NEURAL REGENERATION RESEARCH
Volume 8, Issue 2, January 2013
www.nrronline.org

doi:10.3969/j.issn.1673-5374.2013.02.001 [http://www.nrronline.org; http://www.sjzsyj.org]
Sun ZG, Hu LY, Wen YM, Chen KM, Sun ZJ, Yue HY, Zhang C. Adenosine triphosphate promotes locomotor recovery after spinal cord injury by activating mammalian target of rapamycin pathway in rats. Neural Regen Res. 2013;8(2):101-110.

Adenosine triphosphate promotes locomotor recovery after spinal cord injury by activating mammalian target of rapamycin pathway in rats★

Zhengang Sun[1, 2], Lingyun Hu[2, 3], Yimin Wen[2, 4], Keming Chen[4], Zhenjuan Sun[5], Haiyuan Yue[2], Chao Zhang[2]

1 *Department of Spine Surgery, First People's Hospital of Qingdao Economic & Technological Development Area, Qingdao 266555, Shandong Province, China*
2 *Second Clinical Medical College, Lanzhou University, Lanzhou 730000, Gansu Province, China*
3 *Department of Orthopedics, Nanchong Central Hospital, Nanchong 637000, Sichuan Province, China*
4 *Department of Spine Surgery, Lanzhou General Hospital of Lanzhou Military Region, Lanzhou 730050, Gansu Province, China*
5 *Department of Information, Qingdao Eighth People's Hospital, Qingdao 266100, Shandong Province, China*

Abstract

The mammalian target of rapamycin (mTOR) pathway plays an important role in neuronal growth, proliferation and differentiation. To better understand the role of mTOR pathway involved in the induction of spinal cord injury, rat models of spinal cord injury were established by modified Allen's stall method and interfered for 7 days by intraperitoneal administration of mTOR activator adenosine triphosphate and mTOR kinase inhibitor rapamycin. At 1–4 weeks after spinal cord injury induction, the Basso, Beattie and Bresnahan locomotor rating scale was used to evaluate rat locomotor function, and immunohistochemical staining and western blot analysis were used to detect the expression of nestin (neural stem cell marker), neuronal nuclei (neuronal marker), neuron specific enolase, neurofilament protein 200 (axonal marker), glial fibrillary acidic protein (astrocyte marker), Akt, mTOR and signal transduction and activator of transcription 3 (STAT3). Results showed that adenosine triphosphate-mediated Akt/mTOR/STAT3 pathway increased endogenous neural stem cells, induced neurogenesis and axonal growth, inhibited excessive astrogliosis and improved the locomotor function of rats with spinal cord injury.

Zhengang Sun★, Master, Attending physician.

Zhengang Sun and Lingyun Hu contributed equally to this article.

Corresponding author: Yimin Wen, Chief physician, Professor, Second Clinical Medical College, Lanzhou University, Lanzhou 730000, Gansu Province, China; Department of Spine Surgery, Lanzhou General Hospital of Lanzhou Military Region, Lanzhou 730050, Gansu Province, China, wenyimin007@163.com.

Received: 2012-07-20
Accepted: 2012-11-10
(N20111004001/H)

Key Words

neural regeneration; spinal cord injury; serine/threonine-specific protein kinase; mammalian target of rapamycin pathway; signal transduction and activator of transcription 3; adenosine triphosphate; signal pathway; rapamycin; photographs-containing paper; neuroregeneration

Research Highlights

(1) Adenosine triphosphate (ATP) promotes locomotor recovery after spinal cord injury in rats.
(2) ATP can activate the Akt/mammalian target of rapamycin pathway (mTOR)/signal transduction and activator of transcription 3 (STAT3) pathway after spinal cord injury in rats.
(3) ATP-activated Akt/mTOR/STAT3 pathway contributes to locomotor recovery after spinal cord injury in rats.

Abbreviations

SCI, spinal cord injury; Akt, serine/threonine-specific protein kinase; mTOR, mammalian target of rapamycin; STAT3, Signal transducer and activator of transcription 3; BBB, Basso, Beattie, Bresnahan rating scale; NeuN, neuronal nuclei; NSE, neuron specific enolase; NF200, neurofilament protein 200; GFAP; glial fibrillary acidic protein

INTRODUCTION

At present, strategies to promote neural regeneration and repair include reduction of neuronal secondary damage, promotion of neural regeneration capacity, and elimination of inhibitory factors such as Nogo-A and other myelin associated factors[1-2]. The methods of treating spinal cord injury (SCI), including drug therapy, neurotrophic factor transplantation therapy, gene therapy, exclusion of neuronal growth inhibitory factors, and intervention with signaling pathways, have been trying to be used with encouraging results[2]. Though there is much progress in research regarding SCI treatment by interfering with signaling pathways, the underlying mechanism of these pathways in SCI remains to be revealed.

Serine/threonine-specific protein kinase (Akt) plays a central role in mediating cell survival, proliferation and differentiation through activation or inhibition of substrates[3]. In addition, Akt has recently emerged as an important regulator of neurite outgrowth, neuroprotection and neurogenesis[3]. The mammalian target of rapamycin (mTOR) is a member of the phosphatidylinositol kinase-related family of serine/threonine kinases[4]. Activated mTOR plays a pivotal role in the regulation of cell growth, proliferation, differentiation by integration signals from growth factors, nutrients and cytokines[3]. Its activity can be blocked by rapamycin, a specific inhibitor of mTOR kinase[5]. mTOR has been shown to be a direct substrate of Akt in neurogenesis[3]. Signal transducer and activator of transcription 3 (STAT3) is activated in response to many cytokines and growth factors, and is phosphorylated readily by mTOR kinase[6]. Recent evidence has demonstrated that STAT3 plays an important role in the control of neuronal survival, proliferation, and differentiation owing to its active state in the central nervous system[7]. It is well known that ATP-mediated signaling has widespread actions in the central nervous system from neurotransmission to regulation of cell survival, proliferation and differentiation[8]. Previous studies have demonstrated that extracellular ATP induces Akt activation and stimulates mTOR phosphorylation in cell proliferation, repair and plasticity[9-10].

Many extracellular factors such as neurotrophins and ATP play key roles in the development of SCI[8], whose signaling *via* the Akt/mTOR/STAT3 pathway regulates a number of important biological processes, including neuronal growth and differentiation. Still, the potential for therapeutic intervention of mTOR signaling during pathological state is only now beginning to be explored. It is therefore of considerable interest to decipher the specific action of ATP in pathological states and to explore the possibility of manipulating the ATP-mediated Akt/mTOR/STAT3 pathway in SCI.

