A recent genome-wide association meta-analysis for Alzheimer’s disease (AD) identified 19 risk loci (in addition to APOE) in which the functional genes are unknown. Using Drosophila, we screened 296 constructs targeting orthologs of 54 candidate risk genes within these loci for their ability to modify Tau neurotoxicity by quantifying the size of >6000 eyes. Besides Drosophila Amph (ortholog of BIN1), which we previously implicated in Tau pathology, we identified p130CAS (CASS4), Eph (EPHA1), Fak (PTK2B) and Rab3-GEF (MAD2) as Tau toxicity modulators. Of these, the focal adhesion kinase Fak behaved as a strong Tau toxicity suppressor in both the eye and an independent focal adhesion-related wing blister assay. Accordingly, the human Tau and PTK2B proteins biochemically interacted in vitro and PTK2B co-localized with hyperphosphorylated and oligomeric Tau in progressive pathological stages in the brains of AD patients and transgenic Tau mice. These data indicate that PTK2B acts as an early marker and in vivo modulator of Tau toxicity.

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INTRODUCTION

Since 2009, the Alzheimer’s disease (AD) genetic field has benefited from the advent of genome-wide association studies (GWASs), which revealed numerous genetic risk factors of AD. The International Genomics of Alzheimer’s Project (IGAP) consortium was the latest attempt to improve our knowledge of the genetic landscape of AD by meta-analyzing the available GWAS for common late-onset AD. This study raised the number of genome-wide significant AD risk loci up to 19 in addition to APOE.1

However, while GWASs and IGAP in particular represent major breakthroughs in our understanding of the genetic risk underlying late-onset AD, it is difficult to identify the functional genes and variants within these loci and to understand how they mechanistically contribute to AD pathogenesis. These points are important challenges of the post-GWAS era, and strong efforts are needed to decipher the link between AD genetics and pathogenesis. To date, several studies have indicated that some of these genes, for example, SORL1 or ABCA7, might be involved in amyloid precursor protein metabolism, Aβ peptide production or clearance,2–4 implying that the amyloid cascade hypothesis could be relevant not only in monogenic forms of AD.5 However, beyond the central role of amyloid precursor protein metabolism and its catabolites, we also recently described BIN1 as the first genetic risk factor modulating Tau pathology.6 This implies that pathological pathways directly involving Tau might also be responsible for the development of AD at the early stage. Interestingly, this latter point was reinforced by a recent report from IGAP describing a novel risk locus located near the gene encoding the Tau protein (MAPT) in non-APOE-e4 carriers.7 Within this background, one can argue that, in addition to BIN1, some of the recently discovered genetic risk factors of AD might exert their pathogenic effect by modulating toxicity of the Tau protein and/or neurofibrillary tangle (NFT) pathology.

For our study on BIN1,6 we took advantage of the Drosophila model, which is a small model organism that displays easily scorable and AD-relevant readouts for high-throughput genetic modifier screens.8 In Drosophila, expression of the human MAPT gene in the eye results in small rough eyes associated with vacuolar neurodegeneration without NFT formation.9 It thus constitutes a genetically sensitized system of early-stage pre-NFT AD that allows the identification of genetic modifiers by assessing roughening and size of the eye as readouts of Tau neurotoxicity. In the present study, we decided to screen genes within AD risk loci in a systematic manner for their ability to modify Tau toxicity in flies.

