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Paradoxical Effect of TrkA Inhibition in Alzheimer's Disease Models

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Abstract

An unbiased screen for compounds that block amyloid- β protein precursor (A β PP) caspase cleavage identified ADDN-1351, which reduced A β PP-C31 by 90%. Target identification studies showed that ADDN-1351 is a TrkA inhibitor, and, in complementary studies, TrkA overexpression increased A β PP-C31 and cell death. TrkA was shown to interact with A β PP and suppress A β PP-mediated transcriptional activation. Moreover, treatment of PDAPP transgenic mice with the known TrkA inhibitor GW441756 increased sA β PP α and the sA β PP α to A β ratio. These results suggest TrkA inhibition—rather than NGF activation—as a novel therapeutic approach, and raise the possibility that such an approach may counteract the hyperactive signaling resulting from the accumulation of active NGF-TrkA complexes due to reduced retrograde transport. The results also suggest that one component of an optimal therapy for Alzheimer's disease may be a TrkA inhibitor.

Keywords

A β PPneo; Alzheimer's disease; amyloid- β protein precursor; GW441756; nerve growth factor; transcriptional activation; TrkA receptor

INTRODUCTION

Alzheimer's disease (AD) is characterized by senile plaques, neurofibrillary tangles, and loss of synapses and neurons. The predominant components of senile plaques are the amyloid- β peptides (A β_{40} and A β_{42}), generated from the amyloid- β protein precursor (A β PP). Although the accumulation of A β has been identified as an important mechanism

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underlying AD pathogenesis, neither the details underlying A β toxicity nor the physiological function(s) of A β has been fully defined.

A major neuroanatomical feature of AD is the selective degeneration of basal forebrain cholinergic neurons (BFCN). These neurons provide cholinergic innervation to the neocortex, hippocampus, and entorhinal cortex. BFCN express nerve growth factor (NGF) receptors p75^{NTR} and TrkA, and NGF trophic support is required for the normal function and survival of BFCN. NGF is produced by target regions of the BFCN and retrogradely transported to the cell body in the form of NGF-TrkA complexes. It has been proposed that this retrograde transport deficit is one of the major causes of BFCN degeneration in AD [1].

We have found that, in addition to A β , A β PP generates another cytotoxic peptide, A β PP-C31, by intracytoplasmic cleavage at D664 [2]. In a screen for small molecules that block the production of A β PP-C31, we identified a compound, ADDN-1351, which, surprisingly, turned out to be a TrkA kinase inhibitor. This finding runs counter to the current notion that TrkA promotes anti-AD signaling. TrkA is reported to be reduced in BFCN in AD, and failure of NGF-TrkA signaling has been considered the major cause of BFCN degeneration; therefore, NGF and TrkA agonists have been proposed as potential treatments for AD [3, 4]. Our data, however, suggest the possibility that *both* TrkA hyper-activation—in neuronal processes—and TrkA hypo-activation—in neuronal somata—may feature in AD. Under these conditions, TrkA may promote pro-AD signaling through its kinase activity. Indeed, to our surprise we found that TrkA overexpression induces A β PP-C31 production, which could be prevented by a kinase-dead TrkA mutant or by TrkA inhibitor GW441756 [5]. We also found that TrkA interacts with A β PP, modulating A β PP processing, and that activation of TrkA by NGF induces increased A β production *in vitro*. Further confirmation of the role of TrkA in A β PP processing was provided by *in vivo* testing of GW441756. Treatment with this TrkA inhibitor resulted in increased sA β PP α level and sA β PP α to A β ratio in the PDAPP AD transgenic mouse model. Our data raise the possibility that NGF and NGF mimetics may have detrimental as well as beneficial effects on AD pathophysiology. Selective pharmacological inhibition of TrkA kinase may prove to be part of an optimal therapeutic cocktail for AD, by negating the hyperactivation and resulting toxicity produced from accumulation of active NGF-TrkA complexes, as a result of the retrograde transport deficits that occur early in AD.

MATERIALS AND METHODS

Plasmids

Constructs pCMV5-TrkA and pCMV5-TrkA (K538A) were kindly provided by Dr. Moses Chao. Construct pcDNA3-flag-rat-TrkA was a gift from Dr. Francis Lee. Constructs pCMV5-Mint3, pMst-A β PP, pG5E1B-luc, pCMV-LacZ, and pCMV-Fe65 were generously provided by Dr. Thomas Südhof, Dr. Patrick Mehlen, and Dr. Veronique Corset. Construct pcDNA4-His-MaxB-hYAP1 was a kind gift from Dr. Marius Sudol. Constructs pcDNA3-A β PP-C83, pcDNA3-A β PP-C99, pcDNA3-A β PP695, pcDNA3-A β PP-D664A, pcDNA3-A β PP-C31, pcDNA3-A β PPsi, and pcDNA3-flag-p75^{NTR} were described previously [2, 6].

