Gastrodin Regulates GLUR2 Internalization and Reduces BDNF Expression in the Cerebellum of Diabetic Rats

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Research Article

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Abstract

Cognitive dysfunction is a serious neurological complication of diabetes mellitus (DM). Recent studies have found that in addition to the hippocampus, the cerebellum is also involved in regulating cognitive functions such as learning and memory through long-term depression (LTD), but the molecular mechanism of cognitive impairment resulted from cerebellum dysfunction in diabetes has not been fully elucidated. Moreover, gastrodin has been supposed to reduce the incidence of Alzheimer's disease and improve function in the central nervous system. This study aimed to evaluate the changes of LTD-related factors including GluR2, p-GluR2, PKC, BDNF, and TrkB in the cerebellar cortex of diabetic rats by western blotting and double immunofluorescence, and to explore the therapeutic effect of gastrodin. We found that there was neuronal necrosis and neuronophagia in the cerebellar cortex of diabetic rats, the protein expression levels of GluR2 and PKC in Purkinje cells were significantly decreased, and p-GluR2, BNDF and TrkB were significantly increased. After treatment with gastrodin, the damage of Purkinje cells was ameliorated, and the expression of the above factors was restored to a certain extent. Arising from the above, Thus, diabetes may cause cerebellar LTD by affecting the internalization of GluR2, thereby causing cognitive dysfunction. Gastrodin ameliorates cerebellar neuronal damage and restores the expression levels of GluR2 and BDNF in diabetic rats.

Introduction

Diabetes Mellitus (DM)is a metabolic disease characterized by hyperglycemia, which often involves a variety of complications [1]. The risk for the occurrence of Alzheimer’s disease in the diabetic patients with cognitive impairment is more than twice as likely as normal people [2].

Cognitive impairment occurs earlier in associative memory, learning skills, and attention. These functions are based on the plasticity of the structure and function of the nervous system [3]. Previous studies have found that the hippocampus is an important structure of the central memory circuit. The hippocampus plays an important role in the integration of learning and memory in both humans and animals [4.5.6]. However, recent neurological studies have suggested that the cerebellum is not only an important organ involved in the balance maintaining of the body, but also the function of learning and memory [7. 8]. Learning and memory function of the cerebellum is mainly realized by amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor endocytosis in the postsynaptic membrane of Purkinje cells [9.10].

In the central nervous system, a large number of neurons are connected with each other through synapses to form neural circuits. Glutamate is the main neurotransmitter of excitatory synapses in the central nervous system. The release of glutamate from presynaptic neurons is through two postsynaptic receptors: AMPAR (The α-amino-3-hydroxy-5-methyl-4-isoxaolepropionic acid receptor) and NMDAR (The N-methyl-D-aspartate receptor) [11]. The signals of presynaptic neurons are transmitted to postsynaptic neurons which AMPA receptors bind glutamic acid, which depolarizes postsynaptic neurons. Postsynaptic signals can respond to different stimuli and make corresponding changes in synaptic

structure and function. Synaptic plasticity can be divided into two categories: long-term potentiation (LTP) and long-term depression (LTD). The internalization of AMPA receptor in the postsynaptic membrane of cerebellar Purkinje cells is a common pathway for cerebellar involvement in the process of learning and memory formation [12.13].

In ataxia mutant mice, brain-derived neurotrophic factor (BDNF) level in the cerebellum was reduced obviously, coupled with severe dysfunction in the blink of an eye in classically conditioned reflexes. It is suggested that normal cerebellar BDNF is essential for combined learning and plasticity [14]. In the cerebellum of post-stroke depression rats, the content of BDNF was decreased, which suggests that cerebellar BDNF expression may be related to learning and memory [15]. The positive immune reaction experiment on the expression of BDNF in the cerebellum showed that the positive cells were mainly Purkinje cells in the Pyriform cell layer. BDNF/TrkB signaling pathway has been shown to be involved in regulating the growth, proliferation, and survival of neurons, thus participating in learning and memory [16.17]. Increased BDNF in the cerebellum damaged by high glucose is considered to be neuroprotective. [18] Protein kinase C (PKC) is a second messenger in the cells. The increase of postsynaptic Ca^{2+} concentration can directly activate PKC, resulting in the phosphorylation of serine 880 at the GluR2 end of the AMPA receptor and participating in LTD by mediating AMPAR internalization [19]. The decreased number of receptors on the protruding posterior membrane and reduced sensitivity to glutamic acid can result in the formation of LTD [20]. It's reported that BDNF participates in the enhancement of AMPA receptor function and the activation of synaptic vesicles by regulating the GluR2 endocytosis. However, which pathway promotes the internalization of GluR2 remains unclear [21].

Gastrodin has been proved to promote learning and memory in 2003 by An SJ et al [22]. In the passive avoidance test, gastrodin can promote memory consolidation in the rats. Zeng X et al. found that gastrodin can reduce neuronal death induced by oxygen and glucose deprivation in the hippocampus through reducing intracellular Ca^{2+} concentration and NO synthesis. Gastrodin can also reduce neuronal injury induced by local ischemia through reducing the inflow of Ca^{2+} and inhibiting the synthesis of NO [23]. Gastrodin has been widely used in clinic. Because of its low toxicity and the therapeutic effects, it has high medicinal value. It has been used to headaches (vascular headache, tension headache, migraine, etc.), neurodegenerative diseases, vertigo (neurosis, insufficient cerebral artery blood supply, etc.) and cerebrovascular diseases. This study will observe the damage of cerebellar neurons, the changes of GLUR2, BDNF and other factors in diabetic rats, and the regulatory effect of gastrodin.

Materials And Methods

Induction of diabetes

One hundred twenty male SD rats (10-month-old, weight300-360 g) were used. Three quarters of them were randomized and type 1 diabetes was induced after overnight fasting by a single intraperitoneal (i.p.) streptozotocin administration (60 mg/kg in 10 mM citrate buffer, pH 4.5, Sigma-Aldrich, St Louis, MO, USA). Control rats received the same volume of sterile saline. Blood glucose levels were measured with a

glucometer in non-fasted rats. At 72h after STZ injection, rats with blood glucose concentration higher than 16.7 mmol/L was defined diabetic.

Drug administration

The rats were randomly divided into 4 groups: (1) NC9W group, rats were gavaged with saline daily (4 ml/kg) and fed for 6 weeks;(2) DM9W+S group, diabetic rats were gavaged with normal saline for 6 weeks;(3) DM9W+G60 group, diabetic rats were administered with gastrodin (60 mg/kg daily, dissolved in 0.9% saline) for 6 weeks; (4) DM9W+G120 group, diabetic rats were administered with gastrodin (120 mg/kg daily; dissolved in 0.9% saline) for 6 weeks.

