Neddylation dysfunction in Alzheimer’s disease

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Abstract

Ubiquitin-dependent proteolysis is a major mechanism that downregulates misfolded proteins or those that have finished a programmed task. In the last two decades, neddylation has emerged as a major regulatory pathway for ubiquitination. Central to the neddylation pathway is the amyloid precursor protein (APP)-binding protein APP-BP1, which together with Uba3, plays an analogous role to the ubiquitin-activating enzyme E1 in nedd8 activation. Activated nedd8 covalently modifies and activates a major class of ubiquitin ligases called Cullin-RING ligases (CRLs). New evidence suggests that neddylation also modifies Type-1 transmembrane receptors such as APP. Here we review the functions of neddylation and summarize evidence suggesting that dysfunction of neddylation is involved in Alzheimer’s disease.

Keywords: APP • NAE1 • Alzheimer’s disease • Parkinson’s disease • neurodegeneration • proteasome • neurogenesis • ubiquitination • neddylation • differentiation

Introduction

Ubiquitination tags a target protein for proteolysis when it is misfolded or when it finishes a programmed task. It also plays a role in cell signaling depending on the ubiquitin-linkage type. Ubiquitination is important in maintaining normal cell functions in all types of cells, most relevantly those in the brain. Evidence accumulated in recent years suggests that ubiquitination dysfunction underlies major neurodegenerative diseases. This evidence not only includes the presence of insoluble proteins such as tau tangles and amyloid plaques in Alzheimer’s disease (AD), but also the critical roles certain proteins play in the ubiquitination pathway. One such important pathway protein is APP-binding protein 1 APP-BP1 (BP1), which activates CRLs and regulates Type-1 transmembrane receptor signaling via neddylation. Neddylation has been under intense scrutiny in cancer research (see recent review by Soucy et al. [1]). This current review instead focuses on the emerging field that studies the neuronal functions of APP-BP1 (BP1) and its downstream effectors. We highlight the areas of research that suggests that the BP1-activated neddylation pathway is dysfunctional in AD.

BP1 was first cloned by its interaction with the C-terminus of APP in 1996. It is homologous to Arabidopsis auxin-resistant gene 1 (AXR1) [2, 3]. The ubiquitin-like protein nedd8 was first cloned as a developmentally down-regulated gene expressed in neural precursor cells in 1993 [4]. Both BP1 and AXR1 are homologous to the N-terminus of the ubiquitin-activating enzyme E1 except that they lack the active site cysteine residue required for ubiquitin conjugation and present in the C-terminus of E1. In 1998, Ula1, the yeast counterpart...
of BP1 and AXR1, was shown to form a heterodimer with Uba1 (analogous to mammalian Uba3) to activate Rub1 (related to ubiquitin-1, analogous to medd8) [5]. Based on in vitro studies, BP1 was proposed to be a member of the nedd8-conjugation pathway [6, 7]. In vitro, BP1 together with Uba3 that has an active cysteine and the ATP binding site, behaves like the ubiquitin-activating enzyme E1 and activates nedd8 (Fig. 1). After activation, nedd8 forms a thiolester bond with the nedd8-conjugating enzyme Ubc12. Subsequently, nedd8 is covalently coupled to the substrate in a step mediated by a nedd8 ligase such as the RING domain protein Rbx1 [8, 9], Rbx2 [10], and Tfb3 [11]; an IAP (inhibitor of apoptosis protein) [12, 13]; and Dcn1 (the defective in Cullin neddylation 1) or Dcn1-like protein [14, 15]. The BP1/Uba3 bipartite activating enzyme of nedd8 is also known as NAE (the nedd8-activating enzyme). The series of reactions that lead to the covalent conjugation of nedd8 to the substrate is now known as neddylation.

