alzheimer's research&therapy

REVIEW

Fyn kinase inhibition as a novel therapy for Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a devastating neurodegenerative disorder, afflicting more than one-third of people over the age of 85. While many therapies for AD are in late-stage clinical testing, rational drug design based on distinct signaling pathways in this disorder is only now emerging. Here we review the putative signaling pathway of amyloid-beta (Aβ), by which the tyrosine kinase Fyn is activated via cell surface binding of Aβ oligomers to cellular prion protein. Several lines of evidence implicate Fyn in the pathogenesis of AD, and its interaction with both Aβ and Tau renders Fyn a unique therapeutic target that addresses both of the major pathologic hallmarks of AD. We are currently enrolling patients in a phase Ib study of saracatinib (AZD0530), a small molecule inhibitor with high potency for Src and Fyn, for the treatment of AD. The results of this trial and a planned phase IIa multisite study will provide important data regarding the potential for this therapeutic strategy in AD.

Review

Introduction

While the last few decades have produced significant advances in our understanding of Alzheimer's disease (AD) pathogenesis, clinical therapeutic trials have yet to yield a disease-modifying intervention. AD is invariably associated with diffuse cortical deposition of amyloid-beta (Aβ) plaques and tau-positive neurofibrillary tangles, and considerable resources have therefore been committed to developing therapies to reduce Aβ burden. This approach is supported by a large body of literature suggesting that Aβ plays a critical role in AD. A limitation to this approach, however, has been the lack of a well-defined downstream pathway linking specific misfolded forms of Aβ to neurotoxicity. Many previously developed upstream approaches to reduce Aβ levels have effects on multiple cellular pathways, inadvertently targeting essential biological processes that may counter the intended therapeutic effects. For these reasons, the neuronal signaling pathway triggered by Aβ has been the subject of intense study.

The various elements now thought to comprise a distinct Aβ signaling cascade have come from many sources dating back at least several decades. In this review, we highlight the recent finding that extracellular oligomeric Aβ binds cellular prion protein (PrP^C) with high affinity, activating an intracellular signaling cascade coupled to the protein tyrosine kinase Fyn [1-4]. While several elements of this proposed pathway might be targeted as a novel therapeutic strategy in AD, Fyn kinase is of particular interest given its widely accepted role in AD pathogenesis, and the clinically available drugs targeting this protein. A phase Ib study of saracatinib (AZD0530), a Src family kinase inhibitor with high potency for Fyn and Src kinase, is currently underway to treat patients with AD (NCT01864655), with a planned proof-of-concept phase IIa trial in 2014.

Fyn and Src family kinases

Fyn is a 59 kDa protein belonging to the Src family of nonreceptor tyrosine kinases (SFKs), which also includes Src, Yes, Yrk, Blk, Fgr, Hck, Lck, and Lyn [5]. The prototype member of the family is Src kinase, so named for the critical role of viral Src, contained in the Rous sarcoma virus genome, to transform chick fibroblasts infected with this virus into a sarcoma (src) [6]. The Src family kinases are grouped together based on a shared domain structure consisting of three Src homology domains (SH1 to SH3), a variable N-terminus region, and the SH4 domain that anchors the protein to the cytosolic plasma membrane

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[7]. Variations within the N-terminus region as well as the SH4 domain are thought to be largely responsible for the different physiologic functions among the Src family members [8-10].

The Fyn gene is located on chromosome 6q21, and has three known splice variants [11]. The known active forms are FynT and FynB, with the former expressed mostly in cells of hematopoietic origin while FynB is ubiquitously expressed, with particularly high levels in the brain [11,12]. In addition to FynB, the other Src family members Src, Lck, Lyn, and Yes are also expressed to varying degrees in the central nervous system, with Fyn and Src being the most extensively studied with regard to brain function.

Fyn activity, like that of other Src family kinases, is regulated by intramolecular interactions that depend on equilibrium between tyrosine phosphorylation and dephosphorylation. In the basal state, catalytic activity is constrained by engagement of the SH2 domain by a phosphorylated C-terminal tyrosine 531 [13]. External protein interactions with the SH2 and SH3 domains by phosphorylation at Tyr 420 in the activation loop of the kinase domain, and/or by dephosphorylation of Tyr 531, result in Fyn activation [13]. Dephosphorylation at Tyr 420, such as that seen with striatal-enriched tyrosine phosphatase 61, significantly reduces Fyn activity [14].

