A pilot exploration with Posiphen to normalize amyloid precursor protein in Down syndrome

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Down syndrome (DS) pathology: DS is the most common cause of Alzheimer's disease (AD) and has a known AD-causing genetic variation which is trisomy of the whole or part of *Homo sapiens* chromosome 21 (HSA21) (Chen and Mobley, 2019a). Compared with AD, DS usually present with various symptoms and manifestations which are related to dysfunction of multiple body systems; the extra copies of the many genes present in HSA21 in DS can cause various developmental problems including neurodevelopmental deficits, which underlie the physical features in DS and may also contribute to the AD-related neurological symptoms of DS like cognition decline (Antonarakis et al., 2020). However, DS and AD share many pathological hallmarks including amyloid plaques and neurofibrillary tangles, synaptic and neuronal loss, dysregulation of endosomal pathway and others (Chen and Mobley, 2019a). The plaques and tangles have been studied extensively, while the dysfunction of early endosome has been found to be impacted long before appearance of amyloid deposits and neurofibrillary tangles with amyloid beta (Aβ) of varing length and phosphorylated tau as the main components, respectively (Nixon, 2017; Chen and Mobley, 2019a). Deficient endosome-mediated retrograde axonal transport of neurotrophic signals plays an important role in the neuropathogenesis in both DS and AD as continuing neurotrophic support is required for maintenance of mature neurons including the basal forebrain cholinergic neurons (BFCNs) whose degeneration has been linked to age-related cognitive dysfunction in DS and AD (Chen and Mobley, 2019b). HSA21 contains about 233 protein-coding genes with several encoding protein products demonstrated to contribute to different phenotypes in DS (Antonarakis et al., 2020) from the extensive researches in the different mouse models of DS, including Ts65Dn mouse which are segmentally trisomic for orthologs of about half of the protein coding genes located on HSA21 (Antonarakis et al., 2020). Accumulated evidence have attributed AD pathogenesis to toxic oligomeric Aβ and tau (Chen and Mobley, 2019a), however, recent clinical trials intending to target them in AD have yet to demonstrate considerable efficacy. Importantly, the 99 amino acid C-terminal fragment (β-CTF) of amyloid precursor protein (APP) was recently found to dysregulate endosomal and lysosomal systems as well as induce cholinergic neurodegeneration in an Aβ-independent manner (Xu et al., 2016; Nixon, 2017; Chen and Mobley, 2019b). Thus, reviewing the hypotheses for AD and discovering novel targets will be beneficial to combat AD and AD in DS.

APP contribution to AD in DS: APP as a type 1 transmembrane protein is processed by sequential cleavage via either β- or a-secretase to produce the C-terminal fragments, β-CTF or α-CTF, respectively. The former is then cleaved by γ-secretase to yield the APP intracellular domain (AICD) and Aβ peptides of varying length while cleavage of the latter yields the same AICD and the P3 peptide(Chen and Mobley, 2019a).

Multiple studies using APP knockdown in cell culture, in the Ts65Dn$^{APP+/-}$ mouse in which *App* gene copy number was reduced to two in Ts65Dn, and using APP/β-CTF overexpression all support the contribution of increased *APP* gene dose in inducing early endosome enlargement phenotypes (Salehi et al., 2006; Xu et al., 2016). Importantly, increased *App* gene dose in Ts65Dn mice markedly reduced the retrograde axonal transport of nerve growth factor signaling and caused neurodegeneration of BFCNs (Salehi et al., 2006). Further studies showed that APP/β-CTF-induced Rab5 hyperactivation and early endosome enlargement were linked to the aberrant retrograde axonal transport of neurotrophin signals (Xu et al., 2016). Definitely increased *APP* gene dose has effects beyond changes in early endosome that may also contribute to neurodegeneration, however, these data strongly support dysregulation of early endosome system along with deficits in neurotrophin signal transduction is linked to degenerations of many neuron populations including BFCNs. It is reasonable to speculate that the cumulative effect over decades of gradually disrupted neurotrophin signaling contributes significantly to neurodegeneration (Chen and Mobley, 2019b). Although APP and Aβ peptide have been shown to cause tau pathology, using CRISPR/Cas9 approach, normalization of *APP* gene dose to two in DS induced pluripotent stem cell (iPSC)-derived cortical neurons was reported to reduce both $Aβ_{42}$ and Aβ42/40 ratio, but fail to affect increased tau phosphorylation (Ovchinnikov et al., 2018). As the protein products of other triplicated genes on HSA21 have also been shown to contribute tau hyperphosphorylation in DS context (Antonarakis et al., 2020), whether or not and to what extent *APP* is related to tau pathology in DS need further elucidation.

