Role of p21-Activated Kinase Pathway Defects in Alzheimer's Disease Cognitive Deficits

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Dendritic spine defects are common to multiple forms of cognitive deficits, including mental retardation and Alzheimer’s disease (AD). Because p21-activated kinase (PAK) mutation can lead to mental retardation, and because the PAK kinase-cofilin signaling plays a critical role in dendritic spine morphogenesis and actin dynamics, we hypothesized PAK pathway involvement in AD synaptic and cognitive deficits. Here, we demonstrated that PAK and its activity are markedly reduced in AD, accompanied by reduced and re-distributed phosphoPAK, prominent cofilin pathology and downstream loss of the spine actin-regulatory protein, drebrin, which cofilin removes from actin. Direct implication of β-amyloid (Aβ) in PAK signaling deficits and drebrin loss was shown in APPswe transgenic mice and Aβ oligomer-treated hippocampal neurons. In addition, pharmacological PAK inhibition in adult mice was sufficient to cause similar cofilin pathology, drebrin loss and memory impairment, consistent with a potential causal role of PAK defects in AD cognitive deficits.
Alzheimer’s disease (AD), the most common cause of cognitive deficits associated with aging, shares the key feature of spine loss or dysgenesis with human developmental mental retardation (MR) syndromes\(^1\). Dendritic regression and spine loss per remaining neurite have been observed in AD with traditional Golgi staining\(^2\) and, recently, with multicolor DiOlistic labeling\(^5\). Synaptic and dendritic loss is more closely correlated with cognitive deficits in AD than neuronal loss\(^4\). The analogy between AD and mental retardation is more evident in Down’s syndrome (DS), the most common cause of mental retardation (Supplementary Fig. 1 and Notes). Accumulation of β-amyloid (Aβ) or altered processing of its precursor, amyloid precursor protein (APP) located on chromosome 21, is associated with AD; while increasing the APP gene dosage with complete or segmental trisomy 21 leads to early spine and cognitive deficits and, eventually, plaque and tangle pathology in DS\(^7\).

Although it is possible that dendritic spine defects are merely secondary to upstream deficits in mental retardation\(^4\), a primary role for dendritic spine defects is strengthened by recent identification of a set of mental retardation genes, many X-linked\(^9\). These mental retardation genes reveal a clustering of proteins in the postsynaptic pathways regulating spine actin assembly and disassembly and spine morphogenesis, one of which is p21-activated kinase (PAK), a downstream signaling effector of the rho/rac family of small GTPases. Missense mutation in PAK3 causes severe X-linked nonspecific mental retardation\(^10,11\). Animal models of mental retardation syndromes with dominant negative PAK kinase\(^12\) or knockout of the gene for its downstream kinase, LIMKI (LIM for LIM domain, an acronym of three gene products Lin-11, Isl-1, and Mec-3)\(^13\) show defects in dendritic spines and cognition. Two major isoforms of PAK exist in the brain, PAK1 and PAK3, both of which show diffuse distribution in cell bodies and dendrites, and both are peri-synaptic\(^11,14\). Consistent with the possibility of a critical role of dendritic spine defects in AD cognitive impairment, dendritic postsynaptic proteins in excitatory neurons are disproportionately altered during the progression of AD. For example, although the estimate of neuronal loss in AD hippocampus is around 5-40\%\(^15\), loss of postsynaptic proteins, such as the actin-regulating developmentally-regulated brain protein (drebrin), was found to reach 70-95\%\(^16\). Drebrin is highly localized at spines in adult brains\(^20\), and is required for actin clustering and synaptic targeting of postsynaptic density-95 protein (PSD-95)\(^21\). Drebrin and PSD-95 are concentrated at excitatory synapses\(^22\). Both AD and DS show profound loss of postsynaptic drebrin that far exceeds presynaptic synaptophysin loss at early stages, and this is a specific molecular defect shared by both early- and late-stage DS patients\(^18\). This may reflect selective loss of this class of PSD-95 synapse which is targeted by Aβ oligomers\(^23\). That synaptophysin deficits alone could not account for cognitive deficits was suggested by the observation that synaptophysin knockout mice exhibited no deficits in synaptic plasticity, long-term potentiation or cognitive function\(^24\).

The data presented here implicate Aβ-induced aberrations in the postsynaptic PAK pathway in contributing to the massive drebrin loss and cognitive deficits found in AD. Specifically, we show that the PAK pathway defects in AD are of a magnitude sufficient to cause drebrin and memory loss in adult animals. These defects caused by Aβ oligomers result in active PAK and drebrin loss, which can be prevented by anti-Aβ antibody in vivo or in vitro over-expression of wild type but not kinase-dead PAK.
Results.

PAK Signaling Defects in AD Brains. Because of the overlapping dendritic spine defects occurring in mental retardation and AD, and the implication of the PAK signaling defects in the dendritic spine and synaptic dysfunction of mental retardation\textsuperscript{10,11,25,26}, we determined whether PAK levels are altered in a non-development-related form of cognitive loss, AD. Significant deficits in both PAK1 (35% ± 6 relative to normal, * \(P < 0.001\)) and PAK3 (62% ± 7 relative to normal, * \(P < 0.001\)) were observed in AD hippocampus (Fig. 1a). In contrast, levels of β-actin were unaltered. A PAK deficit was also observed in temporal cortex. Levels of PAK3 (but not PAK1, data not shown) were significantly decreased in AD temporal cortex compared to normal brains (70% ± 7 relative to normal, * \(P < 0.001\)), while the levels of the neuronal marker neuron-specific enolase (NSE) were unchanged (Fig. 1b). Further, when pre-synaptic proteins were determined in AD cortex samples, an asymmetric trend of general pre- and excitatory post- synaptic protein marker loss was observed that may reflect a selective attack. For example, only a modest (approximately 17%) loss of pre-synaptic synaptophysin was seen in the same AD cortex samples\textsuperscript{17}. Therefore, these results demonstrated large deficits in both PAK1 and PAK3 in AD brains, and the specificity of PAK loss relative to other neuronal proteins suggested that the loss of PAK is not simply secondary to neuron loss occurring in AD brains.

