Phosphorylation of Amyloid Precursor Protein at Threonine-668 is Essential for its Copper-Responsive trafficking in SH-SY5Y neuroblastoma cells.

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Background: The function, localization and processing of the amyloid precursor protein (APP) is regulated by phosphorylation.

Results: Copper promotes APP trafficking by phosphorylation at Threonine-668 in SH-SY5Y cells.

Conclusion: By promoting APP phosphorylation, copper regulates its intracellular localization.

Significance: Understanding the role copper plays in regulating APP function in normal neuronal cells will provide insight into the interplay between copper and APP in normal and pathological conditions.

ABSTRACT

Amyloid Precursor protein (APP) undergoes post-translational modification, including O- and N-glycosylation, ubiquitination and phosphorylation as it traffics through the secretory pathway. We have previously reported that copper promotes a change in the cellular localization of APP. We now report that copper increases the phosphorylation of endogenous APP at Threonine 668 (T668) in SH-SY5Y neuronal cells. The level of APPT668-p (detected using a phosphosite-specific antibody) exhibited a copper-dependent increase. Using confocal microscopy imaging we demonstrate that the phospho-deficient mutant, T668 to Alanine (T668A), does not exhibit detectable copper-responsive APP trafficking. In contrast, mutating a serine to an alanine at residue 655 does not affect copper-responsive trafficking. We further investigated the importance of the T668 residue in copper-responsive trafficking by treating SH-SY5Y cells with inhibitors for glycogen synthase kinase 3-β (GSK3-β) and cyclin-dependent kinases (Cdk), the main kinases that phosphorylate APP at T668 in neurons. Our results show that the GSK3-β kinase inhibitors LiCl, SB 216763 and SB 415286 prevent copper-responsive APP trafficking. In contrast, the Cdk inhibitors Purvalanol A and B had no significant effect on copper-responsive trafficking in SH-SY5Y cells. In cultured primary hippocampal neurons, copper promoted APP re-localization to the axon and this effect was inhibited by the addition of LiCl, indicating that a lithium-sensitive kinase(s) is involved in copper-responsive trafficking in hippocampal neurons. This is consistent with APP axonal transport to the synapse, where APP is involved in a number of functions. We conclude that copper promotes APP trafficking by promoting a GSK3-β dependent phosphorylation in SH-SY5Y cells.
Amyloid Precursor Protein (APP) is an integral type I trans-membrane protein that is synthesized in the endoplasmic reticulum and transported through the Golgi network via the secretory pathway where it undergoes post-translational modifications including N- and O-glycosylation, ubiquitination and phosphorylation (1-11). A proportion of APP reaches the plasma membrane where it is rapidly endocytosed and trafficked through endocytic and recycling compartments back to the cell surface or degraded by lysosomes (reviewed in (12,13)). The trafficking process of APP has been intensively studied as it is closely linked to its processing and the generation of the toxic amyloid β peptide (Aβ) peptide central to Alzheimer’s disease (AD) pathogenesis. APP can be cleaved by α, β and γ-secretases, which are localized to specific subcellular compartments. For instance, APP is cleaved by the β-secretase BACE1 in acidic compartments (the trans-Golgi, early endosomes) to generate a soluble ectodomain (sAPPβ) and a C-terminal fragment (β-CTF). C-terminal fragments can be further processed by the γ-secretase complex, which resides in the endocytic compartment or late endosomes to release the Aβ peptide. Processing by β- and γ-secretases to generate Aβ is referred to as the amyloidogenic processing pathway (reviewed in (12,13)). α-secretase cleavage (non-amyloidogenic) occurs primarily at the plasma membrane (PM). There has been much debate as to the normal function of APP. In neurons APP function has been associated with neurite outgrowth, neuronal migration and repair via interaction with extracellular matrix proteins (14-16). APP undergoes rapid kinesin-1 dependent anterograde transport and reaches presynaptic terminals (17,18). At the synapse, APP is involved in synapse formation, synaptic transmission, plasticity and learning and memory (reviewed in (19)). Relevant to this study, APP is also involved in copper homeostasis (20,21). Identifying the cellular signals, which mediate APP trafficking is central to understanding its normal function and processing, including yielding the toxic Aβ peptide of Alzheimer’s disease.

The subcellular localization of APP is regulated by phosphorylation at a number of sites within the intracellular domain. APP is phosphorylated at eight residues (Y653, T654, S655, S675, T668, Y682, T686 and Y687; APP695 numbering), within the APP intracellular domain (AICD). Phosphorylation at these sites has been reported to impact APP processing and cellular localization (reviewed in (22)). The phosphorylation of APP at Thr668-668 (T668) results in a significant conformational change that may affect interactions with binding partners and hence impact its subcellular localization and metabolism (23). Phosphorylation at T668 is a normal process associated with neurite extension, anterograde transport of vesicular cargo and in signaling to the nucleus (24-28). APP is phosphorylated at T668 in vitro and in vivo by a number of kinases including glycogen synthase kinase 3-β (GSK-3β), Jun N-terminal kinase-3 (JNK3), cell division cycle protein (Cde2) and Cyclin-dependent kinase 5 (Cdk5) (7,29-32). Whether and how phosphorylation at T668 impacts APP processing remains controversial with studies showing varying results. For instance, one study reported that phosphorylation of APP at T668 increased Aβ production by enhancing β-secretase cleavage (33) whilst a later study showed a decrease in Aβ due to the inhibition of γ-secretase cleavage (34). In contrast, knock-in mice expressing APP with a threonine to alanine substitution showed no change in APP metabolism including brain levels of Aβ (35). A recent study has shown that non-phosphorylated forms (at T668) of C-terminal APP fragments are associated with lipid raft-like micro-domains where the γ-secretase complex (amyloidogenic) resides, whereas T668 phosphorylated C-terminal fragments reside pre-dominantly in cytoplasmic fractions (36). Hence phosphorylation regulates the localization of APP and thus affects its processing by γ-secretases (36).

