Constraint-induced movement therapy promotes motor function recovery and downregulates phosphorylated extracellular regulated protein kinase expression in ischemic brain tissue of rats

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Abstract

Motor function impairment is a common outcome of stroke. Constraint-induced movement therapy (CIMT) involving intensive use of the impaired limb while restraining the unaffected limb is widely used to overcome the effects of 'learned non-use' and improve limb function after stroke. However, the underlying mechanism of CIMT remains unclear. In the present study, rats were randomly divided into a middle cerebral artery occlusion (model) group, a CIMT + model (CIMT) group, or a sham group. Restriction of the affected limb by plaster cast was performed in the CIMT and sham groups. Compared with the model group, CIMT significantly improved the forelimb functional performance in rats. By western blot assay, the expression of phosphorylated extracellular regulated protein kinase in the bilateral cortex and hippocampi of cerebral ischemic rats in the CIMT group was significantly lower than that in the model group, and was similar to sham group levels. These data suggest that functional recovery after CIMT may be related to decreased expression of phosphorylated extracellular regulated protein kinase in the bilateral cortex and hippocampi.

Key Words: nerve regeneration; constraint-induced movement therapy; mitogen-activated protein kinase signaling system; brain ischemia; locomotion; recovery; cortex; hippocampus; middle cerebral artery occlusion; foot fault test; balance beam walking; rats; NSFC grants; neural regeneration

Introduction

Stroke is a leading cause of disability in the United States, with disability occurring in 75% of stroke survivors (Coffey et al., 2000; Towfighi et al., 2011). Motor function impairment is the most common outcome of stroke. 'Learned non-use' was first reported in post-stroke patients by Taub and colleagues (Sterr et al., 2002). In this disorder, the patient prefers to use the unaffected limb in daily life owing to repeated failure when using the paralyzed limb, resulting in further dysfunction of the paralyzed limb. Constraint-induced movement therapy (CIMT) has been developed to readjust and overcome this disorder. This forced active-movement therapy is performed on the affected limbs, and involves intensive use of the affected limbs while restraining the unaffected limbs (Taub et al., 2002). CIMT has been reported to decrease motor deficits and enhance fine movement of the affected limb in hemiplegic stroke patients (Treger et al., 2012; Taub et al., 2013).

The underlying mechanism of learned non-use and CIMT remains unclear (Janssen et al., 2013). There is some evidence that behavioral improvements after CIMT in cerebral ischemic rats may be associated with increasing stromal cell-derived factor-1 in the cortex and dentate gyrus (Zhao et al., 2009). These data suggest that the cortex and hippocampus are potential target areas for functional recovery after CIMT. The effects of CIMT were also suggested to involve a reduction in intrinsic growth-inhibitory signaling pathways activated in the peri-infarct cortex, including molecules such as Nogo-A/NgR and RhoA/ROCK (Zhao et al., 2013a, b). Further, CIMT can induce neurogenesis in the rat following cerebral ischemia, and increase growth associated protein-43, synaptophysin, vGlut1, and postsynaptic density-95 (Zhao et al., 2013a, b).

The extracellular regulated protein kinase (ERK) pathway plays a key role in cerebral ischemia (Zhao et al., 2014).

ERK belongs to a subfamily of mitogen-activated protein kinases that mediate a wide range of physiological and pathological activities in cells (Sawe et al., 2008). Inhibition of ERK phosphorylation (p-ERK) has been reported to reduce infarct volume and protect neural cells following ischemia (Zhang et al., 2010a). Further, p-ERK can activate the inflammation response and oxidative stress in the acute periods after ischemia (Sawe et al., 2008). By contrast, p-ERK was reported to be involved in neuroprotection following treatments with estrogen, preconditioning, or hypothermia after cerebral ischemia (Choi et al., 2006). Thus, the relationship between inhibitory and excitatory factors and the ERK pathway remains controversial. Further, few studies have examined the long-term role of the ERK pathway after ischemia and chronic treatment interventions.

In the present study, we investigated the motor functions of the affected forelimb and protein levels of total-ERK and p-ERK after 14 days of continuous CIMT in cerebral ischemic rats. The side effects of limb restraint and changes in body weight after CIMT were also examined.

