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PII: S0014-4886(13)00241-0
DOI: doi: [10.1016/j.expneurol.2013.08.001](https://doi.org/10.1016/j.expneurol.2013.08.001)
Reference: YEXNR 11508

To appear in: *Experimental Neurology*

Received date: 7 February 2013
Revised date: 26 July 2013
Accepted date: 1 August 2013

Please cite this article as: Cops, Elisa J., Sashindranath, Maithili, Daglas, Maria, Short, Kieran M., da Fonseca Pereira, Candida, Pang, Terence Y., Lijnen, Roger H., Smyth, Ian M., Hannan, Anthony J., Samson, Andre L., Medcalf, Robert L., Tissue-type plasminogen activator is an extracellular mediator of Purkinje cell damage and altered gait, *Experimental Neurology* (2013), doi: [10.1016/j.expneurol.2013.08.001](https://doi.org/10.1016/j.expneurol.2013.08.001)

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

Authors:

Elisa J. Cops¹, Maithili Sashindranath¹, Maria Daglas¹, Kieran M. Short², Candida da Fonseca Pereira^{5,6}, Terence Y. Pang⁴, Roger H. Lijnen⁷, Ian M. Smyth^{2,3}, Anthony J. Hannan⁴, Andre L. Samson^{1,2*#}, Robert L. Medcalf^{1*#}.

Affiliations:

¹Australian Centre for Blood Diseases, AMREP, Monash University, Melbourne, Victoria 3004, Australia

²Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3800, Australia

³Department of Anatomy and Developmental Biology, Monash University, Clayton, Victoria 3800, Australia

⁴Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Victoria 3010, Australia

⁵Burnet Cell Imaging Facility, Burnet Institute, Melbourne, Victoria 3004, Australia

⁶Monash Micro Imaging and Department of Infectious Diseases, AMREP, Monash University, Melbourne, Victoria 3004, Australia

⁷Center for Molecular and Vascular Biology, University of Leuven, Belgium

*These authors contributed equally to this work.

Correspondence should be addressed to Dr Andre Samson or Prof. Robert Medcalf, Australian Centre for Blood Diseases, Level 6 Burnet Tower, 89 Commercial Road, Melbourne, Victoria 3004, Australia. Email: andre.samson@monash.edu.au (A.L.S.) or robert.medcalf@monash.edu (R.L.M.). Phone: +61 3 99 03 0133 (R.L.M.).

Abstract

Purkinje neurons are a sensitive and specialized 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 or 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.

Keywords: tissue-type plasminogen activator, plasminogen activation, Purkinje neuron, ataxia, calbindin, Huntington's disease, neurodegeneration, cerebellum, astrogliosis, DigiGait.

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

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) (Tang et al., 2010; Matilla-Duenas et al., 2012; Rüb et al., 2012). 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 hypothesized 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; Seeds et al., 2003). A pathological role for tPA in the cerebellum has also been postulated in ataxia. Notably, high cerebellar tPA levels coincide with Purkinje damage in *Nervous* (Li et al., 2006a,b and Li et al., 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 develop 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 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 25ml 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 150mg 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 PBS. 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 (6µm) were mounted on Superfrost Plus slides (Menzel-Glaser; Braunschweig, Germany). Tissues sections were de-waxed, rehydrated and antigen retrieval was performed by incubating tissues in 10mM citrate buffer (pH 6.5) for 20 minutes at 95°C. To prevent non-specific binding, tissue sections were blocked for one hour with 5% goat serum, then incubated with primary antibodies (1:100) to detect Calbindin (Anti-Calbindin D-28K; 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 minutes in three changes of PBS and exposed to AlexaFluor goat anti-rabbit 568 secondary antibody (1:1000; Life Technologies; Carlsbad CA, USA) for one hour, followed by Hoechst (1:1000; Life Technologies) counter stain for five minutes. For dual staining immunofluorescence (Figure 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, 40x 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 sub-divided 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 millimeter.

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 200µl of ELISA blocking buffer (Bethyl Laboratories; Montgomery TX, USA) and plates were then incubated with 20µl of supernatant from centrifuged (13000rpm for 2min 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 Aldrich; St Louis MO, USA) followed by a 1:1000 dilution of a horseradish peroxidase (HRP) tagged anti-mouse secondary antibody (R&D Systems; Minneapolis, MN, USA). All incubation steps were performed at room temperature for an hour, followed by three washes (ELISA Wash Solution; Bethyl Laboratories). Following incubation with the secondary antibody, plates were washed five times and incubated with TMB One Component substrate (Bethyl Laboratories). Once the desired colour development was achieved, sulphuric acid (2M) was added to cease the reaction and plates were analysed by absorption at $\lambda = 450\text{nm}$ using a plate reader (BMG Fluostar Optima). ELISA values were normalised to sample protein concentration, which was determined by performing a bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Thermo Scientific Pierce; Rockford IL, USA). The specificities of the aforementioned calbindin antibodies were confirmed by western blot, which produced a single band at 28kDa in both instances (data not shown; see method below).

