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DEVELOPMENT AND DISEASE

Partial rescue of neural apoptosis in the *Lurcher* mutant mouse through elimination of tissue plasminogen activator

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SUMMARY

Lurcher **is a spontaneous mouse mutant characterized by premature and aberrant apoptosis in the cerebellum. The phenotype has been shown to be caused by a point mutation in the δ2 glutamate receptor subunit gene that results in a large constitutive inward current, which has proved that endogenous excitotoxicity can lead to apoptotic cell death. Additional studies have suggested a direct link between this endogenous excitotoxicity and the activation of intracellular cell death enzymes. We have previously shown that excitotoxic neuronal degeneration elicited through exogenous insults (e.g. excitotoxins, stroke) is promoted by an extracellular cascade involving the serine protease tissue plasminogen activator (tPA). However, whether it is through necrotic or apoptotic mechanisms that this excitotoxic cell death occurs has remained contested. We describe the attenuation of the** ***Lurcher*** **cell death progression in tPA-deficient mice. Elimination of tPA delayed the apoptotic death of Purkinje and granule neurons in** ***Lurcher*** **mice, and reduced the phosphorylation of Jun and the activation of caspase 8. These results indicate that not only does tPA-promoted excitotoxic cell death proceed through a receptor-mediated apoptotic pathway, but that neuronal cell death in the** ***Lurcher*** **mouse is facilitated by extracellular cascades in addition to the already described intracellular pathways. Finally, these findings suggest that therapeutic benefits may be achieved for a wide variety of insults to the CNS by regulating tPA activity to preserve neuronal viability.**

Key words: tPA, Apoptosis, *Lurcher*, Cerebellum, Purkinje, Neurodegeneration, Mouse

INTRODUCTION

Lurcher (*Lc*) is a neurological mutant mouse strain. Homozygous *Lurcher* (*Lc*/*Lc*) mice die within a few hours of birth. Heterozygous *Lurcher* (*Lc*/+) mice display impaired motor control and have a wobbly, 'lurching', mild-to-moderate ataxic gait. This phenotype results from selective, cell-autonomous and apoptotic death of cerebellar Purkinje cells during postnatal development (Norman et al., 1995). The mutation responsible for neurodegeneration in *Lc* mice has been identified as a G-to-A transition that changes a highly conserved non-polar alanine to a polar threonine residue in transmembrane domain III of the mouse δ2 glutamate receptor subunit gene (GluRδ2). As GluRδ2 is localized primarily to the postsynaptic dendritic spines of Purkinje cells during parallel fiber synaptogenesis and because the mutation results in a large, constitutive inward current, it has been concluded that the mechanism of death of the Purkinje cells in *Lc*/+ mice involves excitotoxicity (Zuo et al., 1997). The progression of apoptotic neuronal death in *Lc* mice accordingly provides a physiologically relevant model for excitotoxic cell death. Delayed degeneration of granule neurons occurs secondarily, presumably owing to a failure to establish a sufficient number of contacts with Purkinje cells, which is known to be essential (Wetts and Herrup, 1982a; Wetts and Herrup, 1982b). Both the intrinsic and target-related (secondary) neuronal death in *Lc* mice involve the activation of caspase 3 (Selimi et al., 2000), which suggests that the death pathway proceeds entirely internal to the affected neurons.

Tissue plasminogen activator (tPA), a member of the fibrinolytic system, is a serine protease that converts the zymogen plasminogen (Plg) to the active protease plasmin (Vassalli et al., 1991), thus initiating a potent proteolytic cascade. The fibrinolytic activity of tPA is found in a variety of tissues. In the central nervous system (CNS), tPA is expressed by both neurons and microglia (Tsirka et al., 1997) and plays an important role in neuronal plasticity, reorganization and cell death (Krystosek and Seeds, 1981; Qian et al., 1993; Carroll et al., 1994; Seeds et al., 1995; Tsirka et al., 1995; Wu et al., 2000). Most crucially, mice lacking functional tPA (Carmeliet et al., 1994) are resistant to excitotoxic neuronal death induced by unilateral intrahippocampal injection of kainic acid (Tsirka et al., 1995; Tsirka et al., 1996). However, it is unclear what type of cell death occurs in this model system.

To investigate whether the tPA-facilitated pathway involves apoptosis, we set out to examine the impact of its ablation on the exaggerated-apoptosis-prone mutant mouse strain, *Lurcher*. As tPA functions in an extracellular cascade, this experiment was also a test of whether the *Lurcher* cell death pathway ensues entirely as an intracellular cascade initiated by a constitutively active receptor, or whether a more complex mechanism with extracellular components is involved.

MATERIALS AND METHODS

Mice

Lurcher mice (B6CBACaA^{W-J}/A-Grid2^{Lc}) were purchased from the Jackson Laboratory, and mated with tPA$^{-/-}$ (*Plat*$^{-/-}$ – Mouse Genome Informatics) mice to generate the *Lc* mutation in a tPA-null background. Postnatal (P) day 12 or day 30 *Lc*/+; tPA$^{+/+}$ and control mice used in this study were littermates to *Lc*/+; tPA$^{-/-}$ mice (by mating *Lc*/+;tPA$^{+/-}$ mice together). Mice obtained by the cross were genotyped for both tPA and *Lc* using PCR-based strategy. Genomic DNA were extracted from tails. For tPA, a region of the wild-type tPA allele was amplified using the forward primer 5′-CCACCTGTGGCCTGAGGCAGTACAA-3′ and the reverse primer 5′-ATGCCTCATGCTTGCCGTAGCCAGA-3′. A region of the *neo* cassette was amplified using the forward primer 5′-CATGAGAGCAGCCGATTGTCTGTTGTGCCC-3′ and the reverse primer 5′-TTGAACAAGATGGATTGCACGCAGGTTCTC-3′. To acquire *Lc*/*Lc* DNA as a positive control, the *Lc* mutation-specific PCR product was generated from an *Lc*/+ mouse, ligated into the pCR-TOPO vector, and transformed into One-Shot^{TM} cells. Two primer sets were designed to identify the *Lc* mutation in GluRδ2. One set will only amplify DNA from *Lc* mutant chromosome: forward primer, 5′-CTCTGGCAACCCGAATGATG-3′; and reverse primer, 5′-CGGGTGATAGTGAGGAAAG**T**-3′. The other set will only amplify DNA from wild-type chromosomes with the same forward primer and a different reverse primer 5′-CGGGTGATAGTGAGGAAAG**C**-3′. For *Aci*I-mediated digestion, forward primer 5′-CTCTGGCAACCCGAATGATG-3′ and reverse primer 5′-CGGGTGATAGTGAGGAAAG-3′ were used to perform PCR. At later timepoints, the genotyping of *Lc* mice was confirmed by their ataxic gait. Mice were bred in-house under specific pathogen-free conditions (Division of Laboratory Animals Resources at SUNY at Stony Brook), controlled for temperature (21°C) and maintained with a daily light period of 12 hours.

