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Down regulation of brain cellular prion protein in an animal model of insulin resistance: Possible implication in increased prevalence of stroke in pre-diabetics/diabetics

Nam Pham [1], Arti Dhar [1], Siavash Khalaj, Kash Desai, Changiz Taghibiglou *

Department of Pharmacology, College of Medicine, University of Saskatchewan, Canada

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ABSTRACT

The risk of stroke is drastically increased in diabetic and pre-diabetic patients. The worldwide spread of obesity and insulin resistance increases the incidence of stroke. The direct effect of insulin resistance, as it pertains to stroke, on the central nervous system is not well understood. Since one of the physiological functions of the cellular prion protein (PrP^C) is neuroprotection, we studied effects of brain insulin resistance on the expression of PrP^C in fructose-fed rats as an animal model of prediabetes. Compared with control chow-fed animals, rats fed a high-fructose diet (FF), exhibited compromised tyrosine phosphorylation of insulin receptor β subunit (IRβ) and reduced serine phosphorylation of Akt, PI3K activity, and decreased PIP3 levels in cortices indicating the induction of brain insulin resistance. We also observed that both mRNA and protein expression of the PrP^C was significantly decreased whereas protein level of NR2B subunit of NMDA glutamate receptors profoundly increased in the brain of fructose-fed rats compared to control chow-fed rats. Considering a neuroprotective role for PrP^C and the involvement of NR2B subunit in the excitotoxicity-induced neuronal apoptosis, these phenomena may contribute to the severity and poor prognosis of ischemic stroke in diabetes/prediabetes.

1. Introduction

Several large cohort studies have identified diabetes as a substantial risk factor for stroke [1]. The risk of stroke is increased 1.5 to 3-times in diabetic patients. Pre-diabetes is now also recognized as a stroke risk factor [1,2]. Diabetes is associated with pathophysiological determinants such as dyslipidemia, hypertension, and hyperglycemia, all of which are risk factors in ischemic stroke and neuronal damage [3]. Insulin resistance and/or diabetes exacerbates ischemic brain damage by disrupting the integrity of the blood brain barrier, impairing protective vascular mechanisms, stimulating vascular inflammation, disturbing cellular metabolism, and causing tissue ketoacidosis [4,5]. Elevation of extracellular glucose levels in insulin resistance/diabetes may also provoke the Na^+-dependent depolarization of the membrane through interaction with the sodium glucose transporter type 3 [6]. Neuronal membrane depolarization, similar to what occurs following energy failure and release of excitatory neurotransmitter glutamate in cerebral ischemia, triggers a massive Ca^{2+} influx leading to the activation of deleterious calcium-dependent enzymatic pathways and apoptosis (excitotoxicity) [7–9]. Moreover, in hippocampal neurons the insulin signaling pathway profoundly affects NMDA glutamate receptors trafficking and activation [10–13]. Therefore, pre-diabetic hyperinsulinemia and diabetes, as well as post-stroke hyperglycemia, potentially contributes to and exacerbates neuronal apoptosis in ischemic stroke, apparently through glutamate/NMDA receptors-induced excitotoxicity.

NMDA-sensitive glutamate receptors (NMDAR) are heterotetrameric ligand-gated ion channels composed of the NR1 (GluN1) subunit with at least one type of NR2 (GluN2) (NR2A to D) and/or NR3 (GluN3) subunits. The NMDARs present in the CNS are essential for brain development and innate neuronal physiology [14]. However, the overstimulation of NMDARs, particularly those containing the NR2B subunit, causes neuronal excitotoxicity due to accumulation of intraneuronal Ca^{2+} and from downstream events such as mitochondrial dysfunction, activation of proteases (calpains, caspases), accumulation of reactive oxygen species, and release of nitric oxide [7]. Several important pro-death signaling molecules have been associated with NR2B-mediated toxicity [8]

* Corresponding author. Address: 107 Wiggins Road, Health Sciences Bldg., Department of Pharmacology, College of Medicine, Saskatoon, SK S7N 5E5, Canada.
E-mail address: changiz.taghibiglou@usask.ca (C. Taghibiglou).
[1] Equal contribution.

