The Amyloid- β Precursor Protein Is Phosphorylated via Distinct Pathways during Differentiation, Mitosis, Stress, and Degeneration^D

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Phosphorylation of amyloid- β precursor protein (APP) at Thr⁶⁶⁸ is a normal process linked to neurite extension and anterograde transport of vesicular cargo. By contrast, increased phosphorylation of APP is a pathological trait of Alzheimer's disease. APP is overexpressed in Down's syndrome, a condition that occasionally leads to increased APP phosphorylation, in cultured cells. Whether phosphorylation of APP in normal versus high APP conditions occurs by similar or distinct signaling pathways is not known. Here, we addressed this problem using brainstem-derived neurons (CAD cells). CAD cells that ectopically overexpress APP frequently show features of degenerating neurons. We found that, in degenerating cells, APP is hyperphosphorylated and colocalizes with early endosomes. By contrast, in normal CAD cells, phosphorylated APP (pAPP) is excluded from endosomes, and localizes to the Golgi apparatus and to transport vesicles within the neurites. Whereas the neuritic APP is phosphorylated by c-Jun NH₂-terminal kinase through a pathway that is modulated by glycogen synthase kinase 3 β , the endosomal pAPP in degenerated CAD cells results from activation of cyclin-dependent kinase 5. Additional signaling pathways, leading to APP phosphorylation, become active during stress and mitosis. We conclude that distinct pathways of APP phosphorylation operate in proliferating, differentiating, stressed, and degenerating neurons.

INTRODUCTION

A stated goal of systems biology is to accurately describe and predict how signal transduction pathways function in both normal and diseased cells (Wiley, 2006) and to explain diseases as alterations of the normal signaling pathways or activation of novel pathways that are normally inactive. In this study, we have identified and briefly characterized signaling pathways that all lead to the phosphorylation of a key threonine residue of amyloid- β (A β) precursor protein (APP)—a protein relevant to Alzheimer's disease and Down's syndrome—in the context of normal neuronal function and during degeneration.

Alzheimer's disease, a complex neurodegenerative disorder of old age humans, is characterized by two major brain lesions: the neuritic plaques and the neurofibrillary tangles (Selkoe, 2001). Neuritic plaques contain extracellular deposits of A β peptide generated by proteolytic processing of the transmembrane protein, APP (Price *et al.*, 1995). Neurofibril-

lary tangles consist of intracellular, filamentous aggregates of the microtubule-binding protein, tau (Friedhoff *et al.*, 2000). A β deposition and tau aggregation in Alzheimer's disease are likely the result of altered metabolism and post-translational modification of APP and tau (Selkoe, 2001). Patients with Down's syndrome also develop Alzheimer's disease pathology, including neuritic plaques (Head and Lott, 2004). There is evidence that neurodegeneration and plaque formation in Down's syndrome and some early-onset cases of Alzheimer's disease is likely caused by increased APP levels (Rovelet-Lecrux *et al.*, 2006; Salehi *et al.*, 2006; Sleegers *et al.*, 2006).

Tau aggregation is accompanied by extensive phosphorylation, done mainly by cyclin-dependent kinase 5 (Cdk5) and glycogen synthase kinase 3 β (GSK3 β ; Friedhoff *et al.*, 2000). APP is also hyperphosphorylated in Alzheimer's disease, but the nature of the kinases that perform this phosphorylation is uncertain. The phosphorylated residue is Thr⁶⁶⁸ (numbering for APP695); Cdk5 (Iijima *et al.*, 2000), GSK3 β (Aplin *et al.*, 1996), and c-Jun NH₂-terminal kinase (JNK; Standen *et al.*, 2001; Muresan and Muresan, 2005a) are the candidate kinases. It was proposed that APP phosphorylation alters its metabolism, leading to increased production of carboxy-terminal fragments (CTFs; Lee *et al.*, 2003).

Phosphorylation of APP also occurs normally in differentiated neurons. This phosphorylation has been implicated in the transport of vesicular cargoes into neurites (Muresan and Muresan, 2005b), in neurite extension (Ando *et al.*, 1999; Muresan and Muresan, 2005a), and in signaling to the nucleus (Muresan and Muresan, 2004; Kimberly *et al.*, 2005; Chang *et al.*, 2006). In normal, differentiated neurons, phosphorylation of APP occurs through a specific signaling pathway that uses JNK and the JNK-interacting protein-3 (JIP-3;

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Abbreviations used: APP, amyloid- β precursor protein; Cdk5, cyclin-dependent kinase 5; CTF, carboxy-terminal fragment; GFP, green fluorescent protein; GSK3 β , glycogen synthase kinase 3 β ; JNK, c-Jun NH₂-terminal kinase; JIP, JNK-interacting protein; pAPP, Thr⁶⁶⁸-phosphorylated APP; YFP, yellow fluorescent protein.

Muresan and Muresan, 2005a). Cdk5 and GSK3 β may also contribute to APP phosphorylation, in conditions that remain to be established.

The mechanism of APP phosphorylation is only partially characterized. Recent reports indicate that a primary site of APP phosphorylation in Alzheimer's disease neurons is the endosome (Lee *et al.*, 2003). By extension, it was suggested that the phosphorylated APP (pAPP) found in the neurites of normal neurons might also represent endosomal pAPP (or phosphorylated CTFs). However, we recently reported that pAPP is recruited to Golgi-derived vesicles and is transported by kinesin-1 into neurites (Muresan and Muresan, 2005b). APP is also phosphorylated during cellular stress and during mitosis (a situation relevant to brain development), but little is known about these processes. Here, we characterize the subcellular compartmentalization and the signaling pathways that lead to the production of pAPP in normal neurons (during proliferation, differentiation, and stress) and in neurons that overexpress APP. This condition leads to degeneration of neurons in vivo (Nishimura *et al.*, 1998) and in culture, possibly by increasing sensitivity of APP-overexpressing cells to neurotoxins (Hanson *et al.*, 2003). Our study was conducted in CAD cells (Qi *et al.*, 1997), a CNS-derived, catecholaminergic, neuronal cell line that has emerged as an excellent system for studying neuronal cell biology and pathology (Lee *et al.*, 2003; Muresan and Muresan, 2005b, 2006).

Using CAD cells, we compared the mechanism of phosphorylation of APP during mitosis, normal differentiation, and stress and in cells showing neurodegenerative phenotypes caused by overexpression of APP. We used immunocytochemistry to detect global changes in the amount and localization of pAPP in mitotic and degenerating cells, compared with normal cells. We also used dominant negative approaches and a battery of specific kinase inhibitors to identify the kinases and to define the signaling pathways that lead to APP phosphorylation. We found that, depending on context, at least four mutually exclusive pathways lead to the phosphorylation of Thr⁶⁶⁸ of APP, with distinct consequences on the intracellular localization and function of pAPP.

