Established protocols were followed for immunocytochemistry (87). Transfected CHO cells were fixed in 4% paraformaldehyde for 15 min at room temperature and incubated with a mouse anti-α-tubulin antibody (DM1A, Invitrogen) overnight at 4 °C followed by secondary antibody conjugated with Alexa Fluor 647 (Invitrogen) for 1 h at room temperature. Primary cortical neurons were also fixed as above after virus infection for 4–5 days and incubated with anti-tau (Genscript) antibody. They were then mounted and scanned with a Leica TCS SPE confocal microscope.

Drug treatment

Primary embryonic cortical neurons were incubated with 20 μM roscovitine (Santa Cruz Biotechnology) for 4–8 h or 20 mM LiCl (Enzo Life Sciences) for 2 h prior to cell lysis for immunoblotting. HEK293T cells expressing various constructs alone or in combination (EGFP-tau^{WT}, EGFP-tau^{P301L}, mCherry, CCT5-mCherry) were treated with either 20 μM roscovitine, or 20 mM LiCl for 2 h before assays.

Native PAGE assay

Transfection of HEK293T cells was performed with EGFP-tau^{WT} and mCherry or CCT5-mCherry or EGFP-tau^{P301L} for 48 h. The cells were harvested in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100 supplemented with protease inhibitor mixture as above) on ice. Native electrophoresis was performed at neutral pH 7.5 using the NativePAGE Novex 4–16% Bis-Tris Gel system (Life Technologies) in which G-250 was used as a negative-charge shift molecule maintaining the proteins in their native state. Gels were run at 4 °C at 150 V.

Microtubule-binding assay of tau

Microtubule-binding assay of tau was performed as described previously (88). Briefly, lentivirus-infected primary cortical neurons treated with or without 20 μM roscovitine for 8 h were harvested in reassembly buffer (0.1 M MES, 0.5 mM $MgSO_4$, 1 mM EGTA, 2 mM DTT, pH 6.8) supplemented with 0.1% Triton X-100, 20 μM Taxol, 2 mM GTP, and a

mixture of protease inhibitors at 37 °C. Cell lysates were homogenized with 20 strokes in a warm Dounce homogenizer and then immediately centrifuged at 50, 000 × g for 20 min at 25 °C. The supernatant containing unbound tau was removed and the remaining pellet was resuspended in the same volume of sample buffer as the total volume of supernatant. The ratio of tau bound to microtubules (P) versus soluble or unbound tau (S) was determined by comparing the immunoreactivities of tau in these two fractions.

Microtubule assembly assay

Lentivirus-infected primary cortical neurons were harvested and lysed at 37 °C for 10 min in dark with hypotonic buffer (1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP-40, 20 mM Tris-HCl, pH 6.8) supplemented with protease inhibitors. The cell lysates were centrifuged at 18, 000 × g for 10 min at 25 °C. The supernatants (S) containing the unpolymerized tubulin were saved. The pellets (P) containing the polymerized tubulin were resuspended in an equal volume of hypotonic buffer and sonicated on ice for 2 min. Equal volumes of the two fractions were separated by SDS-PAGE to measure the amounts of tubulin.

Western blotting

The cell lysates were incubated in SDS-PAGE loading buffer for 5 min at 100 °C as before (87, 89). The samples were separated on 4–10% SDS-PAGE gel, transferred, probed with GFP (sc-9996), CCT8 (sc-13891), CDK5 (sc-173), p35 (sc-820), ac-tubulin (6–11B-1) (Santa Cruz Biotechnology), AT8 (MN1020), α-tubulin (DM1A) (62204) (Invitrogen), pT231 (55313), pT181 (54960) (ANASPEC), pS199 (A00894), tau (A01387), Avi (A00674), His (A00174) (GenScript), mCherry (GTX59788) (GeneTex), CCT2 (WH0010576M1) (Sigma), actin (60008–1-Ig) (Proteintech), CCT4 (EPR8495), CCT5 (EPR7562) (Epitomics), GSK3β (12456), pGSK3β(Ser9) (9323) (Cell Signaling), pGSK3β(Tyr216) (13A) (BD), HA.11 (901501) (Biolegend), PHF-1 (a kind gift of Dr. P. Davies, Albert Einstein University, Bronx, NY) antibodies and visualized with Clarity Western ECL Substrate (Bio-Rad). The intensity of specific bands was analyzed using the Image Lab 5.0 software (Bio-Rad). All experiments were repeated at least three times.