Fyn and central nervous system functions

The biological functions of Fyn are diverse and include processes such as T-cell receptor signaling, cell division and adhesion, platelet function, synaptic function and plasticity, and central nervous system (CNS) myelination (Table 1) [5,15]. Of particular interest for AD are multiple lines of evidence linking Fyn kinase to synaptic function. Fyn has been localized to the post-synaptic density (PSD) fraction of the brain, which is the primary post-synaptic site for signaling transduction and processing in neurons. Amongst its substrates are receptors for the major excitatory transmitter, glutamate [16], which plays a critical role in synaptic function. Fyn regulates glutamate receptor trafficking and synaptic plasticity in part by specifically phosphorylating the *N*-methyl-D-aspartate-type glutamate receptor subunits NR2A and NR2B [17-21]. These subunits are critical for both long-term potentiation and long-term depression, which are thought to model the underlying mechanisms of learning and memory [22,23]. Brain slices from mice lacking Fyn expression have blunted long-term potentiation, and mice show impaired contextual fear memory function [19,24], indicating that Fyn plays an important role in regulating synaptic plasticity. This is further corroborated by the finding that overexpression of a constitutively active Fyn transgene reduces the threshold for long-term potentiation induction in mouse brain hippocampal slices [25], and Fyn knockout mice have an age-dependent reduction in dendritic spines, the single contact points between an axon and a dendrite critical for synaptic function [26].

Table 1 Major cellular functions regulated by Fyn

Cellular function	References
Central nervous system	
Synaptic plasticity	[17-21,25]
Synapse density	[26]
Myelination	[27,28]
Noncentral nervous system	
Cell proliferation	[5-7]
Cell migration	[5-7]
Cell differentiation	[5-7]
Platelet function	[72]
T-cell signaling	[5,70,71]

In summary, Fyn regulates key aspects of synaptic physiology. Activation of Fyn enhances synaptic function, which, as discussed in subsequent sections, can have the undesired effect of rendering neurons more vulnerable to synaptotoxicity. A reduction in Fyn activation has the opposite effect, and while this may be neuroprotective, excessive inhibition may lead to impairments in long-term potentiation, and hypothetically affect cognitive function in humans. A therapeutic approach aimed at maintaining the delicate balance between activation and inhibition of Fyn will thus probably optimize the function of individual synapses, as well as the broader neuronal network.

Beyond synaptic function, Fyn has been shown to have a unique role in CNS myelination. Fyn associates with the large myelin-associated glycoprotein, and this interaction plays an important role in the signaling cascade required for the initial stages of myelination [27]. Consistent with this function, mice lacking Fyn expression have significantly reduced brain myelination, a role not shared by other Src family kinases present in CNS oligodendrocytes, including Src and Lyn [27,28].

Fyn kinase and Alzheimer's disease

Fyn is elevated in Alzheimer's disease brain and activated by amyloid-beta

An early focus in AD research was to identify proteins responsible for the pathologic phosphorylation of various tau residues. In 1993, Shirazi and Wood reported that a subset of neurons from AD brain exhibited strong Fyn immunoreactivity compared with control brains, and that these neurons were also positive for abnormally phosphorylated Tau protein [29]. The possible connection between Fyn and AD led to numerous studies focusing mainly on the downstream effects of Aβ on tyrosine kinases in cell culture. An early study suggested that $A\beta_{25–35}$ and

$A\beta_{1-40}$ increased tyrosine phosphorylation in a dose-dependent manner, although the exact Aβ preparation was not well characterized [30]. Other synthetic preparations reported to consist of mostly fibrillar Aβ also showed an increase in tyrosine phosphorylated proteins in cell culture, suggesting the importance of tyrosine kinases in AD [31,32]. While the majority of early studies in AD used fibrillary or mixed preparations of Aβ, it is now widely believed that soluble intermediates of this peptide, termed Aβ oligomer (Aβo), are the most relevant assemblies in AD pathogenesis [33]. These specific conformations of Aβ, initially named Aβ-derived diffusible ligands, were found to cause apoptosis in organotypic mouse brain slices, with full protection conferred by the lack of Fyn expression [34].

