Expression of prosaposin and its receptors in the rat cerebellum after kainic acid injection

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A R T I C L E   I N F O

A B S T R A C T

Prosaposin (PSAP), a highly conserved glycoprotein, is a precursor of saposins A–D. Accumulating evidence suggests that PSAP is a neurotrophic factor that induces differentiation and prevents death in a variety of neuronal cells through the active region within the saposin C domain both *in vivo* and *in vitro*. Recently, GPR37 and GPR37L1 were recognized as PSAP receptors. In this study, we examined the alteration in expression of PSAP and its receptors in the cerebellum using rats injected with kainic acid (KA). The results show that PSAP was strongly expressed in the cytoplasm of Purkinje cells and interneurons in the molecular layer, and that PSAP expression in both types of neurons was markedly enhanced following KA treatment. Immunoblotting revealed that the expression of GPR37 was diminished significantly three days after KA injection compared with control rats; however, no changes were observed through immunostaining. No discernable changes were found in GPR37L1. These findings may help us to understand the role of PSAP and the GPR37 and GPR37L1 receptors in alleviating the neural damage caused by KA.

1. Introduction

Prosaposin (PSAP) is the precursor of four small non-enzymatic glycoproteins, termed saposins A, B, C, and D. Each saposin acts as sphingolipid activator protein and coenzyme, and is necessary for enzymatic hydrolysis of certain sphingolipids in lysosomes (Sano et al., 1989; O'Brien and Kishimoto, 1991; Schulze et al., 2009). In addition, intact PSAP is widely expressed in various tissues, including the Purkinje cell layer of the cerebellum, the spinal cord, testes, ovaries and kidneys (Qi and Grabowski, 2001; Li et al., 2013; Saito et al., 2014), and is secreted into various body fluids including bile, pancreatic juice, breast milk, cerebrospinal fluid, and seminal plasma (Hineno et al., 1991; Kondoh et al., 1991; Hiraiwa et al., 1993; Koochekpour et al., 2012). In the past two decades, PSAP has been identified as a potent neurotrophic factor, protecting neural cells against cellular damage (O'Brien et al., 1995; Kotani et al., 1996; Morita et al., 2001; Ochiai et al., 2008; Gao et al., 2013c) through its active region within the saposin C domain (O'Brien et al., 1994; O'Brien et al., 1995). The PSAP gene contains at least 15 exons, and generates alternatively spliced forms by inclusion or exclusion of 9-bp exon 8 (Pro+9 and Pro+0, respectively) (Lamontagne and Potier, 1994). Pro+9 is secreted preferentially from cells, whereas Pro+0 is trafficked mainly to the lysosomes (Madar-Shapiro et al., 1999).

GPR37 and GPR37-like 1 (GPR37L1) are two orphan G-protein-coupled receptors (GPCRs) that have enhanced expression in the rat brain. GPR37, also known as parkin-associated endothelin-like receptor (Peal-R), is a substrate of the E3 ubiquitin ligase parkin (Imai et al., 2001). GPR37L1 shares 42% identity with GPR37. Phylogenetic analysis indicated that the receptors closest to the endothelin receptors were the bombesin receptors. However, neither the endothelin nor bombesin peptides act as the endogenous ligands for GPR37 and GPR37L1 (Leng et al., 1999). Recent reports

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demonstrate that these two related receptors could be stimulated by prosaposin and its active peptide fragment prosaptide (Leng et al., 1999; Meyer et al., 2013a).

Kainic acid (KA) (2-carboxy-4-isopropenyl-pyrrolidin-3-ylacetic acid), a glutamate analog, is a powerful neurotoxic agent (Olney and de Gubareff, 1978) that stimulates excitatory neurotransmitter release (Ferkany et al., 1982). Excitotoxicity is believed to contribute to the pathogenic process of acute and chronic neurodegenerative disease (Doble, 1999). Systemic injection of KA has been widely used as a tool to explore the mechanism involved in excitotoxicity (Wang et al., 2005; Nabeka et al., 2014; Mohd Sairazi et al., 2015).

In a previous study, we established a rat model using systemic injection of KA, and investigated the expression and function of PSAP in the brain (Nabeka et al., 2014, 2015). However, we did not determine what happened to the cerebellum using this model. Therefore, we examined the expression of PSAP and its two receptors, GPR37 and GPR37L1, in rat cerebellum using the same KA-injected rat model in this study.

2. Experimental procedures

2.1. Animals

Ten-week-old, 220–260-g male Wistar rats (Clea Japan Inc., Tokyo, Japan) were housed at a constant temperature (22 °C) under a 12/12-h light/dark cycle and given food and water *ad libitum*. The experiments were conducted in accordance with the Guide for Animal Experimentation of the Ehime University School of Medicine, Japan. The protocol was approved by the Animal Care Committee of Ehime University (Permit Number: 05A261). All surgeries were performed under chloral hydrate anesthesia (10 mg/kg), and all efforts were made to minimize suffering in accordance with ARRIVE guidelines.

2.2. Specific antibodies for PASP, GPR37 and GPR37L1

Specific polyclonal antibodies against rat PSAP (PSAP-Ab) and its two receptors were generated by Eurofins Genomics Co., Ltd. (Tokyo, Japan), and all the procedures were performed as described elsewhere (Gao et al., 2013a; Shimokawa et al., 2013; Nabeka et al., 2014). Briefly, specific antibodies were created by immunizing rabbits with synthetic oligopeptides based on the rat amino acid protein sequences specific to PSAP (M19936(Collard et al., 1988)), GPR37 (NP_476549.1(Dutta et al., 2014)), or GPR37L1 (NP_665727.2(Leng et al., 1999)). The sequences used were:

PSAP: 409-PKEPAPPKQPEEPKQSALRAHVPPQK-434,
GPR37: 134-REPTDSQLFRQTSE-147 (#12795V),
GPR37L1: 286-CIMKPSADLPESLYS-300 (#12796V), and 34-RAKVQEQQSRPRRG-47 (#13493VP).

The PSAP sequence did not encode any saposins and was acquired from a PSAP amino acid sequence analysis that included protein secondary structure predictions, analyses of accessibility to solvents, flexibility, surface probability, antigenicity, hydrophilicity, and dipole analyses. All the antibodies were tested for specificity using immunoblotting.

Two commercial antibodies for GPR37 (PAB16206, Abnova, Taipei, Taiwan) and GPR37L1 (A-405, LifeSpan BioSciences, Int. Seattle, WA, U.S.A) were also utilized in our study.