MATERIALS AND METHODS

Drosophila genetics and imaging

Files were raised at 25 °C on standard fly medium (Nutri-fly BF, Genesee Scientific, San Diego, CA, USA). We used the same GMR > Tau (2N4R) line as previously described.5 RNA interference (RNAi) stocks were obtained from the National Institute of Genetics Fly Stock Center (NIG collection, Kyoto, Japan), the Vienna Drosophila RNAi Center (VDRC, GD and KK collections, Vienna, Austria) and the Harvard Transgenic RNAi project (TRIP, attP2 and attP40 collections). Additional loss-of-function mutant lines and gain-of-function lines were obtained from the Bloomington Drosophila Stock Center (BDSC, Bloomington, IN, USA) and the fly research community.
Immunohistofluorescence of human and mouse brain tissue
Four parallel, fixed brain tissue samples were obtained from hippocampus and frontal cortices of different age-matched, pathologically confirmed AD patients. Brain stages II–VI (Supplementary Table S4). Human brain samples were obtained from the Lille Neurobank, which was given to the French Research Ministry by the Lille Regional Hospital (CHRU-Lille) on 14 August 2008 under the reference DC-2000-642. The Lille Neurobank fulfills criteria from the French Law on biological resources, including informed consent, ethics review committee and data protection (article L1243-4 of the Code de la Santé publique, August 2007). THY-Tau22 transgenic mice (five animals per age) were killed by decapitation and the brains were rapidly dissected and cryopreserved in PBS supplemented with antioxidant (Life Technologies) and analyzed by Western blotting analysis.

RESULTS

Selection of Drosophila orthologs of candidate AD risk genes
We selected all annotated genes within the 19 IGAP genomic risk regions (with the exception of the APOE locus) as defined by IGAP regional association plots assuming that the functional risk variants are located in the vicinity of the single-nucleotide polymorphism (SNP) producing the top signal and taking into account the linkage disequilibrium patterns within the loci of interest (Supplementary Table S1). We identified 148 human genes within these intervals and determined their corresponding Drosophila orthologs with the Drosophila RNAi Screening Center Integrative Ortholog Prediction Tool (DIOPIT; http://www.flynai.org/diopit).11 We used a DIOPIT cutoff score of $\geq 3$ to select high confidence homologs.11 We found that 54 of the 148 human genes had at least one Drosophila ortholog. From the 19 loci, 6 were excluded for further analysis (31%), because they did not contain any fly orthologs in the genomic region of interest (CR1, CLU, HLA-DRA, MS4A6A, NMB2, SOLR1). Conversely, some human genes had several Drosophila orthologs and we retained 74 Drosophila orthologs corresponding to 54 human genes in the 13 remaining loci (Supplementary Figure S1; Supplementary Table S1). For these 74 orthologs, we obtained 274 corresponding RNAi lines, that is, around 4 RNAi lines per gene of interest, from 5 publicly available collections (Supplementary Figure S1; Supplementary Table S2).

Genetic screen of candidate AD risk genes identifies BIN1 and four novel modifiers of Tau toxicity
To test whether these Drosophila orthologs could modify Tau neurotoxicity, RNAi lines were crossbred with flies expressing the longest isoform of human Tau (2N4R) in the eye (GMR driver). In this model, expression of Tau causes neuronal degeneration without NFT formation and results in smaller rough eyes.12,13 We determined whether RNAi-mediated knockdown of the genes of interest were able to modify this Tau-induced phenotype by quantifying the eye surface (Figure 1a). We discarded all RNAi lines about the protocol are available in Supplementary Information. Proteins were detected by immunoblot using PTK2B antibody (P3209, Sigma-Aldrich) and chemiluminescence.

Western blotting analysis

Drosophila heads were dissected and crushed in LDS sample buffer supplemented with antioxidant (Life Technologies) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4–12% gel NuPAGE Novex, Life Technologies). Proteins were detected by immunoblot using AT8 (phosphoSer202-phosphoThr205 Tau, MN1020 Thermo-Pierce), phosphoThr18 Tau (MM-0194-100, Medimabs, Montréal, Québec, Canada), R22 (phosphoSer231, Dr P Davies), PHF1 (phosphoSer396 phosphoSer404, Dr P Davies), Tau1 (dephosphorylated aa197-205, MAB3420, Millipore), AT270 (phosphoThr181, MN1050, Thermo-Pierce), p5422 (phosphoSer422, 44764G, Life Technologies) and actin (A2066, Sigma-Aldrich) antibodies and chemiluminescence.