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and we recently identified a novel NR2B-dependent signaling pathway following ischemic stroke leading to the activation of lipid transcription factor, sterol response element binding protein 1 (SREBP1), and neuronal death [15]. We further demonstrated that NR2B-dependent nuclear translocation of PTEN (phosphatase and tensin homolog deleted on chromosome TEN) is a delayed step causatively leading to excitotoxic (in vitro) and ischemic (in vivo) neuronal injuries [16]. There is a consensus that NMDARs, particularly those containing NR2B subunits, are involved in excitotoxicity-induced neuronal death [8,17]. Most recently, an interaction between NMDAR and PrP^C has been implicated in the neuroprotective function of PrP^C [18].

The PrP^C is a protein ubiquitously expressed, including throughout the CNS. The PrP^C is best known for its ability, once 'misfolded', to cause neurodegenerative 'prion' diseases affecting both humans and animals [19]. Several physiological functions for PrP^C have been proposed including neurite outgrowth, copper binding, synaptic transmission and plasticity, and neuroprotection [20–25]. A neuroprotective role for PrP^C is strongly supported by the increased neuronal excitotoxicity in prion KO mice, a longer lifespan in mice overexpressing human PrP^C [24–28], and a significantly lower ischemic injury in a stroke rat model by adenoviral overexpression of PrP^C [29]. Animal models of stroke as well as human blood and post-mortem brain samples from stroke victims also provide evidence for a neuroprotective role for PrP^C [29–32], but the underlying molecular mechanisms remain unclear.

In this study, using an animal model of insulin resistance and diet-induced pre-diabetes (fructose-fed rats), we found significant suppression of both mRNA and protein expression of PrP^C as well as elevation of NR2B protein levels in the cortices when compared with normal chow-fed rat brains. These phenomena may contribute to the severity and poor prognosis of ischemic stroke in diabetes/prediabetes.

2. Material and methods

2.1. General antibodies and reagents

Rabbit anti PI3K P85α was obtained from New England Biolabs (Pickering, ON). Mouse anti phosphotyrosine (pY), mouse monoclonal anti PrP^C (5B2), rabbit anti-insulin receptor β-subunit (IRβ), rabbit anti-NR1, rabbit anti-NR2D and rabbit anti insulin receptor substrate 1 (IRS-1) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Akt and p-Akt (Ser 473) were obtained from BD Pharmagen (San Diego, CA). Mouse anti β-tubulin was from Sigma (St. Louis, MO). Anti-PIP_3, anti-PIP2, and PI3K activity ELISA kit were obtained from Echelon Biosciences (Salt Lake City, UT). Rabbit anti NR2B and NR2A were generous gifts from Dr. Yu Tian Wang (UBC, Vancouver, Canada). All lysate buffer components, including phosphatase and protease inhibitors, were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Laboratory animals

Male Sprague–Dawley (SD) rats from Charles River Laboratories (Quebec, Canada) were used according to guidelines of the Canadian Council on Animal Care and approval by the University of Saskatchewan's Animal Research Ethics Board. 5 week old SD rats were randomly divided into two groups of control (normal rat chow), and high-fructose diet (60% of total calories) and fed for 8 weeks. At the end of the feeding period, the rats were anesthetized with sodium pentobarbital (60 mg/kg body weight, i.p.) and subsequently euthanized by cutting the thorax and the heart, and causing exsanguination, as per the guidelines of the Canadian Council on Animal Care. Organs and tissues, including the brain, were cleaned in ice-cold phosphate buffer saline and then immediately frozen in liquid nitrogen and stored at −80 °C until processing.