MATERIALS AND METHODS

Antibodies

The following primary antibodies were used in this study: rabbit anti-Cdk5 (C-8), rabbit anti-p35 (C-19), mouse anti-JIP-1, rabbit anti-JIP-3 (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-early endosome antigen 1 (EEA1; Affinity BioReagents, Golden, CO); mouse anti-Rab5, mouse anti-EB1 (BD Transduction Laboratories, BD Biosciences, Palo Alto, CA); mouse anti- α -adaptin (AP2; Affinity BioReagents); rat anti-CD71 (transferrin receptor; Leinco Technologies, Ballwin, MO); mouse anti-Golgi 58K protein (Sigma, St. Louis, MO); mouse anti-ubiquitin (StressGen Biotechnologies, Victoria, BC, Canada); rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185; Cell Signaling Technology, Beverly, MA); mouse anti-Alzheimer precursor protein A4 (MAB348, Clone 22C11; Chemicon, Temecula, CA); mouse anti-human A β protein (clone 4G8; Signet, Dedham, MA; used in immunocytochemistry); rabbit anti-APP (no. 2452; raised against a polypeptide from the APP cytoplasmic domain; Cell Signaling Technology). The latter antibody recognizes total APP (phosphorylated and nonphosphorylated; Muresan and Muresan, 2004), and was used for immunoblotting. Antibody 44-336Z (BioSource International, Camarillo, CA) was used to detect pAPP. This antibody recognizes specifically the Thr⁶⁶⁸-phosphorylated, but not nonphosphorylated forms of APP (Muresan and Muresan, 2005a,b; see also the BioSource data sheet for this antibody, showing specificity strictly for pAPP). Mouse anti- α -tubulin (Clone B-5-1-1) was from Sigma. The mouse anti-lysosomal membrane glycoprotein (LAMP-1) antibody (1D4B) developed by Dr. Thomas August (Johns Hopkins University, Baltimore, MD) was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Iowa City, IA). Green fluorescent protein (GFP) was detected with mouse (BD Living Colors; BD Biosciences) and chicken antibodies (Aves Labs, Tigard, OR). The anti-GFP antibodies cross-react with YFP.

Cell Cultures and Transfections

The mouse CNS-derived, catecholaminergic cell line, CAD (Qi *et al.*, 1997), was grown in 1:1 F12:DME medium, containing 8% fetal bovine serum and penicillin/streptomycin. Differentiation was induced by culturing cells in serum-free medium.

CAD cells were transfected with FuGene 6 (Roche Diagnostics, Indianapolis, IN). Constructs of human APP695 and GFP-tagged, dominant negative Cdk5 (dnk5-GFP; Niethammer *et al.*, 2000) were obtained from Dr. Li-Huei Tsai (Massachusetts Institute of Technology, Howard Hughes Medical Institute, Cambridge, MA). A JIP-3 construct (pcDNA3-JIP-3-FLAG) was obtained from Dr. Roger Davis (University of Massachusetts Medical School, Howard Hughes Medical Institute, Worcester, MA; Kelkar *et al.*, 2000). A pcDNA3/APP-YFP (human APP695) construct was obtained from Dr. Carlos Dotti (Katholieke Universiteit Leuven, Belgium) and Dr. Christoph Kaether (EMBL, Heidelberg, Germany; Kaether *et al.*, 2000).

SDS-PAGE and Western blot analysis was done as previously described (Muresan and Arvan, 1997).

After transfections, CAD cells were plated in complete medium (to allow attachment), and then transferred to differentiation medium without serum. At 48 h after transfection, cells were fixed for immunocytochemistry. At this stage, CAD cells show extended processes with well-defined growth cones, and axonal transport is robust. Occasionally, CAD cells were fixed after 72–120 h of differentiation. In some cases, CAD cells were fixed without inducing differentiation, to capture cells during mitosis.

Kinase inhibitors were added directly to the culture medium of differentiated or nondifferentiated CAD cells (transfected or not) as follows: the JNK inhibitor, SP600125 (Biomol Research Laboratories, Plymouth Meeting, PA), 25 μ M, 1–8 h (Bennett *et al.*, 2001; Han *et al.*, 2001); the Cdk5 inhibitor, roscovitine (Calbiochem, San Diego, CA), 20 μ M, 6 h or overnight (Niethammer *et al.*, 2000); the GSK3 β inhibitor SB415286 (Sigma), 100 μ M, 1–1.5 h (Wang *et al.*, 2002); and the GSK3 β inhibitor, lithium chloride, 20 mM, 1–1.5 h. Control cultures received equal amounts of dimethyl sulfoxide (DMSO) or 20 mM potassium chloride. For the activation of the JNK stress pathway, cells were treated with sorbitol (0.4 M, 30 min at 37°C; Inomata *et al.*, 2003).

Immunocytochemistry

Transfected or nontransfected CAD cells were fixed for 20 min in phosphate-buffered saline containing 4% formaldehyde and 4% sucrose, permeabilized with 0.3% Triton X-100 (20 min, 20°C), and processed for labeling with antibodies as previously described (Muresan *et al.*, 2000). Secondary antibodies coupled to Alexa dyes (488 and 594) were from Molecular Probes (Eugene, OR). A fluorescein-labeled goat anti-chicken IgY was from Aves Labs. Transfected APP-YFP was detected via the YFP fluorescence. Occasionally, sensitivity of detection was increased by immunolabeling with anti-GFP antibodies. Digital images were obtained with an Olympus IX81 microscope (20 $\times$, 40 $\times$ objectives; Tokyo, Japan) equipped with deep cooled ORCA ER monochrome CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan), and collected using Image-ProPlus software (Media Cybernetics, Silver Spring, MD). Images were processed for contrast and brightness with Adobe Photoshop (San Jose, CA).

The degree of colocalization of various antigen pairs (e.g., pAPP and an endocytic marker; see Figure 3) was quantified as follows: First, the mean pixel intensity for each pair of images was determined in Photoshop CS2 (Sabo *et al.*, 2003). Images were then thresholded at the mean intensity, to eliminate most of the diffuse, nonparticulate labeling. Finally, the thresholded images were multiplied. The resulting image showed nonzero pixel intensities only in regions of colocalization, i.e., regions were pixel intensity differed from zero in both images. Percentages of colocalization were calculated by dividing the number of pixels of nonzero intensities in the product image by the number of pixels of nonzero intensities in the parental images. The degree of colocalization of pAPP with endocytic markers was confirmed by calculating coefficients of colocalization, using Image-ProPlus software (Media Cybernetics; Manders *et al.*, 1993).

The distribution of immunolabeled, fluorescent particles along neurites was analyzed in thresholded, inverted, grayscale images, using the "plot profile" function of the NIH Image 1.63 software. This procedure was used to compare the distribution of two antigens (e.g., pAPP and EEA1; see Supplementary Figure 3) along the neurites.

The percentage of neurites that showed significant accumulation of pAPP at their terminals in cell cultures treated with kinase inhibitors (i.e., SP600125, roscovitine, SB415286, and lithium chloride) was estimated by comparison with DMSO- or potassium chloride-treated cells (see Figure 5 and Supplementary Figure 4). Micrographs, obtained under identical conditions of exposure and image processing, were thresholded in Photoshop CS2, to limit the input levels between 50 and 255. This processing eliminated low intensity labeling. Percentages of neurites that showed pAPP at terminals were derived from the thresholded images (see legend to Figure 5 and Supplementary Figure 4, D–F).

Statistical analysis was done using a two sample *t* test for the two-tailed hypothesis (Zar, 1999). For each experimental condition, data were derived from at least three separate experiments. Between 57 and 150 cells in each experimental group were analyzed.

