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LPS-Induced Inflammation Abolishes the Effect of DYRK1A on IkB Stability in the Brain of Mice

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Abstract

Down syndrome is characterized by premature aging and dementia with neurological features that mimic those found in Alzheimer's disease. This pathology in Down syndrome could be related to inflammation, which plays a role in other neurodegenerative diseases. We previously found a link between the NFkB pathway, long considered a prototypical proinflammatory signaling pathway, and the dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A). DYRK1A is associated with early onset of Alzheimer's disease in Down syndrome patients. Here, we sought to determine the role of DYRK1A on regulation of the NFkB pathway in the mouse brain. We found that over-expression of *Dyrk1A* (on a C57BL/6J background) stabilizes IκBα protein levels by inhibition of calpain activity and increases cytoplasmic p65 sequestration in the mouse brain. In contrast, *Dyrk1A*-deficient mice (on a CD1 background) have decreased IκBα protein levels with an increased calpain activity and decreased cytoplasmic p65 sequestration in the brain. Taken together, our results demonstrate a role of DYRK1A in regulation of the NFkB pathway. However, decreased IκBα and DYRK1A protein levels associated with an increased calpain activity were found in the brains of mice over-expressing *Dyrk1A* after lipopolysaccharide treatment. Although inflammation induced by lipopolysaccharide treatment has a positive effect on calpastatin and a negative effect on DYRK1A protein level, a positive effect on microglial activation is maintained in the brains of mice over-expressing *Dyrk1A*.

Keywords Down syndrome · DYRK1A · NFkB pathway · IkB · Calpain activity · Mouse brain

Introduction

Immune activation in the central nervous system (CNS) is implicated in the pathological mechanisms of neurodegenerative disorders. Glial cells mediate the innate immune response in the CNS, and gliosis accompanies neurodegeneration, which suggests that inflammation is involved. Microglia-mediated neuroinflammation is critical for induction of neurodegeneration

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[1]. Microglia, as resident immune effector cells of the CNS, have the principal function of managing brain homeostasis. They have been proposed to play a pivotal role in the immune surveillance of the CNS [2]. When activated, astrocytes and microglia produce proinflammatory signal molecules. Activation of inflammatory pathways early in neurodegeneration or even preceding the lesions and symptoms might have a causative role, while later activation might accelerate the disease process.

After activation, microglia undergo phenotypic changes including increased or de novo expression of transcription factors like nuclear factor-kappa B (NFkB), which regulates inflammatory processes [3–5]. NFkB (p65/p50) has diverse functions in the CNS depending on the cellular context and is constitutively activated. NFkB is detected mostly in glutamatergic neurons, whereas in glia, NFkB has a lower basal activity, is inducible, and regulates inflammatory processes that exacerbate neurodegenerative diseases [6]. Pro-inflammatory stimuli, such as pathogen-derived lipopolysaccharide (LPS), are strong inducers of NFkB activity in many cell types [7]. Activation of the NFκB transcription factor is associated with nuclear translocation of the p65 component of the complex. The activation mechanism in response to inflammatory stimuli involves the signal induced proteolysis of NFkB-bound IkB proteins [7]. Signal-induced phosphorylation of specific serines in IkB proteins by the IκBα kinase complex (IKK) is a necessary step in IkB proteolysis. Phosphorylated IKKβ consequently phosphorylates IκBα at Ser 32/36, leading to its poly-ubiquitination and proteasomal degradation. Enhanced activity of IKKβ may be caused by inhibition of phosphatase PP2A, a potential negative regulator of IKKβ [8]. IkB can also be degraded by calpain activation [9]. Calpains are calcium-dependent cysteine proteases that are able to degrade IkBα independently of its phosphorylation state [10, 11]. Extensive research revealed the deregulation of calpain activity as a key event in neurodegenerative disorders [12]. LPS also increases calpain activity in microglia [13].

Genome-wide transcription profiles of lymphoblastoid cell lines (LCLs) from individuals with Down syndrome (DS) and euploid individuals showed that NFkB transcription levels are impaired in DS due to reduced IkBα ubiquitination, increased IkBα, and reduced p65 nuclear fraction [14]. DS is characterized by premature aging and dementia with neurological features that mimic those found in Alzheimer's disease (AD). Early onset of AD in individuals with DS has been linked to DYRK1A, the dual-specificity tyrosine phosphorylation-regulated kinase 1A that is found in three copies in this population [15]. Moreover, DYRK1A appears to be involved in neurodegenerative diseases including AD, Parkinson's disease (PD) and Huntington's disease (HD) [16]. We previously found decreased expression of heme oxygenase 1 (*HO-1*) and inhibitor of apoptosis protein 2 (*cIAP2*), two NFkB-regulated genes, in the liver of mice over-expressing *Dyrk1A* [17]. Our objective was therefore to determine the molecular and cellular mechanisms linking DYRK1A and the NFkB pathway in the mouse brain under physiological conditions and after acute inflammation linked to LPS injection.

Materials and Methods

Experimental Mice

All procedures were carried out in accordance with the ethical standards of French and European regulations (European Communities Council Directive, 86/609/EEC). Official authorization from the French Ministry of Agriculture was granted to perform research and experiments on animals (authorization number 75–369), and the experimental protocol was approved by the institutional animal care and use committee of the Paris Diderot University (CEEA40). Mice were housed in a controlled environment with unlimited access to food and water on a 12-h light/dark cycle. The number of mice used and suffering were minimized as much as possible. The murine bacterial artificial chromosome 189N3 (mBACtgDyrk1A) strain was previously constructed by electroporating HM-1 embryonic stem cells with the retrofitted BAC-189N3 [18]. Mice carrying the murine BAC containing one copy of *Dyrk1A* (TgDyrk1A) were maintained on a C57Bl/6J background and genotyped as described [18]. Dp(16)1Yey mice were maintained on a C57Bl/6J background and genotyped as described [19]. The generation of *Dyrk1A* +/− mice has also been previously described [20], and mice were maintained on a CD1 background and genotyped as described [20]. Male mice from the same litter, 2 and 5 months of age, were used.

LPS Treatment

Mice were injected intraperitoneally with 2 mg/kg of LPS (Sigma) dissolved in 0.9% NaCl for 24 h before analysis. Control mice were injected with 0.9% NaCl.

Preparation of Serum Samples, Tissue Collection, and Plasma Assays

Mice were anesthetized, and blood samples were obtained by retro-orbital sinus sampling with heparinized capillaries, collected into tubes containing a one tenth volume of 3.8% sodium citrate, and immediately placed on ice. Plasma was isolated by centrifugation at 2500x*g* for 15 min at 4 °C. Mice were killed by cervical dislocation and brains were harvested, snap-frozen, and stored at − 80 °C until use. Plasma total homocysteine (hcy), defined as the total concentration of hcy after quantitative reductive cleavage of all disulfide bonds, was assayed using the fluorimetric high-performance liquid chromatography (HPLC) method as previously described [21].