Figure 1. Functional screen of AD susceptibility loci in Drosophila. (a) Quantification of the eye size of flies co-expressing Tau and AD candidate gene loss/-gain-of-function constructs. Each symbol in the graph represents the median of the measurement of the size of 10 fly eyes per construct and control (30 values for the 3 pooled controls). Blue circles, squares and triangles represent RNAi, mutant and overexpression constructs, respectively ($\bigtriangledown$, $\square$, $\triangle$), and black circles represent controls ($\bigcirc$). For RNAi, we plotted values normalized according to their origin (see Materials and Methods section). When the size of the eye was statistically different from the control (Wilcoxon test, Bonferroni correction, for RNAi with and without correction), we used red color ($\bigtriangledown$, $\square$, $\triangle$). Positive hits were re-tested and plotted with filled symbols ($\bullet$, $\blacklozenge$, $\blacktriangle$, $\bigstar$, $\bullet$). The two horizontal lines mark the eye size range of the Tau expressing control. Constructs that are above the controls are Tau toxicity suppressors and the ones that are lower are enhancers. Vertical dashed and solid lines separate Drosophila genes and loci. The name of the Drosophila gene targeted by the construct is indicated below the two graphs, as well as the name of their human orthologs and the name of the locus they belong to. Positive hits are shaded in gray. (b) Representative images of the positive hits (scale bar 0.1 mm). Images for the Fak gene are available in Figure 2.
that induced an external eye phenotype on their own when expressed with the GMR driver. Heterozygous genomic loss-of-function alleles and overexpression constructs were also tested when available. Genes were considered positive hits when at least two lines (RNAi, mutant alleles or overexpressing constructs) from different collections modified Tau toxicity (Figure 1a; Figure 1. For caption please refer page 875.)
PTK2B ortholog Fak suppresses Tau toxicity in the eyes and wings

We focused on the PTK2B ortholog Fak, as it behaved as a consistent suppressor of Tau toxicity in our screen suggesting that Fak might be a specific downstream mediator of Tau toxicity (Figures 2a and b). We confirmed the Tau–Fak genetic interaction in the eye with an independent 0N4R Tau construct, the Fak<sup>GT1</sup> null<sup>14</sup> and the Fak<sup>GT2</sup> hypomorphic<sup>15</sup> alleles in heterozygous, homozygous and transheterozygous states. The transheterozygous genotype is important to rule out effects of possible second-site mutations on the chromosome. The 0N4R Tau construct allows assessing the requirement of the 2 N alternative domains of Tau for the interaction. We also observed a Fak gene dose-dependent suppression of Tau toxicity, confirming the Tau–Fak interaction without requirement of the 2 N domains of Tau (Figures 2c and d). Next we wanted to confirm the interaction between Fak and Tau in an independent assay and chose the more specific wing blister phenotype, which links Fak to its established function in the focal adhesion pathway downstream of integrins.<sup>16</sup> Wing blisters are the result of cell adhesion defects between the dorsal and ventral blade of the wing. As reported previously,<sup>16</sup> overexpression of Fak results in wing blisters in 20% of the flies (Supplementary Figure S3). We tested the expression of Tau (2N4R) in the posterior compartment of the wing using the engrailed driver. Overall, 62% and 11% of the flies exhibited blisters in one or their two wings, respectively, in addition to a reduced wing size (Figures 2e and f). When co-expressed with Fak RNAi, the proportion of flies with wing blisters in one or two wings significantly decreased to 53% and 2%, respectively. With this readout, we also wanted to confirm the interaction with an independent Tau construct and Fak loss-of-function mutations. We used the Tau construct expressing the 0N4R Tau isoform. Expression of this construct induced a strong reduction of the wing size with some of them exhibiting blisters (Supplementary Figure S4). In Fak<sup>KG+/–</sup> and Fak<sup>KG+</sup> loss-of-function backgrounds, the size of the posterior compartment of the wing was partially restored but we were unable to quantify the blister frequency owing to the greatly reduced wing size (Supplementary Figure S4). To circumvent this problem, we tested the phosphodeficient Tau<sup>AP</sup> (0N4R) construct, in which 14 Serine or Threonine phosphorylation sites are mutated into alanine and which is known to be less toxic in the eye.<sup>17</sup> Expression of Tau<sup>AP</sup> was also less toxic in the wing and resulted in a mild reduction of the wing size, with 80% of the flies exhibiting typical blisters in their two wings (Figures 2g and h). In Fak<sup>GT1/–</sup> and Fak<sup>GT2</sup> backgrounds, the proportion of flies with blisters in their two wings was significantly reduced to 31% and 62%, respectively. Together, using three different Fak and three different Tau lines, these results indicated that Fak and Tau genetically interact in an independent readout related to focal adhesion.