2.3. Preliminary study to determine the optimal KA dose

According to previous studies, the optimal dose of KA for administration in the hippocampus is 5 mg/kg in the rat model (Nabeka et al., 2014). Accordingly, we first applied several does of KA (0, 5, 8, 10, and 12 mg/kg) to determine the optimum dose for the rat cerebellum. Briefly, rats were anesthetized with diethyl ether, and clonazepam (an anticonvulsant) was injected intraperitoneally (0.2 mg/kg). After 10 min, rats were anesthetized again with diethyl ether and injected subcutaneously with KA dissolved in normal saline at various doses (5, 8, 10, and 12 mg/kg) or with saline as a control. Seven days after KA injection, each rat was anesthetized and perfused transcardially. The cerebellums were dissected, fixed, dehydrated, and embedded in paraffin for microtome serial coronary sectioning (7-μm thickness). Notably, the rats injected with 12 mg/kg KA were not available because two-thirds of them died during the experimental period.

Sections were stained with hematoxylin-eosin (H-E) using standard procedures. In brief, sections were deparaffinized, rehydrated and stained with hematoxylin for 6 min. Sections were then stained with eosin for 30 s and rinsed with ethanol. The slides were subsequently dehydrated and mounted with coverslips.

Microscopically, the Purkinje cells of rats injected with 5 mg/kg KA exhibited normal morphologic structures, whereas some shrunken and condensed Purkinje cells were observed in rats injected with 8 mg/kg KA, and even greater damage was observed in those injected with 10 mg/kg KA (Fig. 1a–d). These data indicated that 5 mg/kg KA was the optimum dose that could stimulate neurons but not kill them at the ordinal light-microscopic level. Rats younger than 9 weeks injected with 5 mg/kg KA occasionally suffered some neuronal damage in the cerebellum, which was similar to that observed in 10-week-old rats injected with 8 mg/kg KA.

2.4. KA injection and tissue preparation

After determining the appropriate dose of KA (5 mg/kg), rats were injected with normal saline or KA as described above. Under these conditions, no animal experienced status epilepticus, even with KA injection. Rats were sacrificed on days 1 and 3 after injection.

In one group of rats, the cerebellums were freshly stored at −80 °C immediately after extraction. The tissues were then homogenized for immunoblotting. In another group of rats, deep anesthetic was given and the rats were perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). The cerebellums were dissected and fixed with the same solution overnight at 4 °C. The samples were then dehydrated and embedded in paraffin for microtome serial coronary sectioning (7-μm thickness), and used for immunohistochemistry (IHC) and immunofluorescence (IF).

For *in situ* hybridization, the rats were anesthetized on the indicated day and their cerebellums were removed quickly and frozen immediately on dry ice. Coronary sections (20-μm thickness) were cut on a cryostat, thaw-mounted onto silane-coated slides, and then stored at −80 °C until use.

2.5. Immunoblotting

Cerebellums were sonicated (1:5 w/v) in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.25% sodium deoxycholate, 1% NP-40, pH 7.4) for 2 min, NaVO$_3$ (0.5%), protease inhibitor cocktail (1%, Nacalai Tesque, Inc., Kyoto, Japan) and phosphatase inhibitor cocktail (1%, Nacalai Tesque, Inc., Kyoto, Japan) were included in the lysis buffer. All procedures were performed on ice. Homogenates were centrifuged for 30 min at 12,000 × g and 4 °C and the supernatants were collected. Protein concentration was examined by DC protein assay (Bio-Rad, Hercules, CA, U.S.A.), with bovine serum albumin (BSA) as the standard using a FlexStation 3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). Equal amounts (21 μg) of total protein
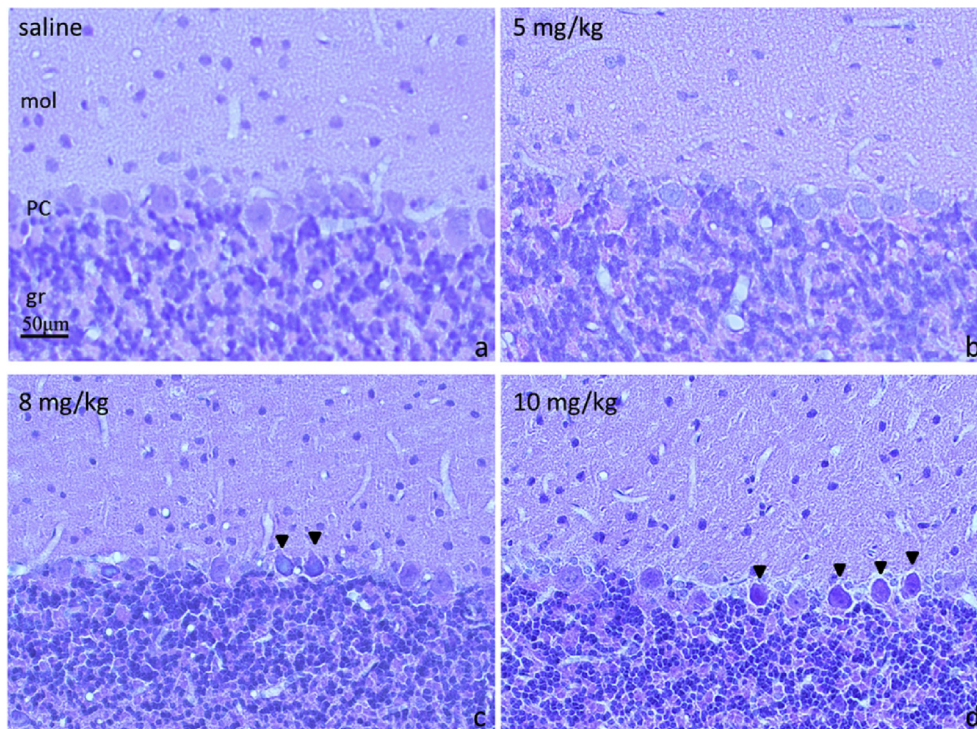
Fig. 1. H–E staining to determine the optimum dose of KA. a–d: Purkinje cells from rats injected with saline (a), or 5 (b), 8 (c), or 10 (d) mg/kg KA on day 7 after KA injection. Arrowheads indicate damaged Purkinje cells. Mol: molecular layer; PC: Purkinje cell layer; gr: granule layer. Scale bar is indicated.

were loaded into Nupage Bis-Tris mini gels following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and transferred to 0.45-μm polyvinyl difluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.). Membranes were blocked by 5% BSA in 1 × Tris-buffered saline +0.1% Tween 20 (TBS-T) and incubated at 4 °C overnight with one of the following primary antibodies: PSAP (1:10,000), GPR37 (1:5000), or GPR37L1 (1:5000). Afterwards, horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:5,000, Dako, Glostrup, Denmark) and ECL prime western blotting detection reagent (GE Healthcare, Buckinghamshire, U.K.) were applied to capture the immunoreactive band using an ImageQuant LAS 4000 imaging system (GE Healthcare, Marlborough, MA, U.S.A). GAPDH (1:5000, ab9485, Abcam, Cambridge, U.S.A.) was utilized on the same membranes as a loading control. Image analysis was performed using Quantity One (version 4.6.2, Bio-Rad) software.