Materials and Methods

Animals
Thirty male Sprague-Dawley rats (weighing 230–280 g, aged 3 months) were obtained from Shanghai SIPPR-BK LAB Animal Ltd., Shanghai, China (license No. SCXK (Hu) 2008-0016). The rats were kept under a 12-hour light/dark cycle with food and water available *ad libitum*. All experimental procedures were in accordance with and approved by the Institutional Animal Care and Use Committee of Fudan University (Shanghai, China). All procedures in this study were in accordance with ethical standards of the Department of Laboratory Animal Science, Fudan University (Approval No. 20140143C001).

Animal grouping
Using a random number list, the rats were randomly assigned to CIMT group ($n = 10$), model group ($n = 10$), and sham group ($n = 10$). Five rats in each group were used for measurement of the infarct areas. The remaining five rats were used for western blot assay to measure the expression of ERK.

Middle cerebral artery occlusion surgery
Rats in the CIMT and model groups underwent middle cerebral artery occlusion. Rats were anesthetized with 10% chloral hydrate (0.36 mL/100 g, intraperitoneal). Surgical procedures for middle cerebral artery occlusion and reperfusion were performed as previously reported (Zhang et al., 2010). Briefly, rats in the model and CIMT groups had their left middle cerebral artery occluded using a filament with a poly-L-lysine coated blunted tip. To occlude the middle cerebral artery at its origin, the length of the filament inserted through the internal carotid artery was approximately 20 mm. After a 1.5-hour occlusion, the filament was withdrawn and reperfusion was established. The body temperature of the rats was maintained at 37°C with a heating pad. Rats in

the sham group underwent the middle cerebral artery occlusion procedures without insertion of the filament and occlusion of the middle cerebral artery.

Neurological defect score
The neurological defect score is a six-grade scale, with 0 points indicating normal, 1 point indicating minor defects (left forelimb flexion while suspended with tail), 2 points indicating moderate defects (turning left side while walking), 3 points indicating moderate defects (lean on left side of the body), 4 points indicating unable to walk and diminished consciousness, and 5 points indicating death related to cerebral ischemia. Neurological impairment of the rats was evaluated at 24 hours and 22 days after surgery, as previously described (Zhang et al., 2012).

CIMT protocol
Rats in the CIMT and sham groups underwent restriction of the unaffected limbs by a plaster cast lined with smooth cotton at 7 days after surgery, for 14 consecutive days. The rats were forced to use the right (affected) forelimb in their daily lives. A task-oriented exercise was adopted. The rats were trained to walk through a horizontal ladder by grasping the rods with their affected limbs, three times per day for 14 consecutive days (**Figure 1**). After 14 days of restricted exercise, the rats were released from the plaster casts for 1 day before final assessments to avoid interference of the restrained limb on the assessment results. Gentle message and a passive range of motion were performed on the restrained limbs to improve recovery from the restriction.

Functional assessment
The balance beam walking test was used to evaluate gross motor function and balance ability. The apparatus was a 2.5-cm-wide beam, 150 cm in length, and elevated 55 cm above a sawdust cushion. A box was placed at the end of the beam for rats to rest between tests. Before the first test, rats were trained to walk on the beam from one end to the other, and then stayed in the box after walking. A 5-point scale was adopted in the test as follows: 0, the rat was able to balance and walk on the beam using its forelimbs symmetrically; 1, the rat was able to balance and walk on the beam using its unaffected limb preferentially; 2, the rat was able to balance and walk on the beam mostly relying on the unaffected limb; 3, the rat was not able to balance on the beam once moved; 4, the rat fell off the beam immediately. The test was repeated three times for each rat by a rater blinded to the group division. The average scores were calculated for statistical analysis. The balance beam walking test was performed before surgery, and at 7 and 22 days after surgery.

The foot-fault test, an objective indicator, was used to evaluate fine motor function of the affected limb. A horizontal ladder with 34 rods (2-cm interval between adjacent rods) was used as previously described (**Figure 1**) (Zhang et al., 2012). A box was placed at the end of the ladder for rats to rest between tests. All animals were trained on this system before the first test. If the forelimb of the affected

side fell off or slipped through the rod, this was counted as one fault footstep. The foot fault rate of the affected limb was obtained by the ratio of the number of fault footsteps to the total number of steps. The test was repeated three times for each rat by a rater blinded to the group division and was performed before surgery, and at 7 and 22 days after surgery.