We have previously reported that copper promotes the re-localization of APP from a predominant Golgi localization to a wider distribution (37) including the PM,
Cu-dependent APP phosphorylation drives its trafficking which is the predominant site of non-amyloidogenic cleavage by α-secretase. Copper-responsive APP trafficking was due to both a stimulation of exocytosis and suppression of endocytosis of APP (37). Our earlier studies on the copper transport protein which is mutated in Menkes disease, ATP7A, demonstrated that copper induces the trafficking of ATP7A via phosphorylation at specific residues in its C-terminus (38). This was demonstrated by targeted mutagenesis of phosphorylatable residues. In the current study we investigated whether phosphorylation at T668, a widely studied phosphorylation site, is required for copper-responsive APP trafficking. We investigated this by (1) studying copper-responsive trafficking of a phospho-deficient mutant T668A (2) studying the level of phosphorylated T668 using a phosphosite-specific antibody following copper treatment and (3) using kinase inhibitors including lithium chloride (LiCl) to inhibit phosphorylation at T668. Our results from these various approaches strongly suggest that copper promotes a re-localization of APP by phosphorylation at T668 in the neuronal cell model SH-SY5Y. This involves GSK3-β and importantly identifies a novel mechanism by which copper can regulate APP function in neuronal cells.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents- The following antibodies were used in this study: GM130 (BD Transduction Laboratories), β-catenin (abcam), Ankyrin-G (NeuroMab, Davis, CA), C20 (C-terminal APP antibody; calbiochem), Phospho-APP (T668 (D90B8); Cell Signaling Technology); β-actin (Sigma) and W0-2. The antibody CT77 was used to detect the copper transport protein, ATP7A, and was a kind gift from Prof.B. Eipper (Neuroscience and Molecular, Microbial and Structural Biology Division, University of Connecticut). GM130, and Ankyrin-G were used as markers for the cis-Golgi network and as an axonal marker in primary hippocampal neurons, respectively. The C-terminal APP antibody C20 specifically recognizes residues 751-770 and will detect full-length APP and C-terminal fragments. The W0-2 epitope lies within the Aβ domain (1-4 amino acids) and will detect full-length APP as well as the sAPP-α ectodomain and Aβ peptide. Lithium Chloride (Sigma) was used as a GSK3-β inhibitor. Other kinase inhibitors for GSK3-β and cyclin-dependent kinases were obtained from the Torcicscreen Kinase Inhibitor Toolbox (Tocris Bioscience). PhosSTOP Phosphatase inhibitor cocktail tablets (Roche) were used to inhibit phosphatase activity following cell lysis. Western lysis buffer was also supplemented with Complete EDTA-free protease inhibitor cocktail tablets (Roche).

Cell culture and generation of stable cell lines- Human neuroblastoma SH-SY5Y cells (American Type Culture Collection catalog no. CRL-2266) were cultured in DMEM (Invitrogen) containing GLUTAMAX™-I (Invitrogen) supplemented with 10% fetal calf serum and 1 mM sodium pyruvate. Cell lines were cultured at 37°C and in the presence of 5% CO2. To generate SH-SY5Y stable cell lines, cells grown in 6-well plates were transfected with 2.4 µg of plasmid DNA using the Lipojsectamine 2000™ reagent (Invitrogen) according to manufacture’s instructions. Stable SH-SY5Y cell lines were selected and maintained with Geneticin (0.5 mg/ml; Invitrogen) 48 h following transfections. The SH-SY5Y cell lines generated express APP695 or APP with point mutations at the threonine 668 or the serine 655 residue with a C-terminal mCherry fluorescent tag in the pcDNA3.1 vector (Invitrogen). The generation of the pcDNA3.1-APP-cherry expression vector has been previously described (37). To obtain an enriched population of APP-mCherry expressing cells, cells lines were subjected to flow cytometry using the FACSAria III cell sorter (Becton Dickinson).

Isolation of mouse hippocampal primary cultures- Hippocampal neuronal cultures were prepared from E17 mouse C57BL/6 embryos as described previously (39,40) in accordance with ethics committee approval of the University of Melbourne. Briefly, hippocampi were removed, dissected free of meninges and
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dissociated in 0.025% (w/v) trypsin. Dissociated cells were plated onto poly-L-lysine coated coverslips in sterile 24 well culture plates in minimal essential medium (MEM) supplemented with 10% fetal calf serum. Cultures were maintained at 37°C in 5% CO₂ for 2 h before the plating medium was replaced with Neurobasal growth medium containing B27 supplements (Life Technologies Inc., USA). Experiments were performed in fresh Neurobasal medium.

