

# Specific JNK Inhibition by D-JNKI1 Protects Purkinje Cells from Cell Death in Lurcher Mutant Mouse

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**Abstract** In the Lurcher mutant mouse (+/Lc), Purkinje cells (PCs) selectively die due to the mutation that converts alanine to threonine in the glutamate ionotropic receptor GRID 2, thus resulting in a constitutively leaky cation channel. This intrinsic cell death determines a target-dependent cell death of granule cells and olivary neurons and cerebellum cytoarchitecture is severely disrupted in the adult Lurcher mutant. Although the +/Lc mutant has been widely characterized, less is known about the molecules involved in +/Lc PC death. We, here, used organotypic cerebellar slice cultures from P0 mice to investigate the role of c-jun N-terminal kinase (JNK) in +/Lc PC death by using D-JNKI1 as very specific tool to inhibit its action. Our results showed that D-JNKI1 treatment increased the

number of +/Lc PC at 14 DIV of 3.6-fold. Conversely, this specific JNK inhibitor cell permeable peptide did not increase PC number in +/+ treated versus untreated cultures. These results clearly indicate that JNK plays an important role in +/Lc PC mechanism of cell death.

**Keywords** Cell death · Lurcher mutant mouse · Purkinje cells · JNK · Cell permeable peptide

## Introduction

In Lurcher heterozygous mouse (+/Lc), one base-pair substitution (Ala/Thr) in the third hydrophobic segment of the orphan glutamate ionotropic receptor GRID 2 results in a constitutively open cation membrane channel that chronically depolarizes Purkinje cells (PCs) in cerebellum [1]. In this mouse model, a PC death occurs during the second post natal week to reach about 90% by P26 [2–4]. Subsequently, a target-dependent cell death results in granule cell and olivary neuron loss [5]. Interestingly, two cell-death mechanisms have been described for this PC death, autophagy and apoptosis (for a review see [6]). Experimental evidences showed that the chronic cation leak current mediated by the GRID2 Lc channel can result in increased oxidative stress that, in turn, can trigger apoptotic cell-death pathways [7].

The occurrence of these two cell-death mechanisms underlines the relevance of this model, in which a well-defined genetic lesion affects a single cell type (PC), since increased autophagy as well as excitotoxic mechanisms have been linked to the neuropathology of a number of neurodegenerative diseases [8, 9]. Previous studies have tested the role of key cell-death molecules on +/Lc Purkinje cell death by overexpression (Bcl2) or deletion (Bax),

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without definitive results. In both cases, PC death was only delayed [10–12].