PAK activation involves phosphorylation at serine 141. To further characterize PAK pathway deficits in AD, an antibody specific to phosphoPAK (ser141) (pPAK) was used to selectively label activated PAK isoforms 1, 2 and 3\textsuperscript{27}. Quantification of pPAK on Western blots revealed a 73% decrease in AD temporal cortex (* \(P < 0.001\), Fig. 1b), indicating the loss of PAK activity exceeded PAK protein loss.

Immunohistochemical DAB staining of pPAK (Fig. 2a) demonstrated normal control hippocampus displayed diffuse neuronal, apical dendritic and neuritic process neuropil staining for active pPAK similar to previously reported results with PAK1 and PAK3 antibodies\textsuperscript{14}. Diffuse but stronger nuclear pPAK staining was also apparent in normal brains, consistent with recently reported nuclear localization of activated PAK\textsuperscript{28}. In contrast, in AD hippocampus, while some pPAK was labeled as in normal brains, neurons showed markedly reduced diffuse pPAK labeling coupled with intense and abnormally focal, apparently vesicular, intra-neuronal pPAK staining (Fig. 2b). Intense flame-shaped pPAK staining was also observed in AD hippocampus (Fig. 2b), but confocal double- labeling studies revealed no co-localization between pPAK and phosphoTau-labeled tangles (not shown). Similar pPAK staining patterns in control and AD brain were also observed with antibody to phospho-PAKThr423 (not shown). This aberrant pPAK staining resembles the intraneuronal Aβ 1-42 that has been reported to accumulate along with APP C-terminal fragments in enlarged endosomal/ lysosomal structures\textsuperscript{29} which are rab-5 and Aβ42 positive and of similar morphology in AD brain\textsuperscript{30}. Thus, aberrant pPAK accumulation in granular intraneuronal structures in AD brain may be related to observations of PAK3 binding to APP C terminal domain in rab-5 positive neuronal endosomes in vitro\textsuperscript{31}.

To investigate whether the large PAK deficits and aberrant pPAK localization in AD are associated with other pathologies, PAK downstream molecules were investigated. An important molecule downstream of PAK signaling and responsible for actin regulation is
cofilin. PAK phosphorylates and activates the intermediate LIM kinase, which in turn phosphorylates cofilin at serine. Unphosphorylated cofilin is active which binds cooperatively to F-actin, weakening the interaction between actin subunits and promoting the severing of F-actin. However, inactivation of cofilin (through phosphorylation by PAK via LIM kinase) causes it to lose the ability to bind to F-actin filaments. Although the normal function of cofilin is critical for maintaining the well-orchestrated mechanism of actin dynamics, cofilin dysregulation, occurring with defective phosphorylation, may lead to pathology. Pathologic intracellular inclusion bodies containing cofilin, actin rods and other actin-binding proteins (Hirano bodies) are prominent features in the hippocampus and cortex of AD brains. Staining of cofilin in AD hippocampus revealed different types of labeling (Fig. 2c) including normal diffuse cofilin staining that is commonly observable in normal brains and classic somatic Hirano body staining resembling that previously observed in AD brains. AD brains also show intense dendritic cofilin labeling, suggesting abnormal cofilin aggregation in neurites, consistent with the observation of cofilin aggregates in dendrites in response to neurodegenerative stimuli. Intense and punctate cofilin labeling was also observed around plaques (Fig. 2c).

Confocal co-labeling of pPAK and cofilin in AD hippocampus revealed different stages of pPAK and cofilin pathology with increasingly intense cofilin labeling associated with progressively decreased diffuse pPAK staining (Fig. 2d), consistent with the hypothesis that loss of pPAK can lead to local pathology related to cofilin aggregate formation and their abnormal redistribution in AD (Fig. 2b,c,d).

The severe pPAK and cofilin pathologies in AD, coupled with the previously reported large loss of the dendritic spine actin-regulating protein drebrin (70-95%), suggests that the defects in this actin-regulatory machinery could be an underlying factor in dendritic and synaptic dysfunction in AD. Comparison between drebrin and cofilin in the same membrano-cytoskeletal samples of human brain hippocampus revealed a reciprocal relationship between drebrin and cofilin; i.e., in the subjects with low drebrin levels, high cofilin levels were observed, and vice versa (Fig. 2e). Since both drebrin and cofilin are actin-binding proteins and because of their reciprocal relationship, we hypothesized that cofilin might remove drebrin from actin. Consistent with this hypothesis, the N-terminus of drebrin’s actin-binding sequence contains a segment of high homology with actin depolymerizing factor (ADF) or cofilin. In addition, cofilin induces conformational changes in actin that prevents other proteins from binding to actin. To test this hypothesis directly, pooled normal human hippocampal samples were split into groups with or without reaction with 10 µg exogenous dephosphorylated cofilin, and then the drebrin-actin binding was determined. Specifically, the samples were immunoprecipitated with an anti-β-actin antibody followed by Western blotting detection of actin-bound drebrin in the immunoprecipitated pellets which contained equivalent actin. Results of this in vitro assay indicated that with addition of exogenous dephospho-cofilin, the drebrin-actin binding was reduced by over 50% (* P < 0.05) (Fig. 2f), consistent with the hypothesis that dephospho-cofilin can prevent drebrin from binding to actin. Therefore, lower PAK activity in AD leading to less phosphorylation of cofilin could potentially increase cofilin binding to actin, which would in turn limit drebrin-actin binding. This would result in drebrin translocation to the cytosol and, because of its many calpain
cleavage sites enriched in proline, glutamate, serine and threonine (PEST sites), possible proteolytic degradation by calpain.\textsuperscript{17,20}