Statistical analyses

All data are presented as the mean ± SEM. Statistical analyses were performed using PRISM (GraphPad Software Inc.) with the two-tailed and paired or unpaired Student's *t*-test or one-way ANOVA. The significance levels were as follows: *p<0.05, **p<0.01 and ***p<0.001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TCP	T-complex protein
TRiC	chaperonin T-complex protein (TCP) 1-ring complex
CCT	chaperonin containing TCP-1
HD	Huntington's disease
mHTT	mutant Huntingtin
PD	Parkinson's disease
AD	Alzheimer's disease
NFT	neurofibrillary tangles
FTDP-17	frontotemporal dementia with parkinsonism linked to chromosome 17
Hsp	heat shock proteins
BDNF	brain-derived neurotrophic factor
CDK5	cyclin-dependent kinase 5
QD-BDNF	quantum dot-labeled brain-derived neurotrophic factor
DIV	days *in vitro*
GSK3β	glycogen synthase kinase 3 beta
shRNA	short hairpin RNA
HEK293T cells	human embryonic kidney 293T cells
CHO cells	Chinese hamster ovary cells

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Synopsis

The cytosolic chaperonin T-complex protein (TCP) 1-ring complex (TRiC) or chaperonin containing TCP-1 (CCT) has been shown to exert neuroprotective effects in Huntington's disease. Herein we demonstrate that CCT enhances retrograde axonal transport of brain-derived neurotrophic factor (BDNF) to impact neuronal function. Our study has showed that upregulation of TRiC/CCT increases the level of cyclin-dependent kinase 5 (CDK5)/p35, resulting in tau phosphorylation. Surprisingly, the net effect on retrograde axonal transport of BDNF is the reduction in pauses with a concomitant increase in transport velocities. We further provide evidence that the effect of CCT on enhancing axonal transport of BDNF is dependent on endogenous tau.

