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ORIGINAL ARTICLE

A cluster of protein kinases and phosphatases modulated in fetal Down syndrome (trisomy 21) brain

Rachel Weitzdoerfer · Nuria Toran · Saraswathi Subramaniyan · Arnold Pollak · Mara Dierssen · Gert Lubec

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Abstract Down syndrome (DS; trisomy 21) is the most frequent cause of mental retardation with major cognitive and behavioral deficits. Although a series of aberrant biochemical pathways has been reported, work on signaling proteins is limited. It was, therefore, the aim of the study to test a selection of protein kinases and phosphatases known to be essential for memory and learning mechanisms in fetal DS brain. 12 frontal cortices from DS brain were compared to 12 frontal cortices from controls obtained at legal abortions. Proteins were extracted from brains and western blotting with specific antibodies was carried out. Primary results were used for networking (IntAct Molecular Interaction Database) and individual predicted pathway components were subsequently quantified by western blotting. Levels of calcium–calmodulin kinase II alpha, transforming growth factor beta-activated kinase 1 as well as phosphatase and tensin homolog (PTEN) were reduced in cortex of DS subjects and network generation pointed to interaction between PTEN and the dendritic spine protein drebrin that was subsequently determined and reduced levels were observed. The findings of reduced levels of cognitive-function-related protein kinases and the phosphatase may be relevant for interpretation of previous work and may be useful for the design of future studies on signaling in DS brain. Moreover, decreased drebrin levels may point to dendritic spine abnormalities.

Keywords Brain · Down syndrome · Fetal · Kinase · Phosphatase · Drebrin

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R. Weitzdoerfer · S. Subramaniyan · A. Pollak · G. Lubec (✉)
Department of Pediatrics, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria
e-mail: gert.lubec@meduniwien.ac.at

N. Toran
Department of Pathology, Hospital Universitari Vall d'Hebron, Barcelona, Spain

M. Dierssen
Genes and Disease Program, Center for Genomic Regulation and CIBERER, Barcelona, Spain

Introduction

Down syndrome (trisomy 21) is the major cause of mental retardation and serious cognitive deficits including memory and learning as well as behavioral changes are regularly observed. A myriad of proteins has been reported to be changed (Engidawork et al. 2001b; Lubec et al. 2001; Weitzdoerfer et al. 2001b, c, 2002; Peyrl et al. 2002; Engidawork and Lubec 2003) and changes of protein kinases were proposed to modulate brain function in DS (Bernert et al. 1996; Peyrl et al. 2002; Sun et al. 2011).

A large amount of protein kinases are expressed in the mammalian brain (Giese and Mizuno 2013) and Dyrk1A, a serine/threonine kinase is encoded on chromosome 21. This protein kinase is involved in dendritic growth, synaptogenesis (Chen et al. 2014) and seems to play a role in the generation of (Ryoo et al. 2008) disease-like changes that inevitably occurs in DS brain from the fourth decade (Arai et al. 1996; Oka and Takashima 1999; Engidawork et al. 2001a; Ryoo et al. 2008). Alterations of the Dyrk1A–actin interaction were detected in newborns and infants with DS (Dowjat et al. 2012). Overexpression of Dyrk1A was suggested to be involved in premature differentiation of neurons and subsequently in altered brain development in DS (Park et al. 2009a, b; Park and Chung 2013; Soppa et al. 2014). Moreover, Dyrk1A has been evaluated for its

capacity to serve as a drug target by modulating its protein kinase activity in various DS models (Becker et al. 2014).

Apart from Dyrk1A higher levels of mTor signaling components were observed in fetal hippocampus (Iyer et al. 2014) and in adult DS several protein kinases were proposed to be probably involved in pathomechanisms leading to Alzheimer disease. These findings are, however, not considered herein as relevant as in studies on adult brain, neurodegenerative changes are already present and assignment of protein dysregulations cannot be clearly assigned to DS per se, but may be linked to neurodegenerative changes including Alzheimer disease-like neuropathology from the fourth decade.

Herein, it was the aim of the study to investigate protein levels of a battery of protein kinases, selected according to their known involvement in brain structure and function including learning and memory mechanisms (Saito and Shirai 2002; Zhu et al. 2007; Wayman et al. 2008; Coultrap and Bayer 2012; Roskoski 2012; Ishitani and Ishitani 2013; Mihalas et al. 2013) and to generate a cluster of kinases that may be relevant for signaling abnormalities in fetal DS at a time point when no morphological changes or neurodegeneration is observed yet (Unterberger et al. 2003).

