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Nerve growth factor metabolic dysfunction in Down's syndrome brains

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Basal forebrain cholinergic neurons play a key role in cognition. This neuronal system is highly dependent on NGF for its synaptic integrity and the phenotypic maintenance of its cell bodies. Basal forebrain cholinergic neurons progressively degenerate in Alzheimer's disease and Down's syndrome, and their atrophy contributes to the manifestation of dementia. Paradoxically, in Alzheimer's disease brains, the synthesis of NGF is not affected and there is abundance of the NGF precursor, proNGF. We have shown that this phenomenon is the result of a deficit in NGF's extracellular metabolism that compromises proNGF maturation and exacerbates its subsequent degradation. We hypothesized that a similar imbalance should be present in Down's syndrome. Using a combination of quantitative reverse transcription-polymerase chain reaction, enzyme-linked immunosorbent assay, western blotting and zymography, we investigated signs of NGF metabolic dysfunction in post-mortem brains from the temporal ($n = 14$), frontal ($n = 34$) and parietal ($n = 20$) cortex obtained from subjects with Down's syndrome and age-matched controls (age range 31–68 years). We further examined primary cultures of human foetal Down's syndrome cortex (17–21 gestational age weeks) and brains from Ts65Dn mice (12–22 months), a widely used animal model of Down's syndrome. We report a significant increase in proNGF levels in human and mouse Down's syndrome brains, with a concomitant reduction in the levels of plasminogen and tissue plasminogen activator messenger RNA as well as an increment in neuroserpin expression; enzymes that partake in proNGF maturation. Human Down's syndrome brains also exhibited elevated zymogenic activity of MMP9, the major NGF-degrading protease. Our results indicate a failure in NGF precursor maturation in Down's syndrome brains and a likely enhanced proteolytic degradation of NGF, changes which can compromise the trophic support of basal forebrain cholinergic neurons. The alterations in proNGF and MMP9 were also present in cultures of Down's syndrome foetal cortex; suggesting that this trophic compromise may be amenable to rescue, before frank dementia onset. Our study thus provides a novel paradigm for cholinergic neuroprotection in Alzheimer's disease and Down's syndrome.

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Keywords: Down's syndrome; Alzheimer's disease; basal forebrain cholinergic neurons; proNGF; matrix metallo-protease 9

Abbreviations: IOD = integrated optical density; MMP2 = matrix metallo-protease 2; MMP9 = matrix metallo-protease 9; proNGF = pro-nerve growth factor; TIMP1 = tissue inhibitor of metalloproteases; tPA = tissue plasminogen activator

Introduction

It is well established that individuals with Down's syndrome are at increased risk of developing premature ageing and Alzheimer's disease dementia (Lott, 2012). Amyloid-β peptides progressively deposit in Down's syndrome brains from early life (Lemere *et al.*, 1996; Mori *et al.*, 2002) and recent studies have shown accumulation of Pittsburgh compound B-positive amyloid plaques in subjects with Down's syndrome already in their 30 s (Handen *et al.*, 2012). By middle-age (40–60 years) almost all Down's syndrome sufferers have the neuropathological hallmarks characteristic of Alzheimer's disease, including senile amyloid plaques surrounded by dystrophic neurites and neurofibrillary tangles (Wisniewski *et al.*, 1985; Mann, 1988). Thus, Down's syndrome brains represent a unique opportunity to explore the molecular changes accompanying the over-production of APP and of its amyloidogenic peptide products.

A major consequence of Alzheimer's disease and Down's syndrome pathology is basal forebrain cholinergic neuron degeneration (Bowen *et al.*, 1976; Davies and Maloney, 1976; Yates *et al.*, 1980; Whitehouse *et al.*, 1982). These cells are highly dependent on target-derived NGF for the phenotypic maintenance of their cell bodies and synaptic integrity at post-natal stages (Thoenen, 1995; Debeir *et al.*, 1999; Sofroniew *et al.*, 2001). In consequence, it has been hypothesized that the atrophy of these neurons in Alzheimer's disease was caused by NGF deficits. However, in post-mortem Alzheimer's disease brains the synthesis of NGF is not affected (Goedert *et al.*, 1986; Jette *et al.*, 1994; Fahnestock *et al.*, 1996) and the levels of its precursor (proNGF) are increased (Fahnestock *et al.*, 2001; Pedraza *et al.*, 2005; Bruno *et al.*, 2009*a*).

Recent data from our lab demonstrated that upon neuronal activity, proNGF is released to the extracellular space, along with the enzymes necessary for its conversion to mature NGF and for its subsequent degradation (Bruno and Cuello, 2006). ProNGF is cleaved and matured by plasmin, which derives from plasminogen by the action of tissue plasminogen activator (tPA). tPA activity is inhibited by neuroserpin in the CNS (Miranda and Lomas, 2006). MMP9 is the main mature NGF degrading enzyme (Bruno and Cuello, 2006). In post-mortem Alzheimer's disease brains, we have shown that there is accumulation of proNGF as a result of a failure in its maturation, as well as increased MMP9 activity (Bruno *et al.*, 2009a). Notably, these changes are also present in mild cognitive impairment brains, a stage in which the increase in proNGF and MMP9 activity positively correlates with the degree of pre-mortem cognitive decline (Peng *et al.*, 2004; Bruno *et al.*, 2009*b*).

Thus, the above opens the question whether an analogous compromise in NGF metabolism would occur in Down's syndrome. In consequence, we embarked on an extensive study involving post-mortem adult Down's syndrome brains, primary cultures of human foetal Down's syndrome cortex and brains from a widely used Down's syndrome mouse model (Lockrow *et al.*, 2012). In line with our findings in Alzheimer's disease and mild cognitive impairment, we report marked deficits in NGF metabolism in Down's syndrome brains, evidenced by increased MMP9 activity and proNGF accumulation. These alterations were accompanied by increased neuroserpin and reduced plasminogen levels and tPA synthesis, changes that will impair the maturation of proNGF. In addition, there is a probable enhanced degradation of mature NGF, reflected by the upregulation of MMP9 activity in human Down's syndrome brains and by the reduction of mature NGF levels in the brains of Ts65Dn mice. Our results may offer new opportunities to prevent or decelerate cholinergic neurodegeneration in individuals with Down's syndrome-related Alzheimer's disease.

Materials and methods

Human brain tissue

Frozen grey matter tissue from the temporal ($n = 14$), frontal ($n = 20$) and parietal ($n = 20$) cortices of adult Down's syndrome subjects and age-matched control cases were obtained from the Alzheimer's Disease Research Centre, University of California, Irvine. Additional frontal cortex tissue ($n = 14$) was obtained from New York University School of Medicine. These additional samples did not result in any demographic difference such as post-mortem interval or age between the three regions studied. For further demographic information see Supplementary Table 1. Clinico-pathological analysis, performed at the respective research centres in Irvine and New York, revealed the presence of Alzheimer's disease dementia, confirmed by the widespread deposition of senile plaques and neurofibrillary tangles in all Down's syndrome brains included in this study. Diagnosis of Alzheimer's disease in Down's syndrome subjects was done following neurological examination using DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, American Psychiatric Association) and ABC criteria (Montine *et al.*, 2012) as well as considering medical and behavioural history obtained through a knowledgeable informant such as a parent/caregiver. Control cases showed no evidence of chromosomal or neuropathological abnormalities and displayed no signs of cognitive decline. The project has been approved by the McGill University Research Ethic Board.

Mixed primary cultures

Primary cultures were established from the cerebral cortex of normal and Down's syndrome foetuses, following previously described protocols (Kerkovich *et al.*, 1999; Pelsman *et al.*, 2003; Helguera *et al.*, 2013) and detailed in the Supplementary material. The protocols for obtaining post-mortem foetal brain complied with all US federal and institutional guidelines, with special respect for donor identity confidentiality and informed consent.

Ts65Dn mice

Hippocampus and frontal cortex tissue from trisomic and age-matched (12–22 months) normosomic littermates were used. See Supplementary material for further details.