Cell Primary Cultures

Mouse pups, 1 to 3 days old, were killed by decapitation, and cells were obtained by dissociation and cultured in minimum essential medium (MEM), supplemented with 10% fetal calf serum (FCS); 0.75% bicarbonate, vitamins, and L-glutamine (to double the initial concentration); penicillin (2000 IU/mL), streptomycin (200 μg/mL); and fungizone (250 pg/mL) (Invitrogen, Life Technologies), at 37 °C and 5% CO_2 [22]. The culture medium was changed every 2 days until cells were confluent, and the concentration of FCS was reduced to 2% to favor proliferation. Glial cell populations, including astrocytes and microglia, were confirmed by surface expression of glutamate transporter 1 (GLT1) and CD11b by fluorescence activated cell sorting (FACS).

Protein Extraction and Analysis

Total protein samples were prepared by homogenizing brains in 500 μL phosphate-buffered saline with a cocktail of protease inhibitors. Cytoplasmic and nuclear proteins were obtained by using the NE-PER Nuclear Protein Extraction Kit (Thermo Scientific). Protein concentrations were detected with the Bio-Rad Protein Assay reagent (Bio-Rad). For immunoprecipitation, 500 μg of protein was incubated 2 h at 4 °C with 2 μg rAb anti-protein. The immunocomplexes were incubated with protein A/G Magnetic Beads (Pierce) overnight at 4 °C, and beads were washed. Then, the immunoprecipitates were resuspended and analyzed by western blotting. To assess the relative amount of proteins, we used a slot blot method after testing the specificity of antibodies by western blotting. Protein preparations were blotted on a Hybond-C Extra membrane (GE Healthcare Europe GmbH) using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad). After transfer, membranes were saturated by incubation in 10% *w*/*v* nonfat milk powder or 5% *w*/*v* BSA in Tris-saline buffer (1.5 mM Tris base, pH 8; 5 mM NaCl; 0.1% Tween-20) and incubated overnight at 4 °C with an antibody directed against IkBα (1/1000, Cell Signaling Technology), calpain 2 domain IV (1/5000, Abcam), calpastatin (1/1000, Cell Signaling Technology), DYRK1A (1/500, Abnova corporation), IBA1 (1/1000, Abcam), NFkB p65 (1/2000, Abcam), and RNF216 (1/2000, Abcam). Binding of the primary antibody was detected by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology). Ponceau-S coloration (Sigma-Aldrich) was used as an internal control. Digitized images of the immunoblots obtained using an LAS-3000 imaging system (Fuji Photo Film Co., Ltd.) were used for densitometric measurements with an image analyzer (UnScan It software, Silk Scientific Inc.).

Enzyme Activities

Calpain activity was measured using the fluorogenic peptide *N*-Succinyl-Leu-Tyr-7-Amido-4-Methylcoumarin as described by Kohli et al. [23]. Briefly, 60 μg of brain extract in a final volume of 40 μL was added to 160 μL of 50 μM N-Succinyl-Leu-Tyr-7-Amido-4-Methylcoumarin dissolved in dimethyl sulfoxide and Tris buffer (100 mM Tris-HCl, 145 mM NaCl at pH 7.3). Proteolysis of the substrate was monitored for 21 min at room temperature with a FlexStation3 multi-mode microplate reader (excitation 380 nm, emission 460 nm; Molecular Devices) in the presence of either 10 mM Ca^{2+} or 10 mM EGTA to determine calcium-independent activity, thus excluding cathepsin activity. A protein phosphatase 2A (PP2A) activity assay was performed using the PP2A immunoprecipitation phosphatase assay kit (Millipore) using 5 mg of protein extracts as described by the manufacturer.

RNA Extraction, cDNA Synthesis, and Real-Time PCR Using SYBR-Green Chemistry

Total RNA was isolated from the brain using a RNeasy Lipid kit (Qiagen). The concentration of RNA samples was ascertained by measuring optical density at 260 nm. The integrity of RNA was confirmed by the detection of 18S and 28S bands after agarose-formaldehyde gel electrophoresis. The quality of RNA was verified by optical density absorption ratio OD 260 nm/OD 280 nm. To remove residual DNA contamination, the RNA samples were treated with RNAse-free DNAse (Qiagen) and purified with an RNeasy mini column (Qiagen). For each sample, 4 μg of total RNA from each sample was reverse transcribed with 200 U of M-MLV Reverse Transcriptase (Invitrogen, Life Technologies) using random hexamer primers. Real-time quantitative PCR amplification reactions were carried out in a LightCycler 480 detection system (Roche) using the LightCycler FastStart DNA Master plus SYBR Green I kit (Roche). The primers used for the target genes were derived from mouse sequences (Table S1). For each reaction, 40 ng of reverse transcribed RNA was used as template. All reactions were carried out in duplicate with a no template control. The PCR conditions were as follows: 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. The mRNA transcript level was normalized against the mean of two genes: Ppib and Hprt. To compare the target gene level, relative quantification was performed as outlined in Pfaffl et al. [24].

Data Analysis

Statistical analysis was done with the Student's *t* test using Statview software. For multiple pairwise comparisons

between genotypes and treatments, statistical analysis was done with two-way ANOVA followed by the Bonferroni/Dunnet post hoc test using Statview software. The results are expressed as mean ± SEM (standard error of the mean). Data were considered significant when $p < 0.05$. Correlations were determined by using Spearman's rank correlation, as data were not normally distributed according to the Shapiro-Wilk test.

Results

RNF216 Protein Level and Nuclear Protein Translocation of p65 Are Linked to DYRK1A Protein Level in the Mouse Brain

Using yeast two hybrids, a highly complex random-primed human adult brain cDNA library was screened to identify genes encoding DYRK1A-interacting proteins. Among the DYRK1A-interacting proteins was found an E3 ubiquitin-protein ligase, RNF216. RNF216, also known as triad domain-containing protein 3 (triad3) or zinc finger protein inhibiting NFkB (ZIN), is an inhibitor of NFkB-dependent signaling pathways induced by TNFα and IL1 [25]. Other secreted or membrane proteins identified in this two-hybrid screen, such as ADAMST1, CYR61, and ITGB8, are more directly involved in inflammation. We first confirmed the interaction between DYRK1A and RNF216 in brain extracts. For this, protein extracts were subjected to immunoprecipitation with anti-RNF216 or anti-DYRK1A antibodies, and the presence of DYRK1A in the immunoprecipitates was analyzed by western blotting. RNF216 was coimmunoprecipitated with DYRK1A in control (WT) mice and mice over-expressing *Dyrk1A* (TgDyrk1A) (Fig. 1a). Moreover, RNF216 protein level was increased in the brains of 5-month-old TgDyrk1A mice compared to WT mice (Fig. 1b) and decreased in the brains of 5-month-old *Dyrk1A*-deficient mice (Dyrk1A +/−) (Fig. 1c), with a positive correlation between RNF216 and DYRK1A protein level ($p < 0.0465$ with a $\rho = 0.630$).