**Copper, copper-chelator and kinase inhibitor treatment**- SH-SY5Y cell lines were treated with copper (CuCl₂) or copper-chelators at a concentration of 150 µM for 3 h in normal growth medium (see above) containing 10% fetal calf serum. The copper chelators used were Bathocuproine disulfonate (BCS) which chelates Cu(I) and D-penicillamine (DPen) for Cu(II), both of which were used at equal concentrations (ie. 150 µM). The copper and copper-chelator concentrations used were based on previously published data and takes into account the presence of copper binding proteins in the serum such as albumin and alpha fetoprotein, which reduce the level of bioavailable copper (37,38,41,42). As hippocampal neurons were cultured in defined Neurobasal medium without serum, a lower concentration of copper was used in these experiments (15 µM). LiCl at 10 mM was used to inhibit GSK3-β activity (43). The potent selective GSK3-β inhibitors, SB 216763 and SB 415286, were obtained from the Tocris inhibitor toolbox and were used at 10 µM as per manufacturer’s instructions. Purvalanol A and Purvalanol B were used to inhibit cyclin-dependent kinases at a concentration of 10 µM (Tocris inhibitor toolbox).

**Immunocytochemistry**- SH-SY5Y stable cells were plated onto 12 mm coverslips at a density of 0.05 x 10⁶/well in 24-well plates. Following copper/copper chelator treatment, cells were fixed in 4% paraformaldehyde in PBS (pH 7.2, Sigma) and permeabilized with 0.1% Triton X-100 (Sigma) and non-specific sites blocked with 1% bovine serum albumin (BSA; Sigma) overnight. Antibodies used for immunocytochemistry were used at the following dilutions: GM130 (1:200), AnkG (1:500), W0-2 (1:20), C20 (1:500) and CT77 (1:500). Primary antibodies were detected using secondary IgG antibodies conjugated to AlexaFluor® 488, 594 or 647 fluorophores (Life Technologies Inc., USA) at a dilution of 1:400. Rhodamine phalloidin (Life Technologies Inc., USA), a fluorescent probe that labels filamentous actin, was used at a concentration of 1:50 to mark the perimeter of individual cells. Images were captured using the Leica SP8 confocal microscope.

**Image analysis**- Images captured were deconvoluted using the Huygens essential software version 4.3 (Scientific Volume Imaging B.B., Hilversum, The Netherlands). For hippocampal neurons, the average intensity of APP fluorescence in a ~200µm length axon (as determined by AnkG fluorescence) and a dendrite segment was quantified per z-stack using the Metamorph Meta Imaging Service Software version 7.1 (Molecular devices, Sunnyvale, CA, USA). The average intensity of APP fluorescence in axons and two dendrites was measured for ten randomly selected hippocampal neurons per treatment.

To quantify copper-responsive trafficking of APP in the exocytic pathway we utilized an analysis we previously used for quantifying the copper-responsive post-Golgi trafficking of the copper-efflux protein ATP7A (44). APP or ATP7A fluorescence intensity was measured from equivalent areas containing the perinuclear regions (defined using nuclear [DAPI] and Golgi [GM130] staining) of at least 10 cells cultured in both copper chelated- and copper- supplemented medium. Fluorescent intensity was measured for the maximum intensity projection per treatment using ImageJ (Wayne Rasband, National Institute of Health, USA).

**Site-directed mutagenesis**- The threonine 668 and serine 655 residues (APP695 numbering) were substituted to an alanine to create a phospho-deficient mutant using the QuikChange Site-directed Mutagenesis Kit (Agilent Technologies) as per the manufacturer’s instructions. Briefly, an amino acid substitution was introduced by
replicating both plasmid strands of the pcDNA3.1-APPcherry expression vector using high fidelity DNA polymerase (PfuTurbo) and two complementary primers, which contained the desired mutation. The synthesized plasmid containing the desired substitution was verified by DNA sequencing.

**Western blot analysis** - Cell lysates were prepared in 50 mM Tris-HCl, 120 mM NaCl, 1% TritonX-100 containing protease and phosphatase inhibitors (Roche). Proteins were separated by SDS-PAGE using a NuPAGE 4-12% Bis Tris gel (Invitrogen) and MES running buffer (Invitrogen), followed by protein transfer to Amersham Biosciences™ Hybond™-ECL 0.2-µm membrane (GE Healthcare). Following transfer, membranes were boiled in PBS for 45 s and blocked in 5% skim milk in Tris-buffered saline (TBS). The membrane was then probed with primary antibodies diluted in TBS containing 0.1% Tween-20. The antibodies and their corresponding dilutions include: W0-2 (1:40); β-catenin (1:2000); Phospho-APP (T668) (1:1000) and β-actin (1:500). Proteins were visualized by probing with the corresponding secondary IgG horseradish peroxidase-conjugated antibody (DAKO; 1:5000). Membranes were developed using the ECL™ Western blotting detection system (GE Healthcare) as per manufacturer’s instructions and visualized using the LAS-3000 Imaging system (Fuji).

**Densitometric analysis** - Protein levels following treatment with copper and copper-chelators (±LiCl) were measured from three independent experiments by densitometry. The pixel intensity (arbitrary unit) per given protein band was quantified using the Multi Gauge software (Fuji). The level of phospho-APPT668 was normalized to total APP detected in the cell lysate.

**Data analysis** - Statistical analyses were performed using One-way ANOVA and Student’s t test expressed as the mean ± SEM. A level of p <0.05 was considered statistically significant (*). To determine whether changes in APP fluorescence in axons and dendrites were statistically significant between treatments, the average APP fluorescence intensity was measured per z-stack for ten randomly selected axons (n=10) and 20 dendrites (n=20; 2 per hippocampal neuron) using the Metamorph imaging software. These values were averaged per axon/dendrite and expressed in relation to axon area (µm²).