Figure 1. APP phosphorylation in degenerating neurons. (A–K) Degenerating CAD cells that overexpress APP also show high levels of pAPP (arrows). CAD cells were transfected with APP-YFP, and the transfected fusion protein was detected via the YFP tag. Note that pAPP largely colocalizes with APP-YFP (D–K), with over 70% of vesicles that contain APP-YFP (green; H–K) also staining for pAPP (red; H–K). (L–N) Transfected CAD cells that show differentiated phenotype do not contain increased pAPP levels compared with nontransfected cells. Arrow points to a cell (extending two processes) that overexpresses APP-YFP. (O) Quantitative measurement of pAPP accumulation in the cell body of CAD cells that overexpress APP-YFP. Cells with differentiated or degenerated phenotype were analyzed separately. The graph shows the percentage of APP-YFP-overexpressing cells in each group that showed increased pAPP levels. Error bars, SEM. (P–R) Nontransfected, degenerating CAD cells, identified here by their rounded shape and the abnormal microtubule cytoskeleton (arrows), show increased levels of pAPP. Microtubules are detected with an anti-tubulin antibody. (C, G, K, N, and R) are phase-contrast micrographs. Scale bars, 40 μ m (A–G and L–N); 20 μ m (H–K and P–R).

RESULTS

Overexpression of APP in CAD Cells Is Accompanied by Abnormal Phosphorylation

Our goal was to investigate APP phosphorylation pathways in neurons during normal function and in conditions of increased APP expression. The latter is a feature of Down's syndrome. Toward this end, we used the CNS-derived cell line, CAD (Qi *et al.*, 1997), a well-characterized model system for studying APP phosphorylation (Muresan and Muresan, 2004, 2005a,b), with relevance to neurodegenerative diseases that involve APP (Lee *et al.*, 2003; Muresan and Muresan, 2006, 2007).

Similar to primary neurons (Muresan and Muresan, 2005b), in differentiated CAD cells endogenous pAPP is largely localized to post-Golgi vesicles that accumulate at neurite endings, and represents only a small fraction of the total, mature APP (Supplementary Figure 1). Because of its low level, this endogenous pAPP fraction is difficult to detect—and to quantify—in immunoblots.

In conditions of APP overexpression, the fraction of APP that becomes phosphorylated is easily detectable. As in normal cells, most of the pAPP, detected in cells transfected with APP, is present in the fully matured, sulfated, and glycosylated APP form (Supplementary Figure 1A). However, the pAPP has altered intracellular distribution. Although endogenous pAPP is seldom detected in the cell body, an increased number of CAD cells overexpressing APP show significant or predominant localization of pAPP in the cell body (Figure 1, A–K). Importantly, many CAD cells with increased levels of pAPP also show dystrophic morphology, such as extensive vesiculation, and short or degenerated neurites (Figure 1, C and G). Such neurites, when present, show increased tortuosity and numerous varicosities (Figure 1, D–G), similar to what has been previously

described for cortical neurons treated with fibrillar A β (Grace *et al.*, 2002). Cells that did not show obvious signs of degeneration did not have increased levels of pAPP in the cell body, in spite of the fact that they expressed high levels of APP-YFP (Figure 1, L–N). Quantitatively, we found a significant correlation between the presence of large amounts of pAPP (concentrated in the cell body) and cell degeneration, in CAD cells overexpressing APP. Thus, ~70% of cells that show vesiculation and dystrophic morphology also show increased levels of pAPP. By contrast, only ~6% of cells with normal, differentiated phenotype show increased pAPP in the cell body (Figure 1O).

Under normal culturing conditions, a small number of CAD cells degenerate and die. These are identified by their shape (i.e., rounded and detached from the substrate) and by their abnormal microtubule cytoskeleton, lacking the typical radial distribution of microtubules emanating from the centrosome. We found that these cells consistently show increased levels of pAPP in the cell body, as detected by immunocytochemistry (Figure 1, P–R).

The increased levels of pAPP detected by immunofluorescence in cells that show features of degeneration is not an artifact caused by the rounded shape of these cells, because other proteins for which we tested—including the microtubule plus-end-tracking protein, EB1 (Figure 2, A–C), the phosphorylated JNK, and JIP-3 (see Figure 6, F–J)—are not up-regulated. We also tested whether the cells with degenerated appearance were undergoing apoptosis. As shown in Figure 2, A–F, no nuclear fragmentation is detected in these cells, indicating that increased phosphorylation of APP likely occurs before the degenerating cells become apoptotic. Moreover, apoptotic CAD cells found occasionally in cell cultures do not show increased pAPP levels (Figure 2, G–I). However, we found that cells overexpressing APP, with

Figure 2. CAD cells that overexpress APP do not show nuclear fragmentation (A–F), but contain ubiquitinated inclusions (J–L). Nontransfected (G–I) or APP-YFP transfected cells (A–F and J–L) were stained for DNA (DAPI; A, D, and G), exogenously expressed APP-YFP (with anti-GFP antibody; B, E, and K), pAPP (H), EB1 (C), and ubiquitin (UBI; J and inset). Note that an apoptotic cell, with fragmented nucleus, does not show increased pAPP levels (G–I). The inset in J shows that ubiquitin is detected in large inclusions, typical for neurodegenerating neurons that contain mis-folded proteins. Also note that the level of EB1 (a cytoplasmic microtubule-binding protein) is not increased in APP-overexpressing cells (A–C). Arrows point to the relevant cells. (F, I, and L) Phase-contrast micrographs. Scale bars, 20 μ m (A–L); 5 μ m (inset).

degenerated appearance, showed increased levels of ubiquitinated proteins (Figure 2, J–L). Neuronal inclusions containing ubiquitinated proteins are commonly detected in many neurodegenerative diseases (Ross and Pickart, 2004). Taken together, these findings indicate that neurons undergoing degeneration show increased levels of phosphorylated APP that is abnormally localized to the cell body.

pAPP Localizes to Different Intracellular Compartments in Normal and Degenerated CAD Cells

Next, we set to identify the subcellular compartments containing pAPP within the cell body of normal and degenerated CAD neurons. In normal, differentiated cells, pAPP is only occasionally detected in the cell body. In such cases, most of it colocalizes with the Golgi marker protein, 58K (Supplementary Figure 2), suggesting its presence within the Golgi apparatus. This result is consistent with the reported preferential phosphorylation of the fully matured, glycosylated and sulfated APP form (processed in the Golgi apparatus), compared with immature forms (Ando *et al.*, 1999; see also Supplementary Figure 1A, lanes 3 and 4).

As previously reported, within neurites, pAPP colocalizes with the JNK scaffolding proteins, JIP-1 and -3; activated, phosphorylated JNK; and the anterograde motor, kinesin-1 (Muresan and Muresan, 2005a). On the basis of these, and other results (Muresan and Muresan, 2005b), we concluded that APP is phosphorylated in the cell body, and accumulates in the distal neurites by anterograde transport. However, a similar distribution pattern would be detected if APP were phosphorylated at the neurite terminals (in endosomes), and then transported retrogradely toward the cell body. Such a retrograde transport of APP, endocytosed along the axonal surfaces, has been previously described

Figure 3. Most neuritic pAPP is not localized to early endosomes or lysosomes, in differentiated CAD cells. Differentiated CAD cells were double labeled for pAPP and one of the following endosomal or lysosomal marker proteins: EEA1 (A–C), Rab5 (D–G), AP2 (H–J), transferrin receptor (TfR; K–M), or LAMP1 (N). Each image shows a distal neurite with the terminal, at high magnification. Note that, although endocytic markers are present at neurite terminals, as reported for primary neurons, they do not significantly colocalize with pAPP. Quantitatively, in each case, <15% of the pAPP present at the terminal or along the neurites colocalized with the endocytic marker (see also Supplementary Figure 3). Insets have been adjusted for contrast and brightness to allow clear visualization of particles containing pAPP and endocytic markers. Panel G corresponds to the region marked by the bracket in F. Insets correspond to the marked regions, indicated by brackets, in (C and J). Scale bars, 5 μ m (G–M, and insets in A–C and J); 10 μ m (A–C and N); 20 μ m (D–F).