Amyloid-β ELISA

Quantification of amyloid-β_{40} and amyloid-β_{42} levels was performed using human amyloid-β ELISAs (Invitrogen), as described in the Supplementary material.

Western blotting

Western blotting was done on human and mouse brain homogenates, conditioned media and cell lysates to examine the expression of APP, NGF, plasminogen, tPA and neuroserpin, as detailed in the Supplementary material.

Real-time polymerase chain reaction

Relative messenger RNA levels were quantified by quantitative real-time-PCR from human and mouse brain homogenates, as described in the Supplementary material.

Gelatin and casein zymography

MMP9/2, and tPA activity were determined by gelatin (MMPs) and casein (tPA) zymography as previously described (Bruno *et al.*, 2009*a*, *b*; Fabbro and Seeds, 2009) and detailed in the Supplementary material.

Statistical analysis

Two-group comparisons were analysed with a 2-tailed Student's *t*-test. Spearman rank analysis was used for correlations (Graph Pad Prism 5.01). Significance was set at $P < 0.05$. Error bars represent mean ± SEM. All experiments were run in triplicate.

Results

Analysis of APP and amyloid-β peptides in adult Down's syndrome brains

To establish whether NGF metabolic deregulation occurs in other amyloid-β pathologies than Alzheimer's disease and mild cognitive impairment, we first confirmed the presence of APP and amyloid-β neuropathology in our cohort of adult Down's syndrome brains. Down's syndrome brains exhibited a significant increase in APP levels (~2.5-fold) in temporal (Fig. 1A), frontal (Fig. 1B) and parietal cortex (Fig. 1C) compared with control cases, in agreement with previous reports (Cheon *et al.*, 2008). Amyloid-β_{40} and amyloid-β_{42} peptides were also highly elevated in Down's syndrome brains (~3–20-fold, depending on the brain region). In temporal cortex, Down's syndrome cases exhibited mean concentrations of amyloid-β_{40} and amyloid-β_{42} of 7.97 and 7.65 μg/g tissue, respectively (Fig. 1D). In frontal cortex, amyloid-β expression was more robust, with mean concentrations of amyloid-β_{40} and amyloid-β_{42} of 19.02 and 9.76 μg/g tissue, respectively (Fig. 1E). Amyloid-β expression was lower in parietal cortex, with mean concentrations of amyloid-β_{40} and amyloid-β_{42} of 4.47 and 2.11 μg/g tissue (Fig. 1F), respectively.

Increased NGF precursor levels in Down's syndrome brains

We next investigated whether post-mortem Down's syndrome brains exhibit proNGF accumulation similar to mild cognitive impairment and Alzheimer's disease brains. The ~32 kDa band quantified here corresponded to that previously identified as proNGF (Fahnestock *et al.*, 2001; Bruno *et al.*, 2009*a*). The immunoreaction was abolished by pre-adsorbing the primary antibody with a proNGF peptide supplied by the manufacturer. ProNGF levels were significantly higher (~2–3 fold) in Down's syndrome temporal (Fig. 2A), frontal (Fig. 2B), and parietal cortex (Fig. 2C). This accumulation was not caused by increased transcription, as NGF messenger RNA levels were not significantly different between control and cases with Down's syndrome, in all regions investigated (Supplementary Fig. 1A–C).

In temporal and frontal cortex, proNGF positively correlated with APP (Fig. 2D and F) and with amyloid-β_{42} levels (Fig. 2E and G). In parietal cortex, there was no significant correlation between proNGF and increased APP expression ($r = 0.309$, $P > 0.05$) or amyloid-β_{42} peptides ($r = 0.342$, $P > 0.05$). These findings suggest a region-specific relationship between the amount of APP and amyloid-β peptides and reduced proNGF processing.

Increased MMP9 activity and TIMP1 expression in Down's syndrome brains

Despite no apparent changes in MMP9 messenger RNA expression between controls and cases with Down's syndrome (Supplementary Fig. 1D–F), we observed an increased MMP9 precursor and MMP9 activity in Down's syndrome temporal (Fig. 3A), frontal (Fig. 3B) and parietal cortex (Fig. 3C). No significant changes in MMP2 zymogenic activity were observed in adult Down's syndrome brains in all areas investigated (Fig. 3A–C).

We found strong associations between MMP9 activity, amyloid-β pathology and proNGF. In temporal cortex, analysis revealed a significant correlation between MMP9 activity and amyloid-β_{42} (Fig. 3D), as well as a strong association between proNGF levels and MMP9 (Fig. 3E). This pattern followed in frontal cortex. MMP9 activity correlated with APP (Fig. 3F), amyloid-β_{42} (Fig. 3G) and proNGF expression (Fig. 3H). We further observed a significant increase (~3-fold) in TIMP1 messenger RNA levels, the endogenous MMP9 inhibitor, in Down's syndrome frontal (Fig. 3J) and parietal cortex (Fig. 3K). TIMP1 messenger RNA positively correlated with MMP9 activity in frontal cortex (Fig. 3I) but not in parietal cortex ($r = 0.093$, $P > 0.05$). A trend reflecting higher TIMP1 expression was observed in Down's syndrome temporal cortex (Supplementary Fig. 2A). There was also a strong association between MMP9 activity and TIMP1 in this region (Supplementary Fig. 2B), excluding one Down's syndrome case with high MMP9 activity and a significant medical history of