**RESULTS**

The phospho-deficient mutant, APPT668A does not traffic in response to copper. We previously reported that copper promotes the trafficking of the copper-transporting P-type ATPase, ATP7A (38), and of APP (37), and the exocytic trafficking pathways appear to be distinct (37). The copper-responsive APP trafficking involved both a stimulation of exocytosis and a suppression of endocytosis. Increased intracellular copper enhanced post-Golgi trafficking of ATP7A to the PM for copper efflux and transport across epithelial cell barriers (44), and this is mediated by phosphorylation at specific sites in the C-terminus of ATP7A (38). Serine 1469, a copper-responsive phosphorylation site, was identified as being required for copper-responsive trafficking of ATP7A from the trans-Golgi Network and the PM. In the current study we investigated whether, similar to ATP7A, copper-responsive APP trafficking (37) is also regulated by phosphorylation at specific sites. Previous studies have reported that APP is phosphorylated at several sites within its intracellular domain, including T668 which is phosphorylated both in vivo and in vitro and has been widely studied (22,32,36,45-47).

To investigate whether phosphorylation of T668 is required for copper-responsive APP trafficking, the T668 residue was substituted to an alanine to create a phospho-deficient mutant at this site. Previous studies have shown that a threonine to alanine substitution at APPT668 effectively mimics the non-phosphorylated state with respect to the helical structure of the cytoplasmic domain (35,36). To determine whether the APPT668A mutant traffics in response to copper in comparison to wildtype APP (APPWT), SH-SY5Y stable cell lines we re generated which express APPT668A and APPWT with a C-terminal cherry fluorescent tag. Both
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of these cell lines were treated with either copper (150 µM CuCl2) or with the copper chelators bathocuproine disulfonate and D-penicillamine (150 µM BCS/DPen) for 3 h. Cells were immunolabeled with the Golgi marker GM130 and the nuclear stain DAPI (Fig. 1). As previously reported APPWTcherry re-localizes from a predominant Golgi localization to a wider distribution following copper treatment (Fig. 1A). By western blot analysis we found that copper-responsive APP re-localization is not accompanied by a change in the protein levels of both endogenous APP protein or the APPWTcherry (data not shown) which we have also demonstrated previously (37).

In contrast to APPWTcherry, APPT668Acherry is localized to a perinuclear localization with partial co-localization with the Golgi marker GM130 following both copper and copper-chelator incubation (Fig. 1B). These results provide strong evidence that copper-responsive trafficking of APP requires phosphorylation at T668 as the phospho-deficient mutant T668A does not exhibit copper-responsive trafficking. We also investigated whether substituting Ser655 to an alanine affects copper-responsive APP trafficking. Fig. 1C shows that the phospho-deficient mutant APPS655Acherry exhibits re-localization similar to wild type APP following copper treatment suggesting S655 is not required for copper-responsive trafficking of APP. Under chelator conditions APPS655Acherry does not appear to exhibit co-localization with GM130 unlike APPT668Acherry and APPWTcherry.

Copper promotes APP phosphorylation at T668. The above results are consistent with copper promoting the phosphorylation of APP at T668. To investigate this using an independent approach, SH-SY5Y cells were incubated with copper or copper-chelators ±10mM LiCl for 3 h. T668 phosphorylation is mediated by the kinases GSK3-β and Cdk5 (29,32). LiCl is an inhibitor of GSK3-β (43) and also indirectly (via the p35/p25 system) an inhibitor of Cdk5 (48,49). Cell lysates were prepared and protein levels were analyzed by western blotting. An antibody to phospho-APP (T668) was used to detect endogenous levels of phospho-APP (T668) (7,25,32,50). The membrane was then stripped and re-probed with W0-2 to determine the total level of APP. APP is detected as three bands by both the phospho-specific antibody and W0-2, which represent glycosylated forms of APP (5). Actin was used as a loading control. Our results show that copper promotes an increase in the level of phospho-APP at T668 in comparison to copper-chelator treatment (Fig. 2A, panel 1). This effect is abolished when cells are treated with both copper and LiCl (n=3; P=0.025). Densitometry analysis was conducted in all three independent experiments comparing the level of pAPP-T668 with that of total APP, which in turn was normalized to actin levels. Results show that copper increased the level of p-APPT668 by 1.65 fold in comparison to copper chelator treatment (n=3; P=0.018; Fig. 2B)).

As substantiation that LiCl was inhibiting GSK-3β activity we analyzed the protein levels of β-catenin, an essential protein of the Wnt signaling pathway, by western blot. Phosphorylation of β-catenin by GSK-3β leads to its degradation via the ubiquitin-proteosome pathway, thus reducing its total levels. Conversely inhibition of GSK3-β leads to an increase in total protein levels of β-catenin (reviewed in (51)). Thus, measuring β-catenin protein levels is a robust read-out of GSK-3β activity. We found that treating SH-SY5Y cells with LiCl for 3 h led to a 1.65 fold increase in the total protein level of β-catenin (data not shown; n=3, P=0.047). These results provide strong evidence that treating with LiCl effectively inhibits GSK3-β activity.

