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A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia

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Neuronal death in cerebral ischemia is largely due to excitotoxic mechanisms, which are known to activate the c-Jun N-terminal kinase (JNK) pathway. We have evaluated the neuroprotective power of a cell-penetrating, protease-resistant peptide that blocks the access of JNK to many of its targets. We obtained strong protection in two models of middle cerebral artery occlusion (MCAO): transient occlusion in adult mice and permanent occlusion in 14-d-old rat pups. In the first model, intraventricular administration as late as 6 h after occlusion reduced the lesion volume by more than 90% for at least 14 d and prevented behavioral consequences. In the second model, systemic delivery reduced the lesion by 78% and 49% at 6 and 12 h after ischemia, respectively. Protection correlated with prevention of an increase in c-Jun activation and c-Fos transcription. In view of its potency and long therapeutic window, this protease-resistant peptide is a promising neuroprotective agent for stroke.

Although ischemic stroke is the third most common cause of death in the United States and Europe, the only currently approved medical treatment is administration of intravenous recombinant tissue plasminogen activator within 3 h of stroke onset, aimed at restoring cerebral blood flow[1]. The permanent occlusion of a cerebral artery leads to a mainly necrotic neuronal death by a complex pathogenic cascade of events that include energy depletion, excitotoxicity and peri-infarct depolarization. There is also a more delayed mechanism involving both inflammation and apoptosis, apoptotic mechanisms becoming more prominent in milder transient ischemias (refs. 2–4; reviewed in ref. 5). Therapeutic interventions targeted to these more delayed events allow a longer therapeutic window, as in the case of caspase-3 inhibition after transient MCAO in the mouse[6]. Although several neuroprotective strategies were able to reduce the lesion volume in animal models, clinical trials have not been successful up to now[7,8]. Several explanations have been proposed for this failure: that animal experiments emphasize infarct volume rather than functional outcome as the primary endpoint, whereas functional outcome is the relevant parameter in patients[7]; that standard histological evaluation takes account of perikaryal damage but ignores axonal injury, which also contributes to the resulting neurological deficit[7,9,10]; that infarct volume is often assessed only at early time points; and that some observed effects may result from delaying rather than preventing cell death[7]. Most importantly, some compounds are protective only when given close to the time of injury, and an extended therapeutic window (the time after injury during which the treatment is still effective) is critical because most stroke patients reach the emergency room more than 3 h after stroke onset, beyond the therapeutic window for thrombolysis[7].

The main mechanism underlying neuronal death in stroke and anoxic and traumatic brain damage is excitotoxicity, which is triggered by the excessive activation of ionotropic glutamate receptors, particularly those of the *N*-methyl-D-aspartate (NMDA) subtype, leading to a rapid influx of Ca^{2+} that triggers cell death[11,12]. Despite the initial promise of glutamate antagonists and Ca^{2+} channel blockers for the reduction of excitotoxic damage, clinical trials with these agents had to be abandoned because of toxic side effects[7]. As glutamate receptor activation and Ca^{2+} entry occur very early, the therapeutic time window is narrow. Furthermore, recent evidence shows that the NMDA antagonist MK-801 does not prevent axonal damage in the white matter, which probably explains its limited efficacy in improving functional outcome after stroke[13]. Targeting signaling events further downstream in the excitotoxic cascade should allow delayed therapeutic intervention to be effective. JNK is activated in the ischemic neurons[14] downstream of the activated glutamate receptors and seems to be involved in mediating neuronal death[15,16]. A role for JNK in the excitotoxic apoptosis of hippocampal neurons *in vivo* has been inferred from JNK-3-deficient mice[17], whose central nervous systems undergo normal development (including developmental cell death, which involves JNK-1 and JNK-2 but not JNK-3; ref. 18). After a systemic injection of kainate, however, adult JNK-3-deficient mice showed a reduction in seizure activity and apoptosis was prevented. Mice with an inactive form of the major JNK target, c-Jun, also showed

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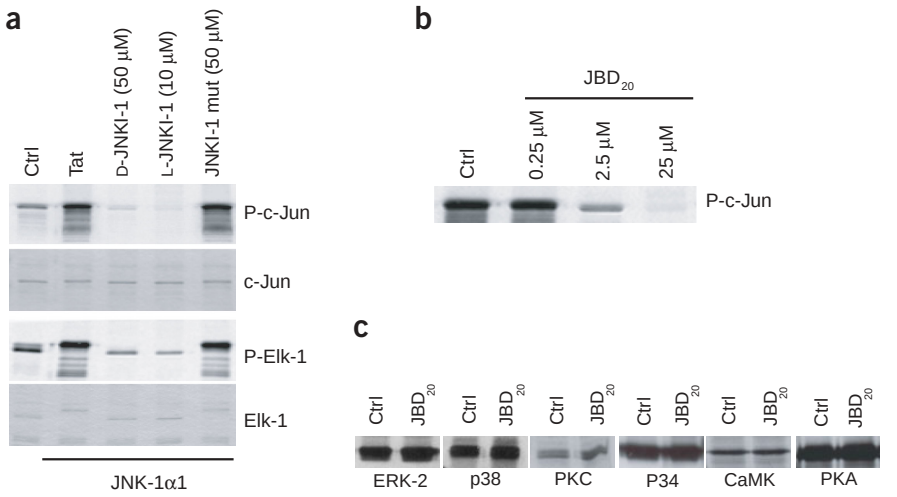