Antibodies and chemical compounds

6E10 anti-A β PP antibody was purchased from Covance. CT15 anti-A β PP C-terminus antibody was a kind gift from Dr. Edward Koo. Anti-A β PPneo antibody was described previously [7]. Anti-TrkA antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). NGF was purchased from Sigma-Aldrich (St. Louis, MO). GW441756 was purchased from Tocris Bioscience (R&D, Minneapolis, MN). PHA739358 was purchased from EMD (San Diego, CA). MTT was purchased from Sigma. ADDN-1351 used for testing was prepared and characterized for Dr. Varghese John by a contract research laboratory.

Cell culture and co-immunoprecipitation

The Chinese Hamster Ovary (CHO) cell line over-expressing human A β PP (7 W) was kindly provided by Dr. Edward Koo. The H4 neuroglioma cell line overexpressing human A β PP (H4A β PPwt) was a kind gift from Dr. Todd Golde. HEK293T and B103 cell lines were described previously [2, 8]. Plasmid constructs were transiently transfected into HEK293T or 7 W cells with Lipofectamine 2000 (Invitrogen). Coimmunoprecipitation and western analysis were performed as previously described [9]. Briefly, 48 h after transfection, cells were harvested and lysed in NP-40 Cell Lysis Buffer (50 mM TrisHCl, pH 8.0, 150 mM NaCl, and 1% NP-40), and then, after centrifugation, incubated overnight with anti-A β PP antibodies 6E10 or CT15. Protein G agarose beads (Santa Cruz Biotech, CA) were then added for 2 h incubation at room temperature. The beads were subjected to five rounds of washing consisting of centrifugation, withdrawal of supernatant, and addition of fresh NP-40 Cell Lysis Buffer. During the final washing step, beads were resuspended in 1X LDS loading buffer (Invitrogen) with 50 mM DTT, and boiled at 10°C for 10 min. After SDS-PAGE and electrotransfer, western blotting was performed using anti-TrkA antibody. Thirty minutes of TBS-Tween wash were followed by incubation with secondary antibodies.

Drug screening and kinase inhibition assay

A high-throughput DELFIA (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay) screen was employed to identify small molecule modulators of the A β PPneo generation. The assay utilizes the DELFIA microtiter plates coated with the 6E10 capture antibody. After stimulation of the H4A β PPwt cells with staurosporine (STS), the assay measures the formation of the A β PPneo fragment using anti-A β PPneo polyclonal antibody and anti-rabbit-Europium (Eu) labeled reporter antibody. The fluorescence measurement is done on a Victor-3 Multilabel Plate Reader (PerkinElmer). The screening of our CNS-focused 5000 compound library was done in a 96-well plate screen format, at a concentration of 10 μ M. The screening assay protocol involves: a) Use of H4A β PPwt cells to seed 96-well dishes with 40,000 cells/well; b) Preincubate with small molecules and treat with STS, lyse and assay cell lysates; c) Coat DELFIA microtiter plates with 6E10 mAb; d) Develop with 100 ng/well anti-A β PPneo Ab and 10 ng/well anti-rabbit-Eu reporter antibody; e) Perform single data point screen of ADDN (Alzheimer's Drug Discovery Network) library; f) Analysis of the data is done as a function of percent activity of positive control (+STS); Set positive control to 100%; g) Cherry pick 'hits' from daughter plates and then assay compounds at 10

μM in triplicate. Kinase inhibition assays were performed by the Reaction Biology Corporation, Malvern, PA.

MTT cell viability assay

CHO-APP (7 W) cells were transfected with pcDNA3, pCMV5-TrkA, or pCMV5-TrkA(K538A) constructs. For GW441756 treatment assay, DMSO or GW441756 (1 μM) was added to the transfected cells. 48 h after transfection, cell viability was measured by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as previously described [10]. Briefly, 100 $\mu\text{g}/\text{ml}$ of MTT was added to the transfected cells and the cells were incubated in the dark for 2 h. Then media were replaced with DMSO and the cells were incubated for 5 min with vigorous shaking. Optical densities were read at 570 nm.