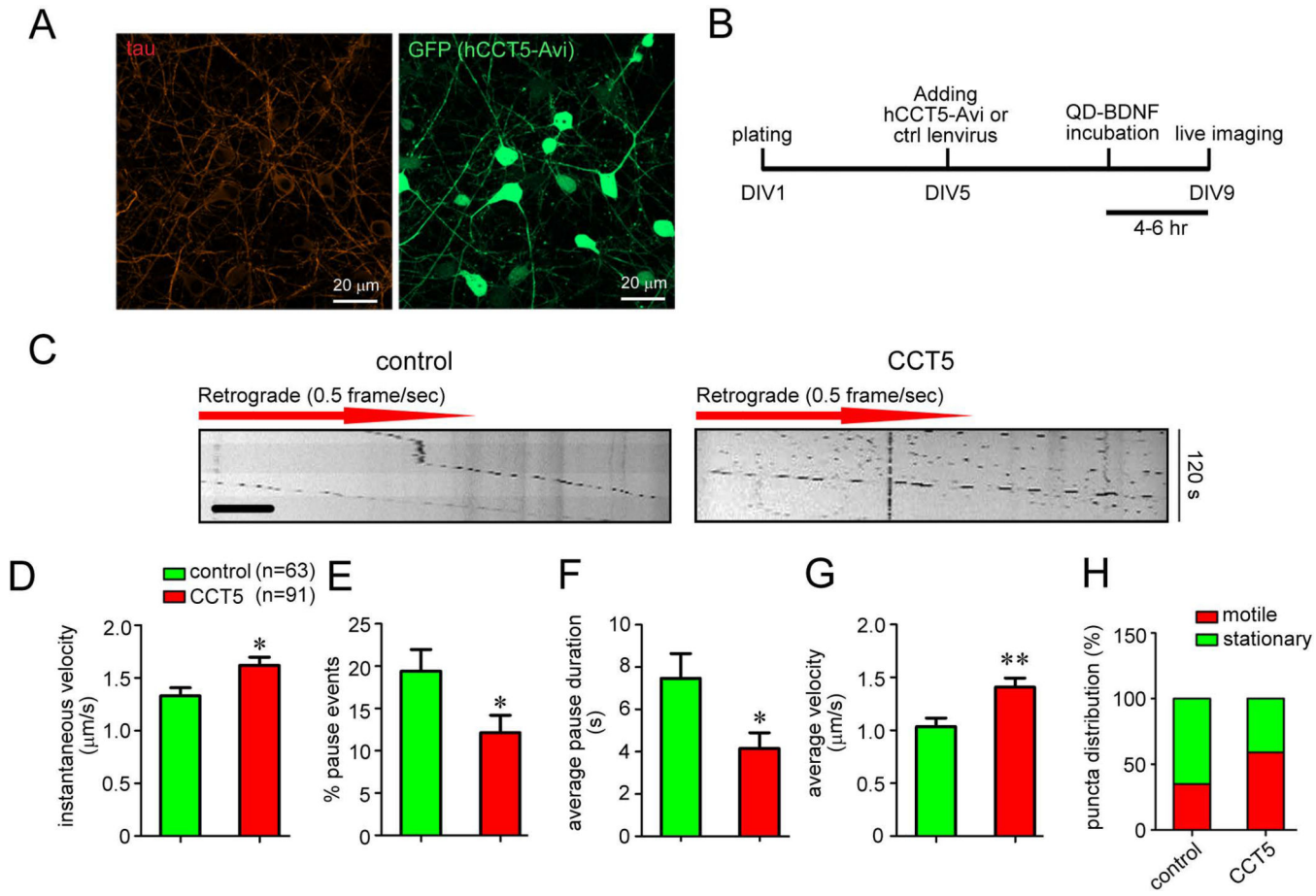

Figure 1. CCT5 promotes retrograde axonal transport of BDNF in rat E18 cortical neurons.

(A) Primary cultured rat cortical neurons at DIV5 were infected with hCCT5-Avi lentivirus at a MOI of 10 for 4 days followed by immunostaining with an anti-tau antibody. Representative images were shown. **(B)** Experimental design. **(C)** Representative kymographs of axonally transported QD-BDNF in cultured rat cortical neurons infected with control or CCT5-Avi lentivirus. Scale bar, 10 μm. The data for retrograde instantaneous velocity, percentage of pause events, average pause duration, average velocity of axonally transported QD-BDNF were quantitated and presented in **D-G**. Results are shown as mean ± SEM from three independent experiments with 60–100 QD-BDNF puncta recorded. The distribution of stationary versus non-stationary of QD-BDNF puncta is presented in **H**. *p<0.05, **p<0.01, unpaired student t-test.