Assay for the amidolytic and fibrinolytic activities of tPA

For quantitative determination of tPA activity, the assay was performed as described (Andrade-Gordon and Strickland, 1986). Briefly, *Lc*/+ and wild-type mice at P12 or P30 were sacrificed. The dissected cerebellar cells were lysed in 0.25% Triton X-100, and incubated in a mix containing either 0.3 mM S-2288 (Chromogenix; a chromogenic substrate specific for tPA), 0.1 M Tris, pH 8.1 and 0.1% Tween-80. Alternatively, the cerebellar extracts were incubated with 0.3 mM S-2251 (Chromogenix; a chromogenic substrate specific for plasmin; this reaction measures the fibrinolytic activity of tPA), 0.1 M Tris, pH 8.1, 0.1% Tween-80 and 0.42 μM plasminogen. The samples were incubated at 25°C. The change in absorbency (ΔA) at 405 nm was measured at different timepoints. tPA activity was calculated from initial rates in the amidolytic assay. Known concentrations of recombinant tPA were used to establish a standard curve. Total protein content was determined from an aliquot of each sample using the Bradford assay (BioRad). These concentrations were used to normalize the amount of tPA present in each sample. The measurements were performed in triplicate.

Histology

Mice were sacrificed at P8, P12 or P30. Dissected cerebella were fixed and dehydrated in 4% paraformaldehyde and 20% sucrose in phosphate-buffered saline (PBS). Sagittal (14 μm) sections were made on a cryostat (Leica) at –20°C, and kept in collecting medium (30% ethylene glycol, 25% glycerol in 50 mM phosphate). Then sections were mounted on microscope slides and Nissl stained with Cresyl Violet.

Immunohistochemistry

Adjacent sections were processed for calbindin and anti-phospho-Jun (JunP) immunohistochemistry to assess potential differences between the wild-type, *Lc*/+ and *Lc*/+; tPA$^{-/-}$ genotypes. Mouse monoclonal anti-calbindin-D (Clone CL-300, Sigma) was used to detect Purkinje cells. Rabbit polyclonal anti-JunP (Cell Signaling Technology) was used to detect apoptotic cells. To perform immunohistochemistry, sections were fixed with 4% paraformaldehyde in PBS, washed in 0.03% H_2O_2 to block endogenous peroxidase, and then incubated overnight at 4°C in anti-Calbindin-D (1:200) or anti-JunP antibody (1:100). After washing in PBS, sections were incubated with biotinylated secondary antibodies (Vector Laboratories). The avidin-biotin-peroxidase complex (ABC reaction) was visualized with diaminobenzidine and hydrogen peroxide (Vector Laboratories), as described previously (Tsirka et al., 1997).

Terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL) reactivity

Frozen sections were used for TUNEL assay (In Situ Cell Death Detection Kit, POD-conjugated, Boehringer Mannheim). Cerebella from P12 wild-type, *Lc*/+ and *Lc*/+; tPA$^{-/-}$ mice were embedded in Tissue-Tek OCT, frozen on dry ice and stored at –80°C until use. Sagittal sections (14 μm) were cut on a cryostat (Leica) at –20°C. Then sections were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP were then added to cover the sections, and incubated in a humidified chamber for 60 minutes at 37°C in the dark. The reaction was terminated by washing with PBS. Then the slides were covered with antifade and analyzed using a fluorescence microscope (Nikon). Anti-fluorescein antibody conjugated with peroxidase (POD) was used later to evaluate the sections under light microscope.

In situ hybridization

In situ mRNA hybridization was performed as described (Tsirka et al., 1997). tPA antisense mRNA probes corresponding to either nucleotides 977-1409 of the tPA-coding region, or 1890-2401 of the tPA 3′ untranslated region were labeled with digoxygenin-11-UTP (Carroll et al., 1994). The two probes generated the same pattern of expression for tPA. No specific expression above background was detected when the corresponding sense probes were used.

Western blotting

Protein extracts were prepared from cerebellum of *Lc*/+ and wild-type mice at P12 or P30 in PBS with 0.05% Triton X-100. Total protein content in each extract was determined using the Bradford assay (BioRad). Proteins (25 μg) were separated by 12% SDS-PAGE and transferred on PVDF membrane. Membranes were blocked using 5% non-fat dry milk in PBS containing 0.05% Tween-20 and incubated overnight at 4°C with rabbit polyclonal anti-mouse caspase 9 antibody (1:1000 dilution, recognize both full length and the cleaved large fragment of mouse caspase 9, Cell Signaling), or rabbit polyclonal caspase 8 antibody (1:500 dilution, reacts with both the p20 subunit and precursor of caspase 8, Santa Cruz). Then the immunocomplex was detected with biotinylated anti-rabbit IgG (Vector Laboratories). The avidin-biotin-peroxidase complex (ABC reaction) was visualized with Lumi-Glo (KPL) and exposed to film.

Quantitative western blotting

Western blot analysis was performed as above. Biotinylated secondary

anti-rabbit antibodies were used (1:500 dilution) and were detected using FITC-labeled ExtrAvidin (1:200; Sigma). Fluorescence emitted by the specific bands was detected by a FluorImager (Molecular Devices) and quantified using the ImageQuant software. Sample loading was visualized by Coomassie Blue staining.

Counts of cellular profiles

Purkinje cellular profiles were performed on 14 μm sagittal cerebellar sections by light microscopy as described (Seeds et al., 1999). Briefly, sections were taken at the midline and at 1/3 and 2/3 lateral to the midline. The hippocampus was used to determine the degree of lateral displacement for the sagittal sections. All cerebellar folia were counted in each of the three sections/hemisphere from six different mice with the same genotype and the same age. *P* values were calculated using single factor ANOVA.

