Early and reversible neuropathology induced by tetracycline-regulated lentiviral overexpression of mutant huntingtin in rat striatum

Etienne Régulier[1], Yvon Trottier[2], Valérie Perrin[1], Patrick Aebischer[1] and Nicole Déglon[1,*]

[1]Institute of Neuroscience, Swiss Federal Institute of Technology Lausanne, EPFL, Lausanne, Switzerland and
[2]Institut de Génétique et Biologie Cellulaire et Moléculaire, CNRS/INSERM/ULP, Illkirch, France

The ability to overexpress full-length huntingtin or large fragments represents an important challenge to mimic Huntington's pathology and reproduce all stages of the disease in a time frame compatible with rodent life span. In the present study, tetracycline-regulated lentiviral vectors leading to high expression levels were used to accelerate the pathological process. Rats were simultaneously injected with vectors coding for the transactivator and wild type (WT) or mutated huntingtin (TRE-853-19Q/82Q) in the left and right striatum, respectively, and analyzed in the 'on' and 'off' conditions. Overexpression of TRE-853-19Q protein or residual expression of TRE-853-82Q in 'off' condition did not cause any significant neuronal pathology. Overexpressed TRE-853-82Q protein led to proteolytic release of N-terminal htt fragments, nuclear aggregation, and a striatal dysfunction as revealed by decrease of DARPP-32 staining but absence of NeuN down-regulation. The differential effect on the DARPP-32/NeuN neuronal staining was observed as early as 1 month after injection and maintained at 3 months. In contrast, expression of a shorter htt form (htt171-82Q) did not require processing prior formation of nuclear aggregates and caused decrease of both DARPP-32 and NeuN neuronal markers at one month post-injection suggesting that polyQ pathology may be dependent on protein context. Finally, the reversibility of the pathology was assessed. Huntingtin expression was turn 'on' for 1 month and then shut 'off' for 2 months. Recovery of DARPP-32 immunoreactivity and clearance of huntingtin aggregates were observed in animals treated with doxycycline. These results suggest that a tetracycline-regulated system may be particularly attractive to model Huntington's disease and induce early and reversible striatal neuropathology *in vivo*.

INTRODUCTION

Huntington's disease (HD) is the most frequent hereditable neurodegenerative disease of a group of nine disorders caused by an expansion of CAG trinucleotide repeats encoding polyglutamine (polyGln) residues (1,2). These diseases have in common autosomal dominant inheritance, ubiquitous expression of the corresponding proteins, the fact that the polyGln tract becomes pathogenic upon reaching a certain size, the presence of nuclear inclusions and the selective degeneration of subpopulations of neurons. Studies performed on transgenic or knock-in mice models of HD have provided important clues on the molecular basis of mutant huntingtin neurotoxicity (3–11). Interestingly, these animal models recapitulate some of the early neuropathological features of HD while the more advanced pathological features are not observed. The transgenic models are also limited by the occurrence of side effects and premature death associated with the widespread overexpression of huntingtin (htt) (3,12,13). To overcome some of these limitations, we have produced lentiviral vectors overexpressing various mutant htt fragments both in rats and primates (14,15). In rat striatum, the overexpression of the first 171 amino acids of the htt protein with 82 CAG repeats was associated with neuronal dysfunction and astrogliosis leading to robust and selective degeneration of GABAergic neurons 12 weeks post-lentiviral injection (15). The severity of the pathology was directly correlated with htt expression level and htt fragment size (15), confirming that these parameters constitute key factors for polyQ diseases modeling (16). Huntingtin being the

*To whom correspondence should be addressed at: Institute of Neuroscience, Swiss Federal Institute of Technology Lausanne (EPFL), Building SG-AAB135, 1015 Lausanne, Switzerland. Tel: +41 216939508; Fax: +41 216939520; Email: nicole.deglon@epfl.ch

largest polyglutamine-containing protein, the targeted over-expression of full-length huntingtin (350 kDa) in animals represents an important challenge to closely mimic the human pathology, analyze the folding and processing of the protein and reproduce all stages of the disease in a time frame compatible with rodent life span.

In the present study, we have developed tetracycline-regulated lentiviral vectors expressing the first 853 amino acids of htt. We have recently shown that in the 'on' state, the expression level reached with dual tetracycline-regulated vectors is 4- to 5-fold higher than the housekeeping and constitutive phosphoglycerate kinase 1 (PGK) promoter (17). This tetracycline-regulated system was used to assess whether an increased htt expression was associated with exacerbated and earlier striatal pathology in adult rats. The neuronal dysfunction was analyzed on DARPP-32- and NeuN-stained sections. In addition, the formation of aggregates as well as the processing and translocation of mutated htt in the nucleus were used to follow the appearance of the pathology. Finally, taking advantage of the tetracycline-regulated system, we have assessed the reversibility of the pathological process by shutting-off htt expression. Yamamoto and coworkers (10) showed that the down-regulation of htt Exon 1 (htt67 with 94 CAG) expression in symptomatic mice can reverse motor deficits and inclusions formation. The present study extended these observations to a larger htt fragment.