Amyloid-beta oligomers activate Fyn via cellular prion protein

A critical early step in AD pathophysiology is the process by which Aβo interacts with the neuronal surface to trigger downstream pathology, and studies of this pathway have further implicated Fyn in AD pathophysiology. In a unique genome-wide unbiased screen for cell surface proteins binding Aβo, we identified PrP^C [1]. Aβ binding to PrP^C has consistently been shown to be of high affinity and is exquisitely specific for the oligomeric form, with little or no affinity for fibrillary or monomeric Aβ peptide [1,35-37]. PrP^C is not essential for all Aβ-related phenotypes [36-39]. However, it is required for cell death *in vitro*, for reduced survival of APP/PS1 transgenic mice, for epileptiform discharges, for synapse loss, for serotonin fiber degeneration and for spatial learning and memory deficits [2,40-46]. The ability of human AD brain-derived Aβ species to suppress hippocampal synaptic plasticity depends upon PrP^C, and human AD brain extracts contain Aβo species capable of interacting with PrP^C as well as Aβ–PrP^C complexes [2,47-49].

Once Aβo forms and binds to PrP^C at the cell surface, changes in neuronal biochemistry occur (Figure 1). Both PrP^C and Fyn were found to be enriched in the PSD, and exposure of cultured mouse hippocampal neurons to Aβo activated Fyn by phosphorylating the Tyr 420 residue [2]. This interaction depends entirely on the normal expression of PrP^C, and in $Prnp^{-/-}$ cultures activation of Fyn by Aβo is eliminated [2]. Brain extracts from patients with AD also stimulate Fyn activation in mouse cortical cultures, but control brain extracts do not, further suggesting that the Aβ–PrP^C–Fyn pathway is relevant in human disease [2].

The connection between Aβo–PrP^C complexes at the cell surface and intracellular Fyn kinase has recently been shown to require the metabotropic glutamate receptor, mGluR5 [3]. This transmembrane protein has a physiological role in linking extracellular glutamate levels to calcium mobilization, to protein translation in dendrites and to tyrosine kinase signaling. For Aβo signaling, we screened transmembrane PSD proteins for the ability to couple Aβo–PrP^C complexes to Fyn activation in transfected non-neuronal cells, and only mGluR5 was positive. In neurons and in mice, mGluR5 is required for downstream neuronal dysfunction caused by Aβ. Extracellular Aβ oligomers thus trigger neuronal signal transduction from PrP^C to mGluR5 to Fyn kinase.

Fyn activation by amyloid-beta oligomer–cellular prion protein activates *N*-methyl-D-aspartate receptors and induces dendritic spine loss

N-methyl-D-aspartate receptors play a key role in synaptic plasticity and AD, and intracellular segments of the NR2A and NR2B subunits contain tyrosine residues phosphorylated by SFKs [50]. We showed that Aβo induces a dose-dependent increase in the phosphorylation of *N*-methyl-D-aspartate receptors, specifically the Fyn-specific phosphorylation of NR2B at Y-1472 [2]. Aβo-induced NR2B phosphorylation at this site is not detected in $Prnp^{-/-}$ or $Fyn^{-/-}$ cultures. Moreover, the role of Fyn is gene-dose dependent, being reduced in heterozygous neurons [2]. Synaptic loss is a hallmark of AD, and several *in vitro* studies have described dendritic spine loss after acute Aβo exposure [51-54]. We found that *in vitro* dendritic spine destabilization by Aβo is eliminated in embryonic hippocampal $Fyn^{-/-}$ neurons, providing further evidence that Fyn plays a critical role in Aβ-induced synaptotoxicity [2].

Fyn is implicated in Alzheimer's disease by interacting with Tau

In addition to its role in Aβ signaling, Fyn is also involved in Tau phosphorylation. It is well known that, in addition to Aβ plaques, AD brain contains diffuse deposits of neurofibrillary tangles, composed of hyperphosphorylated Tau.

Tau may play a critical role in mediating downstream neurodegeneration in AD, and a recent report showed that a 50% reduction in tau expression reversed cognitive impairments in a transgenic AD model [55]. Similar to studies of Aβo, studies of Tau have implicated Fyn mechanistically in AD. Fyn physically associates with Tau, and can phosphorylate tyrosine residues, including Tyr18, near the amino terminus [56-59]. Tyr18 is also phosphorylated in neurofibrillary tangles in human AD brain, suggesting a possible clinical relevance [58]. Activation of Fyn by the Aβo–PrP^C complex also leads to downstream Tau phosphorylation [4].