The profiles of granule cells per cerebellar section was estimated from the total area and granule cell density of the internal granule cell layer (IGL). The area of the IGL was measured from an image captured using NIH Image software and a CCD video camera attached to a Nikon microscope at 20× magnification. The IGL of the captured image was outlined freehand, and the area enclosed was measured using NIH Image software.

RESULTS

Elevation of tPA activity in the *Lc/+* cerebellum during the peak of post-natal Purkinje cell death

The hallmark of the *Lurcher* phenotype consists of a massive wave of apoptotic neuronal death that sweeps through the cerebellum during the first 2 weeks of life. One supportive piece of evidence for the involvement of tPA in the *Lurcher* etiology would be if levels of tPA activity were increased during the peak of cell death, as is observed when excitotoxicity is induced through exogenous stimuli. To begin, we first confirmed that tPA is expressed in the developing mouse cerebellum using in situ hybridization at P12 (Fig. 1A). tPA mRNA was detected in wild-type cerebellum in both Purkinje cells and granule neurons, in agreement with a previous report (Basham and Seeds, 2001). We then performed amidolytic assays on isolated cerebellar extracts to determine quantitatively tPA activity during (P12) and after (P30) the peak of cell death. As shown in Fig. 1B, at P12 the amidolytic activity of tPA in *Lc*/+ cerebellar lysates was 7.1±0.5 ng tPA/μg protein, whereas in wild-type mice it was 5.0± 0.2 ng tPA/μg protein (a 42% increase in *Lc*/+ over wild-type). The activity was decreased at P30 to 3.8±0.4 in *Lc*/+ mice and 2.9±0.4 ng tPA/μg protein in wild-type animals. The difference in tPA activity between the two strains of mice was not statistically significant at P30.

The level of fibrinolytic activity of tPA (using the plasmin-specific chromogenic substrate S-2251) was also measured. We found that at P12, the activity of tPA in the cerebella of *Lc*/+ mice (6.1±0.4 ng tPA/μg protein) was 40% higher than in wild-type mice (4.3±0.3 ng tPA/μg protein). Consistent with the results of Friedman and Seeds (Friedman and Seeds, 1995), tPA activity decreased modestly by P30 in wild-type mice (3.3±0.1 ng tPA/μg protein). A decrease to similar levels was observed in *Lc*/+ mice (2.8±0.4 ng tPA/μg protein), indicating that there was no significant difference in tPA activity between *Lc*/+ and wild-type mice by adulthood. These results demonstrate a correlation between elevated tPA levels and the timing of neuronal cell death in *Lurcher*, which would be consistent with a functional role for tPA in the cell death pathway.

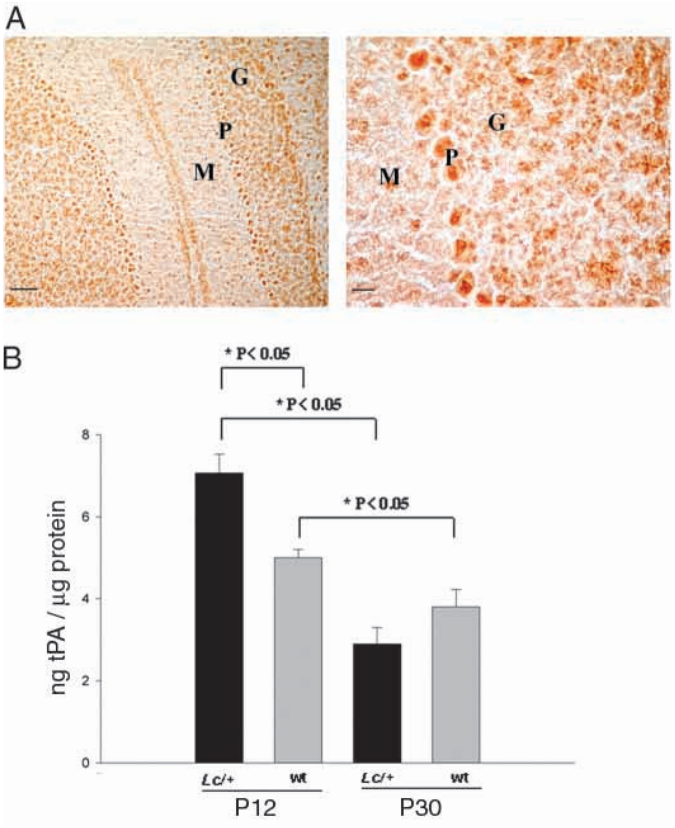

Fig. 1. (A) tPA mRNA is expressed in both Purkinje cells (P) and granule cells (G) in mouse cerebellum during development. In situ hybridization was performed on wild-type mouse cerebellum at P12 using tPA antisense mRNA probes corresponding to either nucleotides 977-1409 of the tPA-coding region (shown), or 1890-2401 of the tPA 3′ untranslated region (not shown). Identical patterns of expression were observed for the probes. No specific expression above background was detected when the corresponding sense probes were used. Scale bars: 100 μm (left); 20 μm (right). (B) Increased tPA activity in *Lurcher* cerebella at P12 but not P30. The amidolytic assay was performed as described in Materials and Methods. Notice that the level of tPA activity in the cerebella of *Lc*/+ at P12 was significantly higher than in wild-type mice, but no significant difference was detected at P30. The data are presented as mean±s.e.m. (*n*=4 or 5 mice). **P*<0.05 by ANOVA.

tPA elimination in *Lurcher* mice confers partial protection from premature neuronal apoptosis

We generated *Lc* heterozygous mice on a tPA-deficient background by first mating *Lc*/+ mice to tPA$^{-/-}$ mice, and then crossing the *Lc*/+; tPA$^{-/+}$ F1s to each other (see Materials and Methods). Mice obtained by the cross were genotyped for both tPA and *Lc* using a PCR-based strategy. A pair of primer sets were designed to distinguish the *Lc* mutation from the wild-type allele in GluRδ2 (as described in the Materials and Methods). One primer set amplified a product only from the *Lc* mutant allele; the other set only from the wild-type allele. An alternative procedure was also used to confirm the PCR genotyping described above. As the G-to-A transition in *Lc* abolishes the recognition sequence for the restriction enzyme

*Aci*I, the wild-type but not the mutant PCR product can be cleaved by *Aci*I (data not shown). At later timepoints, the genotype of *Lc* mice was confirmed by their ataxic gait.

