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Hindlimb suspension-induced cell apoptosis in the posterior parietal cortex and lateral geniculate nucleus: corresponding changes in c-Fos protein and the PI3K/Akt signaling pathway

Wei Huang, Chen Chen and Xufeng Liu*

Department of Military Medical Psychology, Fourth Military Medical University, Xian, Shaanxi Provence, China, Email: xufeng_liu1971@126.com

Recent physiological studies indicate that weightlessness reliably alters ocular structure and function, as well as the ability to process visual-spatial information. The posterior parietal cortex (PPC) and lateral geniculate nucleus (LGN) are two key brain areas implicated in the processing of visual-spatial information. Here, we used the modified tail-suspension rat model to simulate the physiological effects of microgravity. Rats were divided into four groups, which exposed to the simulated microgravity environment for 0 (Control group), 7, 14, or 28 days. We found a significant increase in cellular apoptosis in the PPC and the LGN after 7 days of simulated microgravity. In addition, there was an increase in expression of c-Fos protein in the PPC, and a repression of the PI3K/Akt signaling pathway in the LGN after 7 days. Based on these results, we conclude that short-term simulated microgravity may induce cell apoptosis in the PPC and LGN, and reflect a neural adaptive process to accommodate a microgravity environment.

Key words: simulated microgravity, posterior parietal cortex, lateral geniculate nucleus, apoptosis, c-Fos, PI3K/Akt

INTRODUCTION

Recent physiological studies indicate that weightlessness reliably alters functioning of cardiovascular, osteomuscular and immune systems. Several studies have reported that, during a tracking unpredictable target task, astronauts' reaction times are reliably slower and less accurate during the first week of spaceflight. These impairments are thought to be caused by ongoing visuomotor adaptation to microgravity (Manzey et al. 2000). Indeed, multiple studies suggest that microgravity causes many physiological changes within the visuomotor system, which subsequently affect both visual and motor functions and are associated with various disorders, such as visual disturbances, postural imbalance, and ataxia (Heuer et al. 2003, Manzey et al. 2000). Although several studies have documented microgravity-induced degradation in visual functioning and adaptations in the optic disk, retina, and optic nerve, few have evaluated changes in upper visuomotor cortex. To improve our understanding of how the human visuomotor system accommodates to microgravity, the present study investigates two brain regions - the posterior parietal cortex (PPC) and the lateral geniculate nucleus (LGN). These brain areas are considered to be critical components of the visuomotor system.

The PPC is shown to be critical for spatial orientation and navigation, in both humans and rats (Calton and Taube 2009, Kolb et al. 1994, Tees 1999). The PPC receives afferent signal from the occipitotemporal cortex (Kravitz et al. 2011), and translates visual spatial information into signals that guide complex actions (Culham and Valyear 2006, Niell 2013). One study showed that patients with bilateral damage to the PPC were unable

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to accurately reach toward an object or judge the position of objects (Al-Khawaja and Haboubi 2001). The LGN is one of the main regions that is involved in transmitting and integrating spatial information within the visual system. Several research studies have demonstrated that the LGN receives projections from direction- selective retinal ganglion cells (Kay et al. 2011, Rivlin-Etzion et al. 2011), which respond strongly to an object moving in a preferred direction and weakly to an object moving in the opposite direction (Piscopo et al. 2013).

Microgravity has been shown to cause various cell morphological changes in the central nervous system (CNS), including cell apoptosis, abnormal cell shape, reduced neuron and area, and a significant reduction in the size of the soma (Pani et al. 2013, Sun et al. 2009). Simulated weightlessness has been shown to cause apoptosis in the cortex and the hippocampus *in vivo* (Sun et al. 2009), and to induce apoptosis in glial cells *in vitro* (Uva et al. 2002). Apoptosis, or programmed cell death, also plays a key role in a self-adaptive mechanism when cells are exposed to a new environment (Vaux et al. 1994). To explore the effect of simulated weightlessness on the PPC and LGN, we investigated apoptosis in these regions following different durations of exposure to simulated microgravity to better understand how brain cells acclimate to hypogravity over time.

A few research studies have demonstrated that c-Fos protein is induced by exposure to simulated microgravity in some brain region (Sarkar et al. 2006, Su et al. 2000, Wu et al. 2017). c-Fos is not only a marker of neuronal activation, but also proposed to act as a proapoptotic agent. Multiple studies have suggested that the *c-fos* gene is transiently over-expressed prior to cell apoptosis induced by cellular injury, or in some cancer cells (Zhang et al. 2007). Based on these results, we investigated the expression of c-Fos protein after exposure to simulated microgravity, and test for potential links with cellular apoptosis in the PPC and LGN. We also investigated activity of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway in neural cells of the PPC and LGN. Activated PI3K and Akt have been shown to induce myriad downstream cell survival mechanisms (Brotelle and Bay 2015, Wang et al. 2002), and up-regulate the expression of genes with anti-apoptotic activity (Vara et al. 2004). One study demonstrated that tail suspension repressed activation of the PI3K-Akt signaling pathway within the sensorimotor cortex and the striatum, which may also be observed for neural plasticity induced by microgravity (Mysoet et al. 2014).

