Repulsive guidance molecule a suppresses seizures and mossy fiber sprouting via the FAK-p120RasGAP-Ras signaling pathway

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Abstract. Repulsive guidance molecule a (RGMa) is a membrane-associated glycoprotein that regulates axonal guidance and inhibits axon outgrowth. In our previous study, we hypothesized that RGMa may be involved in temporal lobe epilepsy (TLE) via the repulsive guidance molecule a (RGMa)-focal adhesion kinase (FAK)-Ras signaling pathway. To investigate the role of RGMa in epilepsy, recombinant RGMa protein and FAK inhibitor 14 was intracerebroventricularly injected into a pentylenetetrazol (PTZ) kindling model and Timm staining, co-immunoprecipitation and western blotting analyses were subsequently performed. The results of the present study revealed that intracerebroventricular injection of recombinant RGMa protein reduced the phosphorylation of FAK (Tyr397) and intracerebroventricular injection of FAK inhibitor 14 reduced the interaction between FAK and p120GAP, as wells as Ras expression. Recombinant RGMa protein and FAK inhibitor 14 exerted seizure-suppressant effects; however, recombinant RGMa protein but not FAK inhibitor 14 suppressed mossy fiber sprouting in the PTZ kindling model. Collectively, these results demonstrated that RGMa may be considered as a potential therapeutic agent for epilepsy, and that RGMa may exert the aforementioned biological effects partly via the FAK-p120GAP-Ras signaling pathway.

Introduction

Temporal lobe epilepsy (TLE) is the most common form of adult epilepsy (1). Mossy fiber sprouting (MFS) has been observed in experimental models of TLE and in the epileptic human hippocampus (2-5); however, the mechanisms underlying this structural alteration are not fully understood. Previously, it was demonstrated that axon guidance molecules served important roles in epilepsy (6).

The repulsive guidance molecule (RGM) was originally reported as a membrane-bound protein involved in guiding axons in the developing chick retina (7). As a homolog of RGM, RGMa is mainly expressed in the central nervous system (CNS), particularly in the hippocampus (8). Previous studies have revealed that RGMa served a critical role in neural circuit formation, potentially via focal adhesion kinase (FAK) dephosphorylation at Tyr397 (9-11). Dephosphorylation of FAK (Tyr397) inhibits the interaction between p120GAP-FAK and promotes the interaction between p120GAP and GTP-Ras, downregulating the activation of Ras; p120GAP has been reported a Ras-specific GTPase-activating protein (11). In our previous study, we hypothesized that RGMa may be involved in TLE via the RGMa-FAK-Ras signaling pathway (12); however, the exact role of RGMa and FAK in epileptogenesis and MFS remains unclear. The present study aimed to investigate whether RGMa and FAK dephosphorylation (Tyr397) inhibits epileptogenesis and MFS *in vivo*.

Materials and methods

Intracerebroventricular injection. The present study was approved by the Ethics Committee of Xiangya Hospital of Central South University (Changsha, China). Rats were anesthetized with 10% chloral hydrate (300 mg/kg) intraperitoneally and positioned in a stereotaxic frame (RWD Life Science Co., Ltd., Shenzhen, China). A stainless-steel guide cannula (RWD Life Science Co., Ltd.) was inserted into the lateral ventricle at the following coordinates relative to the Bregma: Antero-posterior-0.9 mm, medio-lateral-1.4 mm and dorso-ventral-3.3 mm. The guide cannula was fixed into the skull with adhesive and dental acrylic cement. A stainless steel cannula served as an injection cannula that was connected via a polyethylene tube to a microsyringe, which was inserted via the guide cannula and extended 2 mm beyond the tip of the guide cannula to reach the lateral ventricle. Following surgery, the rats were allowed to recover for 1 week. Recombinant RGMa protein (R&D Systems, Inc., Minneapolis, MN, USA) dissolved in PBS (0.04 μg/μl), and FAK inhibitor 14 (R&D Systems, Inc.) dissolved in PBS (0.04 mg/μl) (13) were administrated intracerebroventricularly at a volume of 10 μl every 3 days; control rats were injected with PBS.