Amidolytic Assay for Mouse Brain-derived tPA

This method is an adaptation of an amidolytic assay (Verheijen et al., 1982), and was performed as previously described (Sashindranath et al., 2011). In this study, 40µl of centrifuged cerebellum lysates were transferred to a cooled 96-well plate to which, S2251 and CNBr-fibrinogen were added to final concentrations of 2mM and 0.1 mg/ml, respectively. Plasminogen was then added to a final concentration

of 0.5mM and the reactions were made up to a final volume of 70 μ l in PBS. Mineral oil was then placed on the top of every well. Absorbance was measured at λ =405nm every two minutes for eight hours at 37°C using a fluorescence plate reader (BMG Fluostar Optima). As previously described (Niego et al., 2008), best-fit second-order polynomial equations were used for each 'absorbance at λ =405nm Vs time' curve using GraphPad Prism Version 5.04 software. The second-order coefficient of each best-fit polynomial equation was taken as half the initial rate of the amidolytic assay. Fold changes in tPA activity were calculated based on the relationship between relative tPA activity and the rate of the amidolytic assay, as previously described (Sashindranath et al., 2011).

Western Blotting

Western blotting was performed as previously described with minor modification (Samson et al., 2012). Cerebellum tissue lysates were electrophoresed in 12% polyacrylamide gels. Separated proteins were transferred to a polyvinyl difluoride (PVDF) membrane (Merck Millipore) using the Mini-PROTEAN Tetra Cell System (BioRad; Hercules CA, USA), which was then blocked with Odyssey Blocking Buffer (Licor; Lincoln NE, USA) for one hour at room temperature to prevent non-specific binding. Antibody hybridisation was performed by incubating the membrane with a 1:1000 dilution of either anti-glial fibrillary acidic protein (GFAP) monoclonal (13-0300; Life Technologies), anti-calbindin D-28K (AB1778; Merck Millipore), monoclonal anti-calbindin D-28K (C9848; Sigma Aldrich), goat polyclonal to ionized calcium binding adaptor molecule 1 (IBA1; ab5076; Abcam; Cambridge, UK) or anti-actin (I-19; sc-1616; Santa Cruz Biotechnologies, Santa Cruz CA, USA) primary antibody overnight at 4°C. This was followed by a 30-minute incubation with either a goat anti-rat IRDye 800CW (GFAP), donkey anti-rabbit IR Dye 680LT (Merck Millipore calbindin), donkey anti-mouse IRDye 800CW (Sigma calbindin), donkey anti-goat IRDye 800CW (IBA1) or donkey anti-goat IRDye 680LT (actin) secondary antibody (Licor) at room temperature. Following exposure to primary and secondary antibodies, membranes were washed for 30 minutes in three changes of Tris-buffered saline (TBS) + 0.05% Tween-20. Membranes were scanned using an Odyssey Infrared Imaging System (Licor) and densitometry was performed on the resulting bands using ImageQuant Version 5.2 software.

Optical Projection Tomography (OPT)

Following overnight fixation, cerebellum tissues were washed in PBS and dehydrated stepwise in increasing concentrations of methanol/PBS (33%, 66% and 100%). Tissues were clarified by overnight incubation in 15% hydrogen peroxide + 16.7% DMSO in 100% methanol at room temperature. Tissues were then washed in 100% methanol and freeze/thawed five times at -80°C to enhance tissue penetration. Following re-hydration through a TBS/methanol series (33%, 66% and 100%), tissues were incubated for 48 hours in 1µM TO-PRO-3 (Life Technologies) at 4°C. Tissues were washed in multiple changes of TBS + 0.05% Tween-20 overnight at room temperature, then post-fixed in 4% formaldehyde solution. Post-fixed tissues were prepared for Optical Projection Tomography scanning as previously reported (Short et. al., 2010). Samples were imaged in a Bioptonic 3001 Optical Projection Tomography scanner at a resolution of approximately 8.39 µm per pixel, and were reconstructed into volumetric datasets using nRecon (Bruker microCT, Belgium).

OPT quantitation

The reconstructed 3-dimensional stacks were imported into Imaris 7.4.23 software (Bitplane Scientific Software, CT, USA). The Imaris isosurface function was used to do a 3-dimensional reconstruction of the whole cerebellum and the granular cell layer using the residual autofluorescence (488nm channel) and the TO-PRO-3 staining (650nm channel), respectively. One hemisphere per brain was selected for further volumetric analysis. Volumetric analysis of the selected hemisphere was performed with the Imaris MeasurementPro function. All Imaris processing and volumetric analysis of OPT datasets were performed in a blinded-fashion, after which the obtained volumes were unblinded and graphed. An example of the reconstructed granular layer volumes from one hemisphere of wt and T4 mice is presented in Figure 4Aii and Supplementary Video.

