Early defect of transforming growth factor β_1 formation in Huntington's disease

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Abstract

A defective expression or activity of neurotrophic factors, such as brain- and glial-derived neurotrophic factors, contributes to neuronal damage in Huntington's disease (HD). Here, we focused on transforming growth factor-β (TGF-β_1), a pleiotropic cytokine with an established role in mechanisms of neuroprotection. Asymptomatic HD patients showed a reduction in TGF-β_1 levels in the peripheral blood, which was related to trinucleotide mutation length and glucose hypometabolism in the caudate nucleus. Immunohistochemical analysis in post-mortem brain tissues showed that TGF-β_1 was reduced in cortical neurons of asymptomatic and symptomatic HD patients. Both YAC128 and R6/2 HD mutant mice showed a reduced expression of TGF-β_1 in the cerebral cortex, localized in neurons, but not in astrocytes. We examined the pharmacological regulation of TGF-β_1 formation in asymptomatic R6/2 mice, where blood TGF-β_1 levels were also reduced. In these R6/2 mice, both the mGlu2/3 metabotropic glutamate receptor agonist, LY379268, and riluzole failed to increase TGF-β_1 formation in the cerebral cortex and corpus striatum, suggesting that a defect in the regulation of TGF-β_1 production is associated with HD. Accordingly, reduced TGF-β_1 mRNA and protein levels were found in cultured astrocytes transfected with mutated exon 1 of the human huntingtin gene, and in striatal knock-in cell lines expressing full-length huntingtin with an expanded glutamine repeat. Taken together, our data suggest that serum TGF-β_1 levels are potential biomarkers of HD development during the asymptomatic phase of the disease, and raise the possibility that strategies aimed at rescuing TGF-β_1 levels in the brain may influence the progression of HD.

Keywords: transforming growth factor-β • Huntington's disease • brain cortex • peripheral markers • neurodegeneration • neurodysfunction

Introduction

Huntington's disease (HD) is a hereditary progressive and invalidating disease caused by an expanded cytosine-adenine-guanosine (CAG) repeat in the gene encoding the ubiquitous protein, huntingtin [1, 2]. Mutated huntingtin causes neuronal and glial dysfunction in the striatum and cerebral cortex [3, 4] through a cascade of

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events that include excitotoxic mechanisms [5] and abnormal production and transport of neurotrophic factors [6]. Activation of astrocytes and microglia, which reflects a process of neuroinflammation, is already observed during the asymptomatic stage of HD, and correlates with the severity of HD progression [7–9]. Activated astrocytes and microglia critically regulate processes of neuronal death and survival by secreting glutamate, neurotrophic factors, and pro- and anti-inflammatory cytokines [10]. An imbalance between neurotoxic and neuroprotective factors may ultimately be responsible for neuronal dysfunction and cell death in HD. Here, we focused on transforming growth factor-β (TGF-β), which is increasingly recognized as an endogenous neuroprotective factor and has been implicated in the pathophysiology of chronic neurodegenerative disorders [11, 12]. Accordingly, an altered expression of type-II TGF-β receptor has been demonstrated in the brain of patients affected with Alzheimer's disease (AD) [13–16], and a reduced TGF-β_1 signalling increases amyloid deposition and neurodegeneration in transgenic AD mice [17]. In addition, TGF-β_1 loss in transgenic mice enhances neuronal susceptibility to toxic insults [18]. TGF-β_1 production is under the control of extracellular signals, which include oestrogens and excitatory amino acids acting at mGlu3 metabotropic glutamate receptors [12, 19]. Activation of glial mGlu3 receptors is neuroprotective *via* a paracrine mechanism mediated by TGF-β_1 [12, 20, 21]. Interestingly, the expression of mGlu3 receptors is substantially reduced in the striatum of transgenic R6/2 mice, which express exon 1 of the human HD gene with an expanded CAG repeat [22]. We suggested that early abnormalities in the production of TGF-β_1 could contribute to the pathophysiology of neuronal death associated with HD. To examine this possibility, we measured TGF-β_1 levels in the brain and serum of asymptomatic and symptomatic HD patients, as related to the stage and progression of the disease. In addition, we examined the expression and regulation of TGF-β_1 in mutant HD mice or in cultured cells expressing a mutated huntingtin.

Subjects and methods

HD patients

All participants underwent genetic testing after informed consent and neurological examination, including motor, psychiatric, cognitive and functional assessments, by physicians with expertise in HD [23]. Age at onset of symptoms was calculated according to the initial neurological manifestations [23] and the predicted time to onset in years according to a CAG-based model, as described [24]. Motor symptoms, behavioural and cognitive changes were assessed clinically with the Unified Huntington's Disease Rating Scale (UHDRS) [25] and Mini-Mental State Examination (MMSE) [26]. Patients' consent was obtained according to the Declaration of Helsinki. All asymptomatic patients had no or minimal and nonspecific clinical motor or behavioural manifestations with cognitive scores almost unchanged over the previous 2 years [27]. These patients were not taking benzodiazepines or neuroleptics or other drugs for cardiological or

psychiatric pathologies. The rate of independence decline and symptom progression was measured in loss of units per year using the Disability Scale (DS) [23, 28, 29], in patients with a disease history of at least 5 years. The DS combines patients' independence and motor performance, thus taking into account the patients' independence on neurological motor impairment [28]. The disease stage was calculated according to the total functional capacity score [29]. Most patients were taking benzodiazepines; some patients in stages IV-V of disease were receiving low doses of atypical neuroleptics (olanzapine, 2.5–10 mg, risperidone, 1–3 mg or tetrabenazine 12.5–25 mg), associated, in some patients, with benzodiazepines, lithium carbonate or valproate.

Positron-emission-tomography (PET) scanning in HD patients

Twenty-three asymptomatic and 55 symptomatic patients underwent static [fluorine-18]-fluoro- 36 32-deoxy-D-glucose (FDG) FDG-PET scan after injection of 300 MBq of FDG in the resting state, with eyes closed and ears plugged to reduce background noise [27, 30]. Images were acquired about 30 min. after tracer injection with an ECAT EXACT 47 scanner (CTI/Siemens, Knoxville, TN, USA). After a transmission scan lasting 5 min. with a Ge-68 rod source, an emission scan lasting 25 min. was done in two dimensional mode. Emission scan images were reconstructed using the back-projection method with a Shepp-Logan filter (cut-off frequency 0.35), resulting in 47 slices with a 128 $\times$ 128 matrix (pixel size 1.8 mm) and an interplane separation of 3.125 mm. The attenuation effects were corrected with measured transmission images. PET analysis was performed with regions of interest, defined in the normalized space of Talairach using the automated voxel identification routine of the Talairach Daemon software. Data were normalized by mean global counts (measured as [^{18}F]-FDG uptake).

Measurements of TGF-β_1 levels in human and mouse serum

To measure TGF-β_1 levels in human serum, peripheral venous blood samples from HD patients and healthy controls were collected between 8 and 11 a.m. [31]. To minimize possible influence of patients' circadian rhythms on plasma TGF-β_1 concentrations, we also confirmed the cytokine levels in blood samples collected between 5 and 7 p.m. in a group of 10 patients and 10 control patients [31]. No age-related differences in TGF-β_1 serum levels were noticed in control patients, as expected [32], thus excluding the potential bias due to the younger age of asymptomatic patients (Fig. S1). Blood samples were centrifuged at room temperature (3500 $\times$ *g* for 10 min.) and plasma samples were stored at −80°C until processing. Asymptomatic (4 weeks) and symptomatic (12 weeks) transgenic R6/2 mice and asymptomatic YAC128 mice (2 months) were killed to collect blood and brain tissues. Each group included a total of 15 transgenic asymptomatic mice with respective number of wild-type control and age-matched mice. The mice were anaesthetized by intraperitoneal injection of 0.5 ml of Pentoject (sodium pentobarbitone, Animal Ltd., York, UK) and exsanguinated by intracardiac puncture, followed by cervical dislocation. Mouse blood was transferred into a standard 1.5 ml plastic eppendorf tube and allowed to stand for 2 hrs to clot. The blood was spun at 3700 $\times$ *g* for 5 min. and the supernatants were stored at −80°C until processing. Serum TGF-β_1 levels were measured using an ELISA kit (TGF-β_1 Emax Immunoassay System kit Promega, Madison, WI, USA), according to the manufacturer's instructions. Serum TGF-β_1 concentrations were measured in four independent

experiments. Medium TGF-β_1 levels from knock-in cells were measured using the same kit and according to the manufacturer's instructions.

Transgenic R6/2 and YAC128 mice

Breeding pairs of the R6/2 line of transgenic mice (containing 250 CAG repeats in exon-1 of human HD gene; strain name: B6CBATgN(HDexon1) 62Gpb/J) [33] and of the YAC line of transgenic mice (containing 128 CAG repeats in a YAC with human full length HD gene; strain name: FVB-Tg (YAC128)53Hay/J) [34], were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). The lines were maintained by backcrossing to B6CBAF1 X C57BL/6 F1 and to FVB, respectively. The mice were maintained in the animal facilities of the Neurological Institute Neuromed (R6/2), of University of Alberta (YAC128) and of British Columbia (YAC128), and kept under environmentally controlled conditions (ambient temperature = 22°C, humidity = 40%) on a 12-hr light/dark cycle with food and water *ad libitum*. Experiments were performed following the Guidelines for Animal Care and Use of the National Institutes of Health. Hemizygous transgenic mice were used in all experiments, as was confirmed by PCR according to the protocol by Jackson Laboratories (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype¼ protocol& protocol_ id ¼282). Mice were housed in groups of transgenic and non-transgenic littermates.