Figure 1 *In vitro* kinase assays showing sensitivity and specificity of JNK-inhibitory peptides. (**a**) Kinase assays with recombinant JNK-1α1, using GST-Jun and GST-Elk-1 as substrates, as previously described[20]. Elk-1 and c-Jun bands were from Coomassie-stained gels. P, phosphorylated (radiolabeled) forms of substrates. (**b**) Dose-response experiments in conditions similar to those in **a**, with decreasing amounts of JBD_{20} (see ref. 20 for the amino acid sequence). HIV Tat was not linked to JBD_{20} in these experiments because at concentrations above 50 μM, it induces a nonspecific precipitation of proteins in the extracts. (**c**) Kinase assays with different recombinant kinases. JBD_{20} (500 μM) caused no reduction in phosphorylation of more than 1%. Substrates used were Elk-1 for ERK-2, ATF-2 for p38, histone for p34, protein kinases C (PKC) and A (PKA), and casein for calcium/calmodulin-dependent protein kinase (CaMK). Ctrl, control (absence of peptide).

resistance to excitotoxic neuronal death[19], suggesting that preventing JNK from accessing c-Jun might confer protection.

Here we have used cell-penetrating peptides to block selectively the access of JNK to c-Jun and other substrates by a competitive mechanism[20,21]. These peptides were obtained by linking the 10-amino-acid HIV Tat(48–57) transporter sequence[22] to the 20-amino-acid JNK-binding motif (JBD_{20}) of JNK-interacting protein-1/islet-brain 1 (JIP-1/IB1). JIP-1/IB1 and c-Jun share a similar binding motif, but JNK's affinity of binding to JIP-1/IB1 is about 100-fold higher. In addition, we synthesized not only the L-form of the JNK-inhibitory peptide (L-JNKI-1) but also the protease-resistant *all-D-retroinverso* form (D-JNKI-1) to expand its half-life *in vivo*.

Table 1 Regional cerebral blood flow and physiological data[a]

48 h reperfusion	*n*	RCBF (% of baseline) During ischemia	RCBF[b] (% of baseline) After ischemia	Weight (g)	Temp. (°C)
Vehicle	12	10 ± 1 7	6 ± 5	27 ± 2	36.7 ± 0.1
Pretreatment	4	10 ± 4	83 ± 19	26 ± 2	36.5 ± 0.1
Treatment after 3 h	9	14 ± 2	100 ± 16	27 ± 2	36.5 ± 0.1
Treatment after 6 h	4	14 ± 4	75 ± 11	26 ± 1	36.4 ± 0.2
Treatment after 12 h	4	8 ± 2	66 ± 12	29 ± 1	37.0 ± 0.1
14 d reperfusion					
Vehicle	11	13 ± 1	81 ± 7	27 ± 1	36.9 ± 0.1
Treatment after 6 h	9	12 ± 2	81 ± 7	27 ± 1	37.0 ± 0.1

Arterial blood pressures		Before D-JNKI-1[c] (mm Hg)	30 min after D-JNKI-1[c] (mm Hg)
Systolic	3	77 ± 2	76 ± 3
Diastolic	3	70 ± 3	70 ± 4

[a]Data are shown as measurements ± s.d. [b]RCBF, regional cerebral blood flow measured 10 min after end of ischemia. In no case was RCBF statistically different (by *t*-test) from vehicle control. In all except the pretreatment group, these measurements were made before D-JNKI-1 treatment. D-JNKI-1 does not seem to have affected the extent of reperfusion, or RCBF generally, as was confirmed in additional experiments (data not shown) with long-term monitoring (1.5–2.5 h) in pretreated mice and unlesioned controls. [c]Arterial blood pressures measured before, and 30 min after, i.c.v. injection of D-JNKI-1.

The use of D-amino acids seemed particularly crucial for treating neurons, especially because the Tat sequence is essentially made up of pairs of amino acids (six in total) that render it extremely sensitive to the neuronal proteases involved in peptide processing in the nervous system[23,24]. We here show that D-JNKI-1 is an extremely potent neuroprotectant *in vivo* against cerebral ischemia, with a remarkably long therapeutic window and a strong effect both on functional outcome and lesion size.

RESULTS

Effects of the peptides *in vitro* and in cultured neurons

The JNKI peptides used in this study, which have been already described, are aimed at blocking the access of JNK to c-Jun and other substrates by a direct competitive mechanism[20,21]. The inhibitory effect of L-JNKI-1 and D-JNKI-1 on JNK action is shown by their prevention of phosphorylation *in vitro*, by JNK-1α1, of two known JNK targets: c-Jun and Elk-1 (**Fig. 1a**); these decreases in phosphorylation were quantified: 50 μM D-JNKI-1 decreased phosphorylated c-Jun by 95% and phosphorylated Elk-1 by 100%; 10 μM l-JNKI-1 decreased phosphorylated c-Jun by 98% and phosphorylated Elk-1 by 100%. The inhibition can be produced by JBD_{20} alone (L-JNKI-1 without the Tat sequence; **Fig. 1b**). Phosphorylated c-Jun levels with respect to control (100%) were 103% with 0.25 μM JBD20, 10% with 2.5 μM JBD20 and 1% with 25 μM JBD20. Below 50 μM, Tat had no influence on the inhibitory properties of JBD20. JBD_{20} also inhibits other JNK targets including ATF-2, IRS-1, MADD and Bcl-xL, and in all cases the half-maximal inhibitory concentration is about 1 μM (data not shown).