Nissl Staining and Quantitation

Sagittal cerebellum tissue sections (6µm) were mounted on Superfrost Plus slides (Menzel-Glaser). Tissues sections were de-waxed, rehydrated and submerged in 0.25% Cresyl Violet solution for 30 minutes. Sections were then washed in water to clear excess Cresyl Violet solution, dehydrated in changes of 70%, 90% and 100% ethanol, cleared in three changes of xylene and mounted using DPX (Scharlau, Barcelona, Spain). Tissue sections were imaged under bright field at 4x magnification (microscope:

Olympus BX-51). Micrographs were opened in ImageJ 1.46q software and the entire cerebellum tissue section area was isolated and measured using the 'polygon selections' tool and the 'measure' function, respectively. Each component of the tissue (white matter, molecular layer and granular layer) was then subsequently isolated and the area of each region was measured and calculated. Results for each component are represented as percentage area of the whole cerebellum tissue section.

Quantification of Interneuron Density

16-bit tiff files of the anti-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 and subjected to the 'subtract background' function (rolling ball radius of 20 pixels). The molecular layer was isolated from the surrounding tissue using the 'polygon selections' tool and its total area determined using the 'measure' function. Next, the anti-parvalbumin immunofluorescent signal was then thresholded to isolate the interneurons. The thresholded binary image was subjected to a median filter (pixel radius of 2.0) and then processed further with the 'Binary>Close-' and then the 'Binary>Fill Holes' function. Finally, the binary image was subjected to the 'Analyze Particles...' function (size range of 75-500 pixels² and circularity of 0.6-1.0). The number of particles analysed from the analysis was taken as the number of parvalbumin-positive interneurons and expressed as the number of interneurons per 10,000 pixels².

DigiGait analysis

For functional assessment, T4 mice were tested for variations in gait parameters compared to their *wt* littermates using the DigiGait Imaging System (Mouse Specifics, Inc; Quincy, MA USA). As previously described (Sashindranath et al., 2012), paw placement of the mice was recorded digitally through a clear treadmill from the ventral plane. In this study, T4 (n=8) and *wt* (n=6) mice were tested at a speed of 15cm/s for a duration of ~5s at ten weeks of age. The recorded output was analysed using the DigiGait analysis software v.11.5 (Mouse Specifics, Inc). All DigiGait testing was performed in a blinded-fashion. Forelimb and hindlimb data was taken as the average measurement taken across the respective left and right limbs.

Statistical Analysis

Unpaired student t-tests were used to determine significant differences between groups. Significance was taken at $p < 0.05$.

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Results

Correlation between augmented tPA activity and Purkinje neuron loss in the T4 cerebellum

We utilised T4 transgenic mice, which have approximately 4-fold higher tPA activity in cerebellum tissue compared to their *wt* littermates at 12 weeks of age (Sashindranath et al., 2011), to determine if excessive endogenous tPA activity has a degenerative effect on Purkinje cells. Importantly, the murine tPA transgene in T4 mice is driven by the Thy1.2 gene promoter, which in the cerebellum directs strong preferential expression to the Purkinje cells (Radrizzani et al 1995; Supplementary figure S1), with low expression in the granular layer (Supplementary figure S1). To evaluate cerebellar degeneration we detected the calcium-binding proteins, calbindin and parvalbumin, as they are well-established markers of Purkinje neurons. Histological analysis of cerebellum tissue sections from 10 week-old mice illustrated that there was a visible loss of calbindin/parvalbumin-positive Purkinje cell bodies and dendrites branching through the molecular layer in T4 mice compared to *wt* mice (Figure 1A). Quantification of Purkinje cell body density confirmed a significant decrease in cell number within the medial region of the T4 cerebellum ($n=10$) compared to the *wt* cerebellum ($p<0.05$; $n=10$; Figure 1Bi), however there was no difference in cell number in the lateral region of the cerebellum between the two genotypes ($n=13$; Figure 1Bii). Assessment of calbindin protein levels by ELISA confirmed that there were significantly lower levels in the cerebellum of T4 mice compared to their *wt* littermates at ten weeks of age ($p<0.0001$; $n=10$; Figure 1C), supporting the notion that an overall loss of Purkinje neurons occurs in the T4 cerebellum.

Histological analysis of tPA levels in cerebellum tissue sections from 10 week-old mice illustrated immunoreactivity in the Purkinje and the molecular layers of T4 mice (Figure 2A). No comparable tPA-immunoreactivity was observed in the cerebellum of 10 week-old *wt* mice, presumably because basal cerebellar tPA levels were below the detection limits of our immunostaining procedure (Figure 2A). Strong tPA-immunoreactivity in the CA1 hippocampal region (a known region of relatively high tPA expression) in T4 mice and an absence of tPA-immunoreactivity in the CA1 hippocampal region of *tPA*^{-/-} mouse confirmed the specificity of the antibody used for immunostaining (Figure 2A). Assessment of tPA activity in *wt* and T4 cerebellum using an amidolytic assay confirmed that reduced calbindin reactivity was associated with increased tPA activity ($p<0.005$; $n=3$; Figure 2B).

A significant reduction in calbindin immunoreactivity ($p<0.0005$; $n=8$) and a significant increase in tPA activity ($p<0.05$; $n=3$) was also observed in T4 mice at 5 weeks of age (data not shown). These results suggest that targeted overexpression of murine tPA may be sufficient to trigger Purkinje cell degeneration. Future studies using tPA-inhibitors in T4 mice will now need to be performed to establish an unequivocal causal link between excess tPA and Purkinje cell loss.

