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Role of p21-activated kinase pathway defects in the cognitive deficits of Alzheimer disease

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Defects in dendritic spines are common to several forms of cognitive deficits, including mental retardation and Alzheimer disease. Because mutation of p21-activated kinase (PAK) can lead to mental retardation and because PAK-cofilin signaling is critical in dendritic spine morphogenesis and actin dynamics, we hypothesized that the PAK pathway is involved in synaptic and cognitive deficits in Alzheimer disease. Here, we show that PAK and its activity are markedly reduced in Alzheimer disease and that this is accompanied by reduced and redistributed phosphoPAK, prominent cofilin pathology and downstream loss of the spine actin-regulatory protein drebrin, which cofilin removes from actin. We found that β-amyloid (Aβ) was directly involved in PAK signaling deficits and drebrin loss in Aβ oligomer–treated hippocampal neurons and in the *App*swe transgenic mouse model bearing a double mutation leading to higher Aβ production. 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 cognitive deficits in Alzheimer disease.

Alzheimer disease, the most common cause of cognitive deficits associated with aging, shares the key feature of spine loss or dysgenesis with human developmental mental retardation syndromes[1–3]. Dendritic regression and spine loss per remaining neurite have been observed in Alzheimer disease with traditional Golgi staining[4] and recently with multicolor DiOlistic labeling[5]. In Alzheimer disease, synaptic and dendritic loss are more closely correlated with cognitive deficits than with neuronal loss[4,6]. The analogy between Alzheimer disease and mental retardation is more evident in Down syndrome, the most common cause of mental retardation (**Supplementary Fig. 1** and **Supplementary Note** online). The accumulation of Aβ or the altered processing of its precursor amyloid precursor protein (APP), encoded by the gene *App* located on chromosome 21, is associated with Alzheimer disease; and increases in the *App* gene dosage resulting from complete or segmental trisomy 21 lead to early spine and cognitive deficits and, eventually, to plaque and tangle pathology in Down syndrome[7].

Although it is possible that dendritic spine defects are merely secondary to upstream deficits in mental retardation[8], a primary role for dendritic spine defects was strengthened by the recent identification of a set of genes related to mental retardation, 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 these proteins is 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 encoding its downstream kinase, *LIMK1*(LIM for LIM domain, an acronym derived from the three C. elegans gene products *lin-11*, *isl-1* and *mec-3*; ref. 13), show defects in dendritic spines and cognition. Two major isoforms of PAK exist in the brain, PAK1 and PAK3; both show diffuse distribution in cell bodies and dendrites and both are perisynaptic[11,14].

Consistent with the possibility that dendritic spine defects have a critical role in cognitive impairments in Alzheimer disease, dendritic postsynaptic proteins in excitatory neurons are disproportionately altered during the progression of the disease. For example, although the estimated neuronal loss in the hippocampus of persons with Alzheimer disease is around 5–40% (ref. 15), the loss of postsynaptic proteins such as the actin-regulating developmentally regulated brain protein (drebrin) reaches 70–95% (refs. 16–19). 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; ref. 21). Drebrin and PSD-95 are concentrated at excitatory synapses[22]. In both Alzheimer disease and Down syndrome, there is a profound loss of postsynaptic drebrin that far exceeds presynaptic synaptophysin loss at early stages, and this is a specific molecular defect shared by individuals with early- and late-stage Down syndrome[18]. This may

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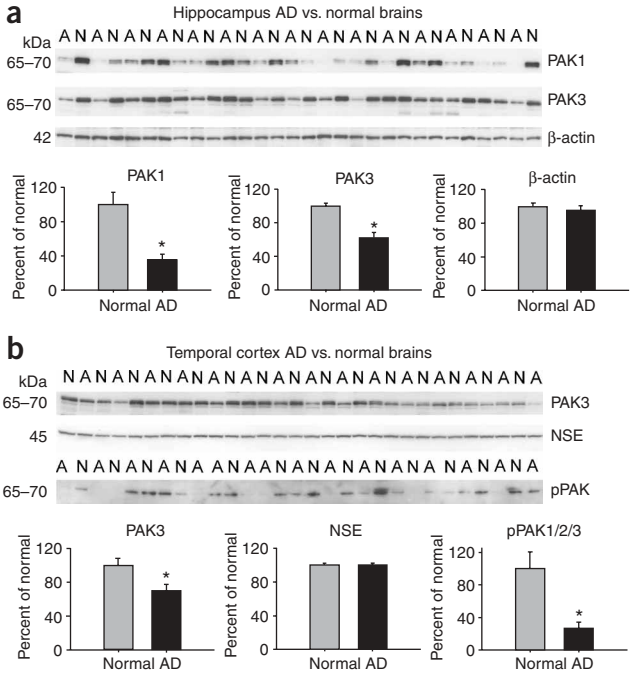

Figure 1 Selective and massive reduction in PAK amounts and activity in the hippocampus and temporal cortex in Alzheimer disease (AD). (**a**) Western blots from 33-lane gels with hippocampal 50,000*g* supernatant Tris-buffered saline (TBS) fractions from 15 normal subjects (N) and 15 subjects with Alzheimer disease (A) reveal major reductions in PAK1 and PAK3 with no changes in the amounts of β-actin. $^*P < 0.001$ compared to normal. (**b**) A similar reduction of PAK3 was observed in temporal cortex of Alzheimer patients, whereas the amounts of a neuron number marker, NSE, were unaltered. Also, the reduction of phosphorylated and active PAK (pPAK) was larger than that of total PAK, suggesting that the activity of PAK in Alzheimer patients was reduced beyond the loss of the PAK protein itself. All data calculated as mean ± s.e.m.

reflect the selective loss of this class of PSD-95 synapse, which is targeted by Aβ oligomers[23]. That synaptophysin deficits alone cannot account for cognitive deficits is suggested by the observation that synaptophysin knockout mice show no deficits in synaptic plasticity, long-term potentiation or cognitive function[24].