RESULTS

Locomotor recovery post-trauma

The Basso, Beattie, Bresnahan (BBB) rating scale (see *Material and Methods*) was used to evaluate locomotor recovery in rats. The BBB scores in the ATP, control, block/interruption groups were increased 1–4 weeks post-trauma compared with pre-trauma ($P < 0.01$). The BBB scores in the ATP group were significantly higher than those in the control and block/interruption groups at 1–4 weeks post-trauma ($P < 0.01$; Figure 1).

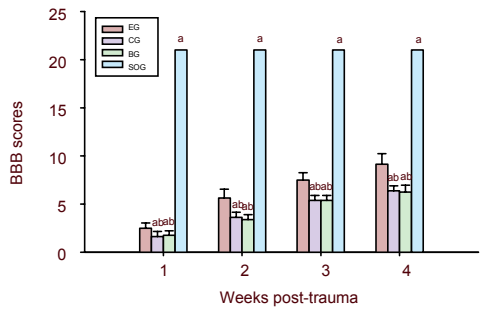

Figure 1 The Basso, Beattie, Bresnahan (BBB) scores of ATP group (EG), control group (CG; sterile saline, i.p.), block/interruption group (BG; ATP + rapamycin treatment, i.p.) and sham-operated group (SOG) at 1–4 weeks post-trauma.

A normal hindlimb is scored 21 by the BBB scale. Higher scores indicate better hindlimb motor function recovery. Results are presented as mean ± SD ($n = 8$). Rats in the EG, CG and BG had a steady increase in the BBB scores at 1–4 weeks post-trauma compared with pre-trauma ($P < 0.01$). $^{a}P < 0.01$, *vs*. EG; $^{b}P < 0.01$, *vs*. SOG. Repeated-measures analysis of variance was used.

Expression of neuronal nuclei (NeuN), neuron specific enolase (NSE), neurofilament protein 200 (NF200), nestin, glial fibrillary acidic protein (GFAP), Akt, mTOR, STAT3 in spinal cord tissue

Immunohistochemical staining showed that expression of NeuN, NSE, NF200, nestin, GFAP, Akt, mTOR, STAT3 protein was mainly localized in neurons, NSCs, and astrocytes of rat spinal cord tissue (Figures 2–4). ATP treatment increased the expression of NeuN (neuron-specific protein marker) and NSE (neuronal marker) positive cells in the border of trauma regions during the whole experimental process. This was higher compared with sterile saline treatment alone at 4 weeks post-trauma.

However, ATP + rapamycin treatment reversed the expression of NeuN and NSE positive cells in the border of traumatized regions (Figures 2A1–H2). The expression of nestin (NSCs marker) in the border of traumatized regions in the ATP group rats was significantly higher than that in rats from the control, block/ interruptionand sham-operated groups at 1 week post-trauma ($P < 0.01$; Figures 2I1–L2). Meanwhile, ATP treatment promoted the production of thick and long axons (NF200 positive axons) in the border of traumatized regions compared with sterile saline treatment at 4 week post-trauma, while ATP + rapamycin treatment generated thin and short axons (Figures 3A1–D2). Surprisingly, ATP treatment produced thinner and shorter astrocytes (GFAP positive cells) scattered around the site of SCI compared with sterile saline treatment at 4 weeks post-trauma, while ATP + rapamycin treatment promoted the formation of thick and abundantly branched astrocytes (Figures 3E1–H2).

As shown in Figures 4A1–L2, the immunoreactivities of Akt, mTOR, and STAT3 were enhanced in the region adjacent to SCI site in ATP-treated rats compared with sterile saline-treated and sham-operated rats at 1 week and increased during the whole experimental process. The immunoreactivities of Akt, mTOR, STAT3 were decreased in the region adjacent to SCI site in ATP + rapamycin-treated rats than in ATP-treated rats.

Expression of NeuN, NSE, NF200, nestin, GFAP, Akt, phosphorylated Akt (P-Akt), mTOR, phosphorylated mTOR (P-mTOR), STAT3, phosphorylated-STAT3 (P-STAT3) protein in spinal cord tissue

To further authenticate the role of ATP in SCI, western blot analysis was used to detect the expression of NeuN, NSE, NF200, nestin, GFAP protein. As shown in Figures 5A–C, F, the expression levels of NeuN, NSE, and NF200 protein were significantly higher compared with those in rats undergoing sterile saline treatment at 4 weeks post-trauma ($P < 0.01$).

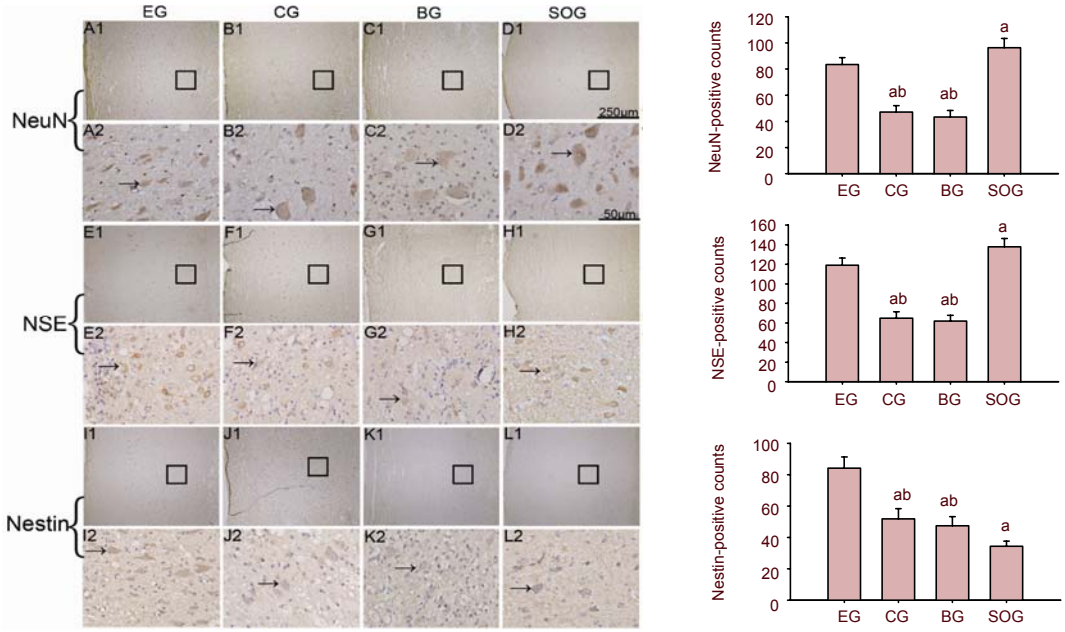

Figure 2 Immunohistochemical staining of neuronal nuclei (NeuN; A1–D2) and neuron specific enolase (NSE; E1–H2) in rostral spinal cord tissues by median cross-section harvested from adenosine triphosphate group (EG), control group (CG), block/interruption group (BG) and sham-operated group (SOG) at 4 weeks post-trauma.