Western blotting analysis

Fresh tissue samples were collected from the cerebellum. To analyze the expression levels of proteins, equal amounts of total proteins were subjected to 10% (w/v) SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Darmstadt, Germany). Membranes were then blocked with 5% skim milk for 2 hours at room temperature and probed with anti-GluR2 (1:2000; Abcam, Cambridge, UK), anti-p-GluR2(phospho S880) (1: 500; Mybiosource), anti-PKC (1:1000; Abcam, Cambridge, UK) anti-BDNF (1:1000; Millipore, Darmstadt, Germany), anti-TrkB (1:750; Abcam, Cambridge, UK), anti-β-tubulin (1:1000; Santa Cruz Biotechnology, Dallas, TX) antibodies at 4°C overnight. Subsequently, membranes were incubated with horseradish peroxidase- (HRP-) conjugated goat anti-rabbit (Thermo Fisher Scientific, Waltham, MA) or rabbit anti-rat (Thermo Fisher Scientific, Waltham, MA) IgG for 2 hours at room temperature and then reacted with a pro-light HRP agent (Santa Cruz Biotechnology, Dallas, TX) after washing. The result of chemiluminescence was recorded with an imaging system and semiquantified using the ImageJ software (National Institutes of Health, Bethesda, MD).

Paraffin Embedding and Sectioning

For histology and double immunofluorescence labeling, four rats in each group were anesthetized with 10% chloral hydrate (4 mL/kg) and perfused with filtered saline (150 mL and 12 mL/min), followed by 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4 (500 mL and 12 mL/min). The cerebellum was removed, dehydrated, embedded in paraffin, and cut into 4 μm thick sections. The sections were transferred to silane-coated microscope slides and dewaxed.

Hematoxylin-Eosin Staining

After dewaxing and hydration, the paraffin sections were processed for routine hematoxylin and eosin (H&E) staining.

Double Immunofluorescence Labeling

The cerebellum was removed, immersed in 4% formaldehyde, dehydrated in ethanol, cleared in xylene and embedded in paraffin blocks. Paraffin-embedded tissue sections of 4μm thickness were deparaffinized and hydrated through a series of graded alcohol. The tissues sections were incubated in citrate buffer for antigen retrieval and the slices were incubated with 5% normal goat serum. The following primary antibodies were used: rabbit anti-Calbindin D-28k antibody (1:1000; Swant) mouse anti-GluR2 antibody (1:500; Abcam) mouse anti-p-GluR2 (phospho S880) (1: 200; Mybiosource), mouse anti-BDNF (BDNF, 1:500, Millipore, Darmstadt, Germany), and TrkB (1:500, Abcam, Cambridge, UK) for Purkinje cell labeling. After incubation and washing, anti-mouse and anti-rabbit Alexa Fluor 488 and 568 second antibodies (1:500; Invitrogen, were added. After mounting, images representing at least one cerebellum section each from four rats at different time points were captured under a confocal fluorescence microscope (Olympus, Tokyo, Japan). Immunofluorescence labeling for the various antibodies directed against the respective cell types was consistent and reproducible across different rats.

Statistical analysis

Data were expressed as the mean ±standard deviation (SD) and analyzed using SPSS 17.0 statistical software (SPSS, USA). Comparisons among groups were performed using one-way analysis of variance and pairwise comparison was performed using the LSD-t test. *$p<0.05$, **$p<0.01$ was considered statistically significant.

Results

Gastrodin Ameliorated necrosis of the Cerebellum in Diabetic Rats

HE staining showed that Purkinje cells in the NC9W group were arranged neatly in a single row (Figure.1). The cells were characterized by a round nucleus and a discrete chromatin mass. Purkinje cells in the DM9W + S group were disorderly distributed, and the cell number decreased, and coupled with nuclear pyknosis. Interstitial edema could be observed in areas that were supposed to be Purkinje cells. Compared with DM9W + S group, the number of Purkinje cells in DM9W + G60 group were increased, which were arranged neatly. However, cell number of in DM9W + S group was still lower than NC9W group, and the effect of DM9W + G120 was lower than that of DM9W + G60 group, but the therapeutic effect was still effective.

Gastrodin Stabilizes LTD by Reducing the Expression of BDNF, TrkB and Inhibiting the Phosphorylation of GluR2 in the Cerebellum of Diabetic Rats

The protein expression of GluR2 and PKC was significantly decreased in DM9W + S group, which was significantly reversed after gastrodin intervention (DM9W + G group) ($P < 0.05$), DM9W + G60 group is more effective. On the contrary, protein expression levels of BDNF, TrkB, p-GluR2 were significantly

upregulation in DM9W + S group in comparison to the NC group ($p < 0.01$), the expression levels of these biomarkers in DM9W + G group were significantly higher than in DM9W + S group (Figure.2)

BDNF, TrkB and p-GluR2 in the Cerebellum of Diabetic Rats Mainly Increased in Purkinje Cells and Reversed after Gastrodin Treatment

Double immunofluorescence labeling showed that GluR2 and PKC was localized primarily in the cell body and projecting apical dendrites of the cerebellar Purkinje cells with Calbindin D-28K, (Fig. 3,5). Note that in NC9W and DM9W + G groups, the Purkinje cells emitted intense GluR2 immunofluorescence that was markedly attenuated in DM9W + S group. Furthermore, the number of Purkinje cells was decreased in DM9W + S group; this was coupled with decreased dendrites and their ramifications. A relative expression changes in BDNF (Fig. 6), TrkB (Fig. 7), P-GluR2 (Fig. 4) immunofluorescence was observed in the Purkinje cells in the above groups. In our study, The expression of GluR2 and PKC in diabetic rats was decreased in the Purkinje cell layer and increased after gastrodin administration. The expression of BDNF and TrkB increased in the Purkinje cell layer in diabetic rats. p-GluR2 increased in Purkinje cell layer and granular layer in diabetic rats, gastrodin decreased p-GluR2 expression in Purkinje cell layer.

Discussion

Our study found that BDNF and TrkB were increased in the cerebellar cortex of diabetic rats. BDNF in hippocampal neurons has been reported to upregulate AMPA receptor subunits and induce AMPA receptor transmission to synapses. [24] Our study also found that GLUR2, one of the AMPAR subunits, was significantly phosphorylated at serine 880 in Purkinje cells, but its upstream PKC was reduced. This suggests that the increase in BDNF/TrkB and the decrease in PKC did not increase the expression of synaptic GluR2, and we speculate that there may be other pathways that promote GLUR2 internalization. Both LTD and LTP as the basis of learning and memory may keep a stable level in a normal state, there is an imbalance between LTD and LTP under pathological or unstable conditions [25]. We suggest that this change may promote cerebellar LTD and weaken LTP in our study, which leads to the impairment of cerebellum and its cognitive function.

The internalization of AMPA receptor in the postsynaptic membrane of cerebellar Purkinje cells is a common pathway for cerebellar involvement in the process of learning and memory formation. Furthermore, it is one of the most critical cerebellar steps functions in the process of learning and memory formation [26]. So, the internalization of AMPA receptor is the key to cerebellar learning and memory function. Diabetic rats exhibited impaired learning and memory ability [27]. We speculate that the cerebellum, an indispensable area for motor learning and memory, may be involved in the occurrence and development of cognitive impairment. At present, there are few reports on the relationship between cognitive impairment and cerebellar lesions in type 1 diabetes mellitus. Only one study detected the effect of gestational diabetes on the distribution of Purkinje and granule cells in the cerebellum of neonatal rats [28].