Measurements of TGF-β_1 levels in mice

Wild-type and R6/2 mice at 4 weeks of age were treated with saline or LY379268 (10 mg/kg, i.p.) and killed 6 hrs later. Both cortical cortices and striata were dissected out and immediately homogenized in radioimmuno-precipitation assay (RIPA) buffer for measurements of TGF-β_1 protein levels. The amount of TGF-β_1 protein was assessed by Western blot analysis. Wild-type and R6/2 mice, 4 week old, were also treated with saline or riluzole (10 mg/kg, i.p.), a drug increasing patients' brain [35] and serum [36] concentration of the brain-derived neurotrophic factor (BDNF), and killed 6 hrs later. Cerebral cortex and striatum were dissected out and used for TGF-β_1 protein assessment. Brain areas were dissected out from transgenic mice and age-matched controls (4 weeks for R6/2 mice and 2 months for YAC128 mice). Tissues were homogenized at 4°C in a buffer composed of Tris-HCl pH 7.4, 10 mM; NaCl, 150 mM; ethylenediaminetetraacetic acid, 5 mM; phenylmethanesulphonylfluoride (PMSF), 10 mM; Triton X-100, 1%; leupeptin, 1 μg/ml; aprotinin, 1 μg/ml with a motor-driven Teflon-glass homogenizer (1700 rev./min.). Tissue extracts were used for protein determinations; 30 μg of proteins were resuspended in SDS-bromophenol blue reducing buffer with 40 mM dithiothreitol (DTT) and used for protein identification. Western blot analyses were carried out using 8% SDS, for mGlu2/3 receptors, and 12.5%, for TGF-β_1, polyacrylamide gels run on a minigel apparatus (Biorad, Milano, Italy; Mini Protean II Cell); gels were electroblotted on ImmunBlot PVDF Membrane (Biorad) for 1 hr using a semi-dry electroblotting system (Biorad, Trans-blot system SD), and filters were blocked overnight in TTBS buffer (100 mM Tris-HCl; 0.9% NaCl, 0.1% Tween 20, pH 7.4) containing 5% non-fat dry milk. Blots were then incubated for 1 hr at room temperature with a primary polyclonal antibody (2 μg/ml) which recognizes mGlu2/3 receptors (Chemicon International, Inc., Tecumela, CA, USA), or overnight at 4°C with a monoclonal anti-human TGF-β_1 antibody (1.5 μg/ml, Chemicon International, Inc.) and then incubated for 1 hr with the secondary antibody (1:5000 or 1:10,000, peroxidase-coupled antimouse, Amersham, Milano, Italy). Immunostaining was

revealed by the enhanced chemiluminescence (ECL) Western blotting analysis system (Amersham). The blots were reprobed with anti-β-actin monoclonal antibody (1:250, Sigma, St. Louis, MO, USA).

Immunocytochemistry for TGF-β_1 in human and mouse brain samples

We examined six cases of HD, one asymptomatic and eight control patients. One patient affected by multiple sclerosis was included as positive control expressing high levels of TGF-β_1. Patients' data were obtained from the databases of the department of Neuropathology of the Academical Medical Center (University of Amsterdam, UVA) in Amsterdam and the Department of Neurology of Leiden University Medical Center (LUMC). Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki (*Br Med J* 1991; 302; 1194). Formalin-fixed, paraffin-embedded tissue was sectioned at 6 μm and mounted on organosilane-coated slides (Sigma). Representative sections of all specimens were processed for haematoxylin eosin, as well as for immunocytochemical reactions. The anti-TGF-β_1 (LC1–30; purified rabbit antibody) was kindly provided by Dr. K. Flanders, (National Cancer Institute, Bethesda, MD, USA) [37, 38]. Immunocytochemistry was carried out on paraffin-embedded tissue as previously described [39]. The sections were incubated with the primary antibody (1:50) overnight at 4°C. Single-label immunocytochemistry was performed with avidin-biotin peroxidase method for the polyclonal goat antibodies and Powervision (Immunologic, Duiven, The Netherlands) for the monoclonal mouse and polyclonal rabbit antibodies. As chromogen, 3,3-diaminobenzidine was used. Sections incubated without the primary antibody were essentially blank.

Mouse brains were dissected out and immediately placed in a solution composed of ethyl alcohol (60%), acetic acid (10%) and chloroform (30%). Twenty hours later brains were placed in 70% ethanol until they were included in paraffin. Ten micrometre serial sections were cut and used for histological analysis. Sections were incubated overnight with rabbit polyclonal anti-TGF-β_1(V) (1:5, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies, and then for 1 hr with secondary biotinylated anti-rabbit antibodies (1:200; Vector Laboratories, Burlingame, CA, USA). 3,3-Diaminobenzidine tetrachloride was used for detection (ABC Elite kit; Vector Laboratories). Control staining was performed without the primary antibodies. Double fluorescence immunohistochemistry was performed by incubating brain sections overnight with polyclonal anti-TGF-β1(V) (1:5) and monoclonal mouse anti-glial fibrillary acidic protein (GFAP) (1:100; Sigma-Aldrich; Milan, Italy) or mouse anti-NeuN (1:100; Chemicon International, Inc.) antibodies, and then for 1 hr with secondary Cy3 anti-rabbit (1:300; Chemicon International, Inc.) and fluorescein antimouse (1:100; Vector, Laboratories) antibodies.

In vitro experiments

Glial cell cultures were prepared from cortex of postnatal CD1 mice (1–3 days after birth), as previously described [40]. Dissociated cortical cells were grown in 60 mm dishes (Falcon Primaria, Lincoln Park, NJ, USA) using a plating medium of minimum essential medium (MEM)-Eagle's salts supplemented with 10% of heat inactivated horse serum, 10% foetal bovine serum, 2 mM glutamine, 25 mM sodium bicarbonate and 21 mM glucose. Cultures were kept at 37°C in a humidified CO_2 atmosphere. Confluent cells were trensfected (see below) and incubated with LY379268 (1 μM for

10 min.), and after the drug washout, cultures were kept for 6–8 hrs (for real time PCR analysis) or 20 hrs (for cytometry analysis) in the incubator.

Striatal knock-in cultures

Striatal knock-in cells, stably expressing full-length huntingtin STHdhQ7/Q7, (ST7/7Q, wild-type cells) or full-length mutated huntingtin STHdhQ111/Q111 (ST111/111Q, mhtt cells) established from HdhQ111 knock-in mice [41], were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 400 μg/ml geneticin (G-418), at 33°C in a humidified 5% CO_2 atmosphere. Cultures cells were transfected (see below) and incubated with LY379268 (1 μM for 10 min.), and after the drug washout, cultures were kept for 6–8 hrs (for real time PCR analysis) or 20 hrs (for cytometry and ELISA analysis) in the incubator.

Cell transfections

All DNA constructs were transfected using Lipofectamine 2000 (Invitrogen, Stockholm, Sweden) as instructed by the manufacturer. Both ST7/7Q, ST111/111Q and primary astrocyte cells were transfected at 50% of confluence. DNA constructs were exon-1-Htt-25Q-EGFP which express 25 CAG triplets and exon-1-Htt-72Q-EGFP which express 72 CAG triplets. The expression of the green fluorescence protein was used as an index of successful transfection.

Real-time RT-PCR analysis

Total RNA isolated with TRIzol®LS (Invitrogen) was treated with DNase I (Qiagen, Milan, Italy) and retrotranscribed into cDNA by using SuperScript III Reverse Transcriptase (Invitrogen). Real-time RT-PCR was performed on a Step One PCR cycler (Applied Biosystems, Foster City, CA, USA). PCR was performed by using Power SYBR Green PCR Master Mix Kit (Applied Biosystems) according to the manufacturer's instructions. Thermal cycler conditions were as follows: 5 min. at 50°C, 1 min. at 95°C, 40 cycles of denaturation (45 sec. at 95°C), and combined annealing/extension (1 min. at 59°C). The sequences of β-actin and TGF-β_1 primers used are: β-actin Forward: 5′-ctggctcctagcaccatga-3′ and Reverse: 5′-tagagccaccaatcca-caca-3′ TGF-β_1 Forward: 5′-acaattcctggcgttacctt- 3′ and Reverse: 5′-ccacgtggagtttgttatct- 3′.

Gene expression measures were derived from samples run in triplicates. Relative expression was calculated by normalization to β-actin expression using the $\Delta\Delta$Ct method [42].