Immunohistochemical staining of nestin (I1–L2) in rostral spinal cord tissues by median cross-section harvested at 1 week post-trauma. The expression level of NeuN and NSE positive cells in the EG was higher than that in the CG and BG, but it was less than that in the SOG. The number of nestin positive cells was significantly higher in the EG than that in the CG, BG and SOG at 1 week post-trauma. Original magnification, × 100 for A1–D1, E1–H1, I1–L1 and × 400 for A2–D2, E2–H2, I2–L2; scale bar, 250 μm for A1–D1, E1–H1, I1–L1 and 50 μm for A2–D2, E2–H2, I2–L2.

NeuN, NSE, nestin positive cells were counted in three sampling frames of 350 μm × 350 μm selected per section at random within the traced region. A2–D2, E2–H2, I2–L2 show enlarged images of A1–D1, E1–H1, I1–L1 boxes, respectively. Arrows in A2–D2 indicate NeuN positive cells; arrows in E2–H2 indicate NSE positive cells; arrows in I2–L2 indicate nestin positive cells. Results are expressed as mean ± SD ($n = 8$). $^{a}P < 0.01$, *vs*. EG, $^{b}P < 0.01$, *vs*. SOG (one-way analysis of variance). Experiments were repeated three times with similar results.

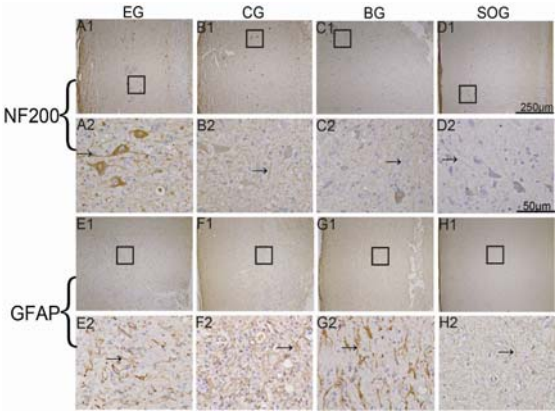

Figure 3 Immunohistochemical staining of neurofilament protein 200 (NF200; A1–D2) and glial fibrillary acidic protein (GFAP; E1–H2) in rostral spinal cord tissues by median cross-section harvested from adenosine triphosphate group (EG), physiological saline group (CG), block/interruption group (BG), and sham-operated group (SOG) at 4 weeks post-trauma.

NF200 positive axons in the EG were thicker and longer than those in the CG, BG and SOG at 4 weeks post-trauma. Original magnification, × 100 for A1–H1, and × 400 for A2–H2. A2–H2 show enlarged images of A1–H1 boxes, respectively. Arrows in A2–D2 indicate NF200 positive cells and arrows in E2–H2 indicate GFAP positive cells. Experiments were repeated three times.

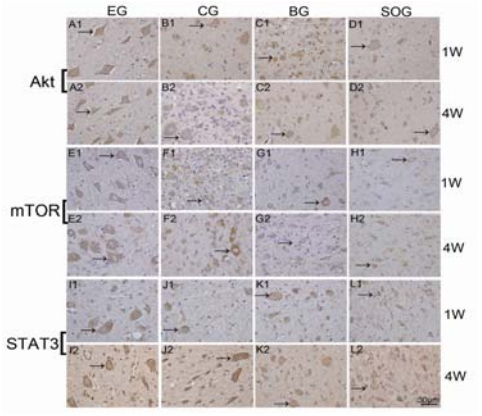

Figure 4 Immunohistochemical staining of Akt (A1–D2), mammalian target of rapamycin (mTOR; E1–H2), and signal transducer and activator of transcription 3 (STAT3; I1–L2) in rostral spinal cord tissues by median cross-section harvested from adenosine triphosphate group (EG), control group (CG), block/interruption group (BG), and sham-operated group (SOG) at 1 and 4 weeks (W) post-trauma.

The immunoreactivities of Akt, mTOR, and STAT3 in the EG were enhanced compared with those in the CG, BG and SOG at 1 and 4 weeks post-trauma. Original magnification, × 400. Arrows ndicate Akt, mTOR, and STAT3 positive cells. Experiments were repeated three times.