Astrogliosis, but not microgliosis, in the T4 cerebellum

Astrogliosis is an atypical increase in astrocyte number due to degradation of adjacent neurons, and hence is a secondary indicator of neuronal loss. To support the notion of Purkinje neuron loss in the T4 cerebellum, we investigated whether levels of the astrocyte marker, GFAP, were higher in T4 mice compared to their *wt* littermates at 10 weeks of age. Histological analysis of tissue sections showed a discernible increase in GFAP immunoreactivity in the T4 cerebellum compared to the *wt* cerebellum (Figure 3A). Western blot analyses confirmed a significant increase in GFAP levels in the T4 cerebellum, relative to the *wt* cerebellum ($p<0.05$; $n=10$; Figure 3B). Therefore, the reduction in Purkinje cells in T4 mice coincides with astrogliosis.

Reactive microgliosis is another host response often observed following acute and chronic neural injury (Ladeby et al., 2005). Western blot analysis of IBA1 expression – a marker of microglial number and activation (Ito et al., 1998) – shows that IBA1 levels were unchanged between the T4 and *wt* cerebellum ($n=12$; Figure 3C). Whilst we cannot exclude the possibility that microgliosis appears at later stages in development, our results showed that the observed reduction in Purkinje cells in T4 mice had not manifested in overt microgliosis by 10 weeks of age.

No change to cerebellar granular layer and a loss of molecular layer volume in T4 mice

We next determined if postnatal overexpression of tPA had altered any other anatomical structures within the cerebellum. There was no significant difference in whole brain or cerebellum weight between the genotypes (Supplementary figure S2A). Optical Projection Tomography (OPT) confirmed that no change in the number of cerebellar lobules (data not shown and Supplementary Video) or the volume of the granular neuron layers (Figure 4Ai, ii and Bi and Supplementary Video) were evident in T4 mice. This data was

supported by area analyses of sagittal cerebellum tissue sections, which recorded no change in the T4 granular layer (Figure 4C). Despite no change in granular layer volume, OPT analysis showed that the T4 cerebellum has a significantly smaller volume relative to *wt* mice (Figure 4Bii). Area analysis of sagittal cerebellum tissue sections confirmed a significant decrease in molecular layer volume in the T4 cerebellum compared to *wt* ($p < 0.05$; Figure 4C); with a reciprocal increase in the area occupied by the white matter (not shown; $p < 0.05$). As no significant change in interneuron density in the molecular layer of the T4 cerebellum was observed (Supplementary Figure S2C), this reduced cerebellar volume in T4 mice most likely reflects a loss of the abundant Purkinje cell bodies and processes from the neuropil of the molecular layer (as seen in Figure 1A). Collectively, these results suggest that the Purkinje cell layer is selectively modified in T4 mice, which in turn reduces molecular layer volume most likely due to a loss of Purkinje dendrites.

Gait variation in T4 mice

Studies that have utilised the DigiGait system to analyse mouse models of cerebellar ataxia exemplify alterations in several gait parameters compared to control mice. Observations include: decreased stance and braking time in the fore and hind limbs (Pallier et al., 2009), increased propulsion time in the fore (Pallier et al., 2009) and hind (Amende et al., 2005) limbs, higher stride length variability in the forelimbs (Amende et al., 2005), opening of hind paw angle (Amende et al., 2005), decreased paw angle variability in the forelimbs (Pallier et al., 2009), change in stance width of the fore and hind limbs (Amende et al., 2005; Pallier et al., 2009; Duvick et al., 2010), increased variability in forelimb stance width (Amende et al., 2005) and decreased paw area at peak stance (Pallier et al., 2009).

In the current study, measurements of stance width indices indicated that T4 mice displayed a shortened 'midline distance' (the distance between the centroid of a paw at peak stance and the central point of the animal) of their forelimbs compared to *wt* mice ($p < 0.05$; Figure 5Ai). A non-significant trend towards a narrower 'forelimb stance width' was also observed in T4 mice compared to *wt* mice ($p = 0.07$; Figure 5Aii). In the hindlimbs, a significantly higher ($p < 0.05$) 'stance width CV' (coefficient of variation; the variability in stance width, divided by the mean stance width) was observed in T4 mice compared to *wt* mice (Figure 5Aiii).

Stride-related indices delineated a significant decrease ($p < 0.02$) in overall 'braking time' in the forelimbs of T4 mice compared to *wt* mice (Figure 5Bi), coupled with a significant compensatory increase ($p < 0.05$) in 'propulsion time' (Figure 5Ci). The percentage of the 'forelimb stride phase' spent in braking was also significantly lower ($p < 0.02$) in T4 mice compared to *wt* mice (Figure 5Bii), while the 'propulsion phase percentage' was also significantly higher ($p < 0.01$; Figure 5Cii). Similarly, the percentage of the 'forelimb stance phase' spent in braking was significantly lower ($p < 0.01$) in T4 mice compared to *wt* mice (Figure 5Biii), and the 'propulsion phase percentage' was significantly higher ($p < 0.01$; Figure 5Ciii). Interestingly, 'stride length variation' (Figure 5Di), and the 'ataxia coefficient' (an index of step-to-step variability and unsteadiness; Figure 5Dii) were significantly lower ($p < 0.05$; $p < 0.02$ respectively) in T4 mice, compared to *wt* mice.