We therefore analyzed the effect of Dyrk1A over-expression and deficiency on subcellular p65 localization in the brains of 2- and 5-month-old mice and found a decreased nuclear p65 protein level in TgDyrk1A mice (Fig. 1d) and an increased nuclear p65 protein level in Dyrk1A +/− mice (Fig. 1f), compared to WT mice. Conversely, cytoplasmic p65 protein level that was increased in TgDyrk1A mice (Fig. 1e) and decreased in Dyrk1A +/− mice (Fig. 1g) was found compared to WT mice. Thus, cytoplasmic sequestration of p65 is increased in the brains of TgDyrk1A mice but decreased in the brains of in Dyrk1A +/− mice.

IkBα Protein Level Is Linked to DYRK1A Protein Level in the Mouse Brain

We analyzed protein and mRNA levels of IkBα in the brains of mice over-expressing and deficient in DYRK1A. We found an increase of IkBα protein in the brains of 5-month-old TgDyrk1A mice compared to WT mice (Fig. 2a), with a positive correlation between IkBα and DYRK1A protein levels ($p < 0.0025$ with a $\rho = 0.660$). We also assayed *IkBα* gene expression by qPCR and, conversely, found a decrease of its expression in the brain of TgDyrk1A mice compared to WT mice (Table 1). This increase in IkBα protein level was also confirmed in the brains of Dp(16)1Yey mice, a mouse model with segmental trisomy of MMU16 and three gene copies of *Dyrk1A* (Fig. 2b). In contrast, IkBα protein level was decreased in the brains of Dyrk1A +/− mice (Fig. 2c). The balance of IKKβ /PP2A has been implicated in IkBα degradation. We quantified the ratios of phospho-IκBα/IκBα and IKKβ protein levels in the brains of TgDyrk1A mice and found no difference compared to WT mice (data not shown). Moreover, no difference in PP2A activity was found between TgDyrk1A and WT mice (Fig. 2d). Taken together, these results demonstrate that greater IκBα protein stability is not dependent on its phosphorylation state in the brains of TgDyrk1A mice.

NFkB Target Genes Were Decreased, and Gene Expression of IL10, an Anti-inflammatory Cytokine, Was Increased in the Brains of Mice Over-expressing *Dyrk1A*

Cytoplasmic sequestration of p65 in the brains of mice over-expressing *Dyrk1A* was evaluated by quantification of target genes. We found a decrease not only of *IκBα* mRNA expression but also of *cIAP2* and *HO-1* in the brains of TgDyrk1A mice (Table 1) [26]. *IκBα* mRNA expression was negatively correlated with DYRK1A protein expression ($p < 0.0247$ with a $\rho = -0.545$). In the CNS, NFκB activation can be negatively regulated by a several cytokines including IL10 [27]. *IL10* mRNA expression was increased in the brains of TgDyrk1A mice (Table 1). All together, these results underline the inhibitory effect of DYRK1A over-expression on the NFkB pathway.

Calpain Activity Is Linked to DYRK1A Protein Level in the Mouse Brain

Another possible mechanism of IκBα degradation involves the balance of calpain/calpastatin. We found a decreased calpain activity in the brains of 5-month-old TgDyrk1A mice compared to WT mice (Fig. 3a), without difference in calpastatin protein level (data not shown). In contrast, calpain activity was increased in the brains of 5-