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Animals and the pentylenetetrazol (PTZ) model. Male Sprague-Dawley rats (n=200; age, 40-45 days-old; weight, 120-180 g) were purchased from the Center for Experimental Animals of Central South University (Changsha, China). Rats were housed under controlled conditions (18-25˚C; 50-60% humidity; 12 h light/dark cycle) with food pellets and water available *ad libitum*. A total of 50 rats were randomly divided into the control (n=15) and PTZ groups (n=35). The PTZ kindling model was established as previously described (12). Briefly, the rats in the PTZ group received an intraperitoneal dose of 30 mg/kg PTZ (10 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) once daily until the rats were kindled or sacrificed. If persistent generalized tonic-clonic seizure lasted ≥20 sec, an intraperitoneal injection of 300 mg/kg chloral hydrate was administered. Following seizure termination, if the rat breathed irregularly, had no autonomous respiration or death appeared imminent, it was sacrificed immediately. Rats in the control group were injected with an equal dose of saline (3 ml/kg). Rats were scored according to the Racine scale: Score 0, behavior arrest; score 1, facial twitches; score 2, chewing and/or head nodding or 'wet dog' shakes; score 3, forelimb clonus; score 4, rearing, falling on forelimbs; score 5, imbalance and falling on side or back (14). A score ≥3 for 5 consecutive days indicated that the rat was kindled. At day 3, and 1, 2, 4 and 6 weeks following the first injection, the rats were sacrificed for co-immunoprecipitation (Co-IP) analysis, Timm staining and western blot analysis. Rats in the day 3 and 1 week group were administrated PTZ/saline daily until they were sacrificed on day 3 and at the end of week 1, respectively. For the 2 weeks, 4 weeks and 6 weeks group, rats were only administrated PTZ/saline daily until they were kindled and then sacrificed at each time-point. If the rat was not kindled, PTZ/saline was administered daily until they were sacrificed at each time-point. A total of 150 rats were divided into experimental and intervention groups. The experimental group (PTZ + PBS) received PTZ intraperitoneally and PBS intracerebroventricularly (n=39, with the exception of 10 rats that succumbed to mortality due to persistent generalized tonic-clonic seizure, and 1 rat that was not kindled). The intervention group were divided into two subgroups, receiving PTZ intraperitoneally and recombinant RGMa protein (PTZ + RGMa; n=36, with the exception of 9 rats that succumbed to mortality due to persistent generalized tonic-clonic seizure, and 5 rats that were not kindled) or FAK inhibitor 14 intracerebroventricularly (PTZ + FAK inhibitor 14; n=39, with the exception of 7 rats that succumbed to mortality due to persistent generalized tonic-clonic seizure, and 4 rats that were not kindled), respectively. The rates of mortality seen in the present study were similar to previous studies (15-17). In our previous study, it was observed that the PTZ group rats were kindled in accordance with the kindling criterion at 23.6±2 days following PTZ injection, and the expression levels of RGMa, FAK (Tyr397) and Ras were the lowest or peaked at 4 weeks (12). The present study was approved and conducted in accordance with the guidelines of the Animal Ethics Committee of Central South University (Changsha, China). All efforts were made to minimize the number of animals employed and their suffering in the present study.

Timm staining. The method of Timm staining was the conducted as previously described (12). In brief, rats were injected with 10% chloral hydrate (300 mg/kg) and perfused intracardially with 300 ml saline, followed by 200 ml 0.4% sodium sulfide in 0.1 M phosphate buffer and 200 ml 4% paraformaldehyde (PFA) at 4˚C. The brains were removed, fixed in 4% PFA at 4˚C overnight and in a 30% solution of sucrose in fixative. Coronal sections (30 μm) were mounted on slides, air-dried and developed for 90 min in the dark at 26˚C in 60 ml gum arabic (50%), 10 ml citrate buffer (2 M), 30 ml hydroquinone (0.5 M) and 0.5 ml silver nitrate (17%). The inner molecular layer of the dentate gyrus, as well as the pyramidal and infrapyramidal CA3 region were analyzed using three randomly chosen fields under an optical microscope (magnification, x200).

