Original Paper

A Novel Role for the Calcium Sensing Receptor in Rat Diabetic Encephalopathy

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Key Words

Calcium sensing receptor • Diabetic encephalopathy • $[Ca^{2+}]_i$ • High glucose

Abstract

Background: Diabetic encephalopathy is a common complication of diabetes, and it may be involved in altering intracellular calcium concentrations ($[Ca^{2+}]_i$) at its onset. The calcium sensing receptor (CaSR) is a G-protein coupled receptor, however, the functional involvement of CaSR in diabetic encephalopathy remains unclear. **Methods:** In this study, diabetic rats were modeled by STZ (50 mg/kg). At the end of 4, 8 and 12 weeks, the CaSR expression in hippocampus was analyzed by Western blot. In neonatal rat hippocampal neurons, the $[Ca^{2+}]_i$ was detected by laser scanning confocal microscopy, the production of reactive oxygen species (ROS) in mitochondria, the level of NO and the mitochondrial transmembrane potential were measured by MitoSOX, DAF-FM and JC-1, respectively. **Results:** Our results showed in hippocampal neurons treated with high glucose, CaSR regulated $[Ca^{2+}]_i$ through the PLC-IP3 pathway. CaSR expression was decreased and was involved in the changes in $[Ca^{2+}]_i$. Mitochondrial membrane potential, NO release and expression of p-eNOS decreased, while the production of ROS in mitochondria increased. **Conclusion:** Down-regulation of CaSR expression was accompanied by neuronal injury, calcium disturbance, increased ROS production and decreased release of NO. Up-regulation of CaSR expression attenuated these changes through a positive compensatory protective mechanism to inhibit and delay diabetic encephalopathy in rats.

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Introduction

Diabetes and its associated complications, such as diabetic cardiomyopathy, diabetic nephropathy, and peripheral arterial disease, are known as major health disorders. The epidemic of obesity as well as sedentary lifestyles are projected to result in over 300 million people with diabetes by 2025 [1]. Diabetes increases the risk of nervous system morbidity and mortality. In the last decade, mounting evidence has indicated that type 1 diabetes has adverse effects on central nervous system (CNS) function and cognition [2].

Diabetic encephalopathy was first proposed by Nielsen [3] as a diabetic complication and was associated with cognitive deficits and an increased risk of dementia, particularly in the elderly. Cognitive dysfunction in diabetes is characterized by lowered performance in several cognitive domains, especially slowing of mental speed and diminished flexibility. Studies of streptozotocin (STZ)-induced diabetes in rats have demonstrated neurobehavioral deficits using the Morris water maze, which is associated with impaired hippocampal long-term potentiation [4]. The multifaceted pathogenesis of diabetic encephalopathy is not yet completely understood, but classical factors such as an imbalance in Ca^{2+} homeostasis [5] may contribute to the pathology of diabetic encephalopathy. In the CNS, Ca^{2+} acts as a second messenger and is known to be associated with almost every cellular event, including ion channel function, cellular proliferation and migration, neurotransmitter secretion, axon pathfinding, and dendritic outgrowth. Therefore, the alterations in $[\text{Ca}^{2+}]_i$ affect the execution of Ca^{2+} -dependent functions. Oxidative stress is also considered to regulate the pathogenesis of diabetic encephalopathy. The brain is especially vulnerable to oxidative damage as a result of its high oxygen consumption rate. Under normal physiological conditions, a balance exists between the production of ROS and antioxidant mechanisms in the CNS. Toxic O_2^- and OH^- radicals have been implicated in diabetes because they cause non-specific glycation of proteins, peroxidation of membrane lipids and protein cross-linking, causing loss of organelle function and cell death [6].

Ca^{2+} and ROS are involved in the response to diabetic encephalopathy. However, it is still not clear how these two compounds play roles in diabetic encephalopathy. In addition, it has been clearly proven that both ROS and Ca^{2+} signals are intimately interconnected, as sub-micromolar concentrations of Ca^{2+} stimulate ROS production by mitochondria [7]. This might be significant in de-energized mitochondria *in vivo*, which are unable to take up Ca^{2+} and to control the cytosolic Ca^{2+} concentration. The mechanisms of the harmful effects of Ca^{2+} on mitochondria are generally assumed to involve high Ca^{2+} loads and excessive ROS generation. However, data available from *in vitro* studies are very contradictory as to the effect of Ca^{2+} on mitochondrial ROS generation [8].