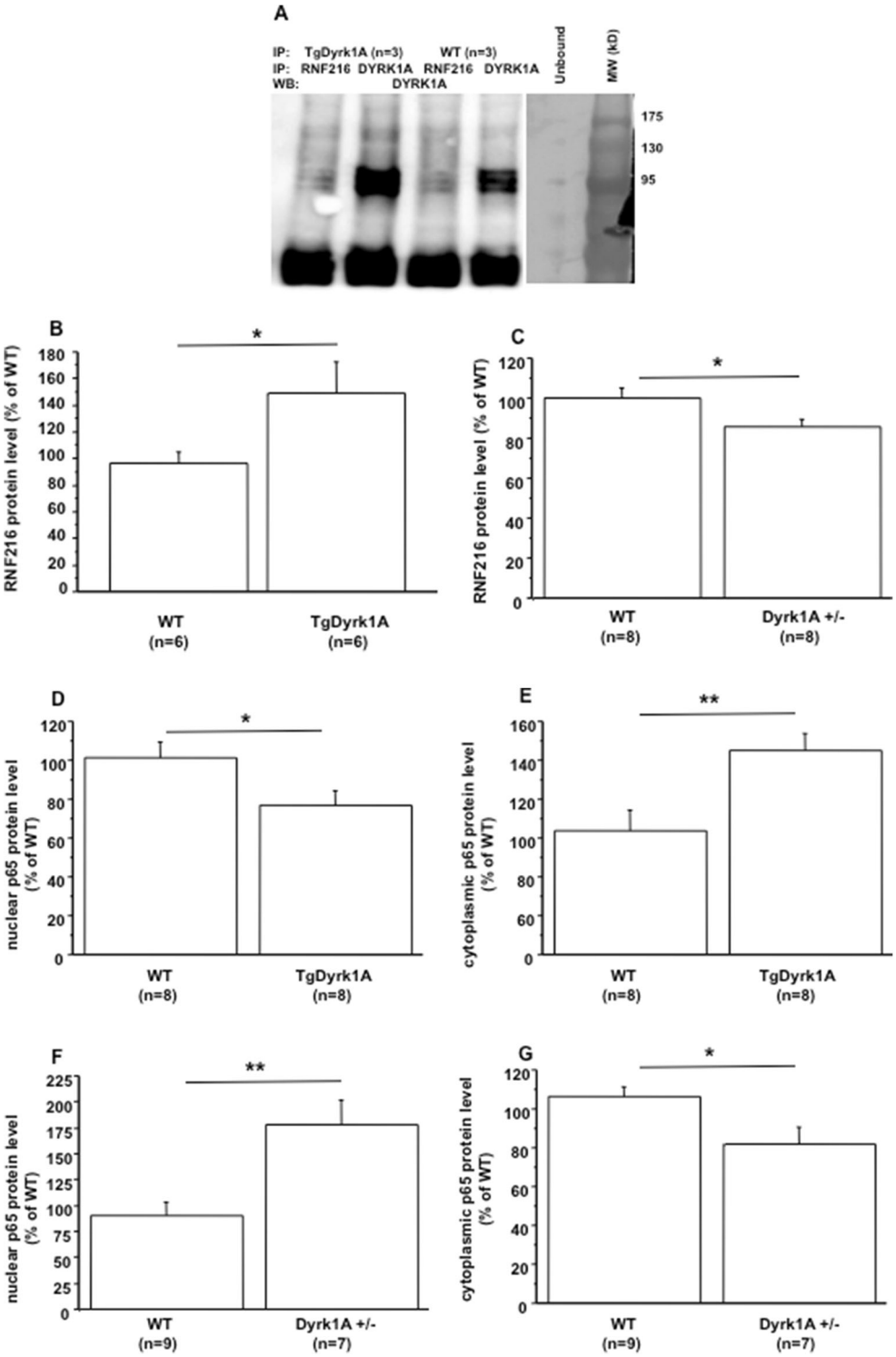

Fig. 1 RNF216 protein interaction with DYRK1A and RNF216 and p65 protein levels in the mouse brain. **a** Western blotting showing DYRK1A after immunoprecipitation with anti-RNF216 or anti-DYRK1A antibodies in the brain of control (WT) and mice over-expressing Dyrk1A (TgDyrk1A). RNF216 (**b**, **c**), nuclear (**d**–**f**), and cytoplasmic (**e**–**g**) p65 levels were determined by slot blotting in the brains of mice over-expressing *Dyrk1A* (TgDyrk1A) (**b**, **d**, **e**) and *Dyrk1A*-deficient mice (Dyrk1A +/−) (**c**, **f**, **g**). Values were obtained by normalization of images from RNF216, nuclear, and cytoplasmic p65 to total proteins marked with Ponceau-S. Statistical analysis was done with the Student's *t* test using Statview software. Data were normalized to the mean of control (WT) mice. The results are represented as means ± SEM. *n* number of mice. $*p < 0.05$; $**p < 0.001$

month-old *Dyrk1A*-deficient mice (Dyrk1A +/−) (Fig. 3b). We therefore analyzed the protein levels of calpain 1 and calpain 2, the two major calpain isoforms in the brain. Calpains are calcium-dependent proteases, with a large subunit containing domains I-IV. No difference was found for domain I, the propeptide domain of calpain 1 and 2, nor for calpain 1 domain IV, a Ca2+-binding domain (data not shown). However, a decrease of calpain 2 domain IV protein level was found in the brains of TgDyrk1A mice compared to WT mice (Fig. 3c). Therefore, decreased calpain activity may be due in part to the decrease of calpain 2 domain IV protein level in the brains of mice over-expressing *Dyrk1A*.

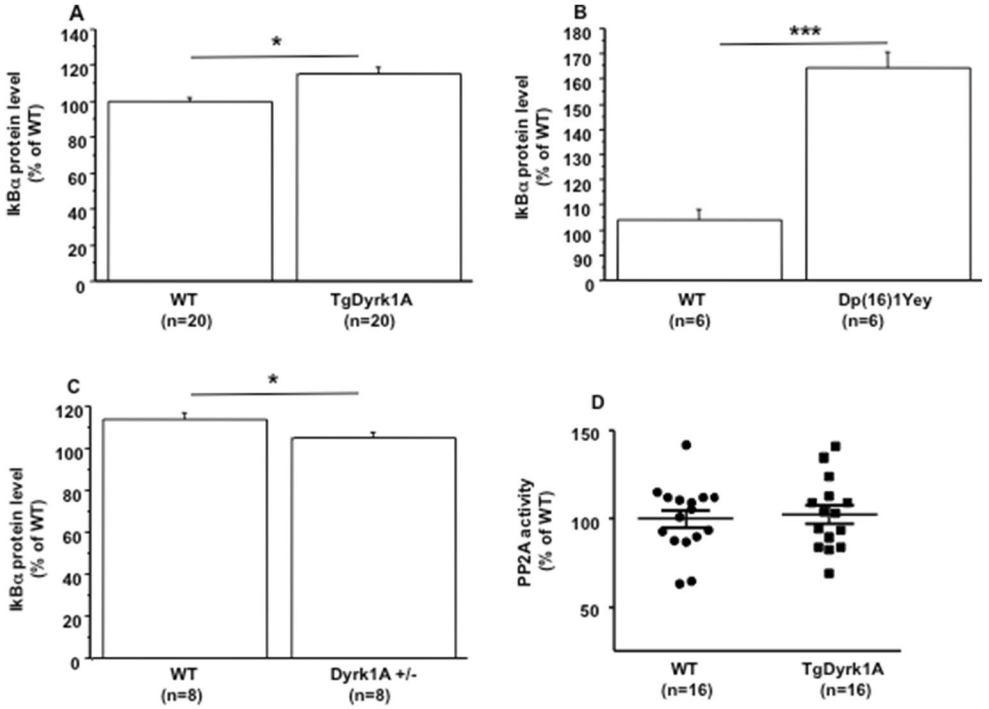

Fig. 2 IkBα protein level and PP2A activity in the mouse brain. Brain IkBα quantification in **a** mBACtgDyrk1A transgenic mice (TgDyrk1A), **b** Dp(16)Yey mice, and **c** *Dyrk1A*-deficient mice. IkBα level was determined by slot blotting, and values were obtained by normalization of images from IkBα to total proteins marked with Ponceau-S. **d** PP2A activity in mBACtgDyrk1A transgenic mice (TgDyrk1A) was determined by an immunoprecipitation phosphatase assay as described in the "Material and Methods" section. Statistical analysis was done with the Student's *t* test using Statview software. Data were normalized to the mean of control (WT) mice. The results are represented as means ± SEM. *n* number of mice. $*p < 0.05$; $***p < 0.001$

IBA1 Protein Level Is Decreased in the Brain of Mice over-Expressing Dyrk1A

Calpain 1 is located primarily in neurons, while calpain 2 is more prominent in glial cells [28]. Interestingly, calpain 2 silencing elicits decreased levels of GFAP [29]. We therefore analyzed the effect of DYRK1A on expression of glial cell markers. We found a decrease in mRNA expression of GFAP (Table 1), an astroglial marker, and of IBA1 protein level, a macrophage/microglial marker, in the brains of TgDyrk1A mice (Fig. 4a), with a negative correlation with DYRK1A protein level ($p < 0.0135$, $r = -0.567$) (Fig. 4b). These results suggest a role of DYRK1A in downregulation of activating signals in glial cells. To confirm the role of DYRK1A in glial cells, protein levels of domain IV of calpain 2 and IBA1 were analyzed in glial cells from newborn mice. Over-expression of *Dyrk1A* was first confirmed in glial cells from TgDyrk1A mice (Table 2). Decreased protein levels of domain IV of calpain 2 and IBA1 were also found in glial cells from TgDyrk1A mice compared to WT mice (Table 2), with a negative correlation between IBA1 and DYRK1A protein level ($p < 0.0233$, $r = -0.857$). We therefore analyzed IBA1 in mice *Dyrk1A*-deficient mice (Dyrk1A +/−) and found that decreased DYRK1A protein level increases IBA1 protein level at 5 months old (Fig. 4c), but not at 2 months old (Fig. 4d).