Materials and methods

Human fetal brain samples

The human biospecimens used in this project were provided by the Fetal Tissue Bank of Vall d'Hebron University Hospital Biobank with appropriate ethics approval. Frontal cortex samples from karyotyped individuals were used for this study.

Biochemical analyses

Sample preparation

A total of 24 samples (12 control brain samples and 12 samples from trisomy 21 were used for analysis: 4 male controls and 4 males with trisomy 21 at 22 weeks of gestation; 8 female controls and 8 females with trisomy 21 at 19–21 weeks of gestation) were homogenized in ice-cold homogenization buffer [10 mM HEPES, pH 7.5, 300 mM sucrose and 1 complete protease inhibitor tablet (Roche Molecular Biochemicals, Mannheim, Germany) per 50 mL] using an Ultra-Turrax homogenizer (IKA, Staufen, Germany). The homogenate was then centrifuged for 10 min at 1000×*g*, and the pellet was discarded. The supernatant was subsequently centrifuged at 50,000×*g* for 30 min in an ultracentrifuge (Beckman Coulter Optima L-90 K), and the resulting supernatant was collected for further experiments and pellets were suspended in 5 mL of wash buffer (homogenization buffer without sucrose), incubated on ice for 30 min and centrifuged at 50,000×*g* for 30 min and supernatants were re-collected and mixed with the supernatants obtained earlier.

The collected cytosolic fraction (supernatant) of the individual homogenized frontal cortex samples was suspended in 2 mL of urea buffer (20 mM TRIS, 7 M urea, 2 M thiourea, 4 % w/v CHAPS, 10 mM 1,4-dithiourea, 4 % PMSF, 1 mM EDTA, 1 tablet of Complete™ from Roche Diagnostics and 0.2 % v/v phosphatase inhibitor cocktail). The suspension was sonicated on ice 5 times for 3 s and samples were then centrifuged at 15,000×*g* for 60 min at 4 °C. Desalting was done using Amicon® Ultracel–4 Centrifugal Units at a molecular cut-off 10,000 Da (Millipore) at 3000×*g* at 4 °C until the eluted volume was around 4 mL and the remaining volume of sample around 100–200 µL. Thus, the extracted cytosolic protein concentration was determined by the Bradford assay (Harlow and Lane 2006).

Western blot analysis

The samples prepared as described above were prepared for SDS-PAGE using the Laemmli Sample Buffer (S3401-10VL, Sigma). Gels for SDS-PAGE were prepared the following way: 10 % running gel, 4 % stacking gel. Samples were then loaded onto these gels and the protein amounts were 20 µg each. The markers used for electrophoresis were Precision Plus Protein™ All Blue Standards (#161-0373, Bio-Rad). The gel running conditions are as follows: 50 V 30 min, 100 V 30 min and 150 V 1 h until the dye front reached the bottom of the gel. After electrophoresis, proteins were transferred onto a Roti®-PVDF membrane pore size 0.45 µm (T830.1, Carl Roth) by a semi-dry transfer system using transfer buffer (48 mM Tris, 39 mM glycine, 0.03 % SDS). The conditions of the transfer were as follows, 20 V for 1 h. After the transfer membranes were blocked with 5 % fat-free Milk in 0.1 % TBST (Tris buffered saline + Tween 20), subsequently the membranes were incubated with diluted primary antibodies overnight at 4 °C. Membranes were washed with 0.1 % TBST buffer, incubated with the secondary antibodies at room temperature and re-washed using 0.1 % TBST buffer. The primary and secondary antibodies used are shown in Table 1. Membranes were incubated with Clarity Western ECL Substrate (#170-5061, Bio-Rad) and imaged using ChemiDoc™ MP System (Bio-Rad).

Pathway analysis/protein interactions

Interactions between significantly modulated protein kinases and protein phosphatase PTEN were analyzed by IntAct Molecular Interaction Database (http://www.ebi.ac.uk/intact/).