Measurements of TGF-β_1 levels by fluorescent-activated cell sorting (FACS) analysis

Intracellular expression of TGF-β_1 in primary astrocyte, ST7/7Q and ST111/111Q cell cultures, was performed by three colour-flow cytometry using a Becton-Dickinson FACS calibur flow cytometer (Franklin Lakes, NJ, USA). Cell suspension was fixed with 2% paraformaldehyde for 20 min. at room temperature, resuspended in permeabilization buffer (0.1% [w/v] saponin, 0.05% [w/v] NaN_3 in phosphate-buffered solution [PBS]) and centrifuged at 200 g for 7 min. Cells were then incubated with PE-conjugated anti-TGF-β_1 monoclonal antibody and phycoerythrin (PE)-conjugated IgG1 isotype (negative control) for 45 min. at room temperature (PharMingen, San Jose, CA USA). Cells were washed and resuspended with PBS before acquisition. The cytokine-positive cells were scored on the basis of isotype control.

Statistical analysis

Student's t-test was used to compare between-group differences in the median age. Statistical differences in serum TGF-β_1 levels among patients at different HD stage and *versus* controls were performed by Student's t-test and ANOVA. Linear dependence of serum TGF-β_1 on CAG repeat expansion, time to onset in years, DS loss of units per year and on ^{18}F-FDG uptake reduction in brain caudate, was determined by a simple regression model. Other statistical analyses were performed by Student's t-test or one-way ANOVA + Fisher's PLSD. Data were considered statistically significant at $P <$ 0.05. Statistical analysis was performed with the StatView 5 software.

Results

Clinical, genetic, serum TGF-β_1 levels and demographic data from all patients are summarized in the Table S1.

TGF-β_1 serum levels in HD patients and healthy controls

Serum TGF-β_1 levels in the total population of HD patients were lower than in non-HD controls ($P = 0.0002$) and this entirely reflected the drop in TGF-β_1 levels found in the patients at the asymptomatic and first HD stages (Fig. 1A, B). No significant changes in serum TGF-β_1 levels were found between controls and symphtomatic patients regardless of their clinical stage (Fig. 1B). In asymptomatic patients, we found a negative correlation between serum TGF-β_1 levels and the number of CAG repeats in the huntingtin gene (Fig. 1C), whereas there was no correlation between TGF-β_1 levels and the number of CAG repeats in symptomatic patients (Fig. 1D). We also found a significant correlation ($P = 0.029$) with the rate of progression in symptomatic patients (Fig. 1E). Asymptomatic HD patients, whose estimated age at onset of symptoms in years was predicted by a mathematical CAG-based model [24], showed a relative increase in serum cytokine levels while approaching manifest HD ($P = 0.0096$; Fig. 1F), although the mean serum concentrations of TGF-β_1 were still reduced as compared to control patients ($P = 0.0004$).

We then examined the relation between changes in TGF-β_1 serum levels and glucose metabolism in the caudate nucleus, as assessed by FDG-PET scan in both asymptomatic and symptomatic patients. In asymptomatic patients, serum TGF-β_1 levels showed a positive correlation with glucose metabolism in the caudate nucleus (Fig. 2).
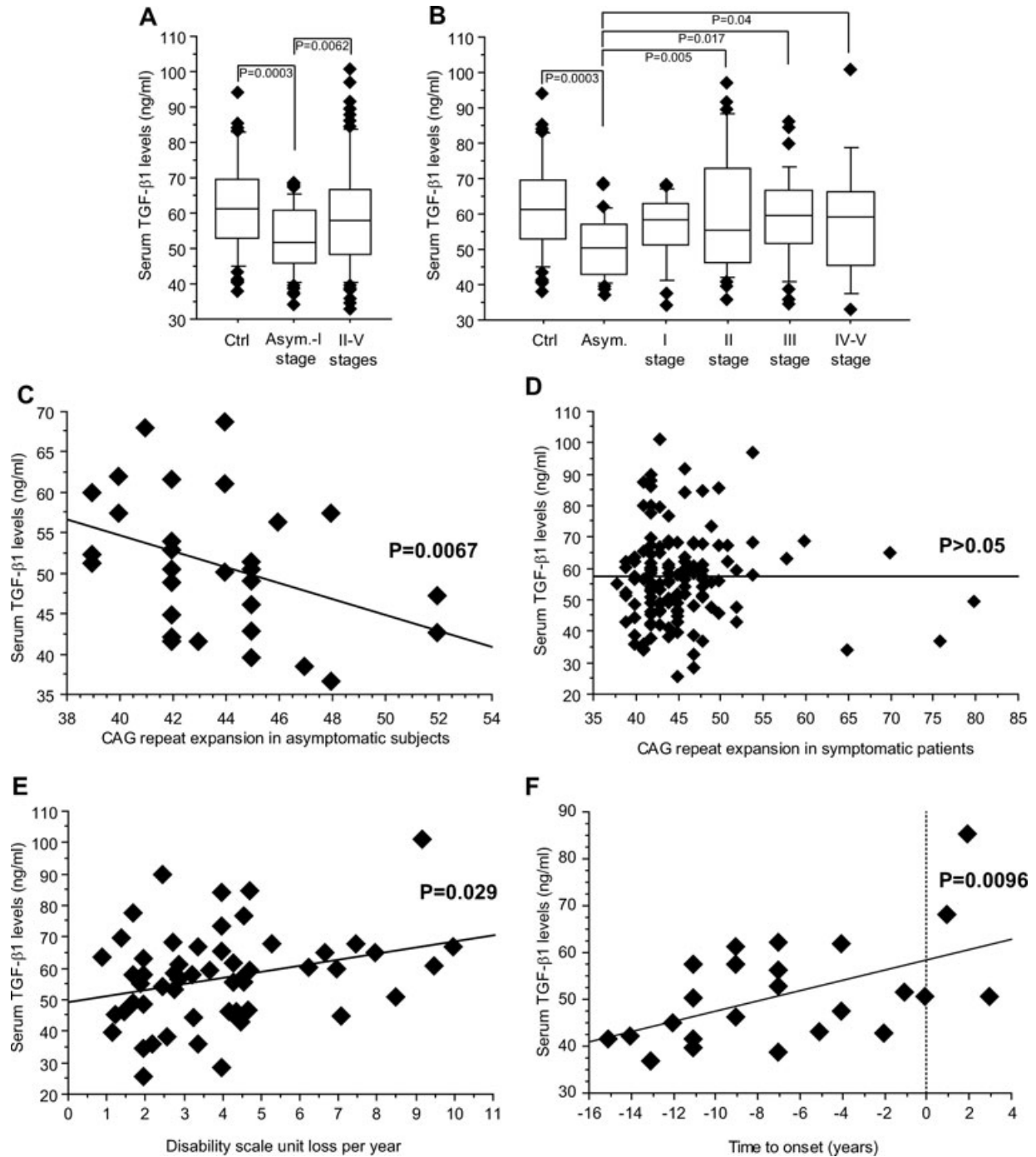
Fig. 1 Serum TGF-β_1 levels in HD patients. Reduced serum TGF-β_1 levels in asymptomatic and stage-I HD patients (**A**). TGF-β_1 levels in control (Ctrl) and all life stage patients are shown in (**B**). Statistical analysis was carried out by Student's t-test in (**A**) and by one-way ANOVA + Fisher PLSD in (**B**). Linear dependence of serum TGF-β_1 levels on expanded CAG repeat number in asymptomatic patients is shown in (**C**) ($n = 30$, $R^2 = 0.16$, $P = 0.0067$). The lack of correlation between serum TGF-β_1 levels and the number of expanded CAG repeats in symptomatic patients is shown in (**D**). (**E**) Patients with disease length beyond 5 years and manifest HD (stages II-V) showed a slight but significant linear dependence of serum TGF-β_1 levels on the rate of progression of the disease, calculated as loss of units of the DS per year ($n = 54$, $R^2 = 0.10$, $P = 0.029$). (**F**) Serum TGF-β_1 in asymptomatic mutation carriers, whose predicted age at onset was estimated within further 15 years, showed an increase linearly correlating with the estimated time to onset ($n = 24$, $R^2 = 0.27$, $P = 0.0096$, simple regression analysis). The few patients with estimated time to onset higher than 0 on x-axis showed an age in years delayed to that expected on the basis of their CAG size.
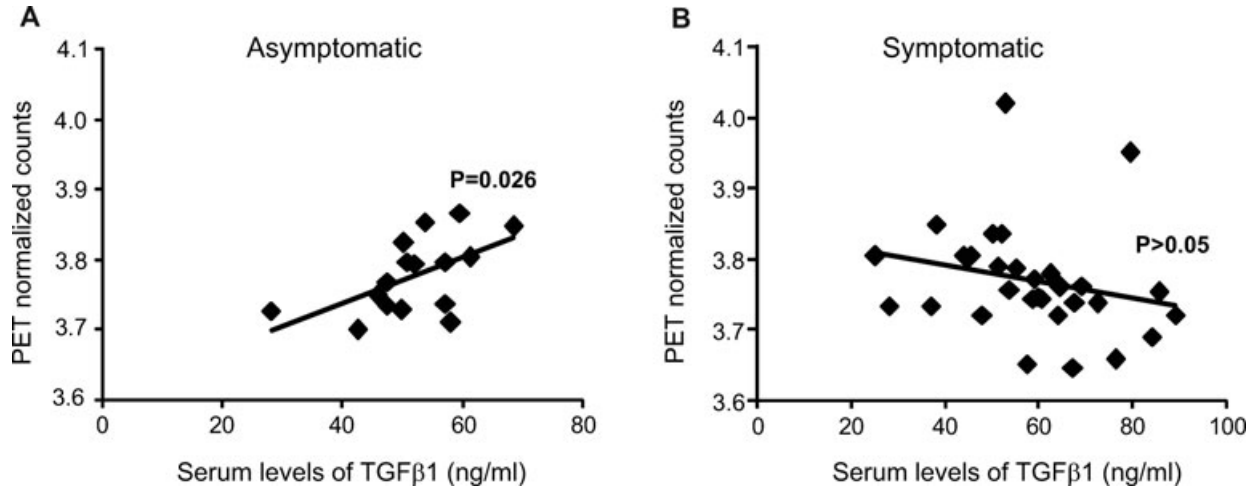
Fig. 2 Correlation between serum TGF-β_1 levels and glucose metabolism in patients at asymptomatic and symptomatic HD stages. The relation between serum TGF-β_1 levels and glucose metabolism in the caudate nucleus of asymptomatic and symptomatic HD patients is shown in (**A**) and (**B**), respectively. Note that a positive correlation is found only in asymptomatic patients ($n = 23$, $R^2 = 0.31$, $P = 0.026$).