No significant variations were observed in the other indices between the genotypes, in either the fore or hind limbs (data not shown). It has been shown by Pallier et al. (2009) that gait measurements can be confounded by differences in body weight. However, no significant difference in body weight was recorded between T4 mice and their *wt* littermates (Supplementary figure S2A), indicating that all aforementioned variations were reflective of a true alteration in gait. Previous studies have also shown that severely ataxic mice perform poorly on accelerating rotarod tests compared to control mice. In this study we observed no difference in performance between T4 and *wt* mice on an accelerating rotarod (Supplementary figure S2B). Altogether, our findings suggest that the observed Purkinje cell loss in T4 mice manifests as altered gait and mild ataxia.

Correlation between augmented tPA activity and Purkinje loss in Huntington's disease mice

HD is a neurodegenerative disorder caused by an expanded polyglutamine tract repeat within the *Huntingtin* gene resulting in neurodegeneration and ataxia. Whilst spiny neurons of the striatum are a principal degenerating cell type in HD, numerous studies document a loss of Purkinje cells in HD patients and mouse models of HD (Shenk and Enters, 1970; Jeste et al, 1984; Rodda, 1981; Turmaine et al, 2000; Dougherty et al, 2012; Dougherty et al, 2013). Notably, no prior study has implicated a role for tPA in pathogenesis of HD. Accordingly, we chose the R6/1 mouse model of HD to further test the hypothesis that

excess tPA is a selective and common downstream mediator to Purkinje degeneration. Histological analysis of tissue sections for calbindin immunoreactivity in the cerebellum of 12 week old R6/1 HD mice - an age when early behavioural deficits are appearing (van Dellen et al., 2000; Mazarakis et al., 2005; Nithianantharajah et al., 2008) - demonstrated a clear loss of Purkinje cell bodies and dendrites branching through the molecular layer, similar to that seen in T4 mice (Figure 6A). Assessment by ELISA also confirmed that there was a significant loss of calbindin expression in HD mice compared to their *wt* littermates ($p<0.01$; $n=5$; Figure 6B). Importantly, this loss of calbindin immunoreactivity was coupled with a significant 200% increase in tPA activity (Figure 6C; $p<0.0005$), once again drawing a parallel between high tPA activity and Purkinje neuron loss. Importantly, no significant change in tPA activity was observed between HD and *wt* littermates in either the cerebral cortex or the striatum (Supplementary figure S2D). Thus, upregulation of tPA coincided with Purkinje degeneration and was confined to the cerebellum. These findings support the notion that excess cerebellar tPA is a selective and common mediator of Purkinje dysfunction.

Discussion

It has long been recognised that tPA is an important mediator of central nervous system function and dysfunction (Samson and Medcalf, 2006; Yepes et al., 2009). While the majority of studies have focused on the role of tPA in the forebrain as a modulator of excitotoxic injury and blood brain barrier permeability, tPA and other components of the plasminogen activation system are also constitutively expressed in the cerebellum (Supplementary figure S1). In *wt* mice, tPA and plasminogen are expressed in the granular and Purkinje layers (Li et al., 2013), with moderate expression of neuroserpin (a tPA-specific inhibitor) in the Purkinje and molecular layers (Hastings et al 1997; Krueger et al 1997; Basham and Seeds, 2001). The role of the tPA in the cerebellum has been linked with motor learning where increased tPA expression in Purkinje cells occurs in rats within one hour of a complex motor task (Seeds et al., 2003). These data supported the notion that Purkinje cell-derived tPA was required for the acquisition of fine motor coordination (Bickford and Seeds, 1995) while additional findings further associated tPA expression with the migration granule neurons (Friedman and Seeds, 1995). However, overexpression of tPA in cerebellar neurons has been associated with Purkinje stress in the *Lurcher* and *Nervous* mouse lines. Indeed, a direct effect of tPA in the *Lurcher* and *Nervous* phenotypes was revealed when deletion of the tPA gene resulted in delayed/attenuated Purkinje cell loss in these mouse models of ataxia (Lu and Tsirka, 2002, Li et al., 2013).

The T4 mouse line represents an elegant model to directly test the influence of tPA on Purkinje function without other confounding influences. Firstly, unlike *Lurcher* mice, which possess a mutated *GluR δ 2* gene and *Nervous* mice, which harbour mutations in the *Defer-rs1*, *tPA*, *Sfrp1* and *Vdac3* genes, the T4 mice line has no overt genetic abnormalities besides transgene insertion (as assessed by microarray analysis; data not shown). Furthermore, T4 mice carry the tPA transgene in a heterozygous state, making it highly unlikely that a non-tPA genetic change is responsible for the observed Purkinje deficits. Secondly, unlike in *Nervous* mice where overexpression of tPA is not restricted to neural tissues (Li et al., 2006), the Thy 1.2 promoter that drives tPA overexpression in T4 mice (Madani et al., 1999), directs strong and preferential expression to post-mitotic neurons (Feng et al., 2000; Porrero et al., 2010). Most importantly, we find that tPA is preferentially overexpressed in the Purkinje cells of T4 mice (Figure 2A). Therefore, the T4 mouse line allows one to study the specific influence of excess tPA on Purkinje cell integrity.