PTK2B and Tau interact biochemically in vitro

We next tested whether PTK2B and Tau are able to physically interact. We performed GST pull down experiments (Figures 3a and b). We were able to pull down PTK2B from PTK2B-transfected HEK293 cell extracts with GST-Tau<sup>1N4R</sup> and GST-Tau<sup>2N4R</sup> constructs (Figure 3a). Reciprocally, we were able to pull down 1N4R and 2N4R Tau isoforms from transfected HEK293 cell extracts with a GST-PTK2B construct (Figure 3b). These results indicate that PTK2B and Tau interact directly or indirectly in a complex.

As PTK2B is a protein tyrosine kinase, we tested whether PTK2B could phosphorylate Tau directly or indirectly through the activation of other kinases. 2N4R Tau isoform has five tyrosine residues (Tyr18, Tyr29, Tyr197, Tyr310, Tyr394) but antibodies have been developed only for Tyr18. In Drosophila, we assessed the effect of the modulation of Fak on the phosphorylation of Tau Tyr18 and on the main AD phosphorylation sites (Figure 3c).

### Table 1. Summary of the functional screen results of the AD risk loci in Drosophila

| IGAP locus | Human genes (with fly orthologs) | Fly genes | Tau-toxicity modifiers (human ortholog) |
|------------|---------------------------------|-----------|----------------------------------------|
| CD2AP      | 3 (1)                           | 1         |                                        |
| BIN1       | 1 (1)                           | 1         | AmpH (BIN1)                             |
| MEF2C      | 4 (1)                           | 1         |                                        |
| PICALM     | 2 (1)                           | 1         |                                        |
| INPP5D     | 3 (1)                           | 1         |                                        |
| SLC24A4    | 2 (2)                           | 1         |                                        |
| CASS4      | 6 (2)                           | 2         | p130CAS (CASS4)                        |
| FERM2      | 6 (4)                           | 6         |                                        |
| EPHA1      | 10 (4)                          | 4         | Eph (EPHA1)                            |
| PTK2B      | 5 (3)                           | 7         | Fak (PTK2B)                            |
| ABCA7      | 12 (6)                          | 7         |                                        |
| CELF1      | 23 (13)                         | 18        | Rab3-GEF (MADD)                        |
| ZCWPW1     | 46 (15)                         | 23        |                                        |
| Total      | 123 (54)                        | 74        |                                        |

Abbreviations: AD, Alzheimer’s disease; IGAP, International Genomics of Alzheimer’s Project.

Figure 2. Genetic interaction between Fak/PTK2B and Tau in the eye and wing of Drosophila. (a) Images of fly eyes expressing the 2N4R Tau isoform (GMR > Tau) in five different Fak conditions (scale bar 0.1 mm). The GMR > images are the five different Fak conditions without Tau expression as control. (b) Quantification of the eye size of the progeny of Tau-expressing flies crossed with the different lines targeting Fak (*P < 1.68 × 10<sup>−6</sup>).* (c) Images of fly eyes expressing the 0N4R Tau isoform or the control mCherryNLS construct in the background expressing decreasing amount of Fak, from wild-type expression of Fak to no expression of Fak in Fak<sup>1N4R</sup> flies (scale bar 0.1 mm). (d) Corresponding quantification of the eye size. (e) Wings co-expressing Tau (2N4R) or GFP and Fak<sup>RNAi7957</sup> or GFP with the engrailed driver. The dashed line in the top left panel marks the border between the anterior and posterior compartment, the driver being expressed in the latter (scale bar 0.5 mm). Arrows label wing blisters (yellow shaded). (f) Quantification of the wing phenotype in flies co-expressing Tau or GFP and Fak<sup>RNAi7957</sup> or GFP. The total numbers of flies over three experiments are indicated above the column. (g) Wings expressing GFP and Tau<sup>AP</sup> (ON4R) with the engrailed driver in wild-type and Fak<sup>1N4R</sup> backgrounds. Arrows label wing blisters (yellow shaded) (scale bar 0.5 mm). (h) Quantification of the wing phenotype in flies expressing GFP and Tau<sup>AP</sup> in wild-type and Fak<sup>1N4R</sup> backgrounds. The total numbers of flies over three experiments is indicated above the column.