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2.6. IHC and IF

Cerebellum sections were dewaxed, rehydrated, and placed in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. After rinsing in PBS (pH 7.4) with 0.1% Tween-20 (PBST), the sections were blocked with 5% BSA and 5% normal goat serum (NGS) in 0.1 M PBS (pH 7.4) for 60 min at room temperature, followed by overnight incubation with PSAP-Ab (1 μg/mL), anti-GPR37 (1 μg/mL), or anti-GPR37L1 (2 μg/mL) at 4 °C.

For IHC, biotinylated swine anti-rabbit IgG (1:500; Dako, Glostrup, Denmark) was then added and incubated for 1 h at 32 °C. Antibody binding was detected with avidin-biotin-peroxidase complex (1:100; Vector Laboratories, Burlingame, CA, U.S.A.) using a DAB Peroxidase Substrate Kit (1:100; Vector Laboratories, Burlingame, CA, U.S.A.) for approximately 5 min. After washing with

distilled water, the sections were mounted and examined under a microscope.

For IF, after washing with PBS, the sections were treated for 30 min at 32 °C with fluorescent anti-rabbit IgG (1:500, Invitrogen) and 4′,6-diamidino-2-phenylindole (DAPI, 1:1000). The sections were then washed with PBST, mounted with Vectashield (1:100; Vector Laboratories, Burlingame, CA, U.S.A.), and examined using a confocal laser scanning microscope.

To ensure accurate and specific staining, normal rabbit IgG (1 μg/mL) was used as a negative control. Nonspecific staining was not observed.

2.7. In situ hybridization (ISH)

We performed ISH to examine PSAP mRNA as previously described (Chen et al., 2008). In brief, two antisense 36-mer probes, AS3 and AS4, and a sense probe S1 were synthesized commercially (Operon Biotechnologies, Inc., Tokyo, Japan). AS3 and AS4 were synthesized to detect Pro+9 mRNA and Pro+0 mRNA, respectively (Collard et al., 1988; Hiraiwa et al., 2003). As the negative control, the sense probe SS1 was complementary to the PSAP cDNA. The sequences of the three probes were:

S1: 5′-GCAGAAGTCGCCTACTTGTGGGTCTAGGGTAATGAA -3′,
AS3: 5′-_CTTGGGTTG_CTGATCCTG_CATGTGCATCATCATCTG_-3′, and
AS4: 5′-TT_CCTTGGGTTG_CATGTGCATCATCATCTG_GACGGC-3′.

The sequence in italics (AS3) was complementary to the 9-base insertion. The underlined sequences in AS3 and AS4 were shared sequences. Meanwhile, computer-assisted homology searches (GenBank) confirmed that each probe had less than 58% homology with any other known sequence. The probes were labeled with [35S]-dATP (46.2 TBq/mmol; PerkinElmer Life Sciences, Boston, MA, U.S.A.) using Terminal Deoxynucleotidyl Transferase (Takara, Tokyo, Japan), and a specific activity of approximately 1.0×10^7 dpm/mL was obtained.

Cryostat sections were fixed in 4% PFA in 0.1 M PB (pH 7.4) for 15 min, washed in 4 × standard saline citrate (SSC, pH 7.4), and dehydrated with ^{35}S-labeled probes in hybridization buffer (50% deionized formamide, 1% Denhardt's solution, 250 μg/mL yeast total RNA, 0.1 g/mL dextran sulfate, 0.12 M PB, 20 mM DTT in 4 × SSC) at 41 °C overnight. After hybridization, sections were rinsed three times in 1 × SSC at 55 °C for 20 min, dehydrated through a graded ethanol series, coated with Kodak NBT-2 emulsion (Eastman Kodak, Rochester, NY, U.S.A.), and exposed at 4 °C for 4 weeks. Finally, the sections were developed in a D-19 developer (Eastman Kodak) and counterstained with hematoxylin. After dehydration and mounting, the sections were observed under a microscope.

2.8. Image capture and analysis

The sections were examined under a Nikon Eclipse E-800M microscope (Tokyo, Japan) linked to a Pro-Series High Performance CCD camera (Sony, Tokyo, Japan). The detection of IF was performed using an A1Rsi confocal laser scanning microscope (Nikon, Tokyo, Japan). For quantification, five randomly selected discontinuous fields per cerebellum were analyzed to determine the average optical density (OD) using Image-Pro Plus version 6.0 software (Media Cybernetics Inc., MD, U.S.A.).

2.9. Statistics

Results are expressed as the mean ± SD, and all statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, U.S.A.). Data were analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test, and statistical significance was set at p < 0.05.

3. Results

3.1. Expression of prosaposin in the rat cerebellum after KA injection

To assess protein expression in the cerebellum, we performed immunoblotting. As shown in Fig. 2a, PSAP was recognized by the specific antibody, and one band of approximately 65 kDa that corresponded to previously reported results was detected (Gao et al., 2013a). Expression was increased markedly at one and three days post-KA injection compared to the control (Fig. 2b).

Next, we performed IHC to determine the distribution of PSAP in the cerebellum. PSAP-positive staining occurred predominantly in Purkinje cells as dot-like staining that was diffuse in the cytoplasm but was not found in nuclei (Fig. 3a–f), as previously described (Gao et al., 2013a; Nabeka et al., 2014). These results were reinforced by the IF results and dot-like particles were distributed between the nuclei and membranes both in control and KA-injected animals (Fig. 3j–l). Similarly, immunoreactivity of PSAP in KA-injected groups was stronger than in the control group, and an increase in staining was detected one and three days after KA injection (Fig. 3m). In addition, we observed that specific labeling occurred in the molecular layer in both control and KA-injected animals. The PSAP-positive neurons had a roughly pyramidal or ovoid shape and were found around or above the Purkinje cells. Based on these results, these positive neurons were termed interneurons, which include basket cells and stellate cells. Immunostaining was also found in the choroid plexus due to the secretion of PSAP from the neurons (Fig. 3g–i).