Treatment with LiCl inhibits copper-responsive APP trafficking. As GSK-3β and Cdk5 phosphorylate APP at T668 in neurons (29), we tested whether 10 mM LiCl decreases the level of phosphorylation at this site. LiCl is a commonly used and efficient inhibitor of GSK3-β and has been shown to down-regulate the expression of Cdk5 (48,52). SH-SY5Y cells grown on coverslips were treated with either copper or copper-chelators and LiCl for 3 h in normal growth medium containing 10%
fetal calf serum (see Fig. 3). As a control, cells were treated with either copper-chelators or copper alone (Fig. 3A). Following the 3 h incubation period, cells were fixed and permeabilized and immunolabeled with the APP C-terminal antibody C20, the Golgi-marker GM130 and rhodamine phalloidin as well as the nuclear DAPI stain. A change in the cellular localization of APP was quantified by measuring APP fluorescence intensity at a defined region of interest at the Golgi as determined by GM130 staining for at least ten cells following copper-chelator and copper treatment (see Experimental Procedures). APP is predominantly localized to a Golgi/perinuclear localization and thus a change in its subcellular localization would lead to a decrease in fluorescence intensity in this region. As previously reported, following incubation with copper, endogenous APP re-localizes from a predominant Golgi localization (see arrows, Fig 3A top panel) to a wider distribution including cell extensions (as shown by arrow heads in Fig. 3A). Following copper treatment, a proportion of APP reaches the PM, as we demonstrated previously (37). Copper treatment lead to a 25% decrease in APP fluorescence intensity at a defined Golgi region in comparison to chelator treatment. These data further verify that APP is exiting the Golgi following copper treatment (n=13, P=0.03). In contrast, cells which were treated with both copper and LiCl did not exhibit APP trafficking with APP remaining in a Golgi/perinuclear localization similar to chelator LiCl conditions (see arrows in Fig. 3B and C).

Moreover, there was no statistically significant difference in APP fluorescence intensity at the Golgi when cells were treated with copper-chelator + LiCl and copper + LiCl, suggesting that there was no change in APP cellular localization between treatments (n=10, P=0.28). Treating SH-SY5Y cells with a range of LiCl concentrations (1-50 mM) for 3 h had no effect on cell viability as measured by the PrestoBlue® cell viability assay (data not shown).

Treatment with LiCl does not inhibit the trafficking of the copper transporter ATP7A. We have previously reported copper-responsive trafficking of the copper-

Copper-responsive trafficking of the copper-transporter ATP7A from the Golgi to the PM (42,44,53). To determine whether, similar to APP, copper-responsive ATP7A trafficking is inhibited by LiCl, SH-SY5Y cells were treated with copper chelators or copper together with 10 mM LiCl. Following treatment, cells were immuno-labeled with the antibody W0-2 and CT77 to visualize endogenous APP and ATP7A respectively. Results show that ATP7A traffics in response to copper in the presence of LiCl, unlike APP (Fig. 4). In chelator (+LiCl) conditions ATP7A was tightly localized to a perinuclear localization and dispersed throughout the cell following copper (+LiCl) incubation (merge panels, see arrow heads). This was quantified by measuring ATP7A fluorescence intensity at a defined perinuclear region for ten cells per treatment. Results showed a 35% decrease in fluorescence intensity following copper + LiCl treatment, suggesting that ATP7A is exiting the perinuclear region in response to copper despite the presence of LiCl (n=10; P=0.039). In contrast, APP was localized to a perinuclear region following both chelator + LiCl and copper + LiCl conditions and there was no statistical difference in APP fluorescent intensity in this region between treatments (n=10; P=0.22). These data provide further evidence that copper promotes APP trafficking via a distinct pathway to that of ATP7A (initial evidence in (37)). The data is also consistent with LiCl treatment not having a generalized effect on protein trafficking.

GSK3-β selective inhibitors, but not Cdk inhibitors, prevent copper-responsive APP trafficking in SH-SY5Y cells. To determine whether copper promotes APP trafficking via GSK3-β or Cdk5, selective inhibitors were used from the Tocris kinase inhibitor toolbox (Tocris Bioscience). The GSK3-β inhibitors used include SB 216763 (Fig. 5; C) and SB 415286 (data not shown), which have been shown to be selective and potent inhibitors of GSK3-β (54-56). Purvalanol A (data not shown) and B (Fig. 5, D) were used to inhibit the activity of cyclin dependent kinases including Cdk5-p35, Cdc2/cyclin B, Cdk2/cyclin E and cdk4/ cyclin D1 (57,58). Kinase inhibitors were used
at a final concentration of 10 µM in the presence of copper-chelators or copper (150 µM) in normal growth media for 3 h. As the above inhibitors were dissolved in DMSO, control cells were treated with copper-chelators and copper in the presence of 10 µM DMSO (Fig. 5, A and B). Following treatment, cells were immuno-labeled with W0-2 and CT77 to visualize the localization of endogenous APP and ATP7A, respectively. We co-labeled with ATP7A to investigate whether copper promoted protein trafficking of APP and ATP7A via the same putative signaling pathway. Results show that cells incubated with copper and GSK3-β inhibitors (SB 216763, panel C) did not exhibit copper-responsive trafficking with APP remaining in the Golgi region in the presence of copper as quantified by measuring APP fluorescence intensity at the perinuclear region. Results show no statistical difference in APP fluorescence between copper-chelator and copper+ SB 216763 treatment (n=10; P=0.13), demonstrating no change in APP cellular localization. In the presence of Purvalanol A (data not shown) and B (Fig. 5 D), copper-responsive trafficking was observed with APP re-localizing throughout the cell including extensions, as indicated by arrow heads. Quantitation showed a 27% decrease in APP fluorescence intensity in the perinuclear region when treated with copper + Purvalanol B in comparison to chelator treatment (n=10; P=0.0023), which is consistent with a re-localization of APP. Importantly, ATP7A traffics from a tight Golgi localization under copper-chelator conditions (A) to a wider distribution in the presence of copper and either GSK3-β or Cdk inhibitors (B-D). Taken together these results strongly suggest that copper promotes trafficking of APP and ATP7A via separate pathways. 