The function of BP1 in cell growth was first characterized in ts41 cell line, a mutant of V-79 cell line. These cells undergo successive DNA replication without cell division at the non-permissive temperature [16]. In 1992, temperature-shift experiments by Handeli and Weintraub confirmed that the ts41 gene product promotes entry into mitosis but also discovered that it inhibits entry into S phase [17]. An informal communication in 1997 suggests that the ts41 gene encodes the hamster BP1 gene [18]. In 2000, we showed that human BP1 rescues the ts41 gene defect at the S-M checkpoint in a manner dependent on the neddylation pathway [19]. This study provided the first evidence that inhibition of BP1 causes cell death, suggesting that it is a promising target for cancer treatment. A small molecule inhibitor of nedd8 called MLN4924 was subsequently developed for cancer growth inhibition as indicated by their enrichment in protein networks [35] and hundreds of substrates [36]. Figure 1 shows a diagram of the well-characterized Cullin1 (Cul1) CRL (also known as SCF complex) consisting of Cul1, the RING protein Rbx1, the adaptor Skp1, and the F-box (Fbx) protein βTrCP1. This SCF complex recognizes phosphorylated β-catenin for ubiquitination after it binds to βTrCP1 [33].

Overview of the ubiquitination-proteasomal pathway

Ubiquitin is a conserved 76-amino acid protein that covalently binds to lysine residues in target proteins and to itself via the ubiquitination pathway [21, 22]. The ubiquitination pathway is carried out through the actions of three enzymes: E1 (the ubiquitin-activating enzyme), E2 (the ubiquitin-conjugating enzyme), and E3 (the ubiquitin ligase) (Fig. 1). The E1 hydrolyzes ATP to form a thiolester bond with the C-terminal glycine of ubiquitin and transfers the activated ubiquitin to a cysteine residue in E2. The E2-ubiquitin can either bind to E3 to directly transfer ubiquitin to a substrate protein, or transfer the ubiquitin to E3 to form an E3-ubiqutin intermediate and then transfer the ubiquitin to the substrate. When a protein is ubiquitinated, the C-terminus of ubiquitin becomes conjugated to a lysine on the substrate protein by an isopeptide bond. Ubiquitination can repeat several times to attach additional ubiquitin molecules to the lysine residue of a former ubiquitin, which introduces an ubiquitin chain onto the substrate protein [23]. Polyubiquitin chains linked by Lys-48 are essential for 26S proteasome recognition, which leads to degradation of the substrate by the proteasome [24]. Polyubiquitin chains linked by Lys-6 inhibit ubiquitin-dependent proteolysis [25]. Membrane receptor monoubiquitination tags the receptor for endocytosis and degradation in the lysosomes [26]. Monoubiquitination of histones is usually associated with signaling and structural marking [27]. Lys-6-linked polyubiquitin chains are involved in signaling [28] and also mark a receptor for endocytosis and lysosomal degradation [29, 30]. The polyubiquitin chain can be disassembled by the activity of deubiquitinating enzymes and the released ubiquitin is reused in the next ubiquitination cycle [31]. The 26S proteasome is responsible for a large fraction of the regulated protein degradation in eukaryotic cells.

Activation of CRLs by neddylation

E3s control the specificity of proteolysis by recognizing specific substrates through protein-protein interactions. They comprise a diverse family of proteins or protein complexes that can be distinguished based on the type of the interaction domain (RING domain, U-box domain, or HECT domain) used to bind E2 ubiquitin-conjugating enzymes and whether they act as a single subunit or multisubunit complex [32]. A major class of RING domain E3s is the multisubunit ubiquitin-ligase CRLs, which consist of a scaffolding Cullin protein, a zinc-chelating RING protein Rbx1 or Rbx2, and a substrate-recognition subunit. In CRLs, the N-terminus of Cullins interacts with Cullin-specific substrate-recognition subunits, usually via adaptor proteins [33, 34]. Seven Cullins (1, 2, 3, 4A, 4B, 5, and 7) in mammalian cells form about 200 unique CRLs. CRLs play essential roles in cell signaling as indicated by their enrichment in protein networks [35] and hundreds of substrates [36]. Figure 1 shows a diagram of the well-characterized Cullin1 (Cul1) CRL (also known as SCF complex) consisting of Cul1, the RING protein Rbx1, the adaptor Skp1, and the F-box (Fbx) protein βTrCP1. This SCF complex recognizes phosphorylated β-catenin for ubiquitination after it binds to βTrCP1 [33].