Several studies, conducted predominantly in rodents, have demonstrated that tail suspension models several of the changes that occur in the brain of larger mammals during microgravity exposure (Morey-Holton and Globus 2002). These changes include alterations in hemodynamics (Zhang et al. 2014), changes in protein levels (Grimm et al. 2011, Ranjan et al. 2014), and in gene expression (Felix et al. 2004, Sarkar et al. 2006). In the present study, we used tail-suspension rat model to induce physiologic changes caused by microgravity, by simulating the effect of cephalad shifts of body fluids that occur in microgravity. Using this model, we investigated apoptosis in the PPC and LGN after short- (7 days), middle- (14 days) and long-term (28 days) exposure to simulated microgravity (Morey-Holton and Globus 2002). We explored the potential mechanisms of apoptosis by measuring c-Fos expression and activity within the PI3K-Akt signaling pathway, to better understand how the visuomotor system accommodates to microgravity.

METHODS

Animals

Eighty-eight male Sprague–Dawley rats supported by the Fourth Military Medical University were used in this study. The rats weighed between 180 and 220 g. Prior to the experiment, all rats were housed in an animal room for seven days to acclimate them to the environment. The room temperature was maintained at 20±2°C, and rats were kept on a 12-h light–dark cycle. All rats had free access to food and water throughout the experiment and acclimation period. After the acclimation period, the rats were randomly assigned to four groups (n=22/group), corresponding with different durations of exposure to simulated microgravity: (1) negative control (CON), (2) 7 d exposure, (3) 14 d exposure, and (4) 28 d exposure. All of the treatments and experiments performed on the rats met Chinese guidelines for experimental animals.

Tail-suspension rat model

The modified tail-suspension rat model was used to simulate the physiological effects of microgravity (Morey-Holton and Globus 2002). Briefly, the tail was first cleaned, air-dried, and then smeared with benzoin and resin. Next, a strip of medical adhesive tape was folded in half, pasted along the opposite sides of the tail, and secured with several short tapes across. A very short plastic tube was then placed at the end of the folded tape, with a chainlet affixed. The chainlet was attached to a swivel that was mounted at the top of the cage, to perform the tail suspension. Finally, the height of the swivel was adjusted to ensure that the rats were in a –30° head-down tilt position, and their hind limbs were un-

loaded. The rats were free to move around in the cage throughout the course of suspension (Fig. 1).

Tissue preparation

After the suspension, the rats in each group were randomly divided into groups. One group (n=40) underwent immunohistochemistry, immunofluorescence and TUNEL staining techniques. The remaining 48 were for used for immunoblotting.

Rats from the first group were anesthetized with pentobarbital intraperitoneally (0.5 ml/100 g), and then quickly perfused transcardially with 500 ml of 0.9% saline, followed by a perfusion of 400 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). Then, the brain was quickly removed into the fixative (4% paraformaldehyde in PBS, pH 7.4) overnight at 4°C, dehydrated in 30% sucrose solution in PBS for 72 h at 4°C, and subsequently frozen. Serial coronal sections containing the PPC and LGN, 10 μm thick, were cut on a cryostat. Sections were selected for analysis at 0.25 mm intervals at five rostrocaudal levels through the PPC [–3.5 to –4.5] mm, and 0.5 mm intervals at five rostrocaudal levels for the LGN [–3.3 to –5.3] mm caudal to bregma. Paxinos and Watson (1986) were used for reference. The first one of every six consecutive sections was used for TUNEL staining. The second slice in each section was reacted with c-Fos antibodies, for a total of five slides from each animal for each technique.

Fig. 1. Tail-suspension rat model. The rat was suspended in a –30° head-down tilt position and their hind limbs were unloaded. The rats were totally free to move around in the cage throughout the course of suspension.

Rats from the second group were perfused with 500 ml of 0.9% saline. Following perfusion, the brain was immediately removed and the brain tissue of PPC and LGN were quickly separated on ice. All of the extraction procedures were performed on ice. The tissues were subsequently preserved at -80°C and prepared for immunoblotting.

TUNEL assay

A TUNEL assay was performed to visualize apoptotic cells using an in Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the protocol given by the manufacturer. Briefly, the brain sections were washed in PBS and then incubated with proteinase K (20 μg/mL) for 15 min at room temperature. Sections were treated in 3% H2O2 and then incubated in terminal deoxynucleotidyl transferase equilibrium buffer for 30 min at room temperature. Next, sections were incubated with labeling reaction mixture for 60 min in a humidified atmosphere at 37°C in the dark. Negative controls were performed with the absence of terminal deoxynucleotidyl transferase. All of the sections were counterstained with 100 ng/mL 4, 6-diamidino-2-phenyl-indole (DAPI, Boster, China) in PBS for 30 min to visualize the nuclei. Sections were then washed and cover-slipped. Stained sections were observed with a LSM5 EXCITER (Carl-Zeiss, Germany), and images were captured. For each slide, six fields were randomly selected in the PPC and LGN to quantify TUNEL-positive cells (green) and nuclei (blue), and to calculate the positive apoptotic rate (i.e., ratio of apoptotic cells to the total nuclei). Finally, overall mean apoptotic rate for each rat was calculated, and mean apoptotic rate of for group was calculated and compared.