**Copper promotes an increase in APP trafficking to axons in primary hippocampal neurons and this is inhibited by LiCl treatment.** To investigate copper-responsive trafficking of APP in primary neurons and the effect of added LiCl, we repeated the above experiments utilizing cultured mouse primary hippocampal cells. Hippocampal neurons isolated from E17 embryos were cultured for 18 days on coverslips to obtain mature neurons (39,40). Neurons were then treated with copper or copper-chelator ± 10mM LiCl for 3 h in Neurobasal medium. Copper was used at 15µM due to the absence of serum in Neurobasal media, as previously described (37,41,59). Following treatment, cells were immuno-labeled with the antibodies C20 and Anti-Ankyrin-G, which visualize respectively endogenous APP and Ankyrin-G, a protein localized at the initial axon segment (60). After copper treatment there was an increase in APP at the axon in comparison to copper-chelator conditions (Fig. 6A-C). To quantify the level of APP at the axon following treatments, the average fluorescence intensity of APP was measured for ten hippocampal neurons per treatment using the Metamorph imaging software (see Experimental Procedures). The addition of LiCl to the copper treatment abolished this effect (Fig. 6A-C). Fig. 6B and C shows the maximum z-projections of 2 representative axons per treatment and further demonstrates that copper increases the level of APP at the axons in comparison to chelator ± LiCl and copper + LiCl axons. Fig. 6D shows that following copper treatment there is a 1.91 fold increase in the level of APP at the axon in comparison to copper-chelator treatment (n=10, P=0.016). There was no increase in APP immunolabelling at the axon when neurons were treated with both copper and LiCl (n=10; copper vs copper + LiCl; P=0.0009).

To determine whether copper promoted significant re-localization of APP to dendrites as well as the axon, the APP fluorescence intensity was measured for two dendrites per hippocampal neuron (n=20). Fig. 6E summarizes our findings that copper promotes trafficking of APP to axons but not dendrites (ie. there is a significantly greater level of APP at the axons but not the dendrites following copper treatment). On the other hand, during chelator ± LiCl or copper + LiCl treatment there is no statistically significant difference between the levels of APP in axon versus dendrites, suggesting APP is evenly distributed throughout neurites. These findings identify a novel mechanism by which copper
may regulate APP function in primary neurons by promoting its trafficking to axons.

**DISCUSSION**

Threonine 668 is a major phosphorylation site within APP which can be phosphorylated by a number of kinases. Cdk5 and GSK3-β phosphorylate APP at T668 in neurons, whereas Cdk1/Cdc2 phosphorylate APP in dividing cells (7,10,29,32,49). Following a cellular stress signal, JNK also phosphorylates APP at T668 (27,30,61). Interestingly, in human and mouse brains it is the mature form (O-glycosylated APP695) that is phosphorylated but not immature APP, suggesting that a fixed pool of APP is constitutively phosphorylated in neurons (32). In this study we used independent approaches to investigate whether copper-responsive trafficking of APP is dependent on phosphorylation, as is the case for ATP7A. The human neuroblastoma cell line, SH-SY5Y, was chosen for this study as it is widely utilized and accepted as a good model for neuronal cells including hippocampal neurons (62,63). In this study, we constructed a phospho-deficient mutant at the T668 site (T668A) to determine whether this residue was important for copper-responsive trafficking of APP. Unlike APPWT, the “phospho-deficient” APPT668A protein did not exhibit trafficking following copper treatment in SH-SY5Y cells. Western blot analysis utilizing an antibody to APP phosphorylated at T668 showed that copper promotes an increase in endogenous APP phosphorylation at this site. In addition, we used LiCl as well as selective potent inhibitors of GSK3-β to determine whether this kinase is involved in copper-responsive phosphorylation of APP at the T668 residue. Our results clearly show that inhibiting the activity of GSK3-β prevents the trafficking of APP from the Golgi to a wider distribution (Fig. 3, 4 and 5). Importantly, inhibiting cyclin-dependent kinases did not inhibit copper-responsive trafficking (Fig. 5). In these studies we have identified a novel mechanism by which copper regulates the localization of APP. In SH-SY5Y cells, this may involve copper signaling upstream of GSK3-β. The activity of GSK3-β is regulated by phosphorylation, modulating its subcellular localization or by protein-protein interactions (reviewed in (79)). Copper may be mediating its effect via a signaling pathway or by directly affecting GSK3-β activity, for example by regulating the activity of a phosphatase that dephosphorylates GSK3-β at its inhibitory residue (Ser9). It has been previously reported that copper is involved in regulating signal transduction pathways (64,65).