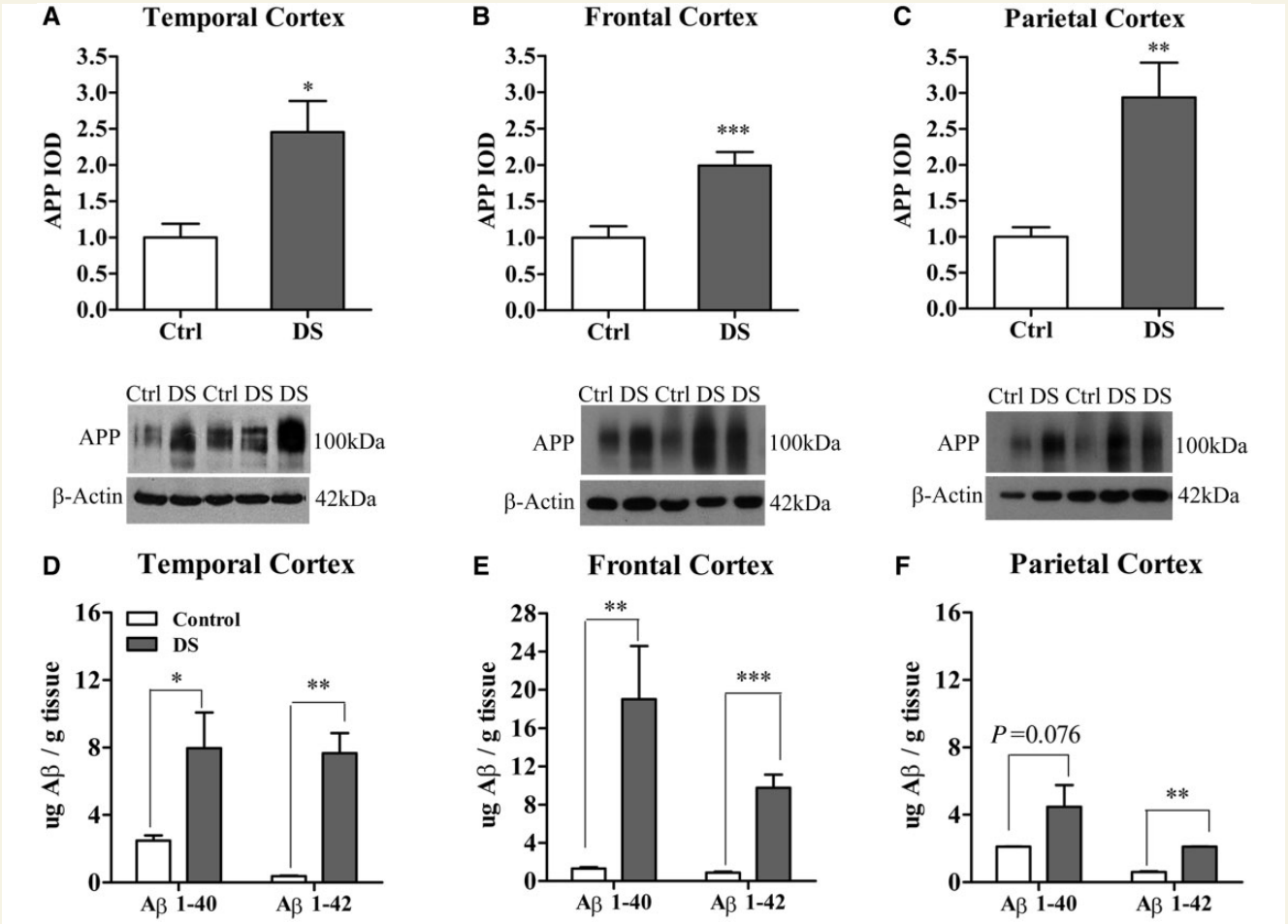

Figure 1 Increased APP and amyloid-β levels in Down's syndrome brains. Western blot analysis from human cortical homogenates revealed increased APP levels in Down's syndrome brains compared to control cases in (**A**) temporal ($P = 0.017$; $n = 14$), (**B**) frontal ($P = 0.0004$; $n = 34$) and (**C**) parietal ($P = 0.004$; $n = 20$) cortex. Representative immunoblots probed with 22C11 and β-actin antibodies are shown. (**D–F**) ELISA analysis of amyloid-β_{40} and amyloid-β_{42} peptides, from guanidine hydrochloride-homogenized brains. Down's syndrome brains exhibited significantly higher levels of amyloid-β_{40} and amyloid-β_{42} peptides in (**D**) temporal ($P = 0.003$, $P = 0.002$) (**E**) frontal ($P = 0.007$, $P < 0.0001$) and (**F**) parietal cortex ($P = 0.076$, $P = 0.005$). Data are expressed as μg amyloid-β/g tissue. Error bars represent mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Student's *t*-test. Aβ = amyloid-β; Ctrl = control; DS = Down's syndrome.

asthmatic bronchitis, an inflammatory disease in which strong elevations in MMP9 activity have been documented (Vignola *et al.*, 1998).

Alterations in neuroserpin, plasminogen and tPA expression in brains from subjects with Down's syndrome

Given that we observed proNGF accumulation in Down's syndrome brains we sought to establish whether Alzheimer's disease-like neurochemical changes in the plasminogen/tPA/neuroserpin proNGF-conversion pathway are also present in this condition. PCR analysis revealed significantly higher neuroserpin messenger RNA levels (~1.5-fold) in Down's syndrome temporal (Fig. 4A), frontal (Fig. 4B) and parietal cortex (Fig. 4C). Neuroserpin protein levels were accordingly elevated (Fig. 4D–F). In frontal cortex amyloid-β_{42} positively correlated with neuroserpin messenger RNA (Fig. 4G) and protein levels (Fig. 4H).

The neuroserpin upregulation in Down's syndrome was accompanied by marked reductions in tPA messenger RNA levels (~70% decrease) in frontal (Fig. 4I) and parietal cortex (Fig. 4J). No significant changes in tPA messenger RNA levels were detected in temporal cortex (Supplementary Fig. 1G). Plasminogen levels were also compromised in Down's syndrome brains, as evidenced by lower protein expression (~40% reduction) in frontal (Fig. 4K), parietal (Fig. 4L) and a strong trend in temporal cortex (Supplementary Fig. 2C). We did not observe any significant reduction in plasminogen messenger RNA synthesis in Down's syndrome temporal (Supplementary Fig. 2D), frontal (Supplementary Fig. 2E) or parietal cortex (Supplementary Fig. 2F).

Importantly, we verified that alterations in NGF metabolism were not influenced by differences in post-mortem interval. We found no significant correlation between protein, messenger RNA

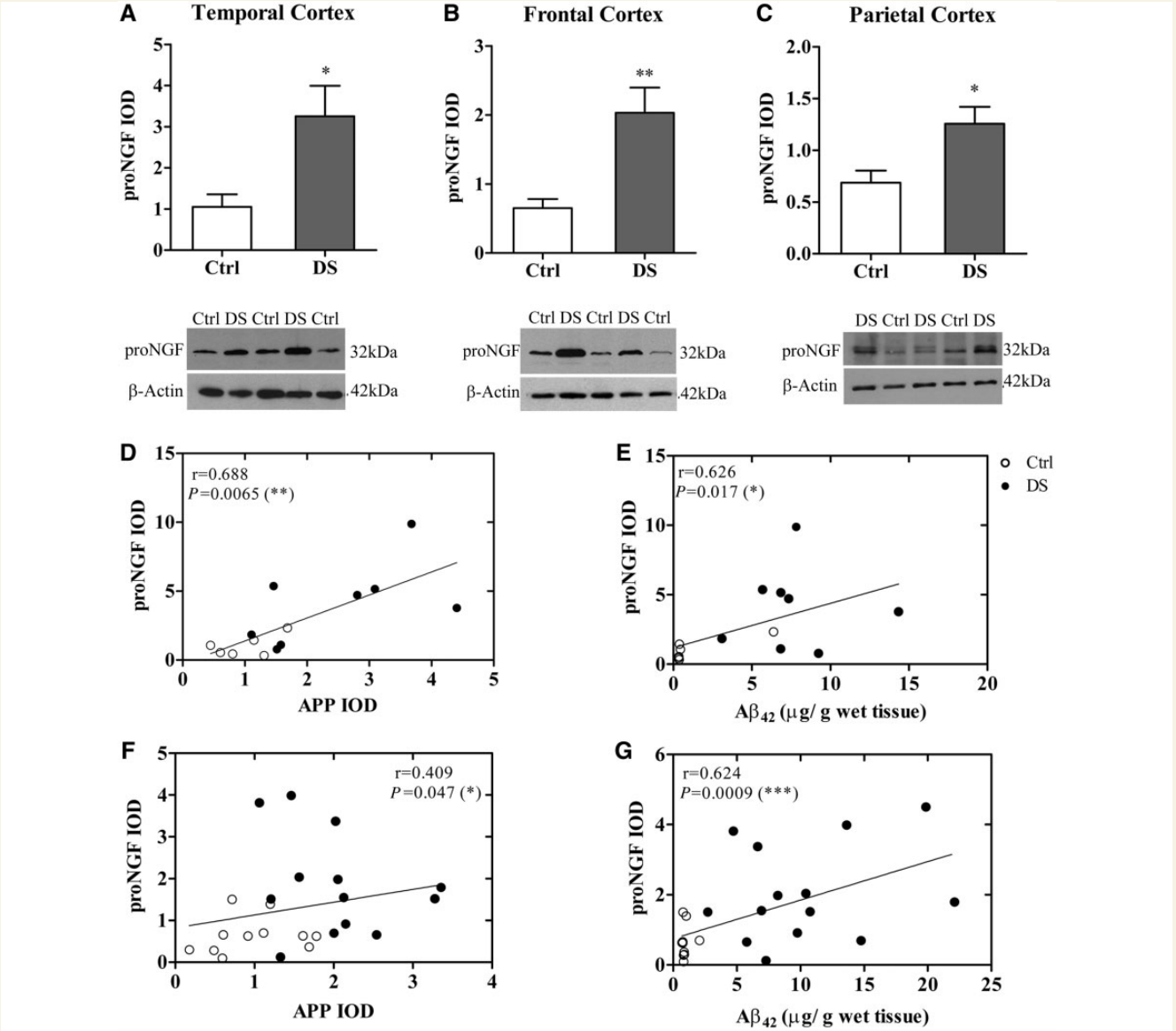

Figure 2 Increased proNGF levels in Down's syndrome brains. Western blot analysis of proNGF in cortical brain homogenates. Down's syndrome subjects exhibited significantly higher proNGF levels compared to age-matched control cases in (**A**) temporal ($P = 0.026$), (**B**) frontal ($P = 0.004$) and (**C**) parietal ($P = 0.018$) cortex. Representative immunoblots probed with proNGF and β-actin antibodies are shown. (**D**–**G**) Scattergrams showing positive correlation between proNGF and APP in (**D**) temporal ($r = 0.688$, $P = 0.007$) and (**F**) frontal cortex ($r = 0.409$, $P = 0.047$) and between proNGF and amyloid-β_{42} in (**E**) temporal ($r = 0.626$, $P = 0.017$) and in (**G**) frontal cortex ($r = 0.629$, $P = 0.001$). Error bars represent mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Student's *t*-test; Spearman Rank analysis for correlations. Aβ = amyloid-β; DS = Down's syndrome.

or enzymatic activity of NGF pathway markers and post-mortem interval, in all of the cortical regions investigated.