Immunohistochemical technique

Brain sections were immersed in 0.3% TritonX-100 (Amresco) for 5 min and then incubated in 3% H2O2 (Histostain – Plus Kits, ZYMED) for 10 min to reduce endogenous peroxidase activity. Next, sections were blocked with normal goat serum (Histostain – Plus Kits, ZYMED) for 20 min, then incubated with anti-c-Fos antibodies (rabbit; 1:300; Abcam) overnight at 4°C. Sections were them incubated in secondary antibodies (Goat, Histostain – Plus Kits, ZYMED) for 40 min at 37°C. After washing with PBS, the sections were then incubated in peroxidase-labeled streptavidin (Histostain – Plus Kits, ZYMED) for 40 min at 37°C. Then, the sections were reacted with nickel-enhanced diaminobenzidine (DAB) to

visualize the immunoreactive nuclei. Following these procedures, the sections were air-dried, dehydrated using sequential washes in ethanol and xylene, and cover-slipped. Negative controls were run by omitting the primary antibody, which resulted in complete absence of nuclear staining. Sections were mounted and observed using an optical microscope. For each slide, six fields were randomly selected in the PPC and LGN to quantify the number of c-Fos-positive cells under high magnification (400×). Finally, mean number of c-Fos-positive cells within each group was calculated and compared between groups.

Western blot

Brain tissue was treated in lysis buffer (50M Tris-HCl, pH 7.6, 0.5% Triton X-100, and 20% glycerol) (Sigma) and subsequently centrifuged for 15 min at 15,000 g at 4 °C. Then, the supernatant was acquired and boiled for 15 min. Protein concentration was estimated using the Bradford method. Proteins were separated with SDS–polyacrylamide gel electrophoresis and then transferred onto nylon membranes. The membranes were blocked in PBS containing 5% nonfat dried milk for 1 h at room temperature and subsequently incubated with specific antibodies for cleaved caspase 3 (17 kDa, Rabbit; 1:1000; Cell Signaling, USA), caspase 3 (35 kDa, Rabbit; 1:1000; Cell Signaling), c-Fos (rabbit; 1:200; abcam), PI3K (Rabbit; 1:100; Santa Cruze), p-Akt (Rabbit; 1:1000; Cell Signaling), Akt (Rabbit; 1:1000, Cell Signaling) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Rabbit; 1:1000, Cell Signaling) overnight at 4°C. Then, membranes were washed in Tris Buffered Saline with Tween (TBST) buffer, and then incubated with HRP-conjugated secondary antibody (Goat; 1:2000; Santa Cruz Biotechnology Inc) for 1 h at room temperature. GAPDH was used as an internal control. Immunoreactive bands were visualized with an ECL system. All blots were scanned with a GS-800 Calibrated Imaging Densitometer (Bio-Rad), and a densitometric analysis was conducted with QuantityOne software. Activity of p-Akt was determined by the ratio of p-Akt/total Akt, and activity of cleaved caspase 3 was determined by the ratio of cleaved caspase 3/total caspase 3. Other proteins were expressed as relative density as compared to GAPDH.

Statistical analysis

All data were analyzed with SPSS 19.0 software and evaluated using one-way ANOVA (Group). For significant main effects, Fishers LSD-t test was subsequently used to assess the differences between individual groups. A value of $P<0.05$ was considered statistically significant in all tests. All data are expressed as mean ± standard error of the mean (SEM).

RESULTS

Simulated weightlessness leads to transitional apoptosis in the PPC and LGN

The number of rats successfully dyed by TUNEL method were 27 for the PPC, and 36 for the LGN. The mean percentage of TUNEL positive cells for thirty pictures randomly selected five slides per rat was calculated and compared between groups. Confocal micrograph showed a significance difference in TUNEL positive cells among the four groups in the PPC ($F_{3,23}=4.03$, $P=0.019$) as well as in the LGN ($F_{3,32}=7.30$, $P<0.001$). There were a few TUNEL positive cells observed in the Con condition (0.74±0.33%). In the 7 d exposure group, there was a higher number of TUNEL positive cells as compared to controls (5.31±1.62%, $P<0.05$, Fig. 2A, C). However, the number of TUNEL positive cells was lower in the 14 d and 28 d exposure groups, with an average of 2.31±0.94% and 1.11±0.25%, respectively. In the LGN, the percentages of TUNEL positive cells in the Con, 7 d, 14 d and 28 d exposure groups were 0.99±0.35%, 3.29±0.54%, 1.40±0.38% and 0.99±0.33%, respectively. The number of TUNEL positive cells was higher in 7 d group compared to the control group ($P<0.01$, Fig. 2B, D), and again, levels were lower in the 14 d and 28 d exposure groups.

Western blotting was successfully performed in 37 rats for the PPC analysis, and 48 rats for the LGN. Western blot analysis showed a significant main effect of group for ratio of cleaved caspase 3 / total caspase 3 in the PPC ($F_{3,33}=8.36$, $P< .01$) as well as in the LGN ($F_{3,44}=7.31$, $P<0.001$). Active caspase 3 was higher in the 7 d exposure group as compared to the control group in the PPC and the LGN (PPC: $P<0.001$, LGN: $P<0.01$, Fig. 3A, B). Active caspase 3 expression did not differ between Con, 14 d, and 28 d groups.