The data presented here suggest that Aβ-induced aberrations in the postsynaptic PAK pathway contribute to the massive drebrin loss and cognitive deficits found in Alzheimer disease. Specifically, we show that the PAK pathway defects seen in Alzheimer disease were sufficient to cause drebrin and memory loss when introduced into adult mice. These defects caused by Aβ oligomers resulted in active PAK and drebrin loss, which could be prevented by an antibody to Aβ (anti-Aβ) and by *in vivo* or *in vitro* overexpression of wild-type, but not kinase-dead, PAK.

RESULTS

PAK defects in Alzheimer disease

Because of the dendritic spine defects common to both mental retardation and Alzheimer disease, and the possible involvement of PAK signaling defects in the dendritic spine and synaptic dysfunction of mental retardation[10,11,25,26], we examined whether PAK levels are altered in a non–development-related form of cognitive loss—Alzheimer disease. We observed significant deficits in both PAK1 (mean ± s.e.m. = 35 ± 6% relative to normal, $P < 0.001$) and PAK3 (62 ± 7% relative to normal, $P < 0.001$) in the hippocampus of postmortem brains from patients with Alzheimer disease (**Fig. 1a**). In contrast, the abundance of β-actin was unaltered. A PAK deficit was also observed in temporal cortex. The abundance of PAK3 (but not PAK1; data not shown) was significantly lower in the temporal cortex of Alzheimer patients than in that of normal controls (70 ± 7% relative to normal, $P < 0.001$), whereas the abundance of the neuronal marker neuron-specific enolase (NSE) did not differ between the two groups (**Fig. 1b**). Further, when the levels of presynaptic proteins were measured in cortical brain samples of Alzheimer disease patients, we observed an asymmetric loss of excitatory postsynaptic protein markers that may reflect a selective attack. For example, only a modest (approximately 17%) loss of presynaptic synaptophysin was seen in the same Alzheimer disease temporal cortex samples[17]. Therefore, these results demonstrated large deficits in both PAK1 and PAK3 in the brains of Alzheimer disease patients, and the specificity of PAK loss relative to other neuronal proteins suggested that the loss of PAK was not simply secondary to neuron loss occurring in these disease vulnerable regions.

PAK activation involves phosphorylation at serine 141. To further characterize PAK pathway deficits in Alzheimer disease, we used an antibody specific to phosphoPAK (ser141) (pPAK) to selectively label activated PAK isoforms 1, 2 and 3 (ref. 27). The quantification of pPAK on western blots revealed a 73% decrease in pPAK in the temporal cortex of Alzheimer patients ($P < 0.001$, **Fig. 1b**), indicating that the loss of PAK activity exceeded the loss of PAK protein.

Upon immunohistochemical diaminobenzidine (DAB) staining for pPAK (**Fig. 2a**), the normal (control) hippocampus showed diffuse neuronal, apical dendritic and neuritic process neuropil staining for active pPAK, similar to previously reported results obtained with PAK1 and PAK3 antibodies[14]. Diffuse but stronger nuclear pPAK staining was also seen in normal brains, consistent with the recently reported nuclear localization of activated PAK[28]. In contrast, in the hippocampi of Alzheimer disease patients, although some pPAK was labeled as in normal brains, neurons showed markedly reduced diffuse pPAK labeling together with intense and abnormally focal, apparently vesicular, intraneuronal pPAK staining (**Fig. 2b**). We also observed intense flame-shaped pPAK staining in the hippocampi of Alzheimer patients (**Fig. 2b**), but confocal double-labeling studies showed no colocalization between pPAK- and phosphoTau-labeled tangles (data not shown). Similar staining patterns in control and diseased Alzheimer brains were also observed with an antibody to phospho-PAKThr423 (data not shown). This aberrant pPAK staining in the Alzheimer patient brains resembled the staining for intraneuronal Aβ1–42 that accumulates along with APP C-terminal fragments in enlarged endosomal and lysosomal structures[29], which are rab-5– and Aβ42-positive in the diseased brain[30]. Thus, aberrant pPAK accumulation in granular intraneuronal structures in Alzheimer disease brains may be related to observations of PAK3 binding to the APP C-terminal domain in rab-5–positive neuronal endosomes in transfected rodent neurons *in vitro*[31].

To investigate whether the large PAK deficits and aberrant pPAK localization in Alzheimer disease are associated with other pathologies, we investigated PAK downstream molecules. Cofilin is an important molecule downstream of PAK signaling and is responsible for actin regulation. PAK phosphorylates and activates the intermediate LIM kinase, which in turn phosphorylates cofilin at serine 3. Unphosphorylated cofilin is active and binds cooperatively to F-actin, weakening the interaction between actin subunits and promoting the severing of F-actin[32]. However, the inactivation of cofilin (through phosphorylation by PAK via LIM kinase) causes it to lose its ability to bind to F-actin filaments[33,34]. Although the normal functioning of cofilin is