(Yamazaki *et al.*, 1995; Marquez-Sterling *et al.*, 1997). We therefore asked whether neuritic pAPP could represent endocytosed, rather than Golgi-derived, exported pAPP. First, we carried out double-immunolabeling experiments of pAPP with protein markers for the early endosomal compartments: AP2 (Ball *et al.*, 1995), EEA1 (Stenmark *et al.*, 1996), and the small GTPase, Rab5 (Gorvel *et al.*, 1991). Figure 3, A–J, shows that pAPP and the endocytic markers are distributed throughout the neurites in a punctate, vesicle-like manner. Although the degree of colocalization of pAPP with the various markers of endocytosis was variable, generally there was minimal overlap between the immunolabeling of pAPP and of either EEA1 (Figure 3, A–C and Supplementary Figure 3), Rab5 (Figure 3, D–G), or AP2 (Figure 3, H–J). Second, pAPP does not colocalize with a

Figure 4. pAPP localizes to endosomes in degenerating CAD cells. CAD cells were transfected with APP-YFP (A–F and K–N) or non-tagged APP (G–J), followed by immunolabeling. APP-YFP was detected via its tag (A, D, K, and M). (C, F, J, and N) Phase-contrast micrographs. Insets in G and H show quasi-identical particulate localization of pAPP and Rab5. Also note the increased level of endocytic markers in cells that overexpress APP, suggesting increased endocytosis in these cells. The Golgi apparatus maintains normal morphology and is only slightly up-regulated in degenerating cells that overexpress APP-YFP (L and M). Note that APP-YFP is distributed throughout the cell, whereas the Golgi apparatus is a compact structure confined to a small area adjacent to the nucleus. Also note that the APP-YFP-expressing cell is vesiculated (N). Arrows point to cells overexpressing APP-YFP (A–F and K–N) or APP (G–J). Scale bars, 20 μ m (A–N); 5 μ m (insets in G and H).

bona fide recycling protein, the transferrin receptor (Harding *et al.*, 1983; Figure 3, K–M). Because a large proportion of APP is metabolized in lysosomes, we also tested whether pAPP might be targeted to lysosomes. As shown in Figure 3N, pAPP does not colocalize with the lysosomal marker, LAMP1 (Chen *et al.*, 1985), which is restricted to cell bodies. We conclude that most pAPP detected within neurites is associated with anterogradely moving vesicles, but not with early endosomes or lysosomes.

A fraction of CAD cells transfected with APP contains an enlarged endosomal compartment, suggesting increased endocytic activity in some cells that overexpress APP (Figure 4, A–J). These cells display increased level of pAPP in the cell body, with a substantial fraction of the pAPP being localized to regions that also contain endosomal markers (Figure 4, G–J). As described above, these cells also show morphological features of degeneration. Importantly, although the endosomal compartment is enlarged (Figure 4, A–J), the Golgi apparatus remains compact, and restricted to a small region adjacent to the nucleus (Figure 4, K–N). We conclude that pAPP localizes primarily to compartments of the secretory pathway in normal CAD cells, whereas it preferentially accumulates in enlarged endosomes within the cell body, in degenerating cells.

Distinct APP Phosphorylation Pathways Operate in Normal and Degenerated CAD Cells

To identify the kinases responsible for APP phosphorylation in normal versus degenerating CAD cells, we used pharmacological inhibitors. We have previously shown that, in differentiated CAD cells, JNK activity is required to phosphorylate APP, and to initiate the vesicular transport that leads to accumulation of pAPP at the neurite terminals (Muresan and Muresan, 2005a). Because other studies have implicated Cdk5 and GSK3 β in APP phosphorylation in neurons, we re-examined the effect of inhibitors targeting these kinases on APP phosphorylation. As previously reported, the JNK inhibitor SP600125 efficiently inhibits APP phosphorylation, to the point that pAPP cannot be detected (Figure 5C). These results were reproduced with CAD cells cultured for both short and long time periods, and at various cell densities (our unpublished results). Confirming our previous report (Muresan and Muresan, 2005a), the treatment of CAD cells with the Cdk5 inhibitor, roscovitine does not prevent phosphorylation of APP and accumulation of pAPP at neurite endings (Figure 5D). On the contrary, ~10% more CAD cells appear to accumulate pAPP at neurite terminals upon roscovitine treatment than without treatment. Such increase in the fraction of differentiated cells that accumulate pAPP within neurites is also detected in cells that express a dominant negative form of Cdk5, dnk5 (Figure 5, E–H). These data suggest that blocking Cdk5 activity normally enhances JNK-dependent APP phosphorylation and accumulation of pAPP at neurite terminals. However, the effect of Cdk5 inhibition on APP phosphorylation may be indirect.

Next, we tested the effect of GSK3 β inhibition on APP phosphorylation in differentiated, normal CAD cells. As shown in Figure 5, A and B, inhibition of GSK3 β by treatment with the specific inhibitor, SB415286 reduces the amount of neuritic pAPP, but does not eliminate it. A mostly similar result is obtained upon treatment of CAD cells with lithium chloride (Supplementary Figure 4). These results indicate that GSK3 β facilitates JNK-mediated phosphorylation of APP, and suggest that GSK3 β might be part of the signaling cascade that includes JNK, possibly through activation of a kinase upstream of JNK (Kim *et al.*, 2003). We further tested the participation of GSK3 β in the phosphorylation of APP using CAD cells transfected with JIP-3, a condition that stimulates APP phosphorylation via JNK (Muresan and Muresan, 2005a). As shown in Supplementary Figure 5A, inhibition of GSK3 β significantly reduces the number of JIP-3-transfected cells showing pAPP labeling, whereas JNK inhibition essentially eliminates APP phosphorylation and accumulation of pAPP at terminals. Confirming results obtained in normal CAD cells (Figure 5), inhibition of Cdk5 consistently increases APP phosphorylation (Supplementary Figure 5).

We next examined the effects of these kinase inhibitors on APP phosphorylation in CAD cells that show phenotypes of degeneration in nontransfected cells (Figure 5, I–Q), or in cells transfected with APP-YFP (Figure 5, R–Z'). When treated with either JNK (SP600125) or GSK3 β (SB415286) inhibitors, degenerating CAD cells show accumulation of pAPP in the cell body similar to untreated, degenerating cells (Figure 5, I–N, R–W, and Z'; compare with relevant images in Figure 1). By contrast, the Cdk5 inhibitor, roscovitine, promotes a significant reduction of pAPP in the cell body of degenerating cells (Figure 5, O–Q, X–Z, and Z'). These results support the notion that pAPP in degenerating neurons is the result of the activity of Cdk5, rather than GSK3 β or JNK.

(E–G and I–Q). (Z') Quantitative measurement of the effect of kinase inhibitors on pAPP accumulation in the cell body of cells transfected with APP-YFP. The graph shows the percentage of transfected cells that showed increased pAPP levels. Error bars, SEM; * $p < 0.005$ (roscovitine vs. DMSO).