In addition, recently β-CTF was shown to mediate APP-induced lysosomal dysfunction through affecting either expression/maturation or activity of lysosomal enzymes (Jiang et al., 2019). The direct evidence for an essential role of APP in AD appearance in DS was from two rare cases of DS subjects who are partial trisomy 21 that is lacked triplication of APP. They are clinically devoid of AD confirmed by several assessments (Prasher et al., 1998; Doran et al., 2017). Thus targeting full length APP (fl-APP) itself becomes an alternative strategy to combat AD pathology in DS and possibly also in AD. This strategy is based on the vast findings supporting the *APP* gene dose hypothesis. Although APP was shown to contribute to several pathologies, whether or not it is feasible to manipulate APP expression in DS with AD *in vivo* to reverse the pathological phenotypes needs to be explored and tested.

APP-targeting strategy: starting with Posiphen: Based on the preclinical studies on the roles of APP in the pathogenesis of AD in DS, several strategies can be envisioned. APP expression can be modulated on the levels of either mRNA or protein. Among them we started to test our hypothesis that normalizing APP protein can rescue AD-relevant endosomal phenotype in Ts65Dn mice (Chen et al., 2020).

Posiphen is an orally available small molecule that targets a conserved regulatory element (iron-response element stem loop) in the mRNAs of several proteins including APP and reduces their translation. Posiphen has been found to decrease fl-APP levels as well as its processing products in several cell lines as well as in AD mouse models. It is noted that Posiphen reduced the levels of APP and its related products, and normalized impairments in spatial working memory, contextual fear learning and synaptic function in the APP/PS1 mouse model of AD (Teich et al., 2018). Importantly, in a Phase I clinical trial Posiphen was proved to be well tolerated and induced reduction in the levels of soluble APP fragments and tau species in the cerebrospinal fluid of mild cognitive impairment subjects (Maccecchini et al., 2012).

We found that *in vitro* Posiphen lowered fl-APP and CTFs in a translation-dependent manner, reversed Rab5 hyperactivation and early endosome enlargement and restored retrograde axonal transport of neurotrophin signaling in Ts65Dn primary neurons. *In vivo*, Posiphen treatment (50 mg/kg/d, 26 days, intraperitoneal delivery) of 16 month-old Ts65Dn mice was also well tolerated and resulted in normalization of the levels of fl-APP, CTFs and induced a reduction in $Aβ_{42}$, reduced Rab5 activity, phosphorylated tau, and more importantly reversed deficits in the activation of tropomyosin receptor kinase B/mitogen-activated protein kinase/cAMP response element-binding protein signaling pathways (**Figure 1**). Choline acetyltransferase (ChAT) is expressed in cholinergic neurons and dysfunction of the cholinergic system contributes to the pathogenesis of AD in DS (Chen and Mobley, 2019b). Posiphen treatment also restored the ChAT levels in Ts65Dn mice to that in normal euploid (2N) mice (Chen et al., 2020). These results provide further evidence supporting the *APP* gene dose hypothesis and also theoretical basis for application of APP-targeting strategy to combat the AD-related pathologies in DS.

In addition to Posiphen, other methods intending to reduce the levels of *APP* mRNA (e.g., antisense oligonucleotides (ASOs)) and the translation of *APP* mRNA (e.g., miRNAs) can also be considered (Chen et al., 2020) (**Figure 1**). Especially the ASO strategy is promising, as ASO has displayed amazing effects to reduce or modify toxic protein expression and enabled clinical trials to treat neurodegenerative diseases including Huntington disease, amyotrophic lateral sclerosis and Parkinson's disease (Leavitt and Tabrizi, 2020).