To determine the specificity of the peptides in blocking JNK action, we first characterized the effects of the peptides on the activity of 40 different kinases (10 μM peptides, 10 μM ATP) toward their respective substrates *in vitro* (the complete list of kinases used in this study can be found at http://www.upstate. com/img/pdf/KinaseProfiler.pdf). Apart from an expected effect on the JNKs and the MKK-4 and MKK-7 kinases (whose susceptibility to the JNKI peptides is predictable because they contain JNK-binding domains), the peptides (L- and D-JNKI-1) were completely unable to interfere with the activities of all other kinases. We also showed that concentrations up to 500 μM of the JBD_{20} peptides did not interfere with the activity of six selected kinases, including extracellular signal–related kinase (ERK), p38, protein kinase C, p34, calcium/calmodulin-dependent protein kinase and

protein kinase A (**Fig. 1c**). This level of specificity is far above those achieved with other small chemical inhibitors of the enzyme[25] and shows the extremely high selectivity of the JNKI peptides.

We then analyzed the effects of the peptides on different JNK targets inside neurons. Activation of JNK in NMDA-treated cortical neurons in culture[26] was estimated with kinase assays of pulled-down JNK using glutathione S-transferase (GST)-c-Jun[27]. The increase in JNK activity was maximal (2.2 fold) after 30 min of NMDA treatment (**Fig. 2a**), and resulted in an elevation in the phosphorylation of JNK (**Fig. 2b**) and c-Jun (Fig. 2c). Addition of the cell-penetrating peptides L-JNK-1 or D-JNKI-1 (both 2 μM) completely prevented the increase in phosphorylated c-Jun after 5 h of exposure to 100 μM NMDA, bringing it below even the control level, but did not affect the phosphorylation of JNK (**Fig. 2a,b**) . NMDA-induced transcription of the Fos gene, under the influence of JNK through the Elk-1 transcription factor[28], was also completely prevented by the peptides (**Fig. 2d**). The peptides completely protected neurons against the excitotoxic effects of NMDA (**Fig. 3**) or 100 μM kainate (data not shown), whereas control peptides had no effect. The D-form of the peptides was superior in protecting neurons for extending periods of time (**Fig. 3**).

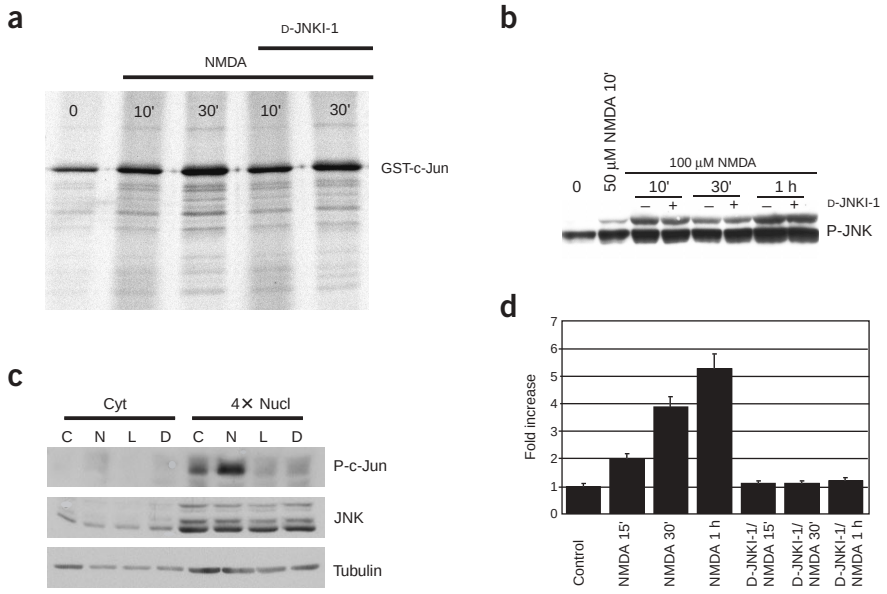

Figure 2 Effects of NMDA on cortical neurons. (**a**) NMDA-induced activation of JNK. JNK activity (incorporation of ^{33}P in GST-c-Jun) is shown in untreated neurons (0) or after 10 min (NMDA, 10') or 30 min (NMDA, 30') of exposure to 100 μM NMDA. Right-hand columns show that activation was essentially unchanged by D-JNKI-1. (**b**) NMDA-induced phosphorylation of JNK was not affected by D-JNKI-1. P, phosphorylated form. (**c**) L- and D-JNKI-1 decrease phosphorylation of c-Jun but not the amount of JNK. Fourfold more protein was loaded from nuclear extracts (Nucl) than from cytoplasmic extracts (Cyt). C, control; N, NMDA; L, L-JNKI-1 + NMDA; D, D-JNKI-1 + NMDA. (**d**) Expression of c-Fos, quantitated by real-time RT-PCR using RNA extracted under the indicated conditions. Data are presented as c-Fos expression relative to actin ($n = 4$). 15' and 30' indicate 15 and 30 min, respectively.