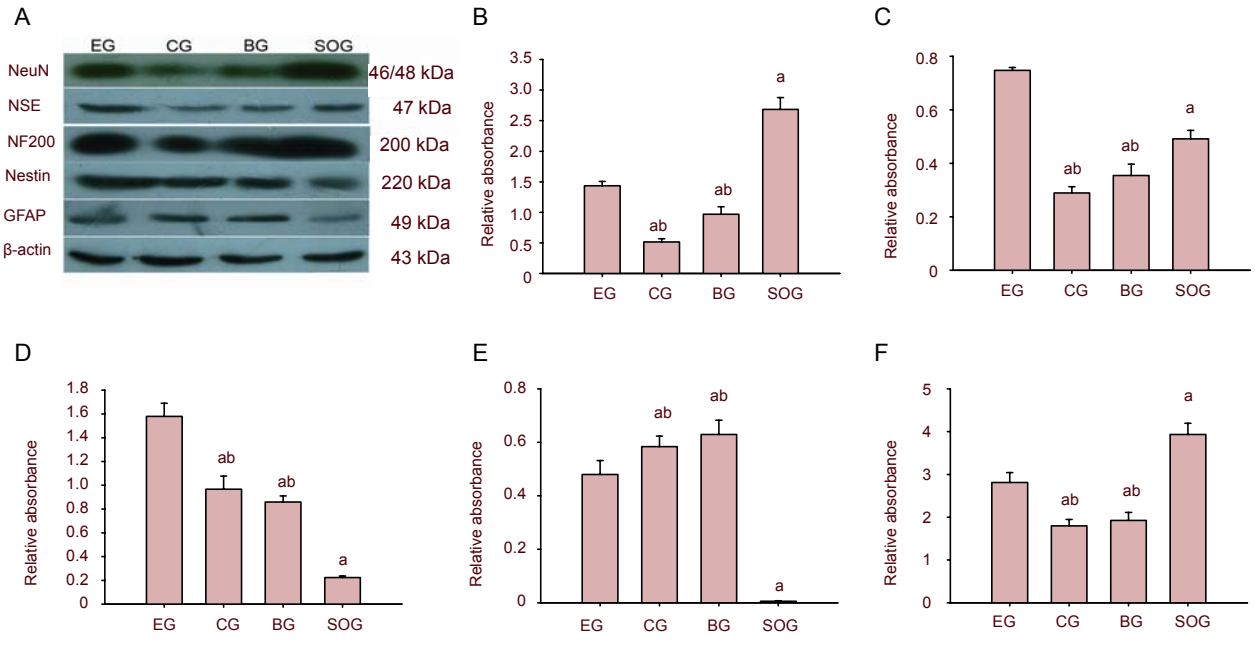

Figure 5 Dynamic changes of neuronal nuclei (NeuN), neuron specific enolase (NSE), neurofilament protein 200 (NF200), nestin and glial fibrillary acidic protein (GFAP) levels in 1/2 caudal spinal cord tissues harvested from adenosine triphosphate group (EG), control group (CG), block/interruption group (BG), and sham-operated group (SOG) at 4 weeks post-trauma.

(A) Western blots of NeuN, NSE, NF200, nestin and GFAP protein levels. β-actin was used as the internal control. (B–F) The semiquantitative data of NeuN, NSE, nestin, GFAP, and NF200. Results were presented as mean ± SD (n = 8), calculated as the integrated absorbance of target protein relative to that of β-actin. [a]P < 0.01, *vs*. EG; [b]P < 0.01, *vs*. SOG (one-way analysis of variance). Experiments were repeated three times with similar results.

Meanwhile, the expression of nestin protein was significantly higher in rats subjected to ATP treatment than in rats subjected to sterile saline treatment or sham operation at 1 week post-trauma (P< 0.01; Figures 5A, D). However, the expression of GFAP protein in rats subjected to ATP treatment was significantly lower than that in rats undergoing sterile saline treatment (P < 0.01), but it was significantly higher than that in rats undergoing sham-operation at 4 weeks post-trauma (P < 0.01; Figures 5A, E). These findings suggest that ATP treatment yielded higher protein expression of NeuN, NSE, NF200, nestin compared with sterile saline treatment alone. After ATP + rapamycin treatment, NeuN, NSE, NF200 and nestin protein expression levels were markedly decreased (P < 0.01), while GFAP protein expression was significantly increased post-trauma (P < 0.01; Figures 5A, E).

To further confirm that the Akt/mTOR/STAT3 pathway was activated following ATP administration in SCI, we examined the expression of Akt, P-Akt, mTOR, P-mTOR, STAT3, and P-STAT3 protein by western blot analysis. ATP treatment remarkably enhanced Akt, P-Akt, mTOR, P-mTOR, STAT3, and P-STAT3 activities compared with sterile saline treatment alone at 1 week post-trauma, but ATP + rapamycin treatment significantly inhibited Akt, P-Akt, mTOR, P-mTOR, STAT3, and P-STAT3 activities compared with ATP treatment alone (Figures 6A–G). As shown in Figure 6D, rapamycin could almost abolish phosphorylation of mTOR activity at Ser2448 induced by ATP. Similar to the effect on STAT3 activation status in the block/ interruption group, a notable decrease in P-STAT3 at Ser727 was observed as early as 1 week after rapamycin treatment (Figure 6F). The mTOR and STAT3 protein levels were also too low to be detected by western blot analysis in the block/interruption group (Figures 6E, G). The expression of P-Akt protein could be inhibited at Ser473 by rapamycin, but P-Akt was not as obviously inhibited as P-mTOR and P-STAT3 by rapamycin (Figures 6B, D, F). Meanwhile, expressions of NeuN and NSE protein (the markers of mature neuron) and NF200 (an axonal marker) significantly increased in SCI after ATP stimulation (Figures 5A–D, F). Down- regulation of the Akt/mTOR/STAT3 pathway by rapamycin induced higher levels of GFAP protein expression (Figures 5A, E).