RESULTS

Tetracycline-regulated vectors based on multiply attenuated lentiviral packaging system (18,19) were used to produce recombinant viruses (Fig. 1A). The cDNA encoding for wild-type (19 CAG) or mutated (82 CAG) htt fragments of 853 amino acids (htt853) was cloned in the SIN-W-TRE backbone (17). The functionality of the vectors was assessed in 293T cells infected with SIN-W-TRE-htt853-19Q (TRE-853-19Q) or SIN-W-TRE-htt853-82Q (TRE-853-82Q) alone or in combination with the transactivator (PGK-tTA1; Fig. 1A and B) (17). Huntingtin fragments corresponding to wild-type and mutated proteins were detected in cells co-infected with the PGK-tTA1 (Fig. 1B, lanes 3 and 5). Importantly, the expression level was 6.8 ± 2.1 times higher than endogenous htt, confirming that the tetracycline system led to strong transgene expression (Fig. 1B). The addition of doxycycline in the culture medium reduced htt expression to background levels (Fig. 1B, lanes 4 and 6). Owing to the intrinsic leakiness of the TRE promoter (20,21), a weak signal was detected in cells infected with the TRE-853-19Q or -82Q viruses alone (Fig. 1B, lanes 1 and 2).

A pilot study was performed in adult rats to verify the hypothesis that tetracycline-regulated htt853-82Q overexpression would lead to an exacerbated pathology compared to a lentiviral vector with the phosphoglycerate kinase promoter (PGK-853-82Q) (15). We have previously shown that a diffuse loss of DARPP-32 staining appeared 3 months post-injection of PGK-853-82Q and that a neuronal dysfunction, with a large DARPP-32 depleted area, is obtained at 6 months (15). In this study, the animals were sacrificed 1 month post-injection. Under these conditions, DARPP-32 expression was unaltered in PGK-853-82Q-injected rats, while a neuronal dysfunction was already detected on striatal sections from TRE-853-82Q-injected rats
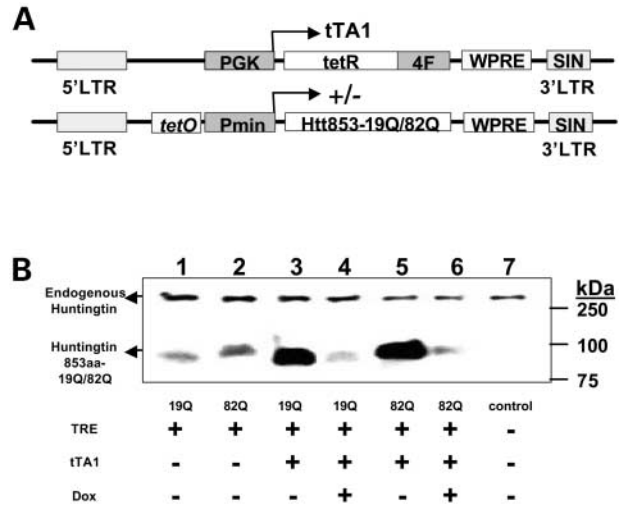

Figure 1. (**A**) Lentiviral vector systems used for the expression of tetracycline-regulated of wild-type (19 CAG) or mutated (82 CAG) huntingtin (htt) proteins; (**B**) western blot of 293T cells infected with (**1**) SIN-TRE-853-19Q; (**2**) SIN-TRE-853-82Q; (**3**) SIN-TRE-853-19Q + SIN-PGK-tTA1/−Dox; (**4**) SIN-TRE-853-19Q + SIN-PGK-tTA1/+Dox; (**5**) SIN-TRE-853-82Q + SIN-PGK-tTA1/−Dox; (**6**) SIN-TRE-853-82Q + SIN-PGK-tTA1/+Dox; (**7**) non-infected 293T cells. The western blot analysis was performed three times.

(Fig. 2). These results support the hypothesis that an increased htt expression level accelerates the pathological process.

To further characterize the onset and progression of the pathology, rats were simultaneously injected with PGK-tTA1 and TRE-853-19Q/TRE-853-82Q vectors in the left and right striatum, respectively. The animals were maintained in the 'on' (−dox) or 'off' (+dox) states for 1, 2 or 3 months. Wild-type and mutated htt proteins were detected around the injection site, in the 'on' groups (Fig. 3A), while htt expression was below detection level in the 'off' groups (data not shown). The residual expression of htt853-82Q in animals ('off' group) and the overexpression of htt853-19Q ('on' group) did not lead to any significant pathology based on DARPP-32 and NeuN expression (Fig. 3B and data not shown). In TRE-853-82Q injected animals ('on' group), a DARPP-32 down-regulation ($13.4 \pm 0.7\%$ of the striatum) was already visible at 1 month (Fig. 3B and C). This pathology is due to striatal dysfunction as indicated by the absence of NeuN down-regulation (Fig. 4B). The differential effect on the DARPP-32/NeuN neuronal markers was still present at 3 months (data not shown) and was also observed in animals injected with the PGK-853-82Q vector (Fig. 4C and D). In contrast, both markers were affected at 1 month in PGK-171-82Q injected rats (Fig. 4E and F). The lesion size, however, suggest that DARPP-32 expression is more sensitive to polyQ toxicity than NeuN (Fig. 4E and F).