**PAK Signaling Defects in Tg2576 and Aβ Oligomer-Treated Neurons.** Neuronal degeneration in AD is related not only to Aβ aggregates but also to intra-neuronal tau aggregates and neurofibrillary tangle formation as well as synuclein aggregates and/or Lewy body formation. This raised the question: are the abnormalities in pPAK, cofilin and drebrin seen in AD causally related to Aβ pathologies? To help address this question, we examined aged 22 month-old APP\textsubscript{swe} transgenic Tg2576 mice with abundant amyloid pathology but lacking classical neurofibrillary or Lewy body pathology. Diffuse labeling of neurons resembling that of control brain was observed in cortex of aged Tg-controls (Fig. 3a), but pPAK labeling in aged Tg+ animals was uneven with a loss of diffuse and nuclear neuronal staining and patches of intense staining, frequently plaque-associated and consistent with a role for Aβ aggregates in inducing PAK pathway defects (Fig. 3b). Labeling with a different active phospho-PAK antibody to PAK1Thr423 confirmed diffuse neuron labeling in Tg negative controls (Fig. 3c). There was a reduction of this diffuse staining in Tg+ animals as well as intense plaque-associated labeling of dystrophic neurites (but not microglia) and clusters of neuron staining, frequently granular or flame shaped (similar to AD, Fig. 2 and plaque-associated, Fig. 3d, Tg+). To further identify the nature of pPAK141 (pPAK) staining around plaques, confocal triple labeling of pPAK (green), amyloid plaques (blue with 10G4 antibody) and markers for plaque cellular components (red) was carried out. We observed frequent co-localization with phospho-tyrosine, a marker for plaque-associated microglia\textsuperscript{40} (Fig. 3e) but no co-localization between pPAK and glial fibrillary acidic protein (GFAP) (Fig. 3f), indicating that peri-plaque pPAK141 staining is in microglia but not astrocytes. The morphology and size of other cells labeled with strong pPAK around plaques was consistent with neurons, suggesting a neuronal nature for some peri-plaque pPAK accumulation which was confirmed by co-localization with neuron specific enolase, NSE (Fig. 3g, arrowheads). Confocal images with SMI 312 antibody to label neurofilament in dystrophic neurites (red) also indicated co-localization between pPAK and dystrophic neurites (Fig. 3h, arrowheads) consistent with PAK1Thr423 in dystrophic neurites (Fig. 3d). Cofilin labeling in and around β-amyloid plaques was also observed in Tg+ hippocampus (Fig. 3i). Triple labeling of pPAK (red), cofilin (green) and amyloid plaques (blue with 10G4 antibody) in Tg+ hippocampus indicated intense peri-plaque pPAK staining sometimes associated with local cofilin puncta, similar to that observed in AD hippocampus (Fig. 3j). Also, our group previously reported a large 62% loss in drebrin in these amyloid-laden (but tangle-free) Tg+ mice\textsuperscript{17}. Therefore, pPAK and cofilin pathology and severe drebrin loss are found in both AD and aged, amyloid-laden tangle-free APP\textsubscript{swe} mice, suggesting that Aβ aggregates could account for these abnormalities. To further determine a role for Aβ in PAK deficits, Aβ levels were reduced with an approach that has been consistently demonstrated to clear Aβ and reverse cognitive deficits in APP\textsubscript{swe} Tg mice, that is, passive immunization with an anti-Aβ antibody recognizing the N-terminal amino acids 1-15 of Aβ\textsuperscript{41,42}. The anti-Aβ antibody significantly increased the levels of pPAK in 11-12 month Tg2576 (Fig. 3k), and the levels of soluble Aβ oligomer (~12-mer) was inversely correlated with pPAK ($r^2 = 0.443$, $P = 0.013$). In contrast, formic acid extracted insoluble Aβ assayed by sandwich ELISA...
was not significantly reduced and not correlated with pPAK ($r^2 = 0.064, P = 0.4$). While global drebrin levels assessed by Western were not significantly reduced at 11-12 months, anti-Aβ infusion still produced a trend toward increased drebrin (Fig. 3i) which was significantly correlated with pPAK($r^2 = 0.315, P < 0.05$). Our results therefore implicate Aβ in PAK and related drebrin deficits, but because the Aβ antibody treatment can reduce multiple forms of Aβ, these results do not exclude the involvement of other forms of Aβ.

In order to directly test the hypothesis that Aβ oligomers can cause PAK signaling defects, we incubated cultured primary hippocampal neurons with 100 ng/ml soluble Aβ1-42 aggregates prepared with an Aβ oligomer-generating protocol. Oligomers are elevated in AD brains, and synaptic failure caused by Aβ oligomer-induced postsynaptic attack at PSD-95 sites on excitatory neurons has been proposed to account for the failure of memory formation in AD patients and in Aβ oligomer infusion models. Aβ1-42 oligomer induced rapid and persistent reduction in PAK activities indicated by the levels of phosphorylation of a PAK substrate, myelin basic protein (MBP) (Fig. 4a). Briefly, the protein samples were immunoprecipitated with a PAK antibody, followed by reaction with exogenous unphosphorylated MBP and detection of phosphorylated MBP (pMBP) on Western blots (Fig. 4a). Loss of viability by lactate dehydrogenase (LDH) assay of media or MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction was not observed until after 56 hours of treatment (data not shown). Also, the PAK activity deficits induced by Aβ were accompanied by rapid and persistent reductions in drebrin levels, while levels of synaptophysin and total PAK were unchanged (Fig. 4b). In addition, pPAK staining in Aβ-treated hippocampal neurons shows a granular focal pattern resembling that in AD (Fig. 4c and 4d).