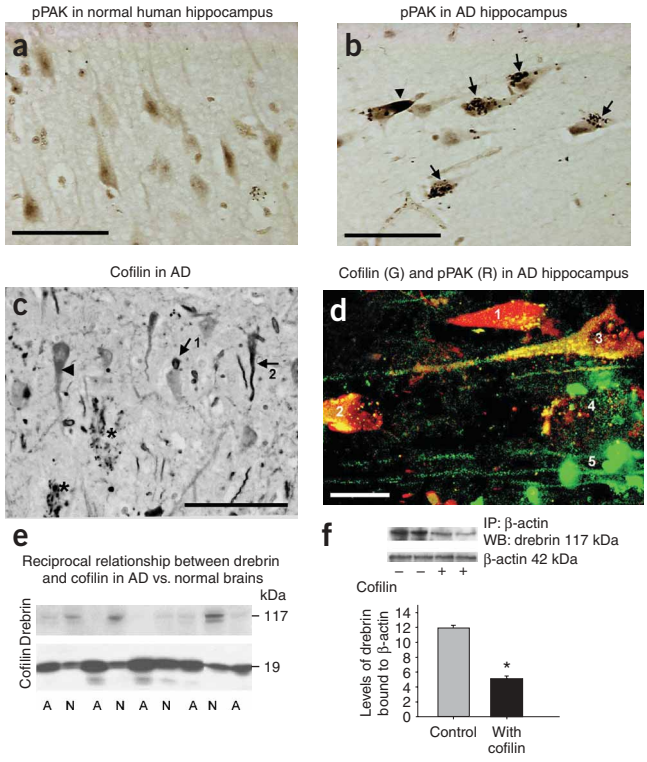

Figure 2 pPAK, cofilin and drebrin changes in the hippocampus of individuals with Alzheimer disease. (**a**) DAB staining showing diffuse pPAK in normal hippocampus. (**b**) Abnormal pPAK staining in the diseased hippocampus labeled focal clumps of pPAK (arrows) and occasional tangle-like accumulations (arrowhead). (**c**) Cofilin diffusely labeled normal neurons (arrowhead) and focally stained Hirano bodies (arrow 1), dendrites (arrow 2) and plaque neurites (asterisks) in 10-μm cryostat sections of diseased hippocampus (magnified 40x OM). (**d**) A confocal image of double labeling of pPAK (red) and cofilin (green) revealed different stages of pPAK and cofilin pathology in the diseased hippocampus (63x OM). Cells labeled 1–5 show increasingly intense cofilin labeling associated with progressively decreased diffuse pPAK. (**e**) Western blot (WB) analyses indicated a reciprocal relationship between cofilin and drebrin. Cofilin increased in the membrano-cytoskeletal pellet lysis buffer fraction, whereas drebrin decreased in diseased brains on the same western blot. (**f**) An *in vitro* cofilin assay with normal human hippocampal TBS fraction followed by immunoprecipitation (IP) with a β-actin antibody and detection with a drebrin antibody. The addition of exogenous cofilin reduced the binding of drebrin to actin. (immunoprecipitation=IP and WB=western blot, OM is original magnification) $^*P < 0.05$. Scale bars in **a**–**c**, 100 μm; in **d**, 25 μm.

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 in Alzheimer disease[35]. Staining of cofilin in the hippocampi of Alzheimer disease model mice revealed different types of labeling (**Fig. 2c**), including normal diffuse cofilin staining that is commonly seen in normal brains and classic somatic Hirano body staining resembling that seen in Alzheimer disease brains[36]. Alzheimer disease 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[37]. We also observed intense and punctate cofilin labeling around plaques (**Fig. 2c**).

Confocal colabeling of pPAK and cofilin in the hippocampus of Alzheimer disease patients revealed different stages of pPAK and cofilin pathology. Increasingly intense cofilin labeling was associated with progressively less diffuse pPAK staining (**Fig. 2d**), consistent with the hypothesis that the loss of pPAK can lead to local pathology related to the formation of cofilin aggregates and their abnormal redistribution in Alzheimer disease (**Fig. 2b–d**).

The severe pPAK and cofilin pathologies in Alzheimer disease, together with the substantial loss of drebrin (70–95%; refs. 16–19), suggested that the defects in this actin-regulatory machinery could be an underlying factor in dendritic and synaptic dysfunction in Alzheimer disease. A comparison between drebrin and cofilin in the same membrano-cytoskeletal samples of the human hippocampus revealed a reciprocal relationship between drebrin and cofilin: in the subjects with low drebrin levels, we saw high cofilin levels, and vice versa (**Fig. 2e**). Because 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)/cofilin[38]. In addition, cofilin induces conformational changes in actin that prevent other proteins from binding to actin[39]. To test this hypothesis directly, we pooled normal human hippocampal samples and split them into two groups, one with and one without 10 μg exogenous unphosphorylated cofilin. We then determined the drebrin-actin binding. Specifically, the samples were immunoprecipitated with an antibody to β-actin and then actin-bound drebrin was detected in the immunoprecipitated pellets that contained equivalent actin. Results of this *in vitro* assay indicated that with the addition of exogenous unphosphorylated cofilin, the drebrin-actin binding was reduced by over 50% ($P < 0.05$) (**Fig. 2f**). This was consistent with the hypothesis that dephosphorylated cofilin can prevent drebrin from binding to actin. Therefore, lower PAK activity in the hippocampi of individuals with Alzheimer disease, 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[17,20].

PAK defects in Tg2576 Alzheimer Model Mice

Neuronal degeneration in Alzheimer disease is related not only to Aβ aggregates but also to intraneuronal tau aggregates, neurofibrillary tangle formation, synuclein aggregates and/or Lewy body formation. This raised the following question: are the abnormalities in pPAK, cofilin and drebrin seen in Alzheimer disease causally related to Aβ pathologies? To address this question, we examined aged (22-month-old) *App*swe transgenic Tg2576 mice that had abundant amyloid pathology but lacked neuron loss and classical neurofibrillary pathology. In the cortex of aged Tg-negative (Tg^-) controls (**Fig. 3a**), we observed diffuse pPAK labeling of neurons, resembling that of neurons in the normal human brain in **Fig. 2**. However, in aged Tg-positive (Tg^+) mice, pPAK labeling was uneven: we observed 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 pPAK antibody to PAK1Thr423 confirmed diffuse neuron labeling in Tg^- controls (**Fig. 3c**). There was a reduction of this diffuse staining in Tg^+ Alzheimer model mice as well as intense plaque-associated labeling of dystrophic neurites (but not microglia) and clusters of neuron staining, which were frequently granular or flame shaped (similar to those in Alzheimer brains, **Fig. 2**, and to the