Neuroprotection against focal cerebral ischemia

To test the feasibility of using cell-permeable peptides *in vivo*, we first evaluated their penetration into the brain, showing that FITC-labeled L-JNKI-1 and D-JNKI-1 were both able to cross the blood-brain barrier and penetrate neurons of adult mice and P5 rats within 1 h after an intraperitoneal injection[29] (data not shown).

In a model of mild ischemia in mice, we occluded the left middle cerebral artery for 30 min, followed by 48 h of reperfusion (**Fig. 4**).

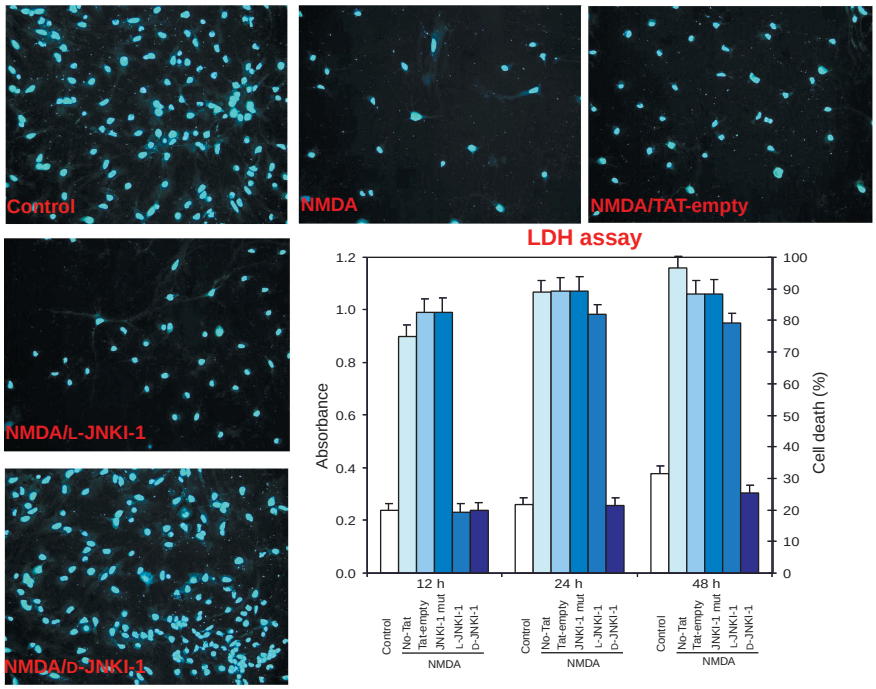

Figure 3 Time course of NMDA neurotoxicity and neuroprotection by L-JNKI-1, D-JNKI-1 and two control peptides, 'Tat-empty' (Tat sequence without JBD_{20}) and 'L-JNKI-1 mut' (L-JNKI-1 with six amino acids mutated to alanine[20]). Micrographs show Hoechst-stained neurons 24 h after treatment. At 24 h after treatment, D-JNKI-1 still gave total protection. Control and NMDA/D-JNKI-1–treated cultures were comparable, although the L-form of JNKI-1 no longer protected the neurons, presumably because of the degradation of the L-peptides already described. Tat-empty peptide did not affect cell death under any conditions. Histogram shows neuronal death at 12, 24 and 48 h after exposure to 100 μM NMDA, as indicated by LDH activity in medium of same Petri dish. Absorbance values (representing LDH concentration) were converted into percentage of neuronal death values by dividing by average absorbance for total LDH (from medium plus lysed neurons). At 12 h, both L-JNKI-1 and D-JNKI-1 peptides inhibited neuronal death, whereas Tat-empty peptide had no effect. At 24 h and 48 h, only D-JNKI-1 still protected; L-JNKI-1 did not prevent neuronal death.