2.3. Western blotting

Western blotting assays were carried out essentially as previously described [33]. Briefly, 40–50 μg of the cortical homogenates solubilized by RIPA buffer (containing protease inhibitors cocktail and 2 mM Na_3VO_4) was separated with SDS–PAGE, transferred onto a polyvinylidene difluoride (PVDF) membrane, and probed with the relevant antibodies. For sequential reprobing of the same blots, the membranes were stripped of the initial primary and secondary antibodies and subjected to immunoblotting with another target antibody. Blots were developed using enhanced chemiluminescence detection (Amersham). Band intensities were quantified using NIH ImageJ software and normalized to the quantity of β-tubulin in each sample lane.

2.4. Dot blotting

Dot blotting analysis was conducted as previously described [16]. Briefly, brain homogenates (cortices) were spotted onto nitrocellulose (NC) membranes using Bio-Rad microfiltration apparatus, as instructed by the manufacturer. The NC membranes were blocked with 5% bovine albumin for an hour at room temperature, washed three times with TBS-T, then probed with an anti-PIP_3 antibody, followed by HRP-labeled secondary antibody. Visualized spots were analyzed by NIH ImageJ software. The NC membranes were later stripped and reprobed for PIP2 as well as a housekeeping protein (β-tubulin) as a loading control.

2.5. Real Time Quantitative PCR (RT-PCR)

Total RNA from the brain was isolated using RNA isolation kit (Qiagen Inc., Toronto, ON). The pre-designed primers for PrP^C, NR1, NR2B, and NR2D were purchased from Qiagen, (Toronto, ON). The real-time PCR was performed in an iCycler iQ apparatus (Life Science, Hercules, CA, USA) associated with the ICYCLER OPTICAL SYSTEM software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada).

2.6. PI3K activity

After immunoprecipitation of brain homogenates from control and FF rats using the anti-PI3K antibody (New England Biolabs, Pickering, ON), PI3K activity was assayed using a competitive ELISA (Echelon Biosciences, Ct#K-1000s, Salt Lake City, UT) in which the signal is inversely proportional to the amount of phosphatidyl inositol 3 phosphate [PI(3,4,5)P3] produced. After the PI3K reactions are complete, reaction products are first mixed and incubated with a PI(3,4,5)P3 detector protein and then added to the PI(3,4,5)P3-coated microplate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric detection is used to detect PI(3,4,5)P3 detector protein binding to the plate. The colorimetric signal is inversely proportional to the amount of PI(3,4,5)P3 produced by PI3K activity. The bar graph data was plotted as relative to the PI3K activity in the chow-fed brain.

2.7. Data analysis

Data are presented as the mean ± SE, and analyzed using a Student's *t*-test for comparison between two groups. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Induction of brain insulin resistance with a high-fructose diet

We previously established the fructose-fed (60% fructose in diet) hamster as model of diet-induced peripheral insulin resistance [33]. This model later revealed impairment of hippocampal insulin signaling [34], based on changes in insulin receptor (IR) β subunit expression, and insulin receptor substrate 1 & 2 (IRS1 & 2) tyrosine phosphorylation (pY) changes in Akt and PI3K activities. In the present study, we used the FF rat, which not only has been widely considered as a model for diet-induced peripheral insulin resistance [35,36], but as depicted in Fig. 1A, presents with impaired brain insulin signal transduction (brain insulin resistance). Both IRβ and Akt showed significant reduction in tyrosine and serine-473 phosphorylation, respectively (Fig. 1A and B). IRβ-pY and Akt p-Serine-473 in FF cortices reduced to 70 ± 5% and 61 ± 10% of those in control chow-fed cortices, respectively. PI3K activity measurements in FF cortices showed a significant reduction and reached to the level of 75 ± 8.3% ($P = 0.03$, $n = 3$) of activity in the chow-fed brain (Fig. 1C). In line of this finding, the catalytic subunit of PI3K, P85α, was also drastically reduced in the FF rat brain. The dot blot analysis of cortical homogenates revealed that fructose diet drastically reduced brain PIP3 levels, while increasing PIP2 levels when compared with those in the control cow fed rat brain (Fig. 1D).