Figure 3A shows that nuclear (NIs) and cytoplasmic (CIs) inclusions were already present one month post-injection of TRE-853-82Q. We have therefore examined whether a proteolytic cleavage was occurring in these animals. Various htt breakdown products have been identified in cellular or animal models of HD and on brain tissue from affected patients (22–25). In this study, we have used antibodies recognizing the N-terminal part of htt (2B4 mAb) (26) or an epitope located between amino acids 115–129 (1H6 mAb) to analyze the
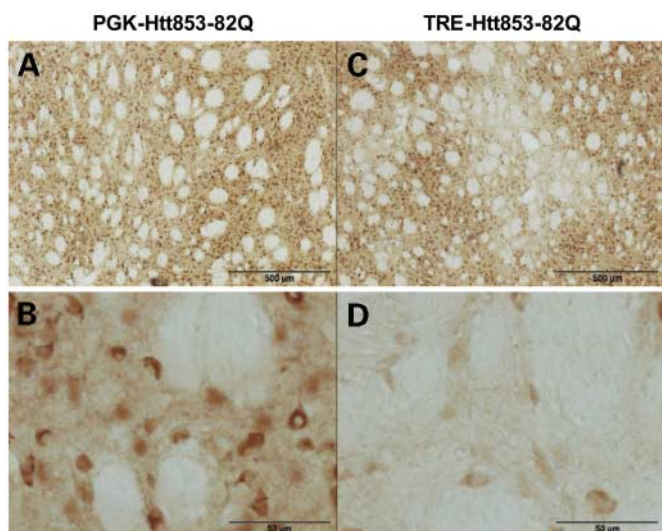
Figure 2. Side-by-side comparison of constitutive versus regulated htt853-82Q expression. DARPP32 down-regulation in rats injected with PGK-853-82Q (**A** and **B**) or TRE-853-82Q + PGK-tTA1 (**C** and **D**) lentiviral vectors.

processing and subcellular localization of various htt fragments (Fig. 5A) (26,27). In animals injected with the TRE-853-19Q construct, a cytoplasmic immunoreactivity was observed with both antibodies (Fig. 5B). In TRE-853-82Q injected animals, nuclear and neuritic inclusions were densely stained with the 2B4 antibody, while a weak cytoplasmic staining was obtained with the 1H6 antibody (Fig. 5B). In agreement with the data previously described in HD patients and *in vitro* systems, this suggest that the majority of NIs and CIs formed by TRE-853-82Q expression are composed of N-terminal htt fragments cleaved between 2B4 and 1H6 epitopes (26). To further analyze the processing and translocation of htt in the nucleus, immunohistochemical staining was performed on striatal sections from PGK-171-82Q injected rats (15). In these animals, NIs were stained with both antibodies, suggesting that, contrary to the htt853 protein, this short htt fragment is not subjected to protease cleavage before being transported into the nucleus (Fig. 5C).

In the last part of this study, we have taken advantage of the tetracycline system to investigate the reversibility of TRE-853-82Q pathology. Huntingtin expression was turned 'on' for 1 month and then shut 'off' for an extra 2 months. Controlled animals were either maintained in the 'on' state for 1 and 3 months or in the 'off' state for 3 months. This experimental paradigm was chosen because TRE-853-82Q overexpression for a month is associated with neuronal dysfunction with no indication of cell death. In the animals switched 'off' for 2 months, a $61.4 \pm 4.3\%$ recovery of DARPP-32 immunoreactivity was observed (Fig. 6A and B). This recovery was accompanied by a clearance of htt aggregates (Fig. 6C). Recent studies have demonstrated that minocycline, a second-generation tetracycline antibiotic, delays the onset of symptoms and prolongs the life span of R6/2 mice (28). To exclude the possibility that the reversibility of the neuropathology was due to a direct effect of doxycycline, rats were stereotaxically injected with viruses constitutively expressing the htt171-82Q

(PGK-171-82Q) (15) and 548-128Q (PGK-548-128Q) fragments and half the animals were treated with doxycycline for 1 month. Neither the cellular dysfunction (Fig. 7A and B) nor the formation of aggregates was affected by the addition of doxycycline (Fig. 7C). These data further confirm that reversal of the pathology was due to htt shut 'off' and not to a direct effect of doxycycline.

DISCUSSION

Rodent models of HD expressing full-length or large htt fragments and reproducing the late stages of the disease have not yet been described. In an attempt to overcome this limitation and closely recapitulate the human pathology, very high transgene expression or extremely long CAG tracts mimicking juvenile form of HD have been proposed (2,11,29,30). We have previously reported that lentiviral vectors represent a powerful and flexible tool for targeted overexpression of disease-causing genes *in vivo* and that the protein levels reached with this system are higher than with transgenic techniques (15). The sustained and local lentiviral-mediated delivery of a short htt fragment of 171 amino acids and 82 CAG repeats was, for example, associated with a severe striatal degeneration at 3 months (15). However, increasing the size of the htt fragments (853 or 1520AA) to include caspases, calpains and/or proteases cleavage sites implicated in HD pathogenesis significantly delays the appearance of a pathology (27,31–35). Neuronal dysfunction with no indication of cell death was obtained up to 6 months post-injection (15).