Dose- response studies with 0, 10, 100 and 500nM Aβ42 oligomer preparations showed parallel dose-dependent loss of drebrin (Fig. 4d) and pPAK (Fig. 4e) after 2 h with a trend beginning at 10nM and highly significant losses at 100nM doses with relative preservation of actin. Supporting an oligomer-induced effect, the parallel drebrin and pPAK deficits induced by Aβ oligomer preparation were prevented by pretreatment with oligomer-specific A11 antibody that fails to recognize contaminating fibrils or monomer. Aβ-treatment of hippocampal cultures resulted in redistribution of pPAK in neuron, as suggested by more granular staining of neurons (Fig. 4f,g).

These in vitro data are consistent with a role for Aβ oligomer in pPAK alterations and drebrin deficits in AD and APPswe transgenic mice, and further support the hypotheses that Aβ oligomers can directly cause PAK signaling defects and drebrin loss.

**Active PAK protects from Aβ oligomer-induced neuronal drebrin loss.** Having shown Aβ oligomers can cause both drebrin and PAK deficits, we sought to use PAK1 transfection in primary hippocampal neurons to prove that increased wild type PAK1 expression can protect from Aβ oligomer-induced drebrin loss. Drebrin antibody strongly labeled cell bodies and neurites in non-transfected neurons (Fig. 5a) but this labeling was reduced after Aβ oligomer treatment (Fig. 5b). Immunostaining with anti-Aβ antibody (Fig. 5c) revealed a punctate staining pattern of the exogenous oligomers distributed along neuronal cell bodies and processes (arrows) similar to the synaptic
pattern observed by Lacor et al. Following transfection of kinase dead PAK1 tagged with green fluorescent protein (GFP), the red drebrin staining (Fig. 5d) was suppressed in the neurites and cell bodies of transfected cells labeled by the green GFP compared with adjacent non-transfected cells (Fig. 5e,f) and kinase dead-PAK1 failed to protect drebrin after Aβ42 oligomer treatment (Fig. 5g-i). Neurons transfected with active wild type PAK1 (Fig. 5j-r) showed drebrin staining equivalent to non-transfected cells but after oligomer treatment, drebrin staining in wild type PAK1 transfected cells was clearly protected compared to non-transfected neighboring neurons (asterisks). These results demonstrate that specifically increasing active (but not inactive) PAK1 can protect against Aβ oligomer-mediated drebrin loss.

**PAK Inhibition Causes Cofilin and Drebrin Pathology and Memory Loss.** Large PAK deficits such as those seen with PAK3 mutations are sufficient to cause severe mental retardation. As with all mental retardation defects, however, these may be largely “developmental” pertaining to aberrant neurogenesis, pathfinding, connectivity and organization during development of the brain and have no direct relation to adult- or age-related cognitive deficits. Since AD is a disease with age-dependent cognitive deficits, and the PAK signaling defects don’t manifest until later in life, the question still remains whether, in adults, PAK down-regulation is sufficient to cause defects in AD including drebrin loss, cofilin pathology and cognitive deficits. To address this question, we infused groups of stereotaxically cannulated 11 month-old retired breeder mice intra-cerebroventricularly (icv) with a PAK inhibitor, PAK18, or a mutant negative control peptide PAK18-R192A (R192A), at 0.18 nmol/hr estimated to produce ~10 µM peptides in the cerebrospinal fluid (Fig. 6a). Similar peptides have been previously used to block PAK activity. Treatment of hippocampal neurons with these peptides showed that 10 µM PAK18, but not the inactive peptide R192A, resulted in a significant reduction in pPAK levels and a loss in drebrin levels accompanied by persistent punctate cofilin labeling, resembling the cofilin rods induced by neurodegenerative stimuli (see Supplementary Fig. 2 and Notes), arguing that the peptides PAK18 and the negative control R192A are valid tools for inhibiting PAK, and that inhibition of PAK activity is sufficient to drive both drebrin loss and cofilin pathology in vitro.

PAK18 infusion for 2 days in adult mice caused a large and significant reduction in pPAK in hippocampus (down 82% compared to vehicle alone, * P < 0.01) while the vehicle and R192A peptide had no impact, validating the use of PAK18 in vivo (Fig. 6b). This ~ 80% reduction in PAK activity caused by PAK18 was found to be associated with a significant reduction in the amount of drebrin bound to actin (39% ± 2 compared to vehicle alone, * P<0.01). The levels of β-actin and synaptophysin remained unaltered (Fig. 6b), consistent with a selective drebrin deficit and the absence of neuronal loss in these animals.