In neurons APP is phosphorylated at T668 by GSK3-β and Cdk5 and phospho-APP_{T668} has been observed in neurites and mostly in growth cones of differentiated neuronal cells (25,29,32,66). Phosphorylation of APP at T668 is associated with neurite extension, anterograde transport of vesicular cargo into neurites and axonal transport (25). Studies have shown that APP is localized to synaptic vesicles in the pre- and post-synaptic compartments (reviewed in (12)). At the synapse full-length APP can be transported to the cell surface where α-secretase cleavage occurs to produce sAPPα, which plays a crucial role in synapse formation and maintenance (67). Only a small fraction of APP is cleaved at the cell surface with the remainder internalized to an early endosome compartment where β-secretase and γ-secretase cleavage occurs to produce sAPP-β and Aβ peptides (68,69). Processing products can then be trafficked back to the plasma membrane for secretion (70). Functions for sAPPβ and Aβ include promoting axonal pruning and inhibition of long-term potentiation which impacts synaptic plasticity, respectively (71). In this study we found that in hippocampal neurons copper promotes the trafficking of APP to axons, and not dendrites, and that this is inhibited by LiCl treatment. APP undergoes rapid kinesin and calssytentin 1-dependent anterograde transport in axons with subsequent retrograde transport to dendrites (72-78). We propose that our data showing a copper-responsive increase at 3h of APP at the axon is consistent with axonal anterograde trafficking associated with an early step in post-Golgi exocytic trafficking.
Indeed, we have previously reported that in SH-SY5Y cells (non-polarised) copper promoted APP exocytosis as shown by live cell imaging (37). On the other hand, as we do not observe a significant increase of APP at dendrites or soma we do not believe the data is consistent with copper stimulating APP retrograde transport.

GSK3-β plays a role in a wide range of cellular processes and has numerous targets including proteins involved in metabolism such as glycogen synthase, cytoskeletal proteins and transduction and transcription factors (reviewed in (79)). In neuronal development, GSK3-β is associated with the regulation of neuronal outgrowth, motility and synaptic plasticity as well as axonal polarity and survival (80-84). Our studies demonstrate that copper plays an important role in the trafficking of APP along the axon, thus potentially regulating the function of APP at the synapse. This process was sensitive to LiCl treatment suggesting that copper-responsive APP trafficking to the axon is dependent on a lithium-sensitive kinase(s). This is consistent with the findings in SH-SY5Y cells. Further studies are required to establish the precise kinase(s) responsible for this effect.

As discussed earlier, whether phosphorylation of APP at T668 regulates its processing remains controversial with various studies yielding contradictory outcomes. The discrepancy between the various studies could be due to the nature of the study. Some studies based their finding on the phospho-mimetic APP protein, whereby the threonine is mutated to an aspartate or glutamate. It has been reported that replacing threonine with either of these amino acids does not accurately mimic the phosphorylated state of T668 (36). In contrast, NMR studies have shown that mutating T668 to an alanine accurately simulates the non-phosphorylated state (36). In the current study we investigated the processing products of APP<sub>T668A</sub> and APP<sub>WT</sub> following copper and copper-chelator treatments after a 3 h incubation period. We investigated the level of C-terminal fragments, sAPPα/β and Aβ by western blot and ELISA analysis, respectively. Within the 3 h time frame when we observed copper-responsive APP trafficking we did not observe a detectable change in the level of APP processing products in APP<sub>T668A</sub> and APP<sub>WT</sub> and between treatments (data not shown). This result is consistent with our previous findings, where we reported that copper does not influence the processing of wild-type APP within a 3 h incubation period (37).

In this study we have used independent approaches to conclude that copper-responsive trafficking of APP requires phosphorylation at T668 in SH-SY5Y cells. Although we have yet to fully elucidate the significance of copper-responsive APP trafficking in neurons, our results using cultured primary hippocampal neurons suggest that copper is promoting APP axonal transport, which may impact APP function at the synapse. Elucidation of the cellular function(s) associated with copper-responsive APP anterograde trafficking will be the subject of future investigations.

The physiological and pathological roles of GSK3-β in the central nervous system are diverse and complex (reviewed in (85)). Understanding the relationship between copper, APP and GSK3-β in normal neuronal cells will provide important insight into the role these play in both normal processes and in pathological conditions like Alzheimer’s disease.
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**FOOTOTES**

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2 The abbreviations used are: APP, amyloid precursor protein; Aβ, amyloid β peptide; AD, Alzheimer disease; CTF, C-terminal fragment; PM, Plasma membrane; BCS, bathocuproine disulfonate; DPen, D-penicillamine; BSA, bovine serum albumin; TBS, Tris buffered saline; GSK3-β, glycogen synthase kinase 3-β; cdc2, cell division cycle protein 2; JNK3, Jun N-terminal kinase 3; Cdk, cyclin-dependent kinase; LiCl, lithium chloride.

**FIGURE LEGENDS**

**FIGURE 1:** The phospho-deficient mutant T668A does not exhibit copper-responsive trafficking. SH-SY5Y cell lines stably expressing either APPWT (A) APPT668A (B) or APPS655A (C) with a fluorescent cherry tag were incubated with either 150 μM of copper or 150 μM of the copper chelators bathocuproine disulfonate and D-penicillamine (BCS/dPEN) for 3 h. (A) Following copper treatment APPWT-cherry traffics from the Golgi to a wider distribution throughout the cell (see arrow heads). (B) APPT668A is localized to a perinuclear localization, with partial co-localization with GM130 under both copper-chelator and copper conditions. (C) Under chelator conditions, the phospho-mutant APPS655A localizes to the perinuclear region (arrows) with no co-localization with the GM130 marker. Following copper treatment APPS655A re-distributes throughout the cell. Scale bar = 20 μm.