In the present study, we have used tetracycline-regulated lentiviral vectors to further push transgene expression level and induce an early pathological onset with a large htt fragment. We have recently demonstrated the potency of this dual tetracycline system to regulate CNTF expression *in vivo* (17). In the 'on' state, the transgene expression was 4- to 5-fold higher than the housekeeping and constitutive phosphoglycerate kinase 1 (PGK) promoter (17). Lentiviral vectors coding for the first 853 amino acids of wild-type and mutated htt driven by the tetracycline operator and minimal CMV promoter were produced (TRE-853-19/82Q). The tetracycline repressor tetR fused to four copies of the minimal transcriptional activation domain of VP16 (36) was cloned in the SIN-W-PGK transfer vector (37). In a side-by-side comparison with PGK-853-19/82Q vectors, we confirmed that higher htt levels (TRE-853-82Q) significantly accelerated the kinetics of appearance of striatal dysfunction. A loss of DARPP-32 expression, NIs and CIs was observed as early as 1 month after the injection of the TRE-853-82Q vector compared with 6 months with the PGK-853-82Q vector. Interestingly, NeuN expression was not decreased under these conditions. This differential effect on neuronal markers (DARPP-32/NeuN) was still present at 3 months whereas both markers were down-regulated at 1 month in animals injected with the short htt171-82Q fragment. These results strengthen the concept of context-dependent polyQ toxicity (38–40) and suggest that the molecular mechanisms leading to striatal dysfunction might differ with the various htt peptides. Data on the processing of htt further illustrate this notion. PolyQ aggregates formed by htt171-82Q or htt853-82Q
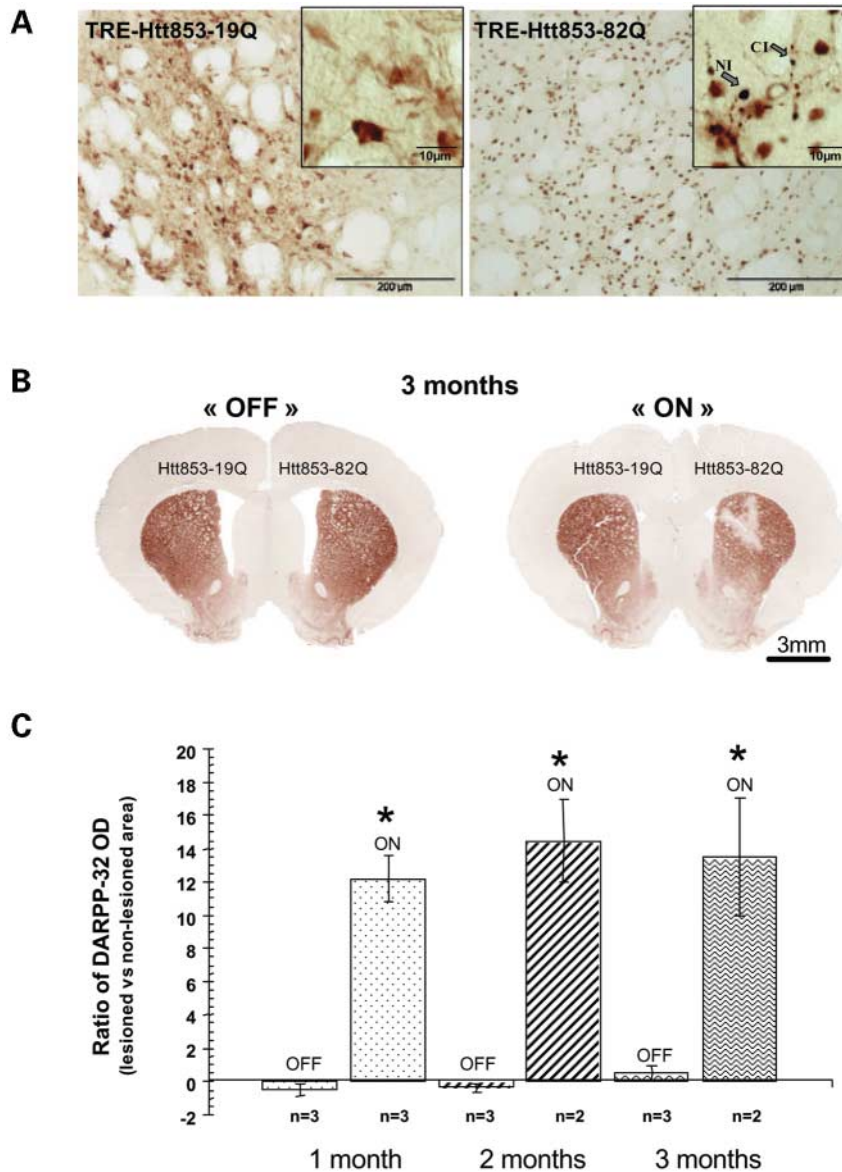
Figure 3. (**A**) 2B4 Ab staining revealing exogenous human huntingtin expression in the striatum of rats injected with PGK-tTA1 and TRE-853-19Q/82Q vectors. High magnification showing the accumulation of cytoplasmic (CI) and nuclear inclusions (NI) in rats injected with the TRE-853-82Q vector as compared with the diffuse staining in animals injected with the TRE-853-19Q vector; (**B**) DARPP-32 staining in the striatum of rats injected with TRE-853-82Q/tTA1 vectors and maintained 3 months in either 'on' or 'off' states; (**C**) quantification of DARPP-32 down-regulation 1, 2 and 3 months post-injection of lentiviruses in animals treated or not with doxycycline. Results are expressed as a ratio of DARPP-32 optical density (OD) between lesioned and non-lesioned areas. One-way ANOVA, $F(5,10) = 27.68$, $P < 0.001$. *Post-hoc* comparison: 'on' versus 'off' for each time point, $*P < 0.001$. *Post-hoc* comparison: 'on' versus 'on' and 'off' versus 'off' was non-significant at all time points.

proteins present differential immunoreactivity with the 1H6 antibody, indicating that NIs and CIs are built up with htt polypeptides of different sizes. A recent report showed that NIs in HD brains and in a cellular model are built up by a short N-terminal htt fragment, named cp-A, which is released by cleavage between the polyQ stretch and the epitope of 1H6 mAb (Fig. 5) (27). In agreement with this, our present result indicates that htt853-82Q was also processed to release fragments similar in size to cp-A that translocate and aggregate in the nucleus. In contrast, in htt171-82Q-injected animals NIs were 1H6 positives, suggesting that the htt171 form is short