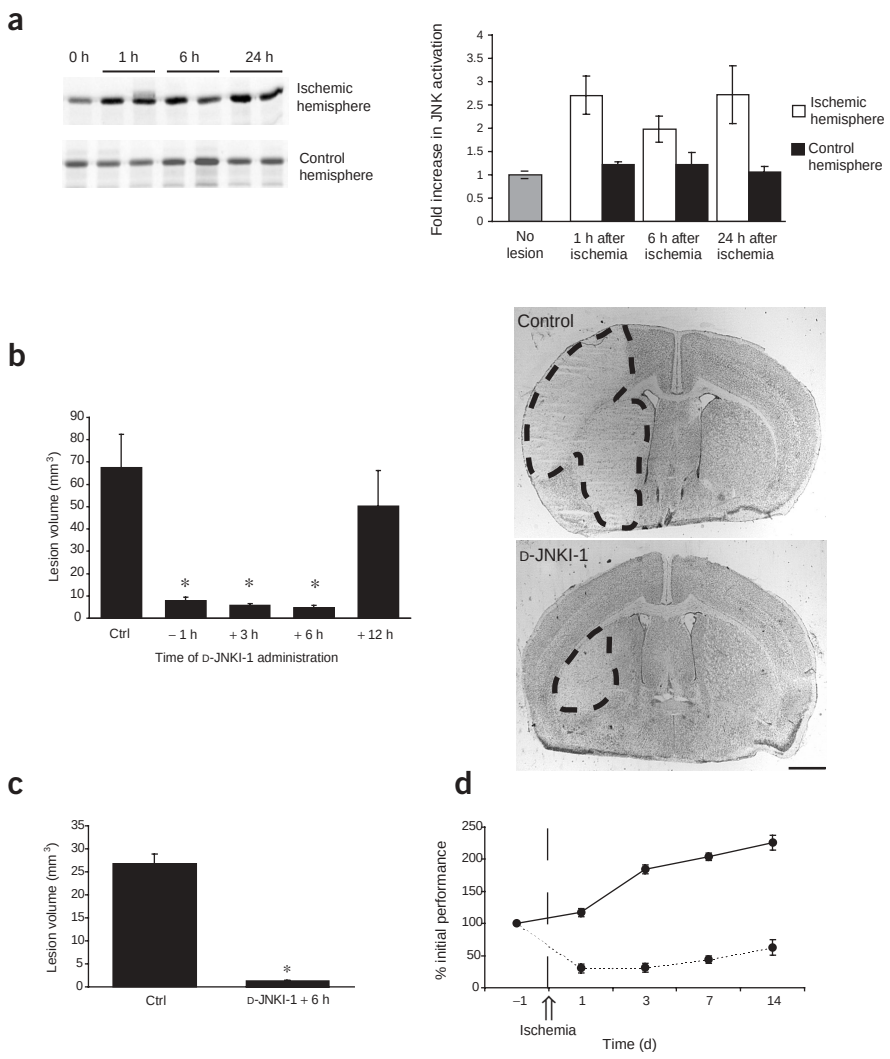

Figure 4 Transient ischemia in mice. (**a**) JNK assay showing increased JNK activation between 1 and 24 h after ischemia. "No lesion" denotes animals not exposed to ischemia. (**b**) Infarction volumes 48 h after i.c.v. injection of 15.7 ng D-JNKI-1, at different times before (– 1 h) or after (+ 3, 6 or 12 h) MCAO. *, $P = 0.0006$ for – 1 h, $P < 0.0001$ for 3 h and $P = 0.0001$ for 6 h, as compared with control. One outlying value (>2 s.d. above mean) was excluded from 3 h group. Cresyl violet–stained sections show typical examples of infarct. Scale bar, 1 mm. Ctrl, vehicle control. $n = 12$ for control, 4 for '– 1 h', 8 for '+ 3 h', 4 for '+ 6 h' and 4 for '+ 12 h'. (**c**) Infarction volume 14 d after MCAO, in animals that were given vehicle (Ctrl; $n = 11$) or 15.7 ng D-JNKI-1 (D-JNKI-1 + 6 h; $n = 9$) i.c.v. 6 h after MCAO. *, $P < 0.001$ compared with control. (**d**) Rotarod evaluation of the two groups of mice in **c**, showing significant behavioral sparing ($P = 0.004$). Upper curve, D-JNKI-1; lower curve, untreated. Error bars represent s.e.m.

Achievement of ischemia, followed by reperfusion, was confirmed in all animals by monitoring regional cerebral blood flow in the area of the left middle cerebral artery (**Table 1**). In the vehicle-treated group, this resulted systematically in a major infarction, containing severely pyknotic cells, predominantly in the cortex, striatum and, in seven of the brains, the hippocampus. The mean volume of the infarction was 67.4 mm^3 ($n = 12$). There was also a twofold increase in JNK activation that was maintained for at least 24 h (**Fig. 4a**). We therefore evaluated the efficacy and therapeutic window of treatment with intracerebroventricular (i.c.v.) injection of D-JNKI-1 (15.7 ng in 2 µl PBS). Pretreatment 1 h before MCAO substantially decreased the infarct volume measured 48 h after reperfusion by 88%, to 7.8 mm^3 ($P = 0.0006$; **Fig. 4a,b** and **Supplementary Fig. 1** online). Administration of the peptide 3 or 6 h after MCAO was still potently protective, reducing the mean infarct volumes to 5.8 mm^3 and 4.8 mm^3, respectively, diminutions of 91% ($P < 0.0001$) and 93% ($P = 0.0001$) compared with untreated animals. In contrast, peptide injection at 12 h was not significantly protective. To assess whether D-JNKI-1 really prevented cell death or only delayed it, we extended our observations to the longer survival time of 14 d after ischemia, with D-JNKI-1 administration at 6 h. Even in untreated mice the lesion shrank more than 50% over this period, but D-JNKI-1 also strongly decreased infarct volume by 93%, just as with the shorter (48 h) survival time (**Fig. 4c**).