Measurement of infarct areas

Infarct area was measured and calculated as previously described (Zhang et al., 2012). Five rats in each group were sacrificed under anesthesia at 22 days after surgery. The brain was removed and frozen at −20°C for 20 minutes. Six slices of coronal brain sections were made with an interval of 2 mm between seven razor blades. The brain sections were incubated in 2% 2,3,5-triphenyltetrazolium chloride solution for 30 minutes at 37°C, and then fixed in 4% paraformaldehyde solution. The infarct volumes and proportion were calculated using Photoshop CS4 11.0 software (Adobe Systems Inc., San Jose, CA, USA). The following formula were used to calculate the infarct proportion of the brain:

Infarct area (each slice, square pixel) = area of the contralateral hemisphere – area of the infarct side.

Infarct proportion (%) = $\sum$(infarct area/area of the contralateral hemisphere × 100%)/6.

Measurement of ERK expression

At 22 days after the surgery, the lesional cortex, contralateral cortex, and bilateral hippocampi were rapidly dissected under direct vision, collected with sterilized instruments and tubes, and stored at −80°C until use. Western blot analysis was performed as previously described (Zhang et al., 2012). Protein was extracted using protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA), and protein concentration was determined with the bicinchoninic acid assay. Protein extract and sample buffer were mixed with equal volumes and boiled in hot water (95°C) for 5 minutes before loading on 10% polyacrylamide gels. The proteins were then transferred onto a Hybond nylon membrane (Amersham, Piscataway, NJ, USA) at 110 V for 1.5 hours with a cold pack. After incubating with 5% solution of non-fat dry milk in TBST, membranes were washed and incubated overnight with rabbit anti-ERK monoclonal antibody (1:3,000; Cell Signaling Technology, Danvers, MA, USA) or rabbit anti-p-ERK monoclonal antibody (1:3,000; Cell Signaling Technology). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Membranes were then incubated in horseradish peroxidase-conjugated secondary antibody (1:5,000; goat anti-rabbit IgG horseradish peroxidase; Promega, Madison, WI, USA) for 1 hour at room temperature. Membranes were developed with an enhanced ECL kit (Millipore Corporation, Billerica, MA, USA) using a ChemicDoc™ system (Bio-Rad Laboratories, Inc., CA, Hercules, USA). Densitometry was performed using Quantity One® 1-D Analysis Software (Bio-Rad Laboratories, Inc.). The results were obtained by calculating the ratio of the grey value of the bands between ERK and GAPDH or between p-ERK and GAPDH.

Statistical analysis

Data are presented as the mean ± SD. Intergroup comparison was performed by one-way analysis of variance followed by the least significant difference *post hoc* test sing SPSS 20.0 software (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered statistically significant.

Results

Effect of CIMT on the adverse effects and body weight of cerebral ischemic rats

In the CIMT and sham groups, there were no deaths after CIMT. However, side effects included joint stiffness (3/10), mainly in the wrist and elbow, and local swelling in the constraint forelimb (7/10). There was no significant difference in the average body weights of cerebral ischemic rats between the groups before surgery ($P > 0.05$). Compared with the sham group, the body weights in the CIMT and model groups decreased significantly at 7 days after surgery. At 14 days of CIMT exercise, the body weights in both the CIMT group and the sham group also reduced. However, at 22 days after surgery there were no differences in body weight between the three groups. The growth rate from day 7 to day 22 was 2.9 g per day for the CIMT group, 0.8 g per day for the sham group, and 7.2 g per day for the model group (**Figure 2**).

Effect of CIMT on functional recovery of cerebral ischemic rats

After surgery, the neurological defect score increased significantly in the CIMT group and the model group at day 1 after surgery when compared with the sham group (**Table 1**). However, at day 22 after surgery the neurological defect scores in the CIMT group and model group decreased to levels similar to the sham group ($P > 0.05$).