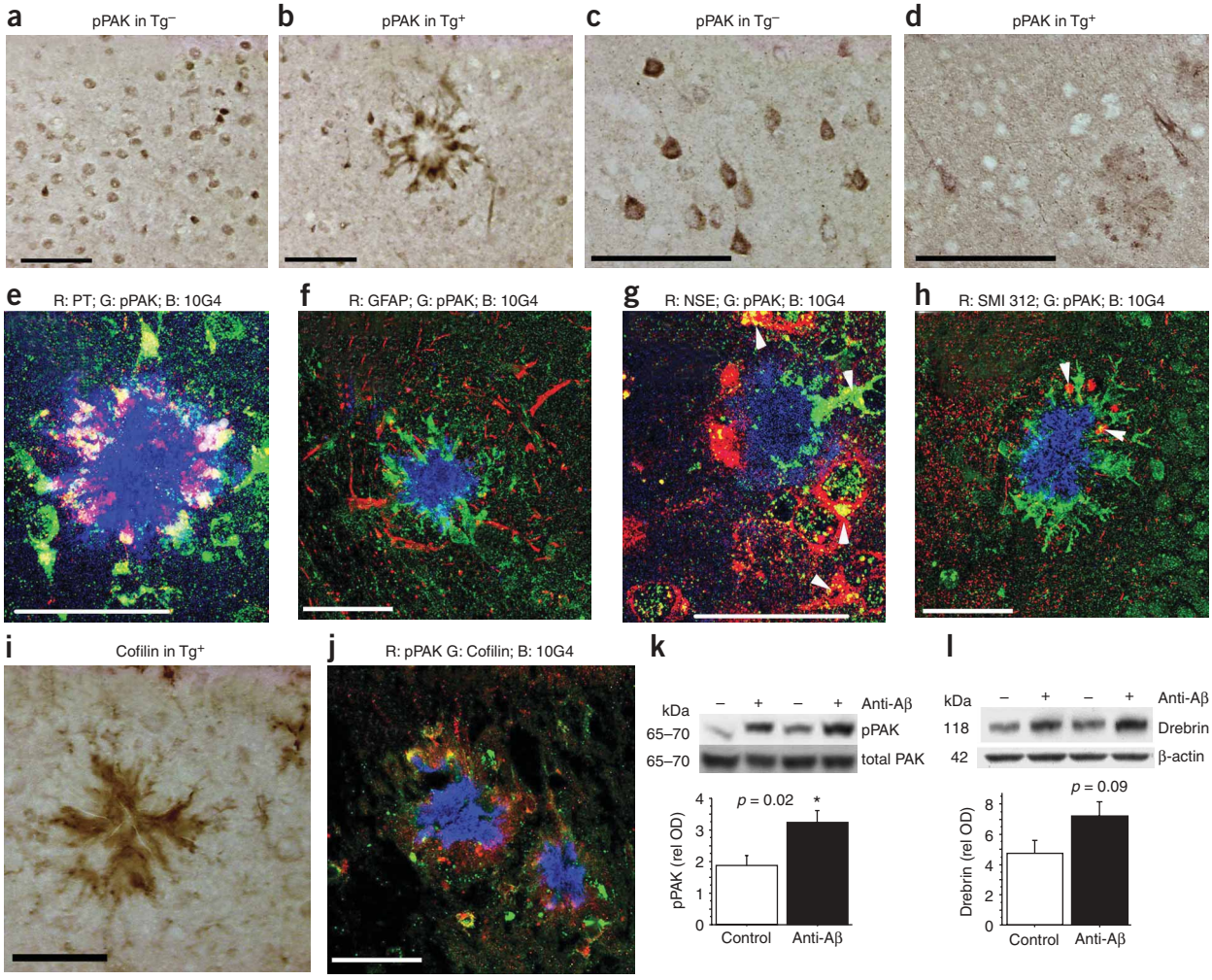

Figure 3 pPAK and cofilin pathologies in 22-month-old *App*swe Tg2576 mice. (**a**–**d**) Compared to Tg⁻ mice, Tg⁺ mice showed strong periplaque pPAK141 (**a** vs. **b**), pPAKThr423 (**c** vs. **d**) staining and less diffuse neuronal and nuclear labeling. However, Tg⁺ mice showed granular and intense neuron staining, often clustered or periplaque (**d**). (**e**–**h**) To assess the cellular pPAK-labeled elements (green), confocal triple labeling of amyloid (10G4, blue) was assessed with markers (red) using (**e**) phosphotyrosine (PT) for microglia, (**f**) GFAP for astrocytes, (**g**) NSE for neurons and (**h**) SMI312 antibody to phosphorylated neurofilaments for dystrophic neurites. Microglia, dystrophic neurites (arrows) and neurons (arrows), but not GFAP-positive astrocytes, showed elevated periplaque pPAK141. (**i**,**j**) 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 periplaque cofilin and punctate pPAK in and around amyloid plaques in aged Tg2576 mice. Scale bar, 100 μm. (**k**,**l**) 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 ($r^2 = 0.315$, $P < 0.05$).

plaque-associated Tg⁺, **Fig. 3d**). To further identify the nature of pPAK141 (pPAK) staining around plaques, we carried out confocal triple labeling of pPAK, amyloid plaques (with 10G4 antibody) and markers for plaque cellular components. We observed frequent colocalization with phosphotyrosine, a marker for plaque-associated microglia (ref. 30; **Fig. 3e**), but no colocalization 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 showing strong pPAK labeling around plaques was consistent with those of neurons, suggesting that some periplaque pPAK accumulation was neuronal in nature, as confirmed by its colocalization with NSE (**Fig. 3g**). Confocal images using SMI 312 antibody to label neurofilament in dystrophic neurites also indicated colocalization between pPAK and these neurites (**Fig. 3h**), consistent with pPAKThr423 in dystrophic neurites (**Fig. 3d**). Cofilin labeling in and around Aβ plaques was also observed in the Tg⁺ hippocampus (**Fig. 3i**). Triple labeling of pPAK, cofilin and amyloid plaques (with 10G4 antibody) in the Tg⁺ hippocampus indicated intense periplaque pPAK staining that was sometimes associated with local cofilin puncta (**Fig. 3j**). This was similar to that observed in the hippocampus of people with Alzheimer disease. Also, our group previously reported a large (62%) loss of drebrin in these amyloid-laden (but tangle-free) Tg⁺ mice[17]. Therefore, pPAK, cofilin pathology and severe drebrin loss are found both in individuals with Alzheimer disease and in aged, amyloid-laden, tangle-free *App*swe mice, suggesting that Aβ aggregates could account for these abnormalities.