Transactivation assay

HEK293T cells were co-transfected with five or six plasmids: (1) pG5E1B-luc, 0.3 μg ; (2) pCMV-LacZ, 0.1 μg ; (3) pMst-A β PP (A β PP-Gal4), 0.3 μg ; (4) pCMV5-TrkA or pCMV5-TrkA(K538A), 1.0 μg ; (5) pcDNA4-His-MaxB-hYAP1 or pCMV-Fe65, 1.0 μg . Where indicated, a sixth plasmid was co-transfected: pCMV5-Mint3, 1.0 μg . For negative controls, the expression vector pcDNA3 was used without insert. Cells were harvested 48 h after transfection in 0.2 ml per well Cell Culture Lysis Buffer (Promega), and their luciferase and β -galactosidase activities were determined with the Promega luciferase assay kit and the Promega β -galactosidase assay kit, respectively. The luciferase activity was standardized by the β -galactosidase activity to control for transfection efficiency and general effects on transcription. Values shown are averages of transactivation assays performed in duplicate or triplicate and repeated at least three times for each constructs combination. All constructs were assayed in HEK293T and B103 cell lines, and representative results from the HEK293T cell lines are shown. Transfections were performed at 80–90% confluency in six-well plates using Lipofectamine 2000 (Invitrogen).

Transgenic mice and in vivo GW441756 treatment

The PDAPP (J20) mice were described previously [11]. 6–6.5 month old J20 mice were treated with DMSO control or GW441756 at 10 mg/kg/day (Sub-Q) for 5 days. 2 h after the last injection, mice were sacrificed and hippocampi were dissected and homogenized. The levels of sA β PP α were detected by AlphaLISA assay (PerkinElmer, Waltham, MA). A β ₄₀ and A β ₄₂ levels were quantified by ELISA (BioSource, Camarillo, CA). Brain levels were measured at 1, 2, 4, 6 and 8 h in brain homogenates after Sub-Q treatment with 10 mg/kg of GW 441756 and analyzed using LC/MS/MS at Integrated Analytic Solutions (Berkeley, CA).

RESULTS

TrkA induces A β PP cleavage at Asp664 and cell death

We have shown that D664 cleavage of A β PP generates the cytotoxic peptide, A β PP-C31 [2]. We further showed that A β binds to its cognate domain, acting as an A β PP anti-trophic ligand and inducing A β PP D664 cleavage, leading to neurite retraction and neuronal damage [8, 12]. We then identified netrin-1 as a novel trophic ligand for A β PP, mediating neurite

extension through A β PP signaling [13]. These findings suggest the possibility that AD results from an imbalance in physiological A β PP signaling, with the anti-trophic A β -A β PP signaling exceeding the trophic netrin-1-A β PP signaling. This notion is supported by data from AD model transgenic mice carrying an uncleavable A β PP D664A mutation: in spite of accumulating high levels of soluble and deposited A β , these PDAPP (D664A) mice do not develop AD-like deficits [11, 14]. Taken together, these findings strongly suggest that the generation of AD-like functional and anatomical deficits may be downstream of A β -A β PP signaling, through a mechanism that involves A β PP processing at D664 [15, 16].

In addition to generating the toxic A β PP-C31 peptide, cleavage of A β PP at D664 removes protein-protein interaction motifs that lie C-terminal to the D664 site. These motifs overlap with the 'YENPTY' internalization signal sequence of A β PP, which is often found in cytoplasmic tails of membrane receptors [17]. The 'YENPTY' motif and some of its neighboring residues act as a binding site for several A β PP-interacting proteins such as kinesin, Fe65, Mint1/X11, Disabled-1, JIP-1, and JNK1. These interactions are thought to define, at least partially, the functions of A β PP in axonal trafficking, in cellular motility and adhesion, in vesicular transport, and in the regulation of synaptic function [18–24]. Cleavage of A β PP at D664 may therefore disrupt these interactions and prevent the activation of trophic signaling cascades emanating from A β PP.

Significantly, the C-terminal domain of A β PP was found in association with chromatin in a complex with Fe65 and the histone acetyltransferase TIP60, strongly suggesting that the C-terminal domain of A β PP is involved in the control of gene expression [20]. The 'VEVD664' caspase recognition site and the 'YENPTY' motif are extremely well conserved across phyla, indicating that they may play key regulatory roles in A β PP intracellular signaling.