Further, results of immunocytochemistry are consistent with the activation of Cdk5, but not JNK, in degenerating CAD cells. Indeed, slightly increased levels of Cdk5 and its activator, p35 (or p25; the antibody detects both p35 and p25) are detected in the cell body of degenerating cells that express APP-YFP (Figure 6, A–E). Most importantly, these cells also showed changes in the distribution of Cdk5 and p35/p25 within the cell body (see inset in Figure 6D). By contrast, no increase in the level of the active, phosphorylated form of JNK or of JIP-3 (which facilitates APP phosphorylation by JNK) is detected in such cells (Figure 6, F–J). Taken together, these results suggest that different kinases control APP phosphorylation in normal versus degenerating CAD cells. Specifically, a GSK3 β /JNK pathway operates in normal cells leading to the transport of pAPP into neurites, whereas Cdk5-dependent APP phosphorylation prevails in degenerating neurons, and leads to accumulation of pAPP in endosomal compartments, within the cell body.

Phosphorylation of APP During Stress and Mitosis

Besides the APP phosphorylation pathways described above, at least two other pathways can be activated in CAD cells. First, stress activates a typical JNK cascade that leads to phosphorylation of many substrates, including APP (Inomata *et al.*, 2003; Muresan and Muresan, 2005a). As shown in Figure

Figure 5. Inhibition of Cdk5 decreases APP phosphorylation in degenerating CAD cells (I–Z') but not in differentiated cells (A–H). (A–D) Detection of pAPP in CAD cell cultures treated with inhibitors of GSK3 β (SB415286), JNK (SP600125), or Cdk5 (roscovitine). A DMSO control is also shown (A). We estimated the number of neurites that contained pAPP above a determined threshold level (see *Materials and Methods* and Supplementary Figure 4). Quantitative data, derived from thresholded images (generated to eliminate low-intensity labeling), showed pAPP accumulation in only 36% of neurites, in cultures treated with SB415286. This compares with 66 and 80% of neurites containing pAPP at terminals, in cultures treated with DMSO and roscovitine, respectively. These data are derived from one set of experiments, but similar results were obtained in two other sets of experiments. Thresholded images are not shown. (E–G) Inhibition of Cdk5 by transfection of CAD cells with the dominant negative construct, dnk5-GFP, does not prevent APP phosphorylation and accumulation at neurite terminals (arrows). (H) Quantitative measurement of the effect of dnk5-GFP expression on pAPP localization at neurite terminals. Control cells were transfected with GFP. Percentages of cells with neurites that showed pAPP at terminals are indicated. Error bars, SEM; * $p < 0.01$. (I–Z) Inhibition of Cdk5 (O–Q and X–Z), but not GSK3 β (I–K and R–T) or JNK (L–N and U–W) inhibits APP phosphorylation in degenerating CAD cells, in nontransfected cultures (I–Q) or cells transfected with APP-YFP (R–Z). In nontransfected cultures, degenerating cells were identified by their spherical shape and abnormal microtubule cytoskeleton. Arrows point to degenerating cells. APP-YFP and dnk5-GFP were detected with an anti-GFP antibody. (G, K, N, Q, T, W, and Z) are phase-contrast micrographs. The different appearance of cells in N is due to the accidental use of an incorrect phase ring. Note that, to avoid saturation of fluorescence images containing brightly labeled cells (I–Z), micrographs have been acquired at exposure times that allow only poor visualization of neuritic pAPP. Scale bars, 40 μ m (A–D and R–Z); 20 μ m

7, A–D, the pAPP generated by osmotic stress is distributed diffusely throughout the cell body, without an increase in the neuritically localized pAPP. On the contrary, generation of pAPP via this stress-activated pathway is accompanied by a significant reduction of pAPP accumulation at neurite terminals (Figure 7, E–I). This result suggests that, under conditions of stress, transport into neurites might be diminished, a hypothesis that remains to be confirmed in future studies.

Second, as previously reported (Suzuki *et al.*, 1994), APP is phosphorylated during the G2/M phase of the cell cycle. In nondifferentiated, proliferating CAD cells, APP is phosphorylated only during mitosis. Figure 7, J–L, shows pAPP localized to large particles throughout mitotic CAD cells. In nonneuronal cells, mitotic phosphorylation of APP is apparently performed by the Cdc2 kinase (Suzuki *et al.*, 1994). Consistent with this result, our preliminary data show that the level of pAPP in mitotic CAD cells is significantly reduced in the presence of inhibitors of cyclin-dependent kinases.

Taken together, our results indicate that the same residue in APP, Thr⁶⁶⁸, is phosphorylated through different kinase pathways that are preferentially activated during certain physiological or pathological conditions. We propose that the context of APP phosphorylation—timing and location—allows for distinct functional interactions of pAPP with

Figure 6. Abnormal distribution of Cdk5 and p35/p25, but not JNK and JIP-3, in APP-YFP-overexpressing CAD cells with degenerated phenotype. Detection of Cdk5 (A–C), p35/p25 (D and E), phosphorylated, active JNK (pJNK; F and G), and JIP-3 (H–J) in CAD cells overexpressing APP-YFP (arrows). The relevant cell in D (arrow) is shown in the inset, at higher magnification. APP-YFP was detected with an anti-GFP antibody. (J) A phase-contrast micrograph. Scale bars, 20 μ m (A–J); 10 μ m (inset in D).

other proteins, a hypothesis that remains to be tested in future studies.

DISCUSSION

This study addresses the problem of APP phosphorylation in neuronal cells, using CAD cells as model system, and is intended as a first step toward a more comprehensive investigation of the role of APP phosphorylation in neuronal function and dysfunction. Although the short cytoplasmic domain of APP contains several residues that can be phosphorylated (Oishi *et al.*, 1997), most studies have concentrated on Thr⁶⁶⁸, mainly because its phosphorylation alters significantly the conformation of APP (Ramelot and Nicholson, 2001) and its interaction with physiologically relevant proteins, such as Fe65 and JIP-1 (Muresan and Muresan, 2005b). This phosphorylation may also alter APP processing (Lee *et al.*, 2003; Ryder *et al.*, 2003). Although phosphorylation of APP at Thr⁶⁶⁸ is thus highly relevant to the pathobiology of Alzheimer's disease, the pathways that lead to this phosphorylation, and their regulation, are subject of controversy. Studies from various laboratories reported that, in differentiated neurons, APP is phosphorylated by Cdk5 (Iijima *et al.*, 2000), GSK3 β (Aplin *et al.*, 1996), or JNK (Standen *et al.*, 2001; Muresan and Muresan, 2005a). Although the lack of consensus with regard to this problem may be due to differences between neurons, it is more likely that APP may be phosphorylated by multiple kinases acting either in concert, or separately, according to a particular physiological state of the cell. In addition, certain signaling pathways not normally active may be activated under pathological conditions that lead to, or are caused by, abnormal expression of APP. One aim of our study was to distinguish between signaling pathways that lead to APP phosphorylation in one cell type (i.e., CAD cells), in the normal versus the degenerated state.

Figure 7. APP is phosphorylated in cells subjected to stress, or during mitosis. (A–H) CAD cells were cultured with (C, D, G, and H) or without (A, B, E, and F) sorbitol, and immunolabeled for pAPP. Note the increased amount of pAPP in the cell body, and the diminished accumulation of pAPP at neurite terminals in sorbitol-treated cells (compare G with E). (I) Quantitative measurement of the effect of sorbitol treatment on pAPP localization at neurite terminals. Percentages of cells with neurites that showed pAPP at terminals are indicated. Error bars, SEM; * $p < 0.005$. The graph does not take into account that the pAPP levels at neurite terminals of sorbitol-treated cells, when present, are significantly lower than in nontreated cells. Moreover, a significantly smaller area of the growth cone is occupied by pAPP in sorbitol-treated, compared with control cells (compare G with E). (J–L) A mitotic cell (arrow) shows intense, punctate distribution of pAPP (J). The mitotic spindle was detected with an anti-tubulin antibody (K). (B, D, F, H, and L) Phase-contrast micrographs. Scale bars, 40 μ m (A–D); 20 μ m (E–H and J–L).