Table 1 Quantitative PCR analysis of *Ikbα*, *cIAP2*, HO-1, *IL10*, and *Gfap* in the brains of mice over-expressing *Dyrk1A*

mRNA level (%)	WT ($n = 8$)	TgDyrk1A ($n = 8$)
Ikbα	100 ± 5	84 ± 5*
cIAP2	100 ± 0.7	90 ± 0.6**
HO-1	100 ± 17	67 ± 8*
IL10	100 ± 4	118 ± 7*
GFAP	102 ± 7	81 ± 8*

Data were normalized to the mean of control (WT) mice. Data are presented as mean ± SEM and analyzed with the Student's *t* test by using Statview software

n number of mice

$*p < 0.05$; $**p < 0.01$

IkBα Protein Level Is Decreased and Calpain Activity Is Increased in the Brains of Mice Over-expressing *Dyrk1A* in Acute Inflammation

To analyze the effect of *Dyrk1A* over-expression on acute inflammation, TgDyrk1A and WT mice were injected with LPS for 24 h. We first confirmed the effect of LPS on markers of inflammation. Plasma hcy, a circulating inflammatory marker, was decreased in TgDyrk1A mice compared to WT

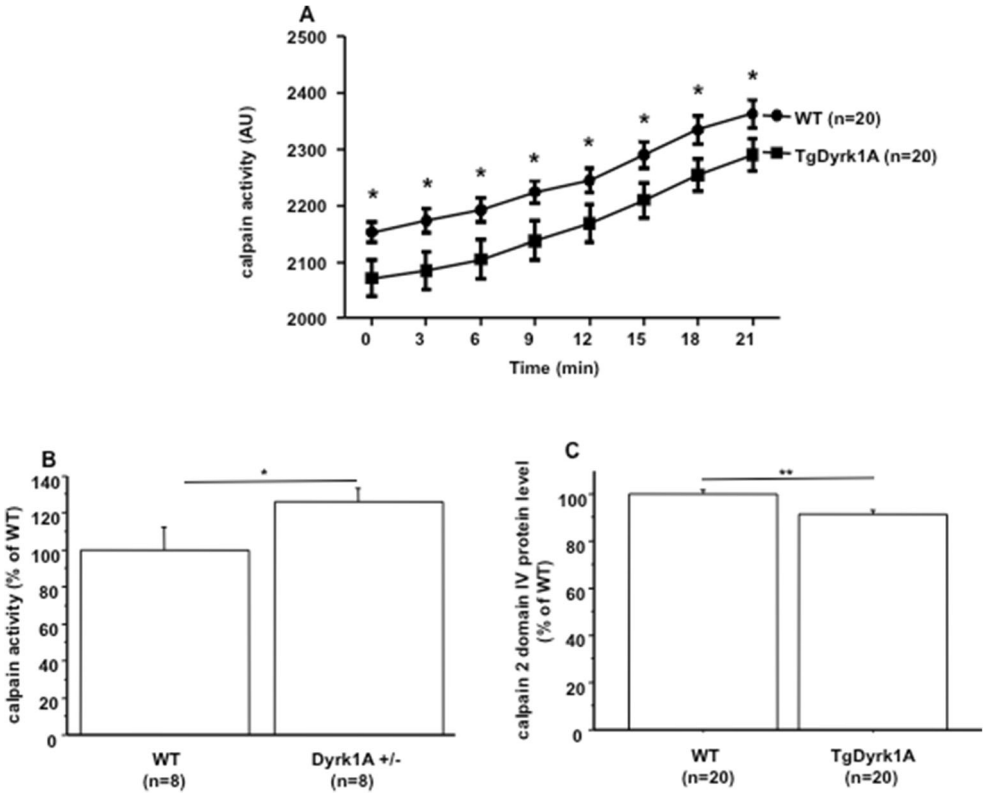

Fig. 3 Calpain activity (**a**, **b**) and calpain 2 domain IV protein level (**b**) in the mouse brain. Calpain activity was determined in **a** the brains of mice over-expressing *Dyrk1A* (TgDyrk1A) and **b** in the brains of *Dyrk1A*-deficient mice (Dyrk1A +/−). **c** Calpain 2 domain IV protein level was determined by slot blotting, and values were obtained by normalization of images from calpain 2 domain IV to total proteins marked with Ponceau-S. Statistical analysis was done with the Student's *t* test using Statview software. Data were normalized to the mean of control (WT) mice. The results are represented as means ± SEM. *n* number of mice. *AU* arbitrary unit. $*p < 0.05$; $**p < 0.01$

mice, as previously described [30]. While we did not find a difference in plasma hcy in WT mice treated with LPS, TgDyrk1A mice showed an increased hcy level after LPS treatment (Table 3). Moreover, mRNA expression of IL6, a major pro-inflammatory cytokine in the CNS [31], was greater in the brains of TgDyrk1A and WT mice after LPS treatment

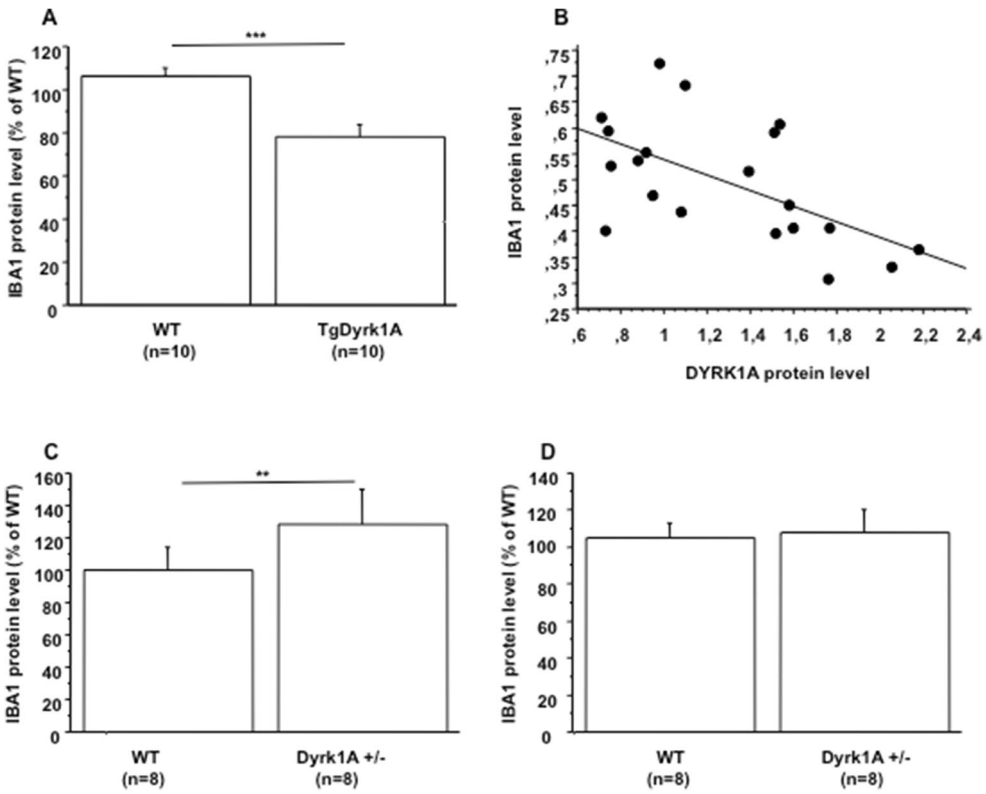

Fig. 4 IBA1 protein level in the brains of mice (**a**, **b**) over-expressing *Dyrk1A* (TgDyrk1A) and (**c**, **d**) *Dyrk1A*-deficient mice (Dyrk1A +/−). Iba1 protein level was determined by slot blotting at 5 months old (**a**, **c**) and 2 months old (**d**), and values were obtained by normalization of images from IBA1 to total proteins marked with Ponceau-S. **b** Levels of DYRK1A protein and IBA1 protein from TgDyrk1A mice and control (WT) littermates were negatively correlated at $p < 0.0135$ with a $\rho = -0.567$. Statistical analysis was done with the Student's *t* test using Statview software. Data were normalized to the mean of WT mice. The results are represented as means ± SEM. *n* = number of mice. $**p < 0.01$; $***p < 0.001$