Passive Aβ antibody treatment increases Tg2576 pPAK

To further examine the possible role of Aβ in PAK deficits, we reduced the amount of Aβ with an approach that has been consistently demonstrated to clear Aβ and reverse cognitive deficits in *App*swe Tg mice—passive immunization with an anti-Aβ antibody recognizing the

N-terminal amino acids 1–15 of Aβ41 and Aβ42[40,41]. The anti-Aβ antibody significantly increased the levels of pPAK in 11- to 12-month-old Tg2576 mice (**Fig. 3k**), and the abundance of soluble Aβ oligomer (~12-mer) was inversely correlated with that of pPAK ($r^2 = 0.443$, $P = 0.013$). In contrast, insoluble Aβ, extracted by guanidine and examined by sandwich enzyme-linked immunosorbent assay (ELISA), was not significantly reduced and was not correlated with pPAK ($r^2 = 0.064$, $P = 0.4$). Although the abundance of global drebrin, as assessed by western blotting, was not significantly reduced at 11–12 months, anti-Aβ infusion still produced a trend toward increased drebrin (**Fig. 3l**) that was significantly correlated with pPAK($r^2 = 0.315$, $P < 0.05$). Our results therefore suggest that Aβ is involved 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.

Aβ oligomers cause PAK signaling defects

To directly test the hypothesis that Aβ oligomers can cause PAK signaling defects, we incubated cultured primary hippocampal neurons with 100 ng ml^{-1} of soluble Aβ1–42 aggregates prepared with an Aβ oligomer–generating protocol[42]. Oligomer abundance is elevated in the brains of persons with Alzheimer disease[43], 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 such individuals[23] and in Aβ oligomer infusion models[44]. The Aβ1–42 oligomer (**Fig. 4a**) induced rapid and persistent reduction in PAK activities as indicated by the levels of phosphorylation of a PAK substrate, myelin basic protein (MBP) (**Fig. 4b**). Briefly, the protein samples were immunoprecipitated with a PAK antibody, and this was followed by reaction with exogenous unphosphorylated MBP and the detection of phosphorylated MBP (pMBP) on western blots (**Fig. 4b**). The loss of viability by lactate dehydrogenase (LDH) assay of media or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction was not observed until after 56 h of treatment (data not shown). Also, the deficits in PAK activity induced by Aβ were accompanied by rapid and persistent reductions in drebrin abundance, whereas the amounts of synaptophysin and total PAK were unchanged (**Fig. 4c**). Dose-response studies with 0 nM, 10 nM, 100 nM and 500 nM of Aβ42 oligomer preparations showed parallel dose-dependent loss of pPAK (**Fig. 4d**) and drebrin (**Fig. 4e**) after 2 h, with a trend beginning at 10 nM and highly significant losses at 100 nM, with relative preservation of actin. In support of an oligomer-induced effect, the parallel drebrin and pPAK deficits induced by the Aβ oligomer preparation (**Fig. 4d,e**) were prevented by pretreatment with the oligomer-specific A11 antibody that does not recognize contaminating fibrils or monomer[42]. Aβ treatment of hippocampal cultures resulted in the redistribution of pPAK in neurons, as suggested by the increased granular staining of these neurons resembling that in diseased hippocampal neurons (**Fig. 4f,g**).

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

Active PAK protects from Aβ-induced drebrin loss

Having shown that 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

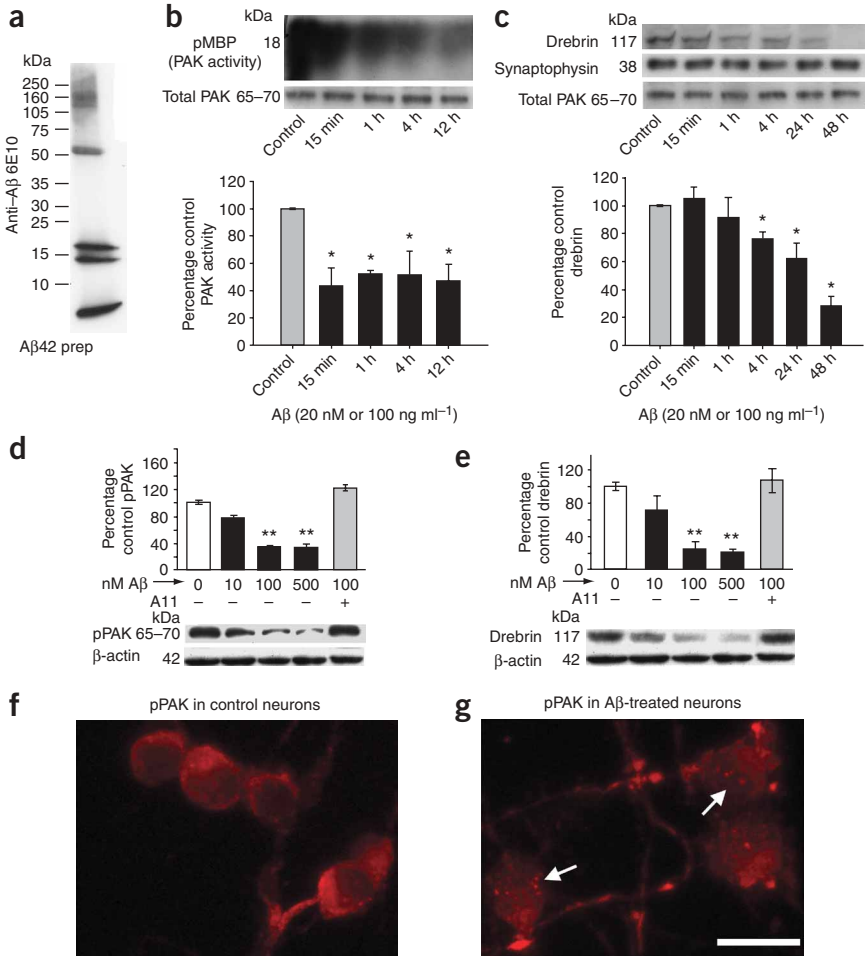

Figure 4 pPAK and drebrin losses were reproduced *in vitro* in soluble Aβ1–42 oligomer-treated hippocampal neurons. (**a**) Western blot detection of soluble Aβ1–42 oligomer with 6E10 antibody. Note ~56-kDa 12-mer. (**b**) PAK kinase assay with 100 ng ml^{-1} of Aβ1–42 oligomer–treated hippocampal neurons showed a rapid PAK activity decrease, whereas the total PAK amount remained unchanged. (**c**) The amount of drebrin was reduced in Aβ1–42 oligomer–treated hippocampal neurons in a time-dependent manner, occurring later than the PAK activity decrease, whereas the amount of presynaptic protein synaptophysin was unaltered. *$P < 0.05$ compared to control. (**d,e**) Dose response for Aβ oligomer preparation induced loss of (**d**) pPAK and (**e**) drebrin in hippocampal neurons harvested after 2 h of treatment and complete protection from 100-nM oligomers with the addition of oligomer-specific A11 antibody. (**f,g**) pPAK staining in control and Aβ-treated hippocampal cultures shows granular pPAK labeling in Aβ-treated neurons (arrows). Scale bar, 25 μm.