TGF-β_1 expression in the HD brain

We examined the expression of TGF-β_1 by immunohistochemistry in post-mortem brain samples of six symptomatic patients (grades III–IV), one asymptomatic patient who was clinically and genetically characterized as described [43], and eight controls (see Table 1). Expression was also examined in the cerebral cortex of one patient with multiple sclerosis, considered as a positive control because of the expected increase in microglia immunoreactivity. TGF-β_1 immunoreactive neurons could be detected in both the cerebral cortex and caudate nucleus of all control patients, whereas no expression was found in cortical or striatal astrocytes, or in the white matter of control patients. The asymptomatic patient was devoid of TGF-β-immunoreactive neurons in the cerebral cortex, and did not show TGF-β_1 immunoreactivity in the white matter (Fig. 3). The caudate nucleus of the asymptomatic patient was not available. In symptomatic patients no expression of TGF-β_1 in cortical neurons and low-to-moderate TGF-β_1 expression in cortical astrocytes, were detected. In contrast, TGF-β_1 was highly expressed in the white matter and caudate nucleus of symptomatic patients, where reactive astrocytes and microglia showed a strong TGF-β_1 immunoreactivity (Fig. 3; Table 1).

Reduced cortical TGF-β_1 levels in asymptomatic HD mice

We examined the expression of TGF-β_1 protein in the cerebral cortex and striatum of asymptomatic YAC128 and R6/2 mice (8 and 4 week old, respectively) and symptomatic R6/2 mice (12 week old). Immunoblot analysis showed a reduction in cortical TGF-β_1 levels and no changes in striatal TGF-β_1 levels in asymptomatic YAC128 and R6/2 mice (Figs 4A and 5A). Symptomatic R6/2 mice also showed a reduced expression of TGF-β_1 levels in the cerebral cortex associated with a slight increase in TGF-β_1 levels in the striatum (Fig. 5D). Immunohistochemical analysis confirmed the reduction in TGF-β_1 levels in the cerebral cortex of asymptomatic R6/2 mice (Fig. 6A), and showed that TGF-β_1 was exclusively localized in cortical neurons of both wild-type and R6/2 mice (Fig. 6B, C). TGF-β_1 expression appeared to be also reduced in the CA1 region of the hippocampus and in the thalamus, but did not change in the striatum and cerebellum (Fig. 6A). TGF-β_1 levels in peripheral blood were substantially reduced in asymptomatic R6/2 mice, but returned back to normal in symptomatic R6/2 mice (Fig. 5D). Serum TGF-β_1 levels were unchanged in asymptomatic YAC128 mice (Fig. 4B). We also examined whether a defect in the pharmacological regulation of TGF-β_1 formation occurred in asymptomatic R6/2 mice. First, we treated mice with the mGlu2/3 receptor agonist, LY379268 (10 mg/kg, i.p.), which is known to up-regulate the expression of TGF-β_1 in brain tissues [12, 20]. A single injection of LY379268 increased TGF-β_1 levels in the striatum and cerebral cortex of wild-type mice but did not change TGF-β_1 levels in 4-week-old R6/2 mice (Fig. 5A). However, asymptomatic R6/2 mice also showed a reduced mGlu2/3 receptor expression in both cerebral cortex and striatum (Fig. 5B), a finding that might contribute to explain their lack of activity of LY379268. Interestingly, an increase in both cortical and striatal TGF-β_1 levels was also observed in mice receiving a single i.p. injection with 10 mg/kg of riluzole (Fig. 5C), a drug that does not interact with mGlu receptors. Similar to LY379268, riluzole was inactive on cortical and striatal TGF-β_1 levels in asymptomatic R6/2 mice (Fig. 5). Taken together these data suggest that HD mice have a defective regulation of TGF-β_1 formation.

Table 1 Pathological, genetic, clinical data and expression of TGF-β_1 in cerebral cortex, white matter and caudate nucleus of eight control subjects, six patients with original pathological diagnosis of HD and one asymptomatic subject

Patients	Age in years	Gender	Vonsattel *et al.*'s Grade	CAG repeats	Brain weight (g)	Clinical diagnosis of HD	TGF-β_1 immunoreactivity*		
							Cerebral cortex	White matter	Caudate nucleus
1	59	F	0	-	1456	-	2	0	2
2	73	M	0	-	1389	-	2	0	1
3	66	F	0	-	1463	-	3	0	2
4	61	M	0	-	1510	-	3	0	1
5	68	F	0	-	1478	-	2	0	1
6	67	M	0	-	1470	-	2	0	1
7	59	F	0	-	1480	-	3	0	1
8	64	M	0	-	1420	-	2	0	2
Mean ± S.E.M.	64.6 ± 1.8						2.4 ± 0.2		1.4 ± 0.2
9	67	M	III	9/43	1185	+	1	2	3
10	61	F	III	22/44	1070	+	1	2	4
11	60	F	III-IV	20/46	825	+	2	3	3
12	61	F	III	25/44	800	+	1	3	3
13	59	M	III	19/42	1330	+	2	2	4
14	67	M	III-IV	20/45	1160	+	1	2	4
Mean ± S.E.M.	62.5 ± 1.6						1.3 ± 0.2	2.3 ± 0.2	3.5 ± 0.2
15	39	F	0	ND#	ND	At risk	0	1	

Subjects 1–8 = Healthy controls; **subjects 9–14** = Symptomatic HD patients, **subjects 15** = asymptomatic subjects (positive genetic test of HD and unreported CAG size #). **HD**: Huntington disease; **(0)** = negative; **(1)** = light; **(2)** = moderate; **(3)** = strong. *$P < 0.05$ (Student's t-test) HD *versus* non-HD patients.

Reduced expression of TGF-β_1 in cultured astrocytes expressing 72Q huntingtin exon 1 (exon-1 htt 72Q)

We used cultured cortical astrocytes to examine whether the presence of huntingtin with an expanded Q repeat could cause a defect in the production and/or regulation of TGF-β_1. Cultures were transfected with exon 1 of the human huntingtin gene encoding for an expanded 72Q repeat or an unexpanded 25Q repeat (the latter used as a control). Real-time PCR analysis showed that basal TGF-β_1 mRNA levels were lower in cultures expressing huntingtin with the expanded Q repeats. Addition of the mGlu2/3 receptor agonist, LY379268, enhanced TGF-β_1 mRNA levels in control cultures, as expected [12, 20], but, interestingly, was ineffective in cultures expressing mutated huntingtin (Fig. 7A). Similar results were obtained by cytofluorimetric analysis, which showed a lower percentage of TGF-β_1-expressing cells in cultures transfected with huntingtin exon-1 containing the expanded Q repeat both under basal conditions and in response to LY379268 (Fig. 7B,C).