Table 2 Protein levels in glial cells from newborn mice over-expressing *Dyrk1A*

Protein level (%)	WT ($n = 4$)	TgDyrk1A ($n = 4$)
DYRK1A	100 ± 0.07	126 ± 0.03**
Calpain 2 domain IV	100 ± 4	89.5 ± 2*
IBA1	100 ± 10	69 ± 3.5*

Data were normalized to the mean of control (WT) mice. Data are presented as mean ± SEM and analyzed with the Student's *t* test by using Statview software

n number of mice

*$p < 0.05$; **$p < 0.01$

(Table 3). Although if LPS treatment had no effect on IkBα protein level in WT mice, IkBα protein level was decreased in TgDyrk1A mice treated with LPS compared to TgDyrk1A mice without treatment (vehicle) (Fig. 5a). No difference in the proteasome pathway was found, regardless of genotype and treatment (data not shown). Calpain activity was increased not only in the brains of WT mice but also in the brains of TgDyrk1A mice after LPS treatment (Fig. 5b). However, the domain IV of calpain 2 protein level was unchanged in the brains of WT mice and TgDyrk1A mice treated with LPS compared to mice without treatment (Fig. 5c). Moreover, we found a decrease of calpastatin protein in the brains of both WT and TgDyrk1A mice after LPS treatment (Fig. 5d). Taken together, our results showed that increased calpain activity in the brains of WT and TgDyrk1A mice may be due in part to a decrease of calpastatin protein level in acute inflammation.

DYRK1A Protein Level Is Decreased in the Brains of Mice Over-expressing *Dyrk1A* in Acute Inflammation

Since we found a negative effect of acute inflammation in the brains of mice over-expressing *Dyrk1A*, we therefore analyzed the effect of LPS treatment on DYRK1A protein level. No difference was found in WT mice. However, DYRK1A protein level was decreased in TgDyrk1A mice treated with LPS compared to untreated TgDyrk1A mice (Fig. 6a). In commensuration with this decreased protein level, we did not find a difference for IBA1 protein level between treated and untreated (Fig. 6b). Moreover, no correlation between IBA1 and DYRK1A protein level was found after LPS treatment.

Discussion

This study was conducted to analyze the role of DYRK1A on regulation of the NFkB pathway in the mouse mice. We found that over-expression of *Dyrk1A* stabilizes IκBα protein level by inhibition of calpain activity. Meanwhile, *Dyrk1A*-deficient mice have decreased IκBα protein level with increased calpain activity in the brain. The direct consequence of *Dyrk1A* over-expression was the increase of cytoplasmic p65 sequestration in the brain. A previous study also showed a reduction of p65 levels in the nuclear fractions and a significant increase of IκBα in the cytoplasmic fractions of DS LCLs [14]. We also demonstrated the increase of IκBα not only in the brain of a murine model of DS, the Dp(16)1Yey mouse, but also in the brain of TgDyrk1A mice with a positive correlation between IkBα and DYRK1A protein levels. Therefore, our study is complementary and underlines the role of DYRK1A on NFkB pathway in the brain of mice. However, Granese et al. also found an increase of IκBα phosphorylation with a reduction of IκBα ubiquitination [14]. Over-expression of DSCR1, which is localized on chromosome 21, stabilizes IκBα by a mechanism that is independent of IKK-mediated phosphorylation of IκBα and decreases the steady-state activity of NFkB in Human U87MG glioblastoma cells, thus inhibiting the induction of genes involved in the inflammatory response [32]. Receptor-interacting protein (RIP) is involved in the induction of NFkB activation by tumor necrosis factor receptor-1 (TNF-R1). RIP is required for activating IKK. RNF216 has been identified by yeast two-hybrid screening to interact with RIP, and RNF216 over-expression inhibits NFkB activation by RIP, IKKβ, TNF,

Table 3 Plasma hcy level and quantitative PCR analysis of *IL6* in the brains of mice treated with LPS and vehicle-treated mice

	WT/vehicle ($n = 8$)	WT/LPS ($n = 8$)	TgDyrk1A/vehicle ($n = 8$)	TgDyrk1A/LPS ($n = 8$)
Plasma hcy level (μM)	3.2 ± 0.4	3.6 ± 0.4	2.1 ± 0.1*	3.6 ± 0.3***
IL6 mRNA (%)	102 ± 6	304 ± 60**	100 ± 17	350 ± 110$^{\#}$

Data from brain IL6 mRNA levels were normalized to the mean of control not treated (WT/vehicle) mice. Data from plasma hcy and brain IL6 mRNA levels are presented as mean ± SEM and analyzed with two-way ANOVA followed by the Bonferroni/Dunnet post hoc test by using Statview software

n number of mice

*$p < 0.01$ vs WT/vehicle; **$p < 0.001$ vs WT/vehicle; ***$p < 0.01$ vs TgDyrk1A/vehicle; $^{\#}p < 0.001$ vs TgDyrk1A/vehicle