enough to passively translocate to the nucleus without the need of proteolysis. Whether the differential build-up of aggregates in htt171-82Q- and htt853-82Q-injected rats has an impact on the pathological process remains to be clarified. *In vitro* and *in vivo* experiments indicate that htt is a substrate for caspases, calpains and aspartyl proteases, suggesting that sequential cleavage of htt may occur (27,28,32–34). To decipher the role of proteolysis in htt toxicity, expression of large htt constructs bearing mutations of the cleavage sites will be required. Lentiviral-based vectors represent a rapid and efficient system to deliver such constructs in the striatum of rat.
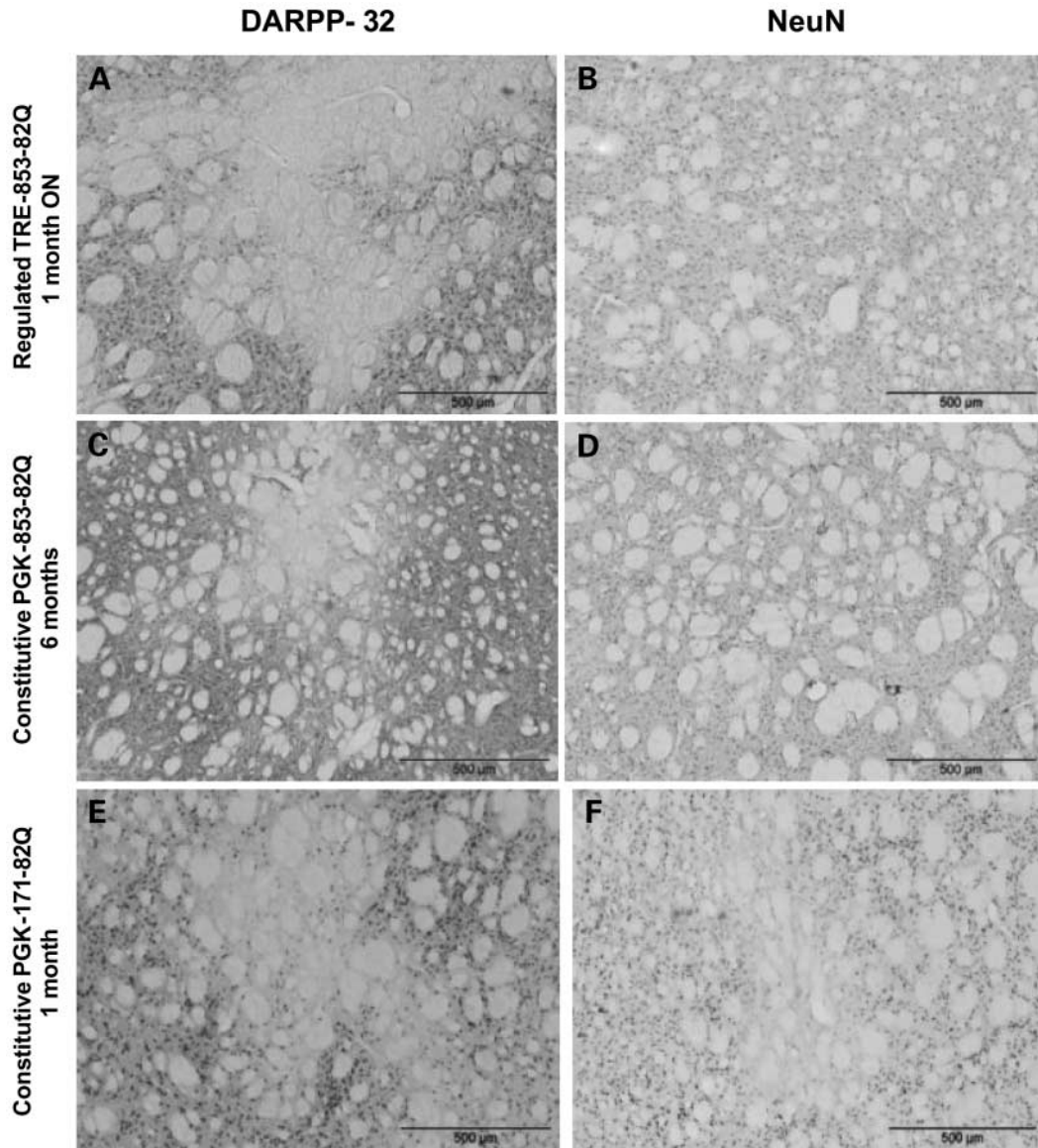
Figure 4. Differential susceptibility of DARPP-32 (**A**, **C**, **E**) and NeuN (**B**, **D**, **F**) markers, 1 month post-injection of the constitutive PGK-171-82Q vector (A and B), 6 months post-injection of the constitutive PGK-853-82Q vector (C and D) and one month post-injection of tetracycline-regulated TRE-853-82Q system (E and F).