Lc/+ and *Lc*/+; tPA$^{-/-}$ mice both exhibited ataxia as early as 12 days after birth; no significant difference in coordination was observed as a consequence of the elimination of tPA. However, very significant differences were observed on the anatomical level. Apoptotic cell death was evaluated using the TUNEL assay. P12 mice were chosen for study because extensive degeneration of Purkinje cells is readily apparent by that point in *Lc*/+ mice when compared with normal littermate controls (Norman et al., 1995). *Lc*/+; tPA$^{-/-}$ exhibited significantly fewer apoptotic neurons (150±8) when compared to *Lc*/+ mice (219±6). Wild-type mice showed the least amount of neuronal cell apoptosis (62±6) (Fig. 2). Thus, the elimination of tPA partially protects *Lc*/+ mice from premature neuronal apoptosis.

tPA deficiency delayed, but could not prevent Purkinje cell death in *Lurcher* mice

Two different types of neuronal death occur in *Lc* mice: an intrinsic Purkinje cell death of genetic origin (GluRδ2 mutation), and a secondary target-related degeneration of granule cells. We examined the effects of tPA deficiency on each type of cell death.

Using an antibody to calbindin D 28K, which is a selective marker for Purkinje cells, we found more calbindin-immunopositive cells remaining in *Lc*/+; tPA$^{-/-}$ mice than in *Lc*/+ mice at both P12 and P30. tPA$^{-/-}$ mice exhibited comparable calbindin (Purkinje) staining pattern as did wild-type mice at both timepoints. Moreover, the Purkinje cells in *Lc*/+; tPA$^{-/-}$ mice displayed less severe dendritic abnormalities; most strikingly, the dendritic trees were more arborized (Fig. 3A). There were significantly fewer Purkinje cells in *Lc*/+ and *Lc*/+; tPA$^{-/-}$ mice than in wild-type (or tPA-deficient) mice (P12, 589±26; P30, 823±50). However, the progressive cell death that resulted in the survival of relatively few Purkinje cells in *Lc*/+ mice (P12, 109±19; P30, 12±3) was attenuated in *Lc*/+; tPA$^{-/-}$ mice both during (P12: 196±21) and after (P30: 46±6) development (Fig. 3B). These data show that tPA deficiency delayed Purkinje cell death in *Lc* mice but could not prevent it.

Decrease in granule cell secondary death in *Lurcher* mice deficient in tPA

Cresyl Violet staining was used to visualize granule cells, which account for 90% of cerebellar neurons. At both P12 and P30, wild-type and tPA$^{-/-}$ cerebella exhibited normal lobular patterns, well-developed folia and robust, densely stained granule layers (Fig. 4). By contrast, the *Lc*/+ cerebella were greatly reduced in size, with poorly formed folia and sparse granule cells. No gross differences were observed between *Lc*/+; tPA$^{-/-}$, tPA$^{-/-}$ and wild-type cerebella at P12. Measuring the granule cells profiles using NIH Image software, 88% of granule cells in *Lc*/+; tPA$^{-/-}$ cerebella survived compared with wild type, as opposed to 56% in *Lc*/+ mice. By P30, *Lc*/+; tPA$^{-/-}$ cerebella were characterized by atrophic folia and a thinner, less cell-dense granule layer in comparison to tPA$^{-/-}$ or wild-type cerebella. However, even fewer granule cells were detected in the *Lc*/+ cerebella (Fig. 4). These results indicate that the secondary apoptotic granule cell death in *Lc* mice was reduced but was only partially prevented by tPA deficiency, presumably reflecting the extended but ultimately limited survival of the Purkinje cells in this setting.

Very little, if any, cell death is observed at P8 in the *Lc*/+ cerebellum; therefore, that timepoint can serve as an internal control to assess whether the absence of tPA results in any intrinsic differences between wild-type and *Lurcher* mice. Using both calbindin immunostaining and Cresyl Violet, no significant differences in the numbers of Purkinje and granule cells were found in wild-type, *Lc*/+ and *Lc*/+; tPA$^{-/-}$ cerebella at P8 (data not shown). These results strengthen the conclusion that the delayed neuronal death observed in *Lc*/+; tPA$^{-/-}$ cerebellum is due to the lack of a tPA-mediated contribution to apoptosis.