We conducted a screen for small molecules that inhibit the production of A β PP-C31, and identified 52 compounds from a 5000-compound CNS-focused library. One of these, ADDN-1351, reduced A β PP-C31 by over 90% (Fig. 1A). Since ADDN-1351 displays structural similarities to some kinase inhibitors, we evaluated it against a panel of 24 kinases at a low dose of 0.1 μ M, to determine whether it interacts with any specific kinase from this panel. Using this approach, we identified TrkA as a specific target for ADDN-1351 (Fig. 1B). A dose-response analysis in the TrkA kinase assay showed that ADDN-1351 is an inhibitor of TrkA, with an IC₅₀ of approximately 358 nM (Fig. 1D). To complement this result, we assessed the effect of TrkA expression on the cleavage of A β PP at Asp664, which leads to A β PP-C31 production. We transiently transfected TrkA into the 7 W cell line, a CHO cell line that stably over-expresses A β PP [25]. TrkA transfection induced a marked increase (more than 10-fold) in A β PP-C31 production, as detected by an antibody specific for the A β PPneo epitope (the epitope exposed after A β PPC31 cleavage; the fragment that we are measuring is the N-terminal (aa 1-664) A β PPneo fragment; note that it is currently not feasible to measure C31 directly because of its short half-life) [7]. This induction did not occur when a kinase dead TrkA (K538A) mutant construct was transfected (Fig. 2A). Moreover, treatment with a potent and specific TrkA inhibitor, GW441756, abolished the TrkA-induced A β PP-C31 production from A β PP (Fig. 2B). These results indicate that TrkA, in a kinase activity dependent manner, activates A β PP cleavage to produce A β PP-C31.

We have previously shown that the A β PP-C31 peptide is toxic, and blocking this cleavage by mutating the cleavage site in transgenic mice ameliorated the AD phenotype, without altering plaque load or A β concentration. Therefore, we asked whether TrkA induced A β PP-C31 production could lead to cell death. Using an MTT assay, we found that TrkA expression significantly decreased cell survival, and this effect required the kinase activity of TrkA (Fig. 2D–F).

Recently, TrkA has been shown to act as a dependence receptor and induce neuronal death in the absence of NGF, while transducing trophic signals in the presence of NGF [26]. We therefore asked whether this TrkA-induced A β PP-C31 production could be blocked by NGF treatment. Surprisingly, NGF treatment further enhanced A β PP-C31 production indicating that NGF-TrkA signaling positively regulates the production of A β PP-C31 (Fig. 2C).

TrkA interacts with A β PP

To explore potential mechanisms behind the induction of A β PP-C31 production by TrkA, we asked whether TrkA interacts with A β PP. Indeed, TrkA not only co-immunoprecipitated with wild type A β PP (Fig. 3A), but also interacted with different mutant forms of A β PP, including the A β PP carrying the Swedish and Indiana familial Alzheimer's disease mutations (Fig. 3A), A β PP carrying the D664A mutation that is resistant to caspase cleavage (Fig. 3B), and a fragment of A β PP with the C-terminal 31 amino acids deleted (Fig. 3B). Interestingly, the C-terminal A β PP fragment created after the β -secretase cleavage (C99) co-immunoprecipitated with TrkA, while the C-terminal fragment created after the α -secretase cleavage (C83) did not (Fig. 3C), suggesting that the region corresponding to the aminoterminal 16 amino acids of A β is required for this interaction. To evaluate the effect of TrkA kinase activity modulation on the TrkA-A β PP interaction, we performed experiments in the presence of NGF or with the kinase dead TrkA(K538A) mutant. In both conditions, TrkA and A β PP co-immunoprecipitated (Fig. 3D, E), indicating that the interaction between A β PP and TrkA is kinase activity independent.

TrkA inhibits A β PP-Gal4 transactivation in a kinase independent manner

While the A β PP-C31 production is associated with cell death, the A β PP intracellular domain (AICD) created following the γ -secretase cleavage has been implicated in various signaling pathways, and has been shown to modulate the expression of numerous genes including KAI1, neprilysin, and A β PP itself [27]. We have established an A β PP-Gal4/Mint3/YAP transactivation assay [9, 28], and using this assay, we found that TrkA inhibited A β PP-Gal4 transactivation by over 95% (Fig. 4A). To confirm this effect, we employed the A β PP-Gal4/Fe65 transactivation assay developed in the laboratory of Dr. Thomas Südhof, and again, we observed potent inhibition of A β PP-Gal4 transactivation by TrkA (Fig. 4B). We then evaluated the effect of TrkA kinase activity modulation on this inhibition, and we saw similar inhibition of A β PP-Gal4 trans-activation with the kinase dead TrkA(K538A) mutant (Fig. 4C), as well as in the presence of NGF (Fig. 4D). These results indicate that the suppression of A β PPGal4 transactivation by TrkA is not dependent on the latter's kinase activity.

TrkA affects A β PP processing

A β PP is processed through two major pathways: the non-amyloidogenic pathway is initiated by α -secretase cleavage, generating sA β PP α and α -CTF (C83), while the amyloidogenic pathway is initiated by β -secretase cleavage, producing sA β PP β and β -CTF (C99). The β -CTF is then cleaved by the γ -secretase, which produces A β and AICD. Since TrkA interacts with A β PP, we evaluated the effects of TrkA overexpression on A β PP processing.