Western blot analysis and Co-IP assay. At the various time-points, rats were deeply anesthetized with chloral hydrate and sacrificed as aforementioned. Proteins of the hippocampus were extracted using radioimmunoprecipitation assay lysate buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentration was determined using a bicinchoninic acid protein assay. For western blotting, equal amounts of protein (40 μg) were subjected to 10% SDS-PAGE. The separated proteins were electro-transferred onto 0.45 or 0.22 μm polyvinylidene difluoride (PVDF) membranes (Pall Life Sciences, Port Washington, NY, USA). The membranes were blocked with 5% dried skim milk in TBS at room temperature for 2 h and were then incubated with the following primary antibodies overnight at 4˚C: Anti-RGMa polyclonal antibodies (1:800; cat. no. ab26287; Abcam, Cambridge, UK), anti-FAK Tyr397 polyclonal antibodies (1:500; cat. no. ab81298; Abcam), anti-FAK polyclonal antibodies (1:1,000; cat. no. sc-558; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Ras polyclonal antibodies (1:500; cat. no. sc-166691; Santa Cruz Biotechnology, Inc.) and anti-GAPDH monoclonal antibodies (1:1,000; sc-66163, Santa Cruz Biotechnology, Inc.). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit/anti-mouse secondary antibodies (1:2,000; cat. no. A0208/A0216; Beyotime Institute of Biotechnology) for 1 h at room temperature. The immunoreactive bands were visualized via enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol, and quantified using Image Lab version 4.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For Co-IP, antibodies were applied to lysates (100-500 μg total protein) plus 1-2 μg rabbit anti-rat FAK polyclonal antibody (cat. no. sc-558; Santa Cruz Biotechnology, Inc.) for 4.5 h at 4˚C and collected by binding to protein G plus- or protein A-agarose beads and washed in lysis buffer (Beyotime Institute of Biotechnology) without SDS and sodium deoxycholate. The precipitated proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes followed by immunoblotting using anti-p120GAP antibodies (cat. no. sc-63; 1:500; Santa Cruz Biotechnology, Inc.) as aforementioned. Experiments were independently repeated three times.

Statistical analysis. The results were presented as the mean ± standard deviation. Analysis was performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was assessed using one-way analysis of variance