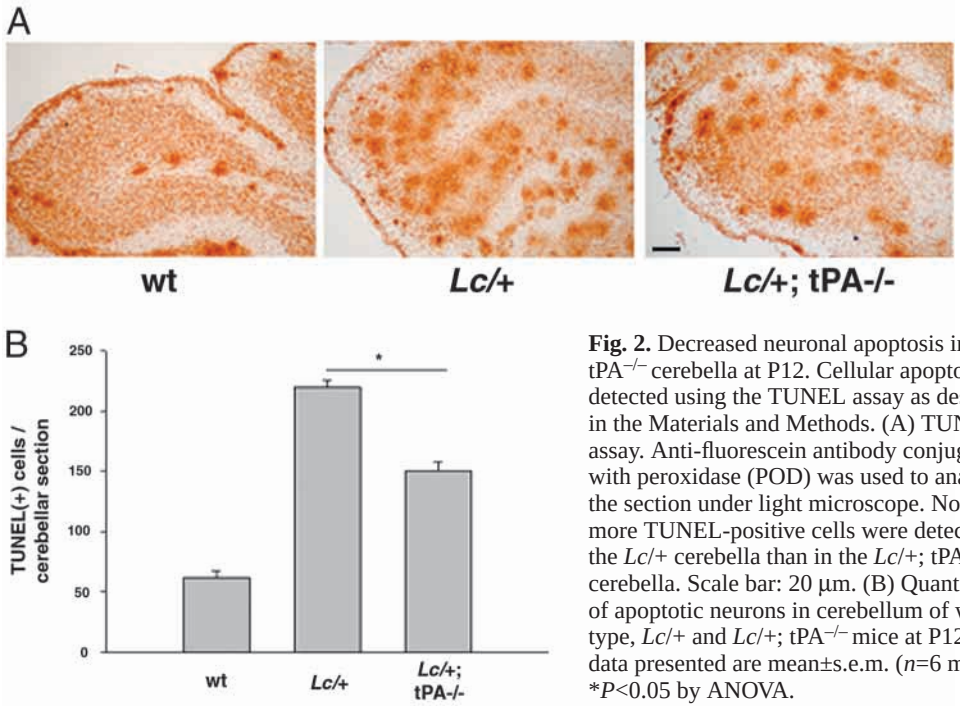

Fig. 2. Decreased neuronal apoptosis in *Lc*/+; tPA$^{-/-}$ cerebella at P12. Cellular apoptosis was detected using the TUNEL assay as described in the Materials and Methods. (A) TUNEL assay. Anti-fluorescein antibody conjugated with peroxidase (POD) was used to analyze the section under light microscope. Notice that more TUNEL-positive cells were detected in the *Lc*/+ cerebella than in the *Lc*/+; tPA$^{-/-}$ cerebella. Scale bar: 20 μm. (B) Quantification of apoptotic neurons in cerebellum of wild-type, *Lc*/+ and *Lc*/+; tPA$^{-/-}$ mice at P12. The data presented are mean±s.e.m. (n=6 mice). *P<0.05 by ANOVA.

Elimination of tPA reduces Jun phosphorylation in *Lurcher* mice

Jun is a component of the transcription factor AP-1. It is proposed that the ability of Jun to activate gene transcription is strongly potentiated by phosphorylation at serine (S)73 (Smeal et al., 1991) by the Jun N-terminal kinases (JNKs), which belong to the MAP kinase family (Kyriakis et al., 1994). JunP may induce FasL expression via binding several AP-1 sites in the Fas-ligand

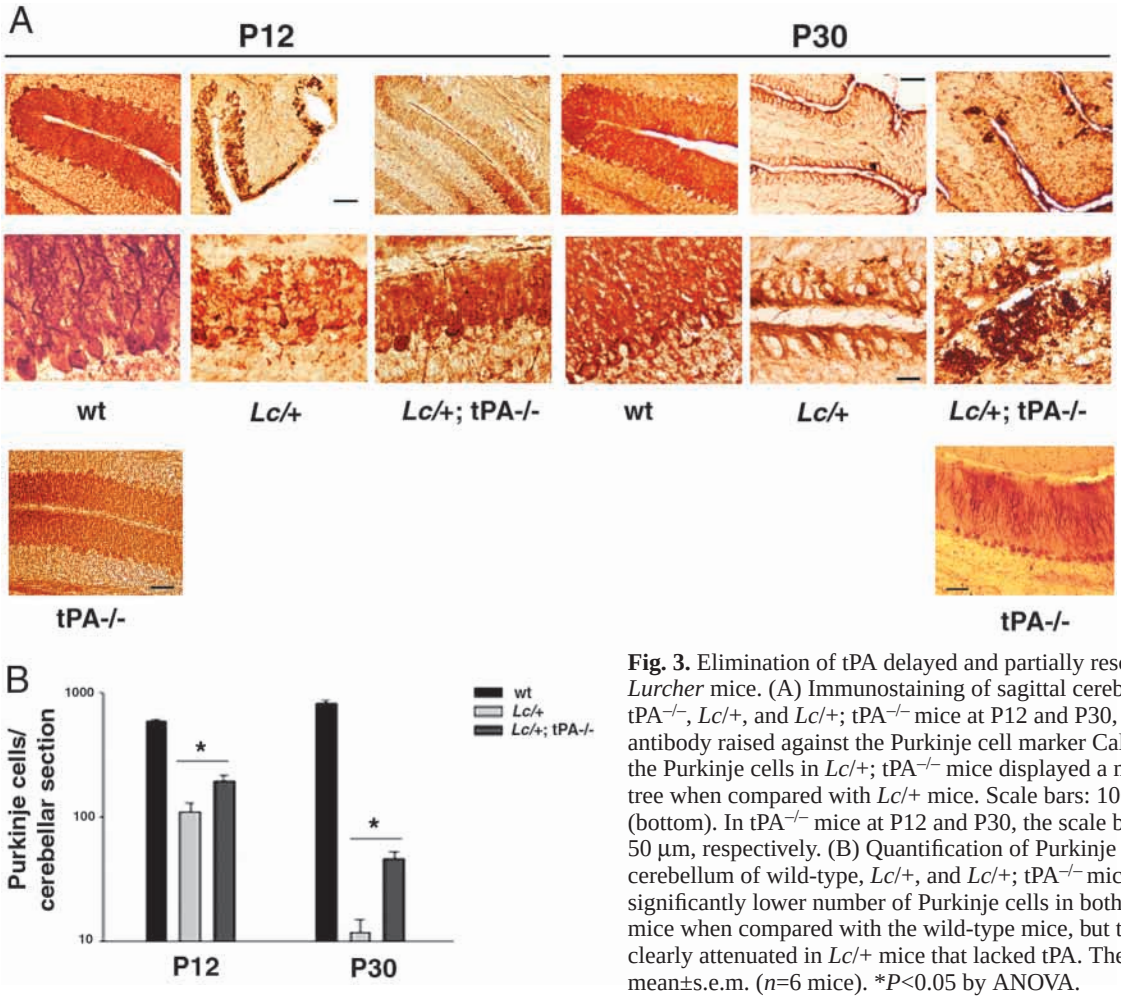

Fig. 3. Elimination of tPA delayed and partially rescued Purkinje cell death in *Lurcher* mice. (A) Immunostaining of sagittal cerebella sections of wild-type, tPA$^{-/-}$, *Lc*/+, and *Lc*/+; tPA$^{-/-}$ mice at P12 and P30, using a monoclonal antibody raised against the Purkinje cell marker Calbindin D 28K. Note that the Purkinje cells in *Lc*/+; tPA$^{-/-}$ mice displayed a more branched dendritic tree when compared with *Lc*/+ mice. Scale bars: 100 μm (top); 20 μm (bottom). In tPA$^{-/-}$ mice at P12 and P30, the scale bars represent 100 μm and 50 μm, respectively. (B) Quantification of Purkinje cell survival in the cerebellum of wild-type, *Lc*/+, and *Lc*/+; tPA$^{-/-}$ mice at P12 and P30. Note the significantly lower number of Purkinje cells in both *Lc*/+ and *Lc*/+; tPA$^{-/-}$ mice when compared with the wild-type mice, but that Purkinje cell death was clearly attenuated in *Lc*/+ mice that lacked tPA. The data are presented as mean±s.e.m. (n=6 mice). *P<0.05 by ANOVA.

promoter (Kasibhatla et al., 1998). FasL is a well-known death factor. Binding of FasL to Fas induces trimerization of the Fas receptor, which eventually triggers the caspase cascade. It has been reported that lasting Jun S73 phosphorylation by JNKs is part of the neuronal stress response in adult-onset neurodegenerative disorders and that FasL may be the apoptotic effector in this pathway (Herdegen et al., 1998). JNK activity and Jun expression have also been reported to mediate apoptosis of cultured mouse cerebellar granule neurons (Watson et al., 1998). Furthermore, apoptosis of hippocampal neurons after excitotoxic injury is closely linked to Jun phosphorylation: genetic elimination of JNK-3 activity and subsequent inhibition of Jun phosphorylation prevent excitotoxin-induced neuronal death (Yang et al., 1997).