3.2. Brain insulin resistance significantly suppresses PrP^C expression in the pre-diabetic rat

In order to investigate whether brain insulin resistance has any effect on the neuroprotective PrP^C protein, we measured both mRNA and protein expression levels in chow-fed and FF rat brains. As shown in Fig. 2A, a high-fructose diet significantly reduced the brain protein expression level of PrP^C to 56.00 ± 8.00% ($P = 0.038$, $n = 4$) of that of control chow-fed rat brain. Moreover, mRNA measurement of PrP^C also revealed a significant reduction in cortices of FF rats and it reduced to 37.93 ± 7.75% ($P = 0.03$, $n = 4$) of the mRNA level in the control rat brain (as 100%) (Fig. 2B). These data suggest that brain insulin resistance significantly compromises both mRNA and protein expression of PrP^C.

3.3. NR2B protein expression increases in the pre-diabetic brain

NMDA glutamate receptors, and particularly overactivity of those extrasynaptic ones containing NR2B and/or NR2D, are involved in stroke-related neuronal apoptosis and brain damage [7]. Moreover, a cross talk and interaction between PrP^C and NMDA glutamate receptor has also been proposed [18]. We therefore measured protein expression levels of NMDAR subunits in cortices of FF prediabetic and control chow-fed rats. As depicted in Fig. 3, we observed no significant protein expression alteration in NR1, NR2A, and NR2D subunits (panels A, B, and C, respectively),