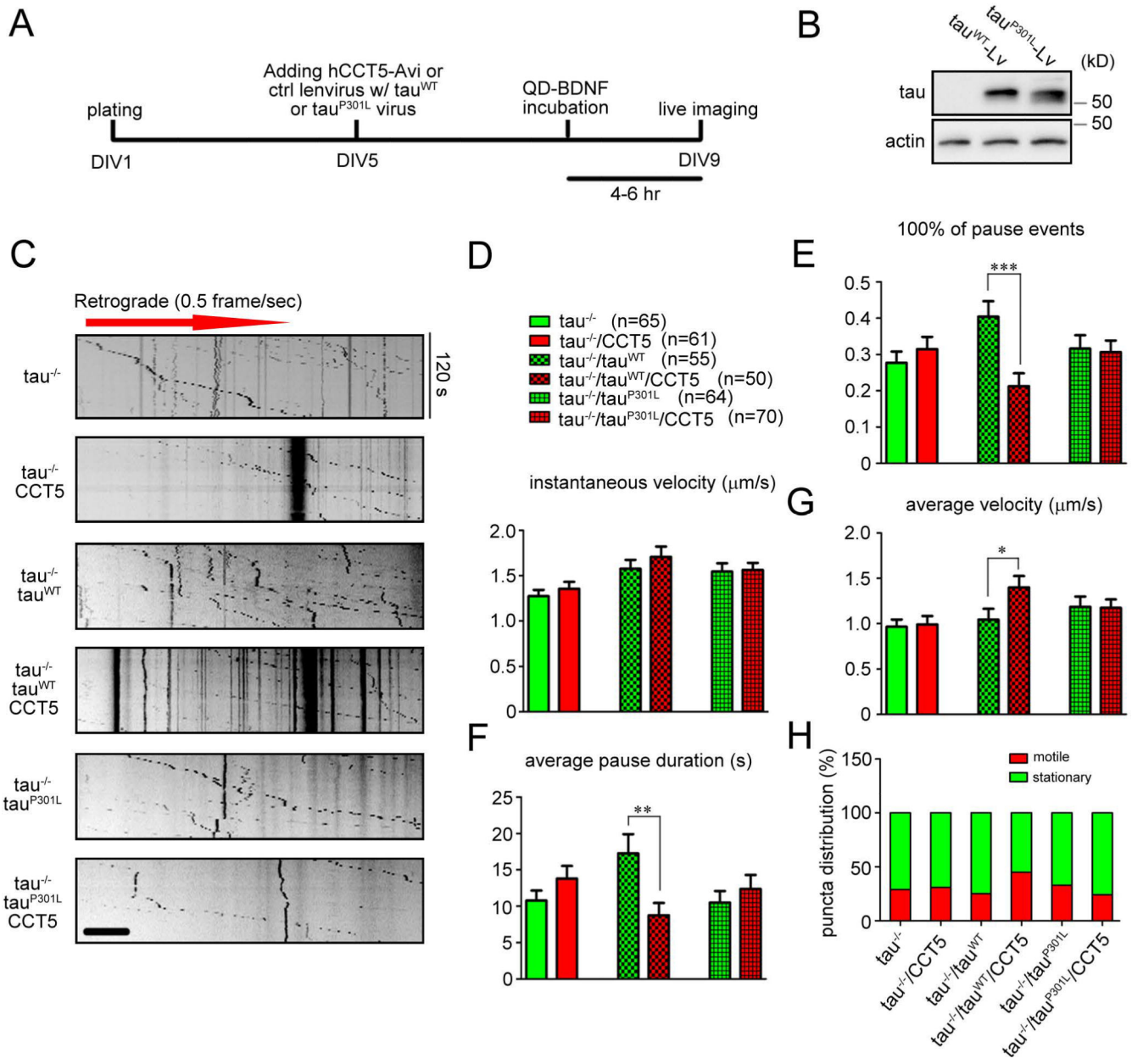

Figure 2. CCT5-mediated enhancement of retrograde axonal transport of QD-BDNF is negated in tau knockout (tau$^{-/-}$) mouse E18 cortical neurons.

(A) Experimental design. Cultured tau$^{-/-}$ mouse cortical neurons were infected with control-, tau^{WT}- or tau^{P301L}-lentivirus for 4 days. **(B)** The expression level of tau^{WT} and tau^{P301L} in tau$^{-/-}$ cortical neurons was detected by immunoblotting. **(C)** Representative kymographs of QD-BDNF retrograde transport in cultured tau$^{-/-}$ mouse cortical neurons infected with control or CCT5-Avi, and either tau^{WT} or tau^{P301L} lentivirus in microfluidic chamber. Scale bar, 10 μm. The data for retrograde instantaneous velocity, percentage of pause events, average pause duration, average velocity of axonally transported QD-BDNF were quantitated and presented in **D-G**. Results are shown as mean ± SEM from three independent experiments with 50–70 QD-BDNF puncta recorded. The distribution of

stationary versus non-stationary of QD-BDNF puncta in each condition is presented in **H,** *p<0.05, **p<0.01, and ***p<0.001, unpaired student t-test.