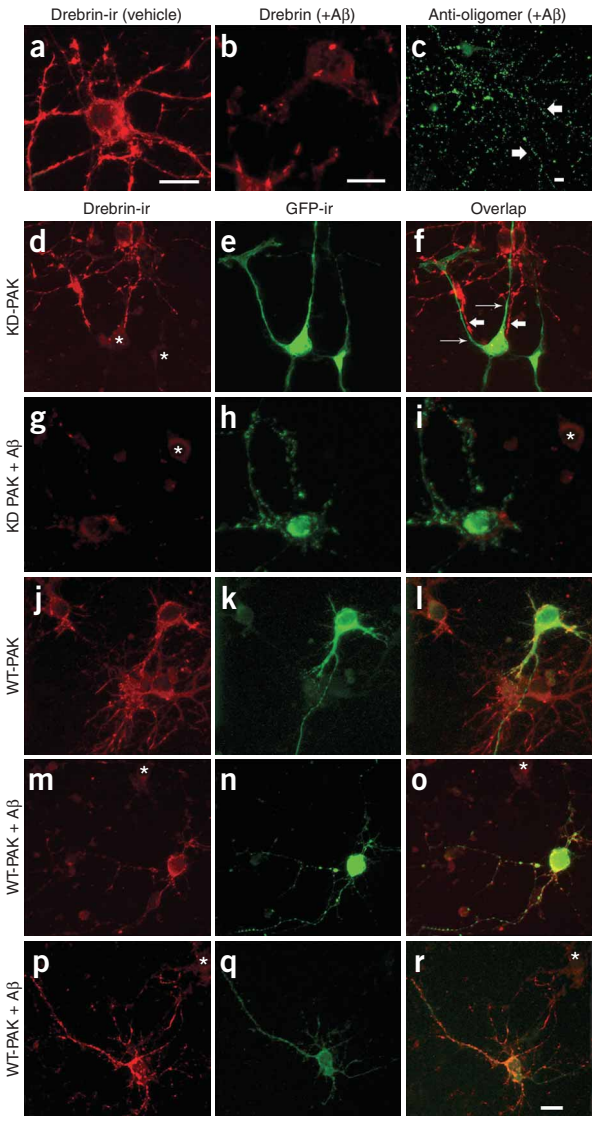

Figure 5 Wild-type (WT), but not kinase-dead (KD), PAK limits Aβ oligomer–induced drebrin loss. (**a**,**b**) Drebrin staining was robust in nontransfected neurons but reduced after oligomer treatment. (**c**) Aβ immunolabeling showed the punctate staining pattern of oligomers distributed along cell bodies and processes (arrows). (**d**–**r**) Primary HP neurons were transfected with kinase-dead *pcDNA3-EGFP-PAK1-K299A* (**d**–**i**) or wild-type *pcDNA3-EGFP-Pak1-WT* (**j**–**r**), treated with 250 nM of Aβ42 oligomer for 2 h (**c**,**g**–**l**,**m**–**r**) and stained for drebrin (red). Transfected cells expressed GFP fluorescence (**e**,**h**,**k**,**n**,**q**), which was merged with drebrin images for **f**,**i**,**l**,**o** and **r**. 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 processes of cells transfected with *pcDNA3-EGFP-PAK1-K299A* (thin arrows) showed little or no drebrin compared to adjacent processes (thick arrows) in nontransfected neighboring cells. Cells transfected with kinase-dead *pcDNA3-EGFP-PAK1-K299A* had drebrin loss similar to nontransfected neighboring neurons (asterisks) after Aβ42 oligomer treatment (**g**–**i**). In contrast, cells transfected with active *pcDNA3-EGFP-Pak1-WT* had drebrin staining (red) that was similar to that of their nontransfected neighbors (**j**–**l**). After Aβ oligomer treatment, drebrin staining in cells transfected with *pcDNA3-EGFP-Pak1-WT* was comparatively preserved relative to their nontransfected neighbors (**m**–**r**, asterisks). Panels **d**–**r**: same magnification. Scale bar, 25 μm.

labeled cell bodies and neurites in nontransfected neurons (**Fig. 5a**), but this labeling was reduced after Aβ oligomer treatment (**Fig. 5b**). Immunostaining with anti-Aβ (**Fig. 5c**) revealed a punctate staining pattern of the exogenous oligomers distributed along neuronal cell bodies and processes, similar to the synaptic pattern observed previously[23]. After the transfection of kinase-dead PAK1 tagged with green fluorescent protein (GFP), drebrin staining (**Fig. 5d**) was suppressed in the neurites and cell bodies of transfected cells labeled by GFP, in comparison with adjacent nontransfected cells (**Fig. 5e**,**f**). Moreover, kinase-dead PAK1 did not protect drebrin after Aβ42 oligomer treatment (**Fig. 5g–i**). Neurons transfected with active wild-type PAK1 (**Fig. 5j–r**) showed drebrin staining equivalent to that in nontransfected cells; however, after oligomer treatment, drebrin staining in cells transfected with wild-type PAK1 was clearly protected compared to that in nontransfected neighboring neurons. These results demonstrate that specifically increasing active (but not inactive) PAK1 can protect against Aβ oligomer–mediated drebrin loss.