![Fig. 1] Neddylation activates ubiquitination of E3 ligase CRLs. A typical SCF complex (purple) is used as an example of a CRL. The activity of CRL is controlled by two major pathways: 1) ubiquitin (Ub) is activated by E1 and transferred to Cul1 by E2, and 2) Cul1 is covalently modified by Nedd8 (N8) via the BP1-activated neddylation pathway. Neddylation changes Cul1 into its active conformation for ubiquitination of the target protein β-catenin, which binds to the substrate receptor βTrCP1. Skp1 is the adaptor that bridges Cul1 and βTrCP1. The neddylation pathway is also regulated by COP9, CAND1, and ASPP2. Green arrows indicate recycling of the components.
Neddylation regulators

Neddylation itself is regulated by several pathways that all affect CRL activity (Fig. 1). The current model is that the dynamic cycling of neddylation and deneddylation is required for CRL activity [52]. Like neddylation, the COP9 signalosomes (CSN) and CAND1 positively regulate CRL activity in vivo [34, 53, 54]. CSN is an evolutionarily conserved complex consisting of eight subunits with similarity to the 19S regulatory particle of the 26S proteasome [34, 53–57]. CSN physically interacts with neddylated Cullins and proteolytically cleaves the isopeptide bond between nedd8 and the Cullin [56, 58, 59]. The neddylation reaction is mediated by the zinc metalloprotease CSN5 (also known as Jab1) [59], but loss of other CSN subunits also leads to destabilization of the entire CSN complex, causing severe development defects in plants [55, 60]. Deneddylation of Cullins by CSN prevents the autocatalytic instability of Cullin substrate-specific adaptors and subunits [61, 62]. CSN appears to differentially regulate Cullins since more CSN is bound to Cul4B than Cul1 under steady-state conditions in 293T cells [35].

Unlike CSN that binds to neddylated Cullins, CAND1 (Cullin-associated and neddylation-dissociated 1) interacts exclusively with unneddylated Cullins [63–65] (Fig. 1). Similar to the manner of regulation by CSN, both decreased and increased CAND1-Cul1 interactions impair the function of SCF complexes in plants [66]. In C. elegans, cand-1 mutants have phenotypes associated with the loss of the SCF complex but lack phenotypes associated with other specific CRLs [67]. Only a small fraction of Cullins are associated with CAND1 [35]. These findings suggest that CAND1 regulates a subset of Cullins and of other currently unknown regulatory mechanisms may exist.

ASP2, another CRL regulator [68], ASPP2, also known as 53BP2 or Bbp, is encoded by TP53BP2 and first cloned as a p53-binding protein [69]. The C-terminus of ASPP2 consists of a proline-rich domain, two ankyrin repeats, and an SH3 domain. Bcl2 and p53 bind ASPP2 competitively to the region that contains the ankyrin repeats and the SH3 domain [70]. ASPP2 interacts with BP1 at two different sites, one near the N-terminus within amino acids 332–483, and the second within the C-terminal amino acids 639–1128 [68]. In HeLa cells, ASPP2 decreases the levels of Cul1 whereas the N-terminal fragment of ASPP2 stabilizes Cul1 [68], which suggests that ASPP2 downregulates Cul1 through the N-terminal domain and that the N-terminal fragment of ASPP2 has a dominant-negative effect. A previous study shows that ASPP2 expression impedes cell cycle progression at G2/M in HEK293 cells [70]. Since ASPP2 interacts with and opposes the effect of BP1 in the cycle progression [17, 19], inhibition of neddylation by ASPP2 is probably the underlying mechanism of ASPP2-mediated cell cycle inhibition and tumour suppression, which was recently reviewed in [71]. We have found that the N-terminal fragments of ASPP2 protect neurons from apoptosis by inhibiting DNA synthesis induced by BP1 overexpression [68]. However, the physiological function of ASPP2 in neurons remains to be determined.