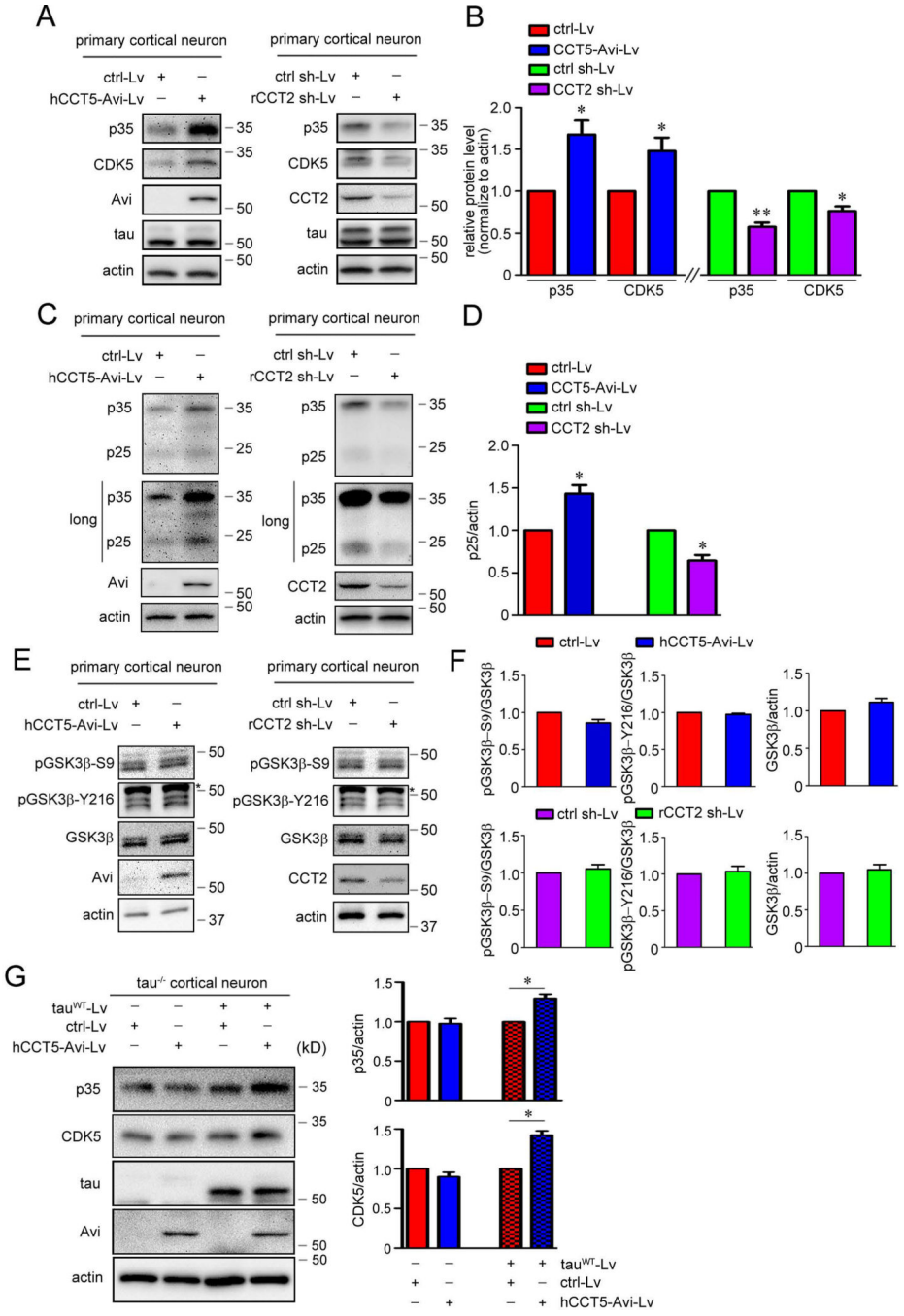

Figure 3. CCT complex regulates the level of CDK5/p35 in a tau-dependent manner.

(A, B) The impact of CCT subunit(s) on the level of CDK5 and p35 in primary rat cortical neurons was analyzed. N=4–5; *p<0.05, **p<0.01, ns, not significant, paired student t-test. **(C, D)** The effect of CCT5 overexpression or knockdown of CCT complex on p25 protein level in primary neurons was analyzed. N=3; *p<0.05, paired student t-test. **(E, F)** As in A, the effects of CCT subunit(s) on the level and activity of GSK3β in primary neurons were analyzed. The activity of GSK3β was tested by two phosphor-specific antibodies to either Ser9 or Tyr216 of GSK3β. Asterisk indicates unspecific band. N=3. **(G)** Cultured tau$^{-/-}$

cortical neurons were infected with CCT5-Avi or control lentivirus and tau^{WT} lentivirus or not for 4 days followed by detection of p35/CDK5 by Western blotting. N=3; *p<0.05, paired student t-test.