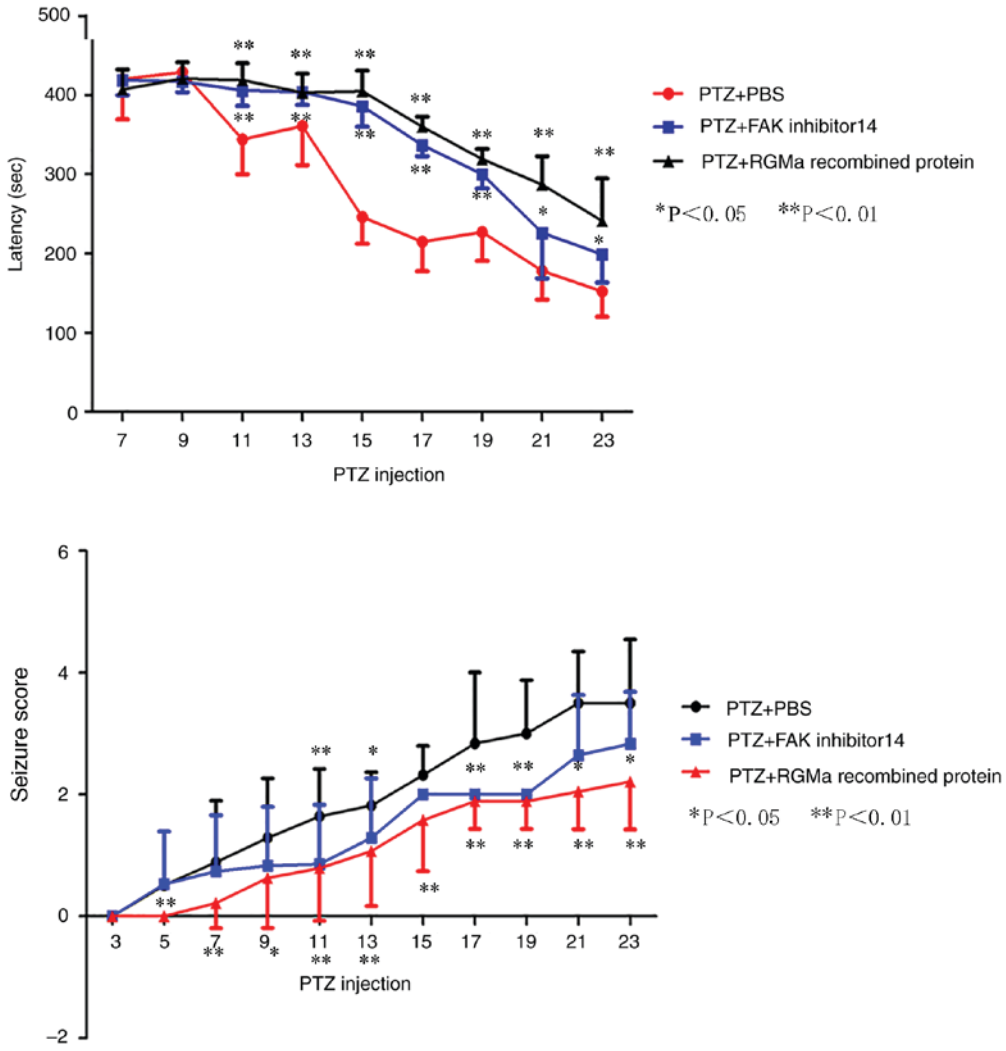

Figure 1. Seizure latency and severity score in PTZ + PBS, PTZ + RGMa and PTZ + FAK inhibitor 14 groups. Intracerebroventricular injection of recombinant RGMa protein and FAK inhibitor 14 increased seizure latency and reduced seizure severity. FAK, focal adhesion kinase; PTZ, pentylenetetrazol; RGMa, repulsive guidance molecule a.

followed by the Least Significant Difference post-hoc test for multiple group comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Behavioral outcomes. To determine the exact role of RGMa and FAK (Tyr397) in the PTZ model, the seizure latency and severity was assessed. With the exception of 10 rats that succumbed to mortality due to persistent generalized tonic-clonic seizure, and 1 rat that was not kindled, rats of the PTZ + PBS group developed seizure activities of varying degrees following continuous PTZ injections for 19-25 days (an average of 22.06±2.32 days) and the success rate was 92.3% (kindled/surviving rats). The PTZ-induced seizure activity was observed to occur 2-8 min following treatment (an average of 420±50.73 sec at 7 days, 429±11.57 sec at 9 days, 344±43.86 sec at 11 days, 361±49.47 sec at 13 days, 246±33.76 sec at 15 days, 215±37.15 sec at 17 days, 227±36.29 sec at 19 days, 178±36.33 sec at 21 days and 152±31.99 sec at 23 days following treatment) (Fig. 1). No epileptic-associated behavior was noted in the control rats.

Similar to the PTZ + PBS group, the seizure severity of the PTZ + RGMa and PTZ + FAK inhibitor 14 groups gradually increased following continuous PTZ administration; however, in the PTZ + RGMa group (with the exception of 9 rats that had succumbed to mortality due to persistent generalized tonic-clonic seizure, and 5 rats that were not kindled), compared with in the PTZ + PBS group, the average kindling duration significantly increased to 29.0±3.13 days (P<0.01; data not shown) and the success rate of PTZ kindling model significantly decreased to 52.6% (P<0.05). The seizure latency was significantly increased from 11 days post-PTZ administrationexhibited no significant difference compared with that of the PTZ + PBS group. The results indicated that intracerebroventricular injection of recombinant RGMa protein and FAK inhibitor 14 exhibited an increase in seizure latency and decreased seizure severity scores (Fig. 1).

Intracerebroventricular injection of recombinant RGMa protein reduces the phosphorylation of FAK (Tyr397), the expression of Ras and suppresses MFS. As reported in our previous study (11), in the PTZ + PBS group, the expression of FAK (Tyr397) increased from 1 week and peaked at 2 weeks, then declined at

Table I. Relative expression of FAK (Tyr397) in PTZ + PBS group, PTZ + RGMa group and PTZ + FAK inhibitor 14 group.