Furthermore, we performed ISH with oligonucleotide probes encoding PSAP sequences to detect mRNA signals in the cerebellums. The analysis showed similar results to the DAB-staining. AS3 (Pro+9) signal was observed mainly in the Purkinje cell layer, and
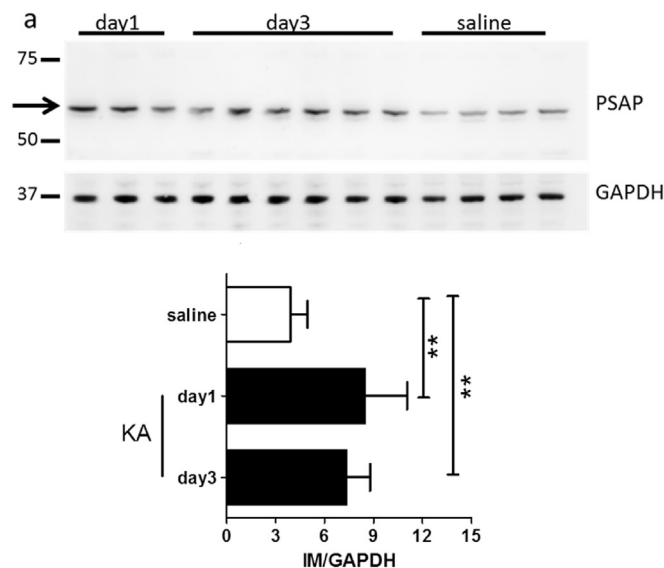

Fig. 2. Immunoblot investigation of PSAP expression in rat cerebellum. **a:** Immunoblot analysis of cerebellums from KA-injected rats. Blots were incubated with a specific anti-PSAP antibody. **b:** Relative value of PSAP intensity normalized to GAPDH. The results were analyzed using one-way ANOVA followed by Bonferroni's post hoc test. Data are presented as the mean ± SD. *p < 0.05, **p < 0.01 versus control.

weaker intensity was detected in the molecular and granule layers in the cerebellums of control animals (Fig. 4a1–2). After KA treatment, AS3 (Pro+9) was elevated markedly in the Purkinje cell layer on days 1 and 3 after injection, compared with the control rats (Fig. 4b1–2, c1–2, and j). High AS3 signal was also obtained in molecular layer in all rat groups, but there was an apparent increase in expression in the KA groups. AS4 (Pro+0) was observed at a high concentration in the Purkinje cell layer of control animals (Fig. 4d1–2). In contrast, the intensity of AS4 labeling in the KA-injected groups was decreased compared to control (Fig. 4e1–2, f1–2, and k). These results suggest an inverted correlation between the expression of AS3 and AS4. Sense probe (S1) showed no specific labeling in any areas (Fig. 4g–i).

3.2. GPR37 and GPR37L1 expression in cerebellums of rats after KA injection

A recent report identified GPR37 and GPR37L1 as receptors of PSAP and prosaptide (Meyer et al., 2013b), which are highly expressed in the brain(Leng et al., 1999). Immunoblotting revealed a single band of about 50 kDa using the antibody specific for GPR37, indicating that GPR37 is present in the rat cerebellum (Fig. 5a). Intensity analysis further showed that the ratio of GPR37/GAPDH decreased after KA treatment with a significant reduction on day 3 (Fig. 5b).

We also used IHC to determine the distribution of GPR37. Labeling was observed predominantly in the cell body and dendrites of Purkinje cells. In control rats, diffuse staining was observed in the cytoplasm of Purkinje cells. In KA-treated rats, intense staining was observed around the nucleus one and three days after injection (Fig. 6a). In addition, IF experiments suggested the same distribution (Fig. 6b). Triple-staining IF further showed that the positive particles were co-localized with PSAP, and aggregated above the nuclei (Fig. 6c). The interneurons were clearly stained in the molecular layer in all rats. We also applied a commercial antibody for GPR37 (PAB16206) using IHC (Fig. 6d). Similar staining patterns were seen, with most of the expression observed in Purkinje cells, and low levels of expression in interneurons. Some Golgi cells were also stained. No changes in GPR37 expression were detected
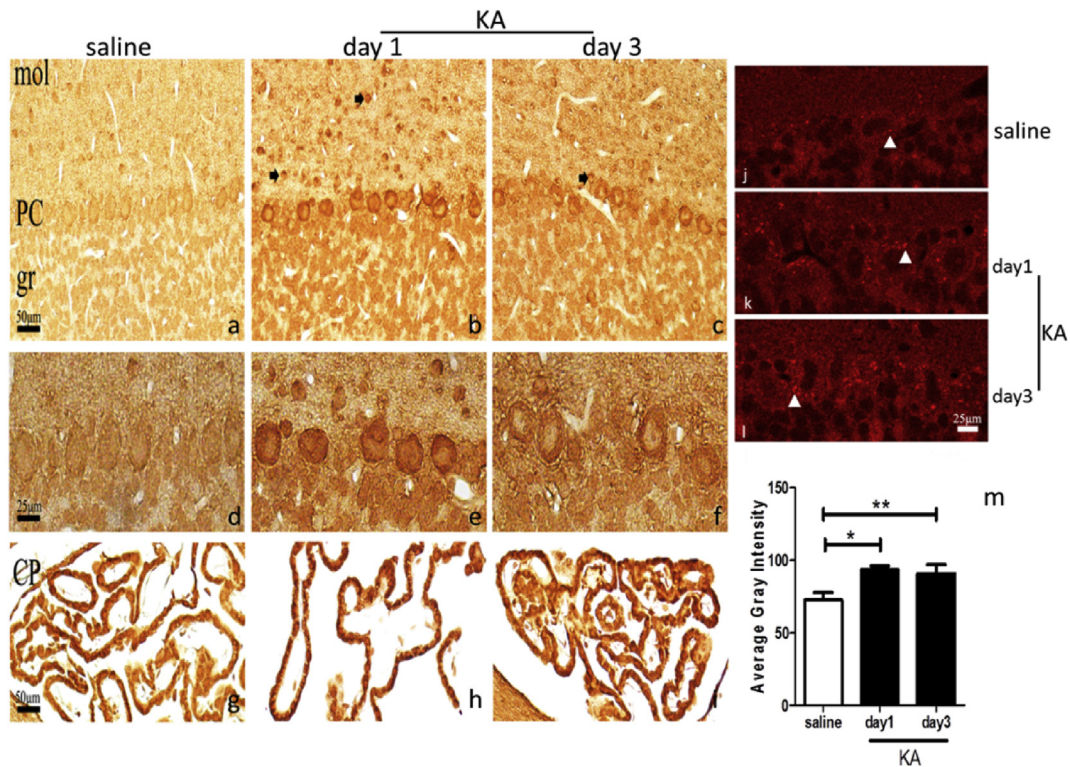
Fig. 3. PSAP expression in cerebellum Purkinje cells in rats. a–f: Paraffin-embodied cerebellum sections from control and KA-injected rats were stained with an anti-PSAP antibody. Positive particles were found in the cytoplasm of Purkinje cells, but not around the nuclei. The solid arrow indicates staining of interneurons in the molecular layer. **g–i:** PSAP is expressed in the choroid plexus in all three groups of rats. Mol: molecular layer; PC: Purkinje cells layer; gr: granule layer; CP: choroid plexus. **j–l:** Immunofluorescence with anti-PSAP antibody. Similarly, signal was also detected in the cytoplasm of Purkinje cells. White triangles indicate the positive PSAP staining. **m:** Quantification with Image-Pro Plus version 6.0 software and analyzed by ANOVA followed by Bonferroni's multiple comparison test. Data are presented as the mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$ versus that in saline group. Scale bar is indicated.

with either antibody between the control and KA-treated groups (Fig. 6e). Staining was also observed in the choroid plexus, and the intensity increased on day three after KA injection compared to the control (Fig. 6a).