Table 1 Identification of proteins and antibodies

Name	Full name	1st Antibody (abcam)	Dilution 1st AB	2nd Antibody (abcam)	Dilution 2nd AB
CaMKIV	Ca^{2+}/calmodulin-dependent protein kinase IV	Ab75874 (Rabbit monoclonal)	1:5000	Ab97069	1:5000
CaMKIIa (Phospho T286)	Ca^{2+}/calmodulin-dependent protein kinase II a	Ab5683 (Rabbit polyclonal)	1:1000	Ab97069	1:5000
DRB	Drebrin	Ab60933 (Rabbit polyclonal)	1:1000	Ab97069	1:5000
LCK (phospho Y505)	lymphocyte-specific protein tyrosine kinase	Ab76304 (Rabbit monoclonal)	1:500	Ab97069	1:2000
MEK1	Dual specificity mitogen-activated protein kinase kinase 1/MAP kinase kinase 1	Ab32091 (Rabbit monoclonal)	1:5000	Ab97069	1:5000
MEK2	Dual specificity mitogen-activated protein kinase kinase 2/MAP kinase kinase 2	Ab140372 (Mouse monoclonal)	1:400	Ab97046	1:5000
NLK	*Nemo-Like Kinase*	Ab97642 (Rabbit polyclonal)	1:1000	Ab97069	1:5000
PIK3IP1	Phosphoinositide-3-kinase interacting protein 1	Ab87094 (Rabbit polyclonal)	1:1000	Ab97069	1:5000
PKC gamma	Protein kinase C gamma	Ab71558 (Rabbit polyclonal)	1:5000	Ab97069	1:5000
PLK1	Polo-like-Kinase 1	Ab17056 (Mouse monoclonal)	1:1000	Ab97046	1:5000
PP2A alpha and beta	Protein phosphatase 2 a	Ab32141 (Rabbit monoclonal)	1:5000	Ab97069	1:5000
PPP1A	protein phosphatase 1, catalytic subunit, alpha isozyme	Ab16476 (Sheep polyclonal)	1:1000	Ab6747	1:5000
PTEN	Phosphatase and tensin homolog	Ab32199 (Rabbit monoclonal)	1:500	Ab97069	1:5000
TAK1	Transforming growth factor beta-activated kinase 1	Ab109526 (Rabbit monoclonal)	1:5000	Ab97069	1:5000

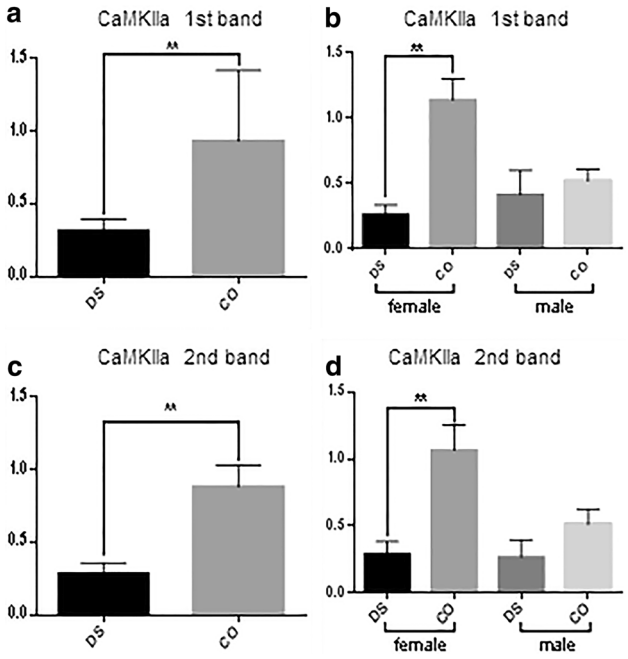

Fig. 1 Graphical demonstration of semi-quantitative analysis of optical density of CAMKIIa (Phospho T286). 1st (**a**, **b**) and 2nd band (**c**, **d**) given as Mean and SEM. *p* values are from the Mann–Whitney test (**a** + **c**) and from the Kruskal–Wallis statistic (**b** + **d**). **a** Difference between Down syndrome and control. $^{**}p \leq 0.005$. **b** Difference between Down syndrome and control according to gender. $^{**}p \leq 0.005$. **c** Difference between Down syndrome and control. $^{**}p \leq 0.005$. **d** Difference between Down syndrome and control according to gender. $^{**}p \leq 0.005$. Down syndrome (*DS*) ($n = 12$), control (*CO*) brain ($n = 12$)

Statistical analysis

Densitometry analysis was carried out using Image Lab 5.0 (Bio-Rad). GraphPad Prism 6 program has been used for statistical analysis of densitometry data. Differences between DS and control brain were analyzed by Mann–Whitney *U* test. In case of significant differences between both groups, differences in gender have been quantified to contribute to the changes by performing ANOVA (analysis of variance) test, Kruskal–Wallis test followed by Dunn's multiple comparisons test. Pearson test was performed to detect correlation between PTEN and drebrin. Data presented are mean and SEM. After quantification, protein samples were normalized to proteins (38–102 kDa) detected on the same gel after Coomassie-blue staining (Welinder and Ekblad 2011; Lee et al. 2013).