Glutamate is an important neurotransmitter in the central nervous system. By binding to its receptors, glutamate regulates almost all the functions of the brain, including learning and memory [29]. Glutamate receptors can be divided into two categories: iGluR (ionotropic glutamate receptor) and mGluR (metabotropic glutamate receptor). Among those receptors, only the receptor subunits GluR2 and mGluR1 are located in the postsynaptic membrane, while mGluR1 and NMDA receptors are linked to playing a synergistic role. Studies have shown that cerebellar LTD in the cerebellum is not seriously affected after the administration of NMDA receptor blockers. Therefore, non-NMDA receptor is the key to inducing cerebellar LTD, including GluR2[30.31].

BDNF plays a key role in neuronal development, differentiation, and neurological disorders by regulating synaptic activity [32]. Synaptic activation leads to glutamate release and activation of NMDA and AMPA receptors in postsynaptic membrane [33]. BDNF can enhance synaptic plasticity [34]. Because the apoptosis of nerve cells in the central nervous system is often associated with some neuropsychiatric diseases, BDNF is often used as a pathological target for neuropathic diseases such as motor neuropathy and neurodegenerative diseases [35.36]. In this study, cerebellar BDNF expression was significantly increased in diabetic rats and decreased after gastrodin treatment.

Gastrodin is one of the main active components of Gastrodiaelata, also known as gastrodin, whose molecular formula is $C_{13}H_{18}O_7$. Gastrodin is distributed in all tissues of the body, especially in the kidney, digestive tract, and lung, and also in the brain. In the central nervous system, gastrodin can pass through the blood-brain barrier (BBB), whose absolute bioavailability is 30% ~ 37% [37.38]. It can also inhibit the excessive proliferation of glial cells. At present, more and more studies have focused on the microenvironment of glial cells and increased nutritional factors and cytokines in the microenvironment can activate neurons playing a supporting and sustaining role. Concerning motor learning, gastrodin can promote the long-term potentiation effect and thus enhance memory. [39.40] Some scholars have pointed out that the effect of gastrodin on learning and memory is exerted through up-regulating the expression of NFE2L2, ADH7, GPX2, and GPX3 proteins [41]. In this study, we found that gastrodin can significantly reduce the phosphorylation of GluR2 in the cerebellum of diabetic rats and restore the expression levels of BDNF and PKC, thereby stabilizing the balance between LTD and LTP in the cerebellum and enhancing learning and memory.

There are still some limitations in this study. Electrophysiological analysis should be conducted to confirm the changes of cerebellar LTD, which invites further investigation. Moreover, as the upstream of BDNF, the expression of NMDAs in the cerebellum of diabetic rats is decreased. Inhibitors or agonists of NMDAs were needed to observe the role of NMDAs in the future study.

Conclusion

Diabetic rats showed obvious neuronal necrosis and neuronophagia on Purkinje cell by observing Hematoxylin-Eosin (HE), and gastrodin had a therapeutic effect that Purkinje cell injury is significantly reduced. Moreover, the damage of Purkinje cells in the cerebellum of diabetic rats may be related to the

enhanced internalization of GluR2 in the cerebellum, and the increase of BDNF and the decrease of PKC may have the effect of preventing further enhancement of LTD and protecting neurons. These proteins may be the therapeutic targets of gastrodin. This study not only provided a theoretical basis for the therapeutic effects of gastrodin, but also the relationship between cerebellar neuronal damage and LTD-related factors in diabetes.

Declarations

Data availability

No datasets were generated or analyzed for this study.

Ethics statement

Animal procedures were reviewed and approved by the Medical Ethics Committee of Kunming Medical University, Kunming, China.

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Declaration of interest

Author Yi-Dan Liu was employed by company Kunming Pharmaceutical Corporation. All other authors declare no competing interests.

Authors' contributions

Y-YZ, Y-HM and YZ designed the project, analyzed the data, and finalized the manuscript. Y-BL and Y-YW performed the majority of the experiments, X-YW performed participated in discussion, analyzed the data, and prepared the first draft of the manuscript. Y-DL provided gastrodin, designed the usage and guided the project implementation. Z-HM performed participated in discussion and revised the manuscript. YZ, CZ, Y-JF, S-LC and GC conducted the part of experiments, helped with removal of tissue samples and took care of the experimental rats. Z-YQ performed the paraffin embedding, sectioning, and H&E staining.