We then generated an ischemic zone in the cerebral cortex of young rats (P14) by subjecting them to permanent distal MCAO, thereby inducing a zone of massive degeneration restricted to the parietotemporal cortex. Because the brain volumes of these rats were somewhat variable, we expressed the lesions as a percentage of the volume of the cerebral hemisphere. D-JNKI-1 (11 mg/kg intraperitoneally, or ~340 µg) was given 30 min before, or 6 or 12 h after, the arterial occlusion, and the rats were fixed at 24 h after occlusion. At all three time points of administration, D-JNKI-1 caused major and statistically significant decreases of 68% ($P = 0.0006$), 78% ($P = 0.0004$) and 49% ($P = 0.02$), respectively, in the infarct volume as compared with control animals (**Fig. 5a–c**). In rats fixed 7 d after occlusion, the infarct had shrunken considerably in both D-JNKI-1–injected and control groups, but there was still significant ($P = 0.02$) protection by D-JNKI-1 (**Fig. 5d,e**).

We used immunohistochemistry to analyze the activation of the c-Jun transcription factor, a major target of JNK, in the brains of rat pups with permanent ischemia. Phosphorylated c-Jun was evident in many neurons in the peri-infarcted cortex up to about 2 mm from the lesion (**Fig. 5f**), although not in the infarct itself. In contrast, in the D-JNKI-1–treated brains, the peri-infarcted cortex was negative and there were only a few detectable positive neurons at the very border of the infarct.

Behavioral evaluation after transient ischemia in mice

We investigated the behavioral effects of transient MCAO in mice treated with D-JNKI-1 6 h after ischemia and in untreated mice, using the rotarod test[30] before MCAO and at 1, 3, 7 and 14 d afterwards. There was no difference between the two groups before ischemia, but at all subsequent time points the D-JNKI-1–treated mice performed better (overall effect, $P = 0.004$ by repeated-measures ANOVA). The untreated mice suffered a major reduction in locomotor performance