The calcium-sensing receptor (CaSR) is a member of the G protein-coupled receptor (GPCR) superfamily [9]. CaSR has been localized to almost all areas of the brain, including the hippocampus [10]. As a GPCR, CaSR activation stimulates phospholipase to generate diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 can trigger the release of calcium from internal stores while DAG activates protein kinases C (PKC) [11].

CaSR has been implicated in a variety of functions in neurons, including neuronal growth, synaptic plasticity, migration, differentiation, injury, neurotransmission and neurodegeneration with long-term memory loss in Alzheimer's disease [12-14]. However, the potential role of CaSR in the progress of diabetic encephalopathy has not been examined.

Neurons are highly specialized for the processing and transmission of cellular signals; they communicate with one another via synapses, where the axon terminal of one cell impinges upon another neuron's dendrite, soma or, less commonly, axon. CaSR has been implicated in neuronal growth, as activation of CaSR in late fetal sympathetic neurons promoted axonal growth, whereas CaSR-deficient or deleted mice failed to exhibit such a change [12].

In this study, we demonstrated that CaSR regulated $[\text{Ca}^{2+}]_i$ and identified its mechanism in hippocampal neurons incubated with high glucose concentrations.

Materials and Methods

Animals

Male Wistar rats (200–250 g) were purchased from Harbin Medical University (Heilongjiang, China). All Wistar rats were 12–16 weeks old. All animal protocols were approved by the Animal Care and Use Committee of Harbin Medical University.

Diabetes model and treatment protocols

Diabetes was induced in rats by tail vein injection of streptozotocin (STZ, 50 mg/kg) dissolved in 0.1 M citrate buffer (pH 4.5), whereas the control group was injected with citrate buffer alone by the same route. Blood glucose levels were measured using blood glucose test strips 72 h after STZ injection, and blood glucose levels over 16.7 mmol/L were considered to indicate diabetes. Wistar rats were randomly divided into 4 groups including control, diabetic-4 weeks (Dia-4W), diabetic-8 weeks (Dia-8W) and diabetic-12 weeks (Dia-12W). Blood glucose measurements were repeated after 2 weeks of treatment in each group.

Cell culture and preparation

Primary cultures of neonatal rat hippocampal neurons were prepared by previously described methods [15]. Briefly, early postnatal rats were decapitated, and the brains dissected from the skull and then transferred into a Petri dish with pre-cooled D-Hanks solution. Brains were cut along the midline, and the hippocampus was extracted from each hemisphere. Hippocampi were incubated in solution of 0.125% crude trypsin (1.5 ml) for 20 minutes at 37°C. This solution was replaced with a 0.125% solution of crude trypsin with Trypsin inhibitor and was incubated for 5 minutes at room temperature. Cells were centrifuged at 1000 r/min for 10 minutes, and then, hippocampi were cultured as monolayers at a density of $4\text{--}5 \times 10^6$ cells/cm² in neurobasal medium (Gibco) supplemented with 2% B-27 and 2 mM glutamine in humidified incubator (5%CO₂ at 37°C).

Neonatal rat hippocampal neurons were randomly divided into the following treatment groups: control (low glucose, 5.5 mM), high glucose (HG, 30.0 mM), HG + Calindol (2 μM, a CaSR activator), HG + Calhex231 (3 μM, a CaSR inhibitor), HG + CaCl₂ (2.3 mM), HG + IBMX (100 μM, 3-isobutyl-1-methylxanthine, a phosphodiesterase (PDE) inhibitor), and HG + L-NMMA (100 μM, a nitric oxide synthase inhibitor). Drugs were dissolved in pre-warmed medium and added directly to the culture for 48 or 72 hours.

Neonatal rat hippocampal neurons loaded with Fluo-4 AM

[Ca²⁺]_i were determined as previously described [16, 17]. In brief, neurons were plated on poly-L-lysine-coated 15 mm glass coverslips at a density of 5,000 cells per coverslip. On the day of imaging, neurons were washed twice with normal saline solution (NSS: 137 mM NaCl, 25 mM Glucose, 10 mM HEPES, 5 mM KCl, 1 mM MgCl₂, and 2 mM CaCl₂ at pH 7.4).