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Figures

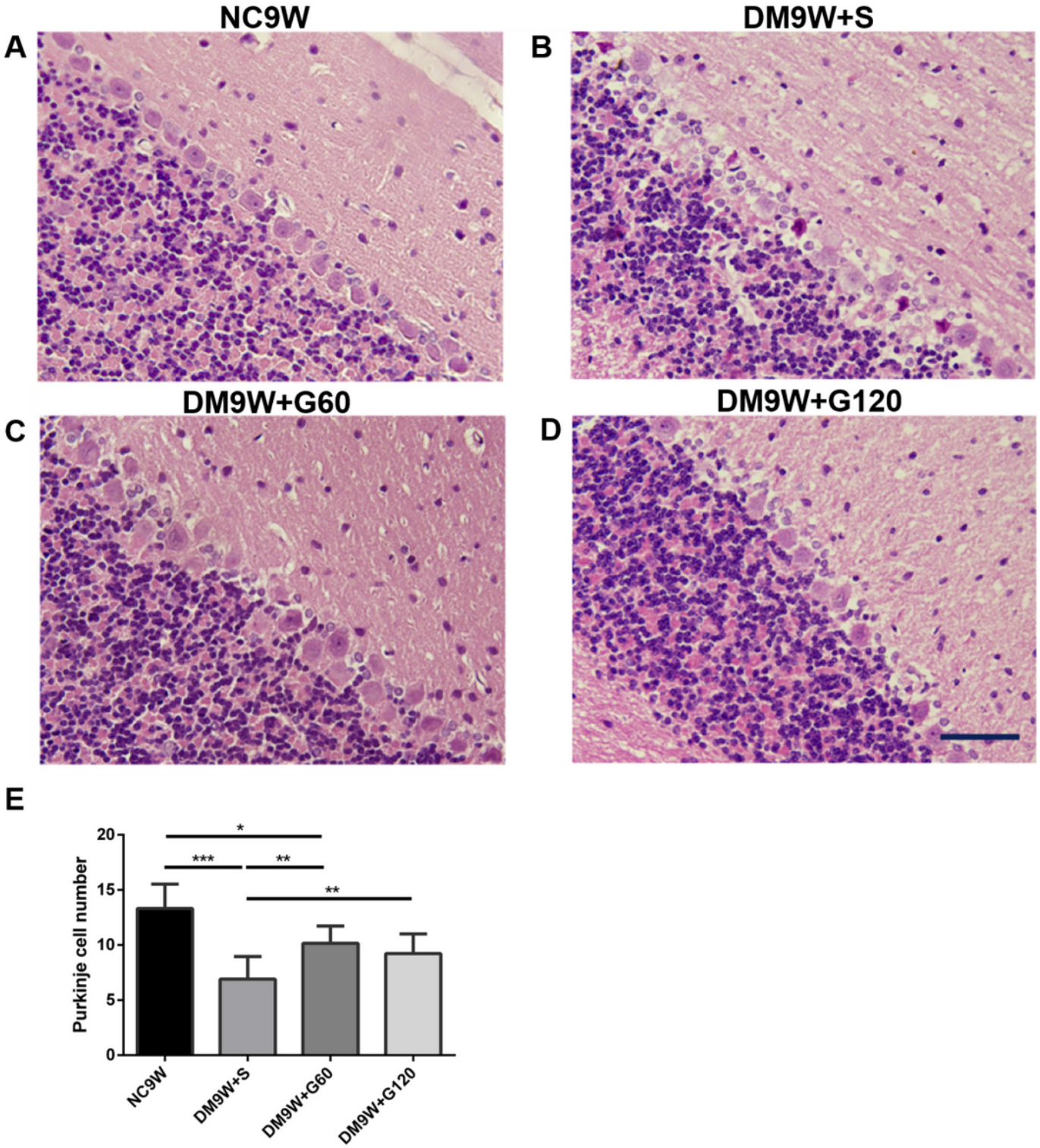

Figure 1

(A-D) Histopathological alterations were evaluated by HE staining (magnification ×400) the NC9W, DM9W+S, DM9W+G60 and DM9W+G120 group in the cerebellum cortex were shown. Purkinje cells are located about the diagonal of each image, with the molecular layer on the upper right side and the granular layer on the lower left side. (E) Quantification of cerebellar Purkinje cells in NC9W, DM9W+S,

DM9W+G60 and DM9W+G120 groups. Bar graph representing Purkinje cell number (mean±SD). Bar = 50 μm. *p⩽0.05, **p⩽0.01 and ***p⩽0.001.

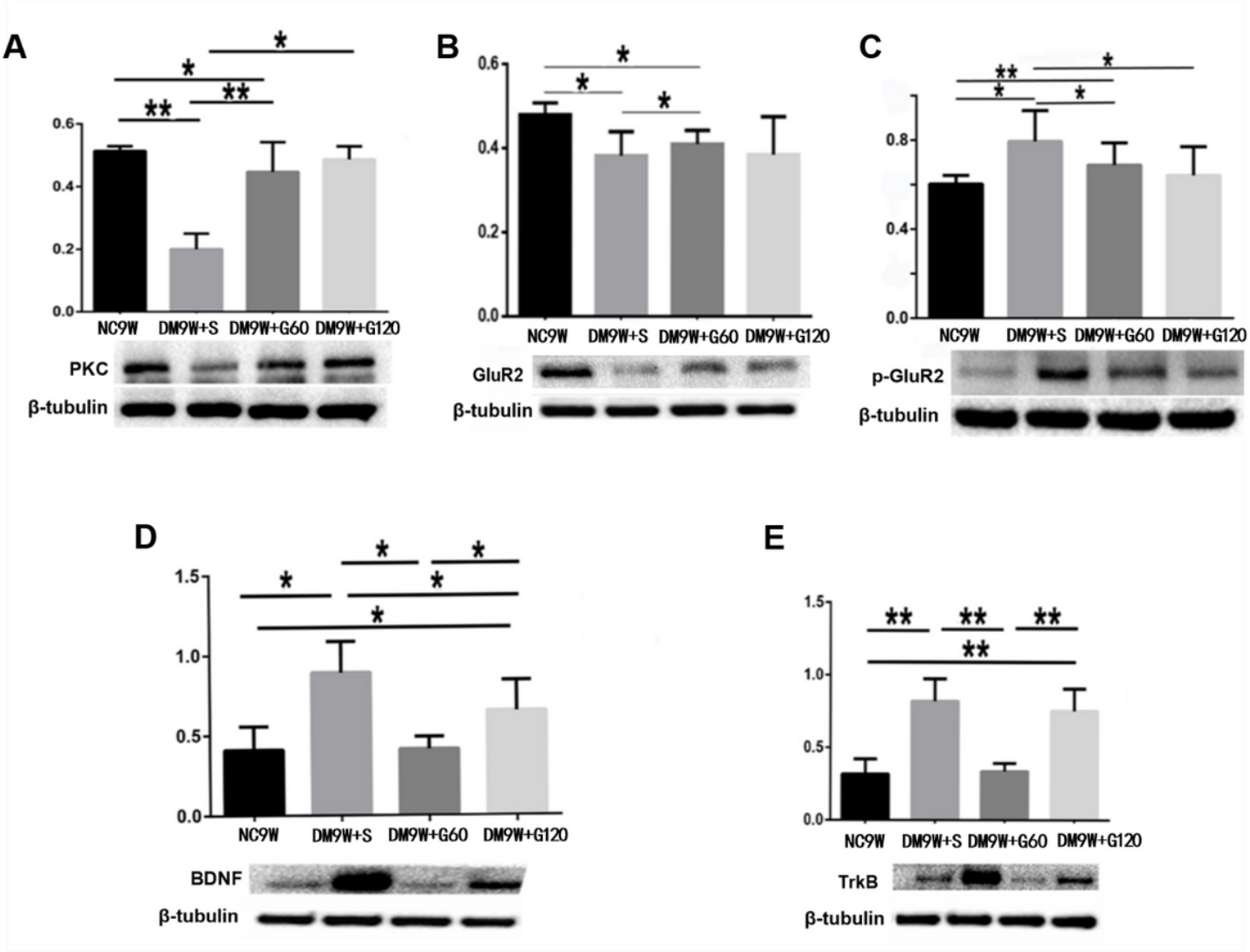

Figure 2

(A-E) Western blot analysis of PKC, GluR2, p-GluR2, BDNF and TrkB expression levels in the cerebellum cortex. immunoreactive bands. Bar graph representing optical density (mean±SD) of PKC, GluR2, P-GluR2, BDNF and TrkB normalized to β-tubulin for sham, *p<0.05, **p<0.01