The GPR37L1 results in this study were complicated. We utilized two anti-rat GPR37L1 antibodies (#12796V and #13493VP, respectively) that bind to different fragments of the protein, and a commercial anti-human GPR37L1 antibody (A-405). As shown in Fig. 7a, immunoblot analysis only with the #12796V antibody revealed a band at 53 kDa, which was close to the predicted size of the receptor. Using antibody #12796V, two bands with molecular weights over 53 kDa were detected, which probably represent oligomeric receptor fragments. Based on these results, we compared the GPR37L1/GAPDH ratio between the three groups using the #12796V antibody (Fig. 7b and c). However, the #12796V antibody failed in IHC and IF and showed obvious nuclear staining (Fig. 8a–c). Therefore, #13493VP and A-405 antibodies were used instead for staining These antibodies showed staining in the cytoplasm of Purkinje cells as previously reported (Gao et al., 2013a; Meyer et al., 2013b) (Fig. 8d–f and g–i).

4. Discussion

In this study, we investigated the expression of PSAP and its receptors, GPR37 and GPR37L1, in the cerebellum utilizing a rat model of KA injection at 5 mg/kg (Qi and Grabowski, 2001; Nabeka et al., 2014). The results showed that PSAP protein expression was augmented after KA treatment, specific PSAP dot-like immunostaining was observed predominantly in the cytoplasm of Purkinje cells, and not in the nucleus. In the molecular layer, some interneurons were also stained by the anti-PSAP antibody, and the intensity increased after KA injection. ISH analysis confirmed that the damage caused by KA resulted in increased expression of intact PSAP (AS3: Pro+9) rather than saposin precursor expression (AS4: Pro+0). Immunoblotting revealed attenuation of GPR37 expression after KA injection. Although the decrease on day 1 was not significant, there was a sharp decrease in expression by day 3. IHC showed that GPR37 expression was highly enriched in the cytoplasm of Purkinje cells. The labeling was also seen in Purkinje cell dendrites and interneurons in the molecular layer. However, the intensity did not differ between KA-treated and control animals.

PSAP is a bi-functional protein that is a precursor of saposin A–D and is released extracellularly as full-length protein, acting as a trophic factor in the nervous system (Sano et al., 1989; Kishimoto et al., 1992; Gao et al., 2013b). In this study, we confirmed that PSAP is expressed in Purkinje cells and interneurons in the molecular layer, which are also known as GABAergic neurons (Hoshino et al., 2005). The results showing that PSAP expression is upregulated after KA injection are similar to results of previous studies that showed that PSAP expression is increased under conditions of cellular stress (Yokota et al., 2001; Hiraiwa et al., 2003). Therefore, the results suggest that PSAP may exert protective effects on cerebellar neurons against KA stimulation.

The increase in PSAP protein expression led us to examine mRNA expression. We designed probes to detect Pro+9 mRNA (AS3) and Pro+0 mRNA (AS4), and examined expression one and three days after KA injection and in control rats (Chen et al., 2008; Shimokawa et al., 2013). The results suggest that KA induces neurons to produce PSAP. These results are supported by a previous study that showed that cerebral ischemia negatively impacted lysosomal processing of PSAP (Costain et al., 2010).
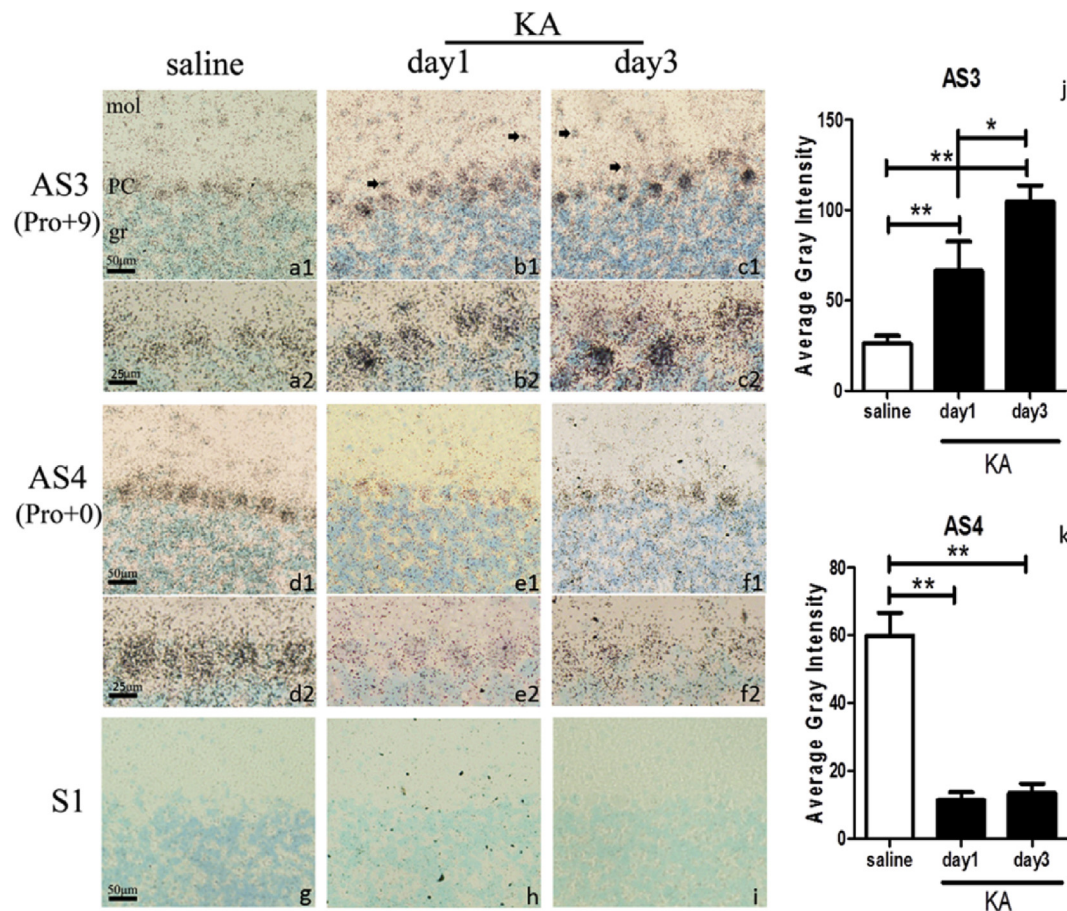
Fig. 4. *In situ* hybridization analysis of PSAP mRNA expression in Purkinje cells of rat cerebellum. a-c: Detection of Pro+9 mRNA using PS-AS3. Black arrows represent the intense signals in the molecular layer. **d–f:** Detection of Pro+0 mRNA using PS-AS4. **g–i:** Sense probe PS-S1 (used as a control). In Purkinje cells, the hybridization signals for PS-AS3 on days 1 and 3 (**b and c**) were more intense, whereas the signals for PS-AS4 (**e and f**) were less intense, compared to the control rats without KA injection (**a and d,** respectively). No specific hybridization signal was detected for the sense probe PS-S1 (**g–i**). **j and k:** Quantification of the results showing the intensity of indicated signals with Image-Pro Plus version 6.0 software and analyzed by one-way ANOVA followed by Bonferroni's post hoc test. Data are presented as the mean ± SD. *$p < 0.05$, **$p < 0.01$ versus that in the saline group. Scale bar is indicated.
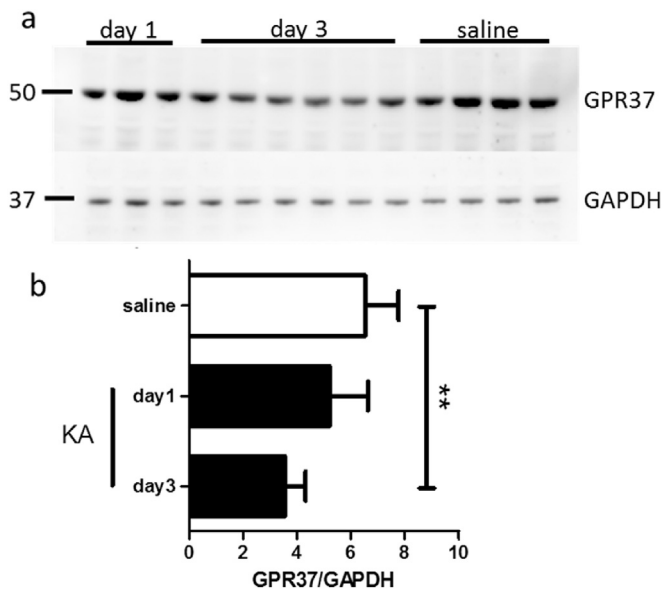