Critically, Fyn and Tau interact genetically to modulate synapse loss, behavioral deficits and electroencephalographic abnormalities in amyloid precursor protein (APP) transgenic mice [60]. Tau was recently reported to have a novel dendritic function to target Fyn to the PSD [61]. Without functional Tau, Fyn is uncoupled from

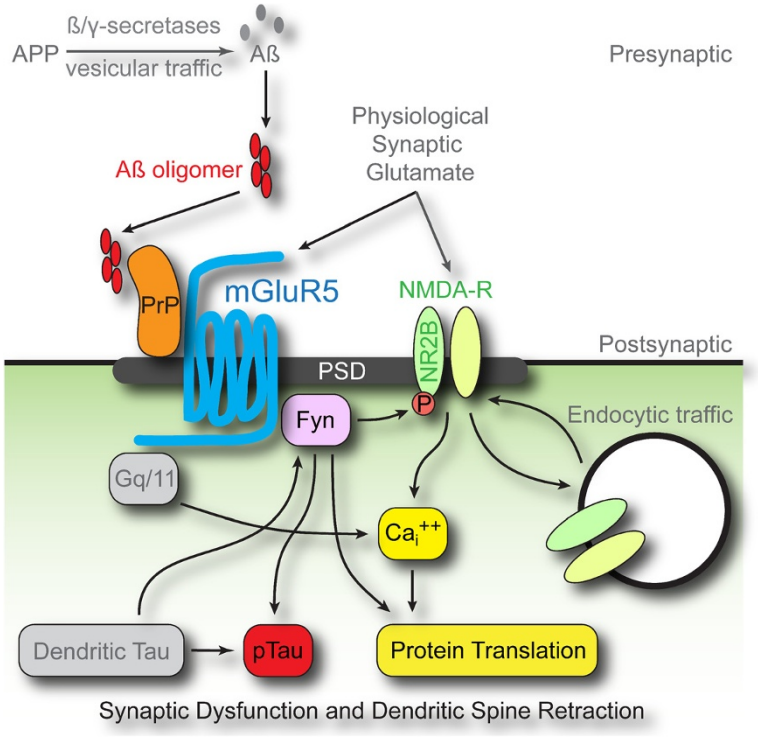

Figure 1 mGluR5 couples amyloid-beta oligomer–cellular prion protein to post-synaptic signaling. Schematic illustrating a central role of Fyn in amyloid-beta oligomer (Aβo) signaling. Binding of Aβo to cellular prion protein (PrP^C) triggers mGluR5-dependent signaling events. Proteins are clustered in the post-synaptic density (PSD) and alter *N*-methyl-D-aspartate receptor (NMDA-R) function, calcium and protein translation. Tau plays a role in localizing Fyn and is a Fyn substrate. The net result of aberrant PrP^C–mGluR5–Fyn signaling is synaptic malfunction and loss. Aβ, amyloid-beta; APP, amyloid precursor protein; PrP, prion protein.

N-methyl-D-aspartate receptors, and Aβ toxicity is rescued [61]. As expected, postsynaptic Fyn levels were reduced in mice with either absent Tau expression or the expression of a truncated Tau transgene, and were increased in mice overexpressing full-length Tau [61]. In both cases, total Fyn levels and activity were normal compared with wild-type littermates [61]. Two studies have reported a genetic association between single nucleotide polymorphisms at the *FYN* locus and cerebrospinal fluid Tau levels in AD, although this was not observed in all cohorts [62,63]. PrP^C-Fyn signaling thus appears to couple Aβ and Tau pathologies, and Fyn can be considered a lynchpin of AD pathophysiology (Table 2).

In vivo studies of the cellular prion protein–mGluR5–Fyn pathway in Alzheimer's disease mouse models

Several groups, including ours, have studied the PrP^C–mGluR5–Fyn pathway in mouse models overexpressing an APP transgene known to cause autosomal dominant AD. Genetic removal or pharmacologic blockade of PrP^C reverses memory impairments and synapse loss in transgenic AD mice [43,44], although these findings have not been successfully replicated in all models [39]. PrP^C does not span the plasma membrane, and thus needs a transmembrane partner to initiate intracellular signaling. This partner is mGluR5, and its blockage with the antagonist MTEP reverses both spatial memory impairments and hippocampal synapse loss in AD mice [3]. Fyn is the third step in the PrP^C–mGluR5–Fyn pathway, and several preclinical studies in AD mice have been reported.