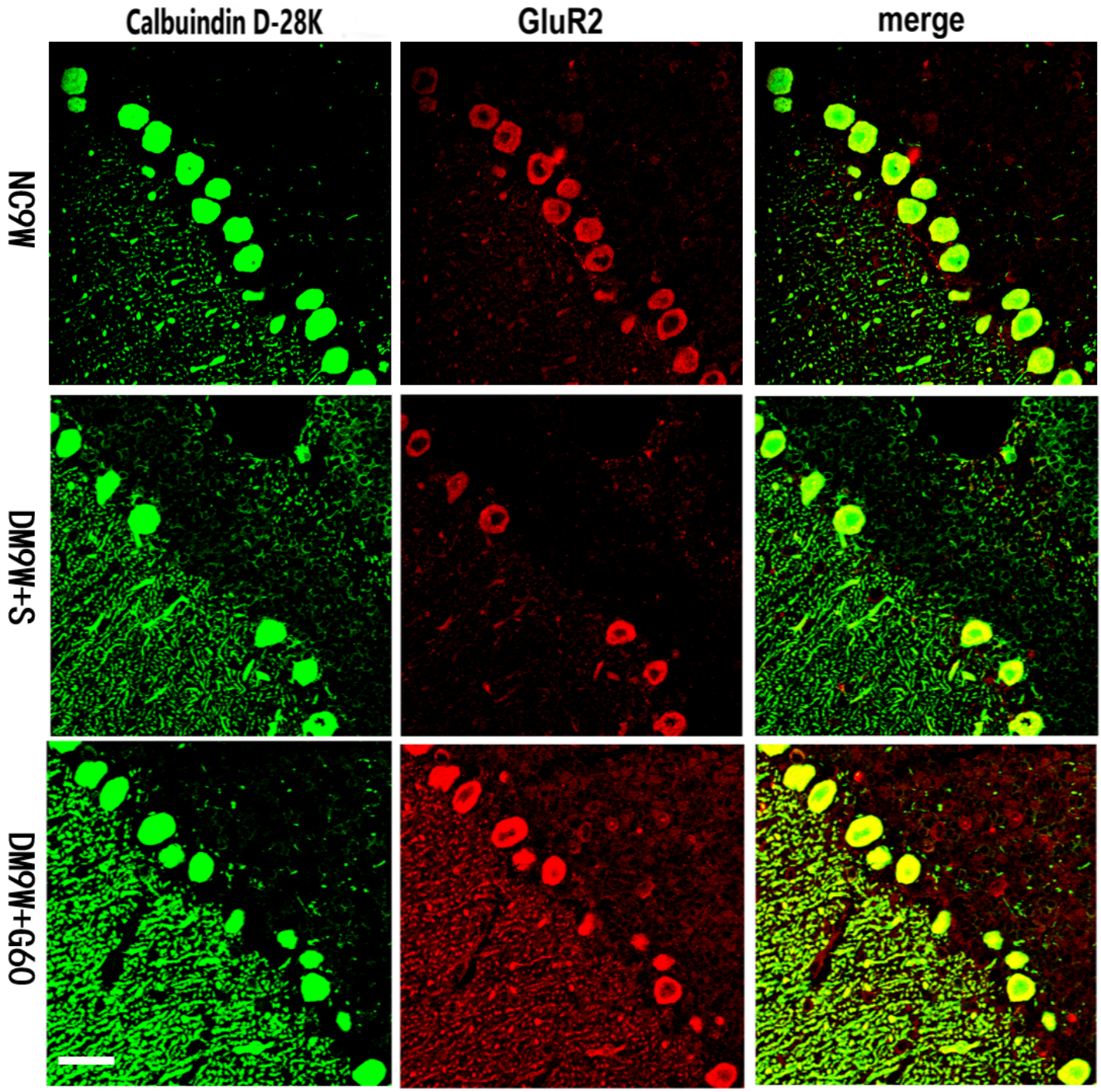

Figure 3

Immunoexpression of GluR2 in cerebellum cortex of the NC6W group, the DM9W+S group, and the DM9W+G group. Calbindin D-28k was shown in green (Alexa 488), and GluR2 are shown in red (Alexa Flour 546), Magnification: ×600. Bar = 50 μm

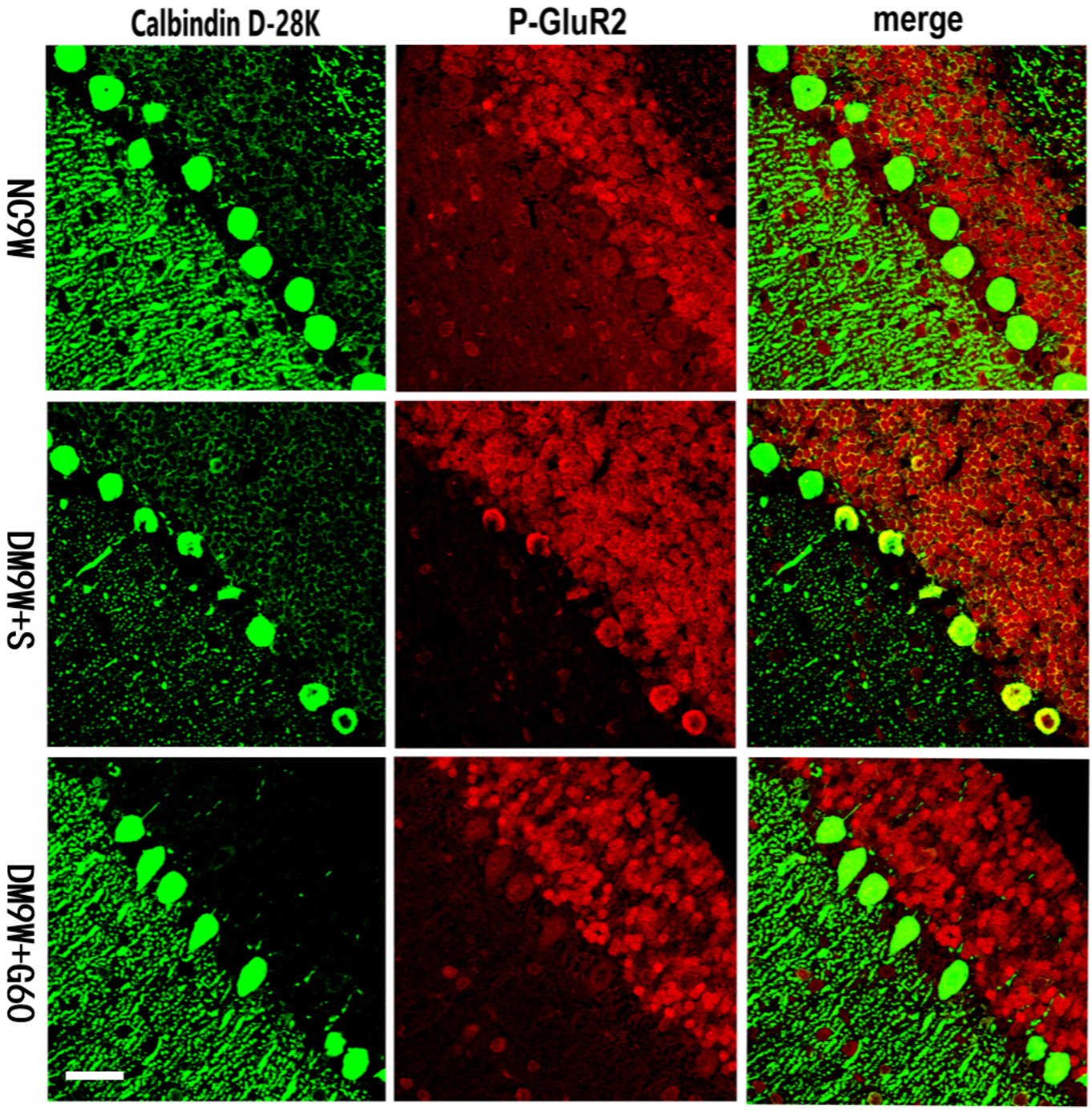

Figure 4

Immunoexpression of p-GluR2 in cerebellum cortex of the NC6W group, the DM9W+S group, and the DM9W+G group. Calbindin D-28k was shown in green (Alexa 488), and P-GluR2 are shown in red (Alexa Flour 546), Magnification: ×600. Bar = 50 μm