Reduced expression of TGF-β_1 in striatal-derived knock-in cells expressing full-length huntingtin with an expanded Q repeat

We used immortalized knock-in cells derived from striatal neurons expressing full-length (ST7/7Q, wild-type cells) or full-length mutated huntingtin (ST111/111Q, mhtt cells). Mhtt cells showed lower basal levels of TGF-β_1 mRNA than wild-type cells (Fig. 8A). Interestingly, wild-type cells expressed detectable levels of mGlu2/3 receptors (not shown) and responded to LY379268 with a substantial rise in TGF-β_1 mRNA levels, an effect that was largely attenuated in mhtt cells (Fig. 8B). This defective production and regulation of TGF-β_1 was confirmed at protein level by combining cytofluorimetric analysis of TGF-β_1-expressing cells (Fig. 8C, D)
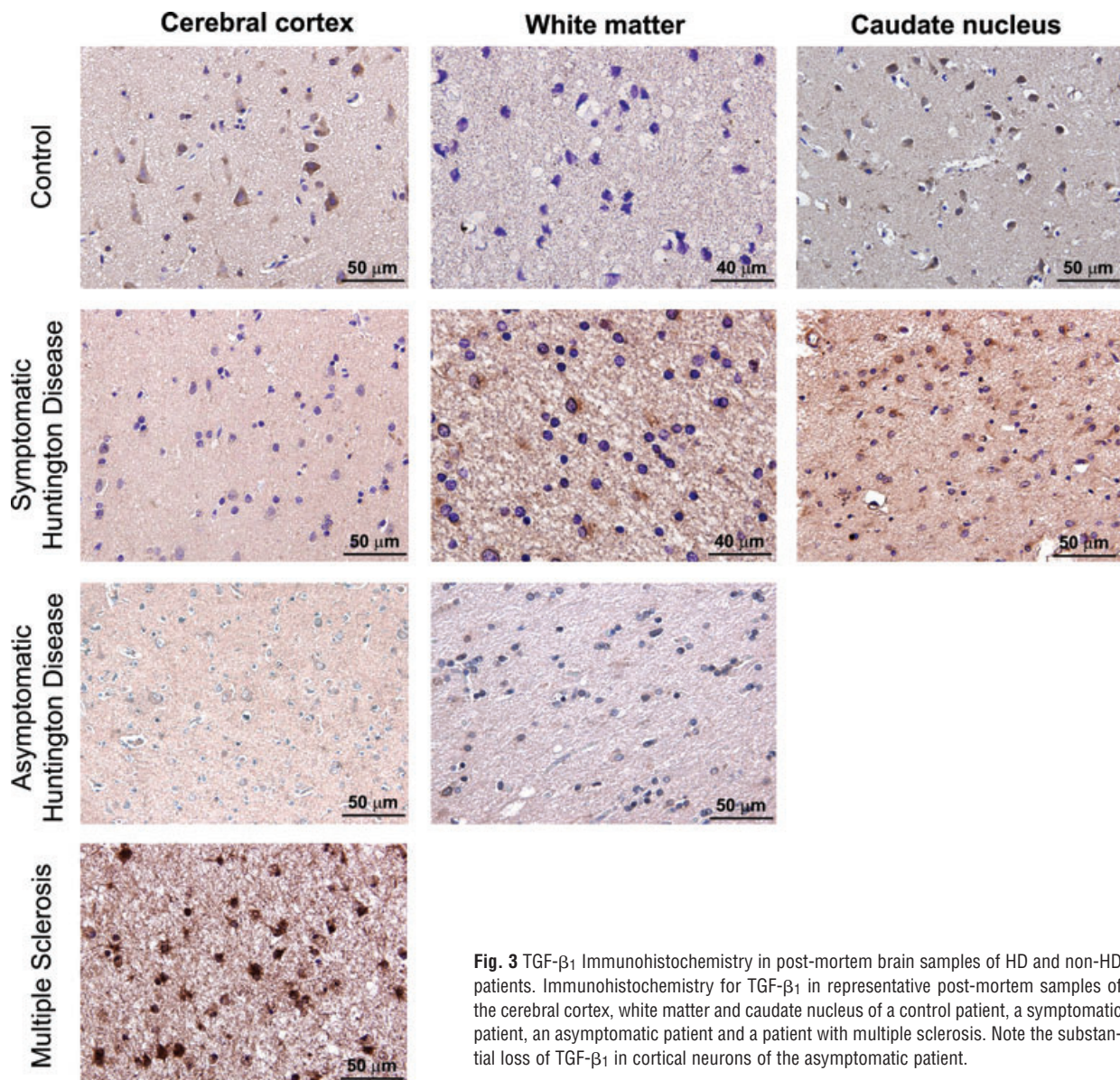
Fig. 3 TGF-β₁ Immunohistochemistry in post-mortem brain samples of HD and non-HD patients. Immunohistochemistry for TGF-β₁ in representative post-mortem samples of the cerebral cortex, white matter and caudate nucleus of a control patient, a symptomatic patient, an asymptomatic patient and a patient with multiple sclerosis. Note the substantial loss of TGF-β₁ in cortical neurons of the asymptomatic patient.

and in response to LY379268 (Fig. 8B, C) and measurements of TGF-β₁ released into the cultured medium under basal conditions and in response to a pulse with LY379268 (Fig. 8E). Both wild-type and mhtt cells expressed mGlu2/3 receptors, as shown by immunoblot analysis exon 1 of huntingtin, and we measured TGF-β₁ mRNA levels by real-time PCR. In wild-type knockin cells, transfection with the expanded exon 1 (72Q) substantially reduced TGF-β₁ mRNA levels (as compared with the unexpanded exon-1). In contrast, expression of the expanded exon-1 did not further reduce TGF-β₁ mRNA levels in mhtt knockin cells (Fig. 8F), suggesting a saturating effect produced by the mutated full-length huntingtin present in these cells. All results from human tissues, cell and animal models are summarized in Table 2.

Discussion

A defective production and/or activity of neurotrophic factors has been implicated in the pathophysiology of HD. Mutated huntingtin looses the ability to increase BDNF gene transcription [44, 45] and to enhance intracellular trafficking of BDNF [46, 47]. In addition, strategies that increase brain levels of BDNF or glial-derived neurotrophic factor (GDNF) are neuroprotective in HD models [36, 48–58]. Recent evidence suggest that the study of neurotrophic factors in HD can be extended to the peripheral blood in an attempt to obtain accessible indicators of disease progression and drug efficacy. Blood levels of BDNF mRNA are progressively reduced in rodent HD models, and can be restored by the neuro-

Table 2 Human data and animal or *in vitro* models used for the study of TGF-β_1 in HD

	HD patients or animal models	Serum	PET scan analysis	CAG repeat length	DS loss per year	Time to onset	Western		Immunohystochemistry	
							Ctx	Str	Ctx	Str
Human beings	Asymptomatic	↓	+	−	+	+			↓	
	Symptomatic	n.c.	n.c.	n.c.	n.c.				↓	↑
R6/2 Mice	Asymptomatic	↓					↓	↔	↓	↔
	Symptomatic	↔					↓	↔		
YAC Mice	Asymptomatic	↔					↓	↔		

	In vitro models	TGF-β_1 mRNA levels	TGF-β_1 protein Levels	TGF-β_1 mRNA levels after transfection (htt exon-1)		TGF-β_1 protein levels after transfection (htt exon-1)	
				25Q	72Q	25Q	72Q
Knock-in cell lines	ST 7/7Q	↔	↔	↔	↓		
	ST 111/111Q	↓	↓	↔	↔		
Cell culture	Astrocytes			↔	↓	↔	↓

(+) positive correlation with serum TGF-β_1 levels; (−) inverse correlation with serum TGF-β_1 levels; (n.c.) lack of correlation with serum TGF-β_1 levels; (↓) decrease; (↑) increase; (↔) unchanged. Ctx: cerebral cortex; Str: striatum.