The tetracycline operator is not only a powerful promoter leading to extremely high transgene levels upon transactivator expression but is also a system allowing suppression of mutant htt synthesis by peripheral doxycycline administration. Conditional mice models of HD expressing the first exon of mutant htt have already provided important information on the reversibility of the pathology and have demonstrated that the progression of the disease relies on a constant expression of htt and that behavioral deficits and neuronal dysfunctions can be blocked and aggregates cleared (10). Determining whether reversible damage is produced with longer htt fragments is important not only for the development of effective therapies but also to determine the time-window of intervention. In TRE-853-82Q animals, doxycycline administration for 2 months abolishes transgene expression and significantly decreases striatal dysfunction and inclusion formation. These effects cannot be attributed to a neuroprotective effect of doxycycline based on data obtained in rat injected with the PGK-171-82Q and PGK-548-128Q vectors and treated with doxycycline for a month. Altogether, these data suggest that a decrease in the cellular load of abnormal polyQ may be sufficient to restore neuronal phenotype and to induce proteasome degradation of cytoplasmic and nuclear inclusions. Strategies aiming at decreasing the disease-related transcript with RNA interference may, therefore, be of therapeutic relevance (41–43). The versatility and efficiency of lentiviral gene transfer in the CNS of adult animals associated with the availability of conditional models of HD provide a unique opportunity to address some of these questions and move further toward a cure for HD.
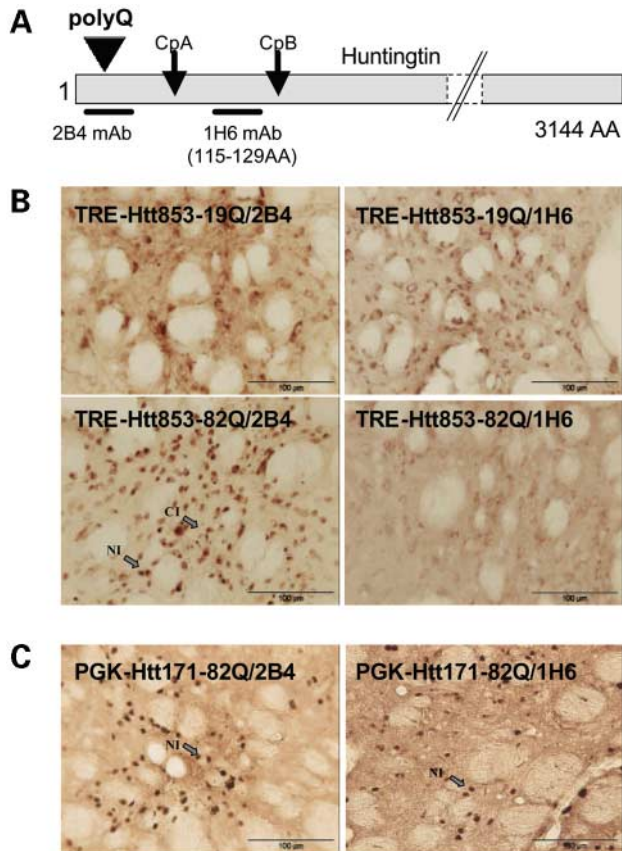
Figure 5. (**A**) Schematic representation of huntingtin protein with two putative protease cleavage sites (CpA and CpB) and 2B4 and 1H6 epitopes; (**B**) 2B4 and 1H6 stained sections from rats injected with PGK-tTA1 and TRE-853-19Q/82Q vectors and sacrificed at 1 month; (**C**) 2B4 and 1H6 stained striatal sections from rats injected with PGK-171-82Q vector and sacrificed at 1 month. NI: nuclear inclusions. CI: cytoplasmic inclusions.

MATERIALS AND METHODS

Lentiviral vector production

The cDNA coding for the first 853 amino acids of wild-type or mutated htt (htt853-19Q or htt853-82Q) was cloned in a SIN-W transfer vector containing the Tet-response element (TRE; BD Bioscience Clontech, Palo Alto, CA, USA) with seven direct repeats of the TetO operator sequence, upstream of a minimal CMV promoter. The cDNA coding for the first 548 amino acids of mutated (128 CAG) htt was kindly provided by Dr M.R. Hayden (44) and cloned in the SIN-W-PGK transfer vector (37). Lentiviral vectors encoding for the first 171 amino acids of mutated (82 CAG) htt (15) and for the tetracycline-controlled transactivator tTA1 (17) [tetracycline repressor tetR fused to four copies (4F) of the minimal transcriptional activation domain of VP16 (36)] were also used in this study. The lentiviral particles were produced and resuspended in phosphate-buffered saline (PBS)/1% bovine serum albumin as previously reported (45,46). The particle content of viral batches was determined by p24 antigen ELISA (PerkinElmer Life Sciences, Boston, USA).

Western blot analysis

293T cells were infected with the lentiviral vectors matched for particle content (200 ng of p24 antigen per well per vector) and cultured with or without addition of doxycycline (1 μg/ml) to the medium. Cellular lysates were harvested 4 days after infection in lysis buffer (125 mM Tris–HCl/0.5% SDS/1% Nonidet P-40) containing protease inhibitors (Roche Pharma, Basel, Switzerland). Protein concentrations of cytoplasmic fractions were determined by the BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). Equal amounts of protein (50 μg) were loaded on 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred onto nitrocellulose membrane (Schleicher and Schuell Bioscience GmbH, Dassel/Relliehausen, Germany). Immunoblotting was performed using a mouse monoclonal antibody (MAb2166; 1:10 000; Chemicon International Inc., Temecula, USA) directed against the 181–810 amino acid sequence of human htt followed by goat anti-mouse horseradish peroxydase (HRP) antibody (1:2000; DakoCytomation AG, Zug, Switzerland). Visualization of the proteins was achieved using ECL and chemiluminescence detection kit (Amersham Biosciences Europe GmbH, Freiburg, Germany) in a Multimage Light Cabinet (Alpha Innotech Corporation, San Leandro, USA).

In vivo experiments

Adult female Wistar rats (Iffa Credo/Charles River, Les Oncins, France), weighing 180–200 g were used. The animals were housed in a temperature-controlled room and maintained on a 12 h light/dark cycle. Food and water were available *ad libitum*. For tetracycline-regulated expression studies, rats were maintained in the 'off' state with the addition of 200 mg/l doxycycline (Dox, Sigma Chemical Co., Saint Louis, MO, USA) in drinking water containing 40 g/l sucrose (corresponding approximately to 20 mg Dox/rat/day), while the 'on' group received only sucrose. The experiments were carried out in accordance with the European Community Council directive (86/609/EEC) for the care and use of laboratory animals.