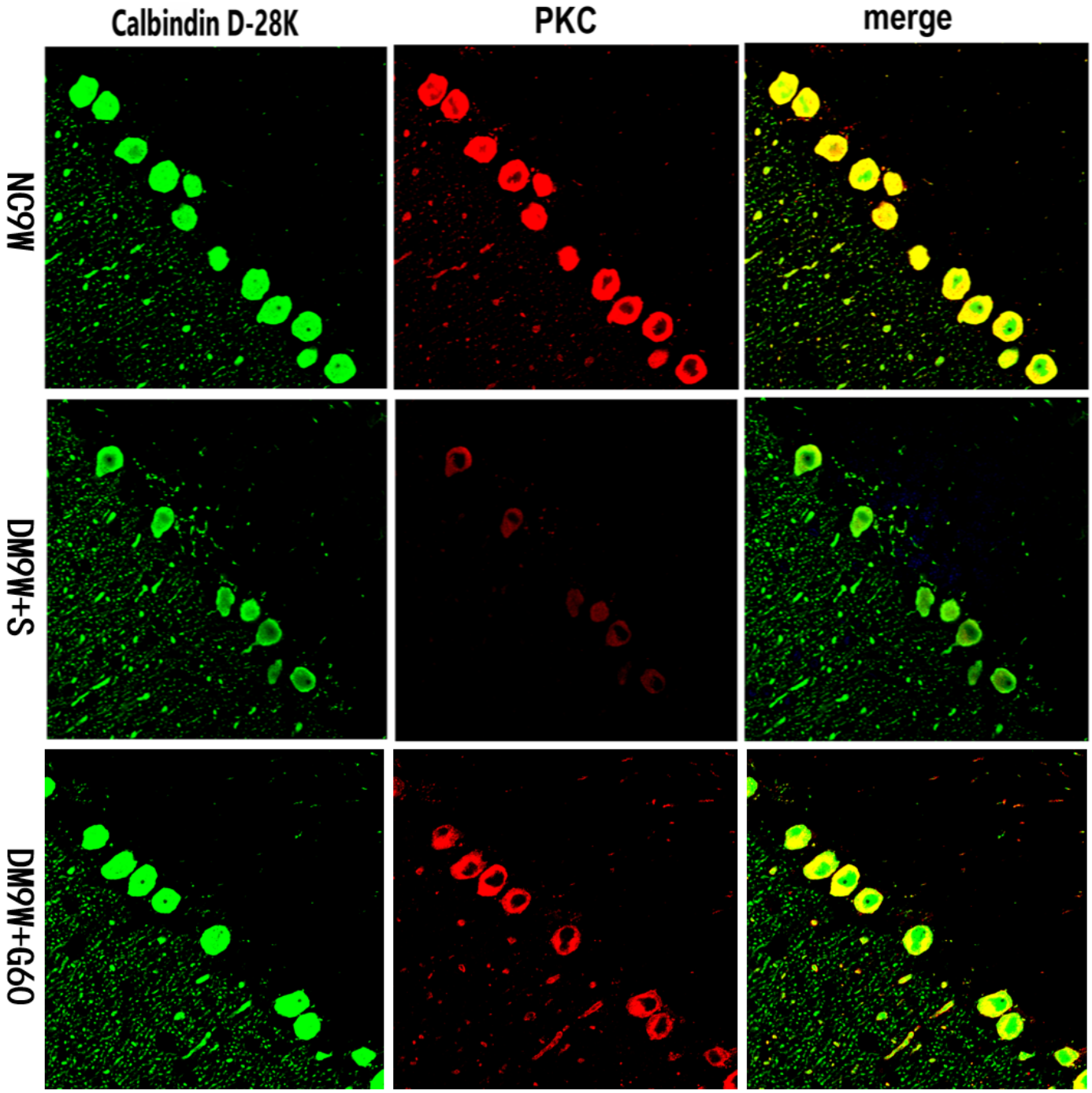

Figure 5

Immunoexpression of PKC in cerebellum cortex of the NC6W group, the DM9W+S group, and the DM9W+G group. Calbindin D-28k was shown in green (Alexa 488), and PKC are shown in red (Alexa Flour 546), Magnification: ×600. Bar = 50 μm

