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# Tissue-type plasminogen activator is an extracellular mediator of Purkinje cell damage and altered gait

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ABSTRACT

Purkinje neurons are a sensitive and specialised cell type important for fine motor movement and coordination. Purkinje cell damage manifests as motor incoordination and ataxia — a prominent feature of many human disorders including spinocerebellar ataxia and Huntington's disease. A correlation between Purkinje degeneration and excess cerebellar levels of tissue-type plasminogen activator (tPA) has been observed in multiple genetically-distinct models of ataxia. Here we show that Purkinje loss in a mouse model of Huntington's disease also correlates with a 200% increase in cerebellar tPA activity. That elevated tPA levels arise in a variety of ataxia models suggests that tPA is a common mediator of Purkinje damage. To address the specific contribution of tPA to cerebellar dysfunction we studied the T4 mice line that overexpresses murine tPA in postnatal neurons through the Thy1.2 gene promoter, which directs preferential expression to Purkinje cells within the cerebellum. Here we show that T4 mice develop signs of cerebellar damage within 10 weeks of birth including atrophy of Purkinje cell soma and dendrites, astrogliosis, reduced molecular layer volume and altered gait. In contrast, T4 mice displayed no evidence of microgliosis, nor any changes in interneuron density, nor alteration in the cerebellar granular neuron layer. Thus, excess tPA levels may be sufficient to cause targeted Purkinje cell degeneration and ataxia. We propose that elevated cerebellar tPA levels exert a common pathway of Purkinje cell damage. Therapeutically lowering cerebellar tPA levels may represent a novel means of preserving Purkinje cell integrity and motor coordination across a wide range of neurodegenerative diseases.



## Introduction

Purkinje cells are the sole efferent neurons of the cerebellum. Impairment/degradation of Purkinje cells manifests as ataxia — characterised by motor incoordination, aberrant limb movement and altered gait (Sarna and Hawkes, 2003). Purkinje cells are highly sensitive to a variety of genetic and environmental perturbations. For example, Purkinje-related deficits are a feature of many human disorders, including the spinocerebellar ataxias, Niemann–Pick disease and Huntington's disease (HD) (Matilla-Duenas et al., 2012; Rüb et al., 2013; Tang et al., 2010). Mutations in numerous unrelated genes also result in Purkinje damage in mice (Sarna and Hawkes, 2003). Moreover, Purkinje damage arises following exposure to toxins (e.g. ibogaine), during brain malignancy and cerebellar hypoxia (Sarna and Hawkes, 2003).

Tissue-type plasminogen activator (tPA) is well known for its role in intravascular proteolysis where its primary function is to convert inactive plasminogen into active plasmin. In blood, tPA-mediated plasmin activation is critical for thrombolysis (Cesarman-Maus and Hajjar, 2005). More recently, however, tPA has been established as a key modulator of neuronal function (Samson and Medcalf, 2006; Yepes et al., 2009). We previously hypothesised that elevated cerebellar levels of tissue plasminogen activator (tPA) contribute to Purkinje damage (Sashindranath et al., 2011). In the cerebellum, tPA performs numerous physiological tasks including the promotion of granule neuron migration (Seeds et al., 1999) and facilitating acquisition of fine motor skills (Seeds et al., 1995, 2003). A pathological role for tPA in the cerebellum has also been postulated in ataxia. Notably, high cerebellar tPA

*Abbreviations:* (tPA), tissue-type plasminogen activator; (SCA), spinocerebellar ataxia; (HD), Huntington's disease; (OPT), Optical Projection Tomography.

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levels coincide with Purkinje damage in *Nervous* (Li et al., 2006a,b, 2013), *Lurcher* (Lu and Tsirka, 2002) and SCA1 mice (Sashindranath et al., 2011) — three genetically unrelated models of ataxia. That increased cerebellar tPA levels are consistently observed in models of ataxia raises the prospect that excess tPA exerts a common form of Purkinje stress.

Consistent with this hypothesis, it has been shown that tPA-deficiency reduces/delays Purkinje loss in *Nervous* (Li et al., 2013) and *Lurcher* mice (Lu and Tsirka, 2002). However, it remains unclear whether an excess of tPA, without other additional genetic aberrations or environmental pressures, causes Purkinje dysfunction and ataxia. To address this question, we utilised T4 transgenic mice that constitutively overexpress murine tPA in postnatal neurons, including Purkinje cells. We found that selective reduction in Purkinje cells develops within 10 weeks of age in T4 mice. Purkinje dysfunction in T4 mice coincides with astrogliosis and manifests as ataxia. Hence, the cerebellar phenotype of T4 mice largely recapitulates that of *Nervous* mice.

Finally, we show that Purkinje degeneration in HD mice also correlates with a 200% increase in cerebellar tPA activity. Based on the consensus that excess tPA levels occur in multiple forms of ataxia, and that neuronal overexpression of tPA likely contributes to Purkinje degeneration, we postulate that tPA is a common extracellular mediator of Purkinje damage. Future studies should address whether therapeutically lowering cerebellar tPA levels can attenuate Purkinje damage across many human disorders.