We found that, in cells that show morphological traits of degeneration, APP is hyperphosphorylated by Cdk5, and the generated pAPP localizes to endosomes within the cell body. This is different from the situation in normal, differentiated CAD cells, where a small fraction of total APP is phosphorylated by JNK through a signaling pathway that requires the participation of JIP-3 (Muresan and Muresan, 2005a) and GSK3 β (as shown in this study). This pAPP is transported into neurites and accumulates at their terminals (Muresan and Muresan, 2005b).

We started by investigating APP phosphorylation in degenerating CAD cells that naturally occur within cultures of normal CAD cells (which serve thus as controls). Spontaneous degeneration in cultures of CAD cells is rare, but is readily detectable because of alterations in the cell shape and organization of the microtubule cytoskeleton. To increase the number of degenerating cells, we used overexpression of exogenous APP, a condition known to cause degeneration in postmitotic neurons *in vitro* and *in vivo* (Yoshikawa *et al.*, 1992; Nishimura *et al.*, 1998; Hanson *et al.*, 2003). This experimental manipulation mimics defects present in Down's syndrome (Head and Lott, 2004) and some early-onset cases of Alzheimer's disease (Rovelet-Lecrux *et al.*, 2006; Slegers

et al., 2006). Our criteria for classifying a CAD cell as undergoing neurodegeneration included vesiculation, abnormal microtubule cytoskeleton, and short (or absent) neurites that showed varicosities and increased tortuosity. These are generally accepted phenotypes of dystrophic neurons (Grace *et al.*, 2002; Grace and Busciglio, 2003).

We note that a large fraction of CAD cells that overexpress APP show normal phenotype and distribution of pAPP. This indicates that high APP levels per se do not generally cause neuronal degeneration. However, high APP levels increase the incidence of neuronal degeneration, a phenotype that occurs concomitant with increased APP phosphorylation by Cdk5, and up-regulation of early endosomes. These results are consistent with a previous report showing that overexpression of APP increases the level of spontaneous degeneration and viability of neuronal cells in culture (Hanson *et al.*, 2003). Future studies are required to determine why some neurons degenerate, whereas others are resistant to degeneration, when APP levels are increased.

An important finding of our study is the link between neuronal damage and APP phosphorylation. We found that cell damage is accompanied by 1) an overall increase in APP phosphorylation, 2) a switch in the pathway that leads to APP phosphorylation, and 3) a change of the subcellular compartment containing pAPP. Two main phosphorylation pathways are revealed by this study: the GSK3 β /JNK pathway, active in normal, differentiated neurons, and the Cdk5 pathway, active in degenerating neurons. Although it is difficult to test whether the Gsk3 β /JNK pathway is turned off when the Cdk5 pathway becomes activated, our data consistently show that inhibition of Cdk5 enhances the GSK3 β /JIP-3/JNK pathway of APP phosphorylation, which supports this scenario. Thus, fluctuations in the activity of Cdk5 may fine-tune phosphorylation of APP at Thr⁶⁶⁸, via distinct pathways.

What is the significance of APP targeting to endosomes, and of phosphorylation in degenerating neurons? It is likely that this represents a mechanism of eliminating excess APP, when APP levels are increased. APP degradation in endosomes may involve the secretases, leading to generation of phosphorylated CTFs. Such a mechanism may account for the increased levels of phosphorylated CTFs detected in Alzheimer's disease brains (Lee *et al.*, 2003) and for the overall increase of endocytosis in sporadic forms of Alzheimer's disease (Cataldo *et al.*, 1997) and Down's syndrome (Cataldo *et al.*, 2004).

The diagram in Figure 8 illustrates the three pathways of APP phosphorylation proposed to be active in normal, stressed, or degenerating neurons. Phosphorylation of APP via the GSK3 β /JIP-3/JNK pathway commits the generated pAPP to transport into neurites. The large number of APP-binding, scaffolding proteins present in neurons may additionally regulate APP phosphorylation by stabilizing the protein against degradation, changing its localization, and recruiting specific signaling complexes. During stress, JNK phosphorylates many substrates (including APP), in a cascade that may ultimately block transport by kinesin-1 (Morfini *et al.*, 2006). In degenerating neurons, increased Cdk5 activity and altered localization leads to hyperphosphorylation of APP at the endosome. The activation of Cdk5 in degenerating neurons is reminiscent of the activation of cyclin-dependent kinases at mitosis, and could result from a failed attempt of neurons to reenter the cell cycle, as previously proposed (Herrup *et al.*, 2004). Taken together, our results indicate that increased activity and mis-localization of Cdk5 may be key events that accompany neuronal injury. Our work thus further validates the hypothesis that aberrant

Figure 8. Distinct pathways of APP phosphorylation in differentiated and degenerating neurons. The left side of the diagram illustrates the JNK-dependent APP phosphorylation that is facilitated by JIP-3, up-regulated by GSK3 β (which may act either upstream or downstream of JNK), and down-regulated by Cdk5. As depicted, the inhibitory effect of Cdk5 occurs through inactivation—likely indirect—of GSK3 β (for a possible mechanism, see Morfini *et al.*, 2004). This pathway leads to recruitment of kinesin-1, and transport of pAPP into neurites (Muresan and Muresan, 2005b). The precise role of GSK3 β in this pathway remains to be established. The right side of the diagram illustrates targeting of APP to endosomes, and its phosphorylation by Cdk5, in degenerating neurons. The precise site of APP phosphorylation by Cdk5 remains to be established. Also shown is the stress-activated pathway that leads to APP phosphorylation by JNK. Activation of this pathway likely blocks axonal transport of pAPP, possibly through phosphorylation of kinesin-1 (Morfini *et al.*, 2006). APP phosphorylation during mitosis is not shown.

rant Cdk5 activation may lead to neurodegeneration (Patrick *et al.*, 1999; Nguyen *et al.*, 2001; Bu *et al.*, 2002; Cruz and Tsai, 2004; Liu *et al.*, 2004; Fischer *et al.*, 2005). Although the exact relationship between Cdk5 activity, level of APP, and APP phosphorylation is not known, a recent study suggested that the soluble ectodomain of APP may inhibit Cdk5 activity and confer neuroprotection (Han *et al.*, 2005). Future studies are required to clarify the role and significance of aberrant APP phosphorylation during neurodegeneration.

In conclusion, we have shown that phosphorylation of Thr⁶⁶⁸ of APP occurs under various normal and pathological circumstances, and is the result of activation of distinct signaling pathways and kinases. Although the goal of our study was to broadly define these signaling pathways, future work will reveal their precise molecular details. This will allow the selective blocking or activation of specific signaling cascades that target APP, with potential therapeutic value in Down's syndrome and Alzheimer's disease.