Group	3 days	1 week	2 weeks	4 weeks
PTZ + PBS	0.111±0.012	0.250±0.018	0.550±0.031	0.359±0.045
PTZ + RGMa	0.100±0.002	0.198±0.014[a]	0.343±0.009[a]	0.248±0.012[a]
PTZ + FAK inhibitor 14	0.084±0.009[a]	0.133±0.004[a]	0.128±0.003[a]	0.116±0.004[a]

[a]P<0.01 vs. PTZ + PBS group. PTZ, pentylenetetrazol; RGMa, repulsive guidance molecule a.

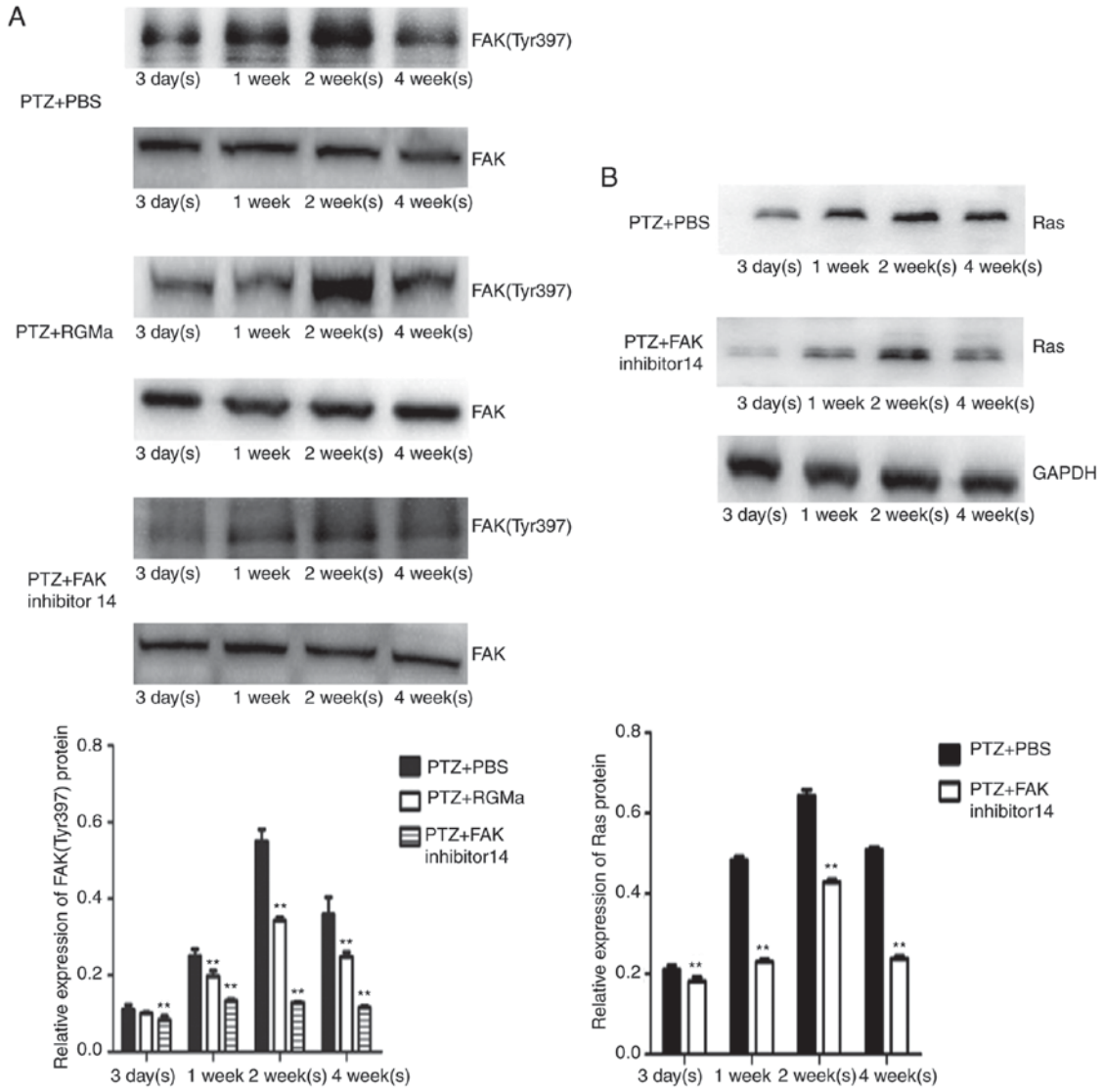

Figure 2. Phosphorylation of FAK (Tyr397) and expression of Ras as determined by western blot analysis. (A) Phosphorylation of FAK (Tyr397) in the PTZ + PBS, PTZ + RGMa and PTZ + FAK inhibitor 14 groups. (B) Expression of Ras in the PTZ + PBS and PTZ + FAK inhibitor 14 group. Data are presented as the mean ± standard deviation **P<0.01 vs. PTZ + PBS group. FAK, focal adhesion kinase; PTZ, pentylenetetrazol; RGMa, repulsive guidance molecule a.