At 7 days after surgery the motor function of the rats on the beam was impaired in both the CIMT group and the model group (scores were increased). However, at 22 days after surgery, the score in the CIMT group was significantly decreased compared with the model group ($P < 0.05$), with levels similar to the sham group, while the score in the model group was significantly higher than that in the sham group and the CIMT group (**Table 2**). Before surgery, a baseline foot fault rate of 10.34 ± 0.93% was observed in normal rats. At 7 days post-surgery, the foot fault rates in the CIMT group (30.45 ± 5.98%) and the model group (35.28 ± 1.63%) were significantly higher than that in the sham group (10.58 ± 8.00%, $P < 0.05$, respectively). By contrast, at 22 days of recovery the fall fault rate in the CIMT group (10.28 ± 6.72%) was similar to the sham group (6.53 ± 6.54%, $P > 0.05$), while that in the model group (22.59 ± 5.48%) remained significantly higher than that in both the sham group and the CIMT group ($P < 0.05$ for both; **Table 2**).

Effect of CIMT on infarct areas of cerebral ischemic rats

At 22 days after surgery, compared with the sham group (1.37 ± 5.60%), the CIMT group and model group had signifi-

cantly higher infarct areas (18.22 ± 8.29%, 17.68 ± 6.45%; *P* = 0.000), while there were no differences between the CIMT group and the model group. The TTC staining results are shown in **Figure 3**.

Effect of CIMT on ERK expression in the bilateral cortex and hippocampi of cerebral ischemic rats

The expression levels of total ERK and p-ERK in the cortex are shown in **Figure 4**. There were no significant differences in total ERK expression between the groups in the cortex at 22 days after surgery (**Figure 4A**). However, p-ERK expression in the model group was significantly higher than that in the CIMT group and sham group in the bilateral cortex (**Figure 4B**). There were no differences in total ERK or p-ERK between the CIMT group and the sham group at 22 days.

The expression levels of total ERK and p-ERK in the hippocampus are shown in **Figure 4**. There were no significant differences in total ERK expression between the groups in the bilateral hippocampi at 22 days after surgery (**Figure 4C**). However, p-ERK expression in the bilateral hippocampi in model group was significantly higher than that in the CIMT group and sham group (**Figure 4D**). There was no difference in total ERK or p-ERK between the CIMT group and the sham group at 22 days.

Discussion

Jones and Schallert (1994) first reported that restriction of the forelimb ipsilateral to the lesion could interfere with normal neural regeneration after ischemia, while animals permitted use of both forelimbs or only the forelimb ipsilateral to the lesion showed the expected increase in neural regeneration. Nevertheless, numerous studies have suggested that existing motor disabilities can be improved by CIMT on affected limbs after cerebral ischemia both clinically and experimentally (Zhao et al., 2009; Hidaka et al., 2012; Lang et al., 2013). Similarly, in the present study, we found that CIMT could improve motor function in rats following cerebral ischemia. We performed immobilization of the intact limb at 1 week after surgery. Restriction of the intact limb at a very early stage after cerebral ischemia can impede recovery, or even lead to death.

Intensive voluntary exercise on affected limbs is a very effective method for facilitating motor function after cerebral ischemia (Ke et al., 2011). In the present study, we used a task-oriented CIMT procedure, where rats in the CIMT group were forced to use their affected limbs in daily living, and were also trained to grasp by walking through a horizontal ladder. We found that the grasp skills of the affected limbs in this task were greatly improved. Thus, in clinical practice, training patients to accomplish task-related activities with their affected upper extremities may be useful for acquisition of practical skills during daily life.

In the present study, we found no correlation between functional recovery and infarct volume. In support, Joo et al. (2012) reported no difference in lesion volumes between CIMT and model groups, despite obvious functional improvements. CIMT has been reported to induce brain plasticity, thus promoting functional recovery (Mark et al., 2006; Joo et al., 2012). The motor areas in the cerebral cortex send projections to other brain areas to form neuronal networks, and the bilateral cerebral hemispheres are connected by the corpus callosum. Further, the hippocampus plays a key role in motor learning and memory. These anatomical structures provide huge functional recovery and compensatory potential for the brain. CIMT provides intensive, forced exercise that induces efferent and afferent stimulation to the central nervous system and extremities. Thus, marked structural changes in the brain may occur under adequate behavioral demand (Jones and Schallert, 1994).