Results

Calcium–calmodulin kinase II alpha (CaMKIIa) phosphorylated on T286: CaMKIIa (Phospho T286) was presented with two bands on WB at 50 and 54 kDa.

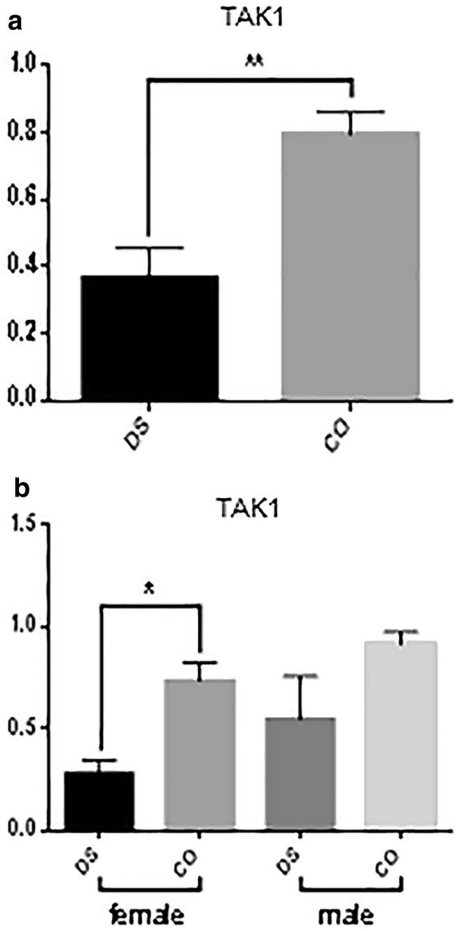

Fig. 2 Graphical demonstration of semi-quantitative analysis of optical density of TAK1. Given as Mean and SEM. p values are from the Mann–Whitney test (**a**) and from the Kruskal–Wallis statistic (**b**). **a** Difference between Down syndrome and control. $^{**}p \leq 0.005$. **b** Difference between Down syndrome and control according to gender. $^{*}p \leq 0.05$. Down syndrome (*DS*) ($n = 12$), control (*CO*) brain ($n = 12$)

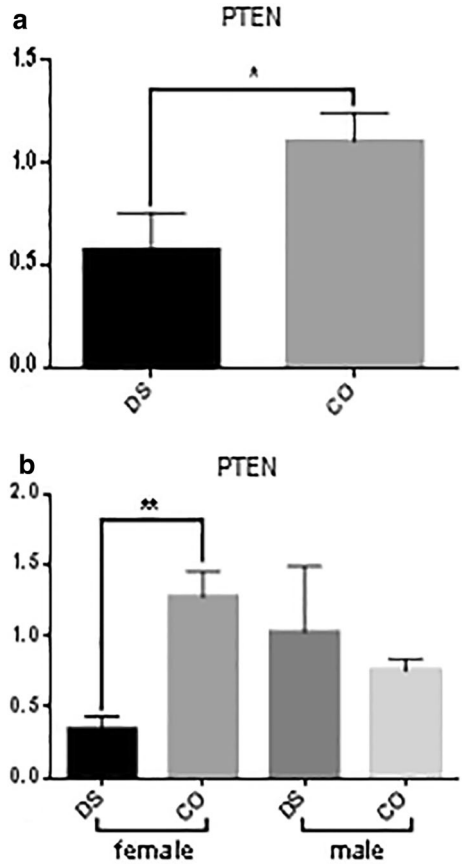

Fig. 3 Graphical demonstration of semi-quantitative analysis of optical density of PTEN. Given as Mean and SEM. p values are from the Mann–Whitney test (**a**) and from the Kruskal–Wallis statistic (**b**). **a** Difference between Down syndrome and control. $^{*}p \leq 0.05$. **b** Difference between Down syndrome and control according to gender. $^{**}p \leq 0.005$. Down syndrome (*DS*) ($n = 12$), control (*CO*) brain ($n = 12$)

As shown in Fig. 1a, c, levels for CaMKIIa (Phospho T286) showed highly significant reduced levels in the DS cohort (males and females) compared to controls (males and females). In Fig. 1b and d, gender differences were revealed: while both bands representing CaMKIIa (Phospho T286) in the female panels were significantly decreased, there was no difference between male controls and DS. Figure 1 represents the mean arbitrary unit of optical density over densitometry of bands from the individual lanes.