## Materials and methods

### *Animals*

All animal procedures were undertaken in accordance with the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia, and were approved by the Institutional Animal Ethics Committees. All experiments were performed with adult male mice on the C57BL/6J background. T4 mice are heterozygous transgenic mice that constitutively produce increased levels of tPA in post-natal neurons. T4 mice and their *wt* controls, which do not express the T4 transgene were obtained from the same colony, $tPA^{-/-}$ mice (Carmeliet et al., 1994) were obtained from a homozygote breeding line and HD mice and their *wt* littermates were obtained from the R6/1 line (Mangiarini et al., 1996), which is transgenic for the 5′ end of the amino-terminal fragment of exon 1 of the human *huntingtin* gene. Transgenic hemizygote males were originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred with CBB6 (CBA6C57/B6) F1 females to establish the R6/1 colony at the Florey Institute. The CAG repeat length of R6/1 mice in the colony at the time of cohort generation was ~135 repeats (Department of Pathology, University of Melbourne, Australia).

### *Tissue collection*

Male T4 transgenic mice and their *wt* littermates (10 weeks of age) were transcardially perfused with 25 ml of phosphate-buffered saline, pH 7.4 (PBS). For ELISA, tPA activity and western blot analyses, brains were removed and the cerebellum was dissected. Each cerebellum was weighed and homogenised to a final concentration of 150 mg wet weight of tissue/ml in PBS + 1% Triton X-100. To prevent protein degradation, protease inhibitors (Complete EDTA-free inhibitor tablets; Roche; Mannheim, Germany) were added to lysates used for ELISA and western blot assays. For immunofluorescence, Optical Projection Tomography (OPT) and Cresyl Violet staining, mice were transcardially perfused with 25 ml of 4% formaldehyde solution following perfusion with Brains were removed and fixed in 4% formaldehyde solution overnight at 4 °C. For OPT, the cerebellum was dissected from the brain before overnight fixation. For immunofluorescence, brains were subsequently processed and embedded into paraffin blocks. HD mice (12 weeks of age) and their *wt* littermates were killed by cervical dislocation and cerebellum tissues were dissected and homogenised as for ELISA and tPA activity assays, above.

### *Immunofluorescence*

Coronal cerebellum and sagittal whole brain tissue sections (6 μm) were mounted on Superfrost Plus slides (Menzel-Glaser; Braunscweig, Germany). Tissue sections were de-waxed, rehydrated and antigen retrieval was performed by incubating tissues in 10 mM citrate buffer (pH 6.5) for 20 min at 95 °C. To prevent non-specific binding, tissue sections were blocked for 1 h with 5% goat serum, then incubated with primary antibodies (1:100) to detect Calbindin (Anti-Calbindin D-28 K; AB1778; Merck Millipore; Billerica MA, USA), tPA (rabbit anti-mouse tPA antibody; a gift from Professor Roger Lijnen, University of Leuven, Belgium) or Glial Fibrillary Acidic Protein (GFAP; Polyclonal rabbit anti-GFAP; Z0334; DAKO; Glostrup, Denmark) overnight at 4 °C. Tissue sections were washed for 15 min in three changes of PBS and exposed to AlexaFluor goat anti-rabbit 568 secondary antibody (1:1000; Life Technologies; Carlsbad CA, USA) for 1 h, followed by Hoechst (1:1000; Life Technologies) counter stain for five minutes. For dual staining immunofluorescence (Fig. 1A), tissue sections were blocked with 5% horse serum, co-incubated with primary antibodies (1:100) to detect calbindin (AB1778; Merck Millipore) and parvalbumin (Sigma monoclonal; P3088), and co-exposed to AlexaFluor donkey anti-rabbit 488 and donkey anti-mouse 568 secondary antibodies (1:1000; Life Technologies). Slides were mounted with fluorescence mounting medium (DAKO) and images of sections were taken on a Nikon A1r-si resonant scanning confocal system (microscope: Nikon Ti; objective: Apo LWD, 40× magnification, 1.15 numerical aperture, water immersion; sequential excitation: 405 nm, and 546 nm laser lines; respective emission filters: 450/50 nm and 595/50 nm; photomultiplier tube detectors; acquisition software: NIS elements Advanced Research). All confocal images were taken in a blinded-fashion and maximum projections were obtained using ImageJ Version 1.45-7k [National Institutes of Health], after which the images were unblinded.

### *Quantification of Purkinje cell body density*

16-bit tiff files of the anti-calbindin/parvalbumin immunofluorescent micrographs of coronal cerebellar sections from the posterior lobe of T4 and *wt* mice (taken on a Zeiss AxioObserver; Burnet Institute) were de-identified and assigned an arbitrary file name. The files were then handed over to an operator who was blinded from the arbitrary file naming process. The micrographs were opened in ImageJ 1.47q software (NIH, USA) and the Purkinje neurons were marked using the 'Cell Counter' plug-in. The marked cerebellar micrograph was then subdivided into lateral and medial portions using the 'polygon selections' tool. The number of Purkinje neurons in the lateral and medial portions was determined by first thresholding the image and then using the 'Analyse Particles…' function. The length of the Purkinje layer in the lateral and medial portions was determined using the 'segmented line' tool and the 'measure' function. The density of Purkinje cell bodies is expressed as the number of Purkinje cell bodies per millimetre.

### *ELISA*

96-well microtiter plates (Nunc; Roskilde, Denmark) were pre-coated with a 1:1000 dilution of a rabbit anti-calbindin primary antibody (AB1778; Merck Millipore) overnight at room temperature. Non-specific binding sites were blocked by the addition of 200 μl of ELISA blocking buffer (Bethyl Laboratories; Montgomery TX, USA) and plates were then incubated with 20 μl of supernatant from centrifuged (13,000 rpm for 2 min at 4 °C) cerebellum lysates, in duplicate. Lysates were then exposed to a 1:1000 dilution of a mouse anti-calbindin primary antibody (Monoclonal Anti-Calbindin-D-28K; C9848; Sigma