Fig. 5. Immunoblotting for GPR37 in KA-injected rat cerebellums. a: Protein from whole cerebellum lysates (21 μg) was incubated with specific anti-rat GPR37 antibody. **g:** Protein levels were normalized to GAPDH. Results were analyzed by ANOVA followed by Bonferroni's post hoc test and are presented as the mean ± SD. **$p < 0.01$ versus control.

Together, these results suggest that KA induces production of full-length PSAP in GABAergic neurons, including Purkinje cells and interneurons, as a protection measure against KA damage (Vielhaber et al., 1996; Hiesberger et al., 1998). However, we could not exclude the possibility that interactions between Purkinje cells and interneurons may enhance defense against KA injury.

GPR37 and GPR37L1 are orphan GPCRs that exhibit distant similarity to the endothelin receptors. Their high level expression and widespread distribution in the central nervous system suggest that they play important roles (Valdenaire et al., 1998; Leng et al., 1999). Several studies have suggested that intracellular GPR37 is commonly misfolded, leading to aggregation in the endoplasmic reticulum (ER), along with other parkin substrates, which then results in significant ER stress and eventually neuronal cell death (Imai et al., 2001; Kubota et al., 2006; Marazziti et al., 2009), In addition, inhibiting GPR37 accumulation in the ER and promoting trafficking to the plasma membrane leads to increased cell viability (Dunham et al., 2009; Mimori et al., 2012; Lundius et al., 2013). In contrast, some studies have shown that GPR37 is expressed at the plasma membrane predominantly in the proteolytically cleaved form, which is related to its natural physiological role (Mattila et al., 2016). Very recently, GPR37 was also found to participate in oligodendrocyte differentiation and myelination as a negative regulator (Yang et al., 2016).