Analysis of soluble APP and amyloid-β peptides in primary cultures of Down's syndrome foetal cortex

We observed a significant increase in soluble APP (Supplementary Fig. 3A) and soluble APP-β levels (~2.5 fold) in Down's syndrome conditioned media compared with control cultures (Fig. 5A). This was accompanied by higher levels of secreted amyloid-β_{40} (Fig. 5B) and amyloid-β_{42} (Fig. 5C) peptides.

Alterations in NGF pathway markers in primary cultures of Down's syndrome foetal cortex

In line with our results in adult brains, proNGF levels were significantly increased in Down's syndrome conditioned media (Fig. 5D). The specificity of the ~40 kDa band corresponding to secreted

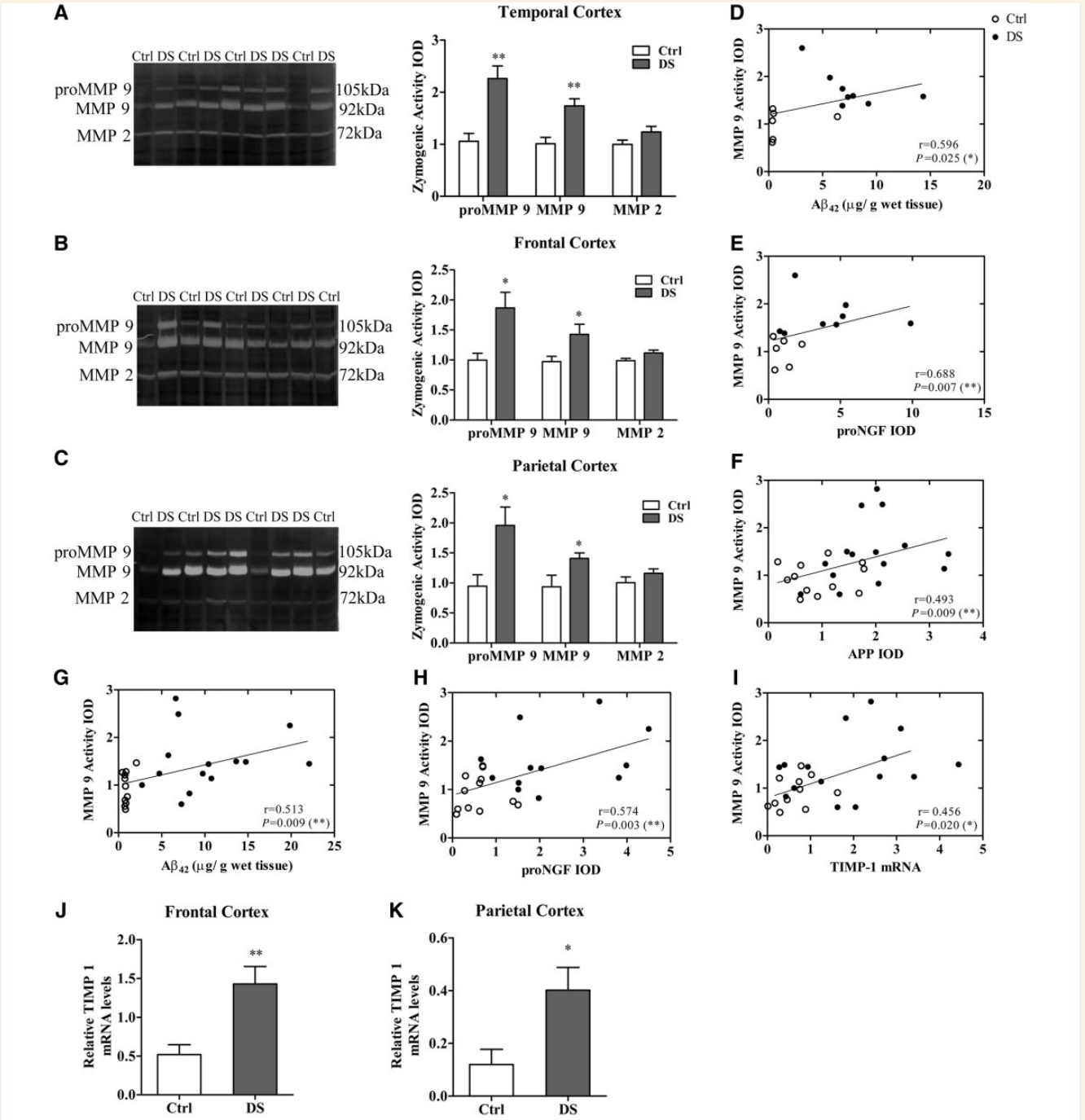

Figure 3 Increased MMP9 activity and TIMP1 expression in Down's syndrome brains. **A–C**) Representative gelatin zymographs depicting MMP9 precursor (proMMP9), MMP9 and MMP2 proteolytic activity. Analysis revealed significantly elevated MMP9 precursor and MMP9 zymogenic activity in Down's syndrome cortical homogenates compared to control cases in (**A**) temporal ($P = 0.002$ and $P = 0.003$, respectively) (**B**) frontal ($P = 0.011$ and $P = 0.043$) and (**C**) parietal cortex ($P = 0.018$, $P = 0.039$). MMP2 activity did not differ between Down's syndrome and control subjects in none of the areas investigated. Values are expressed as fold increase versus control. Independent statistical analysis was done for each metallo-protease, comparing its levels between control and Down's syndrome cases. In temporal cortex there was a positive correlation between (**D**) MMP9 activity and amyloid-β_{42} levels ($r = 0.596$, $P = 0.025$) as well as a strong link between (**E**) MMP9 activation and proNGF accumulation ($r = 0.688$, $P = 0.007$). Correlation analysis in frontal cortex also revealed positive associations between (**F**) MMP9 zymogenic activity and APP levels ($r = 0.493$, $P = 0.009$), (**G**) MMP9 and amyloid-β_{42} ($r = 0.513$, $P = 0.009$) and (**H**) MMP9 and proNGF ($r = 0.577$, $P = 0.003$). Quantitative real-time PCR analysis revealed significantly higher TIMP1 messenger RNA levels in (**J**) frontal ($P = 0.0003$) and (**K**) parietal cortex ($P = 0.015$). TIMP1 messenger RNA expression was normalized to the housekeeping gene *HPRT*. (**I**) Scattergram showing positive correlation between MMP9 activity and TIMP1 messenger RNA levels in frontal cortex ($r = 0.457$, $P = 0.020$). Error bars represent mean ± SEM. $*P < 0.05$; $**P < 0.01$; Student's *t*-test; Spearman Rank analysis for correlations. Aβ = amyloid-β; DS = Down's syndrome.