The washed neurons were then labeled with 5 μM Fluo-4 AM to measure [Ca²⁺]_i for 30 min at 37°C. The neurons were washed twice with NSS and left at 37 °C for another 30 min to allow for complete hydrolysis of the acetoxymethyl (AM) ester moiety, during which time the cells were treated with pipette-applied U73122 (PLC inhibitor, 10 μM), 2-APB (IP₃R inhibitor, 75 μM), Calhex231, MK801 (NMDA receptor inhibitor, 10 μM), or CdCl₂ (calcium channel inhibitor, 0.2 mM). The neurons were then exposed to Calindol, and the fluorescence measurement of Ca²⁺ was captured using a laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm for [Ca²⁺]_i.

Mitochondrial ROS production

Mitochondrial ROS production was measured using MitoSOX Red [18]. Single rat hippocampi were loaded with 5 μmol/L MitoSOX Red at room temperature for 15 min. MitoSOX Red fluorescence was measured at 583 nm following excitation at 488 nm using a Zeiss LSM 510 inverted confocal microscope.

Analysis of mitochondrial transmembrane potential

Treated neurons (48 h) were washed with cold PBS and stained with JC-1 (4 μg/ml, Molecular Probes) for 15 min at 37°C in the dark. The data were acquired using a Zeiss LSM 510 inverted confocal microscope measuring the levels of green and red fluorescence at 514/529 (FL-1) and 585/590 nm (FL-2), respectively.

NO detection by fluorescence

The fluorescent probe DAF-FM (Merck, Darmstadt, Germany) was used to detect intracellular NO [19]. Treated neurons (48 h) were washed with cold PBS and supplemented with 10 μ M DAF-FM for 30 min at 37°C. DAF-FM fluorescence was excited at a wavelength of 480 nm, while emission was detected with an emission filter of 527 nm.

Statistical analysis

Data are expressed as the mean $\pm$ S.E.M. All data involving multiple groups were analyzed by one-way ANOVA. Differences between individual groups were analyzed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results*General features of experimental rats*

Some evidence demonstrates that defective adult neurogenesis is present in individuals with diabetes. However, the mechanisms related to depression and cognitive dysfunction in patients with diabetes have not been elucidated. In our study, a type 1 diabetes model was established by intraperitoneal injection of STZ. Compared with the control group, water and food intake and the concentration of glucose were significantly increased in the diabetes groups (Fig. 1a). These results suggested that the type 1 diabetes model was successfully established.

Ultrastructural changes in the hippocampus of diabetic rats viewed by transmission electron microscopy and the apoptotic rate of the diabetic hippocampal neurons examined by TUNEL

Using transmission electron microscopy, we showed that sections of rat hippocampus in the control group had normal structures, nuclear chromatin was rich, and there was abundant rough endoplasmic reticulum. Synaptic structure was clear, and the mitochondria were typically intact. In the dia-4 w group, the hippocampal morphological structure was damaged, the neurons arranged loosely, degenerative diseases occurred, the mitochondria were swollen and slightly disrupted. However, small vessel architecture was intact, and a minority of synapses were mixed together and fused. With the progression of diabetes, pathological changes gradually became more obvious. In the dia-12 w group, obvious swelling, vacuolating and disruption of mitochondria were observed, and a large number of synapses were destroyed (Fig. 1c). To confirm that diabetes results in apoptosis of hippocampal neurons, we examined apoptosis using the TUNEL assay in hippocampus. The number of TUNEL-positive hippocampal neurons was low in the control group. The percentages of apoptotic cells in the dia-4 w, the dia-8 w and the dia-12 w groups were increased relative to the control group (Fig. 1b).

Measurement of CaSR levels in the rat hippocampus and hippocampal neurons, as detected by Western blot

CaSR is substantially expressed within the hippocampus [20] and has been implicated in a variety of functions in neurons. In our study, the expression of CaSR in the rat hippocampus was detected by Western blot (Fig. 2a). The CaSR protein content in the hippocampus of rats in the dia-4 w, dia-8 w and dia-12 w groups were decreased compared to the control group ($P < 0.05$). These results showed that the expression of CaSR was decreased in the hippocampus of diabetic rats.