The molecular mechanisms involved in functional recovery after CIMT may relate to ERK signaling. We found that total-ERK was maintained at similar level between the three groups, as previously reported (Zhang et al., 2010), while p-ERK expression was increased in the bilateral cortex and hippocampi, at 3 weeks after middle cerebral artery occlusion in the model group. Nevertheless, functional recovery was not completely inhibited by the elevated p-ERK in the model group. After CIMT, p-ERK expression was similar to sham group levels. Elevated p-ERK was previously reported to exert both beneficial and detrimental effects after cerebral ischemia (Sawe et al., 2008). Further, increased p-ERK can contribute to oxidative stress and inflammatory responses, but promote expression of neuroprotective molecules (Noshita et al., 2002; Li et al., 2007). According to our findings, CIMT may initiate beneficial ERK activity, but inhibit damaging ERK activity, although further studies are required to fully elucidate the molecular mechanisms. In addition, the long-term changes in ERK expression are not well established, with the majority of studies examining ERK/p-ERK expression within 24 hours after cerebral ischemia (Zhang et al., 2010).

We also found that CIMT suppressed the growth rate of rats, which may be related to extra weight bearing from the plaster cast and increased activity. The effects of these weight changes on the animals remain to be determined. Nevertheless, functional recovery of the rats in the CIMT group was not affected by the decreased growth rate. There were also some side effects associated with long-term CIMT, including swelling in the restraint limbs and joint stiffness, which may influence behavioral performance. To perform chronic CIMT, we restrained the unaffected limb for 14 consecutive days. Factors involved in addition of the plaster cast, including cast tightness, may have also contributed to these side effects. Nevertheless, although 30% of rats presented with joint stiffness, the behavioral evaluations were not greatly affected as they were largely focused on functions of the paralyzed limb and overall motor function rather than the unaffected side alone. In the future, the quality of the cast should be improved by using softer but restriction-proof materials to minimize stress and side effects in animals (Janssen et al., 2013).

In conclusion, functional recovery after CIMT may be related to decreased p-ERK expression in the bilateral cortex and hippocampi.
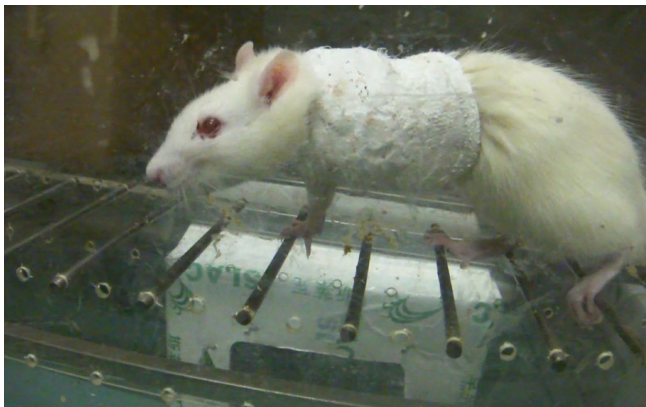
Figure 1 A rat with plaster cast on the horizontal ladder.
The rat walked across the horizontal ladder using its affected limb.
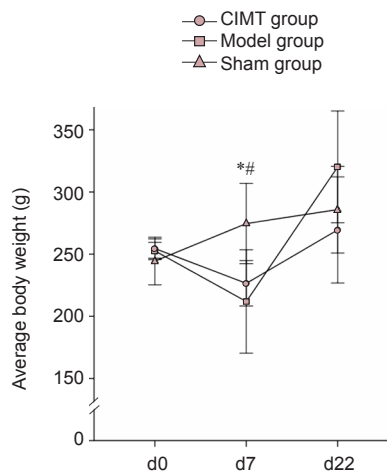
Figure 2 Effect of constraint-induced movement therapy (CIMT) on body weight of cerebral ischemic rats.
There were five rats per group per time point (one-way analysis of variance and the least significant difference *post hoc* test). The body weight of the rats decreased significantly in the CIMT group and model group at 7 days after surgery (d7, #$P = 0.046$, *vs.* CIMT group; *$P = 0.038$, *vs.* model group). However, no significant difference was found among the three groups at 22 days after surgery (d22). $n = 5$ per group per time point (one-way analysis of variance and the least significant difference *post hoc* test). d: Day(s).

Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*

**Table 1 Effect of CIMT on neurological defect scores of rats with cerebral ischemia**

Group	d1	d22
CIMT	2.40±0.89*	0.40±0.55
Model	2.20±0.45*	0.80±0.84
Sham	0.00±0.00	0.00±0.00

Data are expressed as the mean ± SD ($n = 5$ per group per time point; one-way analysis of variance and the least significant difference *post hoc* test).*$P < 0.05$, *vs.* sham group. d: Day(s) after surgery; CIMT: constraint-induced movement therapy.

Table 2 Effect of CIMT on scores of balance beam walking test and proportions (%) of the fault footsteps of cerebral ischemic rats

	Group	Baseline (d0)	d7	d22
Scores of balance beam walking test	CIMT		2.27±0.64§	0.60±0.80†
	Model	0	2.20±0.45§	1.80±0.77§#
	Sham		0.33±0.75	0.60±0.83
Proportions of the fault footsteps	CIMT		30.45±5.98§	10.28±6.72†
	Model	10.34±0.93	35.28±1.63§	22.59±5.48§#†
	Sham		10.58±8.00	6.53±6.54

Data are expressed as the mean ± SD ($n = 5$ per group per time point; one-way analysis of variance and the least significant difference *post hoc* test). §$P < 0.05$, *vs.* sham group; #$P < 0.05$, *vs.* CIMT group; †$P < 0.05$, *vs.* d7 within the group. d: Day(s) after surgery; CIMT: Constraint-induced movement therapy.
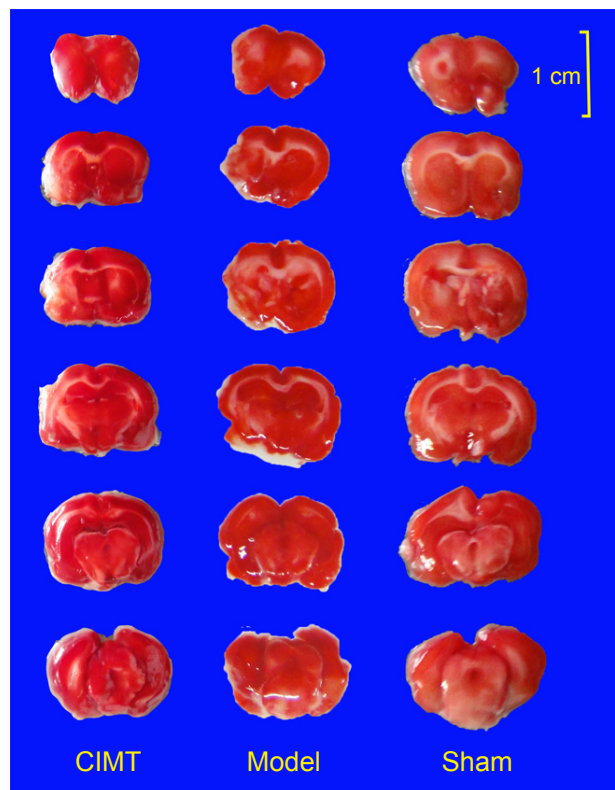
Figure 3 Effect of constraint-induced movement therapy (CIMT) on brain infarct area (white color) of cerebral ischemic rats (2,3,5-triphenyltetrazolium chloride staining).