Neddylation in APP proteolysis and signaling

Besides Cullins, nedd8 modifies multiple lysine residues in APP and the EGFR receptor (EGFR) in the cytoplasmic domain [72, 73]. The function of APP is under intense investigation due to its involvement in AD [74, 75]. EGF stimulates EGFR neddylation, which enhances subsequent ubiquitination and sorting of the receptor for degradation [72]. Like EGFR, neddylation appears to regulate APP endocytosis and degradation although it is not clear if ubiquitination is also involved.

APP is neddylated in all the lysine residues in the APP intracellular domain (AICD) [73] (Fig. 2A). Neddylation of APP is most likely due to the interaction with BP1 since the lysine residues are within the BP1-binding sites in APP [49, 76, 77]. The positioning of these lysine residues suggests that they play important roles in APP function and processing. Three neddylated lysines are found near the γ-secretase cleavage site. Interestingly, the other two lysines are located at an equal distance from Y682. Among the five lysine residues, K649, K676, and K685 are conserved in APLP2, whereas only K650 is conserved in APLP1 (Fig. 2B). In primary neurons, acute BP1 expression downregulates APP, thus having no significant effect on APLP1, and shows a potential effect in stabilizing APLP2 [76]. Besides BP1, many other proteins...
have been found to bind to AICD of APP, but it is not known if these other binding proteins play a role in APP neddylation. For reviews of the other binding partners of APP in AICD, refer to King and Turner [78] and Kerr and Small [79].

APP is sorted to the lysosome for degradation after internalization [80]. We have shown that exogenous expression of a familial AD mutant of APP in neurons causes apoptosis and enlargement of early endosomes, and increases receptor-mediated endocytosis via a pathway dependent on the binding of BP1 to APP [80]. Downregulation of BP1 that inhibits neddylation, results in the accumulation of APP, the β-secretase-cleaved C-terminal fragment of APP, and Aβ in primary neurons [76]. These data suggest that neddylation of APP signals for APP degradation via endocytosis.

Receptor endocytosis attenuates receptor signaling. It has been shown that neddylation of AICD inhibits APP signaling by reducing the interaction between APP and FEB5 [73]. The tyrosine residue Y687 in the NPTY motif regulates rapid APP turnover although it does not appear to be an internalization signal [81]. A recent study shows that Y682 is indispensable for the essential function of APP in developmental regulations [82]. Perhaps neddylation of APP involves a conformational change in this region that affects the tyrosine phosphorylation, stability, and signaling of APP.

**Dysfunction of neddylation in AD**

Neddylation is a delicate biological balance of the cell as demonstrated by the effects of CSN and CAND1. Decreased or increased levels of these regulatory molecules both impair CRL activities. It is expected that BP1 manipulations would display similar complexities. In primary neurons, overexpression of BP1 tips the balance to a direction that induces apoptosis, which involves cell cycle reactivation in neurons [19, 49]. Endogenous BP1 protein and mRNA expressions are abundant in some hippocampal pyramidal cells and dentate granule cells in the brain [77] (Fig. 3A). This suggests that BP1 has a physiological function in these cells, which may be mediated by Cullins and membrane receptors including APP and EGFR as mentioned above. SCCRO (squamous cell carcinoma-related oncogene) also known as DCUN1D1, is a Dcn1-like protein and functions as a ligase for nedd8 in the neddylation pathway [83]. In a recent association study, the GG genotype of DCUN1D1 single nucleotide polymorphism (SNP) was found to be a risk factor for the development of frontotemporal lobar degeneration, carrying a fourfold increased risk association [84]. This along with our previous studies suggests that the BP1 pathway plays a pivotal role in the nervous system.