Given this potential link to excitotoxicity and receptor-mediated apoptotic death, we used double immunofluorescence [against calbindin (red) and JunP (green)] to evaluate apoptosis of Purkinje cells (Fig. 5) in the *Lc*/+, wild-type and *Lc*/+; tPA$^{-/-}$ animals. We observed a small number of JunP-immunopositive Purkinje cells in wild-type (and tPA-deficient; data not shown) cerebella at P12 and fewer at P30, which was not unexpected because apoptosis occurs at a low level during normal development. In *Lc*/+ mice at P12, almost all of the cells were doubly labeled and thus undergoing apoptosis (note the severe shrinkage of Purkinje cell bodies in *Lc*/+), a result consistent with the TUNEL data (Fig. 2). By P30, hardly any Purkinje cells remained in *Lc*/+ mice, which is presumably due to premature death and clearance. There was very limited coincident staining of calbindin and JunP in *Lc*/+; tPA$^{-/-}$ mice at P12. At P30, although many of the Purkinje cells were missing, the ones that remained stained only with calbindin and not with JunP (i.e. they were not undergoing apoptosis). These results suggest that although Purkinje cell death clearly occurs in *Lc*/+;tPA$^{-/-}$ animals, it appears to follow either a pathway of apoptosis that does not involve FasL and JunP, or a different timecourse of apoptotic death.

tPA activity affects receptor-mediated (caspase 8) apoptosis

The two main pathways by which apoptosis proceeds are the mitochondria-mediated and receptor-mediated pathways (Budihardjo et al., 1999). Both pathways converge and proceed via the activation of caspase 3. To evaluate the contribution of tPA in apoptosis upstream of caspase 3, we used quantitative western blotting analysis to investigate the activation of caspase 8 and caspase 9, as markers for the receptor- and mitochondria-mediated apoptotic pathways, respectively.

Activation of caspase 8 results in conversion of the 54-55 kDa precursor form into 30 kDa and 18 kDa active fragments. As evident in Fig. 6, active caspase-8 is strongly detected in *Lc*/+ mice at both P12 and P30. Both the 30 kDa and 18 kDa bands are also present in the *Lc*/+;tPA$^{-/-}$ extracts, but at

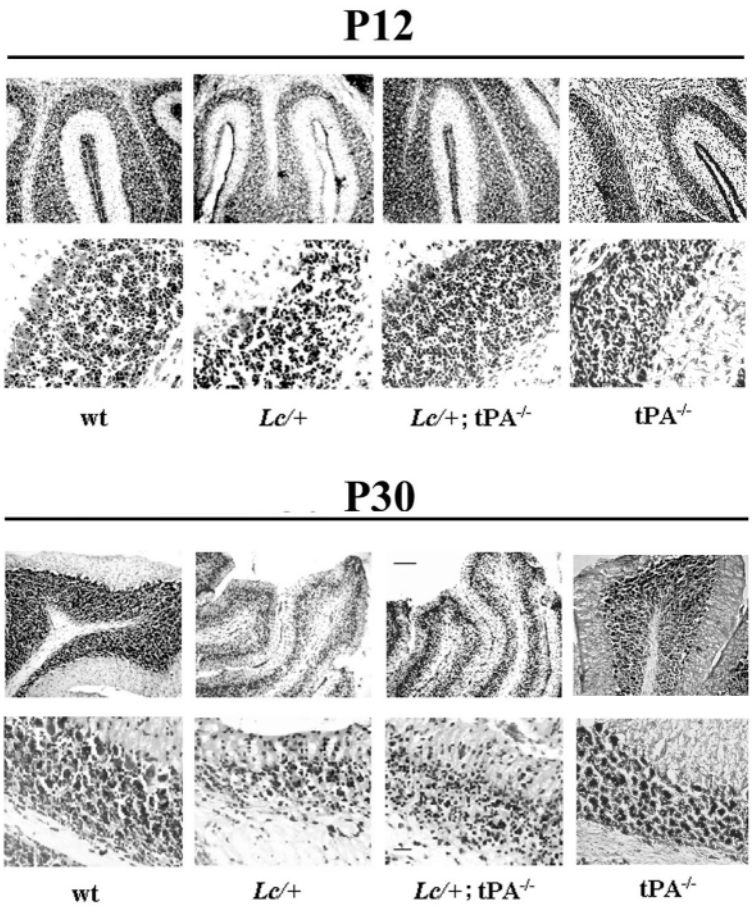

Fig. 4. Secondary death of granule cells is decreased in *Lurcher* mice with tPA deficiency. Sagittal cerebella sections of wild-type, tPA$^{-/-}$, *Lc*/+, and *Lc*/+; tPA$^{-/-}$ mice at P12 and P30 were stained with Cresyl Violet. At P12, both *Lc*/+; tPA$^{-/-}$ and wild-type cerebella showed normal lobular patterns, well-developed folia and densely stained granule layers. By contrast, the *Lc*/+ cerebella were greatly reduced in size, with poorly formed folia and sparse granule cells. By P30, although *Lc*/+; tPA$^{-/-}$ cerebella revealed atrophic folia, more granule cells were still detected there than in the *Lc*/+ cerebella. Scale bars: 100 μm (top); 20 μm (bottom).

significantly lower intensities (Fig. 6A,B), suggesting that tPA-mediated effects contribute to the activation of caspase 8 apoptosis. These results are also consistent with the JunP immunohistochemistry data (Fig. 5), indicating that tPA affects the receptor-mediated pathway of apoptosis.