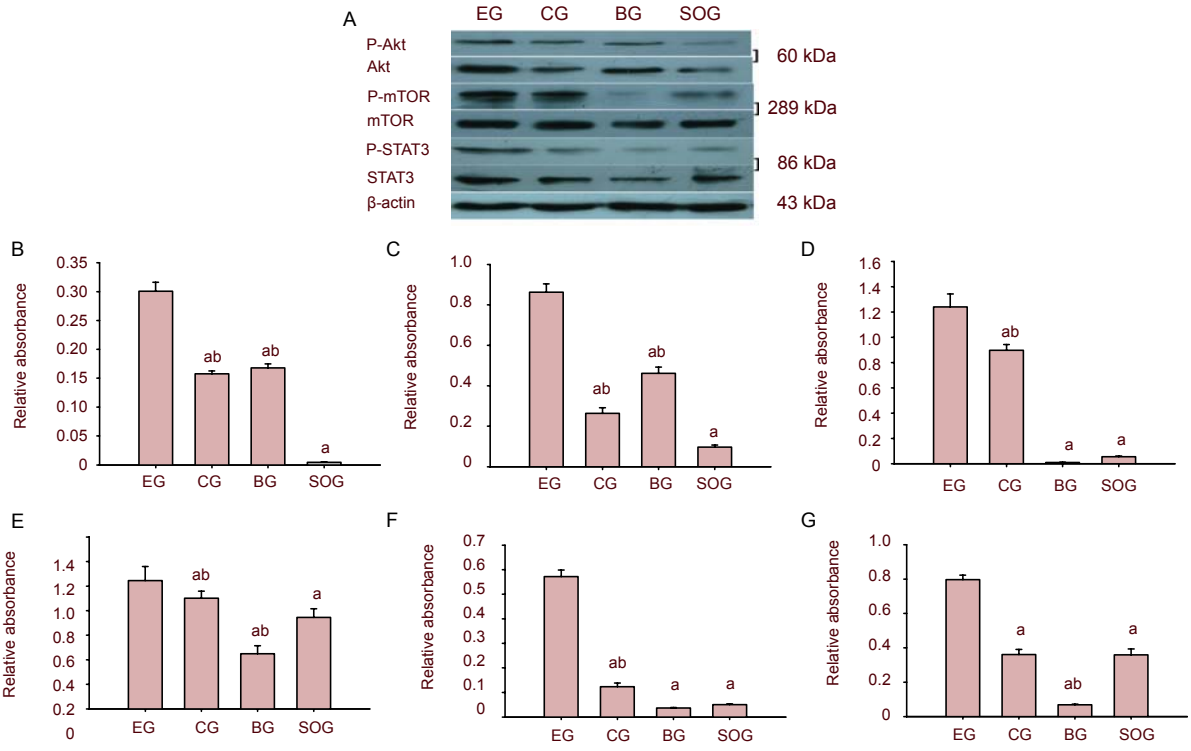

Figure 6 Dynamic changes of Akt, phosphorylated Akt (P-Akt; Ser473), mammalian target of rapamycin (mTOR), phosphorylated mTOR (P-mTOR; Ser2448), signal transducer and activator of transcription 3 (STAT3), phosphorylated STAT3 (P-STAT3; Ser727) protein levels in 1/2 caudal spinal cord tissues harvested from ATP group (EG), control group (CG), block/interruption group (BG), and sham-operated group (SOG) at 1 week post-trauma.

(A) Western blot analysis of P-Akt (Ser473), Akt, P-mTOR (Ser2448), mTOR, P-STAT3 (Ser727), STAT3 protein levels. β-actin was used as the internal control. (B–G)The semiquantitative data of P-Akt, Akt, P-mTOR, mTOR, P-STAT3, and STAT3. Results were presented as mean ± SD (n = 8), calculated as the integrated absorbance of target protein relative to that of β-actin. [a]P < 0.01, *vs*. EG; [b]P < 0.01, *vs*. SOG (one-way analysis of variance). Experiments were repeated three times with similar results.

DISCUSSION

This study investigated the role of ATP-activated mTOR signaling in the physiology and pathology of SCI, with a special focus on molecular targets of upstream, downstream, and inhibitor of mTOR kinase that potentially contributes to neurogenesis and neuropathology. BBB scores, immunohistochemistry, western blot analysis, results showed that ATP can activate Akt/mTOR/STAT3 pathway, increase nestin, NeuN, NSE and NF200 expression but decrease GFAP expression, thereby promoting the locomotor recovery of rats with SCI.

ATP promotes locomotor recovery after SCI in rats

As demonstrated by BBB scores, ATP injections could promote the recovery of locomotor function in SCI, but could not induce complete function recovery in rats. ATP as a neurotransmitter can regulate the function of the nervous system, provide nutrition for spinal cord neurons and promote axonal growth[8]. ATP-mediated electrophysiological changes protect damaged neurons in nerve cells and cell proliferation through spinal cord neurons and the Schwann cell surface P2Y receptor subtype[11]. Furthermore, extracellular ATP promotes differentiation of NSCs through the creatine kinase/phosphocreatine system in the central nervous system[12].

ATP activates Akt/mTOR/STAT3 pathway after SCI in rats

Our results showed that Akt signal can be activated by ATP and will continue to activate the mTOR/STAT3 pathway in neurogenesis in SCI. There are many cytokines that are capable of phosphorylating Akt at Ser473, including ATP and Akt itself[3, 10]. In addition, activated Akt can stimulate the mTOR pathway[13]. mTOR has been shown to possess catalytic activity and phosphorylate several target proteins, including p70 ribosomal S6 protein kinase, eIF–4E binding protein, and STAT3[1, 14].

Our experimental results confirmed that ATP-activated mTOR kinase can phosphorylate STAT3 on Ser727 and phosphorylated mTOR/STAT3 signaling is highly sensitive to rapamycin blocking, which is consistent with a previous study[15]. In mammalian cells, mTOR exists in two distinct multi-protein complexes: mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2)[16]. mTORC1 is sensitive to inhibition by macrolide antibiotic rapamycin[17]. Importantly, mTORC1 can efficiently phosphorylate STAT3 on Ser727, which can promote protein translation, cell growth and differentiation[1]. Although mTORC2 was originally reported to be insensitive to long-term rapamycin treatment, it can be inhibited indirectly by rapamycin in mammalian cells[18]. mTORC2 can phosphorylate and activate another member of the serine/threonine kinase family, Akt at Ser473[19]. Akt promotes cell proliferation and differentiation, so activation of Akt by mTORC2 can be an important mechanism by which activated mTOR/STAT3 pathway promotes neurogenesis. Furthermore, mTOR has been shown to be a direct substrate of Akt in neuronal differentiation[20], indicating that mTOR can act as a substrate, effector and activator of the Akt signaling[21]. In this study, we found that rapamycin can inhibit phosphorylated Akt at Ser473, which might be inhibited indirectly after the inhibition of mTORC2 by rapamycin. In conclusion, ATP can activate the Akt/mTOR/STAT3 pathway in SCI rat models, and rapamycin can inhibit the activated Akt/mTOR/STAT3 pathway *in vivo*.

ATP-activated Akt/mTOR/STAT3 pathway contributes to locomotor recovery after SCI in rats

Akt, mTOR and STAT3 are shown to associate with the growth, proliferation and differentiation of NSCs[1, 22]. NSCs which exist in spinal cord and migrate toward the lesion region during SCI are multipotent progenitor cells that can self-renew and generate new neurons to ameliorate injury of the spinal cord[2]. Recent studies reveal a much broader involvement of mTOR signaling in neuronal development, showing that axon guidance and dendrite development all require its activity[17]. So upgrading proliferation of endogenous NSCs and promoting neurogenesis and axonal growth are effective ways to SCI repair[23]. In addition, inhibiting excessive astrogliosis following SCI is a positive approach towards neurogenesis[24]. In this study, we demonstrated that the levels of activated Akt, mTOR, and STAT3 are directly related to endogenous NSC proliferation, neurogenesis and axonal growth in SCI. Our experimental results have shown that endogenous NSCs (nestin positive cells) present in the spinal cord under normal condition (in SOG), migrate toward the lesions, and significantly increase in SCI after ATP treatment. Meanwhile, neurons (NeuN and NSE positive cells) and neurites (NF200 positive axons) significantly increase, and astrocytes (GFAP positive cells) remarkably decrease in injured spinal cord tissues after ATP activation of the Akt/mTOR/STAT3 pathway. But down-regulation of the Akt/mTOR/STAT3 pathway by rapamycin inhibits the expression of endogenous NSCs, neurons, and neurites induced by ATP and increases the expression of astrocytes.