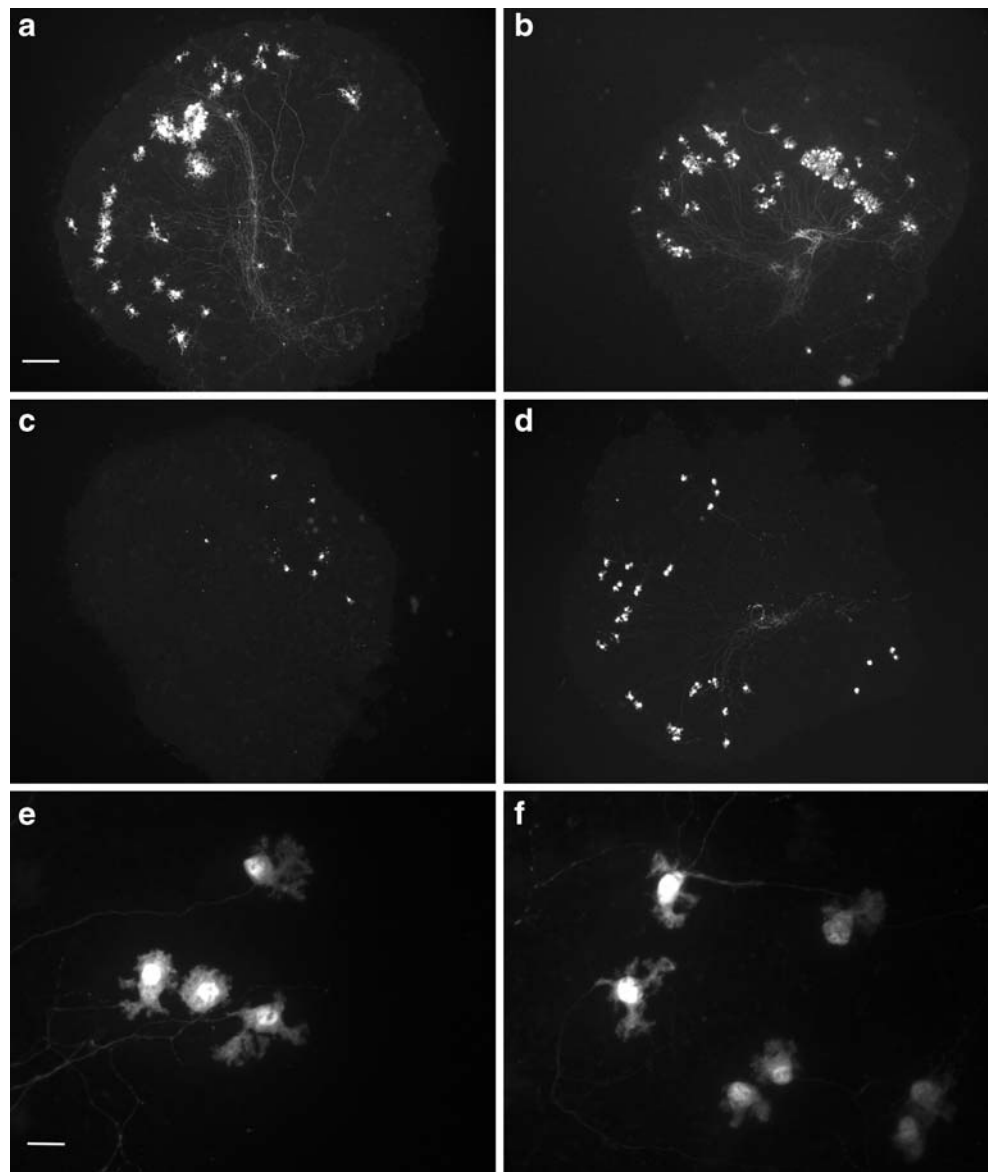
c-jun N-terminal kinase (JNK) pathway is critical for naturally occurring neuronal death during development as well as for pathological death of adult brain following different insults [13, 14]. Several *in vitro* and *in vivo* studies have reported alterations of JNK pathways associated to neuronal death in NMDA excitotoxicity [15], cerebral ischemia [15, 16] as well as in Parkinson's [17], Huntington's and Alzheimer's diseases [18]. Furthermore, in Lurcher mice, an increased activation of JNK in +/Lc PCs occurs (H. Zanjani and M. Vogel, personal communication) and c-jun activation has been shown in PCs at P12 [19], thus suggesting a JNK involvement in Lurcher PC death.

We analyzed the role of JNK in +/Lc PC death by using the specific cell permeable JNK inhibitor peptide D-JNKI1 [20].

## Materials and Methods

+/Lc mutant and wild type ++ pups were generated by mating +/Lc males with wild type B6CBA females (Janvier, Le Genest St. Isle, France) [21]. Cerebellar slices were prepared from P0 (day of birth) mice as described in Dusart et al. [22]. After decapitation, brains were dissected out into cold Gey's balanced salt solution containing 5 mg/ml glucose and meninges were removed. Cerebellar parasagittal slices (350  $\mu$ m thick) were cut on a MacIlwain

**Fig. 1.** Purkinje cell survival in organotypic cultures. Cerebellar slices from P0 ++ (a, b) and +/Lc (c, d) pups were maintained 14 days *in vitro* in the presence (b, d) or absence (a, c) of D-JNKI1. In ++ slice (a) a large number of PCs can be observed after 14 DIV, while in +/Lc slice the PC number is strongly reduced (c). The D-JNKI1 treatment increased the survival of +/Lc PCs (compare c and d). No evident difference in morphology was observed between treated and untreated +/Lc PCs (e, f). Scale bar=200  $\mu$ m in a–d and 25  $\mu$ m in e–f



tissue chopper and transferred onto membranes of 30 mm Millipore culture inserts with 0.4  $\mu\text{m}$  pore size (Millicell, Millipore). Slices were maintained in culture in six-well plates at the interface between the air and the culture media consisting of 50% Basal Medium-Eagle (BME), 25% HBSS, 25% horse serum, 1 mM L-glutamine, and 5 mg/ml D-glucose in a humidified chamber with 5%  $\text{CO}_2$  at 35°C [23]. The media was changed every 2–3 days and cultures were fixed at 14 days in vitro. D-JNKI1 5  $\mu\text{M}$  (Xigen, Lausanne, Switzerland) was added in the culture medium at 7 DIV.