PTEN was presented by a single band on WB at approximately 54kDA.

As shown in Fig. 2a, levels for PTEN showed highly significant reduced levels in the DS cohort (males and females) compared to controls (males and females). In Fig. 2b, gender differences were revealed: while the band representing PTEN in the female panels was significantly decreased, there was no difference between male controls and DS. Figure 2 represents the mean arbitrary unit of optical density over densitometry of bands from the individual lanes.

Transforming growth factor beta-activated kinase 1 (TAK1) was presented by a single band on WB at approximately 75 kDa.

As shown in Fig. 3a, levels for TAK1 showed highly significant reduced levels in the DS cohort (males and females) compared to controls (males and females). In Fig. 3b, gender differences were revealed: while the band representing TAK1 in the female panels was significantly decreased, there was no difference between male controls and DS. Figure 3 represents the mean arbitrary unit of optical density over densitometry of bands from the individual lanes.

Table 2 Data from the protein kinases and interacting proteins without statistical significance

Name	Full name	Mean ± SEM DS	Mean ± SEM CO
CaMKIV	Ca^{2+}/calmodulin-dependent protein kinase IV	0.88 ± 0.20	0.40 ± 0.07
LCK (phospho Y505)	lymphocyte-specific protein tyrosine kinase	1.15 ± 0.17	1.61 ± 0.31
MEK1	Dual specificity mitogen-activated protein kinase kinase 1/MAP kinase kinase 1	0.69 ± 0.11	0.82 ± 0.17
MEK2	Dual specificity mitogen-activated protein kinase kinase 2/MAP kinase kinase 2	2.34 ± 0.57	2.36 ± 0.47
NLK	Nemo-like Kinase	1.84 ± 0.21	1.52 ± 0.13
PIK3IP1	phosphoinositide-3-kinase interacting *protein* 1	0.92 ± 0.31	1.06 ± 0.29
PKC gamma	Protein kinase C gamma	1.80 ± 0.27	2.44 ± 0.35
PLK1	Polo-like-Kinase 1	0.34 ± 0.08	0.53 ± 0.11
PP2A alpha and beta	Protein phosphatase 2 alpha and beta	0.89 ± 0.09	1.11 ± 0.12
PPP1A	protein phosphatase 1, catalytic subunit, alpha isozyme	0.72 ± 0.07	0.67 ± 0.11

Given as mean and SEM

DS down syndrome, *CO* control brain

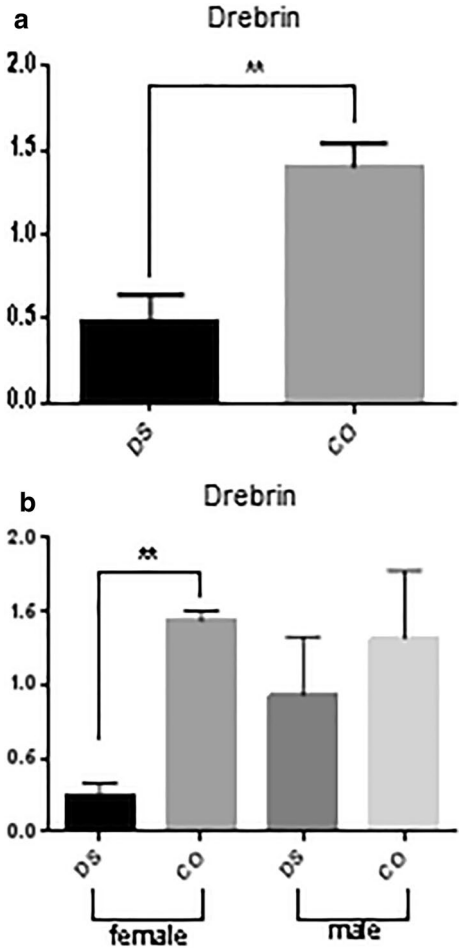

Fig. 4 Graphical demonstration of semi-quantitative analysis of optical density of Drebrin. Given as Mean and SEM. *p* values are from the Mann–Whitney test (**a**) and from the Kruskal–Wallis statistic (**b**). **a** Difference between Down syndrome and control. $^{**}p \leq 0.005$. **b** Difference between Down syndrome and control according to gender. $^{**}p \leq 0.005$. Down syndrome (*DS*) ($n = 12$), control (*CO*) brain ($n = 12$)

Additional results on protein kinases and phosphatases that were comparable between groups are provided in Table 2.