Infusion of PAK18, but not R192A (Fig. 6c) for one month resulted in hippocampal CA1 cofilin pathology often extending into dendrites (Fig. 6d), resembling that found in CA1 in AD (Fig. 6e). Lower power views show the CA1 cofilin pathology in PAK18 inhibitor-infused animals (Fig. 6f) and the similarly distributed fractin (caspase-cleaved actin, a marker for caspase activation) (Fig. 6g), consistent with our previous reports of similar co-localization of caspase activation and Hirano pathology in AD and focal dendritic caspase activation associated with drebrin loss.
Following infusion of PAK18 for 15 days, a measure of hippocampal function was assessed with a hippocampus-dependent social recognition memory task. The ratio between the time the test mouse spends investigating a juvenile mouse it encountered 3 or 24 hr earlier and the initial investigation time was measured as an index of memory acquisition and memory consolidation respectively. PAK18 infusion caused a significant increase in the time the test mouse spends investigating the juvenile mouse following the intervals of both 3 hr and 24 hr (* *P* < 0.01), indicating adult-onset memory deficits can be induced by PAK inhibition (Fig. 6h).

Overall, our results implicate PAK deficits in AD using multiple *in vivo* and *in vitro* models, and support a pathway from PAK activity loss to cofilin pathology to drebrin loss and cognitive deficits as one final common pathway for AD and some forms of mental retardation (Supplementary Figs 1,3 and Notes). Our data with PAK18-icv-infused mice showed that even in adults, PAK inhibition could directly cause cofilin pathology and drebrin loss similar to that found in AD, suggesting a direct causal relationship between PAK and these events. More interestingly, pharmacological inhibition of PAK activity caused a significant inhibition of social recognition memory (3hr and 24hr delay tests), arguing for a critical role of PAK in cognitive function. That PAK mutation can lead to mental retardation, coupled with the role of PAK in cognition, suggests that PAK defects have the potential to play a critical role in AD cognitive deficits (Supplementary Fig. 1 and Notes).

The pharmacological PAK inhibition-caused social recognition memory deficits are consistent with the memory deficits shown by Hayashi et al. using dominant negative PAK (dnPAK) transgenic mice. With an approximately 40% downregulation in PAK activity in cortex and hippocampus of dnPAK mice, they showed PAK downregulation led to selective spine defects and synaptic plasticity impairment in cortex associated with impairment of memory consolidation, but with no observable synaptic defects in hippocampus because of the high basal hippocampal PAK levels. The >80% inhibition in PAK activity in our study with continuous infusion of PAK18 was sufficient to cause drebrin loss and cofilin pathology in hippocampus, and social recognition memory impairment which is strongly dependent on hippocampal function.

In summary, the current study identified defects in dendritic spine PAK pathway regulation of actin dynamics as a shared feature of some forms of mental retardation and AD. It demonstrated that adult-onset PAK signaling defects lead to cofilin pathology, major drebrin loss and memory deficits resembling AD and that Aβ oligomers implicated in AD cause PAK defects leading to drebrin loss and further that expression of active PAK can protect against Aβ oligomer. The prominent PAK pathway alterations in AD, therefore, should contribute to the synaptic and cognitive deficits and represent a new potential therapeutic target.
Methods

Material. Unless otherwise noted, reagents were obtained from Sigma. PAK18 peptide (RKKRRQRRR-G-PPVIAPRPEHTKSVYTRS) and PAK18-R192A peptide (RKKRRQRRR-G-PPVIAPRAPEHTKSVYTRS) were custom synthesized by Bioworld. The PAK18 peptide was synthesized with a specific PAK kinase inhibitory domain coupled to a cell permeant TAT peptide sequence. The negative control peptide, PAK18-R192A, was synthesized containing the same TAT sequence and the PAK18 sequence with an R192A substitution of a single amino acid that results in a loss of PAK inhibitory activity. Antibodies were purchased from the following: anti-PAK1 (Zymed Laboratories Inc.), anti-PAK3 (Stressgen Biotechnologies Corp.), anti-total PAK (PAK1/2/3, Cell Signaling Technology), anti-β-actin (Chemicon International), anti-NSE (Research Diagnostics Inc.), anti-pPAK serine141 and anti-oligomer A11 (Biosource International Inc.), anti-PAKThr423 (Cell Signaling), anti-cofilin (BD Transduction Labs), anti-drebrin (clone M2F6, MBL), anti-synaptophysin (MAB368, Chemicon International), anti-SMI312 (Sternberger Monoclonals Inc.) and anti-pMBP (Upstate Biotechnology). Affinity purified anti-fractin48 and anti-Aβ (10G4)17 rabbit polyclonal antibodies were developed and characterized in our laboratory. Wortmanin and LY294002 were purchased from Calbiochem.

Human Tissue. Postmortem tissue from temporal cortex and hippocampus was obtained from the University of Southern California and the UCLA AD Research Center Pathology Cores. Controls (n = 15) were compared to AD patients (n = 15) with moderate disease. The age of death, gender and post-mortem interval were comparable in both groups.

Aβ1-42 Oligomer Preparation. Soluble Aβ1-42 oligomers were prepared following the previously published method43. A11 anti-oligomer antibody immunoneutralization protocol is described in the same paper.

Primary Hippocampal Neuronal Culture. Primary cultures of hippocampal neurons were prepared from the brains of embryonic day 18 Sprague Dawley rat fetuses as described previously49.

Plasmids and Transient Transfection

Green fluorescent protein tagged PAK1 kinase constructs with wild type (WT) pcDNA3-EGFP-PAK1 WT or kinase dead (KD) pcDNA3-EGFP-PAK1 K299R were kind gifts of Dr. Bokoch GM (The Scripps Research Institute, La Jolla, CA). Primary hippocampal neurons (7 days) were plated onto coverslips in 15.6-mm dishes and were transfected for 20 h using Lipofectamine™2000 (Invitrogen) according to the manufacturer’s protocol. 1.2 µg of expression plasmid and 2µl of Lipofectamine 2000 were used for transfection per dish. After transient transfection for 20 h, 250 nM of Aβ oligomers were directly added to cells and incubated for 2 h at 37° C. Then cells were fixed with 95% methanol for 5 min at 4° C and stained with drebrin or polyclonal rabbit anti-Aβ1-13 antibody, “DAE”, named for the first 3 amino acids of the immunogen.