Striking similarities between the phenotypes of *Nervous* and T4 mice were evident. Both lines exhibit tPA-mediated selective dysfunction of Purkinje cells, coinciding with astrogliosis, a reduced molecular layer volume and no discernible cerebellar microgliosis. Interestingly, the distribution of Purkinje cells differs between T4 and *Nervous* mice, with reduced Purkinje density most evident in the medial zone of the T4 cerebellum and in the lateral zones of the *Nervous* cerebellum (Li et al., 2013). The basis for this discrepancy is unknown, but may relate to regional differences in tPA overexpression. Several additional salient points arise when further comparing the *Nervous*, *Lurcher*, SCA1 and T4 mice. tPA levels increase (between ~1.5-fold and ~10-fold) in the *Nervous*, T4, *Lurcher* and SCA1 cerebellum; with Purkinje neurons being the principal degenerating cell type in all four mouse lines. Our novel finding that a marked 200% increase in cerebellar tPA activity occurs together with Purkinje cell loss in HD mice provides further compelling evidence for a casual role of tPA in Purkinje-related pathologies. Future studies should assess whether crossing HD mice onto a tPA-deficient background will attenuate cerebellar degeneration and ataxia in these mice.

Despite the commonality of high cerebellar tPA expression in these different neurodegenerative mouse models, the extent and rate of Purkinje loss and ataxia in the *Nervous*, *Lurcher*, HD and SCA1 mice is significantly greater than that observed in T4 mice. Hence, an increase in tPA alone does not fully recapitulate the cerebellar phenotypes of these other models. DigiGait analyses delineated an ataxic phenotype of T4 mice, which displayed variations in gait however lacked the more prominent motor deficits observed in *Nervous*, *Lurcher*, SCA1 and HD mice (Norman et al., 1995; Amende et al., 2005; Li et al. 2006; Pallier et al., 2009). Furthermore, homozygosity of the *Lurcher* and *Nervous* mutations results in profound hindbrain aberrations and premature death, whereas homozygous T4 mice have a normal life-span (Madani et al., 1999). Therefore, although excess tPA alone is sufficient to cause a mild form of Purkinje dysfunction, other more severe non-tPA-dependent injury mechanisms likely operate in the *Lurcher*, *Nervous*, HD and SCA1 mouse cerebellum. This conclusion is supported by the findings that crossing *Nervous* and *Lurcher* mice onto a tPA-deficient background only delays/partially reduces cerebellar degeneration (Li et al., 2013, Lu and Tsirka, 2002).

In stark contrast to the contribution of tPA to Purkinje toxicity in *Nervous*, *Lurcher* and T4 mice, tPA-deficiency results in no overt change to the granular cell layer in adult mice (Seeds et al., 1999) and fails to attenuate granule cell loss in *Weaver* mice (Mecenas et al., 1997) or mice following methylazoximethanol injection (Ferrer et al., 2001). In these two examples, the primary degenerating cell type is the granular neuron. Therefore, the neurodegenerative pathway triggered by excess tPA in the cerebellum may specifically target Purkinje cells. Our finding that no change in the granular neuronal layer was evident in T4 mice, and that no change in interneuron density was detected in the molecular layer of T4 mice, further supports this notion.

This study, and that of others, firmly establishes tPA as an extracellular mediator of Purkinje degeneration. Notably, the mechanisms of tPA-mediated cerebellar atrophy that operate in T4 mice have not been addressed here-in and warrant future elucidation. In *Lurcher* mice, tPA selectively potentiates caspase-8-mediated apoptosis by an undefined mechanism (Liu and Tsirka, 2002). In *Nervous* mice, excess tPA activates multiple deleterious mechanisms that contribute to Purkinje dysfunction: 1) by dampening dendrite development via triggering PKC γ signalling, 2) by impeding synaptogenesis via a decrease in BDNF-TrkB signalling, 3) by perturbing mitochondrial status and causing necrosis (Li et al., 2013). The mechanism for how tPA impinges upon the mitochondria of Purkinje cells may involve direct interaction between plasmin and voltage-dependent anion channels (Li et al., 2013; Gonzalez-Gronow et al, 2003; Gonzalez-Gronow et al, 2013). However, it is unknown whether these injurious tPA-dependent mechanisms in the *Nervous* mouse also operate in T4 mice and other instances of cerebellar ataxia. In addition, whether the deleterious actions of tPA in the cerebellum are truly plasmin(ogen)-dependent still remains an open question.