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Loss and gain of Fak did not change the phosphorylation of these sites. Of note, this is in agreement with the suppressive effect of Fak on the phosphodefficient TauAP-induced phenotypes in the wing (Figures 2g and h). As we cannot exclude that Fak modulates other tyrosine or non-tyrosine Tau phosphorylation sites directly or indirectly, we tested whether the kinase activity of Fak is required for the suppression of Tau toxicity. We tested a FakY430F mutant in which the Tyr430 autophosphorylation site is mutated...
into the non-phosphorylable Phe residue, a mutant that has been used as a mutant of Fak kinase activity.\textsuperscript{15,18,19} We observed that FakY430F also suppressed Tau toxicity (Figure 3d). This suggested that the suppressive effect of Fak on Tau toxicity is independent of its kinase activity.

Neuronal cell bodies harboring hyperphosphorylated and oligomeric Tau accumulate PTK2B in the brains of AD patients and transgenic Tau mice.

To further validate a potential link between PTK2B and Tau pathology, we assessed whether the mRNA levels of PTK2B in the

Figure 3. For caption please refer page 880.
temporal AD cortex were altered in relation to the progression of neurofibrillary pathology (Braak staging). No statistically significant differences between different Braak groups were observed (Supplementary Figures S5a and b). Next we co-labeled Tau and PTK2B in the human AD brains (Figure 4a). Already in early-stage AD brains (Braak stage II), PTK2B staining was more abundant and overlapped strongly with AT8 indicating that PTK2B co-accumulates with NFTs (Figure 4a; Supplementary Table S4). To determine whether PTK2B accumulation occurs early during Tau pathology, we performed a co-staining for PTK2B and Tau oligomers detected with the TOC1 antibody. We observed a colocalization between PTK2B and TOC1 staining, indicating that PTK2B accumulation is an early event in AD pathogenesis (Figure 4b). Accumulation of phosphorylated PTK2B has been observed in the brains of the Tau transgenic pR5 mouse model. Using our THY-Tau22 mouse model showing hippocampal NFTs starting at 2-4 months, we assessed the neuronal presence of PTK2B at 2, 5 and 13 months to establish whether PTK2B and Tau accumulate in the same neurons and whether this is an early or late event. Accumulation of PTK2B started at 2 months and progressively co-accumulated within the cytoplasm of a growing number of AT8-positive degenerating neurons (Figure 4c). These data suggest that the accumulation of PTK2B represents an early pathological marker tightly coinciding with progressive Tau pathology in AD.

DISCUSSION
In the postgenomic era, the identification of the causal genes and their pathogenic function within GWAS-defined genomic risk intervals for common diseases constitutes a major challenge. Indeed, most signals discovered by GWASs do not clearly define the responsible causal genes but typically implicate a broad genomic interval for common disease susceptibility that constitutes a major challenge. Indeed, most signals discovered by GWASs do not clearly define the responsible causal genes but typically implicate a broad genomic interval for common disease susceptibility. Among these, we observed strong interactions between Tau and PTK2B with progressive Tau pathology in AD. PTK2B represents an early pathological marker tightly coinciding with AT8 indicating that PTK2B co-accumulates with NFTs (Figure 4a). Already in early-stage AD brains (Braak stage II), PTK2B was clearly detectable in Tau-negative neurons (Figure 4c). These data suggest that the accumulation of PTK2B represents an early pathological marker tightly coinciding with progressive Tau pathology in AD.