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REFERENCES

Ando, K., Oishi, M., Takeda, S., Iijima, K., Isohara, T., Nairn, A. C., Kirino, Y., Greengard, P., and Suzuki, T. (1999). Role of phosphorylation of Alzheimer's

- amyloid precursor protein during neuronal differentiation. *J. Neurosci.* 19, 4421–4427.
- Aplin, A. E., Gibb, G. M., Jacobsen, J. S., Gallo, J. M., and Anderton, B. H. (1996). In vitro phosphorylation of the cytoplasmic domain of the amyloid precursor protein by glycogen synthase kinase-3 β . *J. Neurochem.* 67, 699–707.
- Ball, C. L., Hunt, S. P., and Robinson, M. S. (1995). Expression and localization of alpha-adaptin isoforms. *J. Cell Sci.* 108, 2865–2875.
- Bennett, B. L. *et al.* (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. USA* 98, 13681–13686.
- Bu, B., Li, J., Davies, P., and Vincent, I. (2002). Deregulation of cdk5, hyperphosphorylation, and cytoskeletal pathology in the Niemann-Pick type C murine model. *J. Neurosci.* 22, 6515–6525.
- Cataldo, A. M., Barnett, J. L., Pieroni, C., and Nixon, R. A. (1997). Increased neuronal endocytosis and protease delivery to early endosomes in sporadic Alzheimer's disease: neuropathologic evidence for a mechanism of increased beta-amyloidogenesis. *J. Neurosci.* 17, 6142–6151.
- Cataldo, A. M., Petanceska, S., Terio, N. B., Peterhoff, C. M., Durham, R., Mercken, M., Mehta, P. D., Buxbaum, J., Haroutunian, V., and Nixon, R. A. (2004). Abeta localization in abnormal endosomes: association with earliest Abeta elevations in AD and Down syndrome. *Neurobiol. Aging* 25, 1263–1272.
- Chang, K. A. *et al.* (2006). Phosphorylation of amyloid precursor protein (APP) at Thr668 regulates the nuclear translocation of the APP intracellular domain and induces neurodegeneration. *Mol. Cell. Biol.* 26, 4327–4338.
- Chen, J. W., Murphy, T. L., Willingham, M. C., Pastan, I., and August, J. T. (1985). Identification of two lysosomal membrane glycoproteins. *J. Cell Biol.* 101, 85–95.
- Cruz, J. C., and Tsai, L. H. (2004). Cdk5 deregulation in the pathogenesis of Alzheimer's disease. *Trends Mol. Med.* 10, 452–458.
- Fischer, A., Sananbenesi, F., Pang, P. T., Lu, B., and Tsai, L. H. (2005). Opposing roles of transient and prolonged expression of p25 in synaptic plasticity and hippocampus-dependent memory. *Neuron* 48, 825–838.
- Friedhoff, P., von Bergen, M., Mandelkow, E. M., and Mandelkow, E. (2000). Structure of tau protein and assembly into paired helical filaments. *Biochim. Biophys. Acta* 1502, 122–132.
- Gorvel, J. P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991). rab5 controls early endosome fusion in vitro. *Cell* 64, 915–925.
- Grace, E. A., and Busciglio, J. (2003). Aberrant activation of focal adhesion proteins mediates fibrillar amyloid beta-induced neuronal dystrophy. *J. Neurosci.* 23, 493–502.
- Grace, E. A., Rabiner, C. A., and Busciglio, J. (2002). Characterization of neuronal dystrophy induced by fibrillar amyloid beta: implications for Alzheimer's disease. *Neuroscience* 114, 265–273.
- Han, P., Dou, F., Li, F., Zhang, X., Zhang, Y. W., Zheng, H., Lipton, S. A., Xu, H., and Liao, F. F. (2005). Suppression of cyclin-dependent kinase 5 activation by amyloid precursor protein: a novel excitoprotective mechanism involving modulation of tau phosphorylation. *J. Neurosci.* 25, 11542–11552.
- Han, Z., Boyle, D. L., Chang, L., Bennett, B., Karin, M., Yang, L., Manning, A. M., and Firestein, G. S. (2001). c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. *J. Clin. Invest.* 108, 73–81.
- Hanson, A. J., Prasad, J. E., Nahreini, P., Andreatta, C., Kumar, B., Yan, X. D., and Prasad, K. N. (2003). Overexpression of amyloid precursor protein is associated with degeneration, decreased viability, and increased damage caused by neurotoxins (prostaglandins A1 and E2, hydrogen peroxide, and nitric oxide) in differentiated neuroblastoma cells. *J. Neurosci. Res.* 74, 148–159.
- Harding, C., Heuser, J., and Stahl, P. (1983). Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.* 97, 329–339.
- Head, E., and Lott, I. T. (2004). Down syndrome and beta-amyloid deposition. *Curr. Opin. Neurol.* 17, 95–100.
- Herrup, K., Neve, R., Ackerman, S. L., and Copani, A. (2004). Divide and die: cell cycle events as triggers of nerve cell death. *J. Neurosci.* 24, 9232–9239.
- Iijima, K., Ando, K., Takeda, S., Satoh, Y., Seki, T., Itohara, S., Greengard, P., Kirino, Y., Nairn, A. C., and Suzuki, T. (2000). Neuron-specific phosphorylation of Alzheimer's beta-amyloid precursor protein by cyclin-dependent kinase 5. *J. Neurochem.* 75, 1085–1091.
- Inomata, H., Nakamura, Y., Hayakawa, A., Takata, H., Suzuki, T., Miyazawa, K., and Kitamura, N. (2003). A scaffold protein JIP-1b enhances amyloid precursor protein phosphorylation by JNK and its association with kinesin light chain 1. *J. Biol. Chem.* 278, 22946–22955.
- Kaether, C., Skehel, P., and Dotti, C. G. (2000). Axonal membrane proteins are transported in distinct carriers: a two-color video microscopy study in cultured hippocampal neurons. *Mol. Biol. Cell* 11, 1213–1224.
- Kelkar, N., Gupta, S., Dickens, M., and Davis, R. J. (2000). Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3. *Mol. Cell. Biol.* 20, 1030–1043.
- Kim, J. W., Lee, J. E., Kim, M. J., Cho, E. G., Cho, S. G., and Choi, E. J. (2003). Glycogen synthase kinase 3 beta is a natural activator of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1 (MEKK1). *J. Biol. Chem.* 278, 13995–14001.
- Kimberly, W. T., Zheng, J. B., Town, T., Flavell, R. A., and Selkoe, D. J. (2005). Physiological regulation of the beta-amyloid precursor protein signaling domain by c-Jun N-terminal kinase JNK3 during neuronal differentiation. *J. Neurosci.* 25, 5533–5543.
- Lee, M. S., Kao, S. C., Lemere, C. A., Xia, W., Tseng, H. C., Zhou, Y., Neve, R., Ahljanian, M. K., and Tsai, L. H. (2003). APP processing is regulated by cytoplasmic phosphorylation. *J. Cell Biol.* 163, 83–95.
- Liu, T., Perry, G., Chan, H. W., Verdile, G., Martins, R. N., Smith, M. A., and Atwood, C. S. (2004). Amyloid-beta-induced toxicity of primary neurons is dependent upon differentiation-associated increases in tau and cyclin-dependent kinase 5 expression. *J. Neurochem.* 88, 554–563.
- Manders, E. M. M., Verbeek, F. J., and Aten, J. A. (1993). Measurement of co-localization of objects in dual-colour confocal images. *J. Microscop.* 169, 375–382.
- Marquez-Sterling, N. R., Lo, A. C., Sisodia, S. S., and Koo, E. H. (1997). Trafficking of cell-surface beta-amyloid precursor protein: evidence that a sorting intermediate participates in synaptic vesicle recycling. *J. Neurosci.* 17, 140–151.
- Morfini, G., Pigino, G., Szebenyi, G., You, Y., Pollema, S., and Brady, S. T. (2006). JNK mediates pathogenic effects of polyglutamine-expanded androgen receptor on fast axonal transport. *Nat. Neurosci.* 9, 907–916.
- Morfini, G., Szebenyi, G., Brown, H., Pant, H. C., Pigino, G., DeBoer, S., Beffert, U., and Brady, S. T. (2004). A novel CDK5-dependent pathway for regulating GSK3 activity and kinesin-driven motility in neurons. *EMBO J.* 23, 2235–2245.
- Muresan, Z., and Arvan, P. (1997). Thyroglobulin transport along the secretory pathway. Investigation of the role of molecular chaperone, GRP94, in protein export from the endoplasmic reticulum [published erratum appears in *J. Biol. Chem.* 1997 Nov 28;272(48):30590]. *J. Biol. Chem.* 272, 26095–26102.
- Muresan, Z., and Muresan, V. (2004). A phosphorylated, carboxy-terminal fragment of β -amyloid precursor protein localizes to the splicing factor compartment. *Hum. Mol. Genet.* 13, 475–488.
- Muresan, Z., and Muresan, V. (2005a). c-Jun NH2-terminal kinase-interacting protein-3 facilitates phosphorylation and controls localization of amyloid-beta precursor protein. *J. Neurosci.* 25, 3741–3751.
- Muresan, Z., and Muresan, V. (2005b). Coordinated transport of phosphorylated amyloid-beta precursor protein and c-Jun NH2-terminal kinase-interacting protein-1. *J. Cell Biol.* 171, 615–625.
- Muresan, Z., and Muresan, V. (2006). Neuritic deposits of amyloid- β peptide in a subpopulation of central nervous system-derived neuronal cells. *Mol. Cell. Biol.* 26, 4982–4997.
- Muresan, Z., and Muresan, V. (2007). Seeding neuritic plaques from the distance: a possible role for brainstem neurons in the development of Alzheimer's disease pathology. *Neurodegen. Dis.* 4, 18.
- Muresan, Z., Paul, D. L., and Goodenough, D. A. (2000). Occludin 1B, a variant of the tight junction protein occludin. *Mol. Biol. Cell* 11, 627–634.
- Nguyen, M. D., Lariviere, R. C., and Julien, J. P. (2001). Deregulation of Cdk5 in a mouse model of ALS: toxicity alleviated by perikaryal neurofilament inclusions. *Neuron* 30, 135–147.
- Niethammer, M., Smith, D. S., Ayala, R., Peng, J., Ko, J., Lee, M. S., Morabito, M., and Tsai, L. H. (2000). NUDEL is a novel Cdk5 substrate that associates with LIS1 and cytoplasmic dynein. *Neuron* 28, 697–711.
- Nishimura, I., Uetsuki, T., Dani, S. U., Ohsawa, Y., Saito, I., Okamura, H., Uchiyama, Y., and Yoshikawa, K. (1998). Degeneration in vivo of rat hippocampal neurons by wild-type Alzheimer amyloid precursor protein overexpressed by adenovirus-mediated gene transfer. *J. Neurosci.* 18, 2387–2398.
- Oishi, M., Nairn, A. C., Czernik, A. J., Lim, G. S., Isohara, T., Gandy, S. E., Greengard, P., and Suzuki, T. (1997). The cytoplasmic domain of Alzheimer's amyloid precursor protein is phosphorylated at Thr654, Ser655, and Thr668 in adult rat brain and cultured cells. *Mol. Med.* 3, 111–123.