4 weeks following PTZ administration; however, high phosphorylation levels were maintained (Fig. 2A; Table I). Conversely, in the PTZ + RGMa and PTZ + FAK inhibitor 14 groups, the phosphorylation levels of FAK (Tyr397) were significantly decreased at all investigated time-points (P<0.01) except at 3 days compared with the PTZ + PBS group (Fig. 2A; Table I).

The expression of Ras also increased at weeks 1 and 2, but decreased at 4 weeks (Fig. 2B); the PTZ + FAK inhibitor 14 group exhibited a similar phosphorylation profile to that of the PTZ + PBS group; however, the expression levels of Ras were significantly reduced (Fig. 2B).

Similar to the PTZ + PBS group (Fig. 3A), the PTZ + RGMa group (Fig. 3B) at day 3 and 1 week following PTZ administration, exhibited weak black Timm staining in the stratum pyramidale of the CA3 region. During the progression of PTZ-induced kindling, MFS in the PTZ + RGMa group rats was

Table II. Timm score analysis in PTZ + PBS group, PTZ + RGMa group and PTZ + FAK inhibitor 14 group.

Group	3 days	1 week	2 weeks	4 weeks	5 weeks
PTZ + PBS	1.00±0.00	2.20±0.45	3.20±0.84	4.25±0.50	4.5±0.58
PTZ + RGMa	0.75±0.50	1.66±0.58	1.75±0.50[a]	2.50±0.58[a]	3.0±0.71[a]
PTZ + FAK inhibitor 14	1.00±0.00	1.50±0.58	2.25±0.50	2.75±0.50[a]	4.00±0.82

[a]P<0.05 vs. PTZ + PBS group. PTZ, pentylenetetrazol; RGMa, repulsive guidance molecule a.

Figure 3. Timm staining in the CA3 region in PTZ + PBS, PTZ + RGMa and PTZ + FAK inhibitor 14 groups. (A) Timm granules in PTZ + PBS group in the CA3 areas. (B and C) Timm granules in PTZ + RGMa and PTZ + FAK inhibitor 14 group in the CA3 area, respectively. Magnification, x200. FAK, focal adhesion kinase; PTZ, pentylenetetrazol; RGMa, repulsive guidance molecule a.

notably suppressed than that in PTZ + PBS group. Timm score analysis demonstrated that the PTZ + RGMa group possessed a low density band of Timm staining at 2, 4 and 5 weeks (Table II). The results indicated that recombinant RGMa protein may reduce the phosphorylation of FAK (Tyr397) and suppress MFS. Whether inhibited FAK (Tyr397) phosphorylation may suppress MFS requires further investigation. In contrast to the PTZ + RGMa group, black Timm staining gradually increased at 2 weeks (Fig. 3C) and significantly increased at 4 weeks in PTZ + FAK inhibitor 14 group compared with in the PTZ + PBS group (Table II), there was no significant difference compared with in PTZ + PBS group except at 2 weeks.