In this study, we observed the single band at approximately 50 kDa. Although the predicted molecular weight of GPR37 is
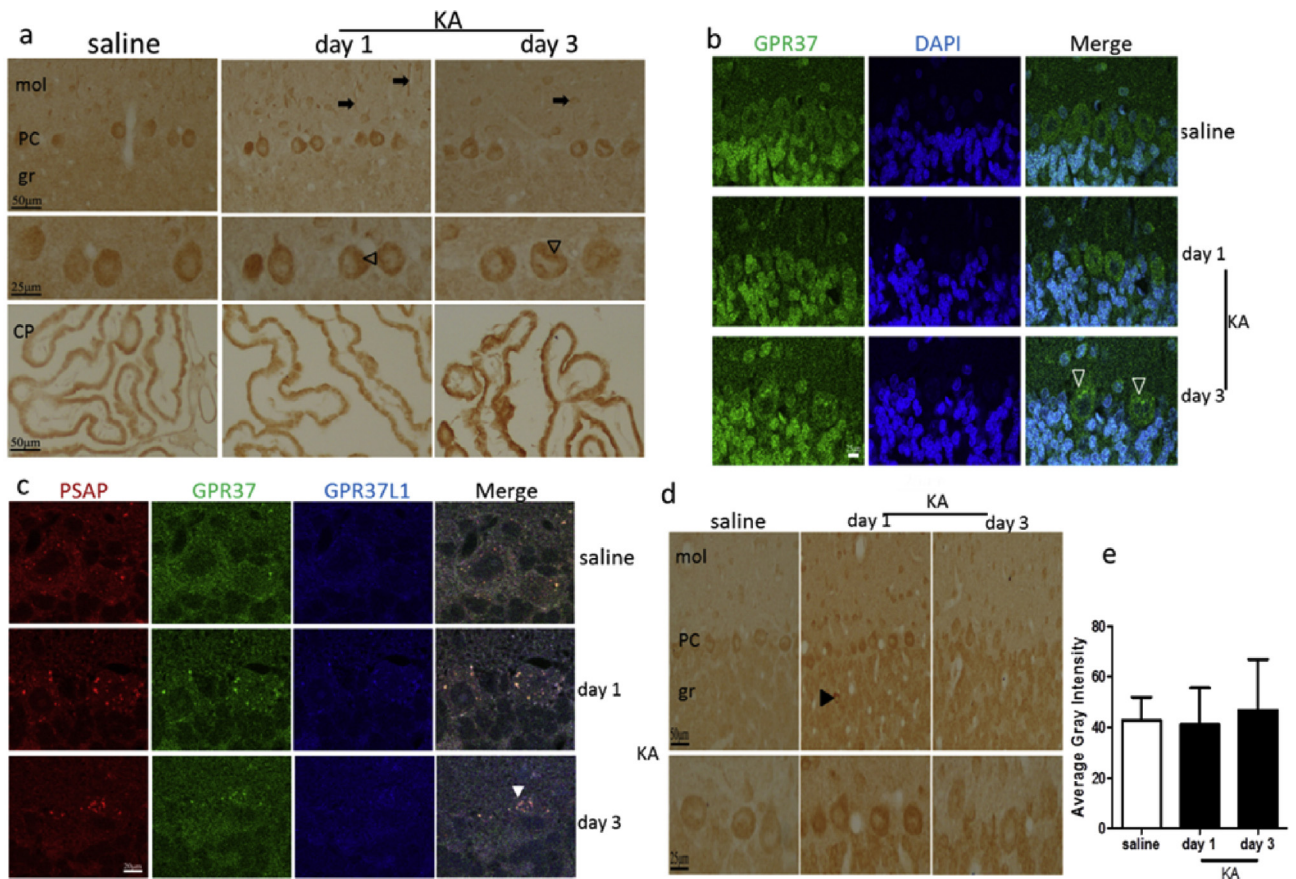
Fig. 6. Immunostaining analysis of GPR37 expression in Purkinje cells with or without KA injection. a and b: Cerebellar immunostaining with specific anti-rat GPR37 (#12795V). Black arrows show labeling of Purkinje cell dendrites in the molecular layer. Empty triangles point to the positive particles accumulating around the nuclei of Purkinje cells. **c:** Triple staining of PSAP (red), GPR37 (green), and GPR37L1 (#12796V, blue) in cerebellums with or without KA injection. White triangles indicate the co-localization of PSAP and GPR37. **d:** Immunohistochemistry using commercial anti-GPR37 (PAB16206). Black triangles represent the positive stained Golgi cells. **e:** The staining intensity was calculated using Image-Pro Plus version 6.0 software and analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. Data are presented as the mean ± SD. Scale bars are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
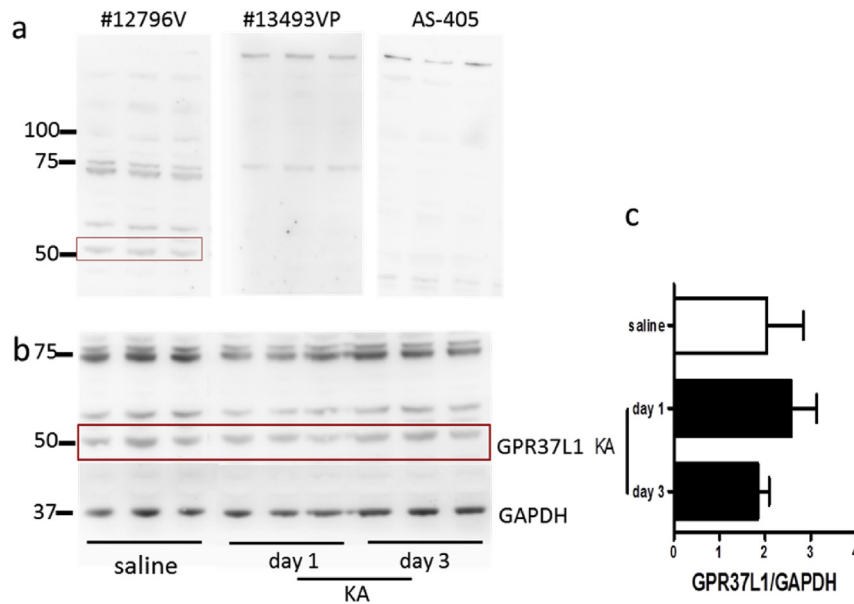

Fig. 7. Immunoblot analysis of GPR37L1 expression in cerebellums with or without KA injection. a: Three antibodies for GPR37L1 were used for immunoblotting of normal cerebral tissue. **b:** Immunoblotting with GPR37L1 antibody (#12796V). Empty rectangles indicate bands at approximately 53 kDa **c:** Quantification of protein levels using the GPR37L1/GAPDH ratio. Data were analyzed using one-way ANOVA followed by Bonferroni's post hoc test.
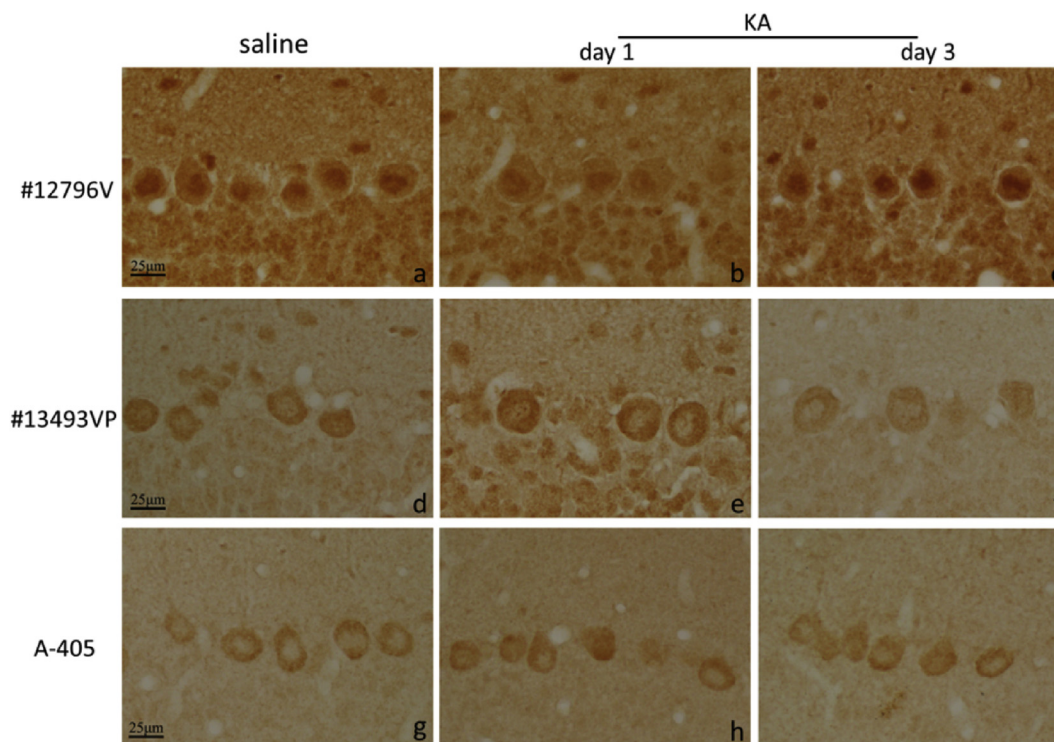
Fig. 8. Immunostaining of GPR37L1 in Purkinje cells of the rat cerebellum. a–c: Labeling of GPR37L1 (#12796V) was observed in nuclei. **d–f and g–i:** Using #13493VP and A-405 antibodies, positive signals were found in the cytoplasm, but not in the nuclei of Purkinje cells. Scale bars are indicated.