Increased c-Fos expression in the PPC during simulated weightlessness

Immunohistochemical analyses were performed successfully dyed in 40 rats for the PPC, and 36 for the LGN. The mean number of c-Fos positive cells was calculated using thirty pictures randomly selected from five slides per rat, and subsequently compared between groups. Photomicrograph results suggested a significant main effect of group for number of c-Fos positive cells in the

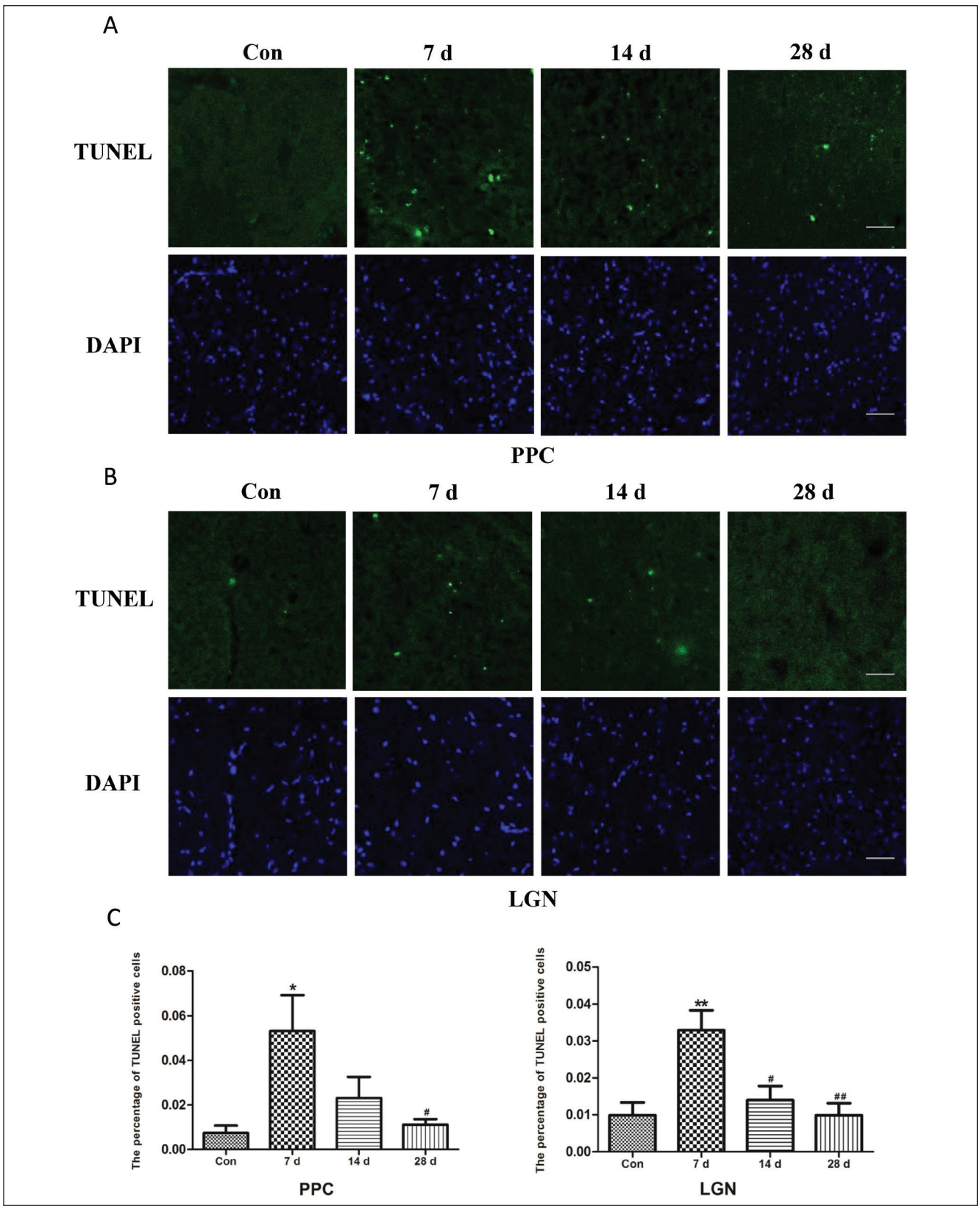


Fig. 2. TUNEL staining in PPC and LGN during simulated microgravity. (A) and (B) A few of TUNEL positive cells was showed in 7 d group. Scale bar=100 μm. (C) Graphical representation of the percentage of TUNEL positive cells in PPC and LGN. Values presented are mean ± SEM (n=6–10 per group). *$P < 0.05$, **$P<0.01$, compared with the Con group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the 7 d group.

PPC ($F_{3,36}$=7.44, P<0.001). Follow-up analyses showed a few dark brown c-Fos positive cells scattered in the PPC of the Con, 14 d and 28 d exposure groups, whereas there was a significantly higher number of c-Fos positive cells in 7 d group as compared to the other groups (Fig. 4A, B, P<0.001 compared to control and P<0.01 compared to 14 d and 28 d groups).

Western blot analyses were successfully performed in 37 rats for the PPC and 48 rats for the LGN. Western blot analysis also showed a significant main effect in the PPC ($F_{3,33}$=9.13, P<0.001). C-Fos protein levels were higher in the 7 d exposure group as compared to the control group (P<0.01, Fig. 4C). C-Fos levels were lower in the 14 d group as compared to the 7 d group (P<0.05, Fig. 4C). Of note, there were no group differences in c-Fos expression in the LGN.