Altogether, a significant upregulation of tPA coincides with Purkinje degeneration in five mice models – the T4, *Nervous*, *Lurcher*, SCA1 and HD mice. In at least three of these models (*Lurcher*, SCA1 and HD mice), the disease-causing mutations have no known association with tPA. Yet, since we observe an increase in cerebellar tPA levels in these different neurodegenerative models, we hypothesize excess tPA is a common downstream event that directly contributes to Purkinje dysfunction and damage.

In conclusion, this study demonstrates that excess cerebellar tPA in T4 mice coincides with specific aberrations of the Purkinje cell layer; including reduced Purkinje projections into the molecular layer, reduced Purkinje cell density and a mild ataxia. These aberrations - together with the similar tPA-dependent phenotype of the *Nervous* mouse (Li et al., 2013) - leads us to propose that excess tPA in the cerebellum may provide specific stress sufficient to cause Purkinje degeneration. Indeed, high cerebellar tPA may be a common effector of Purkinje damage across various neurodegenerative scenarios, including HD, a genetic disorder not previously linked to cerebellar tPA levels. Studies assessing the occurrence of excess cerebellar tPA in human Purkinje-related neurodegenerative disorders are now necessary. Therapeutic strategies that lower cerebellar tPA levels should be tested for their potential to reduce Purkinje dysfunction and ataxia.

Acknowledgements

This study was supported by grants awarded to R.L.M. and A.L.S. by the NH&MRC of Australia, by a Monash University Faculty of Medicine, Nursing and Health Sciences Early Career Strategic Grant awarded to A.L.S., by a NH&MRC Principal Research Fellowship awarded to R.L.M. and by an Australian Research Council Future Fellowship (FT3) and a NH&MRC grant awarded to A.J.H. We thank Mr Benjamin T. Porebski for blinding the optical projection tomography datasets. We thank the Burnet Institute (Victoria, Australia) for use of their Zeiss Cell Observer system. We thank Mr Steve Cody (Monash University) for assistance with microimaging and data analysis.

Figure Legends

Figure 1. Purkinje neuron loss and decreased calbindin immunoreactivity in the T4 mouse cerebellum. **A)** Representative images depicting the granule layer (GL), Purkinje cell layer (PL) and molecular layer (ML) of coronal cerebellum tissue sections from *wt* and T4 mice. Tissues were hybridized with a calbindin antibody to detect Purkinje neurons (i), a parvalbumin antibody to detect Purkinje neurons and interneurons (ii), and counterstained with Hoechst nuclear dye (iii). Micrographs are maximum projections of confocal 3-dimensional stacks. **B)** Purkinje cell bodies in the medial (i) and lateral (ii) regions of the cerebellum were counted from micrographs of coronal tissue sections from *wt* (n=10-13) and T4 (n=13) mice, which had been hybridized with calbindin/parvalbumin antibodies. Dot plots illustrate each tissue section analyzed and the values represent the number of cells per mm. The line and error bars represent the mean $\pm$ SEM for each genotype. The diagram inset indicates the medial (M) and lateral (L) regions of the cerebellum that were counted. P-value was determined by one-tailed student t-tests. **C)** Whole cerebellum lysates from T4 (n=10) and *wt* littermate (n=10) mice were analysed for calbindin immunoreactivity by ELISA. Box plots represent the median value (mid-line), interquartile spread (box) and minimum and maximum values (whiskers). P-value was determined by two-tailed student t-tests

Figure 2. Increased tPA levels and activity in the T4 mouse cerebellum. **A)** Representative images depicting coronal cerebellum tissue sections from *tPA*^{-/-}, *wt* and T4 mice, and sagittal tissue sections of the CA1 region of the hippocampus from *tPA*^{-/-} and T4 mice. Tissues were hybridised with a tPA antibody (i) and counterstained with Hoechst nuclear dye (ii). Micrographs are maximum projections of confocal 3-dimensional stacks. **B)** An amidolytic assay was used to measure tPA activity in whole cerebellum lysates from T4 (n=3) and *wt* littermate (n=3) mice. Graphs represent non-linear regression analyses expressed as 'best-fit second order coefficients' (amidolytic assay rate) $\pm$ SEM (i), and the corresponding calculated fold change in tPA activity $\pm$ SEM (ii). P-values were determined by two-tailed student t-tests.

Figure 3. Purkinje neuron dysfunction promotes astrogliosis in the T4 cerebellum. **A)** Representative images depicting the granule layer (GL) and molecular layer (ML) of coronal cerebellum tissue sections from *wt* and T4 mice. Tissue were hybridised with a GFAP antibody to detect astrocytes (i) and counterstained with Hoechst nuclear dye (ii). Micrographs are maximum projections of confocal 3-

dimensional stacks. **B)** Representative immunoblot of whole cerebellum lysates from *wt* (n=3) and T4 (n=3) mice analysed for GFAP and actin. Densitometry was performed on GFAP signals and normalised to actin signals collated from immunoblots of *wt* (n=10) and T4 (n=10) mice. Box plots represent the median value (mid-line), interquartile spread (box) and minimum and maximum values (whiskers). P-values were determined by two-tailed student t-tests. **C)** Representative immunoblot of whole cerebellum lysates from *wt* (n=3) and T4 (n=3) mice analysed for IBA1 and actin. Densitometry was performed on IBA1 signals and normalised to actin signals collated from immunoblots of *wt* (n=10) and T4 (n=10) mice. Box plots represent the median value (mid-line), interquartile spread (box) and minimum and maximum values (whiskers). No significant differences were observed in IBA1 levels between T4 and *wt* as determined by two-tailed student t-tests.