In conclusion, the Akt/mTOR/STAT3 pathway may regulate the fate of SCI repair by ATP or rapamycin after

SCI in rats. A previous report indicates that ATP has significant effects on self-renewal and early lineage progression of NSCs in the central nervous system[25]. In support of this idea, ectogenous ATP contributes to up-regulation of mTOR signaling, induces endogenic NSC proliferation, promotes neurogenesis and axonal growth, and inhibits excessive reactive astrogliosis, which promote the locomotor recovery of rats with SCI. Therefore, we confirmed that in vivo ATP promoted locomotor recovery by activating the Akt/mTOR/STAT3 pathway after SCI in rats.

Akt/mTOR/STAT3 pathway prospects

Results from this study showed that the NeuN, NSE, or NF200 expression levels were increased after ATP treatment, which may be due to an activation of the Akt/mTOR/STAT3 pathway inhibiting autophagy to inhibit neuronal apoptosis. Autophagy is an evolutionary conserved process of catabolic cell response to poor nutrient conditions that employ the lysosomal pathway, which can be induced by several conditions, including starvation and a number of protein kinases[26]. Apoptosis is normally regulated by a complex network of signaling pathways[27]. In addition to regulation of cell size, proliferation and differentiation, mTOR signaling has been recently implicated in controlling apoptosis in response to nutrient availability, oxygen, and energy-dependent ATP[28]. Therefore, activated mTOR signaling by ATP may inhibit neuronal autophagy to decrease apoptosis. Our future research should focus on whether ATP can inhibit neuronal phagocytosis or not, and the degree of inhibition of phagocytosis after ATP activation of the Akt/mTOR/STAT3 pathway.

In addition, there is a theory that P2X7 receptor is up-regulated and not conducive to improving locomotor recovery after SCI[29] and P2 receptor activation, and this is critically associated with activated mTOR signaling by ATP stimulation[9]. But whether extracellular ATP-activated mTOR signaling occurs through P2X7 receptors in the injured spinal cord remains unclear. Further studies are needed to clarify these questions.

As mTOR signaling is important for neuronal survival, investigators must consider new therapeutic approaches that prolong mTOR signaling activation appropriately in order to create conditions conducive to proper neuronal proliferation and differentiation. A better understanding of molecular mechanisms of downstream and upstream of mTOR signaling actions, and their activators and inhibitors in SCI, can contribute to developing more accurate pharmacological interventions. The search for ATP, which positively regulates neurogenesis by activating mTOR signaling, may serve as a good example of such a strategy. Further clarifying how the Akt/mTOR/STAT3 molecular network regulates neurogenesis could help design more rational novel drugs to promote locomotor recovery after SCI.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

This study was performed at the Department of Orthopedics, Central Research Institute of Lanzhou General Hospital, Lanzhou Military Command of Chinese PLA, from October 2008 to December 2009.

Materials

Totally 128 adult specific pathogen-free female Sprague-Dawley rats, weighing 200–250 g, were provided by Laboratory Animal Center of Gansu College of Traditional Chinese medicine (license No. SCXK (Lu) 20030010) and housed in facilities maintained at 22 ± 2°C under a 12-hour light/dark cycle. Before surgery, all animals were acclimatized for 5–7 days and allowed free access to food and water. All animal experiments were performed in strict accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[30].

Methods

Spinal cord injury model preparation

Rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) and placed in a prone position on a heating pad to maintain a constant body temperature. Under aseptic conditions, a longitudinal incision (about 5 cm) was made at the midline of the back exposing the paravertebral muscles. These muscles were dissected to expose $T_{7\text{-}11}$ vertebrae. The dura mater of the spinal cord was exposed *via* a three-level $T_{8\text{-}10}$ laminectomy, and covered with a metal gasket consistent with the length and curvature of spinal cord $T_{8\text{-}10}$. Spinal cord injury was induced by epidural weight drop using a modified Allen's stall with damage energy of 50 g-cm force (4 g × 12.5 cm). The wound was closed and disinfected. A successful animal model of spinal cord injury should meet the following criteria: at the moment of the ball impacting the dura mater of spinal cord, animals showed body jitter, hindlimb retraction, tail spastic swing, and hindlimb flaccid

paralysis. The sham-operated group animals underwent a T_{8-10} laminectomy without weight- drop injury.

Animal treatment and sample preparations

In the ATP group, following spinal cord injury induction, rats were daily administered ATP (40 mg/kg, i.p.; Qilu Pharmaceutical Corp, Shandong, China) for 7 successive days. In the control group, following spinal cord injury induction, rats were daily administered sterile saline (40 mg/kg, i.p.) for 7 successive days. In the block/interruption group, following spinal cord injury induction, rats were daily administered ATP (40 mg/kg, i.p.; Qilu Pharmaceutical Corp) and rapamycin (3 mg/kg, i.p.; Sigma, St. Louis, MO, USA)[31] for 7 days. In the sham-operated group, rats were subjected to laminectomy but without spinal cord injury induction and then received physiological saline administration (40 mg/kg, i.p.) for 7 successive days (Table 1).
All rats received gentamicin solution administration daily starting 30 minutes post-trauma followed by repeated injections (b.i.d., 2 mg/kg) for 7 successive days. After spinal cord injury induction, rats were housed for a survival period of 1–4 weeks. During this time period, all rats was subjected to treadmill training for 30 minutes per day at 8:00 p.m. and the bladder of each rat was manually emptied twice a day until the rats regained normal bladder function. Rats were anesthetized with an overdose of sodium pentobarbital (60 mg/kg) at 1–4 weeks post-trauma in each group, respectively (n = 8) to harvest 1.5 cm length spinal cord samples centered at the epicentre site. Rostral samples were removed by median cross-section, fixed overnight with 4% paraformaldehyde solution prepared in PBS at 4°C for 24 hours and further processed for histological examination. Caudal samples were divided into two copies from the mid-sagittal plane and kept in liquid nitrogen for protein measurements.

Behavioral analysis

Locomotor activity was evaluated using the BBB locomotor rating scale[32]. Functional tests were performed before surgery and each week during the 4-week survival period. Considering the significant difference between day and night rat activities, all rats were scored in an open field at 8:00 p.m. for 4 minutes. Two independent examiners blinded to experimental design were asked to evaluate the locomotor function of each animal.