Simulated weightlessness represses the PI3K/Akt signaling pathway in the LGN

Western blot analyses were performed successfully in 41 rats for PI3K detection and 37 rats for Akt detection. Western blot analysis showed a main effect of group on PI3K ($F_{3,37}$=5.83, P=0.002) and p-Akt ($F_{3,33}$=4.70, P=0.008) expression in the LGN. Follow-up analyses demonstrated a repression of PI3K in the 7 d group as compared to the other three groups (P<0.05). Similar effects were observed for p-Akt expression (P<0.05). Expression levels were higher in the 14 d group, and were comparable to levels observed in the control group after 28 d exposure to simulated microgravity (Fig. 5).

DISCUSSION

The current study documented an increase in the number of apoptotic cells in the PPC and LGN after 7 days of simulated microgravity as compared to control. Interestingly, there was no difference in number of apoptotic cells between the 14 d group, 28 d group and control group. These results suggest that short-term simulated microgravity induces neural cell apoptosis in brain regions implicated in visuo-motor functions. Previous studies have also reported neural cell apoptosis following simulated microgravity (Pani et al. 2013, Sun et al. 2009, Uva et al. 2002). In particular, studies of short- and middle-term simulated microgravity have reported a subsequent shrinkage of brain cells *in vitro*, a reduction of cell locomotion, and a significant increase in apoptosis, followed by fast recovery processes (Meloni et al. 2006, 2011, Pani et al. 2013). These data indicate a CNS adaptation period in the first days of simulated microgravity, which result

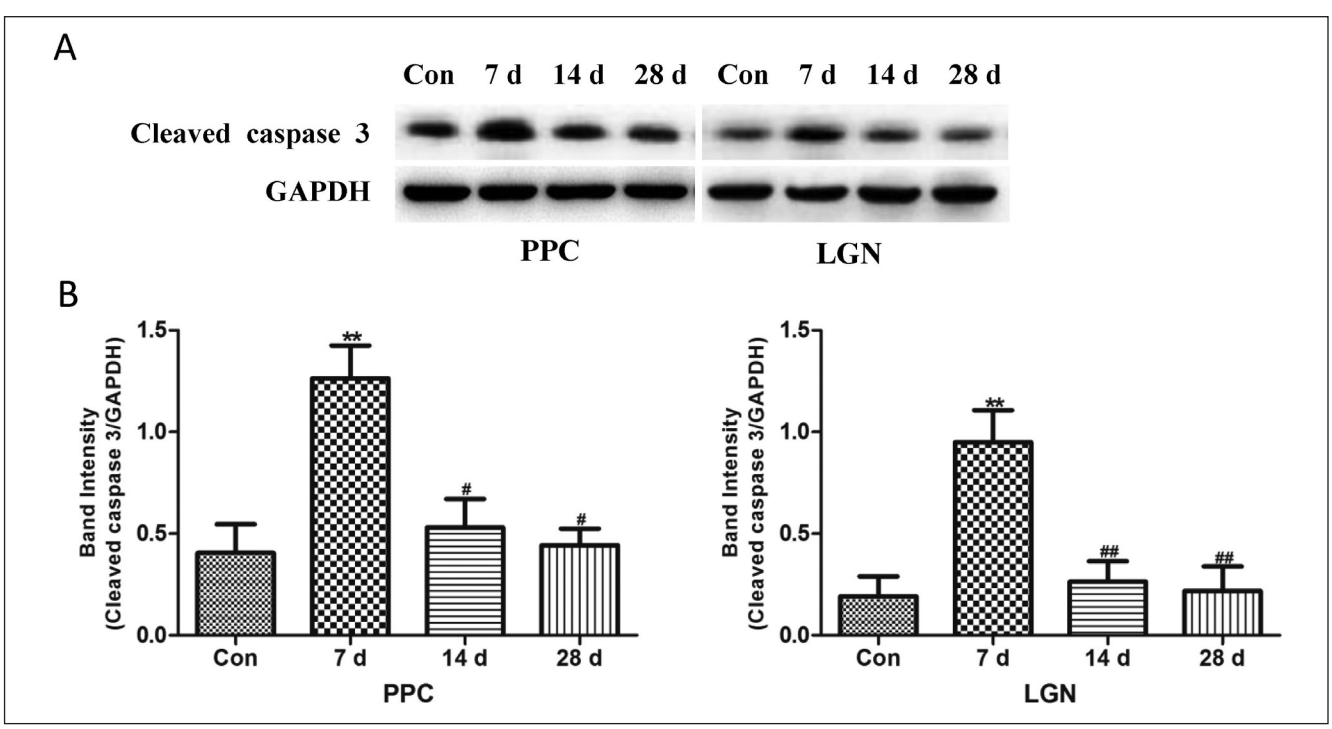
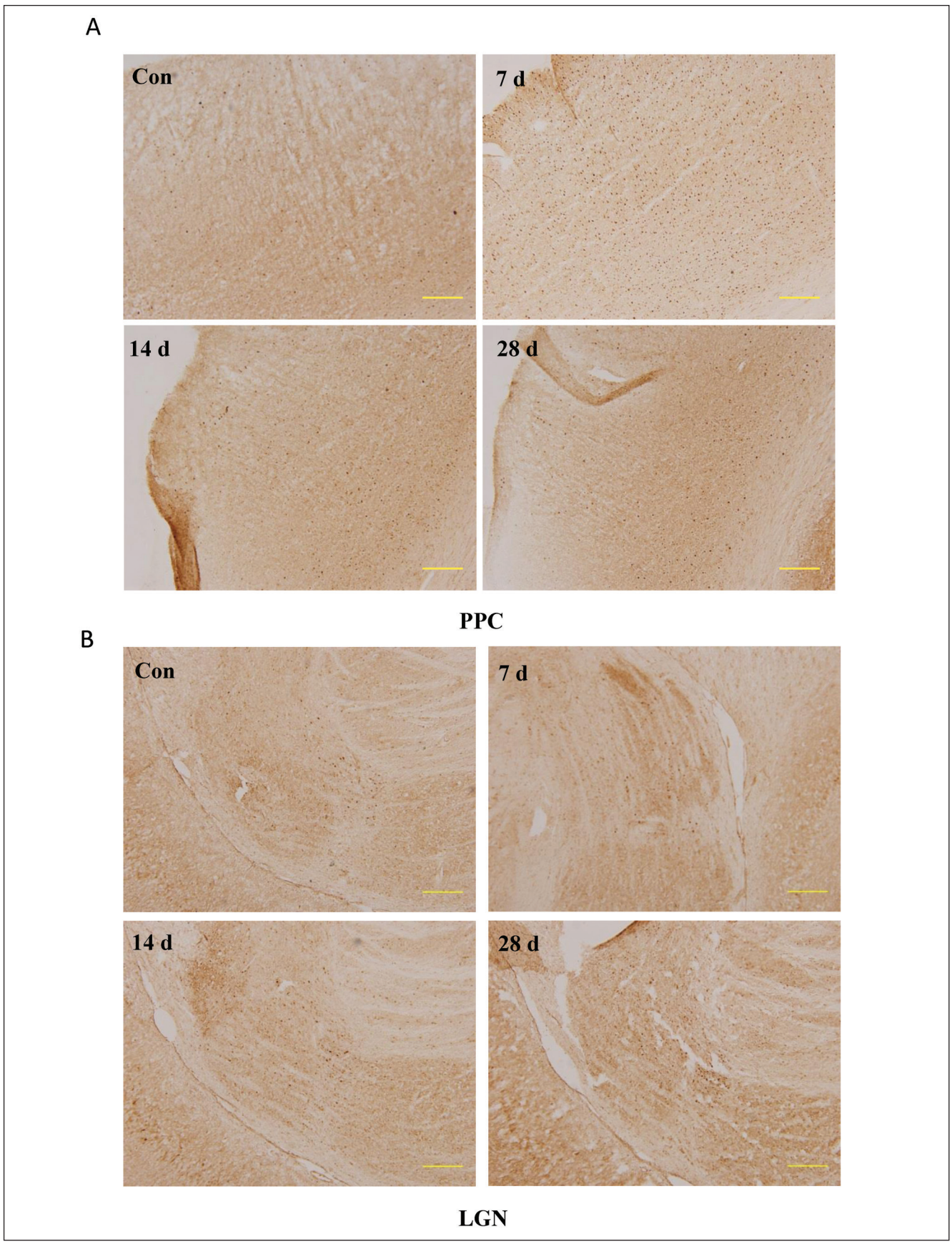