Fig. 4 TGF-β_1 in transgenic YAC128 mice brain and peripheral serum. Immunoblot analysis of TGF-β_1 in the cerebral cortex and striatum of wild-type and asymptomatic YAC128 mice (8 weeks of age) is shown in (**A**). Densitometric values are means ± S.E.M. of five to seven determinations. *$P < 0.05$ (Student's t-test) *versus* the corresponding values obtained in wild-type mice. Serum TGF-β_1 levels in wild-type and asymptomatic YAC128 mice are shown in (**B**), where values are means ± S.E.M. of five to seven determinations.
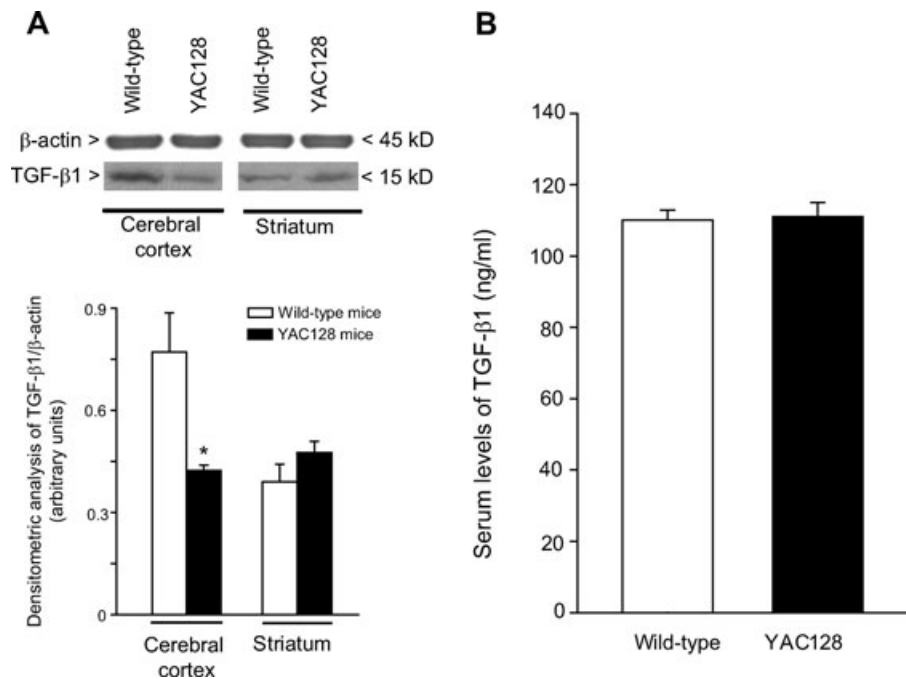
protective drug, CEP-1347 [59]. In HD patients, serum BDNF levels are lower than in age-matched controls, and the reduction correlates with the length of CAG repeats in the huntingtin gene, and duration and severity of HD [31]. Thus, the reduction of BDNF levels in peripheral blood may reflect the reduced cortico-striatal production of BDNF associated with HD [60–62]. Our data provide the first evidence that changes in TGF-β_1 are associated with HD and raise the intriguing possibility that a defective regulation of
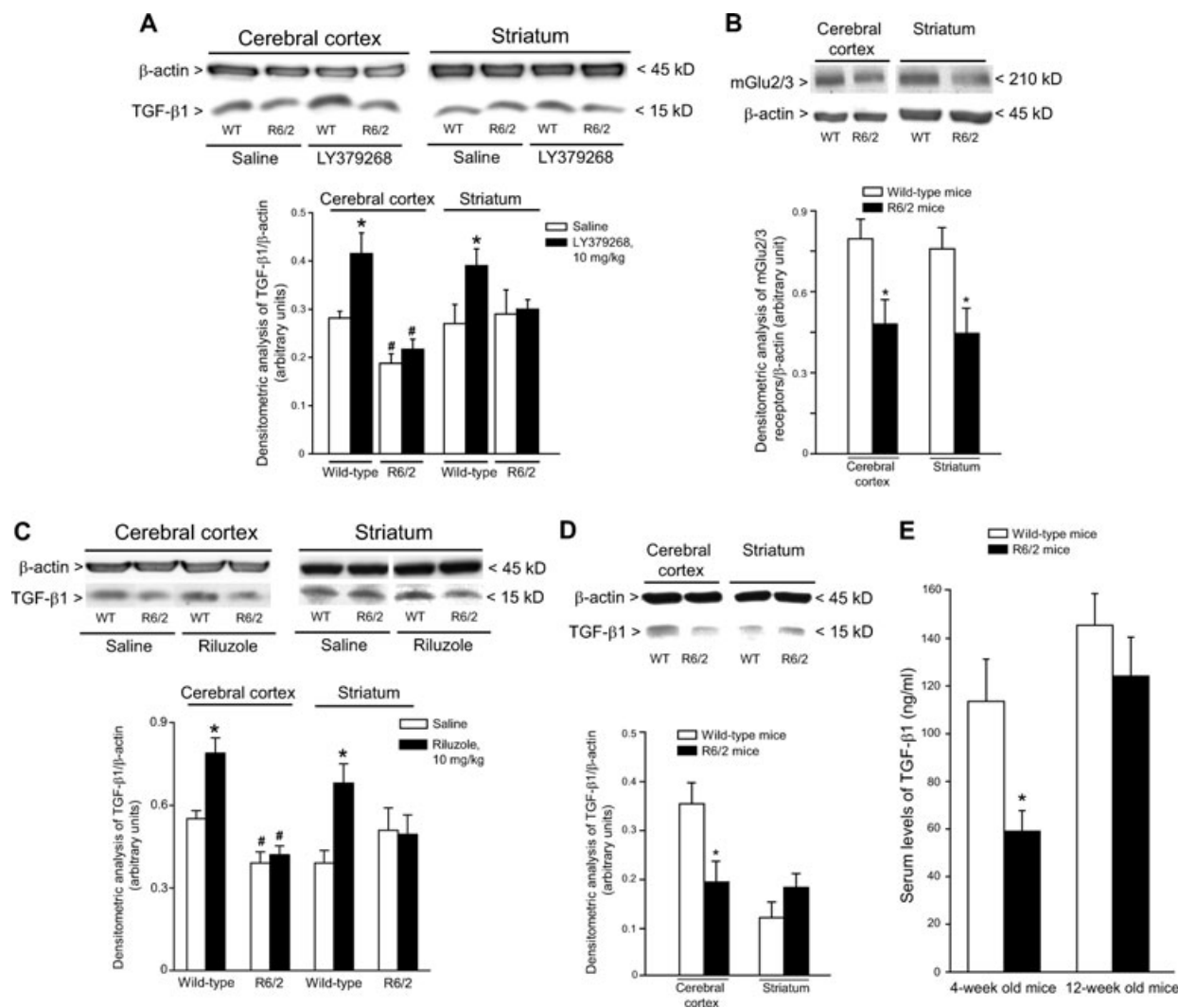
Fig. 5 TGF-β_1 levels in the cerebral cortex, striatum, and peripheral blood of asymptomatic or symptomatic R6/2 mice. Immunoblot analysis of TGF-β_1 in the cerebral cortex and striatum of wild-type, and asymptomatic (4 weeks of age) R6/2 mice is shown in (**A**). Mice were also treated with a single i.p. injection of saline or LY379268 (10 mg/kg). Values are means ± S.E.M. of six determinations. $P < 0.05$ *versus* the respective values obtained in mice treated with saline (*) or *versus* the respective values obtained in wild-type mice (#) (one-way ANOVA + Fisher's PLSD). A representative immunoblot is also shown. Immunoblot analysis of mGlu2/3 receptors in the cerebral cortex and striatum of asymptomatic R6/2 mice is shown in (**B**), where values are means ± S.E.M. of six determinations. *$P < 0.05$ *versus* the respective values obtained in wild-type mice (Student's t-test). Immunoblot analysis of TGF-β_1 in the cerebral cortex and striatum of wild-type and asymptomatic R6/2 mice treated with a single i.p. injection of saline or riluzole (10 mg/kg) is shown in (**C**). Values are means ± S.E.M. of five to six determinations. $P < 0.05$ *versus* the respective values obtained in mice treated with saline (*) or *versus* the respective values obtained in wild-type mice (#) (one-way ANOVA + Fisher's PLSD). Immunoblot analysis of TGF-β_1 in the cerebral cortex and striatum of wild-type and symptomatic (12 weeks of age) R6/2 mice is shown in (**D**). Values are means + S.E.M. of five to six determinations. *$P < 0.05$ (Student's t-test) *versus* the respective values obtained in age matched wild-type mice. Serum TGF-β_1 levels in wild-type, asymptomatic and symptomatic R6/2 mice are shown in (**E**), where values are means + S.E.M. of six determinations. $P < 0.05$ *versus* values obtained in age-matched wild-type mice (*) (Student's t-test).