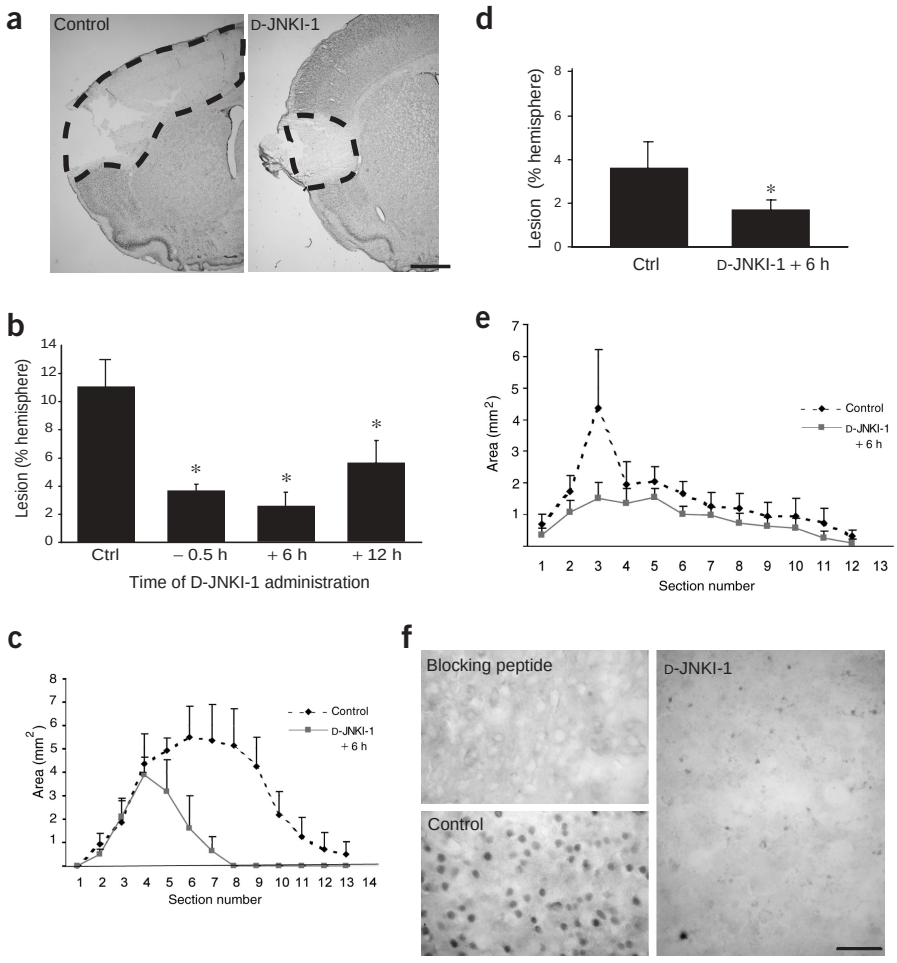

Figure 5 Protection by D-JNKI-1 against permanent focal ischemia in P14 rats. Rats were perfused 24 h (**a**,**b**,**c**,**f**) or 7 d (**d**,**e**) after MCAO. (**a**) Cresyl violet-stained sections showing examples of lesions from a control rat and from one treated with D-JNKI-1, 6 h after occlusion. Dorsal side is up. Scale bar, 1 mm. (**b**) Infarct volumes (expressed as percentage of hemispheric volume) after intraperitoneal injections of D-JNKI-1 at different times before (– 0.5 h) or after (+ 6 h; + 12 h) occlusion. $n = 7$ for control (Ctrl), 6 for '– 0.5 h', 5 for '+ 6 h' and 5 for '+ 12 h'. *, $P = 0.0006$ for '– 0.5 h', $P = 0.0004$ for '+ 6 h' and $P = 0.02$ for '+ 12 h', as compared with control. (**c**) Areas of individual sections in a '+ 6 h' rat and in a control, both fixed at 24 h. (**d**) Infarct volumes 7 d after MCAO in untreated (Ctrl) and D-JNKI-1–treated rats. $n = 5$ for control and 8 for D-JNKI-1–treated. *, $P = 0.02$. (**e**) Areas of individual sections in a '+ 6 h' rat and a control, both fixed at 7 d. (**f**) Immunohistochemistry for phosphorylated c-Jun (using antibody against Ser63 phosphorylation site of c-Jun) in ischemic region of cortex of control (untreated) rats, with or without a blocking peptide for the Ser63 site, and of rats treated with D-JNKI-1 at – 0.5 h. Scale bar, 100 μm.

1 d after ischemia and then improved only slowly (**Fig. 4d**). D-JNKI-1–treated mice improved substantially over the 14-d period ($P = 0.008$ by linear regression test), implying that D-JNKI-1 did not prevent motor learning. We also showed that motor coordination was unimpaired with an i.c.v. dose of D-JNKI-1 ten times above the standard dose (**Supplementary Table 1** online)

DISCUSSION

We have established here that a cell-penetrating peptide selectively blocks the interaction between JNK and its substrate(s) and is effective *in vivo*, potently decreasing brain lesions in both transient and permanent ischemia. Most notably, the level of protection is still very high when D-JNKI-1 is administered as late as 6 or 12 h after ischemia in our animal models. Extrapolating to human stroke, such a window would be sufficient for many patients to be treated in time to prevent cell death[7]. Although 30–50% neuroprotection has previously been reported with various compounds administered up to 24 h after ischemia[6,31], the protection at 6 h after ischemia in both of our models is by far the strongest ever reported at this time point. In the transient ischemia model, late administration (at 6 h) also reduced the behavioral consequences of the lesion for at least 14 d.

In contrast to many neuroprotective studies that intervene at the level of glutamate receptors or events immediately downstream of those receptors, such as free radical generation, our JNKI-1 peptides act further downstream. It seems certain that the pathways responsible for the neuroprotection by L- and D-JNKI-1 do not depend on feedback onto glutamate receptors, because the peptides protect against excitotoxicity mediated by two different receptors (NMDA and kainate) and do not affect the activation or phosphorylation of JNK, but only its action on its targets. The exact mechanism by which late (after 6 or 12 h) administration of D-JNKI-1 protects despite early activation of JNK is unknown, but our data indicate that continuous JNK activation for at least 6 h is required for efficient cell death in cerebral ischemia. In addition, the reduction in infarct volume obtained with D-JNKI-1 is larger than that reported with inhibition of glutamate receptors[32], which are upstream of JNK and whose activation is the major factor responsible for ischemic damage. One possible explanation for our higher level of protection is that our peptides might inhibit additional ischemia-related stress signals that are independent of glutamate receptors, such as calcium entry through other kinds of calcium channels or loss of trophic factors. Alternatively, the lesser protection by glutamate antagonists might be a result of their inhibition of survival pathways not affected by the peptides[33]. Nor can we completely rule out indirect effects mediated by the peptides such as local hypothermia, or diminished death signals from non-neuronal cells.

Despite many reports of compounds showing significant neuroprotection in experimental models of stroke, no major clinical trial of a neuroprotectant has shown improved outcome. Many trials, such as those of the NMDA receptor antagonists Selfotel (CGS19755) and Aptiganel (Cerestat), have been halted because of poor risk-benefit ratios, and those that completed phase 3 (such as the GABAergic facilitator Zendra (Clomethiazole) and the ICAM-1-specific monoclonal antibody Enlimomab) did not show significant improvement of primary outcome measures[7,30]. The lack of efficacy of a number of neuroprotectants in clinical trials may be a consequence of their poor therapeutic ratios, calculated as the ratio between the minimum effective doses for significant impairment in rotarod performance and for significant neuroprotection[30]. In view of the high therapeutic ratio

(much greater than 10 in this study) and long therapeutic window of D-JNKI-1, intervention in the JNK pathway seems to be a plausible approach for the development of stroke therapy. Although caution is required in view of JNK's involvement in metabolic regulation[34] and neuronal plasticity and regeneration[35], inhibition of only some of JNK's actions (as with D-JNKI-1) for only a few days (as would be required for stroke therapy) may turn out to be feasible. It is encouraging that mice continued to improve their rotarod performance under the influence of D-JNKI-1 while showing no negative effects.

Finally, the therapeutic potential of JNK-inhibitory treatment may extend to other pathologies with an excitotoxic component. These include all hypoxic, ischemic and traumatic brain damage[12], and neuronal death arising from epileptic seizures[36]. In addition, an excitotoxic component has been suggested for several neurodegenerative diseases[37,38].