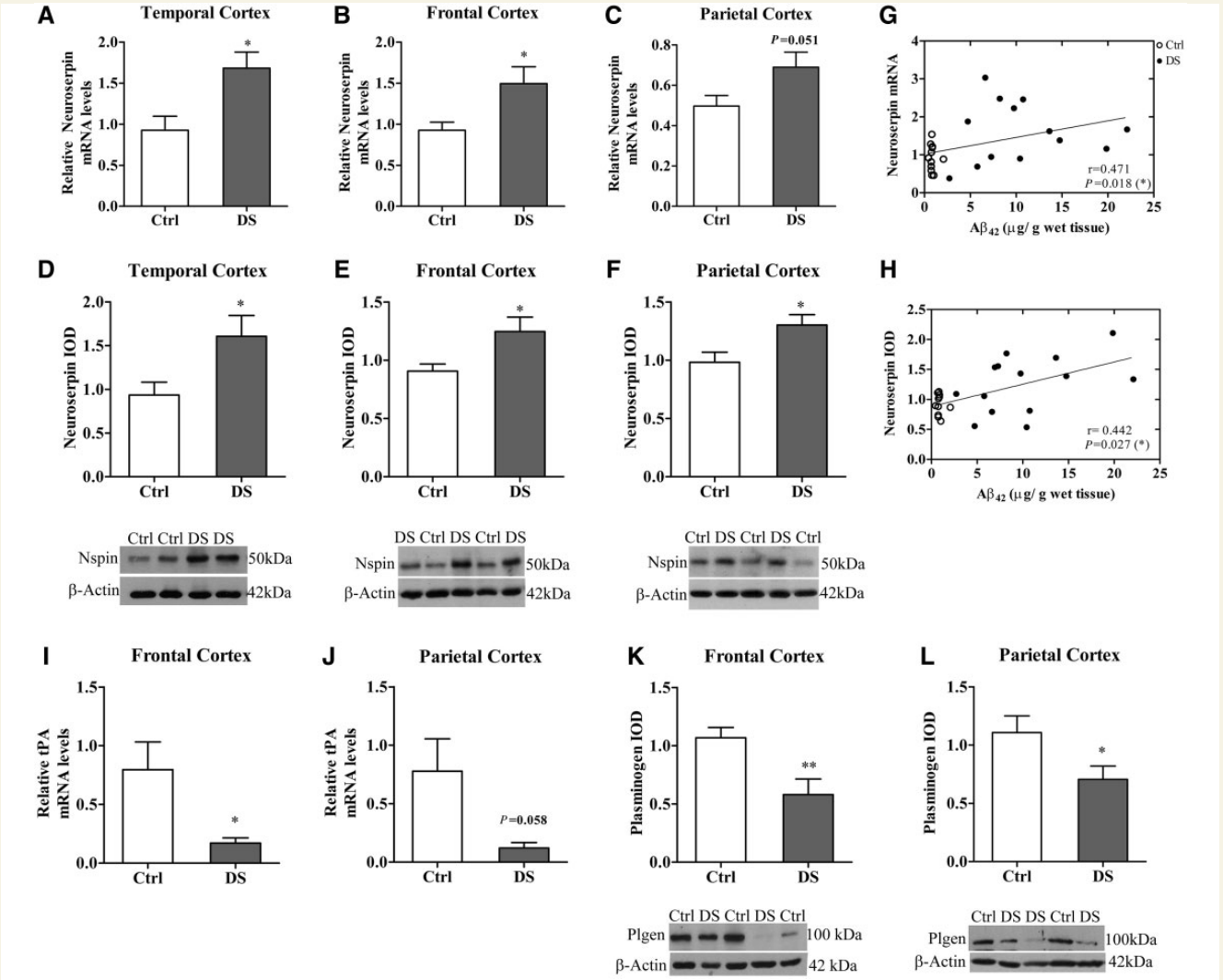

Figure 4 Alterations in neuroserpin, tPA and plasminogen in Down's syndrome brains. Down's syndrome brains exhibited significantly higher neuroserpin messenger RNA levels in (**A**) temporal ($P = 0.017$), (**B**) frontal ($P = 0.029$) and (**C**) parietal cortex ($P = 0.051$), compared with age-matched control cases. (**D–F**) Western blot analysis revealed a significant increase in neurosepin protein levels in (**D**) temporal ($P = 0.047$), (**E**) frontal ($P = 0.045$) and (**F**) parietal cortex ($P = 0.031$). (**G–H**) Scattergrams showing positive correlation between (**G**) neuroserpin messenger RNA and amyloid-β_{42} ($r = 0.471$, $P < 0.05$) and (**H**) neuroserpin protein levels and amyloid-β_{42} ($r = 0.442$, $P < 0.05$) in frontal cortex. PCR analysis revealed marked reductions in tPA messenger RNA levels in (**I**) frontal ($P = 0.016$) and (**J**) parietal ($P = 0.058$) cortex. Down's syndrome brains also exhibited reduced plasminogen protein levels in Down's syndrome (**K**) frontal ($P = 0.0079$) and (**L**) parietal cortex ($P = 0.045$). (**D–F** and **K–L**) Representative immunoblots probed with neuroserpin, plasminogen and β-actin antibodies are shown. (**A–C**, **I** and **J**) PCR data are expressed as the normalized ratio between each protein of interest and *HPRT*. Error bars represent mean ± SEM. $*P < 0.05$; $**P < 0.01$; Student's *t*-test; Spearman Rank analysis for correlations. Aβ = amyloid-β; DS = Down's syndrome.

proNGF (Bruno and Cuello, 2006) was confirmed by its disappearance after incubation of the proNGF primary antibody with a proNGF peptide, provided by the manufacturer. Down's syndrome conditioned media also exhibited marked deficits in tPA proteolytic activity (Fig. 5E), indicating a likely compromise of proNGF maturation, at this early stage.

We next examined MMP9 activity by gelatin zymography and observed a significant increase in MMP9 and MMP2 activity (Fig. 5F) in Down's syndrome conditioned media (extracellular milieu). The changes in MMP9 activity were also accompanied with increases in protein expression (Supplementary Fig. 3B). In accordance with our analysis in adult brains, MMP9 activation positively correlated with amyloid-β_{42} levels (Fig. 5H) and with increased proNGF expression (Fig. 5I). Likewise, western blot analysis revealed significantly elevated TIMP1 protein levels (~3-fold) in Down's syndrome-conditioned media compared with control cultures (Fig. 5G). There was a strong correlation between MMP9 activity and TIMP1 levels (Fig. 5J). Importantly, the changes we observed in Down's syndrome cells are not a result of increased cell death and subsequent release of cellular contents