67 kDa (Marazziti et al., 1997), we attribute this size difference to the different GPR37 fragment targets. Immunoblotting revealed that GPR37 expression was decreased three days after KA injection; however, IHC did not reveal any changes in GPR37 expression. One possible explanation for this is that PFA fixation and paraffin embedding may have prevented some proteins from being detected. Additionally, the expression of GPR37 in IHC was constrained to Purkinje cells, while WB illustrated the gross expression of GPR37 within all structures in the rat cerebellum. Therefore, the total level of GPR37 may have been reduced in the cerebellum after KA treatment, which may have been accompanied by receptor aggregation around Purkinje cell nuclei. GPR37 co-localization with PSAP may be the reason the KA dose used did not damage, but only stimulated, neurons.

However, we cannot exclude the possibility that our antibody failed to recognize the aggregation of GPR37, which should be with apparently much bigger molecular masses (Rezgaoui et al., 2006). Stimulation with KA might cause the unfolded GPR37 to accumulate and aggregate in the cytoplasm of Purkinje cells.

GPR37L1, named for its similarity to GPR37, plays a critical role in the regulation of sonic hedgehog signaling during postnatal cerebellar development (Marazziti et al., 2013). GPR37L1 deficiency is also involved in hypertension (Min et al., 2010). However, less is known about GPR37L1 compared to GPR37, which is likely due to lack of a specific antibody for experimentation. Unfortunately, we could not obtain consistent data for GPR37L1 despite using three separate antibodies.

5. Conclusion

In summary, this study revealed the relationship between PSAP and its receptors, GPR37 and GPR37L1, in rat cerebellum after KA-induced cytotoxicity. However, many questions remain regarding this model. For example, although the function of GPR37 is disputed (Smith, 2015), the mechanism by which PSAP and GPR37 interact during KA toxicity requires further investigation. The two GPR37 antibodies both target the N-terminal fragment. Our data are not consistent with the finding that GPR37 undergoes N-terminal cleavage between E167 and Q168, followed by ectodomain shedding (Mattila et al., 2016). In addition, further effort is needed to develop a more reliable antibody for GPR37L1. Given the limited information available about this receptor, further study on GPR37L1 function is essential to better understand the role of PSAP in neurodegeneration.

Another interesting finding of this study is the distinct RNA expression patterns of AS3 and AS4 in the cerebellum and hippocampus for the same KA-injected rat model. As shown in Fig. 9, the AS4 signals became stronger in the hippocampus and choroid plexus, but weaker in the cerebellum after KA-injection. In contrast, the AS3 signals became slightly weaker in the hippocampus and choroid plexus following KA injection, but much stronger in the cerebellum. These conflicting results suggest different roles for PSAP in different neurons (excitatory neurons and inhibitory neurons); however, this requires further investigation. Despite its preliminary nature, the current study provides important information for future research on the role of PSAP in the neurodegenerative process.
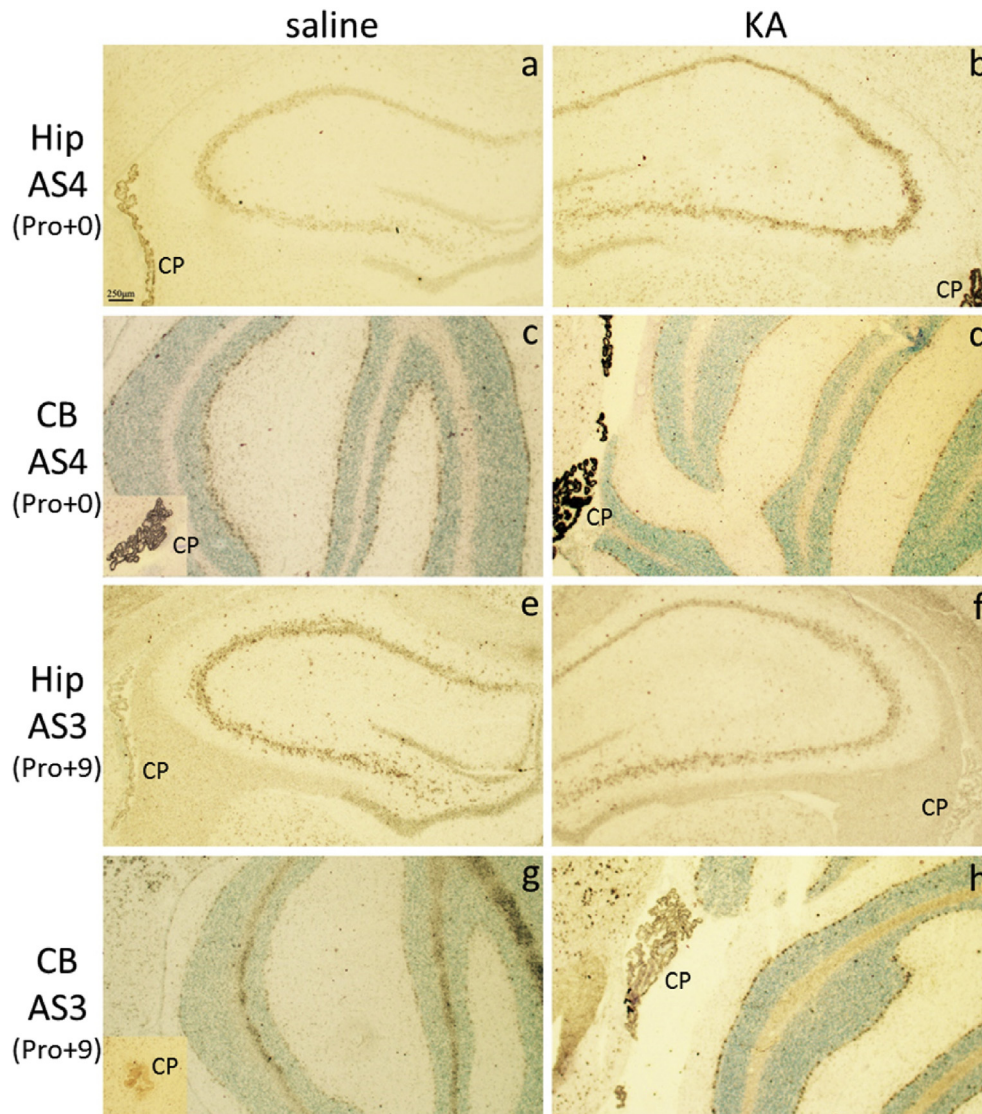
Fig. 9. *In situ* hybridization of PSAP in rat hippocampus and cerebellum. AS4 (Pro+0) signals in the Hippocampus (Hip) and choroid plexus (CP) increased following the KA injection (**a and b**) but decreased in the cerebellum (CB) (**c and d**). AS3 (Pro+9) signals in the Hip and CP were weaker after the KA injection (**e and f**) but stronger in the CB (**g and h**). Scale bar, 250 μm.

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