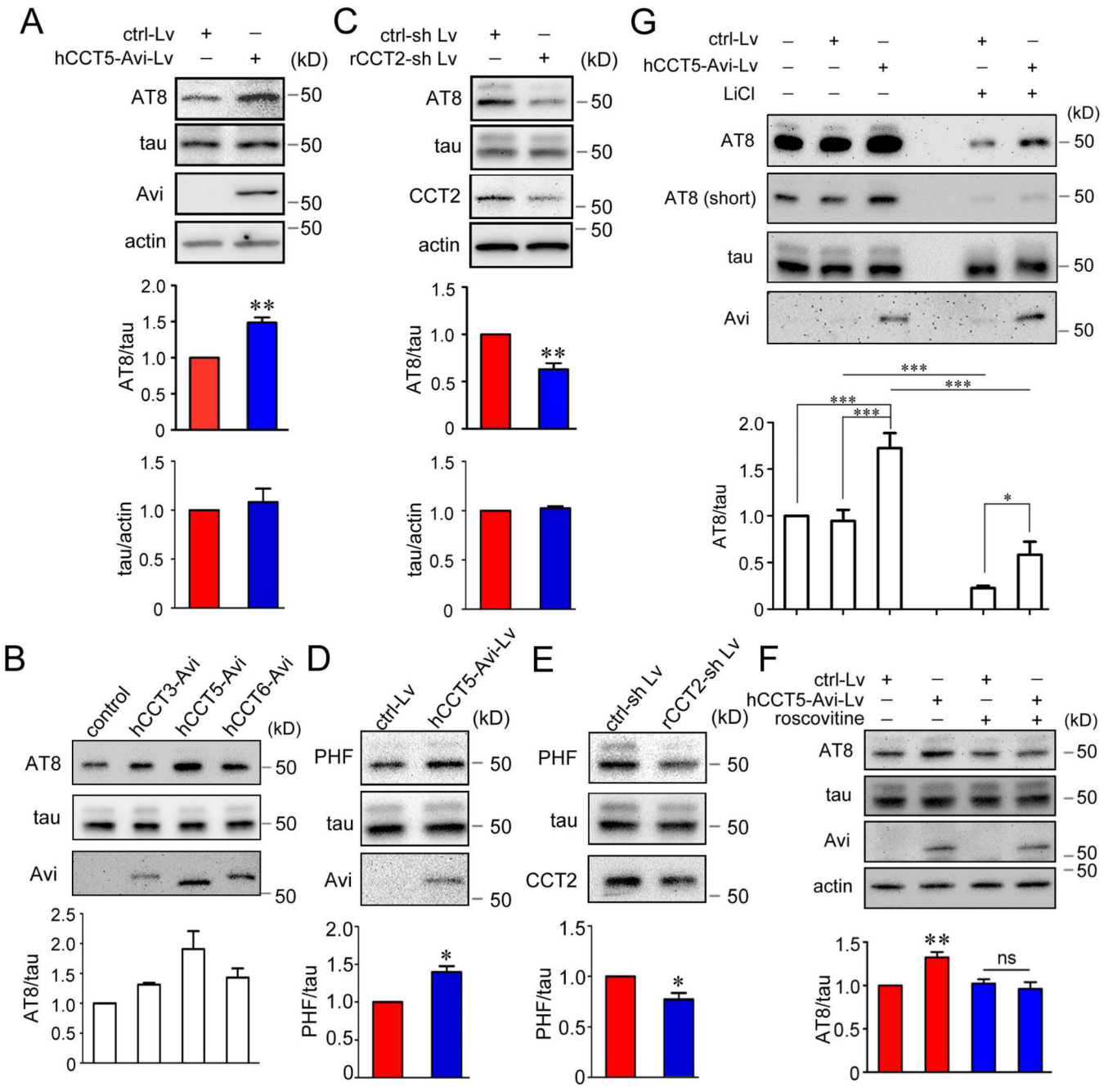

Figure 4. CCT complex modulates the phosphorylation level of endogenous tau in primary rat E18 cortical neurons.

(A) Rat cortical neurons infected with hCCT5-Avi or control lentivirus for 4 days were immunoblotted for ptau (AT8), tau and Avi. **(B)** The effects of lentivirus-mediated introduction of CCT3 and CCT6 on tau phosphorylation were analyzed. **(C)** Cultured rat cortical neurons were infected with control or rCCT2 shRNA-expressing lentivirus for analysis of AT8 levels as well as tau and CCT2. **(D, E)** The effect of CCT5 overexpression or knockdown of CCT complex on PHF signal in primary neurons was analyzed. **(F)** Rat E18 cultured cortical neurons that were infected with control or CCT5-Avi lentivirus for 4

days were treated with 20 μM roscovitine for 8 h. Neurons were harvested and analyzed by immunoblotting with indicated antibodies. **(G)** Rat cultured cortical neurons infected with or without CCT5-Avi or control lentivirus for 4 days were treated with 20 mM LiCl for 2 h or not before lysis for immunoblotting. N=3–5; *p<0.05, **p<0.01, paired student t-test in **A, C, D, E, F**. N=4; *p<0.05, ***p<0.001, one way-ANOVA test followed by Newman-Keuls multiple comparison test in **G**.