Fig. 3. Western blot analysis of cleaved caspase 3 in PPC and LGN during simulated microgravity. (A) Cleaved caspase 3 protein level increased in 7 d group and decreased afterwards in PPC and LGN. (B) Densitometric analysis of the ratio of cleaved caspase 3/GAPDH in PPC and LGN. Values presented are mean ± SEM (n=9–12 per group). **P<0.01, ***P<0.001, compared with the Con group; ##P<0.01, compared with the 7 d group.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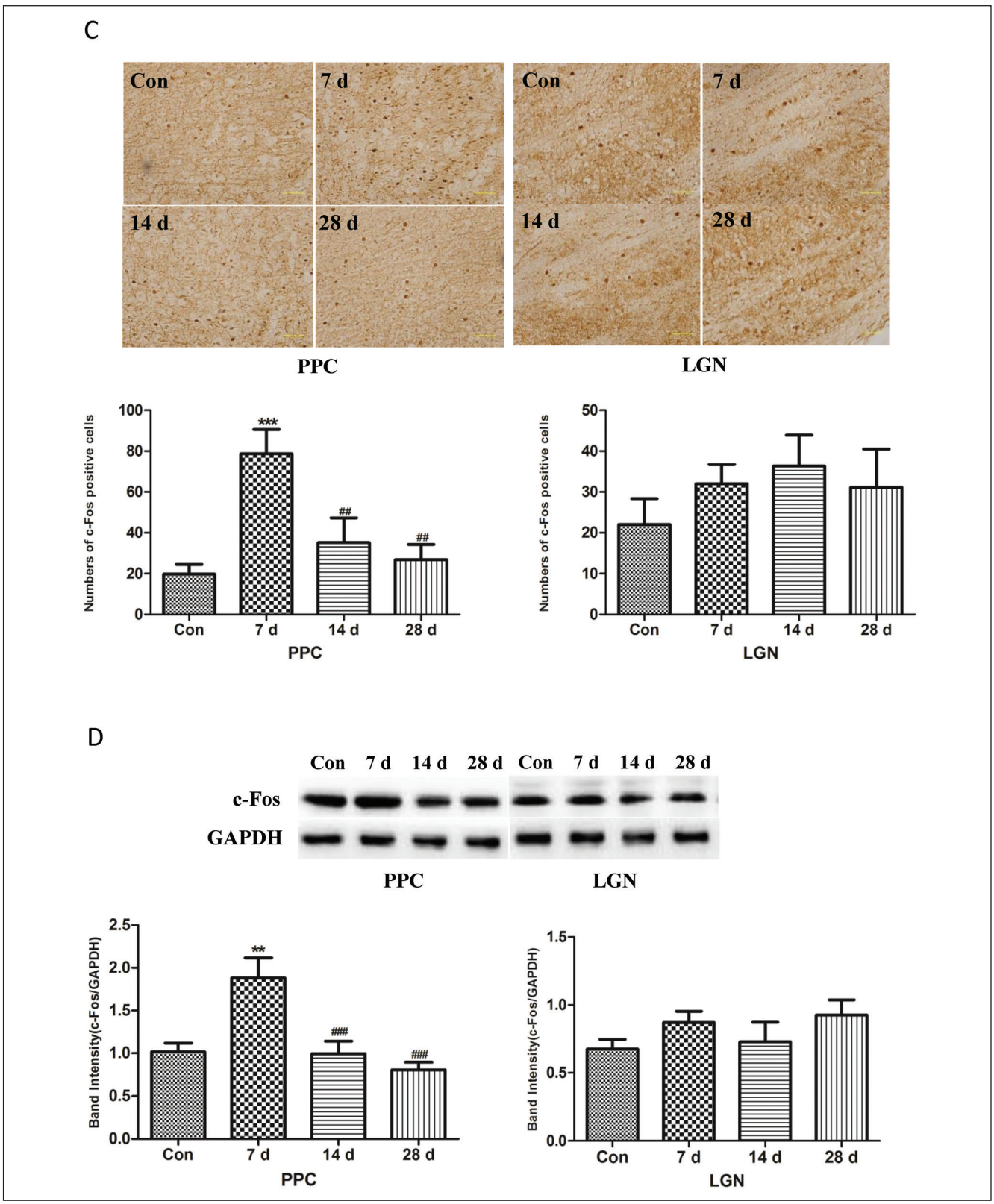