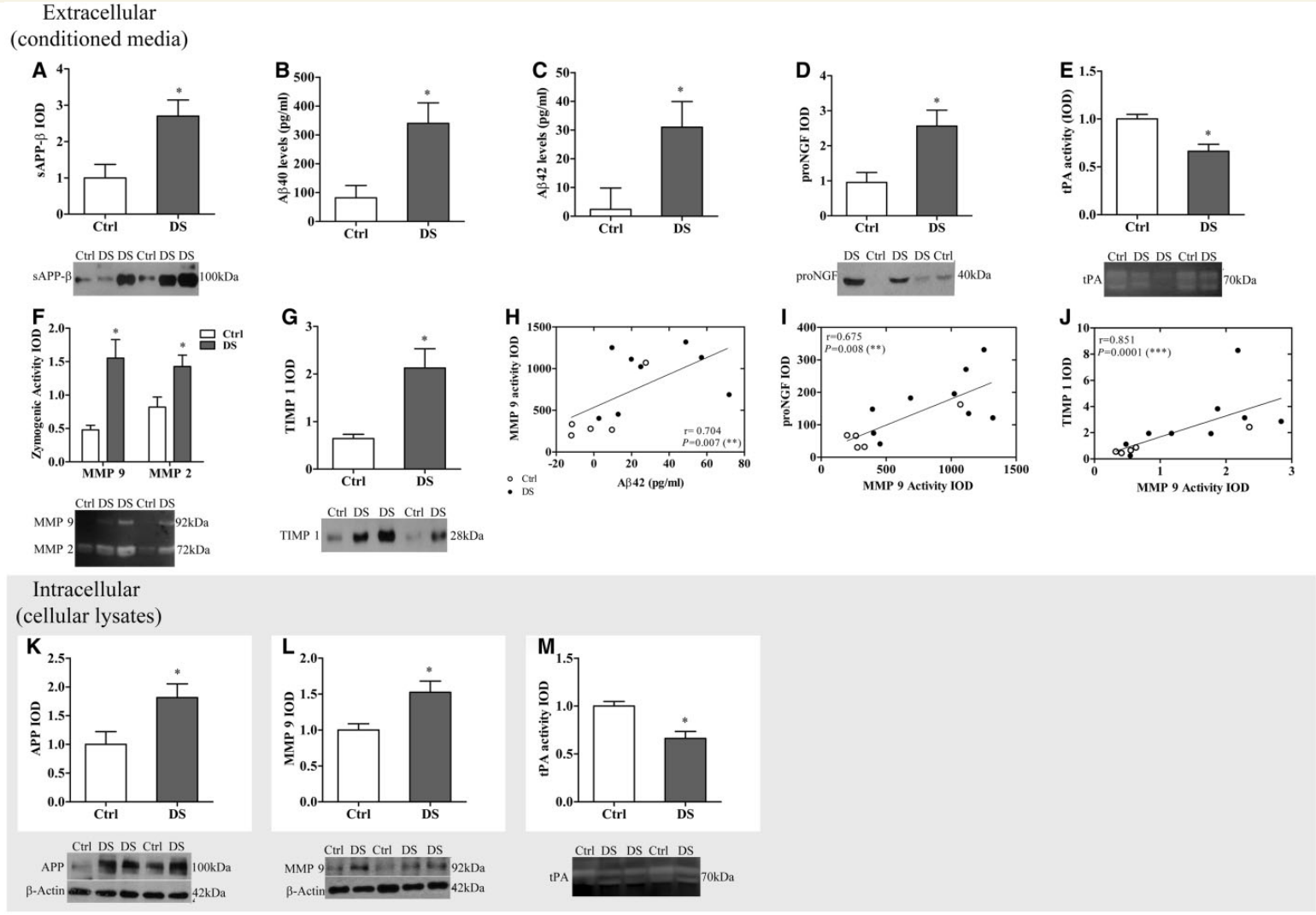

Figure 5 Early signs of NGF dysmetabolism in primary cultures from foetal Down's syndrome cortex. (**A**) Western blot analysis revealed significantly higher soluble APP-β levels ($P = 0.015$) in conditioned media from Down's syndrome cultures. Down's syndrome cultures exhibited significantly higher levels of secreted (**B**) amyloid-β_{40} ($P = 0.036$) and (**C**) amyloid-β_{42} ($P = 0.047$), compared with controls. The concentration of amyloid-β peptides is expressed in pg/ml. (**D**) Western blot analysis revealed a significant increase in proNGF levels in conditioned media from Down's syndrome cultures ($P = 0.029$). (**E**) Reduced tPA zymogenic activity in Down's syndrome conditioned media ($P = 0.046$). (**F**) The activity of MMP9 and MMP2 was significantly increased in Down's syndrome conditioned media ($P = 0.011$ and $P = 0.026$, respectively). (**H**) Scattergram showing positive correlation between MMP9 activity and amyloid-β_{42} ($r = 0.718$, $P = 0.009$). (**I**) Positive correlation between proNGF and MMP9 activity ($r = 0.676$, $P < 0.011$). (**G**) Western blot analysis revealed significantly higher TIMP1 levels in Down's syndrome conditioned media ($P = 0.029$). (**J**) Positive correlation between TIMP1 and MMP9 activity ($r = 0.851$, $P = 0.0001$). For western blot and zymography analysis, IOD values were normalized by total protein concentration in each conditioned media sample. (**K–M**) Western blot analysis from cortical cell homogenates revealed (**K**) increased APP ($P = 0.042$) and (**L**) MMP9 protein levels ($P = 0.030$) in Down's syndrome. (**M**) There was a marked reduction in tPA activity in Down's syndrome cells, as determined by casein zymography ($P = 0.017$). Representative tPA zymogram and immunoblots probed with 22C11, MMP9 and β-actin antibodies are shown. Error bars represent mean ± SEM, ($n = 5$ for control and $n = 9$ for Down's syndrome cultures). $^*P < 0.05$; $^{**}P < 0.01$; Student's *t*-test, Spearman Rank analysis for correlations. Aβ = amyloid-β; DS = Down's syndrome; IOD=Integrated optical density.

to the media, as revealed by low, comparable lactate dehydrogenase levels in control and Down's syndrome cultures (Supplementary Fig. 3C).

In accordance with our results in conditioned media, Down's syndrome cortical cell homogenates exhibited higher APP (Fig. 5K) and MMP9 protein levels (Fig. 5L), (by ~2-fold) compared with control lysates. We also found a significant reduction in tPA proteolytic activity in Down's syndrome primary cortical culture homogenates (Fig. 5M). However, we found no significant differences in the intracellular levels of proNGF between control and Down's syndrome cell culture lysates (Supplementary Fig. 3D).

NGF dysmetabolism in a genetic mouse model of Down's syndrome

Ts65Dn mice, trisomic for a segment of chromosome 16 (orthologue to chromosome 21), possess three copies of the APP gene (Reeves *et al.*, 1995) and exhibit increased APP expression (messenger RNA and protein levels) in cortex and hippocampus by 8–12 months of age (Granholm *et al.*, 2003; Hunter *et al.*, 2003, 2004; Seo and Isacson, 2005; Salehi *et al.*, 2006; Choi *et al.*, 2009; Lockrow *et al.*, 2009).

In this study we examined whether similar NGF-metabolic alterations are evident in Ts65Dn Down's syndrome mice. Trisomic mice (12–22 months of age) exhibited increased hippocampal proNGF (Fig. 6A) and a significant reduction in mature NGF levels (Fig. 6B). We also found marked deficits in plasminogen (Fig. 6C) and tPA (Fig. 6D) suggesting a compromise in proNGF maturation in the basal forebrain target tissue. Such a decrease in plasminogen protein occurred without a reduction in its messenger RNA levels (Supplementary Fig. 2G), consistent with the results obtained in human Down's syndrome brains. Representative immunoblots are shown in Fig. 6E. Trisomic mice also exhibited significantly increased neuroserpin (Fig. 6H), MMP9 (Fig. 6F) and TIMP1 (Fig. 6G) messenger RNA expression in frontal cortex at this time-point, in accordance with our results in human Down's syndrome brains.

Discussion

This comprehensive study is, to the best of our knowledge, the first demonstration that human post-mortem Down's syndrome brains exhibit robust deficits in NGF metabolism that are replicated in cultured Down's syndrome foetal cortical cells and in Ts65Dn trisomic mice. Figure 7 illustrates schematically the normal NGF metabolic pathway (Fig. 7A) and its deregulation in Down's syndrome brains (Fig. 7B).

A link between NGF metabolic deregulation and central nervous system cholinergic neuron dysfunction

In this study we report accumulation of proNGF (in human Down's syndrome brains) in basal forebrain target tissue (cortex), with concomitant alterations in the plasminogen–tPA–neuroserpin metabolic loop responsible for proNGF maturation. These changes suggest an impaired conversion of proNGF into its mature, biologically active molecule, resulting in an accumulation of proNGF. We also demonstrate an enhanced activity of the metalloprotease responsible for mature NGF degradation (MMP9), which should further diminish the brain availability of mature NGF, aggravating the imbalance between proNGF and mature NGF. Despite the scarcity of human Down's syndrome post-mortem brain material, we were able to investigate tissue samples from three different brain regions from each individual (i.e. temporal, frontal and parietal cortex) from two highly reputable brain banks. We have further validated the neurochemical alterations reported in human brains in two additional Down's syndrome experimental paradigms (i.e. in the cell culture system and in the genetic mouse model), confirming our observations.