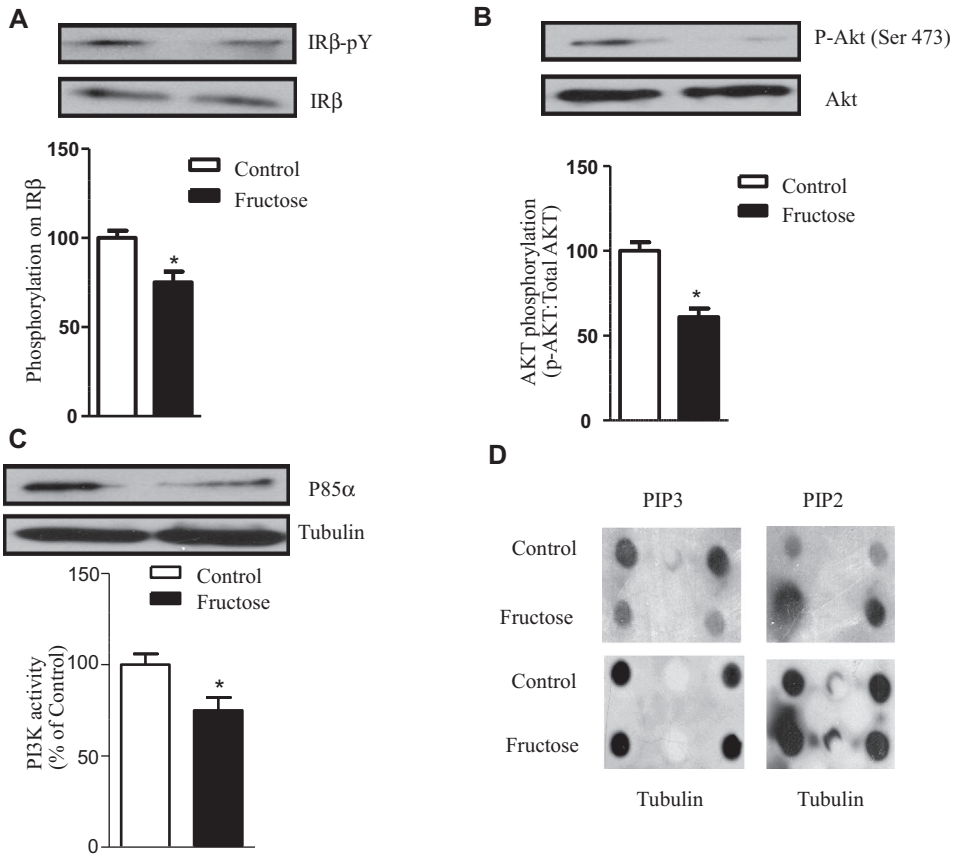

Fig. 1. Induction of brain insulin resistance in with a high-fructose diet. (A) A significant reduction in IRβ-pY in FF cortices. 500 mg of cortex homogenates from control and FF rats were subjected to immunoprecipitation for IRβ and immunoprecipitates subsequently immunoblotted for pY. Results are shown as IRβ-pY/ IRβ. (B) Sequential blotting of cortical homogenates for phospho-Akt Ser473 and total Akt revealed that the high-fructose diet caused a dramatic reduction in phospho-Akt Ser473; levels. (C) Fructose feeding reduces cortical P85α protein expression and PI 3-kinase activity. (D) A representative of sequential dot blotting for PIP3, PIP2 and β-tubulin (as loading control) revealed that the fructose feeding caused a marked drop in PIP3 levels whereas increased PIP2 levels in the cortical tissue. *N* of 3 per group and $P < 0.05$.

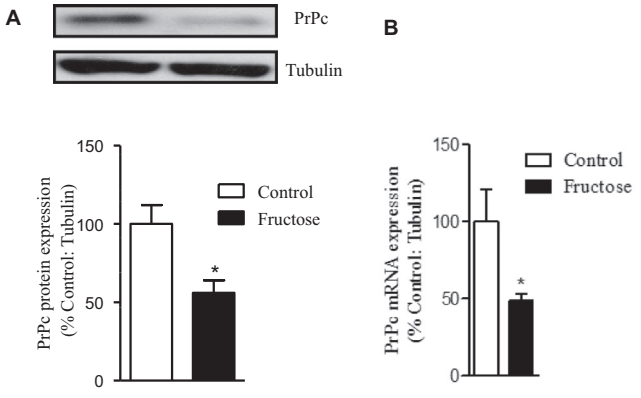

Fig. 2. Significant suppression of PrP^C in the insulin resistant brain. (A) Sequential blotting of cortical homogenates for PrP^C and β-tubulin (as loading control) revealed that the high-fructose diet caused a significant reduction in PrP^C protein levels. (B) PrP^C mRNA in insulin resistant brain is decreased nearly to half of that in control. *N* of 4 per group and $P < 0.05$.

whereas NR2B subunit expression (panel D) increased about 132.0 ± 7.0% ($P = 0.04$, $n = 4$) in the prediabetic cortices compared with that in control rat brain. As shown in Fig 3E, brain insulin resistance did not significantly alter NR2B mRNA level.

4. Discussion

The incidence of ischemic stroke in individuals with insulin resistance and diabetes is significantly higher when compared with non-diabetic population. Although some molecular and cellular mechanisms have been proposed for the higher risk of ischemic stroke in insulin resistance and diabetes, there is likely a variety of factors contributing to this issue. Investigating brain pathological alterations in prediabetes and diabetes at the molecular levels will provide better understanding on how brain insulin resistance may contribute to higher incidence of stroke. In our study using fructose-fed rats as an animal model of diet-induced pre-diabetes, we focused on possible alterations in two proteins, PrP^C and the NR2B subunit of NMDAR, the latter of which is linked to the pathogenesis of stroke. We found that in FF rat cortices both mRNA and protein levels of neuroprotective PrP^C were significantly suppressed whereas protein level of NR2B subunit of NMDAR increased compared with those in control rat brains.

Compared with control rat brains, compromised IRβ tyrosine phosphorylation, Akt serine-473 phosphorylation, lower PI3K activity, higher PIP2, and lower PIP3 levels confirmed the induction of brain insulin resistance in fructose-fed rats. This was in agreement with our previous observation in FF hamsters [34] as well as others in different rodent models [37,38].