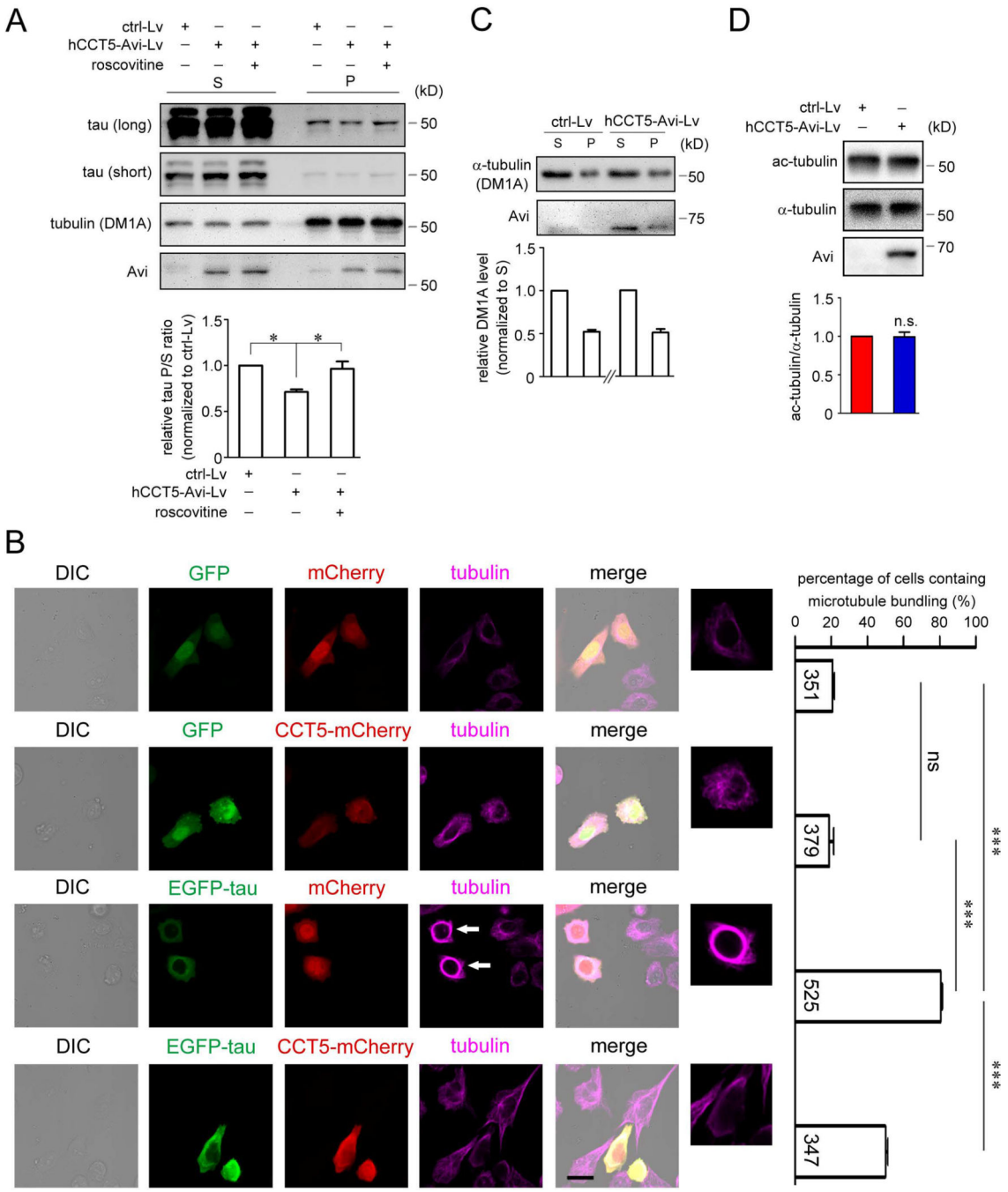

Figure 5. Upregulation of CCT5 reduces tau binding to microtubules and prevents tau-induced microtubule bundling.

(A) Rat E18 cultured cortical neurons that were infected with control or CCT5-Avi lentivirus for 4 days were treated with vehicle or 20 μM roscovitine for 8 h followed by microtubule-binding assay as **A**. The supernatant (S) and pellet (P) fractions were analyzed by SDS–PAGE followed by immunoblotting with tau antibody to visualize tau and DM1A antibody to detect tubulin. The ratios of tau in P versus S fractions were quantitated. N=3; **p*<0.05, one way-ANOVA test followed by Bonferroni multiple comparison test. **(B)** mCherry or

CCT5-mCherry was co-transfected with either EGFP or EGFP-tau into CHO cells. Microtubules were stained with the DM1A antibody against α-tubulin and presented in magenta color. Arrows indicate cells containing tau-induced microtubule bundling. ***p<0.001, ns, not significant, one way-ANOVA test followed by Bonferroni multiple comparison test. **(C)** Lentivirus-infected primary neurons were lysed with hypotonic buffer. Equal volumes of supernatant (S) and pellet (P) proteins were separated by SDS-PAGE and immunoblotted with antibodies against α-tubulin (DM1A) and Avi. N=3. **(D)** Rat cortical neurons infected with hCCT5-Avi or control lentivirus for 4 days were analyzed for tubulin acetylation. N=3, n.s., not significant, paired student t-test.