Neddylation has been found to be deregulated in AD. In human brain tissues, BP1 protein levels are elevated in the Triton-insoluble and SDS-soluble fraction in AD tissues compared to that in age-matched controls [19, 49]. In addition, AD conditions change nedd8 localization. In normal hippocampal pyramidal cells and granule cells, nedd8 is located in the nucleus, but in AD hippocampal neurons, most nedd8 immunostaining is present in the cytoplasm (Fig. 3B) [49]. Anti-nedd8 stains neurofibrillary tangles and senile plaques from patients with AD [85, 86]. Moreover, it has been shown that the tau in the tangles is polyubiquitinated [87]. Evidence in the literature also shows that nedd8 can be erroneously incorporated into ubiquitin chains by the ubiquitination enzymes, which may occur under stress conditions and when the nedd8/ubiquitin ratio is increased [88, 89]. It is conceivable that erroneous nedd8 incorporation into tau ubiquitination may also occur due to elevated cytoplasmic nedd8 in AD neurons. Currently, we are investigating if increased nedd8/ubiquitin ratio will lead to impaired function of the targeted proteins such as tau. A potential pathogenic mechanism involving faulty neddylation may underlie the major pathological features of AD.

APP undergoes γ-secretase cleavage to generate Aβ. Presenilin is the enzymatic component of the γ-secretase complex that makes the second cut of APP in the production of Aβ [90]. We have shown that APP, BP1, and PS1 co-migrate to a Triton-insoluble, low density, lipid-raft fraction in brain protein extracts [49, 76]. This fraction of lipid rafts mediates Aβ42 genesis [91]. Lipid rafts consist of the fully assembled γ-secretase complex [92]. Besides interacting with APP, BP1 also interacts with Presenilin [76]. This tripartite interaction not only affects signaling as shown by β-catenin regulation [49-51], but also regulates APP processing [76, 90]. BP1 knockdown stabilizes PS1-CTF [76], which appears to be caused by the reduced activity of a SCF complex since BP1 activates SCF by modifying Cul1 with nedd8. In C. elegans, the Presenilin homologue Sel-12 is downregulated by the Fbx protein Sel-10, a subunit of the SCF complex [93].
Sel-10 interacts with PS1 and enhances PS1 ubiquitination, thus decreasing cellular levels of γ-secretase. Co-expression of Sel-10 and APP in HEK293 cells alters the metabolism of APP and increases the production of Aβ peptides [94]. Along this line of evidence, it has been demonstrated that BP1 suppression dramatically increases Aβ levels, especially the levels of Aβ42, in neurons [76]. Together, these data suggest that BP1-activated neddylation of Cul1 and activation of SCF is necessary for the proper processing of APP.

Besides the role of BP1 in modulating γ-secretase activity, BP1 may also control Aβ42 degradation by activating a parkin-containing ligase. Mutations in parkin cause autosomal recessive early onset PD characterized by the loss of midbrain dopamine neurons [95]. Parkin is a RING protein in the ubiquitin ligase that includes Cul1, Skp1, and the Fbx/WD repeat protein hSel-10 [96, 97]. This Parkin-containing ligase also polyubiquitinates misfolded proteins through a Lys-63-linked ubiquitin chain [98, 99]. Parkin deletion in mutant tau mice causes extensive amyloidosis [100]. Parkin expression has also been shown to ubiquitinate intracellular Aβ and promote Aβ clearance through proteosomal degradation and activation of autophagy [101–103]. These data suggest that activation of the parkin-containing ligase may be beneficial.

The effect of the interaction between APP and BP1 likely depends on cell and tissue environments and developmental stages. BP1 has been shown to inhibit G1/S transition in ts41 cells [17], which suggests that the BP1 pathway is critical for maintaining homeostasis of neurons in response to signaling. However, this finding in ts41 cells was challenged by a recent study showing that BP1 suppression impedes G1/S transition in foetal neural stem cells [104]. The different role of BP1 in the G1/S checkpoint of the cell cycle has not been replicated by independent laboratories in ts41 cells or neural stem cells. In Drosophila, overexpression of the APP-like protein APPL inhibits neddylation whereas augmenting the BP1 pathway rescues APPL-induced apoptosis [105]. This contrasts with our studies using rodent primary neurons in which APP induces apoptosis in a BP1-dependent pathway [49, 80]. These results may reflect the difference between APPL and APP and/or experimental systems.