this factor contributes to the pathophysiology of neurodegeneration associated with HD. This hypothesis is supported by the emerging role of TGF-β_1 in mechanisms of neuroprotection (see below), and by the evidence that TGF-β_1 is required for the neuroprotective activity of GDNF [63] and of BDNF [64, 65]. In HD patients, we found serum TGF-β_1 levels reduced in asymptomatic and first HD stages, and particularly in asymptomatic patients with lower brain glucose metabolism. As brain glucose metabolism progressively decreases during the asymptomatic phase of HD [27, 30, 66] we expect that blood TGF-β_1 levels are maximally
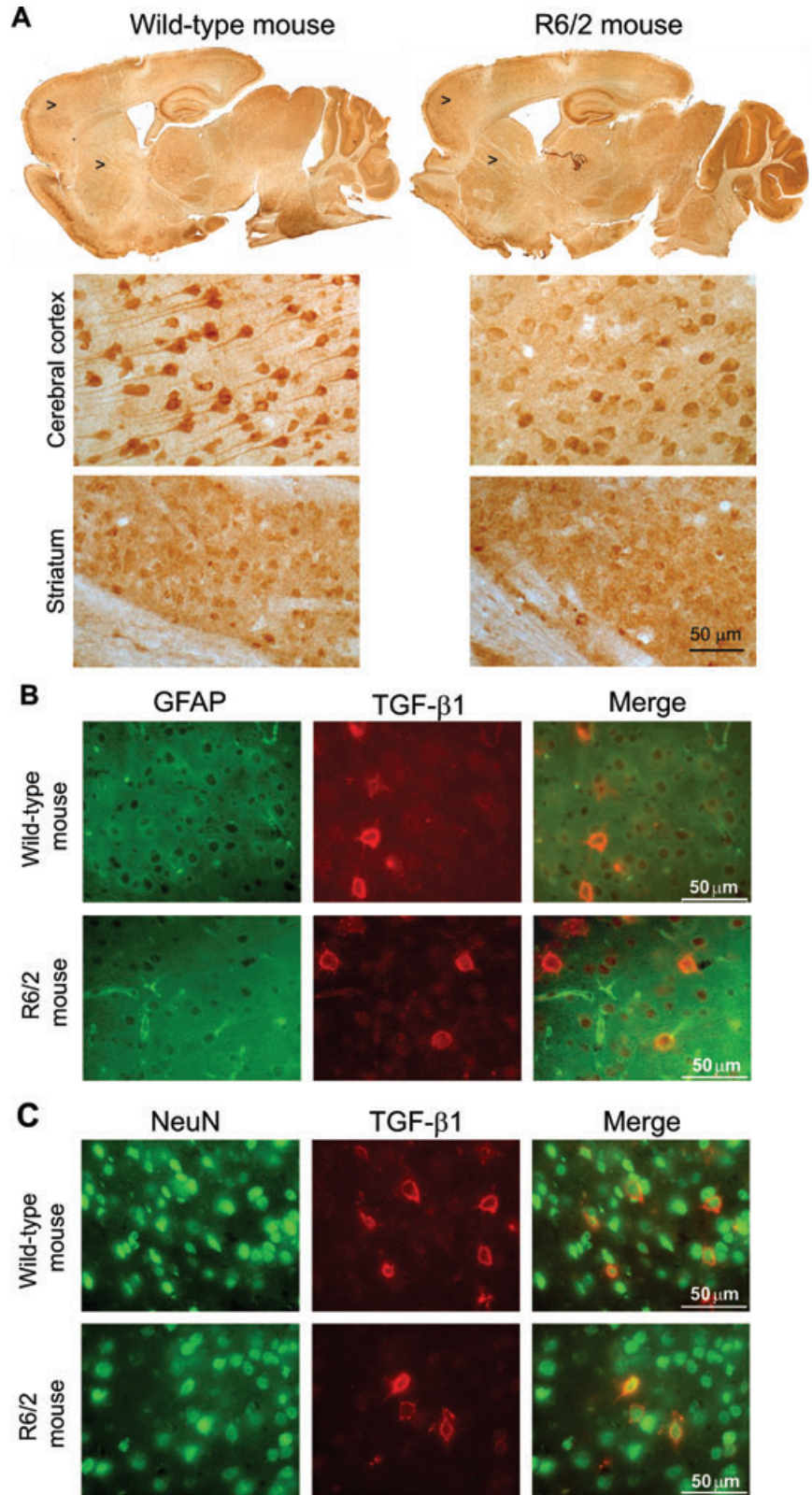
Fig. 6 Immunohistochemical analysis of TGF-β₁ in wild-type and asymptomatic R6/2 mice. Immunohistochemical analysis of TGF-β₁ in a representative wild-type and asymptomatic R6/2 mouse is shown in (**A**). Arrowheads indicate the zone showed at higher magnification. Double fluorescent staining for TGF-β₁ and GFAP, or TGF-β₁ and NeuN in cortical neurons of a representative wild-type and asymptomatic R6/2 mouse is shown in (**B**) and (**C**), respectively. Note that TGF-β₁ is expressed in neurons and not in astrocytes.
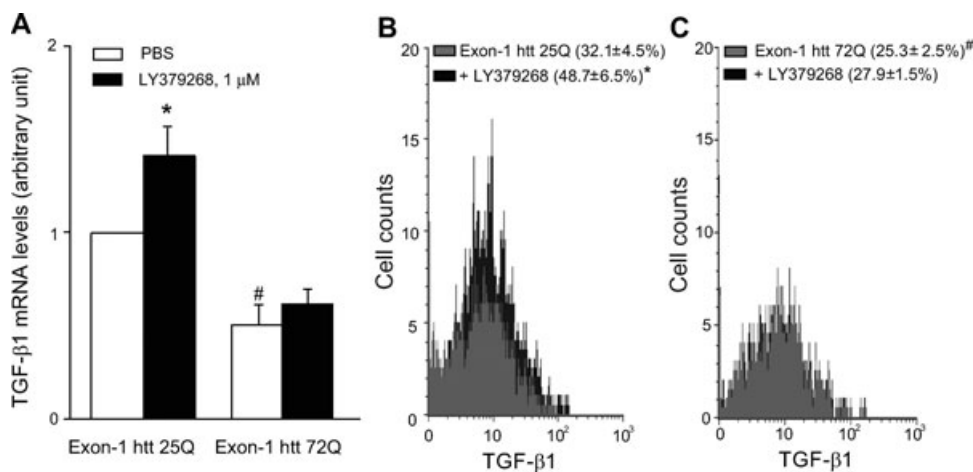
Fig. 7 Analysis of TGF-β_1 in astrocytes expressing exon-1 htt 72Q. Real Time RT-PCR of TGF-β_1 mRNA analysis (**A**) and FACS analysis of intracellular TGF-β_1 protein (**B**, **C**) in astrocytes transfected with exon-1 of human huntingtin (htt) containing an expanded 72Q repeat or an unexpanded 25Q repeat used as a control, under basal conditions and after treatment with LY379268, 1 μM for 10 min. The mean fluorescence intensity (MFI) of TGF-β_1 in astrocytes transfected with exon-1 htt 25Q or exon-1 htt 72Q and treated with PBS or LY379268 was unchanged (MFI: 22.4 $\pm$ 5.8, 22.1 $\pm$ 5.7, 24.5 $\pm$ 6.7, 20.6 $\pm$ 6.3, respectively). Values are means $\pm$ S.E.M. of five to six determinations. $P < 0.05$ *versus* the respective values obtained in transfected astrocytes treated with PBS (*) or *versus* the respective values obtained in astrocytes transfected with exon-1 htt 25Q (#) (one-way ANOVA + Fisher's PLSD).

reduced in the proximity of the clinical onset of HD. We could examine brain TGF-β_1 expression only in one asymptomatic HD patient. Interestingly, TGF-β_1 was absent in cortical neurons of this patient, whereas it was always detected in cortical neurons of age-matched controls. Data obtained in post-mortem samples of symptomatic HD patients confirmed the reduction of TGF-β_1 levels in cortical neurons. In contrast, the examination of the white matter and the caudate nucleus of symptomatic patients were confounded by the high expression of TGF-β_1 in reactive astrocytes and microglia, which overwhelmed any possible change in neuronal TGF-β_1 expression. This was not unexpected because TGF-β_1 has a prominent role in the regulation of the immune response, and is up-regulated in processes of neuroinflammation (see the strong immunoreactivity in the cerebral cortex of the patient with multiple sclerosis in Fig. 3). The substantial increase in TGF-β_1 associated with reactive gliosis might, in part, contribute to explain why blood TGF-β_1 increase from the low levels at asymptomatic stage to normality in the symptomatic HD phase, finally correlating with HD severity and progression rate in severely affected patients. Recent evidence indicates that microglia is activated in the striatum of asymptomatic HD gene carrier individuals, and activation correlates with their predicted 5-year probability of developing HD [67]. It is possible that the amount of TGF-β_1 produced by activated microglia is not sufficient to compensate for the loss of TGF-β_1 in neurons during the asymptomatic phase of the disease, and, therefore, low serum TGF-β_1 levels are detected in spite of microglia activation in asymptomatic HD patients. It should be highlighted that TGF-β_1 can be produced outside the CNS during inflammation and platelet aggregation [32], and whether these processes are altered in the asymptomatic phase of HD and peripheral pathology is contributing to HD severity, is currently under study [68–70]. However, mutant huntingtin might also directly affect peripheral TGF-β_1

production by cells of the immune system. It should be highlighted that plasma levels of the pro-inflammatory cytokine, interleukin-6 (IL-6), are higher before clinical onset of HD [68]. Reduction of TGF-β_1 may be causally related to the increase in IL-6 because targeted disruption of the TGF-β_1 gene in mice results into an enhanced expression of pro-inflammatory cytokines in peripheral organs [71]. It is possible that a reduced expression of the anti-inflammatory cytokine, TGF-β_1, in the context of an increased expression of pro-inflammatory cytokines, such as IL-6 [68], contributes to immune dysfunction in asymptomatic HD patients.

The reduction in cortical TGF-β_1 levels associated with the asymptomatic phase of HD was confirmed using R6/2 and YAC128 mice. YAC128 mice express the full length human huntingtin gene containing 128 CAG repeats, whereas R6/2 mice express exon 1 of the human huntingtin gene with 250 CAG repeats [33, 34]. We only examined asymptomatic HD mice to avoid the bias of the extensive atrophy of the striatum and cortex observed in symptomatic YAC128 mice [34, 72], and the atrophy of striatal neurons observed in symptomatic R6/2 mice [73].

Interestingly, both strains of asymptomatic HD mice showed reduced TGF-β_1 levels in cortical neurons. This reduction might reflect an intrinsic defect of TGF-β_1 formation, or, alternatively, a defect in mechanisms that regulate TGF-β_1 formation in response to environmental factors. Data obtained in transfected astrocytes or in mhtt knock-in cells suggest that expression of mutated huntingtin causes a reduced expression of the TGF-β_1 gene. However, both HD mice and cells expressing pathological huntingtin showed also an impaired response to extracellular stimuli that enhance TGF-β_1 production. Previous studies have shown that activation of mGlu2/3 metabotropic glutamate receptors [see 74 for a review] enhances TGF-β_1 formation in cultured astrocytes and brain tissue [20]. Activation of these receptors with