PAK inhibition impacts cofilin, drebrin and memory

Large PAK deficits such as those seen with PAK3 mutations are sufficient to cause severe mental retardation[10,11]. 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 may have no direct relation to adult- or age-related cognitive deficits. Because Alzheimer disease is a disease with age-dependent cognitive deficits and the PAK signaling defects are not manifest until later in life, the question still remains whether, in adults, PAK downregulation is sufficient to cause the defects seen in Alzheimer disease, including drebrin loss, cofilin pathology and cognitive deficits. To address this question, we infused groups of stereotaxically cannulated 11-month-old retired breeder mice intracerebroventricularly (i.c.v.) with a PAK inhibitor, PAK18, or a mutant negative control peptide, PAK18-192A (R192A), at 0.18 nmol h^{-1}—a rate estimated to produce ~10 μM of peptides in the cerebrospinal fluid (**Fig. 6a**). Similar peptides have been previously used to block PAK activity[45]. The treatment of hippocampal neurons with these peptides showed that 10 μM of PAK18, but not the inactive peptide R192A, resulted in a significant ($P < 0.01$) reduction in pPAK levels and a loss of drebrin; this was accompanied by persistent punctate cofilin labeling, which resembled the cofilin rods induced by neurodegenerative stimuli *in vitro* (ref. 37; **Supplementary Fig. 2** online and **Supplementary Note**). This suggested that the peptides PAK18 and R192A were valid tools for inhibiting PAK and that inhibition of PAK activity was sufficient to drive both drebrin loss and cofilin pathology *in vitro*.

PAK18 infusion for 2 d in adult mice caused a large and significant reduction in pPAK in the hippocampus (reduced by 82% compared to vehicle alone, $P < 0.01$), whereas 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 1 month resulted in hippocampal CA1 cofilin pathology that often extended into the dendrites (**Fig. 6d**), resembling that found in diseased patients (**Fig. 6e**). Lower-power views showed that CA1 cofilin pathology in PAK18 inhibitor–infused animals (**Fig. 6f**) and fractin (caspase-cleaved actin, a marker for caspase activation; **Fig. 6g**) were similarly distributed, consistent with previous reports of the similar colocalization of caspase

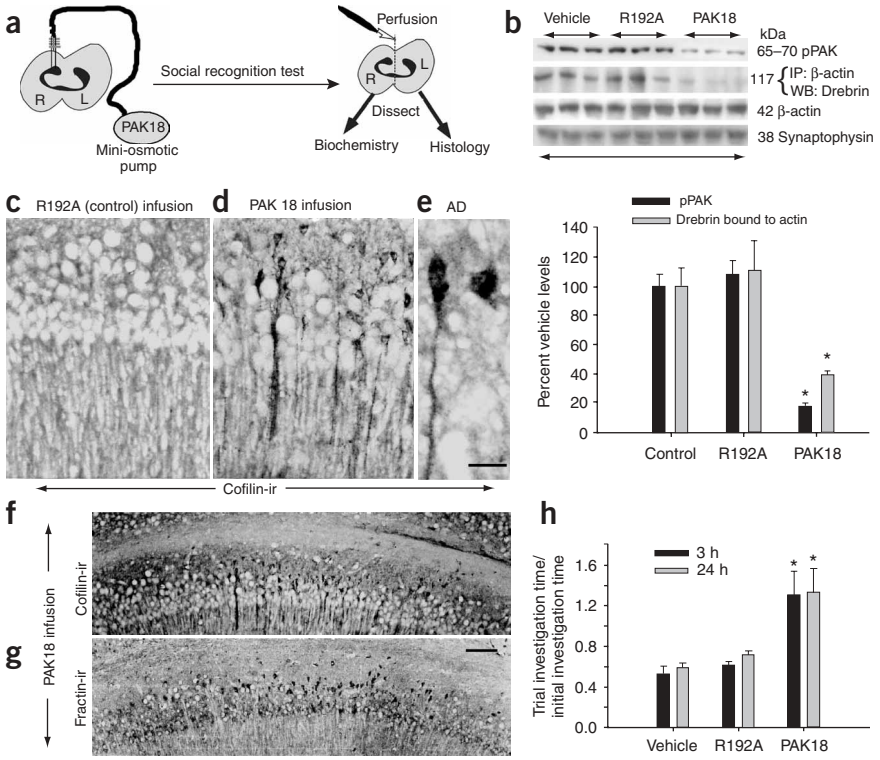

Figure 6 PAK inhibition causes drebrin and cofilin pathologies and behavioral deficits. 11-month-old adult mice were infused with vehicle, the PAK18 inhibitor or the control R192A peptide ($n = 10$ for each group). (**a**) A schematic of the procedure for i.c.v. infusion of PAK18 peptides and the social recognition behavior test, followed by perfusion and death. (**b**) PAK18 infusion i.c.v. for 2 d caused a massive reduction in the amounts of pPAK and actin-bound drebrin in the hippocampus, whereas the amounts of β-actin and synaptophysin were unchanged. (**c**–**e**) Hippocampus CA1 area of PAK18-infused, but not R192A-infused, normal mouse and Alzheimer disease model mouse had very similar cofilin labeling of Hirano body pathology, with cofilin often extending into the dendrites. (**f**,**g**) CA1 cofilin pathology and fractin labeling in PAK18-infused mice were distributed similarly, suggesting an apparent colocalization of these two pathological events. Vehicle- and R192A-infused mice and untreated mice did not show these changes (data not shown). Scale bar in **c**–**e**, 25 μm; in **f** and **g**, 100 μm. (**h**) In social recognition behavior testing after a 15-d infusion with PAK18 (but not control peptide), there were significant memory deficits at both 3 h (memory acquisition) and 24 h (memory consolidation), after initial mouse interaction. $^*P < 0.01$ compared to vehicle.

activation and Hirano pathology in human Alzheimer disease brain[46] and focal dendritic caspase activation associated with drebrin loss[17].

After the infusion of PAK18 for 15 d, we assessed hippocampal function using a hippocampus-dependent social recognition memory task[47]. The ratio of time a test mouse spent investigating a juvenile mouse it encountered 3 or 24 h earlier compared to the initial investigation time provided measures of memory acquisition and memory consolidation, respectively. PAK18 infusion caused a significant increase in the time the test mouse spent investigating the juvenile mouse after both the 3-h and 24-h intervals ($P < 0.01$), indicating that adult-onset memory deficits can be induced by PAK inhibition (**Fig. 6h**).