We co-expressed TrkA and A β PP in 293T cells, and assayed the levels of A β , full-length A β PP, sA β PP α , and β -CTF. The level of full-length A β PP increased, while the levels of A β ₄₀, A β ₄₂, and sA β PP α decreased (Fig. 5A–C). These effects are similar to the effects of Mint1, a protein that interacts with and stabilizes A β PP [29]. In contrast to the effects of TrkA on A β and sA β PP α , the level of β -CTF was increased (Fig. 5E). We next sought to determine the role that TrkA kinase activity plays in these effects. It has been shown that NGF signaling can enhance sA β PP α production through the PKC pathway [30], and, as expected, NGF treatment increased the level of sA β PP α , but the increase was not enough to return sA β PP α to its level in the absence of TrkA expression (Fig. 5D). Surprisingly, NGF also increased the levels of both A β ₄₀ and A β ₄₂ (Fig. 5A, B). When we used the kinase-dead TrkA(K538A) mutant, the inhibition of A β and sA β PP α production was not significantly altered. Interestingly, the effect of NGF on A β ₄₂ production was abolished in the presence of TrkA(K538A) (Fig. 5B), suggesting that the increase in A β production is mediated through TrkA kinase activity and the NGF-TrkA signaling pathways.

TrkA inhibitor treatment reduces A β in an AD mouse model

Given that NGF-TrkA signaling increased A β PPC31 and A β production in a TrkA kinase activity dependent manner, we evaluated the effect of TrkA inhibitor treatment in an AD mouse model, PDAPP (J20) mice. Unfortunately, ADDN-1351 showed very poor blood-brain barrier penetration; therefore we used another TrkA kinase inhibitor, GW441756 (IC₅₀ ~ 50 nM, Fig. 1D). Six-month-old J20 mice were evaluated, and 5 days of treatment with GW441756 at 10 mg/kg increased the level of sA β PP α (Fig. 6). There was a trend toward the reduction of both A β ₄₀ and A β ₄₂, but the trend did not reach statistical significance (Fig. 6). However, the sA β PP α to A β ₄₂ ratio was increased to 1.85 times over control (Fig. 6). At this dose, GW441756 treatment gave measurable brain levels with a maximum brain concentration (C_{max}, 1 h) of ~1×IC₅₀ being obtained. These results indicate that, in transgenic mice, this potent TrkA inhibitor altered the ratio of A β PP amyloidogenic, pro-apoptotic signaling versus non-amyloidogenic, anti-apoptotic signaling in favor of the latter pathway [16].

DISCUSSION

Surprisingly, ADDN-1351, the potent A β PP-C31 cleavage inhibitor identified through library screening, was found to be a TrkA kinase inhibitor; this is somewhat counter-intuitive because an NGF-TrkA signaling deficit has been considered as a potential cause of the cholinergic deficit that is characteristic of AD. However, in both *in vitro* and *in vivo* studies, we found that TrkA hyper-activation not only induces A β PP-C31 production but also increases A β production, and that anti-TrkA treatment in an AD transgenic mouse

model increased sA β PP α to A β ratio. Therefore, we conclude that TrkA inhibition may represent a novel therapeutic approach to AD, and that potent brain-permeable TrkA kinase inhibitors may prove to be therapeutically beneficial in AD. To our knowledge, this is the first report directly indicating TrkA kinase inhibition as a potential goal in a therapeutic approach to AD.

Our results show that TrkA affects A β PP processing through two major pathways: first, the kinase-independent pathway stabilizes A β PP and decreases the production of both sA β PP α and A β , while increasing full-length A β PP. It also inhibits the AICD signaling of A β PP. This pathway is likely to be mediated through the interaction between A β PP and TrkA. Secondly, the kinase-dependent pathway activates A β PP-C31 cleavage and increases A β production. The NGF-induced increase in A β production was surprising, given that NGF is a trophic factor, while A β functions as an anti-trophin [16]. However, the intracellular kinase domain of TrkA, artificially activated through fusion to the TPR protein oligomerization motif, has been shown to activate A β PP-AICD signaling, which is dependent on the β and γ cleavages of A β PP [31, 32]. It is worth noting, however, that we used the full-length TrkA, which interacted with A β PP, and it is therefore likely that the marked inhibition of A β PP-AICD signaling in our experiments may have been due to the interaction between full-length TrkA and A β PP.