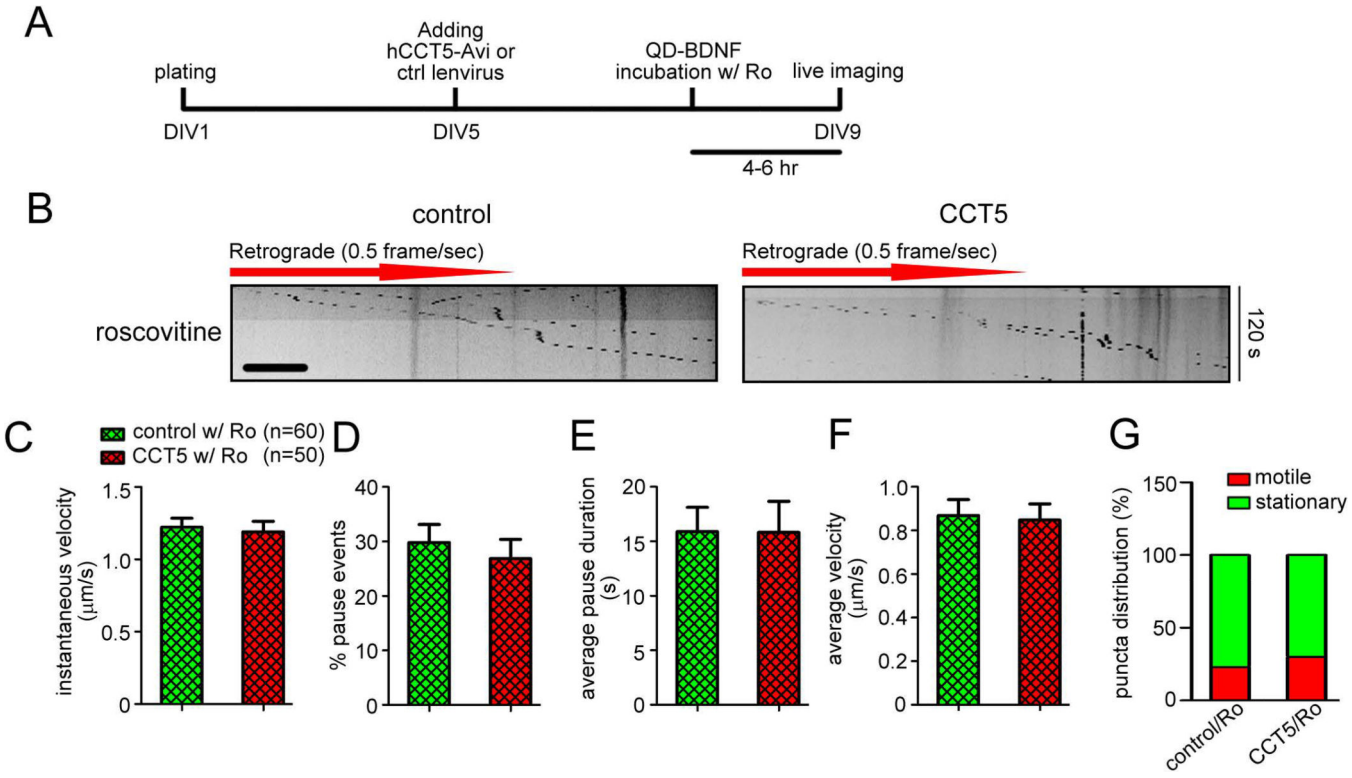

Figure 6. CCT5 mediates promotion of retrograde axonal transport of BDNF likely through CDK5 pathway in rat E18 cortical neurons.

(A) Experimental design. **(B)** Representative kymographs of axonally transported QD-BDNF in cultured rat cortical neurons infected with control or CCT5-Avi lentivirus. These neurons were treated with 20 μM roscovitine (Ros) for 4 h in microfluidic chamber. Scale bar, 10 μm. The data for retrograde instantaneous velocity, percentage of pause events, average pause duration, average velocity of axonally transported QD-BDNF were quantitated and presented in **C-F**. Results are shown as mean ± SEM from three independent experiments with 50–60 QD-BDNF puncta recorded. The distribution of stationary versus non-stationary of QD-BDNF puncta is presented in **G**.