Information by networking indicated a link between PTEN and drebrin and, therefore, this actin-bundling protein was determined.

Drebrin was presented by a single band on WB at approximately 100 kDa.

As shown in Fig. 4a, drebrin levels were highly significantly reduced in the DS cohort (males and females) compared to controls (males and females).

In Fig. 4b, gender differences were revealed: while the band representing drebrin in the female panels was significantly decreased, there was no difference between male controls and DS. Figure 4 represents the mean arbitrary unit of optical density over densitometry of bands from the individual lanes.

Drebrin was significantly correlating with PTEN levels indicating functional or physical interaction ($r = 0.569$; $P = 0.0041$).

Immunoblots of the above-mentioned significant kinases, phosphatase and drebrin are given in Fig. 5.

WB identification and quantification of binding partners proposed by IntAct (nemo-like kinase NLK; protein phosphatase PPP1A; phosphoinositol-3-kinase interacting protein) showed that levels were comparable between DS and controls (Table 2).

Discussion

The major finding of the study proposes changes of CaMKIIa (Phospho T286), a major protein kinase known to be involved in synaptic plasticity and cognitive-function, protein kinase TAK1 and a protein phosphatase PTEN.

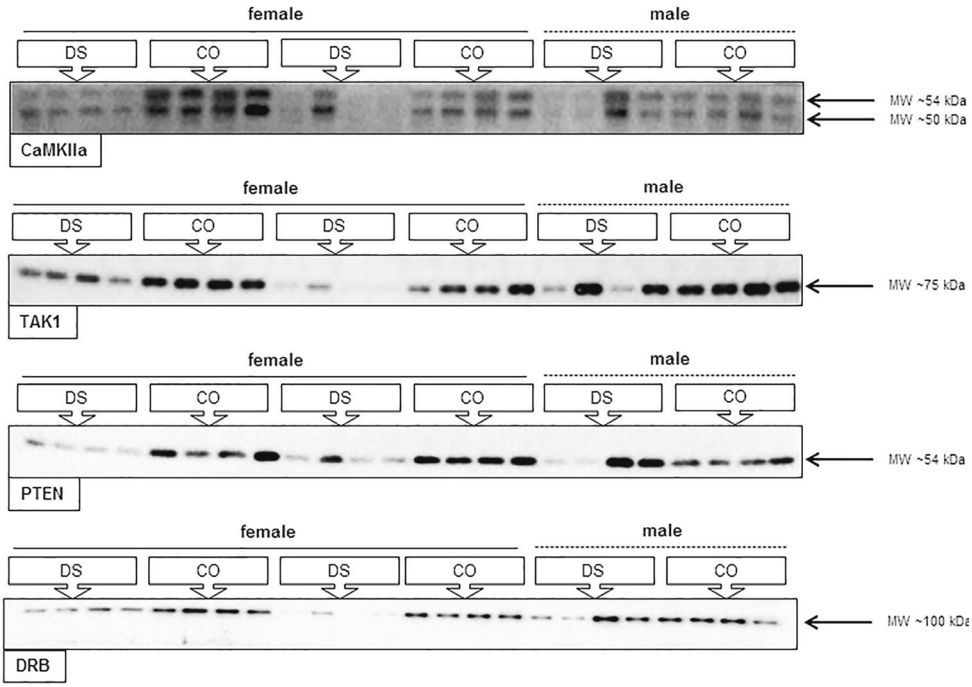

Fig. 5 Representative immunoblot images of the significant protein kinases and phosphatase and drebrin in Down syndrome and control brain. Target bands at the expected apparent molecular weight were observed. *CaMKIIa* Ca^{2+}/calmodulin-dependent protein kinase II a, *PTEN* phosphatase and tensin homolog, *TAK1* transforming growth factor beta-activated kinase 1, *DRB* drebrin. Down syndrome (*DS*) ($n = 12$), control (*CO*) brain ($n = 12$)

These signaling compounds have never been reported in fetal DS brain and own previous work demonstrating that dendritic spine protein drebrin is reduced in fetal DS is confirmed herein. Moreover, drebrin correlated with PTEN levels, as shown by Pearson correlation test, providing evidence for physical interaction or even common pathways.