The +/Lc or +/+ genotype of P0 pups was identified by PCR and single-stranded conformation polymorphism (SSCP) as described previously [24].

Slice cultures were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature and immunohistochemistry was performed as described previously [25]. Mouse monoclonal antibody against calbindin (dilution 1:5,000, Swant, Bellinzona, Switzerland) was used to label PC and was revealed with CY3-conjugated Donkey anti-mouse antibody (dilution 1:400, Jackson Immuno-Research Laboratories, Inc). After 2 h incubation in buffer containing the secondary antibody, the slices were washed several times with PBS, counterstained with DNA fluochrome Hoechst 33258 (diluted 1/50,000, Sigma) and mounted in Mowiol (Calbiochem, La Jolla, CA, USA).

The total number of Purkinje cells per animal was determined by systematically counting on all slices calbindin fluorescent labeled Purkinje cells by using either a  $\times 20$  or  $\times 40$  objectives. Purkinje cell average number for each condition is reported as the mean  $\pm$  standard error.

For statistical analysis we used SAS software (SAS Institute Inc, Cary, NC, USA). Data were tested for the normality of distribution and rank transformation was done. ANOVA followed by multiple comparisons between means (Tukey's test) was used to check significant difference between groups ( $n=8$  for both +/Lc+D-JNKI1 and +/+D-JNKI1 animals,  $n=8$  for both +/Lc and +/+ untreated animals).

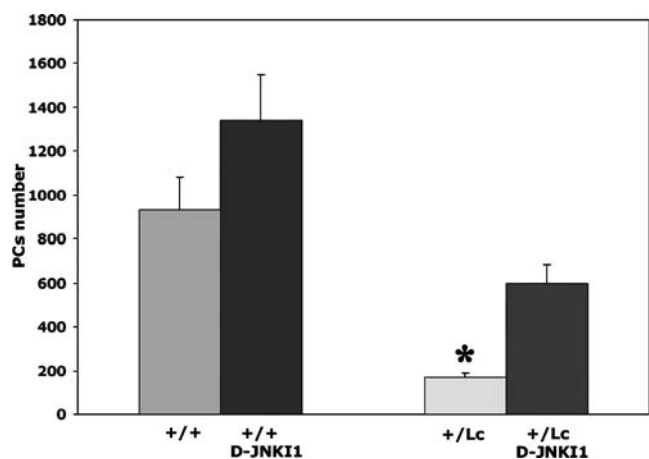
## Results

Preliminary results from Zanjani and collaborators showed that PC from P0 +/Lc pups die in organotypic culture, mostly during the second week in vitro. Indeed, they have shown that at 7 DIV there is no significant difference between Purkinje cell number in wild type (+/+) and +/Lc mice, while at 14 DIV there is a large reduction (about 80%) in the survival of +/Lc Purkinje cells, meaning that PC death starts after 7 days in vitro [26]. Thus, to evaluate the JNK role in +/Lc PC death, cerebellar slice cultures

from P0 pups were treated with D-JNKI1 5  $\mu\text{M}$  starting at 7 DIV through DIV 14. As expected, cerebellar slices from P0 mice (+/+) maintained in culture for 14 DIV presented numerous PC (Fig. 1a) whereas only very few PCs were survived in slices from +/Lc cerebellum (Fig. 1c).