Figure 4. Decreased molecular layer volume, with no change in the granular layer in the T4 cerebellum. **A)** Sagittal section of the TO-PRO-3-stained cerebellum (i) and dorsal view of surface-rendered granular layers of four lobules (ii) from a representative T4 and *wt* mouse cerebellum as determined by OPT. Note, TO-PRO-3 preferentially stains the dense cerebellar granular layer. **B)** Average volume of a granular layer per lobule (i) and average volume of half the cerebellum (ii) in T4 (n=5) and *wt* (n=5) mice as determined by OPT. Each point represents the mean volume for a single mouse and the dotted line represents the cohort average. P-value was determined by two-tailed student t-tests. Note, Supplementary Video shows surface-rendered OPT datasets of the half cerebellar volume and granular layer volumes of a representative T4 versus *wt* mouse, respectively. **C)** Molecular layer and granular layer areas were measured from micrographs of Cresyl Violet-stained sagittal cerebellum sections from *wt* (n=4) and T4 (n=4) mice. Results are represented as a percentage of the whole cerebellum area, and graphs depict the mean value + SEM for each layer and genotype. P-values were determined by two-tailed student t-tests.

Figure 5. Variations in gait indices between *wt* and T4 mice. Gait indices of *wt* (n=6) and T4 (n=8) mice were assessed using the DigiGait imaging system. **A)** forelimb midline distance (i), forelimb stance width (ii), coefficient of variation (CV) of hind limb stance width (iii). **B)** Braking in the forelimbs: overall time spent braking (i), percentage of stride spent in the braking phase (ii), percentage of stance spent in the braking

phase (iii); **C**) Propulsion in the forelimbs: overall time spent in propulsion (i), percentage of stride spent in the propulsion phase (ii), percentage of stance spent in the propulsion phase (iii); **D**) forelimb stride length variation (i), ataxia coefficient of the forelimbs (ii). Data signifies pooled readouts from left and right limbs, with box plots representing the median value (mid-line), interquartile spread (box) and minimum and maximum values (whiskers). P-values were determined by two-tailed student t-tests.

Figure 6. Purkinje neuron loss and decreased calbindin immunoreactivity in the HD cerebellum coincides with increased tPA activity. **A)** Representative images depicting the granule layer (GL), Purkinje cell layer (PL) and molecular layer (ML) of coronal cerebellum tissue sections from *wt* and HD mice. Tissues were hybridised with a calbindin antibody to detect Purkinje neurons (i) and counterstained with Hoechst nuclear dye (ii). Micrographs are maximum projections of confocal 3-dimensional stacks. **B)** Whole cerebellum lysates from HD (n=5) and *wt* littermate (n=5) mice were analysed for calbindin immunoreactivity by ELISA. Box plots represent the median value (mid-line), interquartile spread (box) and minimum and maximum values (whiskers). P-value was determined by two-tailed student t-tests **C)** An amidolytic assay was used to measure tPA activity in whole cerebellum lysates from HD (n=5) and *wt* littermate (n=5) mice. Graphs represent non-linear regression analyses expressed as 'best-fit second order coefficients' (amidolytic assay rate) $\pm$ SEM (i), and the corresponding calculated fold change in tPA activity $\pm$ SEM (ii). P-values were determined by two-tailed student t-tests.

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Fig. 1

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Fig. 3

Fig. 4

Fig. 5

Fig. 6

Graphical Abstract

Highlights:

- Purkinje damage coincides with excess tPA levels in many different models of ataxia
- Neuronal postnatal overexpression of tPA results in reduced Purkinje cell number
- tPA is a common extracellular mediator of Purkinje dysfunction and ataxia
- Therapies that lower cerebellar tPA may slow human Purkinje degeneration and ataxia

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Author/s:

Cops, EJ;Sashindranath, M;Daglas, M;Short, KM;Pereira, CDF;Pang, TY;Lijnen, HR;Smyth, IM;Hannan, AJ;Samson, AL;Medcalf, RL

Title:

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

Date:

2013-11-01

Citation:

Cops, E. J., Sashindranath, M., Daglas, M., Short, K. M., Pereira, C. D. F., Pang, T. Y., Lijnen, H. R., Smyth, I. M., Hannan, A. J., Samson, A. L. & Medcalf, R. L. (2013). Tissue-type plasminogen activator is an extracellular mediator of Purkinje cell damage and altered gait. EXPERIMENTAL NEUROLOGY, 249, pp.8-19. <https://doi.org/10.1016/j.expneurol.2013.08.001>.

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<http://hdl.handle.net/11343/44172>