The effects of kinase-dependent TrkA signaling on sA β PP α production are multifactorial: on the one hand, through NGF-mediated PKC activation, α cleavage is activated [30]; on the other hand, through A β PP Y682 phosphorylation, α cleavage is inhibited [33–35]. The net effect of TrkA inhibition on sA β PP α is therefore variable and dependent on the experimental system. In our PDAPP mice, TrkA kinase inhibition increased the level of sA β PP α , suggesting that the second mechanism dominates *in vivo*, at least in this transgenic mouse model of AD.

Cholinergic deficit is a major abnormality in AD, and it is associated with BFCN degeneration. However, the level of NGF, the trophic factor required for the survival of BFCN, is not decreased in AD patients, and, interestingly, some studies have found that NGF is actually increased [1]. NGF is synthesized in the target regions of the BFCN. BFCN synapses take up NGF, and the NGF-TrkA complexes are transported retrogradely to the cell bodies, while continuing to signal. However, this retrograde transport system is dysfunctional in AD, and the disrupted retrograde transport of NGF-TrkA complexes results in insufficient trophic support for BFCN, and eventually BFCN degeneration in AD [1].

One major hypothesis of AD pathogenesis assumes that NGF signaling deficit and BFCN degeneration are primary causes of AD. In support of this hypothesis is the transgenic mouse line AD11, which expresses anti-NGF antibodies and thus reduces NGF signaling chronically (while leaving pro-NGF unaffected); this mouse develops both neurofibrillary tau tangles and A β plaques [36]. Another series of studies showed that NGF withdrawal induces delayed TrkA hyper-activation and A β production, the former dependent on the latter [37]. These results might partially explain the AD phenotype seen in the AD11 mice, where NGF removal induces both A β and tau pathology. Thus, if A β is capable of causing

hyper-activation of TrkA, a positive feedback loop (i.e., prionic loop) may form, and one approach to disrupting this loop may be TrkA inhibition.

Recently, TrkA was demonstrated to be a dependence receptor, mediating pro-death signals following a reduction in NGF [26]. Moreover, it is well established that the over-activation of NGF-TrkA signaling pathway induces cell death in medulloblastoma cell lines [38, 39]. Therefore, TrkA, when hyper-activated, can induce pro-death signals. What is surprising, however, is that this hyper-activation can apparently occur following NGF withdrawal, in that case mediated by A β [37].

Although our studies do not offer a mechanism for the ameliorative effect of TrkA inhibition on the biomarkers A β and sA β PP α in the PDAPP mouse model of AD, we speculate that the retrograde axoplasmic transport deficit associated with AD may lead to the accumulation of NGF-TrkA complexes, and resultant hyper-activation of signaling. The early and major targets of AD are brain regions innervated by the BFCN, including the entorhinal cortex, hippocampus, olfactory bulb, and neocortex. If the reduction of NGF support that leads to BFCN degeneration is caused by the disruption of the retrograde transport of NGF-TrkA complexes, then those impeded NGF-TrkA complexes should accumulate along the axons of the BFCN. If these accumulated, active NGF-TrkA complexes are harmful and contribute to AD pathogenesis, then a dichotomous pathophysiology might ensue: reduction of NGF in BFCN cell bodies may mediate BFCN degeneration, while the accumulation of active NGF-TrkA complexes causes AD pathology in the target regions of the BFCN.

In support of this notion, downstream signaling markers of the NGF-TrkA pathway have been reported to be increased in AD: for example, ERK1 and ERK2, downstream signaling molecules of the NGF-TrkA pathway involved in the phosphorylation of tau, have been shown to be elevated in the cortex and the hippocampus of AD patients [40–43]. Similarly, PLC γ , a key player of another major downstream pathway of NGF-TrkA signaling, increases in AD, in the hippocampus and superior and middle temporal gyri, but not in the cerebellum [44].

In conclusion, the current study indicates that TrkA interacts with A β PP, suppresses A β PP-dependent signaling, and that hyper-activation induces A β PP-C31 cleavage and increases A β production. We propose that the chronic hyper-activation of TrkA, due to the retrograde axoplasmic transport deficit associated with AD, and resultant accumulation of active NGF-TrkA complexes, may be an early mechanism in the pathogenesis of AD (Fig. 7). Thus, potent and brain-permeable TrkA kinase inhibitors may represent one component of an optimal treatment for AD. Ongoing efforts in our laboratory are being made to develop analogs of ADDN-1351 with potent TrkA kinase inhibition and better blood-brain barrier penetration.

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Fig. 1.