Spinal cord immunohistochemistry

Samples were fixed and embedded in paraffin. Serial para-sagittal sections (5 μm) were made from spinal cord centered on the injury epicenter and mounted on poly-L-lysine-coated glass slides. Sections were dewaxed in xylene, rehydrated in gradient ethanol, and washed in 0.1 M PBS (pH 7.5). Endogenous peroxidase activity was quenched with 3% H_2O_2 at room temperature for 10 minutes, sections were washed three times in distilled water, and then heated in 10 mM citrate buffer (pH 6.0) for antigen retrieval. After cooling, sections were washed three times in PBS and then blocked in 5% bovine serum albumin (Boster, Wuhan, China) for 1 hour at room temperature. Incubation with the indicated primary antibodies (Table 2) was performed overnight at 4°C. inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Sigma) for 30 minutes at 4°C. After three washes in PBS for 5 minutes each, the sections were reacted with biotin-conjugated goat anti-rabbit/ mouse IgG (Boster) at room temperature for 30 minutes, and then incubated with horseradish peroxidase- conjugated streptavidin (Boster) for 30 minutes at room temperature. Following washes in PBS, sections were incubated with 0.05% diaminobenzidine (Boster), counterstained with hematoxylin, sealed by DPX mountant, covered, and then visualized using an Olympus light microscope (DP71, Olympus, Japan) at 100 × and 400 × magnification. Negative control sections were treated with the identical procedure except that the primary antibodies were omitted. Cell counts were determined using unbiased sampling: three sampling frames of 350 μm × 350 μm were selected per section at random within the traced region.

Table 1 Animal experimental design

Group	Treatment	Postoperative time to sacrifice			
		1 week	2 weeks	3 weeks	4 weeks
ATP	SCI+ATP (40 mg/kg daily)	n = 8	n = 8	n = 8	n = 8
Control	SCI+sterile saline (40 mg/kg daily)	n = 8	n = 8	n = 8	n = 8
Block/interruption	SCI+ATP (40 mg/kg daily)+rapamycin (3 mg/kg daily)	n = 8	n = 8	n = 8	n = 8
Sham-operated	Laminectomy+physiological saline (40 mg/kg daily)	n = 8	n = 8	n = 8	n = 8

SCI: Spinal cord injury; ATP: adenosine triphosphate. There were 32 rats in each group.

Table 2 Species of different antibodies used in western blot analysis and immunohistochemical staining*

Antibody	Species	Dilution for immunohistochemistry	Dilution for western blot	Source	Stacking gel (%)	Resolving gel (%)
Nestin	Mouse, polyclonal	1:200	1:1000	Abcam, Cambridge, UK	5	15
NeuN	Mouse, monoclonal	1:500	1:1000	Millipore Corp Billerica, MA, USA	6	17
NSE	Rabbit, polyclonal	1:100	1:1000	Abcam, Cambridge, UK	6	17
GFAP	Rabbit, polyclonal	1:100	1:1000	Abcam, Cambridge, UK	6	17
Akt	Rabbit, polyclonal	1:400	1:1000	CST, Danvers, MA, USA	6	17
P-Akt	Rabbit, polyclonal	–	1:1000	CST, Danvers, MA, USA	6	17
mTOR	Rabbit, polyclonal	1:50	1:1000	CST, Danvers, MA, USA	5	15
P-mTOR	Rabbit, polyclonal	–	1:1000	CST, Danvers, MA, USA	6	17
STAT3	Rabbit, polyclonal	1:100	1:1000	CST, Danvers, MA, USA	6	17
P-STAT3	Rabbit, polyclonal	–	1:1000	CST, Danvers, MA, USA	6	17
β-actin	Mouse, monoclonal	–	1:400	Zhongshan, Beijing, China	5–6	15–17
NF200	Mouse, monoclonal	1:200	1:1000	Abcam, Cambridge, UK	5	15

*The concentration of PAGE when electrophoresis. NeuN: Neuronal nuclei; NSE: neuron specific enolase; GFAP: glial fibrillary acidic protein; Akt: serine/threonine-specific protein kinase; P-Akt: phosphorylated Akt; mTOR: mammalian target of rapamycin; P-mTOR: phosphorylated mTOR; STAT3: signal transducer and activator of transcription 3; P-STAT3: phosphorylated STAT3; NF200: neurofilament protein 200.

Western blot analysis

Samples were quickly removed from liquid nitrogen, homogenized by adding lysate buffers (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1mM ethylenediamine tetraacetic acid; 1% NP-40; 0.25% Na-deoxycholate;1 mM phenylmethyl sulfonylfluoride; 0.1% sodium dodecyl sulfate) and supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Sigma) for 30 minutes at 4°C. After homogenates were centrifuged at 12 000 r/min for 15 minutes at 4°C, the supernatants were collected and total protein contents were determined using commercial background-corrected absorbance assay kits (BIOS, Beijing, China). The samples (8 μg protein) were added to 1/2 sample buffer (2% sodium dodecyl sulfate, 10% glycerol, 0.1% bromophenol blue, 2% 2-mercaptoethanol, and 50 mM Tris-HCl) and subjected to 5–17% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Table 2). After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (0.45 μm) (Millipore Corp, Billerica, MA, USA), and blocked in 2% nonfat dry milk buffer for 2 hours at room temperature. After one wash with PBS + 0.05% Tween-20 (PBS-T), the membranes were incubated overnight at 4°C with primary antibodies (Table 2) diluted in antibody dilution (PBS-T + 2% nonfat dry milk + 1% bovine serum albumin). After three washes with PBS-T, the membranes were incubated for 2 hours with horseradish peroxidase-conjugated secondary antibody (sheep anti-rabbit/mouse, 1:5 000) in the dilution described above. Following extensive washes in PBS-T, the signals were detected with an immobilon western chemiluminescent horseradish peroxidase substrate (Millipore Corp, Billerica, MA, USA) and exposed on X-ray film (Kodak, China). For semiquantitative analysis of the expression level of Akt, P-Akt, mTOR, P-mTOR, STAT3, P-STAT3, NeuN, NSE, nestin, NF200, and GFAP, the integrated absorbance of each band was measured by Image-Pro PLUS (Meyer Instruments, Houston, TX, USA). The integrated absorbance was normalized to that of β-actin (Zhongshan, Beijing, China) and expressed as arbitrary units. Each sample was assayed in triplicate and averaged.