At 14 DIV, PC survival was reduced by over 80% in +/Lc compared to +/+ PCs ( $167 \pm 21$  +/Lc PC,  $n=8$  vs.  $932 \pm 150$  WT PCs,  $n=8$ ; Fig. 2, [26]). Furthermore, +/Lc PC in organotypic culture showed the morphological features already described in vivo, namely characteristic dendritic abnormalities with less branched dendritic trees and axonal varicosities (Fig. 1e). These results as the ones of Zanjani and collaborators [26] demonstrate that organotypic culture represents an appropriated model to investigate the signaling pathways involved in the Lurcher Purkinje cell death.

After the specific JNK inhibition via D-JNKI1 during the second week of culture, the difference in PC number between +/+ and +/Lc slices was still visible (Fig. 1b,d). However, PC survival was increased in +/Lc slices treated with D-JNKI1 compared to the untreated ones (compare Fig. 1c and d). Quantitative analysis revealed that D-JNKI1 increased +/Lc PCs survival by more than 3.5-fold ( $599 \pm 86$ ,  $n=8$ ;  $P<0.05$ ) compared to untreated +/Lc. D-JNKI1 treatment also resulted in a slight increase of PC number (1.4-fold) in +/+ treated versus untreated slice cultures. Statistical analysis of our data strongly confirmed the selective neuroprotective D-JNKI1 action on +/Lc PC, since the difference between +/+ and +/+ D-JNKI1 PC number was not statistically significant ( $P>0.05$ ; Fig. 2). No major difference was observed in the PC morphology between D-JNKI1 treated and untreated animals in both +/+ and +/Lc groups (Fig. 1e–f). These results show that JNK plays an important role in +/Lc PC mechanism of cell death.



**Fig. 2.** Quantitative analysis of CaBP positive cells in the different slice cultures after 14DIV. The PC number is expressed, for each condition, as the mean  $\pm$  SEM ( $n=8$  for all groups). The D-JNKI1 treatment increased +/Lc PC number of 3.6-fold ( $P<0.05$ ) but did not have a significant effect on +/+ PC

## Discussion

Excitotoxicity is a major mechanism in many human CNS disease states such as cerebral ischemia, spinal cord injury, epilepsy, and many neurodegenerative diseases. Even if the initial event, an excessive glutamate release that activates post-synaptic receptors, is well deciphered, molecular mechanisms of glutamate toxicity are still unclear. To date, it is well known that JNK MAPK plays an important role in cell response to glutamate, and specific JNK inhibition has revealed important neuroprotective features in several models of acute excitotoxicity.

We, here, investigated JNK role in a model of chronic excitotoxicity, the Lurcher mutant mouse, by using D-JNKI1 as very specific tool to inhibit its action.

Experimental evidences have clearly shown that PCs and consequently GC and IO neuron death in Lurcher mouse are not tight correlated to one specific death pathway, but are instead the result of various and coexistent processes of cell death [6, 27, 28].

Our results confirmed data obtained by Zanjani et al. [26]: +/Lc PC die in slice cultures taken from P0 pups. We then proved that D-JNKI1 increases PCs survival by 3.6 fold. This effect is strictly related to Lurcher “pathological cell death” and is not present on +/+ PCs.

Neuroprotective D-JNKI1 action suggests a JNK role in one or both processes that contribute to PCs death in Lc mouse: apoptosis and autophagic cell death.

It has already been shown that D-JNKI1 completely prevents death of primary cortical neurons exposed to high NMDA concentrations [29] and D-JNKI1 action results in a prevention of caspase 3 activation after NMDA excitotoxicity in vitro and cerebral ischemia in vivo [29, 16]. JNK activity may also be involved in autophagy [30, 31] and autophagic cell death of pyramidal hippocampal neurons triggered by NMDA is JNK mediated [32].

Wang et al. recently put in evidence that the axonal swellings of +/Lc PC due to the autophagic process present a local accumulation of MAP1B-P bound to microtubule-associated protein light chain 3 (LC3) [33]. MAP1B is a cytoplasmic JNK target [34, 35]. Experimental evidences showed that MAP1B-P accumulates in pre-apoptotic neuronal somata axotomized by spinal cord injury, and that MAP1B phosphorylation is correlated with activation of JNK [36]. MAP1B may thus represent a link between JNK and autophagic cell-death pathway in Lurcher mutant mice. Further studied are needed to deeper clarify JNK mechanism of action in +/Lc PC death.

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