AβPP-C31 cleavage inhibition screening identified a TrkA inhibitor. A) Scattergraph of results from AβPP-C31 cleavage inhibition screen of a CNS-focused small molecule library. 5000 compounds were screened and 52 inhibited the AβPPneo signal (the neo epitope exposed after AβPP-C31 cleavage, AβPP1-664) by over 70%. One of them, ADDN-1351, inhibited AβPPneo signal by over 90%. B) ADDN-1351 is a TrkA inhibitor. At a low concentration of 0.1 μM, ADDN-1351 was tested against a panel of 24 kinases. Only TrkA was inhibited by ADDN-1351 in this kinase inhibition assay. C) Chemical structures of TrkA inhibitors ADDN-1351, PHA-739358, and GW441756. D) IC₅₀-Data for TrkA inhibition: ADDN-1351, PHA-739358, and GW441756.

Fig. 2.

TrkA induced AβPP-C31 cleavage is mediated through TrkA kinase activity. A) TrkA induced AβPP-C31 cleavage was blocked by the kinase dead TrkA(K538A) mutation. CHO cells stably overexpressing human AβPP were transiently transfected with empty vector, TrkA or TrkA(K538A) mutant constructs, and the level of AβPPneo was detected by western blot. TrkA overexpression induced strong AβPP-C31 cleavage, and this induction was absent when kinase dead TrkA(K538A) was overexpressed. B) TrkA induced AβPP-C31 cleavage was blocked by the TrkA inhibitor GW441756. CHO cells stably overexpressing human AβPP were transiently transfected with empty vector or TrkA, and treated with vehicle (DMSO) or GW441756 (1 μM). The level of AβPPneo was detected by western blot. TrkA overexpression induced strong AβPP-C31 cleavage, and this induction was abolished by GW441756 treatment. C) NGF treatment enhanced TrkA induced AβPP-C31 cleavage. CHO cells stably over-expressing human AβPP were transiently transfected with empty vector, TrkA, TrkA + p75^{NTR} or p75^{NTR}, in the presence or absence of NGF (5 nM). The level of AβPPneo was detected by western blot. TrkA induced AβPP-C31 cleavage was enhanced by NGF treatment, in the absence or presence of TrkA co-receptor p75^{NTR}. D) TrkA overexpression induced cell death was blocked by the kinase dead TrkA(K538A) mutation. CHO cells stably overexpressing human AβPP were transiently transfected with empty vector, TrkA or TrkA(K538A) mutant constructs, and MTT assay was performed to evaluate the rate of cell survival. While TrkA overexpression induced significant cell death, TrkA(K538A) overexpression did not. E) TrkA overexpression induced cell death was blocked by GW441756. CHO cells stably overexpressing human AβPP were transiently transfected with empty vector or TrkA, in the presence or absence of GW441756 (1 μM), and MTT assay was performed to evaluate the rate of cell survival. While TrkA

overexpression induced significant cell death, GW441756 significantly increased cell survival. *** $p < 0.001$, ** $p < 0.01$, $n = 3$, Student's t -test. Error bars indicate SD. F) CHO-A β PP cells were transfected with pcDNA3 (Vector), TrkA or TrkAK538A, representative images of the cells 48 h after transfection are shown.

Fig. 3.

TrkA interacts with AβPP. (A, B) TrkA co-immunoprecipitated with wild type AβPP (A), AβPP carrying the Swedish and Indiana familial AD mutations (AβPP_{si}, A), AβPP carrying the D664A mutation (B) and AβPP with the C-terminal 31 amino acids deleted (B). 293T cells transiently expressing TrkA and wild type AβPP (A), AβPP_{si} (A), AβPP(D664A) (B) or AβPP C31 (B) were lysed and the crude lysates were subjected to immunoprecipitation with anti-AβPP antibody Protein G agarose beads. Cells expressing TrkA only were used as controls. Immunoprecipitated samples were subjected to western blotting with anti-TrkA antibody. Total cell lysates were used as input controls. C) TrkA co-immunoprecipitated with AβPP-C99 but not AβPP-C83. D) TrkA(K538A) mutant co-immunoprecipitated with AβPP. E) TrkA co-immunoprecipitated with AβPP in the presence of NGF (5 nM).

Fig. 4.

TrkA inhibits AβPP-AICD signaling. A) Potent transactivation of transcription was achieved with AβPP fused to the Gal4 DNA binding domain when both Mint3 and YAP were present. This transactivation was abolished by TrkA. p75^{NTR} also inhibited the transactivation, but to a lesser extent. B) Significant transactivation of transcription is achieved with AβPP fused to the Gal4 DNA binding domain when Fe65 was present, and this transactivation was also abolished by TrkA. C) YAP directed AβPP-Gal4/Mint3 transactivation was inhibited by both TrkA and TrkA(K538A) mutant. D) YAP directed AβPP-Gal4/Mint3 transactivation was abolished by TrkA alone or TrkA plus p75^{NTR}. This inhibition was not significantly affected by 5 nM NGF treatment. Diagrams exhibit representative experiments in which cells were co-transfected with a Gal4-luciferase reporter plasmid (to measure transactivation), a β-galactosidase plasmid (to normalize for transfection efficiency), and the test plasmids identified below the bars. The normalized luciferase activity is expressed as fold induction over transcription by AβPP-Gal4 alone (A, B), or as a percentage of the AβPP-Gal4/Mint3/YAP control (C, D). Error bars indicate SD.