Figure 3. In vitro biochemical interaction between PTK2B and Tau. (a) Pull down of PTK2B with GST, GST-TauN14R and GST-Tau2N4R protein constructs. Upper panel: detection of PTK2B in the pull down extract by western blotting. Lower panel: corresponding Coomassie blue gel used as loading control of GST constructs. (b) Pull down of TauN14R and Tau2N4R with GST and control GST-PTK2B protein constructs. Upper panel: detection of Tau (arrows) in the pull down extract by western blotting (an unspecific band is labeled with a star, MW, molecular weight). Lower panel: corresponding Coomassie blue gel used as loading control of GST constructs. As the GST-PTK2B constructs were difficult to produce and visualize with Coomassie blue staining (arrows), we checked the GST-PTK2B construct through detection of PTK2B by western blotting (middle panel). All results shown in panels (a and b) are representative of three independent experiments. (c) Western blotting analysis of Tau phosphorylation in different Fak conditions in the Drosophila eye. Actin is used as a loading control (n=2–4). (d) Images and quantification of fly eyes co-expressing the 2N4R Tau isoform and a mC8:GFP construct used as control or wild-type Fak or mutant Fak(N397F) (scale bar 0.1 mm).
the human Tau, especially the 4R domain, and PTK2B proteins. Finally, PTK2B co-localized with hyperphosphorylated and oligomeric Tau in progressive pathological stages in the brains of AD patients and transgenic Tau mice. Altogether, these data strongly support PTK2B as a genetic risk factor of AD likely involved in the pathophysiological processes implicating Tau. In line with this hypothesis, the time course analysis of Tau pathology in the THY-Tau22 mouse model indicated that the accumulation of PTK2B occurred simultaneously with the appearance of the first AT8-positive labeling in neurons, and in the brain of AD patients, the accumulation of PTK2B occurred in neurons exhibiting Tau oligomers. This implies PTK2B as an actor of very early events in

Figure 4. PTK2B colocalizes with neurofibrillary degeneration in the brains of AD patient and Tau transgenic mouse. Co-labeling of PTK2B with phospho-Tau (a) and PTK2B with oligomeric Tau (b) in the hippocampus of AD patients at Braak stages II and VI (scale bar 50 μm). (c) Co-labeling of PTK2B with phospho-Tau in the hippocampus CA1 region of 2-, 5- and 13-month-old THY-Tau22 transgenic mice (scale bar 50 μm). AD, Alzheimer’s disease.
Tau pathology. Of note, a redistribution of activated PTK2B to the perinuclear region of pyramidal neurons has been described in chronically stressed rats and is associated with retraction of dendritic arbors. However, whether the accumulation of PTK2B in the soma of degenerating neurons is a cause or a consequence of Tau pathology remains to be explored.

Although the exact mechanism remains uncertain, it is interesting to note that three of our positive hits Fak/PTK2B, p130CAS/CASS4 and Eph/EPHA1 have previously been implicated in the focal adhesion pathway. PTK2B and CASS4, respectively, belong to the FAK and CAS families of proteins, which are known to directly interact in the cell adhesion pathway. PTK2B has been reported to physically interact with members of the CAS family downstream of integrins. The EPH receptor family is less directly connected. The activation of EPHA4, a paralog of EPHA1, inhibits β1 integrin downstream signaling pathways, including BCA1 (a paralog of CASS4) and PTK2B, and regulates dendritic spine morphology in hippocampal pyramidal neurons. Ephrin-A1 ligand induces carcinoma cell retraction through EPHA2, a paralog of EPHA1, and downstream SRC and FAK, a paralog of PTK2B. Hence, our results suggest that a cell adhesion pathway based on PTK2B and CASS4, and possibly regulated by EPHA1, could be involved in Tau pathology and AD pathogenesis (Supplementary Figure S6). Accordingly, Drosophila screening of pre-IGAP AD risk genes also identified genes related to the focal adhesion pathway as modulators of Tau toxicity. Of note, the cell adhesion pathway has already been involved in AD as a potent link between β- and Tau pathogenesis. In conclusion, our work highlights the role of PTK2B as a major actor in AD pathogenesis at the level of Tau toxicity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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