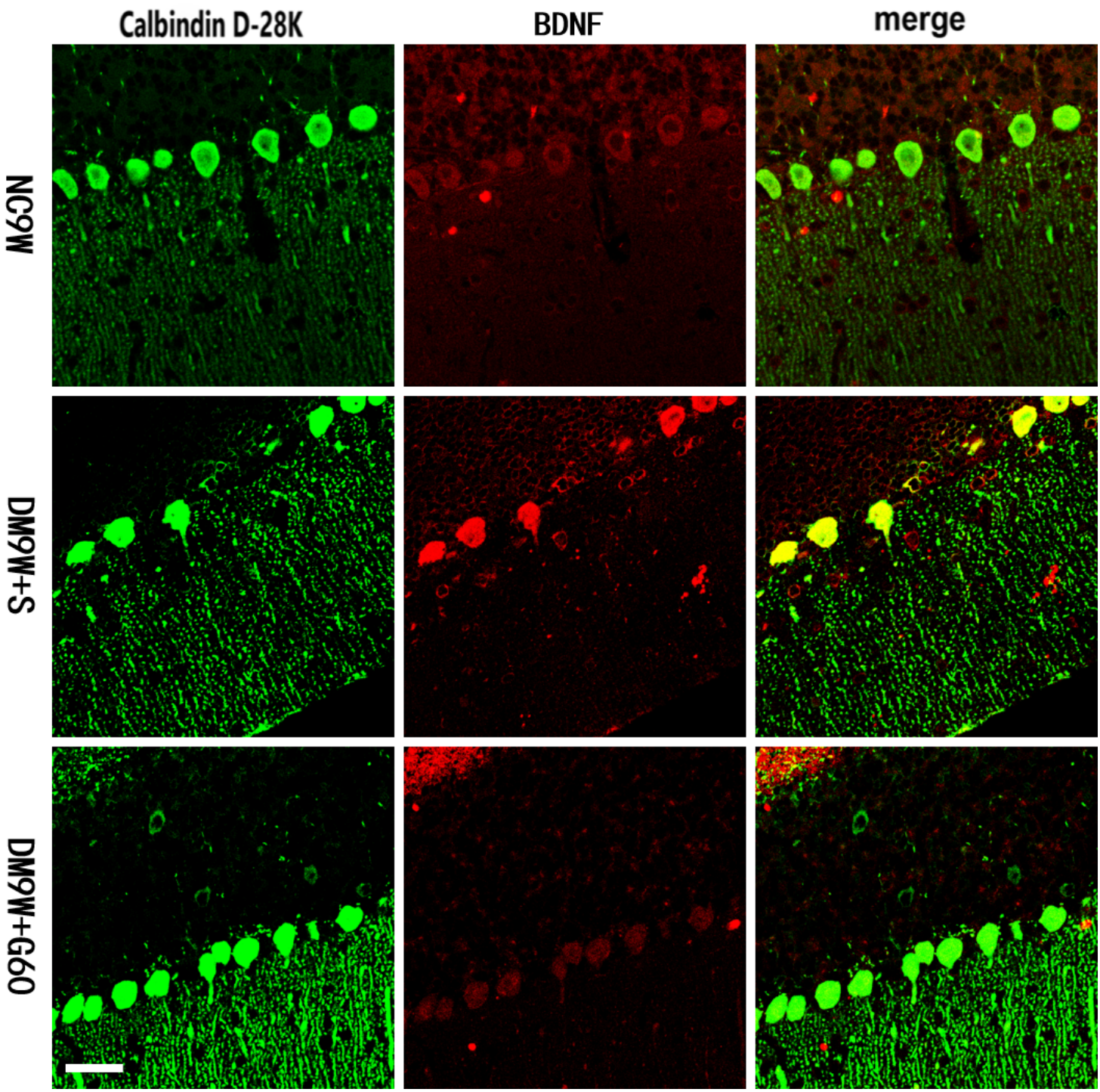

Figure 6

Immunoexpression of BDNF in cerebellum cortex of the NC6W group, the DM9W+S group, and the DM9W+G group. Calbindin D-28k was shown in green (Alexa 488), and BDNF are shown in red (Alexa Flour 546), Magnification: ×600. Bar = 50 μm

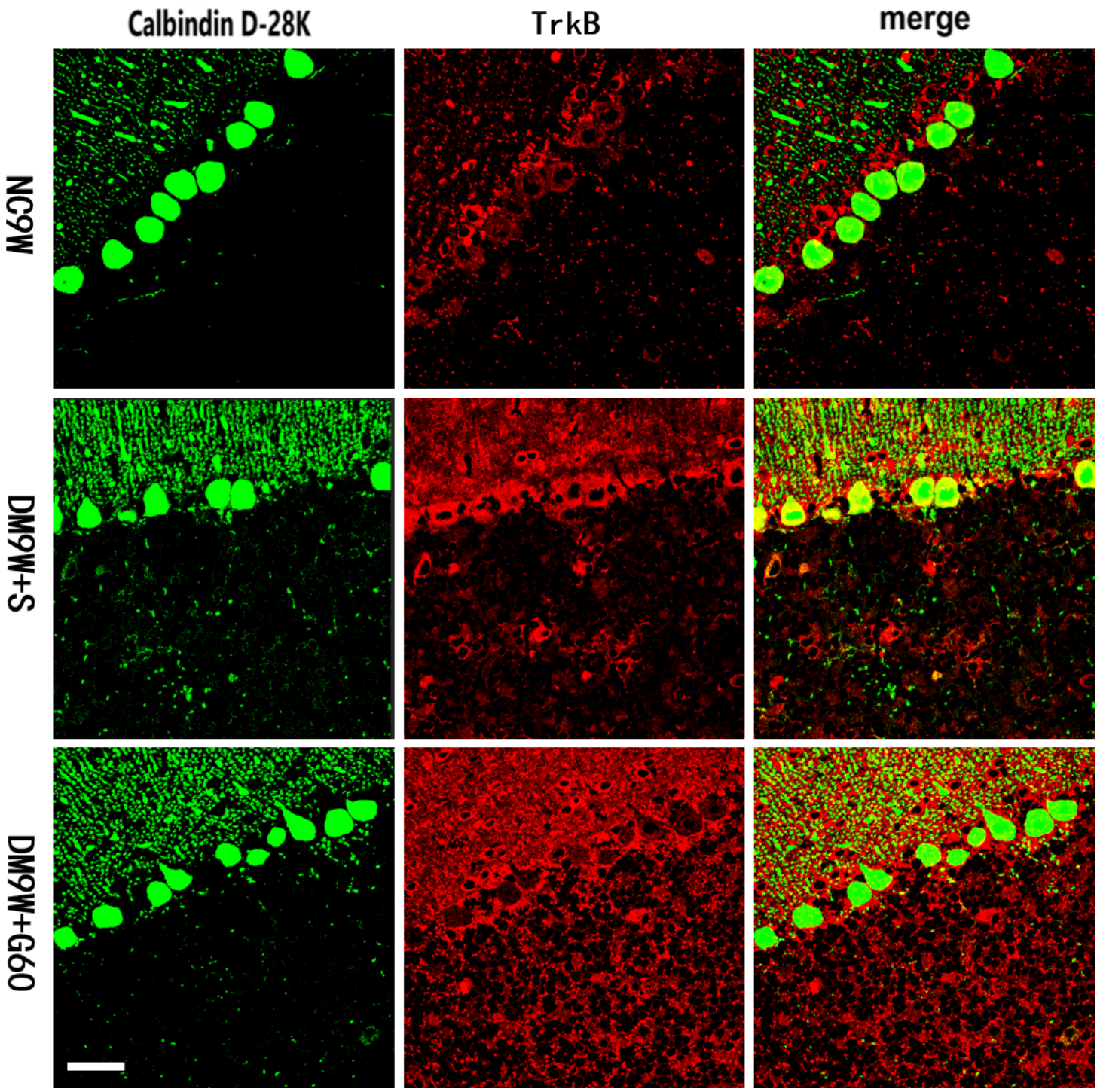

Figure 7

Immunoexpression of TrkB in cerebellum cortex of the NC6W group, the DM9W+S group, and the DM9W+G group. Calbindin D-28k was shown in green (Alexa 488), and TrkB are shown in red (Alexa Flour 546), Magnification: ×600. Bar = 50 μm