To further confirm the role of CaSR in hippocampus of diabetic rats, cultured neonatal rat hippocampal neurons were treated with 30 mM glucose to mimic cellular diabetic encephalopathy. The levels of CaSR protein were detected by Western blot (Fig. 2b). The CaSR protein content in HG group was decreased compared to the control group ($P < 0.05$). CaSR protein levels in the HG+Calindol group were increased compared to the HG group ($P < 0.05$),

Fig. 1. (a). General characteristics of control and diabetic rats. (b). Representative image of TUNEL staining in the hippocampus of diabetic rats. Quantitation of TUNEL staining for the different groups is shown. Values represent group means $\pm$ SEMs. ** $P < 0.01$ vs. Control group, *** $P < 0.001$ vs. Control group. (c). Ultrastructural alterations in rat hippocampus in the different groups. "☆" indicated apoptosis; "△" indicated mitochondrion damage; "→" indicated synapsis damage. (A) Control group, (A1: 4000X; A2: 25,000X; A3: 25,000X). (B) Dia-4 W group, (B1: 4000X; B2: 25,000X; B3: 20,000X). (C) Dia-8 W group, (C1: 4000X; C2: 40,000X; C3: 15,000X). (D) Dia-12 W group, (D1: 4000X; D2: 25,000X; D3: 15,000X).

while CaSR levels in the HG+Calhex231 group were decreased ($P < 0.05$). These results demonstrated that Calindol could up-regulate the expression of CaSR, while Calhex231 could down-regulate the expression of CaSR.

Measurement of $[Ca^{2+}]_i$ by laser confocal microscopy

Ca^{2+} plays an important role in regulating nuclear functions, including cell growth, and death. Our results revealed that Calindol increased the $[Ca^{2+}]_i$. Pretreated with Calhex231, U73122 and 2-APB, Calindol couldn't increase the $[Ca^{2+}]_i$, the basal $[Ca^{2+}]_i$ and the amplitude of Ca^{2+} transients remained unchanged. These results indicated that hippocampal neurons could regulate $[Ca^{2+}]_i$ via the CaSR-PLC-IP₃ pathway. However, compared to the control group, $[Ca^{2+}]_i$ was significantly decreased (Fig. 2c,2d).

Fig. 2. (a) Protein levels of CaSR in the hippocampus were analyzed by Western blot. Basal levels of CaSR were detected in the control group, which served as the baseline for computing the fold increase for the other groups. The quantitation of the Western blot analyses for CaSR is shown in the lower panel. The fold change values represent group means $\pm$ SEMs. * $P < 0.05$ vs. Control. (b) Protein levels of CaSR in hippocampal neurons were analyzed by Western blot. Basal levels of CaSR were detected in the control group, which served as the baseline for computing the fold increase for the other groups. The quantitation of the Western blot analyses for CaSR is shown in the lower panel. The fold change values represent group means $\pm$ SEMs. * $P < 0.05$ vs. Control; ** $P < 0.01$ vs. Control; *** $P < 0.001$ vs. Control; § $P < 0.001$ vs. HG group. (c) Changes in the

Ca²⁺ gradient in hippocampal neurons was detected by laser scanning confocal microscopy with Fluo-4AM staining. Fluorescence images in A, B, C and D represent 0 s, 30 s, 180 s and 300 s, respectively. Scale bar is 200µm. (d) The changes in fluorescence intensities of intracellular calcium were recorded continuously with laser scanning confocal microscopy for each of the different treatments. Intracellular calcium was recorded after 48 h of high glucose incubation.

Fig. 3 Protein levels of p-CaMKII/CaMKII in the hippocampus were analyzed by Western blot. Basal levels of p-CaMKII/CaMKII were detected in the control group, which served as the baseline for computing the fold increase for the other groups. The quantitation of the Western blot analysis for p-CaMKII/CaMKII is shown in the lower panel. The fold change values represent group means $\pm$ SEMs. * $P < 0.05$ vs. control; § $P < 0.05$ vs. HG group.