The observation that proNGF maturation is affected is highly relevant in the context of Alzheimer's disease pathology in Down's syndrome. NGF is a key neurotrophin for basal forebrain cholinergic neurons. This neuronal system is highly dependent on endogenous NGF supply to maintain synapse numbers and TrkA messenger RNA expression (Venero *et al.*, 1994; Figueiredo *et al.*, 1995; Debeir *et al.*, 1999). The basal forebrain cholinergic system progressively degenerates in Alzheimer's disease and Down's syndrome, deficits which contribute to the manifestation of cognitive impairments (Yates *et al.*, 1980; Bartus *et al.*, 1982; Pepeu and Giovannini, 2004). Therefore, the present findings provide a mechanistic explanation for the atrophy of basal forebrain cholinergic neurons in Down's syndrome, related to an impaired NGF metabolism and a concomitant trophic disconnection.

In line with the above, we have shown that pharmacological inhibition of proNGF maturation with α_2-antiplasmin resulted in atrophy and reduction of pre-existing cholinergic synapses, leading to cognitive impairments in young rats (Allard *et al.*, 2012). Likewise, transgenic mice expressing a furin cleavage-resistant form of proNGF exhibit learning and memory deficits and cholinergic cell loss in the medial septum (Tiveron *et al.*, 2013). Importantly, a likely aggravating factor to proNGF accumulation and basal forebrain cholinergic neuron degeneration in Down's syndrome is impaired NGF retrograde transport as a result of endosomal dysfunction (Cooper *et al.*, 2001; Salehi *et al.*, 2006). Such deficits in NGF trophic support and subsequent cognitive impairment can be rescued with exogenous NGF application, in Ts65Dn mice (Cooper *et al.*, 2001), emphasizing the importance of NGF availability to the maintenance of the basal forebrain cholinergic phenotype.

NGF metabolic dysfunction in Down's syndrome

The increase in proNGF in Down's syndrome is consistent with the finding of proNGF accumulation in mild cognitive impairment and Alzheimer's disease brains (Fahnestock *et al.*, 2001; Peng *et al.*, 2004; Bruno *et al.*, 2009*a*). Importantly, the deficits in tPA and plasminogen levels illustrated in this study reproduce the changes that we have reported in post-mortem Alzheimer's disease brains

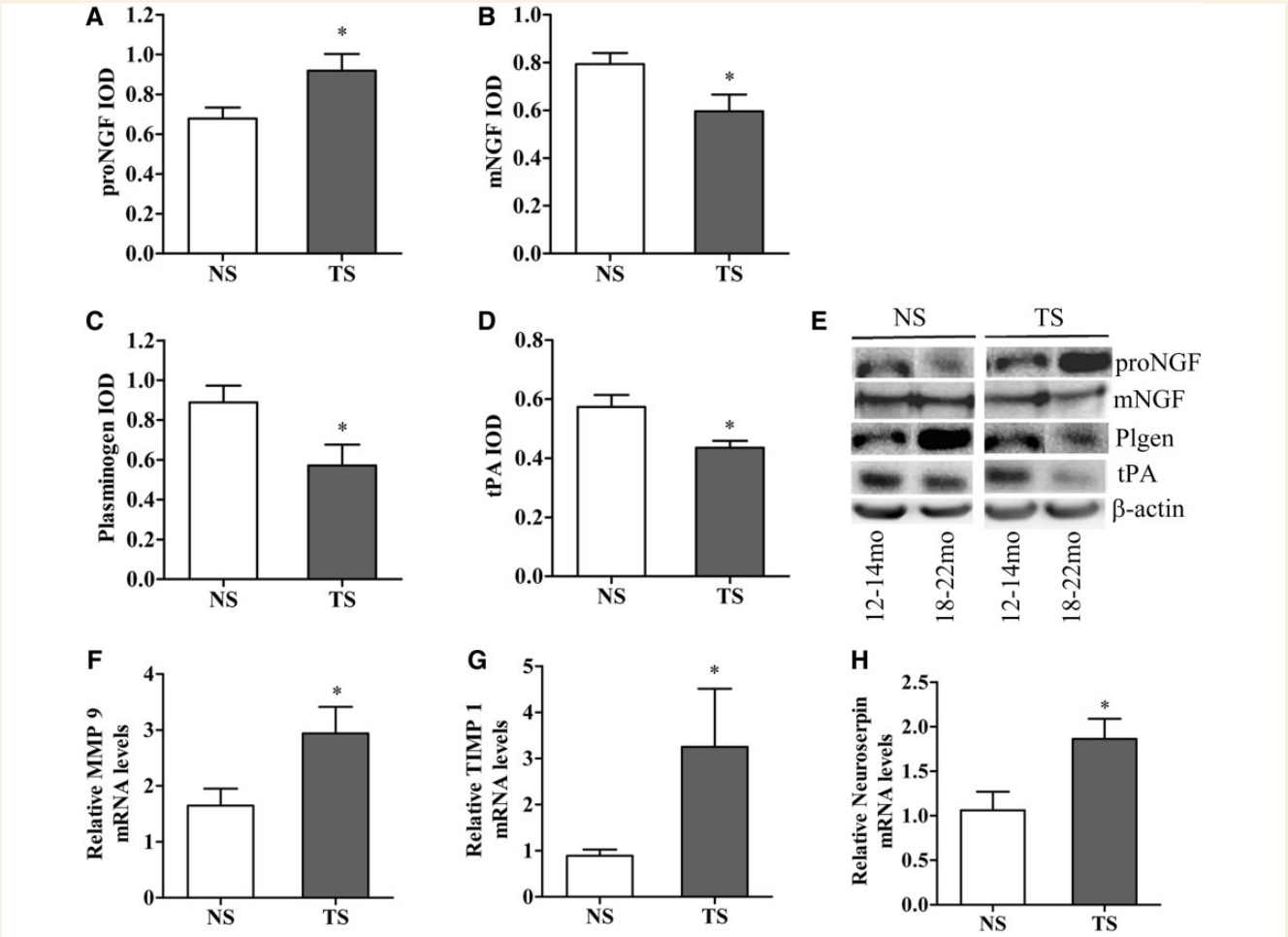

Figure 6 Deficits in proNGF cleavage in Ts65Dn mice, a genetic mouse model of Down's syndrome. Western blot analysis of NGF pathway markers in basal forebrain target tissue (hippocampus) of 12–14 and 18–22 month-old mice. Trisomic mice exhibited significantly higher (**A**) proNGF levels ($P = 0.035$) and lower (**B**) mature NGF levels ($P = 0.010$), compared with normosomic littermates. Analysis also revealed a significant reduction in (**C**) plasminogen ($P = 0.031$) and (**D**) tPA ($P = 0.026$) protein levels. Graphs depict data combined from the two time points. (**E**) Representative immunoblots probed with NGF, plasminogen, tPA and β-actin antibodies are shown. (**F–H**) Quantitative real-time PCR analysis of NGF pathway markers in frontal cortex from trisomic mice and normosomic littermates. Trisomic mice exhibited higher (**F**) MMP9 ($P = 0.044$) and (**G**) TIMP1 messenger RNA levels ($P = 0.049$). (**H**) Increased neuroserpin messenger RNA levels in trisomic mice ($P = 0.039$). Data are expressed as the normalized ratio between each protein of interest and the housekeeping gene *Hprt*. Error bars represent mean ± SEM. $^*P < 0.05$; Student's *t*-test, NS = normosomic; TS = trisomic.

(Bruno *et al.*, 2009*a*). Besides affecting proNGF maturation, a reduction in tPA synthesis can further contribute to neurodegeneration given that endogenous tPA has been shown to be neuroprotective in the ischaemic brain and in hypoxic conditions (Wu *et al.*, 2013).

Higher neuroserpin protein levels have also been reported in post-mortem Alzheimer's disease brains (Fabbro and Seeds, 2009) and CSF (Nielsen *et al.*, 2007). The fact that increased neuroserpin might have deleterious effects in the CNS is suggested by knock-out studies in J20 hAPP transgenic mice, resulting in a reduction of amyloid-β_{40} and amyloid-β_{42} peptides, a decline in amyloid plaque burden and rescue of cognitive deficits (Fabbro *et al.*, 2011). It is likely that increased neuroserpin levels in Down's syndrome are also related to the pathological accumulation of amyloid-β peptides. The strong positive correlation between neuroserpin and amyloid-β_{42} reported in this study reinforces such observations.