**FIGURE 2:** Copper increases the level of phosphorylation at T668. SH-SY5Y cells were cultured in 6-well plates and treated with copper (150 μM) or copper-chelators (150 μM) ± 10 mM LiCl for 3 h. (A) Protein levels were analyzed by western immunoblotting, whereby the membrane was probed with the antibody anti-Phospho-APP (T668). The membrane was stripped and then re-probed with W0-2 to detect total APP. The three bands detected in the top two panels represent different glycosylated APP isoforms. Actin was used as the loading control. (B) Western blot results were analyzed from three independent experiments by densitometry. The analysis shows copper increased the level of APP phosphorylation by an average of 1.65 fold in comparison to chelator treatment (n=3; P=0.018). Incubating cells with both copper and LiCl abolished this effect (n=3; P=0.025).
**FIGURE 3: Treatment with LiCl prevents copper-responsive APP trafficking.** SH-SY5Y cells were treated with (A) either copper or copper-chelators for 3 h. Endogenous APP was visualized by staining with the C-terminal APP antibody C20. Cells were co-stained with the nuclear marker DAPI, Golgi marker GM130 and the actin cytoskeleton stain rhodamine phalloidin to visualize the cell surface. Merged images show a partial co-localization of APP and the Golgi marker. Under copper-chelator conditions APP has a predominant Golgi-localization (see arrows). Following copper treatment APP distributes throughout the cell, including extension as shown by arrow heads. (B) Cells were treated with 10 mM LiCl and copper-chelators or copper for 3 h. LiCl did not affect the localization of endogenous APP following chelator conditions (compare top panel A and B). However, in the presence of LiCl, copper did not induce a change in APP localization from the Golgi to a wider distribution. (C) Magnification of merged images showing that copper treatment induces a re-localization of APP to extensions and this is inhibited by LiCl (refer to arrows). Scale bar = 20 µm.

**FIGURE 4: LiCl treatment does not inhibit the copper-responsive trafficking of the copper efflux protein ATP7A.** SH-SY5Y cells were treated with either 150 µM copper-chelators or 150 µM copper and LiCl (10 mM) for 3 h and endogenous APP and ATP7A immuno-labelled with the antibodies W0-2 and CT77, respectively. Unlike APP, which does not exhibit copper-responsive trafficking in the presence of LiCl, ATP7A trafficked from a tight Golgi-localization to a wider distribution (as shown by arrow heads). Scale bar = 20 µm.

**FIGURE 5: SB 216763, a potent and selective GSK3-β inhibitor prevents copper-responsive APP trafficking but not Purvalanol B, a Cdk inhibitor.** To determine whether GSK3-β or Cdk5 activity is required for copper-responsive trafficking SH-SY5Y cells were treated with copper (150 µM) in the presence of 10 µM SB 216763 (C) or Purvalanol B (D), a GSK3-β and Cdk inhibitor respectively. (A) Control panel showing that APP localizes to a Golgi region with partial co-localization to ATP7A in chelator and DMSO conditions. (B) Both APP and ATP7A traffic to a wider cellular distribution following the addition of copper and DMSO. (C) In the presence of copper and SB 216763, APP fails to traffic in response to copper. (D) Purvalanol B does not inhibit APP re-distribution in the presence of copper. ATP7A traffics in response to copper in the presence of both SB 216763 and Purvalanol B. Scale = 10 µm.

**FIGURE 6: Copper promotes the trafficking of APP to axons and this is inhibited by treatment with LiCl.** (A) Hippocampal neurons were isolated from E17 embryos and cultured for 18 days prior to incubation with either 15µM copper or 15µM copper-chelators ±LiCl (10mM) for 3 h in serum free Neurobasal medium. Neurons were immunolabelled with the C20 and Anti-Ankyrin-G antibody to visualize APP (green) and Ankyrin-G (yellow), a protein localized to axon initial segments, respectively. Ankyrin-G was used as an axonal marker. A ~200µm section of the axon and two dendrites (1D and 2D) is outlined by boxes. (B) Maximum-intensity z-projections of axonal regions following chelator/ chelator + LiCl (C) and copper/ copper + LiCl treatment. Images were taken of ten hippocampal neurons per treatment and the average intensity of APP fluorescent labeling along a ~200µm axonal segment (n=10) and two dendrites per neuron (n=20) was measured using the Metamorph imaging software. (D) Shows the average APP fluorescence intensity of ten axonal regions per µm², per treatment. Copper promotes a 1.91 fold increase in the level of APP at the axon in comparison to chelator condition (n=10; P=0.016), and this effect is abolished in the presence of LiCl (n=10; P=0.0009). (E) Shows a comparison between APP fluorescent intensity in the axon and dendrites following various treatments. Copper does not promote an increase in APP fluorescence at the dendrites (n=20; P=0.0026). Scale bar = 10 µm.
Figure 1

Cu-dependent APP phosphorylation drives its trafficking
Figure 2

Cu-dependent APP phosphorylation drives its trafficking
Figure 3

Cu-dependent APP phosphorylation drives its trafficking

A

+Chelator

+ Copper

+Chelator +LiCl

+ Copper +LiCl

B

C

+Copper

+ Copper +LiCl
Cu-dependent APP phosphorylation drives its trafficking

Figure 4
Cu-dependent APP phosphorylation drives its trafficking

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Figure 6

Cu-dependent APP phosphorylation drives its trafficking
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Phosphorylation of Amyloid Precursor Protein at Threonine 668 Is Essential for Its Copper-responsive Trafficking in SH-SY5Y Neuroblastoma Cells

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