Neddylation in AD neurogenesis

If the functions of BP1 in cell cycle progression are applicable to neuronal populations, one may expect that the neddylation pathway regulates stem cell renewal. Currently, a BP1 KO animal model is not available, but Uba3 deletion leads to embryonic lethality at the peri-implantation stage [106]. This, together with the fact that BP1 is necessary for cell cycle progression in ts41 cells, suggests that BP1 KO mice may be embryonically lethal and that humans who harbor mutations that jeopardize the function of BP1 in neddylation may not survive beyond the fetus stage. Despite that one cannot observe the effect of BP1 on neurogenesis in vivo and limited information is available from in vitro studies, evidence suggests that the BP1 pathway plays pivotal roles in neurogenesis by interacting with APP and PS1 (Fig. 4).
BP1-activated neddylation attenuates the signaling of EGFR and APP and affects neuronal differentiation. EGFR attenuation inhibits the cell cycle [107], which may be necessary for a neural progenitor cell (NPC) to undergo differentiation. Growth factor withdrawal is a prerequisite for NPC differentiation [108]. In this context, a higher rate of neuronal differentiation is found in NPCs isolated from APP KO mice when grown in culture [109]. Deletion of PS1 that abolishes Aβ40 production during development also increases neuronal differentiation, which depletes NPCs [110]. Similarly, reduction of PS1 in the adult brain increases neuronal differentiation [111]. These data suggest that deletion of APP and PS1 abolishes the replication potential of the NPCs and leads to the default fate of differentiation. Since BP1-activated neddylation downregulates EGFR and APP signaling, neddylation may increase the potential for neuronal differentiation. This appears to be the case since BP1 knockdown is shown to reduce neuronal differentiation [112]. BP1 has also been shown to promote neurogenesis induced by focal transient ischemia [113].

A characteristic of the APP KO mice is reduced body weight in the adult animals [114, 115], which indicates impaired cell cycle progression and reduced NPC self-renewal. Perhaps APP deletion results in the loss of a proliferative signal. The secreted, α-secretase cleavage product of APP, sAPPα, behaves like a growth factor and stimulates NPC proliferation (see recent review [116]). In addition, αβ peptides also induces a strong proliferative effect in NPCs [117]. Meanwhile low levels of monomeric Aβ40 promotes neuronal differentiation whereas low levels of Aβ42 induces gliogenesis [117]. In contrast to these positive effects of sAPPα and αβ peptides in NPCs, AICD has been shown to attenuate NPC differentiation [109, 112]. To summarize the data, the α-secretase cleavage pathway generates sAPPα that favors NPC self-renewal (Fig. 4A) whereas the β- and γ-secretase pathway mainly produces Aβ40 that promotes NPC differentiation (Fig. 4B). BP1 downregulates AICD and ensures non-amyloidogenic processing of APP, both of which promote neuronal differentiation. Currently, how neurogenesis is affected in AD brains remains controversial [118–120]. Clearly more research is warranted before we can successfully apply stem cell therapies for treating neurodegeneration.

Conclusions

A major conclusion of this review is that dysfunction of neddylation is involved in neurodegenerative diseases, especially in AD. BP1 resides in the center of a large network of proteins that organize the postsynaptic proteome by activating neddylation. Major substrates of neddylation consist of CRLs and Type 1 transmembrane receptors. BP1 and CRLs play major roles in APP trafficking and processing. Specifically, by neddylation APP, BP1 regulates APP turnover and signaling in neurons and NPCs.

This review also summarizes the intricate relationship between Presenilin and BP1 in the regulation of APP processing and suggests that NPCs are another major cell type affected by the disease conditions. More research in this area will inform potential stem cell therapies since neurogenesis is likely impaired by AD conditions. The BP1 inhibitor MLN4924 is currently under clinical trials and its potential impact on neuronal survival, neuronal signaling, and NPC proliferation and differentiation should also be examined if such drugs are used for prolonged treatments of cancer.

Overall, this review suggests that a better understanding of the neddylation pathway is needed for discovering invaluable biomarkers and specific drug targets against AD. At the molecular level, the available data suggest that APP regulates ubiquitination, but it remains to be established what exactly APP does in the ubiquitination pathway. Once we know the physiological function of APP, we may have concrete strategies to prevent and treat AD.

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Conflict of interest

The authors declare that there are no conflicts of interest.
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