Passive Immunization. The passive immunization experiment was conducted with eleven to twelve month-old male and female Tg2576 Tg (+) mice as described in detail50. The mice were randomly divided into two groups (n = 6 for control group; n = 7 for
Abeta-antibody treatment group). Control and anti-Aβ IgG2b antibodies were intracerebroventricularly (icv) infused into mice. After 14 days, mice were deeply anesthetized and sacrificed, and the brains were removed and brain regions were dissected. Biochemical measurements were performed on the cortical tissue. Oligomers were assayed by Westerns and ~12-mer quantified. 6E10, biotinylated 6E10 and 4G8 gave similar results, but only 6E10 data is shown. Formic acid extracted, detergent insoluble total Aβ was assayed as previously described17.

PAK Kinase Assay. PAK kinase assay was conducted according to the previously published method45 and manufacturer’s instruction from Upstate Biotechnology, from which the ATP, MBP and 1x assay dilution buffer were purchased.

Surgery for Intracerebroventricular (icv) Infusion of PAK18 Peptides. Custom length, stainless steel icv catheters (Plastics One) were implanted to the desired depth (dorsal/ventral: -2.0 mm from skull) at defined stereotaxic coordinates (Medial/lateral: +1.0 mm, anterior/posterior: -0.5 mm) in 11-month old male retired breeder B6D2F1 mice. The icv cannula was connected to an Alzet osmotic mini-pump (# 1002, Durect Corporation) via polyethylene tubing. The projected concentration of PAK18 and PAK18-R192A in the CSF was 10 µM, a dose found effective in vitro. This value is based on a release rate of 0.18 nmol/hr PAK18 or PAK18-R192A (240 µg/100 µl delivered at 0.25 µl/hr) into a ventricular volume of 35 µl (140-fold dilution) with production of 18 µl/hr of new cerebrospinal fluid.

Social Recognition Behavior. Social recognition experiments were conducted as previously reported47. The ratios of test trial inspection time (3 hr or 24 hr) to initial inspection time were used to measure social recognition memory.

Data Analysis. Data were presented as group means ± s.e.m. Immunoblot data were expressed as the percentage relative to controls (unstimulated or vehicle-treated neurons, or normal brains) run in the same experiment or their OD values. Statistical analysis was performed by one-way analysis of variance followed by Fisher’s PLSD post hoc analyses.

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Supplementary Notes

Common Postsynaptic Mechanisms for Developmental Cognitive Deficits and AD. Despite the strong correlation between AD cognitive deficits and synaptic marker loss, arguing for synaptic defects underlying cognitive decline\(^5\), the molecular basis for these cognitive deficits has been unclear. Many previous reports on AD and animal models focused on presynaptic synaptophysin deficits\(^5\). Synaptophysin knockout mice exhibited no deficits in synaptic plasticity, long-term potentiation or cognitive function\(^2,5\), suggesting there can be compensation for synaptophysin loss; although synaptophysin is a useful marker for underlying synaptic defects, synaptophysin loss per se did not seem to account for cognitive and synaptic plasticity deficits in AD. Therefore, we sought to gain insight into potential synaptic defect mechanisms by investigating the overlap between AD cognitive deficit syndromes with known genetic causes (Supplementary Fig. 1). Human developmental cognitive deficits (mental retardation syndromes including Down’s syndrome\(^1\) overlap AD and AD animals\(^4,5,5\) in the common compartmental arena of dendritic spine defects. That increased APP dosage can lead to both AD and Down’s syndrome cognitive deficits makes this comparison even more intriguing. DS appears to have core similarities to mental retardation, AD and AD transgenic mice with respect to early loss and degeneration of dendrites and spines, and accompanying cognitive deficits (Supplementary Fig. 1), strengthening the hypothesis of a mechanistic connection between developmental cognitive disorders and AD. Because PAK mutation causes mental retardation, and large PAK deficits occur in AD, PAK seems to be the missing link to lead to spine defects in these cognitive disorders.

PAK Inhibition with PAK18 Peptide Causes Drebrin Loss and Cofilin Pathology in Hippocampal Neurons. The parallel changes in PAK, cofilin and drebrin in AD, APP\(^\text{swe}\) transgenic mice and A\(\beta\)1-42 oligomer-treated hippocampal neurons raise the question whether the large PAK deficits are causally related to the cofilin pathology and drebrin loss. To address this question in vitro, we treated cultured hippocampal neurons with a PAK inhibitor, PAK18, or an inactive mutant peptide, PAK18 R192A. Incubation of primary neurons with PAK18, but not R192A, resulted in a significant reduction in pPAK levels (31\(\%\) ± 3 compared to control, *P < 0.05) and a loss in drebrin levels (64\(\%\) ± 11 compared to control, *P < 0.05) in hippocampal neurons (Supplementary Fig. 2). PAK18 peptide treatment also induced a marked loss in the levels of drebrin bound to actin as shown by immunoprecipitation with β-actin. The loss of drebrin was accompanied by persistent punctate cofilin labeling, resembling the cofilin rods induced by neurodegenerative stimuli such as ATP depletion, peroxide and glutamate (Supplementary Fig. 2)\(^37\). Collectively, these results argue that the peptides PAK18 and the negative control R192A are valid tools for inhibiting PAK, and that inhibition of PAK activity is sufficient to drive both drebrin loss and cofilin pathology in vitro.