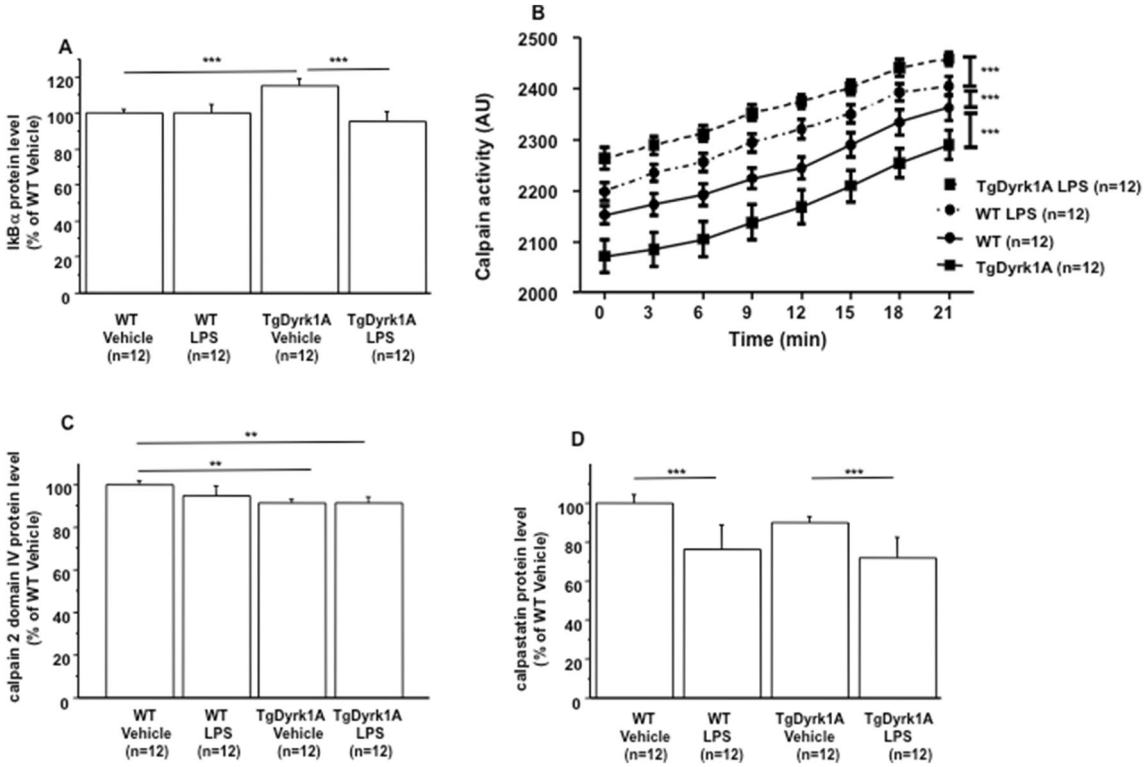

Fig. 5 IkBα protein level (**a**), calpain activity (**b**), calpain 2 domain IV protein level (**c**), and calpastatin protein level (**d**) in mBACtgDyrk1A transgenic (TgDyrk1A) mice with (LPS) or without (vehicle) treatment. IkBα, calpain 2 domain IV, and calpastatin protein levels were determined by slot blotting, and values were obtained by normalization of images from IkBα, calpain 2 domain IV, or calpastatin to total proteins marked with Ponceau-S. Calpain activity was determined by measuring the proteolysis of the substrate for 21 min. Statistical analysis was done with two-way ANOVA followed by the Bonferroni/Dunnet post hoc test by using Statview software. Data were normalized to the mean of control (WT) mice. The results are represented as means ± SEM. *n* number of mice. $^{**}p < 0.01$; $^{***}p < 0.001$

and IL1 [25]. We found not only an interaction between RNF216 and DYRK1A but also an increased RNF216 protein level in the brains of mice over-expressing *Dyrk1A* and a decrease in the brains of *Dyrk1A*-deficient mice. Taken together, these results underline the role of DYRK1A on the NFkB pathway.

In commensuration with the increased p65 sequestration, we found a decreased mRNA expression of NFkB-target genes, *cIAP2* and *HO-1* [33], in the brains of mice over-expressing *Dyrk1A*. HO-1 participates in the cell defense against oxidative stress. HO-1 protein is a cellular marker of oxidative stress [34]. NFkB transcription factor also controls

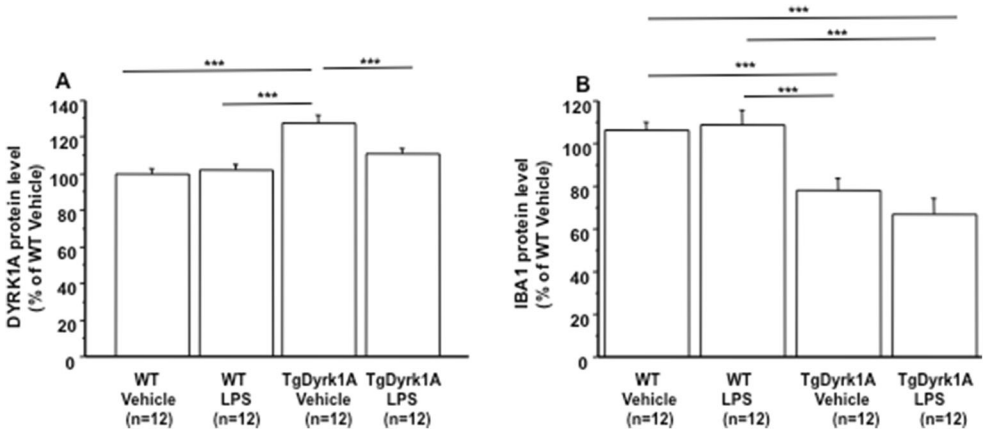

Fig. 6 DYRK1A (**a**) and IBA1 (**b**) protein levels in mBACtgDyrk1A transgenic (TgDyrk1A) mice with (LPS) or without (vehicle) treatment. DYRK1A and IBA1 levels were determined by slot blotting, and values were obtained by normalization of images from DYRK1A and IBA1 to total proteins marked with Ponceau-S. Statistical analysis was done with two-way ANOVA followed by the Bonferroni/Dunnet post hoc test by using Statview software. Data were normalized to the mean of control (WT) mice. The results are represented as means ± SEM. *n* number of mice. $^{***}p < 0.001$

the transcription of anti-apoptotic genes [33], such as *cIAP2*. The expression of these two NFkB regulated genes has also been previously found to be decreased in the livers of mice over-expressing *Dyrk1A* [17]. The CNS environment is anti-inflammatory, featuring high local concentrations of inflammation-suppressive cytokines such as IL10 [35]. NFkB activation can be negatively regulated by cytokines like IL10 [27]. IL10 mRNA expression was also found to be increased in the brains of mice over-expressing *Dyrk1A*. Interestingly, inhibition of NFkB plays a role in the anti-inflammatory function of IL10 [36].

NFkB signaling in the CNS is essential in several physiological processes, such as synaptic processes and neurotransmission. Constitutive NFkB activity is found in glutamatergic neurons of the CNS [37], and it can be suppressed by pharmacological inhibitors such as glutamate antagonists and L-type calcium channel blockers [38]. Therefore, constitutive NFkB activity results from physiological basal synaptic transmission. We recently found that molecular alterations in synaptic plasticity pathways in the brains of mice over-expressing *Dyrk1A*, particularly expression changes in GABAergic and glutaminergic related proteins, were induced, suggesting lesser calcium into postsynaptic neurons [39]. NFkB is also an important regulator of neuronal morphology and shapes brain structures that are important for learning and memory. Mouse models with reduced NFkB activity in the brain have lower performance in different behavioral tests, which were also seen following more precisely repression of NFkB specifically in glutamatergic neurons [6]. NFkB function in glutamatergic neurons is responsible for learning and memory. We previously also found by behavioral analyses of mice over-expressing Dyrk1A a reduction in motor learning and motor function (rotarod), short-term memory (Y-maze), and long-term memory and spatial learning (Morris water maze) [39].