Measurement of p-CaMKII and CaMKII levels in hippocampal neurons by Western blot

Calmodulin-dependent kinase (CaMKII) plays a critical role in mediating calcium-induced dendritic growth in cortical neurons [21]. The levels of phosphorylated CaMKII (p-CaMKII) and total CaMKII protein in hippocampal neurons were analyzed by Western blot. The data showed that the levels of p-CaMKII in the HG, HG+Calindol, HG+Calhex231 and HG+KN93 groups were decreased compared to the control group ($P < 0.05$). Compared to the HG group, the p-CaMKII levels were increased in the HG+Calindol group and decreased in the HG+Calhex231 and HG+KN93 groups ($P < 0.05$). Compared to the control group, the level of total CaMKII in the HG, HG+Calindol and HG+KN93 groups were increased ($P < 0.05$). The levels of p-CaMKII and CaMKII in hippocampal neurons from the CaCl₂ group remained nearly unchanged (Fig. 3).

NO release detection by fluorescence and p-eNOS measurement by Western blot

NO is an essential gas messenger in diverse neural processes such as the regulation of dendrite and dendritic spine morphologies [22, 23]. Fluorescence was utilized to detect NO generation in hippocampal neurons (Fig. 4a). Compared with the control group, the NO fluorescence intensity was lower in the HG, HG+Calhex231 and HG+L-NMMA groups ($P < 0.05$). In contrast, the HG+Calindol group and the HG+CaCl₂ group had increased levels of NO compared to the control group and the HG group ($P < 0.05$). The levels of phosphorylated eNOS (p-eNOS) in hippocampal neurons were analyzed by Western blot, which showed that p-eNOS levels in the HG group were decreased compared to the control group ($P < 0.05$). Compared with the HG group, the expression of p-eNOS in the HG+Calindol group and the CaCl₂ group were increased, while the p-eNOS protein content of the HG+Calhex231 group and the L-NMMA group were decreased ($P < 0.01$) (Fig. 4b).

Fig. 4. (a) The diversity of NO production in different groups. (A) Control, (B) HG, (C) HG+Calindol, (D) HG+Calhex231, (E) HG+L-NMMA, (F) HG+CaCl₂. The quantitation of NO generation for each of the different groups is shown in the lower panel. Values represent group means $\pm$ SEMs. *** $P < 0.001$ vs. Control group; § $P < 0.05$ vs. HG. Scale bar is 100 μ m. (b) The protein levels of p-eNOS in hippocampal neurons were analyzed by Western blot. Basal levels of p-eNOS were detected in the control group, which served as the baseline for computing the fold increase for the other groups. The quantitation of the Western blot analysis for p-eNOS is shown in the lower panel. The fold change values represent group means $\pm$ SEMs. * $P < 0.05$ vs. control; ** $P < 0.01$ vs. HG.

Neurite outgrowth

To investigate the influence of high glucose on the neurite outgrowth of hippocampal neurons, the total neurite length for hippocampal neurons of each condition was analyzed by immunofluorescence labeling for anti- β III-tubulin. Compared with the control group ($3569 \pm 315.2 \mu$ m), the total neurite length in the high glucose (HG) and HG+Calhex231 groups was

Fig. 5. Total neurite length for rat hippocampal neurons in each condition was detected by immunofluorescence labeling with anti- β -tubulinIII (200X). (A) Control, (B) HG, (C) HG+Calindol, (D) HG+Calhex231. The quantitation of the total neurite length for neurons in each condition is shown in the lower panel. The fold change values represent group means $\pm$ SEMs. * $P < 0.05$ vs. control; § $P < 0.05$ vs. HG. Scale bar is 200 μ m.

decreased ($2698 \pm 371 \mu$ m and $2374 \pm 600 \mu$ m, respectively) compared to the control group ($P < 0.05$), while the HG+Calindol group was increased ($3614 \pm 525.2 \mu$ m) ($P < 0.05$). The total neurite length in the HG+Calindol group was increased relative to the HG group ($P < 0.05$), while the HG+Calhex231 group was decreased ($P < 0.05$) (Fig. 5).