PAK Signaling Pathway in Regulation of Actin Dynamics. Supplementary Fig. 3 summarizes the PAK signaling pathway and indicates the possible sites of A\(\beta\) and PI3 kinase intervention. PAK is densely concentrated at peri-synaptic locations\(^14\) where it phosphorylates and activates LIM kinase, which in turn phosphorylates ser3 of the actin-binding protein cofilin and prevents its actin-binding and actin-severing activity (Supplementary Fig. 3). When PAK activity is reduced, cofilin is active and bound to actin, which, according to our in vitro cofilin assay, removes drebrin off actin. This could be due to direct competition because homologous actin-binding domains are shared by cofilin and drebrin\(^38\). A second or alternative possible mechanism for cofilin-induced drebrin-actin dissociation involves conformational changes in cofilin-bound actin\(^39\). Drebrin loss in both AD and Down’s syndrome\(^16,18\) and in normal aging\(^19\) would therefore be predicted as a consequence of reduced PAK activity and cofilin pathology. Cofilin bound to actin is found in pathological intracellular inclusions of Hirano bodies which are prominent in AD\(^46\). Although it is unclear whether Hirano bodies are directly involved in AD progression, these intracellular inclusions might cause cytoskeletal and morphological changes leading to altered neuritic transport and synaptic dysfunction. Recent evidence suggests cofilin exists in two forms, monomers and dimers or oligomers\(^5\). The requirement of oxidizing conditions for the formation of cofilin oligomers and actin bundles provides a link between oxidative stress and cofilin pathology, both of which are prominent characteristics in AD. Our observation that cofilin pathology is co-localized with caspase-activity is consistent with our previous reports of similar co-localization of caspase activation and Hirano pathology in AD\(^46\) and focal dendritic caspase activation associated with drebrin loss\(^17\). This co-localization can now
be accounted for because dephospho-cofilin migrates to mitochondria and induces cytochrome C release and caspase activation56.