Fig. 4. C-Fos protein expression in PPC and LGN during simulated microgravity. (A) and (B) Immunohistochemistry of c-Fos protein in PPC and LGN. Scale bar=200 μm. (C) Graphical representation of the number of c-Fos positive cells. Values presented are mean ± SEM (n=9–12 per group). Scale bar=50 μm. (D) Western blot analysis of the c-Fos protein expression in PPC and LGN. Values presented are mean ± SEM (n=9–10 per group). $^{**}P<0.01$, $^{***}P<0.001$, compared with the Con group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared with the 7 d group.

in a new equilibrium. Hence, the cell apoptosis we observed following the 7-day simulated microgravity treatment may reflect a self-adaptive reaction to a new environment that functions to eliminate nonfunctional neural cells and connections, and re-establish new neuronal connections. Consistent with this interpretation, cell apoptosis is thought to play a key role in self-adaptive mechanisms by eliminating mutated cells when exposed to a new environment, and allowing for tissue to be repopulated with the progeny of more resistant cells (Krasnov 1994, Raff et al. 1993, Thompson 1995, Vaux et al. 1994).

Our results showed that the number of c-Fos positive cells increased after 7 days of simulated weightlessness as compared to the control group, and then dropped off again after 14 days. These results are consistent with previous studies that similarly demonstrated an induction of c-Fos protein by exposure to simulated microgravity in some brain region (Sarkar et al. 2006, Su et al. 2000). Importantly, the observed time-course of elevated c-Fos expression was consistent with the time-course of cell apoptosis in the PPC. Based on these data, we hypothesized that c-Fos protein plays a vital role in the activation of apoptosis in the PPC. Several lines of evidence suggest that c-Fos protein cab act as a proapoptotic agent in various kinds of cell types, including nerve cells (Asim et al. 2010, Preston et al. 1996). Given that c-Fos accumulation is considered to be a hallmark of cell death (Piechaczyk and Blanchard 1994, Zhang et al. 2007), we hypothesized that the elevated c-Fos protein expression was related to cell apoptosis in the PPC. However, how c-Fos protein induces apoptosis or which apoptotic pathway is activated by c-Fos protein in simulated microgravity is still unclear. This is a future direction for our research. At the same time, however, simulated microgravity had no apparent effect on c-Fos expression in the LGN. Rather, there was a repression within the PI3K/Akt signaling pathway in the LGN after 7 days of simulated microgravity. This repression is in line with a previous study showing that tail suspension down-regulates brain IGF-1 levels, and thus down-regulates activity in the PI3K/Akt signaling pathway in the sensorimotor cortex and striatum (Mysoet et al. 2014). Of note, the PI3K/Akt signaling pathway is known to modulate cell survival and proliferation. Based on results of a previous study (Jie et al. 2015), it is possible that repression of the PI3K/Akt signaling pathway contributes to the activation of cell apoptosis in the LGN. However, another study found no effet of hindlimb unloading on the expression of most genes, including apoptotic factors (*Bcl-xl, Bax*) (Kulikova et al. 2017). These discrepant results may be due to evaluation of different genes and different brain regions.