The level of ROS measured by MitoSOX Red

Some studies show that mitochondrial dysfunction and subsequent ROS production are involved in the development of diabetes. Because hyperglycemia is known to disturb mitochondrial function and because mitochondria are likely the major source of ROS, we measured the level of ROS using MitoSOX Red in neonatal rat hippocampal neurons from each group (Fig. 6a). ROS production significantly increased in the HG group compared with the control group ($P < 0.001$). Compared with the HG group, fluorescence intensity of ROS production in the HG+Calindol, HG+Calhex231, HG+L-NMMA and HG+IBMX groups was clearly decreased ($P < 0.05$).

Measurement of mitochondrial membrane potential by JC-1 staining

In our study, the mitochondrial membrane potential was detected using a membrane potential-specific dye, JC-1. Healthy non-apoptotic cells maintain a high mitochondrial membrane potential, and exhibits red fluorescence. In contrast, unhealthy, apoptotic cells exhibits green fluorescence. As seen in Fig. 6b, the HG group showed a clearly decreased mitochondrial membrane potential relative to control neurons, as observed through changes in the amount of green fluorescence ($P < 0.001$), whereas the HG+Calindol, HG+L-NMMA and CaCl_2 groups exhibited increased mitochondrial membrane potential compared to the HG group

Fig. 6. (a) The diversity of ROS production in mitochondria from different groups. (A) Control, (B) HG, (C) HG+Calindol, (D) HG+Calhex231, (E) HG+L-NMMA, (F) HG+CaCl₂. Quantitative results of MitoSOX Red fluorescence for each of the different groups is shown in the lower panel. Values represent group means $\pm$ SEMs. *** $P < 0.001$ vs. Control; § $P < 0.05$ vs. HG. Scale bar is 200 μ m. (b) The diversity of mitochondrial transmembrane potential in each of the different groups. (A) Control, (B) HG, (C) HG+Calindol, (D) HG+Calhex231, (E) HG+L-NMMA, (F) HG+CaCl₂. The quantitation of JC-1 staining for each of the different groups is shown in the lower panel. Values represent group means $\pm$ SEMs. *** $P < 0.001$ vs. Control; § $P < 0.05$ vs. HG. Scale bar is 200 μ m.

at 30 min ($P < 0.05$). Because a decrease in mitochondrial membrane potential indicates an early stage of apoptosis, our results suggested that high glucose treatment might result in neuronal apoptosis. However, Calindol treatment attenuated the decrease in mitochondrial membrane potential in neonatal rat hippocampal neurons.

Discussion

Diabetic encephalopathy is a serious DM complication. To explore the molecular mechanisms underlying diabetic encephalopathy, the establishment of a reliable animal model is critical. In this study, intraperitoneal injection of STZ was used to generate a rat diabetes model. Water and food intake and the serum concentration of glucose were significantly increased in diabetic model groups compared to the control group, indicating the successful establishment of the diabetic model. Ultrastructural injuries and the level of apoptosis in diabetic hippocampal neurons corresponded to the characteristics of diabetic encephalopathy.

To investigate the roles of CaSR in the mechanism of diabetic encephalopathy, a cellular diabetes model was established using hippocampal neurons treated with 30 mM glucose. Our results suggested that the expression of CaSR significantly decreased and was accompanied by a decrease in $[Ca^{2+}]_i$ in these neurons. CaSR stimulation elicits PLC-mediated IP_3 formation, leading to an elevation in $[Ca^{2+}]_i$ [24]. Down-regulation of CaSR expression was involved in rat diabetic encephalopathy. Calindol, a CaSR agonist, could attenuate the high glucose effects and maintain the status of the cells.

The reduction in the levels of CaSR protein caused disturbances in Ca^{2+} homeostasis, induced the alteration of a series of downstream signaling pathways, or directly damaged cellular structure through mitochondrial dysfunction. A substantial number of studies have indicated that the destruction of mitochondrial transmembrane potential is one of the earliest events in the apoptotic cascade, as it occurs before the nuclear features of apoptosis become apparent, and once it collapses, cellular damage becomes irreversible. The results of JC-1 staining suggested that the ratio of aggregate/monomer in high glucose-treated cells was markedly decreased, whereas 2 μ M Calindol and 2.3 mM $CaCl_2$ could stabilize the mitochondrial transmembrane potential.