Injection of lentiviruses

Concentrated viral stocks were thawed and resuspended by repeated pipetting. Htt171-82Q-, htt548-128Q-, htt853-19Q-, htt853-82Q- and tTA1-expressing lentiviral vectors were stereotaxically injected into the striatum of ketamine (75 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthetized animals using an Hamilton syringe with a 34-gage blunt-tip needle (Hamilton, Reno, NV, USA). A 1:4 ratio in particle contents was used for lentiviral co-injection with 200 000 ng of p24/ml of PGK-tTA1 and 50 000 ng of 24/ml of TRE-853-19Q/82Q. Particle content was matched to 20 000 ng of p24/ml for single lentiviral injections. The viral suspensions (4 μl) were injected at 0.2 μl/min by means of an automatic injector (Stoelting Co., Wood Dale, USA) and the needle was left in place for an additional 5 min. The stereotaxic coordinates were: 0.5 mm rostral to bregma; 3 mm lateral to midline; and 5 mm from the skull surface. The skin was closed using a 6–0 Prolene® suture (Ethicon, Johnson and Johnson, Brussels, Belgium).
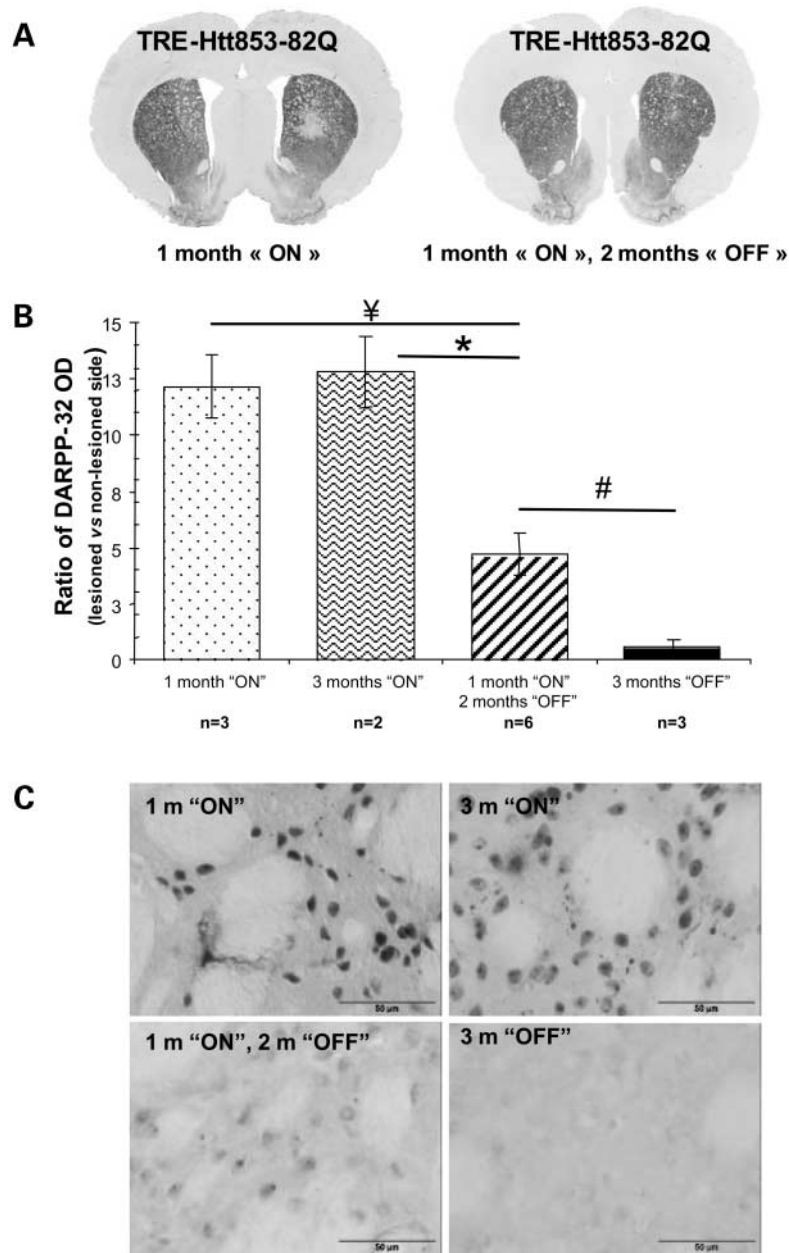
Figure 6. (**A**) Effect of htt853-82Q shut-off on DARPP-32 expression; (**B**) quantification of DARPP-32 down-regulation in rats injected with TRE-853-19Q/82Q ± Dox at 1, 2 and 3 months. One-way ANOVA, $F(3,10) = 22.08$, $P < 0.001$. *Post-hoc* comparison: 1 month 'on' versus 1 month 'on' and 2 months 'off', ¥$P < 0.01$; 3 months 'on' versus 1 month 'on' and 2 months 'off', *$P < 0.01$; 1 month 'on' and 2 months 'off' versus 3 months 'off', #$P < 0.05$; 1 month 'on' versus 3 months 'on', non-significant. (**C**) Htt stained brain sections (2B4) from rats injected with TRE-853-82Q showing the absence of htt expression in animals maintained 3 months 'off', the accumulation of aggregates in 1 and 3 months 'on' rats and finally the clearance of neuritic and neuronal inclusions in animals expressing htt for 1 month and switched 'off' for 2 months.