Overexpressing Fyn was found to accelerate synapse loss and the onset of cognitive impairment in the J9

Table 2 Evidence implicating Fyn in amyloid-beta oligomer action of Alzheimer's disease

Observation	Detail	References
Increased Fyn in AD	Co-localized with Tau	[29]
Amyloid-beta activates Fyn	Oligomer specific and PrP mediated	[1-4]
Fyn impairs synapse	*N*-methyl-D-aspartate receptor and dendritic spine loss	[2-4]
Fyn interacts with Tau	Localizes and phosphorylates Tau	[61-63]
Fyn alters AD transgenics	Fyn loss protects and Fyn gain accelerates	[61,64,65]

AD, Alzheimer's disease; PrP, prion protein.

($APP_{swe/Ind}$) transgenic AD mouse model, while removing Fyn expression rescued synapse loss in the J20 ($APP_{swe/Ind}$) transgenic AD model [64,65]. Because J20 mice manifest PrP^C-independent dysfunction [39], Fyn represents a common downstream effector required in both PrP^C-dependent and PrP^C-independent pathways. This further supports Fyn as an attractive target in AD therapy. In the APP23 (APP_{swe}) transgenic model, overexpression of a truncated Tau protein, which competes with full-length Tau for the Fyn binding site, rescued cognitive impairments, and was associated with a reduction in PSD Fyn levels [61]. The findings from these two transgenic lines were not replicated when reducing Fyn expression in a different AD model expressing a tau transgene, in addition to APP and Presenilin 1 (3xTg-AD) [66]. However, the negative study focused on subtle and sex-specific deficits. Interestingly, inhibiting striatal-enriched tyrosine phosphatase 61, which results in Fyn activation, reversed impairments in spatial memory in the 3xTg-AD model [67].

While these results differ from the majority of studies to date, they highlight the complexity of Fyn homeostatic regulation, and the importance of ongoing efforts to characterize these important signaling pathways in humans. While a specific Fyn inhibitor has not yet been studied in an AD model, the SFK inhibitor dasatinib was administered to APP/PS1 transgenic mice in one study [68]. The authors focused on Aβ-induced SFK signaling in microglial cells and reported that dasatinib reduced microgliosis, lowered tumor necrosis factor-alpha levels in the brain and improved behavior in a T-maze test. These data further support the use of SFK inhibitors in AD, and suggest that the benefits may derive from modifying microglial toxicity as well as altering Aβo synaptotoxic signaling in neurons.

Targeting Fyn as a novel therapy in Alzheimer's disease

Potential adverse effects of Fyn inhibition

Taken together, the preclinical evidence strongly supports targeting Fyn as a promising therapeutic intervention in AD. However, Fyn is involved in a diverse set of physiologic processes important to normal function, and disrupting some of these processes could potentially have unintended consequences. Most important is the role of Fyn in synaptic function and plasticity. While the relationship between long-term potentiation and human memory function continues to be debated, excessive inhibition of Fyn could have an adverse effect on memory and cognitive function. These effects have not been reported in clinical studies of SFK inhibitors for cancer, but this will need to be monitored closely in a more vulnerable population with little cognitive reserve, such as patients with AD. Preclinical data strongly suggest that Fyn is activated by Aβ, and thus a therapeutic strategy aimed at lowering this activity to a physiologic level is likely to represent the most effective approach, with the least serious adverse effects.

While Fyn also regulates CNS myelination, this function may not be as relevant in the fully mature CNS, where myelination is complete. Fyn has been shown to be part of a signaling pathway important for myelin regeneration in chronic demyelinating diseases [69]. While partial inhibition of Fyn seems unlikely to have a clinically meaningful effect on myelination in AD, the possible side effects of excessive Fyn inhibition on CNS myelination in humans must be considered when evaluating new therapies aimed at reducing Fyn activity.

FynT is part of the T-cell receptor, and is an important mediator of both T-cell signaling and differentiation of $CD4^+$ cells into T-helper type 2 cells in rodents, the latter cells being critical for the humoral immune response [70,71]. Both processes are essential to normal immune function, and while they are not exclusively regulated by Fyn, monitoring immune function in patients receiving SFK inhibitors is critical.

Finally, Fyn also affects the hematologic system, and associates with glycoprotein IIb–IIIa on platelets, a well-known integrin that has been targeted in cardiovascular disease and stroke [72]. Removal of Fyn expression in mice decreases platelet function and increases the bleeding time [72]. A similar function in humans is not known, and the same effects on platelets have not been demonstrated when Fyn is partially removed. Nevertheless, these findings must be considered when inhibiting Fyn in human subjects.