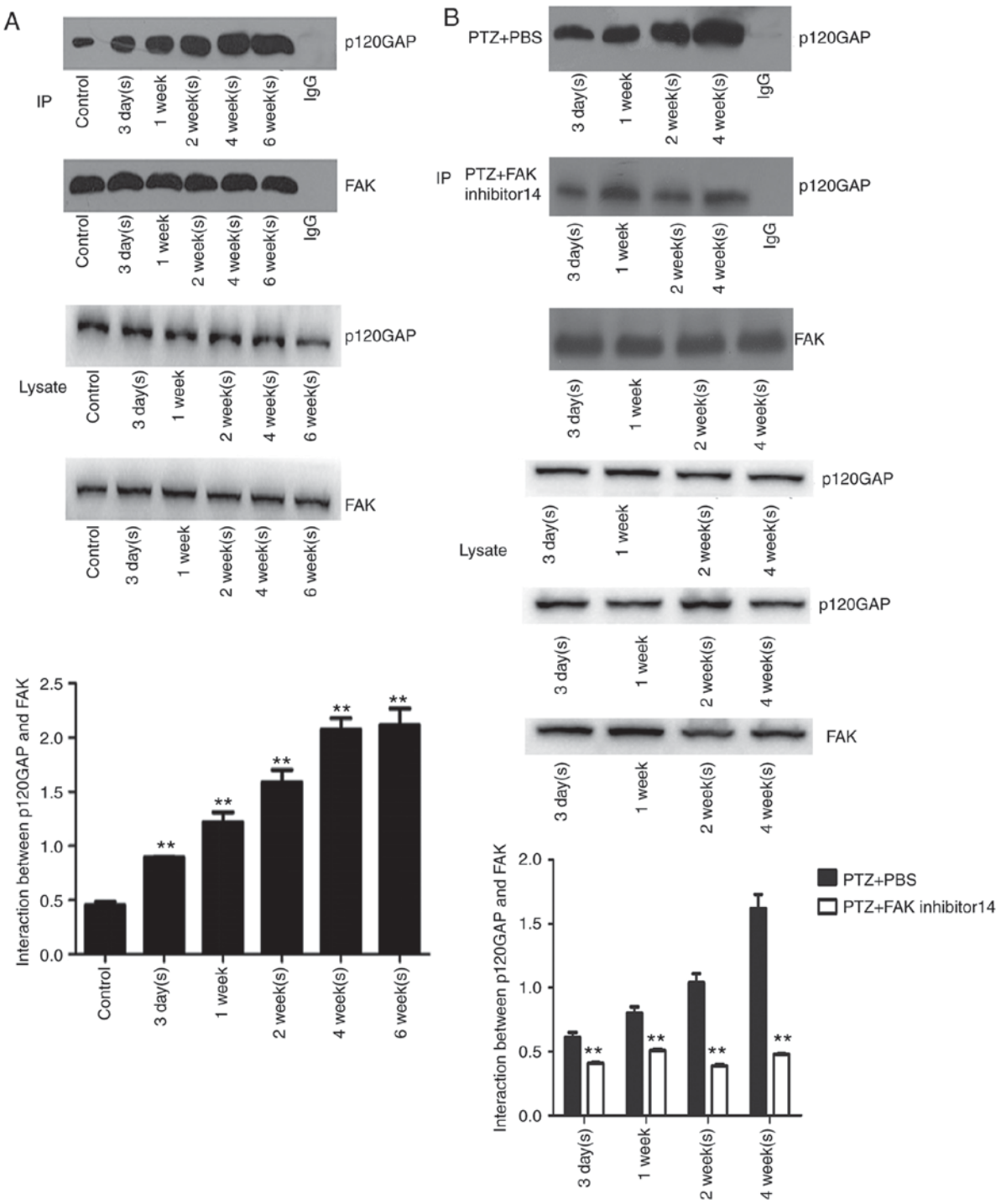

Figure 4. Interaction between FAK and p120GAP in PTZ group, PTZ + PBS group and PTZ + FAK inhibitor 14 groups by co-immunoprecipitation. (A) Interactions in the PTZ group at different time points. (B) Interactions in PTZ + PBS group and PTZ + FAK inhibitor 14 group respectively. The data are expressed as mean ± standard deviation. **P<0.01 vs. control or PTZ + FAK inhibitor 14. FAK, focal adhesion kinase; PTZ, pentylenetetrazol.

Intracerebroventricular injection FAK inhibitor 14 inhibits the interaction between FAK and p120GAP, but does not suppress MFS. From the Co-IP assay, the interaction between FAK and p120GAP was detected in the PTZ and control groups, which was significantly upregulated (P<0.01) from day 3 in the PTZ group than that of control group, and peaked at weeks 4 and 6 week (Fig. 4A). Similar to the PTZ group, interactions between FAK and p120GAP increased at 1 week, and peaked at 4 weeks in the PTZ + PBS group (Fig. 4B); however the interactions between p120GAP were significantly reduced in the PTZ + FAK inhibitor 14 group compared with in the PTZ + PBS group.