Fig. 5.

TrkA modulates AβPP processing. A, B) NGF stimulated Aβ₄₀ and Aβ₄₂ production in the presence of TrkA, while TrkA overexpression inhibited the production of Aβ₄₀ and Aβ₄₂. 293T cells were co-transfected with AβPP and empty vector or TrkA. Conditioned media were collected for Aβ₄₀ and Aβ₄₂ assays. Overexpression of TrkA inhibited the production of Aβ₄₀ and Aβ₄₂. NGF treatment (5 nM) increased Aβ₄₀ and Aβ₄₂ production in the presence of TrkA. TrkA(K538A) mutant also inhibited the production of Aβ₄₂, but the NGF mediated Aβ₄₂ increase was abolished by the kinase dead TrkA(K538A) mutation. $**p < 0.01$, $n = 3$, Student's *t*-test. Error bars indicate SD. C) TrkA overexpression inhibited the production of sAβPPα, while increasing the level of full-length AβPP. 293T cells were co-transfected with different forms of AβPP and empty vector or TrkA. Conditioned media were collected for sAβPPα western blot, and cell lysates were collected for full length AβPP western blot. For wild type AβPP, AβPP C31 and AβPP(D664A), TrkA overexpression decreased sAβPPα production and increased the levels of full length AβPP. D) TrkA(K538A) overexpression also inhibited the production of sAβPPα. NGF treatment (5 nM) increased sAβPPα production in the presence of TrkA but not TrkA(K538A). E) TrkA overexpression increased the level of β-CTF (AβPP-C99).

Fig. 6.

TrkA inhibitor GW441756 treatment increases sAβPPα and sAβPPα/Aβ₄₂ ratio in an AD transgenic mouse model (PDAPP J20). 6–6.5 month-old J20 mice were treated with DMSO control (7 mice) or GW441756 (7 mice) at 10 mg/kg/day subcutaneously for 5 days. Mice were sacrificed and hippocampal regions of the brains were dissected and homogenized. Brain lysates were subjected to ELISA for Aβ₄₀ and Aβ₄₂ assays. AlphaLISA was employed for the detection of sAβPPα. There was a trend toward the reduction of both Aβ₄₀ (A) and Aβ₄₂ (B), but the trend did not reach statistical significance. However, sAβPPα (C) and sAβPPα/Aβ₄₂ ratio (D) were significantly increased. ***p* < 0.01, *n* = 7, Student's *t*-test. Error bars indicate SEM.

Fig. 7.

Chronic hyper-activation of TrkA in the target regions of basal forebrain cholinergic neurons (BFCN) as an early mechanism in Alzheimer's disease (AD). NGF is produced in the target regions of BFCN, and synapses of BFCN take up NGF and retrogradely transport it back to the cell bodies in the form of NGF-TrkA complexes. In non-AD controls, sufficient amounts of NGF trophic support maintain the normal function of BFCN. With aging, the retrograde transport system efficiency is reduced, which leads to lower levels of active NGF-TrkA complexes in the BFCN, and higher levels of NGF-TrkA complexes in the target regions of BFCN, including the entorhinal cortex, hippocampus and neo-cortex. Accumulation of active NGF-TrkA complexes in the target regions of BFCN leads to increased Aβ and AβPP-C31 production, which results in progression into the mild cognitive impairment stage (MCI). In the MCI stage, hyper-activation of TrkA in the target regions of BFCN continues to facilitate the progression of AD. In the mild AD stage, TrkA levels are decreased in the target regions of BFCN, but the cholinergic functions are still normal. In the severe AD stage, due to prolonged NGF-TrkA signaling deficit, BFCN degeneration and cholinergic deficits are prominent. Although total levels of TrkA are decreased in the target regions of BFCN, NGF levels are not, and it is likely that local hyper-activation of TrkA in the target regions of BFCN still exists. Therefore anti-TrkA treatment will still have anti-AD effects in the target regions of BFCN. However, because of the prominent BFCN degeneration at this stage, a combination of anti-TrkA treatment and targeted BFCN NGF delivery may be ideal.