In the brains of mice over-expressing *Dyrk1A*, the increased IκBα protein level is associated with a reduction of calpain activity, which is not dependent on calpastatin activity. On the contrary, *Dyrk1A*-deficient mice showed increased brain calpain activity. Most calpain in cells is latent, and its activity is regulated by local calcium levels, phosphorylation and association with calpastatin. Excitotoxic glutamate has been shown to activate neuronal expression of NFkB through calpain by cleaving IkBα [40]. Concomitant with the decreased calpain activity, we found decreased protein level of domain IV of calpain 2, a calcium-binding domain implicated in calpain activity [41], in the brains of mice over-expressing Dyrk1A and in glial cells from newborn mice. A previous study of cortical neurons showed that calpain 2 has a neurotoxic effect through extra-synaptic receptors, while calpain 1 has a neuroprotective effect through NMDAR activation [42]. Calpain 1 activation is necessary for certain forms of synaptic plasticity and learning and memory, while calpain 2 activation during a brief consolidation period limits the extent of plasticity/learning. Similarly, calpain 1 is neuroprotective, while calpain 2 is neurodegenerative [43]. Calpain 1 is located primarily in neurons, while calpain 2 is more prominent in glial cells [28]. Inhibition of calpain 2 has been shown to lead to decreased GFAP expression [29]. We found decreased mRNA expression not only of *Gfap* but also of IBA1 protein level in the brains of mice over-expressing *Dyrk1A*. Moreover, decreased IBA1 protein level was also found in glial cells from newborn mice. Microglia represent a major cellular component of the innate immune system in the CNS [44] and support CNS homeostasis and plasticity by promoting learning-related synapse formation through brain-derived neurotrophic factor (BDNF) signaling [45]. BDNF, a neurotrophic factor critically involved in synaptic plasticity, was found to activate calpain-2 through ERK-mediated phosphorylation [46]. BDNF mRNA and protein levels have been found to be decreased in the brains of mice over-expressing Dyrk1A [47, 48]. Taken together, these results underline the role of DYRK1A on calpain activity.

Neurodegeneration occurs in part because the environment is affected during disease in a cascade of processes collectively termed neuroinflammation. Neuroinflammation is characterized by a reactive morphology of glial cells, including both astrocytes and microglia, accompanied by inflammatory mediators. When subjected to abnormal stimulation, microglia become activated, with the release of proinflammatory cytokines, which further aggravate neuroinflammatory injury [49]. The NFkB signaling pathway in glial cells has been related to inflammatory responses [50], and glial-inducible NFkB activation is implicated in neuroinflammation-associated pathogenesis related to neuronal damage. NFkB signaling regulates inflammatory processes that exacerbate inflammation-induced neurodegeneration. NFkB signaling in the CNS is essential in pathological processes associated with neurodegeneration. Calcium dyshomeostasis is also implicated in the pathogenesis of several neurodegenerative disorders [51]. Several kinases implicated in learning and memory, including calcium-calmodulin kinase II (CaMKII), activate NFkB [52]. CamKII activation was previously found to be decreased in the brains of mice over-expressing *Dyrk1A* and increased in the cortex of Dyrk1A-deficient mice [39]. Interestingly, IBA1 protein level was increased in the brains of 5-month-old mice. However, at 2 months old, nuclear translocation of p65 was increased in the brains of *Dyrk1A*-deficient mice, without increased IBA1 level. We can hypothesize that DYRK1A plays a role in the NFkB pathway before microglial activation.

Given that inhibiting the activation of microglia in inflammatory responses represents a potentially neuroprotective treatment strategy that may influence neurodegeneration processes, we also investigated the effect of *Dyrk1A* overexpression on acute inflammation by IP injection of LPS in mice. High levels of activated calpain disinhibit NFkB from its translocation to the nucleus and induce gene expression of

several pro-inflammatory cytokines, including IL6, a pleiotropic inflammatory cytokine mainly produced by activated microglia and astrocytes in different brain regions [31]. In addition, IL6 can stimulate glial cells to release a cascade of pro-inflammatory cytokines and acute-phase proteins. We first analyzed the effect of LPS treatment on IL6 expression and found an increased expression not only in control mice but also in mice over-expressing *Dyrk1A*. The increased IkBα protein level associated with the decreased calpain activity in the brains of mice over-expressing *Dyrk1A* was not found after LPS treatment. However, LPS treatment did not appear to have an effect on domain IV of calpain 2 and IBA1 protein levels in the brains of control mice and mice over-expressing *Dyrk1A*. Considerable evidence points to the essential role of calpain hyperactivation in driving neurodegenerative diseases [53]. Increased calpain activity is associated with decrease of its endogenous inhibitor, calpastatin, itself a known calpain substrate, in the AD brain [54]. We therefore analyzed the protein level of calpastatin and found that it was decreased in the brains of control mice and mice over-expressing *Dyrk1A*. A number of potential calpain targets have been proposed to play crucial roles in certain forms of neurodegeneration. Calpain truncates glycogen synthase kinase 3β (GSK-3β), resulting in its increased kinase activity [55]. GSK3β directly interacts with and is phosphorylated by DYRK1A, inhibiting GSK3β kinase activity [56]. We therefore analyzed the level of DYRK1A and found that it decreased in the brains of mice over-expressing *Dyrk1A* after LPS treatment. We previously found a deleterious effect of calpain activation on DYRK1A protein levels in the liver of hyperhomocysteinemic mice [57]. Calpain activity is also found increased in the aorta of hyperhomocysteinemic mice [58], with a reduction of DYRK1A protein level [59]. Like IkBα, DYRK1A protein also contains a PEST sequence, a signal peptide for protein degradation, which may be mediated via calpains [11].

In summary, the negative effect of *Dyrk1A* over-expression on NFkB pathway activation is abolished after acute inflammation due to LPS treatment. Although LPS treatment has a positive effect on calpastatin and a negative effect on DYRK1A protein level, the positive effect on microglial activation is maintained in the brains of mice over-expressing *Dyrk1A*.

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Author Contributions AL, YG, and NJ designed the study; AL, YG, NK, FD, CC, and BG performed experiments; SM, VH, JCR, and SM performed yeast two-hybrid analysis; JLP performed HPLC analysis; NJ, AL, YG, JLP, JMD, EY, MA, and MM analyzed the data; NJ and AL wrote the paper; NJ and MM correct the manuscript.

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Compliance with Ethical Standards

All procedures were carried out in accordance with the ethical standards of French and European regulations (European Communities Council Directive, 86/609/EEC). Official authorization from the French Ministry of Agriculture was granted to perform research and experiments on animals (authorization number 75–369), and the experimental protocol was approved by the institutional animal care and use committee of the Paris Diderot University (CEEA40).

Conflict of Interest The authors declare that they have no conflict of interest.

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