Caspase 9 exists in a precursor form of 49 kDa, which is activated during apoptosis and cleaved into a 37 kDa product. The precursor form of caspase 9 was present at equal levels in all three genotypes. Similarly, activated caspase 9 was detected in *Lc*/+ but not in *Lc*/+; tPA$^{-/-}$ mice at P12. At P30 there was no difference at the levels of the band corresponding to active caspase 9 in *Lc*/+ and *Lc*/+; tPA$^{-/-}$ mice, suggesting that caspase 9 activated in *Lc*/+ mice regardless of the presence or absence of tPA (data not shown), indicating that tPA does not modulate the endogenous, mitochondria-mediated apoptosis.

DISCUSSION

tPA mediates excitotoxic neuronal cell death in the mouse hippocampus (Tsirka et al., 1995). However, it has not been clear whether tPA functions in the apoptotic or necrotic cell death pathway after such injury. This report adds support to the hypothesis that the tPA/plasmin proteolytic cascade mediates apoptotic neuronal death. We show here using *Lc*/+ mice as a physiologically relevant model for excitotoxic neuronal apoptosis (Zuo et al., 1997), that tPA participates in the receptor-mediated apoptotic cell death pathway.

One possible explanation for our finding of different cell death outcomes for *Lc*/+; tPA$^{-/-}$ and *Lc*/+ mice could be that there are genetic modifiers that were revealed by crossing the *Lc* and tPA$^{-/-}$ strains. This is not likely to be the case, however, because the *Lc*/+; tPA$^{+/+}$ and wild-type control mice used in this study came from the same litters as the *Lc*/+; tPA$^{-/-}$ mice used. As additional support, the presence or absence of tPA does not appear to affect the number or morphology of cells in the cerebella at P8, a timepoint just

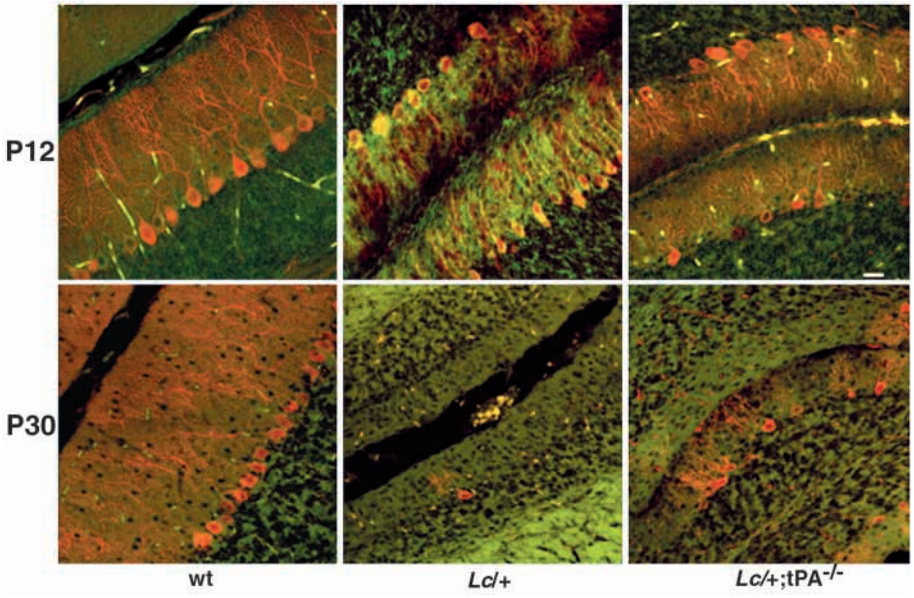

Fig. 5. Elimination of tPA reduces Jun phosphorylation in *Lurcher* mice. Double immunofluorescence [against calbindin (red) and JunP (green)] was used to evaluate apoptosis of Purkinje cells (see quantitative data in Fig. 6A,B). Almost all of the cells were doubly-labeled in *Lc*/+ mice at P12, note the severe shrinkage of Purkinje cell bodies. By P30, hardly any Purkinje cells remained in *Lc*/+ mice. In *Lc*/+;tPA$^{-/-}$ cerebella, there were few JunP-positive Purkinje cells at P12, and at P30 the Purkinje cells observed stained only with calbindin and not with JunP. Scale bar: 20 μm.

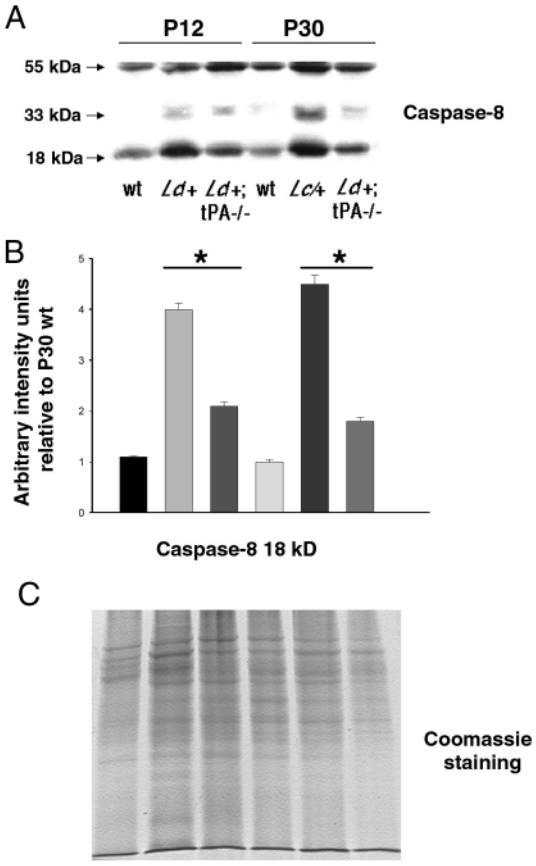

Fig. 6. Caspase 8 activation is decreased in *Lurcher* mice that lack tPA. (A) Western blotting was performed for wild-type (wt), *Lc*/+, and *Lc*/+; tPA$^{-/-}$ mice at P12 and P30, using either rabbit polyclonal caspase 8 antibody (1:500 dilution, reacts with both the p18 subunit and precursor of caspase 8). (B) Quantification of caspase 8 activation by western blot analysis. The activated caspase 8-specific bands were quantitated using a fluorimager, as described in the Materials and Methods. Note that the decreased caspase 8 activation (18 kDa band) in the *Lc*/+; tPA$^{-/-}$ cerebellum was detected at both P12 and P30. The data presented are from four different experiments. (C) Coomassie Blue staining to show protein loading. *P<0.001 by ANOVA.

before the apoptotic program becomes evident for *Lc*/+ mice (data not shown), which serves as an internal control to assess whether the absence of tPA results in any intrinsic differences between wild-type and *Lc* mice.