- Patrick, G. N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L. H. (1999). Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature* 402, 615–622.
- Price, D. L., Sisodia, S. S., and Gandy, S. E. (1995). Amyloid beta amyloidosis in Alzheimer's disease. *Curr. Opin. Neurol.* 8, 268–274.
- Qi, Y., Wang, J. K., McMillian, M., and Chikaraishi, D. M. (1997). Characterization of a CNS cell line, CAD, in which morphological differentiation is initiated by serum deprivation. *J. Neurosci.* 17, 1217–1225.
- Ramelot, T. A., and Nicholson, L. K. (2001). Phosphorylation-induced structural changes in the amyloid precursor protein cytoplasmic tail detected by NMR. *J. Mol. Biol.* 307, 871–884.
- Ross, C. A., and Pickart, C. M. (2004). The ubiquitin-proteasome pathway in Parkinson's disease and other neurodegenerative diseases. *Trends Cell Biol.* 14, 703–711.
- Rovelet-Lecrux, A. *et al.* (2006). APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. *Nat. Genet.* 38, 24–26.
- Ryder, J., Su, Y., Liu, F., Li, B., Zhou, Y., and Ni, B. (2003). Divergent roles of GSK3 and CDK5 in APP processing. *Biochem. Biophys. Res. Commun.* 312, 922–929.
- Sabo, S. L., Ikin, A. F., Buxbaum, J. D., and Greengard, P. (2003). The amyloid precursor protein and its regulatory protein, FE65, in growth cones and synapses in vitro and in vivo. *J. Neurosci.* 23, 5407–5415.
- Salehi, A. *et al.* (2006). Increased App expression in a mouse model of Down's syndrome disrupts NGF transport and causes cholinergic neuron degeneration. *Neuron* 51, 29–42.
- Selkoe, D. J. (2001). Alzheimer's disease: genes, proteins, and therapy. *Physiol. Rev.* 81, 741–766.
- Slegers, K., Brouwers, N., Gijssels, I., Theuns, J., Goossens, D., Wauters, J., Del-Favero, J., Cruts, M., van Duijn, C. M., and Van Broeckhoven, C. (2006). APP duplication is sufficient to cause early onset Alzheimer's dementia with cerebral amyloid angiopathy. *Brain* 129, 2977–2983.
- Standen, C. L., Brownlee, J., Grierson, A. J., Kesavapany, S., Lau, K. F., McLoughlin, D. M., and Miller, C. C. (2001). Phosphorylation of thr(668) in the cytoplasmic domain of the Alzheimer's disease amyloid precursor protein by stress-activated protein kinase 1b (Jun N-terminal kinase-3). *J. Neurochem.* 76, 316–320.
- Stenmark, H., Aasland, R., Toh, B. H., and D'Arrigo, A. (1996). Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. *J. Biol. Chem.* 271, 24048–24054.
- Suzuki, T., Oishi, M., Marshak, D. R., Czernik, A. J., Nairn, A. C., and Greengard, P. (1994). Cell cycle-dependent regulation of the phosphorylation and metabolism of the Alzheimer amyloid precursor protein. *EMBO J.* 13, 1114–1122.
- Wang, X., Janmaat, M., Beugnet, A., Paulin, F. E., and Proud, C. G. (2002). Evidence that the dephosphorylation of Ser(535) in the epsilon-subunit of eukaryotic initiation factor (eIF) 2B is insufficient for the activation of eIF2B by insulin. *Biochem. J.* 367, 475–481.
- Wiley, H. S. (2006). Systems biology beyond the buzz. Lessons from EGFR research show how to kick-start a systems approach for other areas of biology. *The Scientist. Magazine of the Life Sciences* 20, 53–57.
- Yamazaki, T., Selkoe, D. J., and Koo, E. H. (1995). Trafficking of cell surface beta-amyloid precursor protein: retrograde and transcytotic transport in cultured neurons. *J. Cell Biol.* 129, 431–442.
- Yoshikawa, K., Aizawa, T., and Hayashi, Y. (1992). Degeneration in vitro of post-mitotic neurons overexpressing the Alzheimer amyloid protein precursor. *Nature* 359, 64–67.
- Zar, J. H. (1999). *Biostatistical Analysis*, Upper Saddle River, NJ: Prentice Hall.