CaMKIIa in its phosphorylated form (Phospho T286) (Lucic et al. 2008; Barcomb et al. 2014) was significantly reduced in fetal DS brain and may be compatible with impaired cognitive functions in DS as CaMKII is a prominent kinase in the central nervous system that is involved in long-term potentiation and neurotransmitter release (Hinds et al. 2003; Stein et al. 2003; Tao-Cheng et al. 2006; Buard et al. 2010; Lisman et al. 2012; Coultrap et al. 2014). As a member of the NMDAR (N-methyl-D-aspartate receptor) signaling complex in excitatory synapses, it may regulate NMDAR-dependent potentiation of the AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and synaptic plasticity (Gustin et al. 2011, Sanhueza and Lisman 2013). In addition, in an animal model of DS (mBACtgDyrk1a mice), the phosphorylated/non-phosphorylated CaMKIIa ratio was markedly reduced (Thomazeau et al. 2014).

TAK1 levels were about twofold reduced and Yu et al. (2014) have shown that TAK1, activated by TGF-β signaling controls axonal growth during brain development. Furthermore, it was proposed that TAK1 as a member of the mitogen-activated protein kinase kinase kinase family is a key regulator in apoptotic signaling pathways (Zhang et al. 2013). Although speculative, one may consider reduced TAK1 levels in fetal DS brain as one of the signaling proteins in brain development preceding neurodegeneration that occurs in fetal DS from the third trimester (Unterberger et al. 2003).

Herein, reduced cortical levels of PTEN were observed in fetal DS brain and indeed, conditional deletion of PTEN impairs synaptic transmission and synaptic plasticity at excitatory synapses in the hippocampus suggesting that PTEN may be involved in mechanisms that control development of neuronal and synaptic structures and subsequently synaptic function (Fraser et al. 2008). This would be in line with the eminently important function of protein phosphatases as key signaling molecules for LTP and cognitive functions *per se* and may be relevant as a protein phosphatase preceding histopathological changes observed later in prenatal development of the brain in DS. Drebrin was predicted by database IntAct to interact with PTEN: phosphorylation/de-phosphorylation of actin-bundling protein drebrin, thus forming dendritic spines is mediated by neuronal activity and PTEN at S647 (Kreis et al. 2013) and drebrin with subsequent synaptic plasticity may be, therefore, regulated by PTEN. This data is now supported in the herein shown significant correlation of PTEN and drebrin levels.

In previous work, reduced cortical levels of drebrin in DS fetal brain were reported using a gel-based proteomics method (Weitzdoerfer et al. 2001a), but this finding could not be supported by an immunohistochemical approach in a comparable panel (Unterberger et al. 2003).

A series of gender-dependent changes was observed and it is worth mentioning that reduction of the above-mentioned proteins was demonstrated in DS females. There is information that gender differences in brain development and synaptic composition (LaVeck and LaVeck 1977; Downes et al. 2008) as well as onset of Alzheimer-like neuropathology (Raghavan et al. 1994; Schupf et al. 1998; Lai et al. 1999) in DS can be observed.

Conclusion

In the current study, evidence for deranged protein kinase and phosphatase levels in fetal DS cortex was provided and significant gender differences were observed.

Moreover, protein phosphatase PTEN was correlating with a dendritic spine protein, drebrin, which has been already shown to be decreased in fetal DS brain in previous work (Weitzdoerfer et al. 2001a). Findings herein are relevant for interpretation on previous studies as well as for the design of future work on signaling proteins in DS brain.

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

Ethical approval and informed consent All procedures performed in studies involving human samples were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All samples were obtained by THE CIBERER BIOBANK (CBK), which is a public, non-profit-making biobank that was set up by the Biomedical Network Research Centre for Rare Diseases (CIBERER), located at the Centro de Investigación en Salud Pública (CSISP). Ethics Committee. This committee has the task of guaranteeing compliance with the ethical principles applicable to biomedical research projects incorporating human origin samples of the CBK, as well as the use made of these. CBK is attached to the Ethics Committee of the CSISP.

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