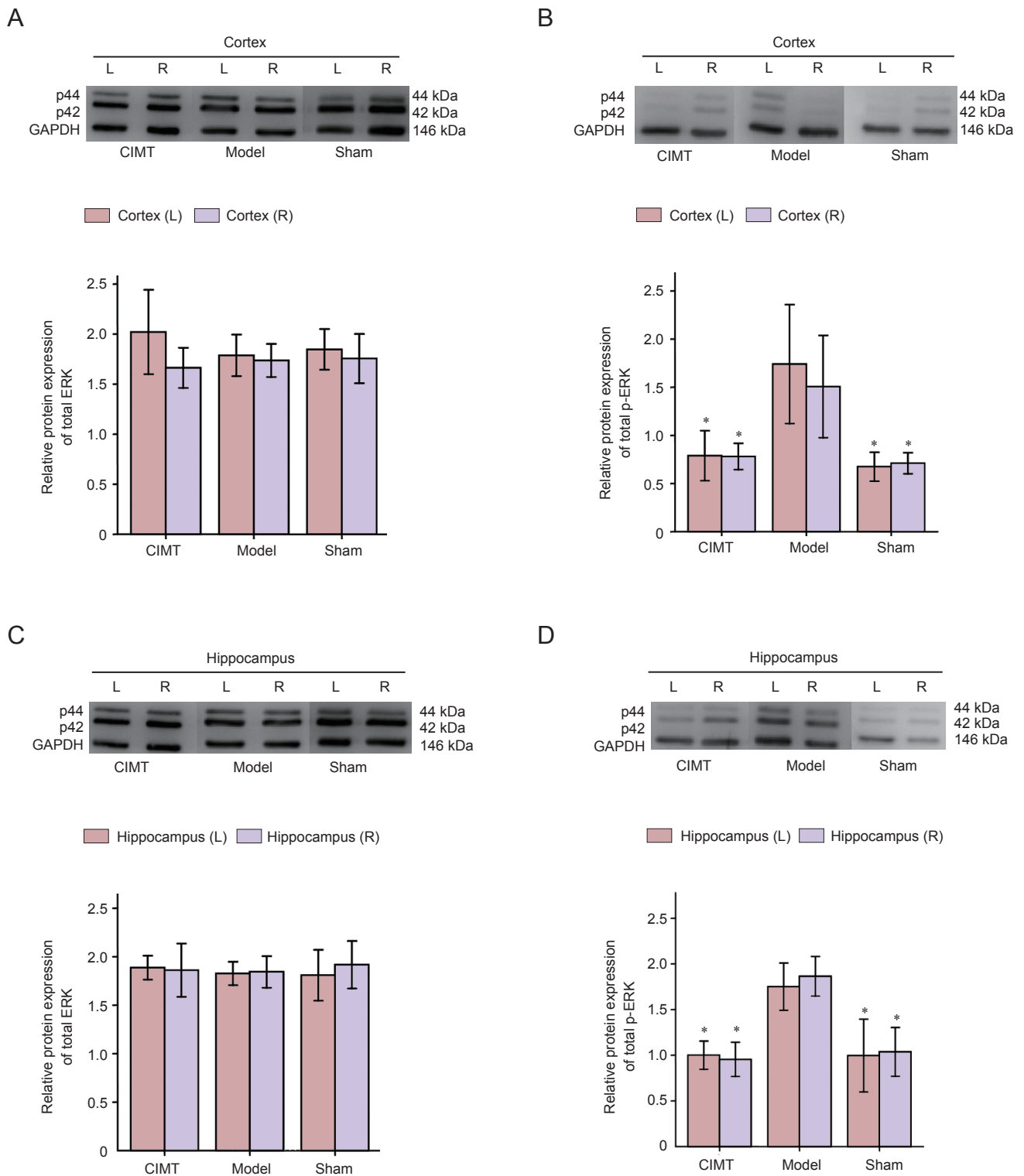
Figure 4 Effect of constraint-induced movement therapy (CIMT) on the expression of extracellular regulated protein kinase (ERK) in the bilateral cortex and hippocampi of cerebral ischemic rats.
Protein expression of total ERK and phosphorylated ERK (p-ERK) in the cortex and hippocampus were obtained by calculating the ratio of the grey value of the bands between ERK and GAPDH or between p-ERK and GAPDH. (A) No significant differences were observed for total ERK protein expression between the groups in the cortex. (B) p-ERK protein expression in the model group was significantly higher than that in the CIMT group and sham group in the bilateral cortex. (C) There were no significant differences in total ERK expression between the groups in the bilateral hippocampi at 22 days after surgery. (D) p-ERK expression in the bilateral hippocampi in model group was significantly higher than that in the CIMT group and sham group. *$P < 0.01$, *vs.* model group at same side. Data are expressed as the mean ± SD ($n = 5$ per group; one-way analysis of variance for comparisons among groups and the least significant difference *post hoc* test). The molecular weights of ERK1 and ERK2 were 44 kDa and 42 kDa, respectively. DAPDH: Glyceraldehyde 3-phosphate dehydrogenase; L: Left; R: right.

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