Additional References for the Supplementary Information

and massive reduction in PAK levels and activities in AD hippocampus and temporal cortex. Western blots from 33 lane gels with hippocampal 50,000 x G supernatant TBS fractions from 15 normals (N) and 15 AD (A) reveal a major loss in PAK1 and PAK3, with no changes in the levels of β-actin (a). * P < 0.001 compared to normal. A similar reduction of PAK3 was observed in temporal cortex of AD brains, while the levels of a neuron number marker neuron-specific enolase (NSE) were unaltered (b). Also, the reduction of phosphorylated and active PAK (pPAK) was larger than that of total PAK, suggesting the activity of PAK in AD was reduced beyond the loss of PAK protein itself. All data calculated +/- standard error of the mean (s.e.m.)
Figure 2. pPAK, coflin, and drebrin changes in AD hippocampus. DAB staining showing diffuse pPAK in normal hippocampus (a). Abnormal pPAK staining in AD hippocampus labeled focal clumps of pPAK (arrows) and occasional tangle-like accumulations (arrowhead) (b). Cofilin diffusely labeled normal neurons (arrowhead), and focally stained Hirano bodies (arrow 1), dendrites (arrow 2), and plaque neurites (asterisks) in 10µm cryostat AD hippocampal sections (40x) (c). A confocal image of double labeling of pPAK (red) and coflin (green) revealed different stages of pPAK and coflin pathology in AD hippocampus (63x) (d). Cells labeled 1 through 5 exhibit increasingly intense coflin labeling associated with progressively decreased diffuse pPAK. Western analyses indicated a reciprocal relationship between coflin and drebrin (e). Cofilin increased in the membranocytoskeletal pellet lysis buffer fraction while drebrin decreased in AD brains on the same Western blot. An in vitro coflin assay with normal human hippocampal TBS fraction followed by immunoprecipitation with a β-actin antibody and detection with a drebrin antibody (f). The addition of exogenous coflin reduced the binding of drebrin to actin. * P < 0.05. Scale bars in panels A, B, C = 100µm, scale bar in panel D = 25µm.
Figure 3. pPAK and cofilin pathologies in 22-month-old APPswe Tg2576 mice. Compared to Tg- mice, Tg+ mice show strong peri-plaque pPAK141 (a vs. b) and pPAKThr423 (c vs. d) staining and reduced diffuse neuronal and nuclear labeling, but with granular and intense neuron staining, often clustered or peri-plaque (d). To assess the cellular pPAK labeled elements (green), confocal triple labeling of amyloid (10G4, blue) was assessed with phosphotyrosine for microglia (e), GFAP for astrocytes (f), neuron specific enolase for neurons (g), and SMI312 antibody to phosphorylated neurofilaments for dystrophic neurites (h). Microglia, dystrophic neurites (arrows) and neurons (arrows), (but not GFAP positive astrocytes) exhibited elevated peri-plaque pPAK141. Cofilin staining in Tg+ hippocampus (i) and confocal triple labeling of cofilin (green), pPAK (red) and Aβ (10G4 antibody, blue) in Tg+ cortex (j) demonstrated peri-plaque cofilin and punctate pPAK in and around amyloid plaques in aged Tg2576 mice. Scale bar = 100µm. pPAK (k) and drebrin (l) levels were increased in mice passively immunized with an anti-Aβ antibody to clear Aβ. pPAK levels were inversely correlated with soluble Aβ (~12-mer) oligomer ($r^2 = 0.443$, $P = 0.013$) and drebrin ($r^2 = 0.315$, $P < 0.05$).
Figure 4. pPAK and drebrin loss were reproduced \textit{in vitro} in soluble Aβ1-42 oligomer-treated hippocampal neurons. Western blot detection of soluble Aβ1-42 oligomer with 6E10 antibody (a). Note ~56kD 12-mer. PAK kinase assay with 100 ng per ml Aβ1-42 oligomer-treated hippocampal neurons showed a rapid PAK activity decrease, while the total PAK levels remained unchanged (b). Drebrin levels were reduced in Aβ1-42 oligomer-treated hippocampal neurons in a time-dependent manner, occurring later than the PAK activity decrease, while the levels of presynaptic protein synaptophysin were unaltered (c). * $P < 0.05$ compared to control. Dose response for Aβ oligomer preparation induced loss of pPAK (d) and drebrin (e) in hippocampal neurons harvested after 2h treatment and complete protection from 100nM oligomers with addition of oligomer-specific A11 antibody. pPAK staining in control and Aβ-treated hippocampal cultures with the arrows denoting granular pPAK labeling in Aβ-treated neurons (f, g).
Figure 5. Wild type but not kinase dead PAK limits Aβ oligomer-induced drebrin loss. Primary HP neurons were transfected with kinase dead pcDNA3-EGFP-PAK1-K299A (d-i) or wild type pcDNA3-EGFP-Pak1-WT (j-r), treated by 250nM of Aβ42 nM oligomer for 2 h (c, g-l, m-r), and stained for drebrin (red). Transfected cells expressed green GFP fluorescence (e,h,k,n,q) which was merged with drebrin images for (f,i,l,o,r). Drebrin staining was robust in non-transfected neurons (a) but reduced after oligomer treatment (b). Aβ immunolabeling (c) revealed the punctate staining pattern of oligomers distributed along cell bodies and processes (arrows). After kinase dead pcDNA3-EGFP-PAK1-K299A transfection, drebrin staining (d) was markedly reduced in the transfected cells (asterisks) revealed by green GFP tag (e, f). In the merged (f), the pcDNA3-EGFP-PAK1-K299A transfected cell processes (thin arrows) showed little or no drebrin compared to adjacent processes (thick arrows) in non-transfected neighboring cells. Kinase dead pcDNA3-EGFP-PAK1-K299A transfected cells had drebrin loss similar to non-transfected neighboring neurons (asterisks) after Aβ42 oligomer treatment (g-i). In contrast, active pcDNA3-EGFP-Pak1-WT transfected cells had similar drebrin staining (red) to non-transfected neighbors (j-l). After Aβ oligomer treatment, drebrin staining in pcDNA3-EGFP-Pak1-WT transfected cells was comparatively preserved relative to non-transfected neighbors (m-r, asterisks). Panels (d-r) same magnification. Scale bar = 25µm.
Figure 6. PAK inhibition causes drebrin and coflin pathologies and behavioral deficits. 11-month-old adult mice were infused with vehicle, PAK18 inhibitor or control R192A peptide, \( n = 10 \) for each group. A schematic indicates the procedure for intracerebroventricular (icv) infusion of PAK18 peptides and the social recognition behavior test followed by perfusion and sacrifice (a). PAK18 infusion icv for 2 days caused a massive reduction in pPAK levels and in actin-bound drebrin in hippocampus, while the levels of \( \beta \)-actin and synaptophysin were unchanged (b). Hippocampus CA1 area of PAK18, but not control R192A-infused normal mouse and AD brain had very similar coflin labeling of Hirano body pathology with coflin often extending into dendrites (c-e). CA1 coflin pathology in PAK18-infused mice was similarly distributed as fractin (caspase-cleaved actin) labeling, suggesting an apparent co-localization of these two pathological events (f, g). Vehicle and control R192A infused and untreated mice did not show these changes (data not shown). Scale bar in panels (e, d, e) = 25\( \mu \)m, scale bar in panels (f, g) = 100\( \mu \)m. In social recognition behavior testing after PAK inhibitor, PAK18 (but not control peptide), for 15 days there were significant memory deficits at both 3 hr (memory acquisition) and 24 hr (memory consolidation) following initial mouse interaction (h). * \( P < 0.01 \) compared to vehicle.
Supplementary Figure 1. A schematic diagram, summarizing the overlapping features of mental retardation (MR), Alzheimer’s disease (AD) and human APPswe Tg2576 transgenic mice. Dendritic spine and cognitive defects characterize all three conditions. Down’s syndrome appears to encompass the attributes of all three because of its shared features with AD (Aβ accumulation and tangles, oxidative stress, etc) and APPswe Tg2576 mice (overexpression of the APPswe mutation, early cognitive deficits), and because it is the most common cause of MR syndrome.
Supplementary Figure 2. PAK inhibition by PAK18 in hippocampal neurons. PAK activity was reduced in PAK18-treated hippocampal neurons indexed by the levels of pPAK in the Western blot, while the inactive control peptide PAK18-R192A (R192A) had no impact, demonstrating the activity of the inhibitor (a). PAK inhibition by PAK18 also led to reduced drebrin levels, while synaptophysin, total PAK and coflin levels were unchanged. * $P < 0.05$ compared to control.

Immunoprecipitation with a β-actin antibody and detection with a drebrin antibody on Western blot revealed a marked decrease in drebrin-actin binding after PAK inhibition by PAK18. Error bars indicate s.e.m. (b). PAK18 caused punctate neuritic coflin immunocytochemical staining suggesting coflin pathology (c). Scale bar = 50μm.
Supplementary Figure 3. A schematic diagram, summarizing the PAK-LIM kinase-cofilin-drebrin signaling pathways. A negative impact of Aβ on the PAK pathway is also indicated.