Histological processing

One, 2 or 3 months after lentiviral injection, the animals were given a sodium pentobarbital overdose and transcardially perfused with saline and 4% paraformaldehyde, 10% picric acid. The brains were removed and postfixed in 4% paraformaldehyde, 10% picric acid for ∼24 h and finally cryoprotected in 25% sucrose/0.1 M phosphate buffer for 48 h. The brains were frozen in dry ice and 25 µm coronal sections were cut on a sliding microtome cryostat (Cryocut 1800, Leica Microsystems AG, Glattbrugg, Switzerland) at −20°C. Slices throughout the entire striatum were collected and stored in 48-well trays (Costar, Cambridge, MA, USA) as free-floating sections in PBS supplemented with 0.12 µM sodium azide. The trays were stored at 4°C until immunohistochemical processing. Striatal sections from injected rats were processed by immunochemistry for dopamine and cAMP-regulated phosphoprotein of a molecular mass of 32 kDa (DARPP-32, 1 : 8000; Chemicon International
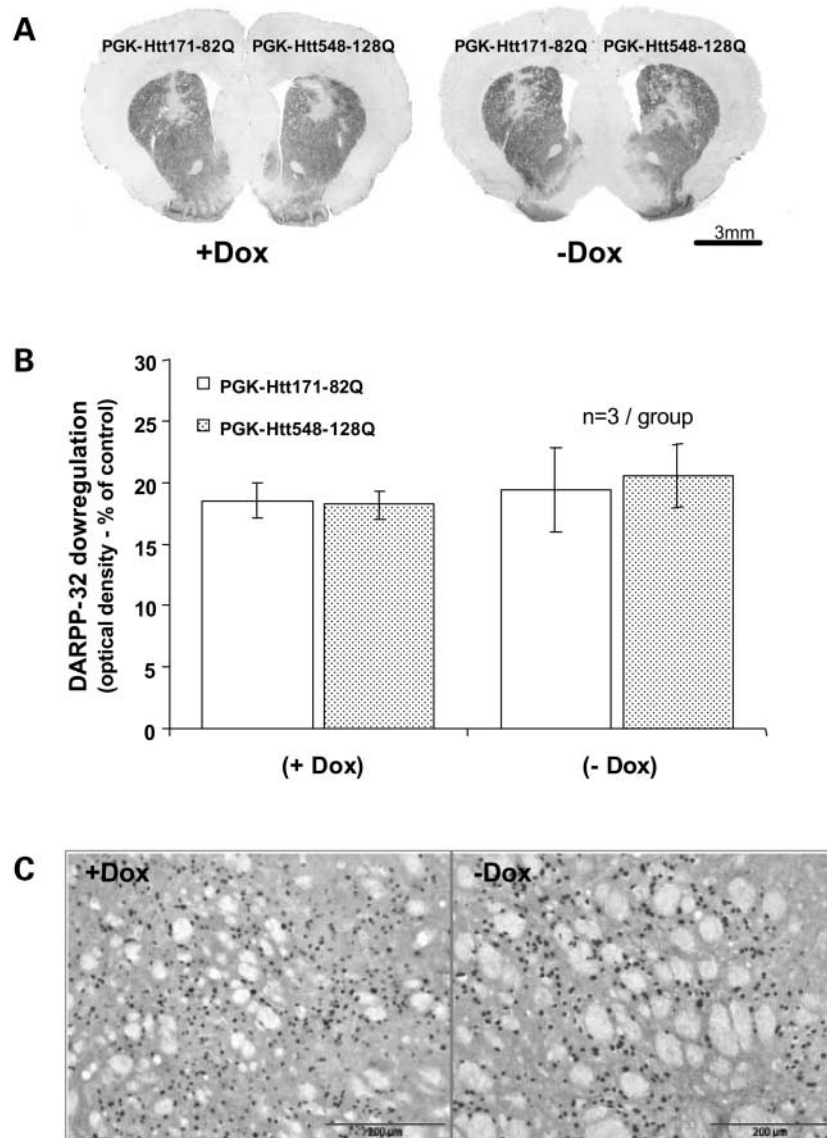
Figure 7. (**A**) Doxycycline treatment in rats injected with the constitutive PGK-171-82Q and PGK-548-128Q vectors has no effect on DARPP-32 down-regulation; (**B**) quantification of DARPP-32 staining at one month. The difference between Dox and saline injected groups was not significant on the measure of DARPP-32 down-regulation [group × down-regulation, $F(3,8) = 0.2$, $P = 0.89$]. (**C**) the addition of doxycycline in the drinking water of rats injected with PGK-171-82Q vector has no effect on the formation of Htt inclusions.

Inc., Temecula, USA), Neuronal nuclei (NeuN, 1 : 400; Chemicon, CA, USA) htt (2B4, 1/200; 1H6, 1/100) as previously described (46). The sections were mounted, dehydrated by passing twice through ethanol and toluol and cover-slipped with Merckoglas® (EM Science, Gibbstown, USA).

Image analysis

The htt lesions were analyzed by digitizing from 14 DARPP-32-stained sections per animal (200 µm between sections) with a slide scanner and by quantifying the optical density with an image analysis program (NIH-image, Version 1.6.2, National Institute of Health, USA). Sections throughout the entire striatum were analyzed. Data are expressed as the ratio of

evaluated DARPP-32 optical density. The optical density represents the average grey value of all pixels measured on the lesioned area divided by the total number of pixels of a non-lesioned area. The corpus callosum and the anterior commissure were used to delineate the striatal area.

Data analysis

Immunostaining quantifications are expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by a Newman–Keuls *post-hoc* test (Statistica 5.1, Statsoft Inc., USA). The significance level was set at $P < 0.05$.

ACKNOWLEDGEMENTS

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