Statistical analysis

All data were presented as mean ± SD. The differences were analyzed by one-way analysis of variance and repeated-measures analysis of variance. Results were considered significantly different at $P < 0.05$. All analyses were performed using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA).

Author contributions: Zhengang Sun, Lingyun Hu participated in conception and design of the study, data collection and analysis, wrote the manuscript, provided critical revision of the manuscript for intellectual content, and was responsible for statistical expertise. Yimin Wen was in charge of conception and design of the study and obtained funds. Keming Chen, Zhenjuan Sun, Haiyuan Yue and Chao Zhang provided technical or material support, data collection, analysis and interpretation. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee of Lanzhou University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

REFERENCES

[1] Wang B, Xiao Z, Chen B et al. Nogo-66 promotes the differentiation of neural progenitors into astroglial lineage cells through mTOR-STAT3 pathway. PLoS One. 2008;3: e1856.

[2] Sango K, Yanagisawa H, Komuta Y, et al. Neuroprotective properties of ciliary neurotrophic factor for cultured adult rat dorsal root ganglion neurons. Histochem Cell Biol. 2008; 130:669-679.

[3] Hu LY, Sun ZG, Wen YM, et al. ATP-mediated protein kinase B Akt/mammalian target of rapamycin mTOR/p70 ribosomal S6 protein p70S6 kinase signaling pathway activation promotes improvement of locomotor function after spinal cord injury in rats. Neuroscience. 2010;169: 1046-1062.

[4] Yu W, Chen Z, Zhang J, et al. Critical role of phosphoinositide 3-kinase cascade in adipogenesis of human mesenchymal stem cells. Mol Cell Biochem. 2008; 310:11-18.

[5] Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006;124:471-484.

[6] Dominguez E, Mauborgne A, Mallet J, et al. SOCS3-mediated blockade of JAK/STAT3 signaling pathway reveals its major contribution to spinal cord neuroinflammation and mechanical allodynia after peripheral nerve injury. J Neurosci. 2010;30:5754-5766.

[7] Yu Y, Ren W, Ren B. Expression of signal transducers and activator of transcription 3 (STAT3) determines differentiation of olfactory bulb cells. Mol Cell Biochem. 2009;320:101-108.

[8] Cotrina ML, Nedergaard M. Physiological and pathological functions of P2X7 receptor in the spinal cord. Purinergic Signal. 2009;5:223-232.

[9] Gerasimovskaya EV, Tucker DA, Weiser-Evans M, et al. Extracellular ATP-induced proliferation of adventitial fibroblasts requires phosphoinositide 3-kinase, Akt, mammalian target of rapamycin, and p70 S6 kinase signaling pathways. J Biol Chem. 2005;280:1838-1848.

[10] Neary JT. Protein kinase signaling cascades in CNS trauma. IUBMB Life 2005;57:711-718.

[11] Dalziel HH, Westfall DP. Receptors for adenine nucleotides and nucleosides: subclassification, distribution, and molecular characterization. Pharmacol Rev. 1994;46:449-466.

[12] Andres RH, Ducray AD, Schlattner U, et al. Functions and effects of creatine in the central nervous system. Brain Res Bull. 2008;76:329-343.

[13] Shin DH, Min HY, El-Naggar AK, et al. Akt/mTOR counteract the antitumor activities of cixutumumab, an anti-insulin-like growth factor I receptor monoclonal antibody. Mol Cancer Ther. 2011;10:2437-2448.

[14] Dominguez E, Rivat C, Pommier B, et al. JAK/STAT3 pathway is activated in spinal cord microglia after peripheral nerve injury and contributes to neuropathic pain development in rat. J Neurochem. 2008;107:50-60.

[15] Dunlop EA, Tee AR. Mammalian target of rapamycin complex 1: signalling inputs, substrates and feedback mechanisms. Cell Signal. 2009;21:827-835.

[16] Miyazaki M, Esser KA. Cellular mechanisms regulating protein synthesis and skeletal muscle hypertrophy in animals. J Appl Physiol. 2009;106:1367-1373.

[17] Swiech L, Perycz M, Malik A,et al. Role of mTOR in physiology and pathology of the nervous system. Biochim Biophys Acta. 2008;1784:116-132.

[18] Sarbassov DD, Ali SM, Sengupta S, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell. 2006;22:159-168.

[19] Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. Cancer Cell. 2007;12:9-22.

[20] Grider MH, Park D, Spencer DM, et al. Lipid raft-targeted Akt promotes axonal branching and growth cone expansion via mTOR and Rac1, respectively. J Neurosci Res. 2009;87: 3033-3042.

[21] Memmott RM, Dennis PA. Akt-dependent and -independent mechanisms of mTOR regulation in cancer. Cell Signal. 2009;21:656-664.

[22] Read DE, Gorman AM. Involvement of Akt in neurite outgrowth. Cell Mol Life Sci. 2009;66:2975-2984.

[23] Narayanan SP, Flores AI, Wang F, et al. Akt signals through the mammalian target of rapamycin pathway to regulate CNS myelination. J Neurosci. 2009;29:6860-6870.

[24] Fitch MT, Silver J. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. Exp Neurol. 2008;209:294-301.

[25] Inoue K, Koizumi S, Tsuda M. The role of nucleotides in the neuron--glia communication responsible for the brain functions. J Neurochem. 2007;102:1447-1458.

[26] Rubinsztein DC, Gestwicki JE, Murphy LO, et al. Potential therapeutic applications of autophagy. Nat Rev Drug Discov. 2007;6:304-312.

[27] Qiao L, Wong BC. Targeting apoptosis as an approach for gastrointestinal cancer therapy. Drug Resist Updat. 2009; 12:55-64.

[28] Williams A, Sarkar S, Cuddon P, et al. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat Chem Biol. 2008;4:295-305.

[29] Peng W, Cotrina ML, Han X, et al. Systemic administration of an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. Proc Natl Acad Sci U S A. 2009;106:12489-12493.

[30] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.

[31] Meikle L, Pollizzi K, Egnor A, et al. Response of a neuronal model of tuberous sclerosis to mammalian target of rapamycin (mTOR) inhibitors: effects on mTORC1 and Akt signaling lead to improved survival and function. J Neurosci. 2008;28:5422-5432.

[32] Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma. 1995;12:1-21.

(Edited by Zheng XY, Qu Y/Song LP)