Lc/*Lc* homozygous mutants die shortly after birth (Phillips, 1960). So far we have been unable to generate adult mice with the *Lc*/*Lc*; tPA$^{-/-}$ genotype. Only one mouse *Lc*/*Lc*; tPA$^{-/-}$ pup was born. It survived just for 4 days. This may be due to the fact that the tPA deficiency only delays, but does not ultimately prevent, most of the *Lc* apoptotic cell death, or possibly because at least one functional copy of the GluRδ2 channel mutated in *Lc* is required for viability independently of the afflicted neurons observed in the hippocampus of the heterozygotes.

In spite of the fact that the neuronal degeneration in *Lc*/+; tPA$^{-/-}$ mice was significantly delayed when compared with *Lc*/+ mice, the ataxic gait characteristic of *Lc*/+ was not drastically improved in the absence of tPA (assessed using footprint/gait analysis, data not shown).

Phosphorylation of Jun has been linked to excitotoxicity-induced neuronal apoptosis (Yang et al., 1997). We have previously shown that tPA$^{-/-}$ and plasmin(ogen)$^{-/-}$ (*Plg*$^{-/-}$) mice are more resistant to excitotoxic neurodegeneration, which indicates that tPA and plasmin promote this excitotoxic death. tPA$^{-/-}$ and *Plg*$^{-/-}$ mice do not show a TUNEL-positive signal over their hippocampal pyramidal neurons after excitotoxin injection, in contrast to their wild-type counterparts (Tsirka et al., 1997). In addition, there are decreased numbers of phosphorylated Jun and activated caspase 3-immunoreactive neuronal cells in tPA$^{-/-}$ and *Plg*$^{-/-}$ mice after excitotoxic injury, whereas such cells are abundantly found in wild-type mice subsequent to injury, suggesting that the tPA/plasmin cascade induce apoptosis in excitotoxin-challenged neuronal cells via the Jun S73 phosphorylation pathway (W. L. and S. E. T., unpublished). Similarly, in this model, although cell death was evident in the *Lc*/+;tPA$^{-/-}$ cerebella, the cell death process did not seem to follow the JunP pathway (Fig. 5). Alternatively, this apoptosis process in the *Lc*/+;tPA$^{-/-}$ cerebella could be following a different timecourse than in wild-type or *Lc*/+ mice.

The premature cell death that occurs in the *Lc*/+ cerebellum very possibly proceeds by both intracellular and extracellular cascades. One working model is as follows (Figure 7): the *Lc* mutation results in constitutively open GluRδ2^{Lc} channels and thus depolarized resting membrane potentials in Purkinje cells (Wollmuth et al., 2000), which then trigger the intracellular apoptotic pathway mediated by Bax, mitochondrial dysfunction, and activation of caspase 9, caspase 3 and other components (Doughty et al., 2000; Selimi et al., 2000). This depolarization should also induce secretion of tPA from Purkinje cells, because in other systems (PC12 and chromaffin cells) membrane depolarization induces the Ca^{2+}-dependent secretion of tPA (Gualandris et al., 1996; Parmer et al., 1997). The secretion of tPA would then initiate an extracellular tPA/plasmin cascade and result in extracellular matrix protein cleavage [e.g. laminin (Chen and Strickland, 1997)], and

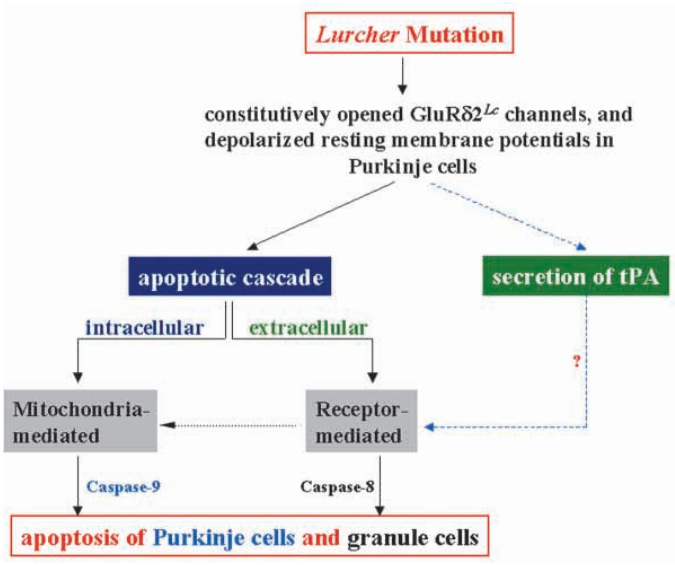

Fig. 7. Proposed model for tPA involvement in the Lurcher apoptotic neuronal death pathway.

induction of a receptor-mediated cell death pathway, which involves activation of caspase 8 and phosphorylation of Jun. This pathway would in turn facilitate the initial Purkinje cell degeneration observed. Furthermore, the caspase 8 pathway could stimulate the mitochondria death pathway by activating caspase 9 (Budihardjo et al., 1999). Secondary apoptotic death of granule neurons is target related, presumably owing to lack of essential contacts with Purkinje cells (Wetts and Herrup, 1982a; Wetts and Herrup, 1982b). The role of tPA in this secondary death is not clear, but as tPA is highly expressed in granule cells and affects their migration and differentiation (Seeds et al., 1999), it may also be involved directly here.

The elimination of tPA from *Lc* mice resulted in change in the rate of apoptotic developmental death of the neurons at risk as assessed by immunohistological and anatomical analyses, but did not confer substantial long-term survival. Our findings here indicate that although eliminating tPA ameliorated the *Lc*-triggered neuronal death by delaying the receptor-mediated pathway through which apoptosis progresses, this particular genetic mutation ultimately triggers other apoptotic pathways in parallel that suffice to elicit cell death. Nonetheless, manipulation of tPA activity did ameliorate the progression and might be of therapeutic utility in other diseases where activation of the intracellular pathway is less vigorous.

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