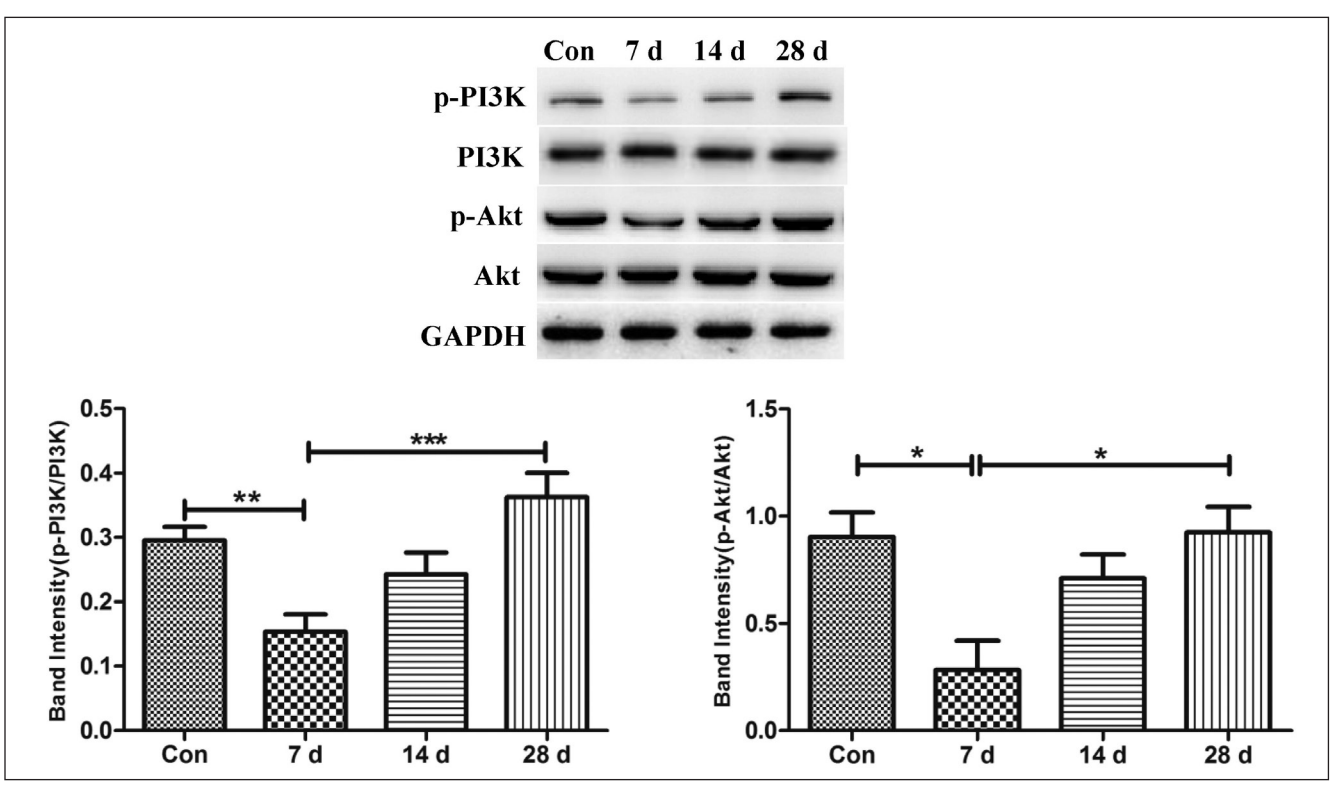

Fig. 5. Western blot analysis of p-PI3K and p-Akt expression in LGN during simulated microgravity. The ratio of p-PI3K/PI3K and p-Akt/Akt were decreased in 7 d group, and slowly rose up afterward. Values presented are mean ± SEM (n=9–11 per group). *P<0.05, **P<0.01.

The data we present here must be interpreted with appropriate caution, given the following limitations of our study. First, the tail-suspension rat model used in this study was created to not only simulate microgravity conditions, but also to induce stress. Stress may also induce cell damage in the brain. However, evidence from real spaceflight supports the notion that real microgravity results in dysregulation in genetic control of neuronal apoptosis, which may be a risk factor for spaceflight-induced behavioral abnormalities (Naumenko et al. 2015). Data from spaceflight also suggest spaceflight-induced alteration in cerebral arteries elevate partial pressure of CO_2, which could induce regional hypoxia and apoptosis in brain (Nadia et al. 2015, Taylor et al. 2013). In addition, several functional magnetic resonance imaging (fMRI) studies have linked a real microgravity environment with lower gray matter volume, as well as in vestibular connectivity in the human brain (Demertzi et al. 2016, Koppelmans et al. 2016). These findings suggest that a simulated microgravity might be the most probable reason for the observed decline in brain function apoptosis of neurons in our study. Nonetheless, future studies should consider adding a positive control group which only immobilizes but not suspends the rats with their tails to measure the effects of stress. Another limitation of the study is that we did not test visuomotor functioning of rats after simulated microgravity, which would allow us to test whether visuomotor ability is related to the level of neural cell apoptosis in the LGN and/or PPC. However, because tail-suspension rat model caused hind limbs muscle atrophy, it would be difficult to compare performance in the visuomotor test between tail-suspended and control rats, whose muscles were not harmed.

In conclusion, our data show that short-term simulated microgravity induces cell apoptosis in the PPC and the LGN, which may reflect a neural adaptive process. The observed overexpression of c-Fos protein, and a concurrent repression of the PI3K/Akt signaling pathway, may contribute to the activation of cell apoptosis in the PPC and LGN.

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