In recent years, considerable circumstantial evidence has indicated a role for diabetes-induced oxidative stress, which is routinely accompanied by diabetic encephalopathy [25]. Oxidative stress contributes to increased neuronal damage and death through protein oxidation, DNA damage, and peroxidation of membrane lipids [26]. Oxidative stress is thought to play a crucial role in the development of memory impairment in diabetes [27]. The activation of CaSR by Calindol inhibited ROS production in a manner similar to IBMX, an endogenous PDE inhibitor.

CaMKII is regulated by the Ca^{2+} /calmodulin complex and has been involved in many signaling cascades and is thought to be an important mediator of learning and memory [28]. When there is insufficient calcium or calmodulin present to bind to neighboring subunits, auto-phosphorylation does not occur. As greater amounts of calcium and calmodulin accumulate, auto-phosphorylation occurs, leading to persistent activation of the CaMKII enzyme for a short period of time [29]. KN-93 is an effective inhibitor of CaMKII activity and auto-phosphorylation of the α - and β -subunits of CaMKII. In our study, the level of p-CaMKII decreased in neurons treated with high glucose. However, the level of p-CaMKII increased in the HG+Calindol group and decreased in the HG+Calhex231 and HG+KN93 groups compared with the HG group, approximating the changes in $[Ca^{2+}]_i$. The level of total CaMKII in the high glucose-treated neurons was increased compared to the control group, suggesting a compensatory increase in protein synthesis. The levels of p-CaMKII and CaMKII in hippocampal neurons in the $CaCl_2$ group was largely unchanged, suggesting that CaSR may improve the status of neurons treated with high glucose through regulating $[Ca^{2+}]_i$.

Nitric oxide (NO) is found in biological systems following synthesis from the amino acid L-arginine by nitric oxide synthase (NOS). In the brain, in addition to relaxing the cerebral vasculature, NO plays a role as a neurotransmitter. The function of NO in the hypothalamus has been implicated in the learning process and in memory formation [30]. Long-term potentiation (LTP) and long-term depression (LTD) are two major forms of activity-dependent synaptic plasticity in the brain. The NO-cGMP pathway has been implicated in the induction of hippocampal LTP and LTD, which are known to be the predominant mechanisms for learning and memory [31].

Ca^{2+} and calmodulin (CaM) can activate the NOS in the endochylema, which can catalyze the formation of NO. Factors that can elevate $[\text{Ca}^{2+}]_i$ may also activate NOS, but long-lasting increases in $[\text{Ca}^{2+}]_i$ may down-regulate NO formation, as *in vitro* phosphorylation by CaMKII decreases the V_{max} of NOS. In addition to the conversion of L-arginine, nNOS generates H_2O_2 and reduces cytochrome c (P450) in a Ca^{2+} /calmodulin-dependent manner [32]. In our study, in addition to decreases in NO in the HG group, the levels of p-eNOS were significantly decreased compared with the control group. These results were consistent with a change in the expression of CaSR and $[\text{Ca}^{2+}]_i$. Furthermore, NO and p-eNOS increased upon treatment with Calindol and CaCl_2 but decreased with Calhex231 and L-NMMA treatment, suggesting that CaSR promoted the synthesis of NO in the high glucose environment.

In this study, CaSR regulated neurite outgrowth might be related to the levels of p-CaMKII and the production of NO. CaCl_2 had two roles: a CaSR agonist and the source of $[\text{Ca}^{2+}]_o$. In hippocampal neurons treated with high glucose, Ca^{2+} homeostasis was disturbed, but the finding that Calindol treatment was nearly identical to CaCl_2 treatment could demonstrate that CaSR might protect hippocampal neurons from the effects of incubation with high glucose through positive compensatory protection to inhibit and delay rat diabetic encephalopathy. Therefore, it may be possible for diabetic patients to use exogenous CaSR agonists such as calcium, magnesium and zinc to ease the symptoms of diabetic encephalopathy.

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Disclosure Statement

The authors declare no conflict of interest. The authors have not published or submitted the manuscript elsewhere.

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