METHODS

Cortical neuronal culture. We dissected small pieces of cortex from the brains of 2-d-old rat pups, incubated them with 200 units of papain for 30 min at 34 °C and plated the neurons at densities of approximately 1×10^6 cells/plate on dishes precoated with 100 μg/ml poly-D-lysine. The culture medium was B27/Neurobasal (Life Technologies) supplemented with 0.5 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Lactate dehydrogenase (LDH) cytotoxicity assay. We measured LDH released into the culture medium with the Cytotox 96 nonradioactive cytotoxicity assay kit (Promega).

GST-c-Jun pull-down kinase assay. We prepared extracts from neuronal cultures by scraping cells in lysis buffer[20]. Cerebral hemispheres were homogenized, after removal of the frontal and occipital poles, in a Dounce homogenizer in lysis buffer. Samples (25 μg) were incubated for 1 h at room temperature with 1 μg GST-c-Jun (amino acids 1–89) and 10 μl of glutathione-agarose beads (Sigma). After four washes in lysis buffer, we resuspended the beads in the same buffer and did kinase assays as described below.

Kinase assays. We performed *in vitro* kinase assays using recombinant JNK-1α1 (0.5 μg; Upstate Biotechnology) and 0.5 μg of substrates (GST-fusion proteins, casein or histone; Sigma; see **Fig. 1** and ref. 20).

Separation of nuclei from cytoplasm. To isolate nuclei for western blot analyses (**Fig. 2b**), we lysed neurons for 15 min in lysis buffer[20], centrifuged the samples at 300 *g* for 10 min at 4 °C, reconstituted the nuclear pellets in lysis buffer and sonicated them.

Real-time RT-PCR. We did real-time RT-PCR using specific primers on a LightCycler apparatus (Roche). We used the housekeeping actin transcript to normalize the amount and quality of the RNA that were extracted by the Chomczynski method[39]. We used the following primers: c-Fos forward, 5′-GCTGACAGATACACTCCAAG-3′; c-Fos reverse, 5′-CCTAGATGATGCCGGAAACA-3′; actin forward, 5′-AACGGCTCCGGCATGTGCAA-3′; actin reverse, 5′-ATTGTAGAAGGTGTGGTGCCA-3′.

Transient ischemia in adult mice. Using male ICR-CD1 mice (~6 weeks old; 18–37 g; Harlan), we provoked ischemia by introducing a filament from the common carotid artery into the internal carotid and advancing it into the arterial circle, thereby occluding the middle cerebral artery[3,40]. We measured regional cerebral blood flow by laser Doppler flowmetry, with a probe fixed on the skull throughout the ischemia until 10 min after reperfusion. Rectal temperature was measured and maintained at 37 °C. Vehicle (PBS; 2 μl) or D-JNKI-1 solution (containing 15.7 ng D-JNKI-1; JNKI-1 peptides available from Alexis) were injected free-handedly into the lateral ventricle, using a 10-μl Hamilton syringe, 0.9 mm laterally, 0.1 mm posteriorly and 3.1 mm deep relative to the bregma[41]. The mice were killed 48 h or 14 d after reperfusion. Serial cryostat sections 20 μm thick were traced using a computer-microscope system equipped with the Neurolucida program (MicroBrightField) and the volumes of the ischemic area and of the whole brain were calculated (blinded) with the Neuroexplorer program. We measured systolic and diastolic blood pressure with an arterial catheter in three additional mice from 10 min before D-JNKI-1 injection until 30 min afterwards (**Table 1**). We respected the guidelines of the Swiss Federal Veterinary Office in all experiments (1324-1).

Permanent focal ischemia in P14 rats. MCAO was induced by electrocoagulating a main branch of the middle cerebral artery close to its origin at the junction with the olfactory branch. Wistar rats (Harlan), weighing 27–35 g, were killed 24 h later with an overdose of chloral hydrate and were perfused through the left ventricle with Zamboni fixative. Their brains were postfixed for 2 h in the same solution and infiltrated overnight in 30% sucrose for cryoprotection. The areas of the ischemic lesion and of the whole brain were traced from 50-μm serial cryostat sections stained with cresyl violet using the Neurolucida program and the volumes were calculated by the Neuroexplorer program. All experimental procedures on live rats were done according to the guidelines for care and use of laboratory animals as published by the Italian Ministry of Health (DDL 116/92).

Assessment of motor performance by the rotarod method. The mice were trained twice a day for 5 d and on the morning of the experimental day in order to reduce the variability between subjects. Both training and test sessions were identical for control and injected mice. We examined the motor function immediately before the injection (**Table 2**) and 1, 3, 6 and 14 d later. The mice were placed on the rotarod, set to accelerate uniformly from 4 to 40 r.p.m. over 5 min, and their latency to falls was recorded.

Statistics. Data from both models of ischemia were transformed logarithmically when necessary to satisfy the Gaussian criterion, and analyzed with overall ANOVA ($P < 0.0001$ for both models; **Figs. 4b** and **5b**), followed by one-tailed unpaired *t*-tests.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details.

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