Pharmacologic compounds targeting Fyn in Alzheimer's disease

Masitinib – a tyrosine kinase inhibitor selective for c-Kit, platelet-derived growth factor receptor and, to a lesser degree, Fyn and Lyn – was recently studied in a 24-week, phase II dose-ranging trial in France, involving 34 patients with mild-to-moderate AD [73]. Masitinib was reasonably well tolerated, and was associated with improvements in cognition and daily function at 12 and 24 weeks, thus supporting tyrosine kinase inhibition as a treatment strategy in AD. A large international phase III trial was launched earlier this year to evaluate the efficacy and safety of two doses of masitinib compared with placebo (ClinicalTrials.gov: NCT01872598).

Saracatinib (AZD0530) is a small molecule inhibitor of Src family kinases, blocking Src, Fyn, Yes and Lyn, with 2 to 10 nM potency [74]. At 10-fold to 100-fold higher concentrations the compound also inhibits Abl family kinases, but there is no detectable activity in this concentration range against other kinase families, including c-kit, Csk and platelet-derived growth factor receptor. AZD0530's specific inhibition of Fyn and SFKs has led to its development as therapy for solid tumors, because Src

family kinases regulate tumor cell adhesion, migration and invasion, and also regulate proliferation [74]. Clinical tolerability and oral bioavailability have been demonstrated, but phase II studies have shown limited benefit as a single agent in specific oncological indications [75-79]. For tumor cell migration and for oncological applications, it is estimated that >98% kinase inhibition is required so clinical doses have targeted concentrations >20-fold above the kinase half maximal inhibitory concentration, in the 200 to 1,000 nM concentration range.

Preliminary studies indicate that the dose needed to disrupt the Aβ–PrP^C–Fyn signaling cascade is significantly lower than that needed for cancer therapy. We recently launched a phase Ib study of saracatinib in patients with AD (ClinicalTrials.gov: NCT01864655). The goal of the study is to establish safety, tolerability, and CNS penetration in patients with AD, in preparation for a phase IIa proof-of-concept clinical trial beginning in 2014. Our data will provide important data on the effectiveness of Fyn and Src family kinases inhibition for the treatment of AD. Future approaches may target more specific aspects of the Aβ-PrP^C–mGluR5–Fyn pathway, particularly addressing mechanisms of Fyn activation unique to this signaling cascade.

Conclusions

Despite the enormous and growing burden of AD, there remains no effective disease-modifying therapy today. The approaches now in clinical trials are limited in their mechanisms of action, with most centering on efforts to alter Aβ itself, or its production, clearance or aggregation. No major trial has centered on the signal transduction downstream of toxic Aβ species, and while there are efforts to develop approaches targeting Tau and Tau kinases in AD, none provides a unified approach to Aβ and Tau. Fyn represents a unique therapeutic target in AD as it is central to Aβ signal transduction, and has major functional interactions with Tau, thereby unifying the two major pathologies in AD. Developing a disease-modifying therapy in AD would revolutionize the care of millions of patients worldwide, with a major impact on rapidly escalating healthcare costs. The hope is that of the numerous discoveries that are being made in models of AD, such as the Aβ–PrP^C–Fyn signaling pathway, some will reach patients with AD over the next decade.

Abbreviations

AD: Alzheimer's disease; APP: Amyloid precursor protein; Aβ: Amyloid-beta; Aβo: Amyloid-beta oligomer; CNS: Central nervous system; PrP^C: Cellular prion protein; PSD: Post-synaptic density; SFK: Src family of nonreceptor tyrosine kinase.

Competing interests

SMS is a co-founder of Axerion Therapeutics, seeking to develop Nogo-receptor-based and PrP-based therapeutics. In relation to AD therapeutics, CHvD has served as a scientific advisor or consultant to Bristol-Myers Squibb, Janssen AI, Pfizer, Glaxo Smith Kline, Elan Pharmaceuticals, Roche Pharmaceuticals, and Abbott/AbbVie Inc. CHvD has received research support from Bristol-Myers Squibb, Elan Pharmaceuticals, Janssen Alzheimer's Immunotherapy, Pfizer Inc., Eli Lilly, Merck, Baxter Pharmaceuticals, GlaxoSmithKline, Abbott Laboratories, Medivation, Inc., Biogen Idec, Eisai, Inc., Genentech, Inc., and Roche Pharmaceuticals. HBN declares that he has no competing interests.

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