Discussion

RGMa is a membrane-associated glycoprotein that regulates axonal guidance and inhibits axon outgrowth. (7). In a case report, a child with deletion of the RGMa gene exhibited epilepsy and mental deficiency (18). In a previous study, we reported that the expression of RGMa was significantly

downregulated in the PTZ kindling model, suggesting that decreased expression of RGMa may be associated with the development of epilepsy (12). In the present study, it was demonstrated that intracerebroventricular injection of RGMa suppressed MFS, increased seizure latency and decreased seizure severity score, which indicated that RGMa reduced MFS and inhibited epileptogenesis in a PTZ-induced rat model. The results of the present study were consistent with a previous report that recombinant RGMa protein could inhibit hyperexcitability-induced MFS in cultured slices (19); the underlying mechanism remains unknown.

A previous study revealed that RGMa may exert its biological effects by dephosphorylating FAK at Tyr397. Dephosphorylation of FAK (Tyr397) decreased the interaction between p120GAP-FAK and increased frequency of interactions between p120GAP and GTP-Ras (p120GAP is a Ras-specific GTPase-activating protein), which reduced the activation of Ras (11). Our previous study reported that FAK (Tyr397) and Ras were significantly upregulated during the progression of PTZ-associated kindling (12). In the present study, it was demonstrated that the interactions between FAK and p120GAP were significantly increased during PTZ-associated kindling progression. Intracerebroventricularly injected recombinant RGMa protein reduced the phosphorylation levels of FAK at Tyr397; the interactions between FAK and p120GAP, and the expression of Ras were significantly decreased in response to intracerebroventricularly injected FAK inhibitor 14. Collectively, these results indicated that by decreasing the phosphorylation levels of FAK (Tyr397), p120GAP may mediate RGMa-induced Ras inactivation, thereby inducing MFS inhibition. However, the present study reported that seizures, but not MFS, may be suppressed by intracerebroventricular injection of FAK inhibitor 14. The possible reasons were as follows: i) Tyr397 phosphorylation may serve an important role in FAK-dependent signaling; however, FAK is also phosphorylated at numerous serine residues, including Ser-722, 732, 910 and 843. The proximity of these phosphorylated serine residues to sites at which FAK interacts with other proteins suggests a possible function in the regulation of the assembly of FAK signaling complexes (20). Semaphorin induced Ser-732 and Tyr397 phosphorylation of FAK serves a previously unreported role in regulating the dendritic development of newborn neurons, and the phosphorylation process of these two residues were independent of each other (21). The association between these phosphorylated serine residues and MFS, and the interaction between tyrosine and serine phosphorylation residues requires further investigation; and ii) in addition to MFS, hippocampal neuronal apoptosis is another common pathological phenomenon (22). When neuronal loss or apoptosis is present in the CA3 region, the mossy fibers of granule cells lose normal connections with CA3 neurons, or otherwise develop collaterals in an abnormal location, such as inner molecular layer subfields and/or stratum oriens of the CA3 (23). A previous study revealed that tyrosine phosphorylation of FAK may confer anti-neuronal apoptosis; the inhibition of Tyr397 phosphorylation may induce hippocampal neuronal apoptosis, which may partly offset the function of inhibiting axonal growth by Ras.

In conclusion, the present study demonstrated that intracerebroventricular injection of recombinant RGMa protein attenuated PTZ-induced seizures and ameliorated MFS. Additionally, RGMa may exert these effects, partly via the FAK-p120GAP-Ras signaling pathway. Thus, the present study proposed that RGMa may be considered as a potential therapeutic agent in the treatment of epilepsy. Furthermore, intracerebroventricular-injected protein and inhibitor may not only affect the hippocampus; however, as TLE is characterized by several histological aberrations in the hippocampus, the effects of RGMa on MFS in a TLE model were investigated only in this region in the present study. The particular effects of RGMa in other brain tissues on TLE require further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MS and FT conceived and designed the study. MS, HX and YX performed the experiments and data analysis. MS wrote the manuscript. FT reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of Xiangya Hospital of Central South University (Changsha, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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