We have gathered significant biochemical evidence supporting an early proNGF accumulation and MMP9 activation in cortical foetal Down's syndrome primary cultures. These changes were accompanied by increased soluble APP-β and amyloid-β peptides, under culture conditions that favour survival, avoid oxidative stress and degeneration. We have found strong correlations between MMP9 activity, proNGF and amyloid-β_{42} pathology, both in adult and in foetal tissue. Notably, Cho and colleagues (2011) have recently reported an elevation of MMP2 levels in amniotic fluid from Down's syndrome pregnancies (Cho *et al.*, 2011). Thus, MMP9 and MMP2 could be early molecular signals responding to Alzheimer's disease pathology accumulation in Down's syndrome.

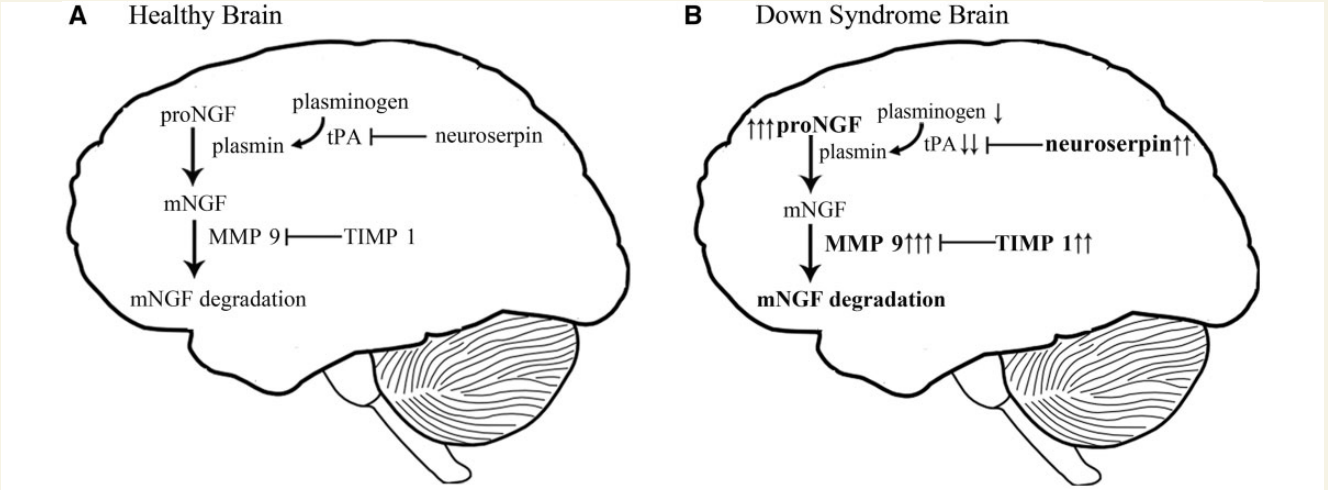

Figure 7 Schematic representation of the NGF metabolic pathway in healthy brains and its deregulation in Down's syndrome. (**A**) The NGF precursor is released to the extracellular space along with the convertases and zymogens necessary for its maturation and subsequent degradation. ProNGF is cleaved extracellulary and converted to mature NGF by plasmin. Plasmin derives from plasminogen by the action of tPA. Neuroserpin is the endogenous tPA inhibitor in the CNS. Mature NGF (mNGF) is degraded by MMP9, which is also released from neurons along with its endogenous inhibitor TIMP1. (**B**) In Down's syndrome brains there is a failure in proNGF maturation due to reduced plasminogen and tPA as well as enhanced neuroserpin levels. Down's syndrome brains also exhibit increased MMP9 activity and higher TIMP1 levels, likely contributing to enhanced mature NGF degradation.

We did not observe an upregulation of proNGF levels in Down's syndrome cellular lysates. The fact that a build-up of proNGF was only detected extracellularly (i.e. in the conditioned media, the secreted fraction) supports the hypothesis that this is a result of diminished proNGF conversion, rather than caused by an increased NGF expression. In this regard, the culture system has the added advantage that the cellular and extracellular fractions can be physically separated. These findings reinforce the concept that proNGF is released to the extracellular space where it is cleaved and converted to its mature form (Bruno and Cuello, 2006).

Down's syndrome-related NGF metabolic dysfunction is further supported by the fact that trisomic Ts65Dn mice also exhibited NGF-metabolic deficits. In agreement with previous studies (Cooper *et al.*, 2001; Salehi *et al.*, 2006), proNGF levels were higher in basal forebrain target tissue (hippocampus). We further report a significant reduction in mature NGF, plasminogen and tPA levels in trisomic mice, together with increased MMP9 and TIMP1 messenger RNA synthesis. These changes suggest that proNGF cleavage is compromised in the brains of trisomic mice, reinforcing the observations in adult and foetal human Down's syndrome tissue. Importantly, at the time point examined, trisomic mice exhibit marked cholinergic deficits and neurodegeneration (Chen *et al.*, 2009; Lockrow *et al.*, 2012).

Possible causes of NGF metabolic dysfunction in Down's syndrome, mild cognitive impairment and Alzheimer's disease

We have previously shown that amyloid-β oligomers injected in the hippocampus of naïve rats are sufficient to induce NGF metabolic alterations, including an increase in proNGF levels, upregulation of MMP9 activity and increased expression of classical inflammatory markers (Bruno *et al.*, 2009*a*). Moreover, pro-inflammatory mediators such as interleukin-1β, TNF-α and nitric oxide are known to be potent stimulators of TIMP1 production and MMP9 activation (Pagenstecher *et al.*, 1998; Gu *et al.*, 2002). Notably, several genes in chromosome 21 (i.e. *S-100β*, *Cxdar*, *Tiam1*; for review see Wilcock, 2012) may contribute to an over-activated pro-inflammatory response in Down's syndrome brains and as such, may be implicated in deregulating MMP9 and TIMP1 expression at foetal stages.

Supporting a role for amyloid-β in the occurrence of NGF metabolic deficits is the fact that in human Down's syndrome brains this compromise was most evident in frontal cortex, the region with the greatest extent of amyloid-β pathology. Notably, amyloid deposits in Down's syndrome appear first in the frontal and entorhinal cortex and further progress to other areas with advancing age (Azizeh *et al.*, 2000). As such, regional differences in NGF metabolism could be a consequence of differential Alzheimer's disease pathology severity. However, we do not exclude the possibility that other non-amyloid-β pathways are involved in compromising NGF's extracellular metabolism. Mitochondrial dysfunction and oxidative stress are well established features of Down's syndrome brains which can induce alterations in APP metabolic processing (Busciglio *et al.*, 2002) and may similarly impair NGF metabolism in Down's syndrome.

Conclusion

This study revealed novel and robust CNS alterations in NGF metabolism in Down's syndrome brains. Overall, these results

strengthen the concept that NGF dysfunction is a relevant component of the amyloid-β pathology. This knowledge may provide new strategies to prevent—or arrest—the compromised NGF metabolism and hence protect CNS cholinergic neurons. The fact that NGF metabolic deficits were evident in human Down's syndrome foetal tissue indicates that alterations in proNGF cleavage and in MMP9 activation occur before the full-blown Alzheimer's disease neuropathology. This may suggest that successful neuropreventive therapy could be initiated decades before frank memory loss in adults with Down's syndrome. Finally, a further relevant observation is the strong association between proNGF, MMP9 and amyloid-β_{42} in Down's syndrome brains, also evident in foetal tissue. These findings are relevant to consider the investigation of novel biomarkers signalling a progressive CNS trophic factor deregulation.

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Supplementary material

Supplementary material is available at *Brain* online.

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