Fig. 8 Analysis of TGF-β_1 in striatal-derived knock-in cells expressing full-length huntingtin with an expanded Q repeat. Real Time RT-PCR of TGF-β_1 mRNA analysis (**A**, **B**) in ST7/7Q, wild-type cells or full-length mutated huntingtin ST111/111Q, mhtt cells, under basal conditions and after treatment with LY379268, 1 μM for 10 min. FACS analysis of intracellular TGF-β_1 protein (**C**) and ELISA analysis of extracellular TGF-β_1 protein wild-type and mhtt cells, under basal conditions and after treatment with LY379268, 1 μM for 10 min (**D**). The mean fluorescence intensity (MFI) of TGF-β_1 in ST7/7Q cells (MFI: 18.1 $\pm$ 4.3, 18.0 $\pm$ 5.6, respectively) or in ST111/111Q cells (MFI: 27.3 $\pm$ 7.3, 18.0 $\pm$ 8.0, respectively) treated with PBS or LY379268 was unchanged (**E**). Values are means $\pm$ S.E.M. of five to six determinations. $P < 0.05$ *versus* the respective values obtained in wild-type cells treated with LY379268 (*) or *versus* the respective values obtained in ST7/7Q cells (#) (one-way ANOVA + Fisher's PLSD). Real Time RT-PCR of TGF-β_1 mRNA analysis (**F**) in ST7/7Q and ST111/111Q knock-in cells transfected with the unexpanded exon-1 htt 25Q or the expanded exon-1 htt 72Q. Values are means $\pm$ S.E.M. of five to six determinations. $P < 0.05$ *versus* the respective values obtained in ST7/7Q cells transfected with exon-1 htt 25Q (one-way ANOVA + Fisher's PLSD).
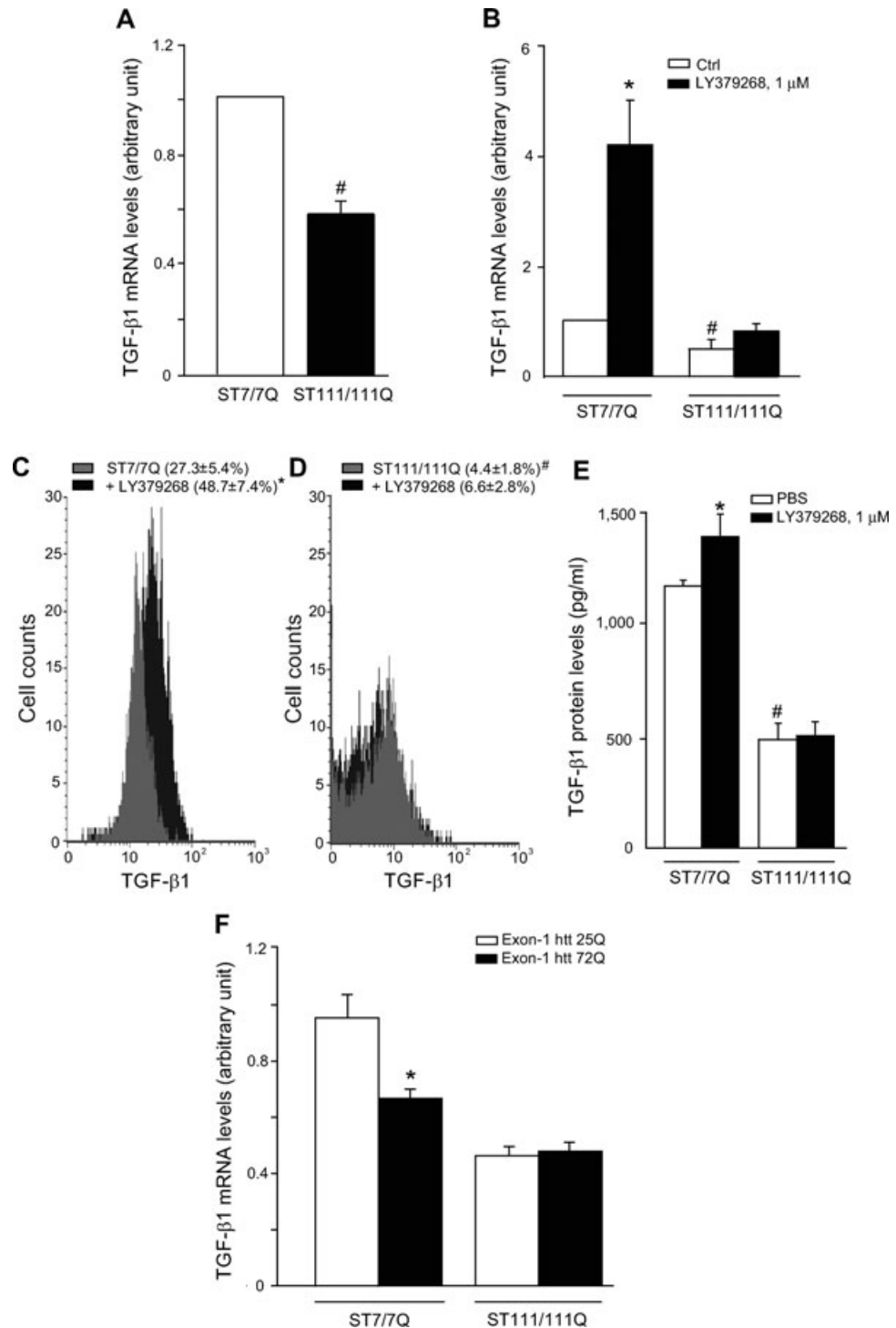
compound LY379268 failed to stimulate TGF-β_1 production in R6/2 mice and in astrocytes or knockin cells expressing huntingtin with an expanded CAG repeat. As these cells showed normal levels of mGlu2/3 receptors (although receptors were reduced in R6/2 mice), we can conclude that the intracellular machinery regulating the production of TGF-β_1 in response to receptor activation is defective in the presence of mutated huntingtin. The hypothesis that the defect is downstream of mGlu2/3 receptors was

supported by the use of riluzole, a drug that is known to enhance the formation of a number of trophic factors, including NGF, BDNF and GDNF [35, 36, 75]. Interestingly, riluzole was able to enhance the formation of TGF-β_1 in wild-type mice, but was inactive in R6/2 mice.

Knowing that TGF-β_1 is anterogradely transported by neurons [76], we speculate that, in the early phase of HD, the striatum is deprived of the amount of TGF-β_1 that is physiologically secreted by cortico-striatal neurons. This might increase the vulnerability of striatal neurons because TGF-β_1 regulates a number of intracellular events that are relevant to processes of neurodegeneration/ neuroprotection. TGF-β_1 protects neurons against different insults, including excitotoxicity, hypoxia, ischemia, deprivation of trophic factors and Aβ toxicity [77, 78]. TGF-β_1 has also a constitutive role in the suppression of inflammation, and appears to control the degree of microglial activation in the CNS [79]. TGF-β_1 acts synergistically with other neurotrophins and is required for a full neuroprotective activity of BDNF, and GDNF [63–65], and enhances the expression of BDNF and TrkB in neuronal cultures [64]. At cellular level, TGF-β_1 prevents apoptotic neuronal death by inhibiting caspase-3 [80], enhancing the expression of the anti-apoptotic proteins, Bcl-2 and Bcl-xl [81], and inhibiting the pro-apoptotic protein, Bad [82]. The anti-apoptotic activity of TGF-β_1 in cultured neurons involves the activation of the mitogen-activated protein kinase pathway and the phosphatidylinositol-3-kinase pathway [83]. Established target genes of TGF-β are the 'check points' p27 and p21, which produce cell cycle arrest by inhibiting the activity of cyclin-dependent kinases [84, 85]. A lack of TGF-β_1 might facilitate the activation of an abortive mitotic cycle in neurons, an event that has been implicated in the pathophysiology of neuronal death associated with chronic neurodegenerative disorders, including HD [86–90]. In addition, the lack of TGF-β_1 might enhance the expression of type-2 cyclooxygenase, an enzyme that contributes to mechanisms of neuronal degeneration [91–93].

At this stage, we cannot prove whether decreased TGF-β_1 dosage in human serum may represent an asymptomatic marker or its relative increase in advanced stages may suggest a novel marker of HD progression and severity. Additional cross-sectional and longitudinal studies on large populations of HD patients clinically stratified according to HD stage are required to demonstrate such role of TGF-β_1 as potential biomarker.

In conclusion, our data show that (*i*) a reduction of TGF-β_1 levels in peripheral blood occurs during the asymptomatic phase of HD and correlates with the reduction in brain glucose metabolism; (*ii*) HD is associated with an early loss of TGF-β_1 in cortical neurons and (*iii*) this loss might reflect a defect in the intrinsic TGF-β_1-producing machinery that makes the factor unresponsive to different stimulating agents. If the early loss in TGF-β_1 contributes to the pathophysiology of neuronal death associated with HD, then strategies that rescue TGF-β_1 levels should limit the progression of HD-related pathology. This hypothesis can be tested in HD mice using either by implantable cells engineered to overexpress TGF-β_1 or viral vectors encoding TGF-β_1.

Acknowledgements

Disclosure

The authors report no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Correlation between age and serum TGF-β_1 levels in control subjects, asymptomatic and symptomatic subjects. (**A**) Age of controls subjects and TGF-β_1 levels ($n = 47$; $R^2 = 0.02$; $P = 0.3315$); (**B**). Age of asymptomatic subjects and TGF-β_1 levels ($n = 30$; $R^2 = 0.006$; $P = 0.6779$); (**C**) Age of symptomatic patients and TGF-β_1 levels ($n = 95$; $R^2 = 0.01$; $P = 0.7703$). Statistical analysis was carried out by ANOVA + Fisher PLSD. Data were considered statistically significant at $P < 0.05$. Statistical analysis was performed with the StatView 5 software.

Table S1 Demographics of Huntington's disease subjects and healthy controls

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