Overall, our results implicate PAK deficits in Alzheimer disease via several *in vivo* and *in vitro* models. These results support the idea that a pathway from PAK activity loss to cofilin pathology to drebrin loss and cognitive deficits is one common pathway for Alzheimer disease and some forms of mental retardation (**Supplementary Fig. 1** and **3** online and **Supplementary Note**). Our data from mice infused i.c.v. with PAK18 showed that, even in adults, PAK inhibition can directly cause cofilin pathology and drebrin loss similar to those found in Alzheimer disease, suggesting a direct causal relationship between PAK and these events. Notably, the pharmacological inhibition of PAK activity caused a significant inhibition of social recognition memory (3-h and 24-h delay tests), implying that PAK is critical to cognitive function. That PAK mutation can lead to mental retardation, together with the role of PAK in cognition, suggests that PAK defects could be critical in the cognitive defects seen in individuals with Alzheimer disease (**Supplementary Fig. 1** and **Supplementary Note**).

The social recognition memory deficits caused by pharmacological PAK inhibition are consistent with the memory deficits shown in a previous study using dominant-negative *Pak* (dn*Pak*) transgenic mice[12]. With an approximately 40% downregulation in PAK activity in the cortex and hippocampus of dn*Pak* mice, this study showed that PAK downregulation leads to selective spine defects and synaptic plasticity impairment in cortex that are associated with the impairment of memory consolidation, but with no observable synaptic defects in the hippocampus (because of the high basal hippocampal PAK levels). The >80% inhibition of PAK activity in our study (resulting from the continuous infusion of PAK18) was sufficient to cause drebrin loss and cofilin pathology in the hippocampus and social recognition memory impairment, which is strongly dependent on hippocampal function[47].

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 Alzheimer disease. We demonstrated that adult-onset PAK signaling defects led to cofilin pathology, major drebrin loss and memory deficits resembling those in Alzheimer disease, that Aβ oligomers implicated in Alzheimer disease caused PAK defects leading to drebrin loss and, further, that the expression of active PAK could protect against the Aβ oligomer. The prominent PAK pathway alterations seen in Alzheimer disease should therefore 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-PPVIAPAPEHTKSVYTRS) were custom-synthesized by Bioworld. The PAK18 peptide was synthesized with a specific PAK 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. We used the following antibodies: antibody to PAK1 (Zymed Laboratories), antibody to PAK3 (Stressgen Biotechnologies), antibody to total PAK (PAK1,2 and 3, Cell Signaling Technology), antibody to β-actin (Chemicon International), antibody to NSE (Research Diagnostics), antibody to pPAKserine 141 and antibody to oligomer A11 (Biosource International), antibody to PAKThr423 (Cell Signaling), antibody to cofilin (BD Transduction Labs), antibody to

drebrin (clone M2F6, MBL), antibody to synaptophysin (MAB368, Chemicon International), antibody to SMI312 (Sternberger Monoclonals) and antibody to pMBP (Upstate Biotechnology). Affinity purified antibody to fractin[48] and anti-Aβ (10G4; ref. 17) rabbit polyclonal antibodies were developed and characterized in our laboratory. Wortmannin 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 Alzheimer Disease Research Center Pathology Cores. Controls ($n = 15$) were compared to patients with moderate Alzheimer disease ($n = 15$). The age of death, gender and postmortem interval were comparable in both groups.

Aβ1–42 oligomer preparation. Soluble Aβ1–42 oligomers were prepared in accordance with the previously published method[42]. 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 previously[49].

Plasmids and transient transfection. GFP-tagged PAK1 constructs with wild-type (WT) *pcDNA3-EGFP- PAK1 WT* or kinase-dead (KD) *pcDNA3-EGFP- PAK1 K299R* were gifts from G.M. Bokoch (Scripps Research Institute, La Jolla, California). Primary hippocampal neurons (7-d-old) 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. We used 1.2 μg of expression plasmid and 2 μl of Lipofectamine 2000 for transfection per dish. After transient transfection for 20 h, we added 250 nM of Aβ oligomers directly to the cells and incubated them for 2 h at 37 °C. Then the cells were fixed with 95% methanol for 5 min at 4 °C and stained with drebrin or polyclonal rabbit antibody to Aβ1–13 (named 'DAE' for the first three amino acids of the immunogen).

Passive immunization. The passive immunization experiment was conducted with 11- to 12-month-old male and female Tg2576 Tg^+ mice as described in detail[50]. The mice were randomly divided into two groups ($n = 6$ for control group; $n = 7$ for Aβ-antibody treatment group). Control antibody and antibody to Aβ IgG2b were i.c.v. infused into mice. After 14 d, mice were deeply anesthetized and killed, and the brains were removed and brain regions were dissected. Biochemical measurements were performed on the cortical tissue. Oligomers were assayed by western blotting and ~12-mer quantified. We obtained similar results using 6E10, biotinylated 6E10 and 4G8, but only 6E10 data is shown. Guanidine–extracted, detergent-insoluble total Aβ was assayed as previously described[17].

PAK assay. We conducted the PAK assay in TBS soluble fractions according to the previously published method[45] and the instructions of the manufacturer (Upstate Biotechnology), from whom the ATP, MBP and 1× assay dilution buffer were purchased.

Surgery for i.c.v. infusion of PAK18 peptides. Custom-length, stainless-steel i.c.v. catheters (Plastics One) were implanted to the desired depth (dorsal and ventral: –2.0 mm from skull) at defined stereotaxic coordinates (medial and lateral: +1.0 mm, anterior and posterior: –0.5 mm) in 11-month-old male retired breeder B6D2F1 mice. The i.c.v. 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 h^{-1} PAK18 or PAK18-R192A (240 μg per 100 μl delivered at 0.25 μl h^{-1}) into a ventricular volume of 35 μl (140-fold dilution) with production of 18 μl h^{-1} of new cerebrospinal fluid.

Social recognition behavior. Social recognition experiments were conducted as previously reported[47]. The ratio of test trial inspection time (3 h or 24 h) to initial inspection time was used to measure social recognition memory.

Data analysis. Data are 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 optical density (OD) values. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Fisher's protected least squares difference (PLSD) *post-hoc* analyses.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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