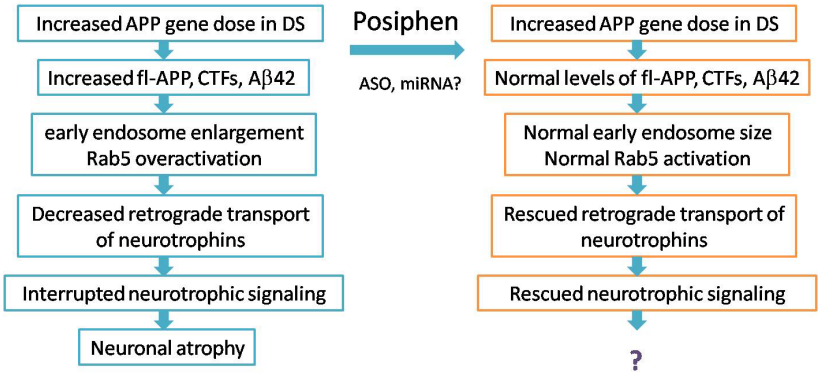

Figure 1 | **Posiphen reverses the endosome-related pathologies in the Ts65Dn mouse model of DS.**
In the brains of Ts65Dn mouse, due to increased *App* gene dose, there are increased *APP* mRNA and protein levels of fl-APP along with its processing products including α-CTF, β-CTF and Aβ peptide species. Increased levels of β-CTF can lead to Rab5 hyperactivation and abnormal early endosome enlargement. Aberrant early endosome may contribute to the deficits in the retrograde axonal transport of neurotrophic signaling in several neuron populations including BFCNs and cortical neurons thus compromising their trophic support for these neurons and leading to neuronal degeneration especially the vulnerable BFCNs. Posiphen treatment can reduce the translation of *APP* mRNA and normalize the protein levels of fl-APP both *in vitro* and *in vivo*, thus correcting the levels of its downstream products including CTFs and $A\beta_{42}$. Consistently, Posiphen treatment also reversed Rab5 hyperactivation and early endosome enlargement induced by APP/β-CTF, and restored the retrograde transport of neurotrophin and neurotrophic signal transduction. It should be noted that *in vivo* Posiphen also normalized the levels of both phosphorylated tau and ChAT protein whose dysregulation contribute to the pathogenesis of DS, further supporting the beneficial effects of Posiphen in DS. Thus, Posiphen rescued the endosomal pathologies in Ts65Dn mice through normalizing the protein levels of fl-APP and its processing products. Whether or not Posiphen treatment can correct the abnormal behaviors especially those related to cognitive function needs further elucidation. In addition to Posiphen, other strategies including ASO and miRNA against *APP* mRNA could be also envisioned to achieve the same goal of normalizing fl-APP levels in DS. Aβ: Amyloid beta; APP: amyloid precursor protein; ASO: antisense oligonucleotide; BFCN: basal forebrain cholinergic neuron; ChAT: choline acetyltransferase; CTF: C-terminal fragment; DS: Down syndrome; fl-APP: full length-amyloid precursor protein.

Limitations and perspective: Although Posiphen has been shown to reverse AD-related early endosome phenotypes in Ts65Dn mice, we failed to rescue the cholinigeic neuron numbers and deficits in behavior assessments including Y-maze, open field and nest building (Chen et al., 2020). Actually, older mice of both 2N and Ts65Dn showed worse performance compared to younger mice in behavior tests like Y-maze, thus age is a possible limiting factor to demonstrate a positive reflection after Posiphen treatment in mouse models. Considering the age of Ts65Dn mice used in our study, we speculate that young animals with treatment for longer periods are needed in combination of more sensitive markers to test the potential protection of Posiphen or other APP-targeting strategies against cholinergic dysfunction and deficient behaviors especially those related to cognitive function. However, our findings motivated renewed attention to how APP and its processing products drive AD-relevant pathologies in DS and consideration of evaluating Posiphen in clinical trials in DS both with and without AD.

Whether or not it is feasible to reverse the other pathologies induced by the year-long increased expression of APP and its products need further exploration. As DS demonstrate AD pathology by 40 years old and dementia by about age 60 years (Chen and Mobley, 2019a), thus when to commit APP-directed treatment in DS need extensive clinical trials. HSA21 owns more than 200 protein-coding genes (Antonarakis et al., 2020). APP exerts significant effects on the transcription of multiple genes in human DS iPSC-derived neurons (Ovchinnikov et al., 2018). Although APP has been proven to have an important role in driving AD-like pathologies in DS, other gene products may also have impact on the pathogenesis of AD in DS with varing degrees. Indeed, normalizing *App* gene dose in Ts65Dn mouse ($Ts65Dn^{APP++-}$) only partially rescued the deficits in retrograde axonal transport of neurotrophin signals (Salehi et al., 2006; Chen et al., 2020), implying that other genes beyond APP may also contribute to this specific pathology. It should also be noted that although we failed to find any effect of Posiphen on the expression of other proteins, the potential off-targeting effects of Posiphen can be limited by drug modification/optimization or by replaced by a more specific strategy like ASO as mentioned above.

Although *APP* gene dose has been argued not to contribute to tau pathology in human DS iPSC-derived cortical neurons (Ovchinnikov et al., 2018), we found that Posiphen not only reduced APP/CTF/$A\beta_{42}$, but also decreased the levels of phosphorylated tau in aged Ts65Dn mice (Chen et al., 2020). Like AD, DS also develops different neuropathologies including inflammation at different stages, thus combined treatments against different pathologies to combat AD in DS can be considered in both preclinical studies and clinical trials.

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