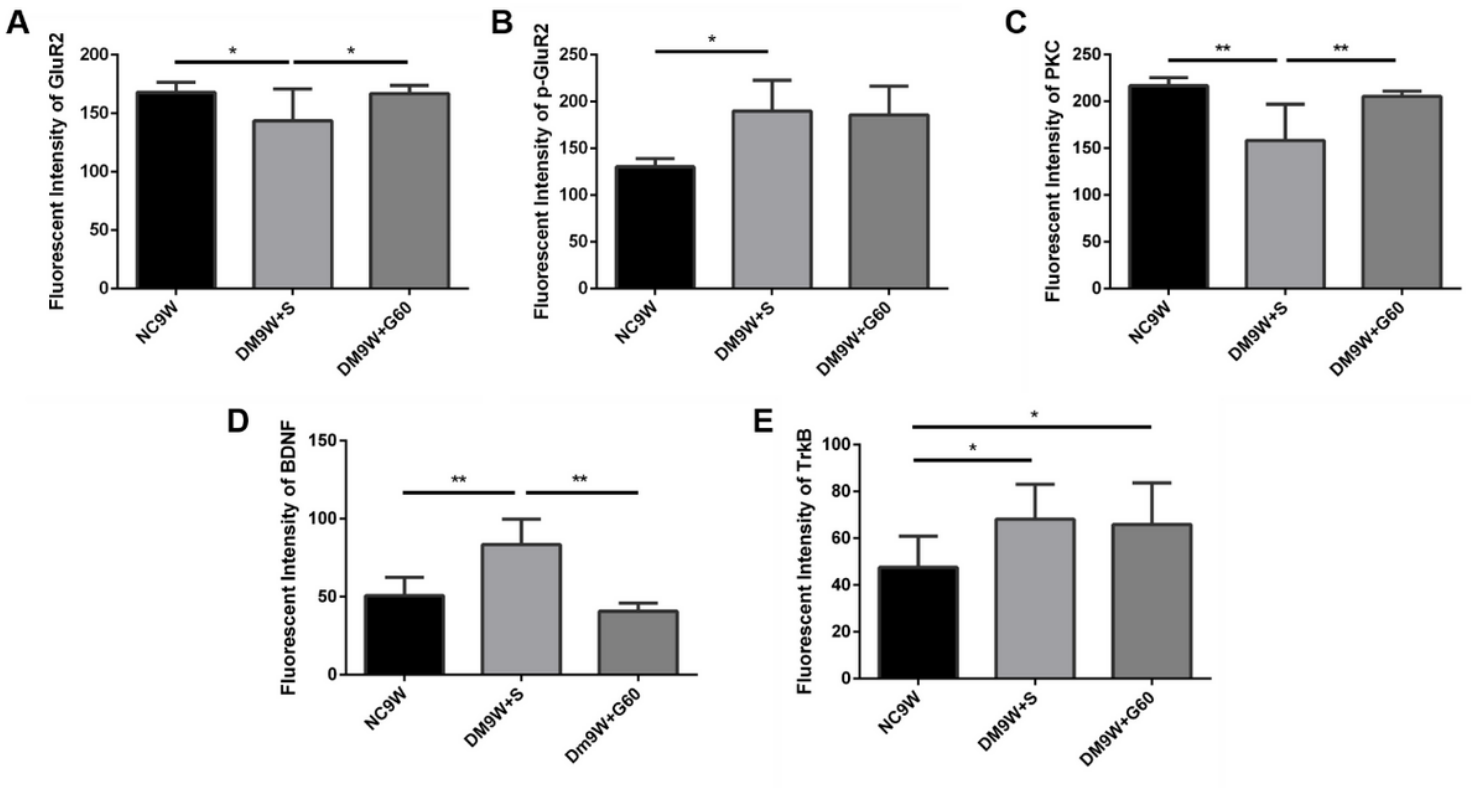

Figure 8

(A-E) Semi-quantification of GluR2, p-GluR2, PKC, BDNF and TrkB by immunofluorescence. Bar graph representing fluorescent intensity (mean±SD) of PKC, GluR2, p-GluR2, BDNF and TrkB. *p<0.05 and p<0.01.

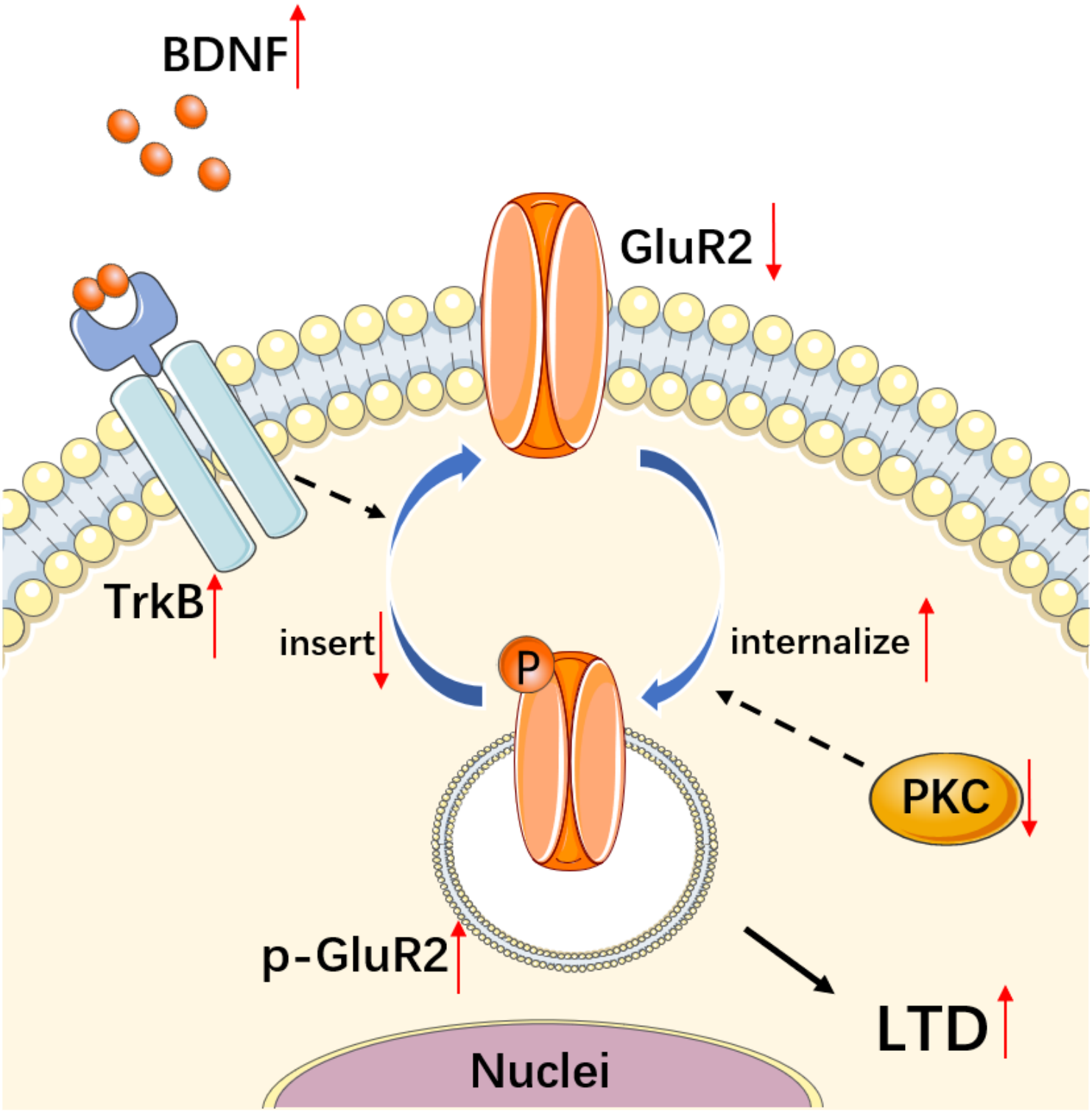

Figure 9

Diagram illustrating the process of diabetes causing cerebellar neuronal damage through the LTD-related factors, which has been indicated by red arrows. Diabetes can enhance the internalization of GLUR2, increase BDNF and TrkB, and decrease PKC expression.