Among several physiological functions attributed to the PrP^C, neuroprotection is the one that neuroscientists appear to have consensus on. We interestingly found that in the insulin resistant brain, both mRNA and protein expression of PrP^C were significantly suppressed compared with those in the control rat brains. Although it has been previously reported in cell line cultures that exogenous growth hormones such as insulin, IGF-1, human growth hormone (hGH), nerve growth factor (NGF) induce PRNP gene expression, to our knowledge this is the first report demonstrating that impaired brain insulin signaling pathway interferes and suppresses PrP^C expression in an animal model of diet-induced prediabetes [39–43]. In fact, our results are consistent with a recent report by Liu

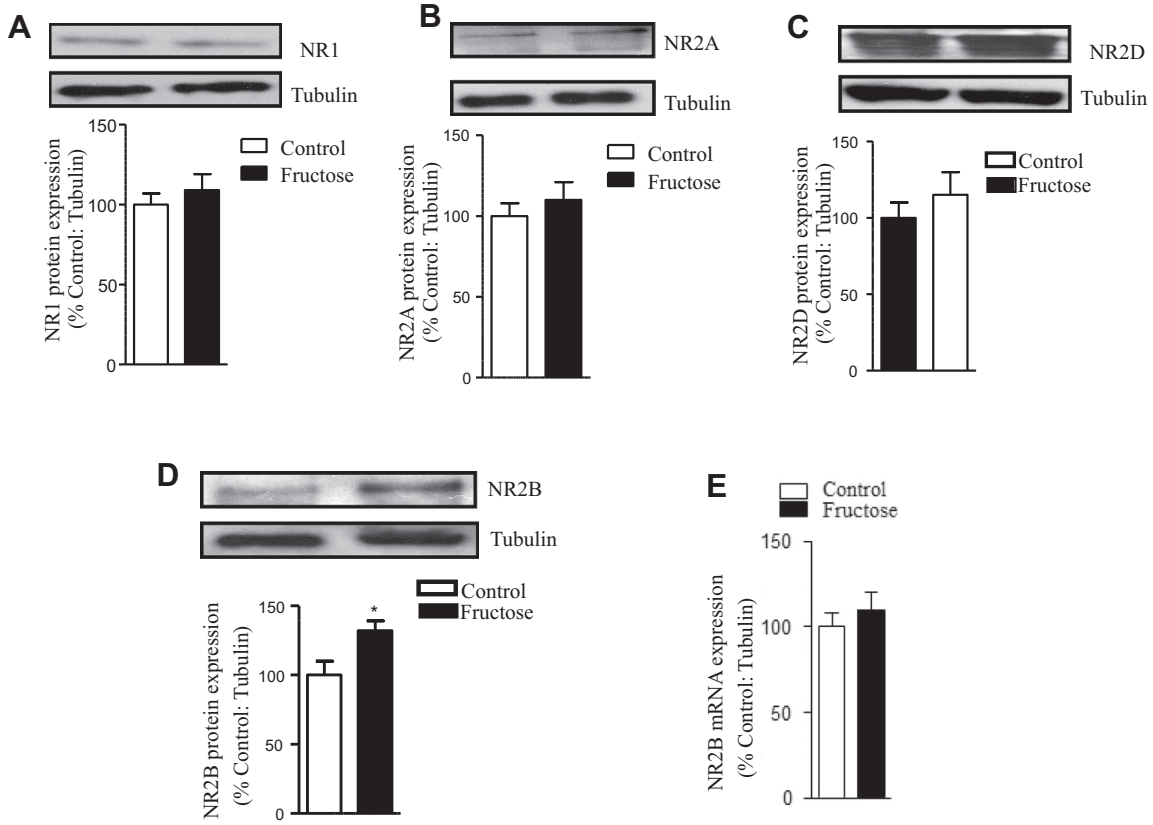

Fig. 3. NMDAR subunits expression levels in the insulin resistant brain. (A–C) Sequential blotting of cortical homogenates for NR1 (panel A), NR2A (panel B), NR2D (panel C) subunits and β-tubulin (as loading control) revealed that the high-fructose diet caused no significant alteration in their protein expression levels. (D) Whereas sequential blotting of cortical homogenates for NR2B subunit and β-tubulin (as loading control) showed that the high-fructose diet caused a significant increase in NR2B protein expression levels. (E) No significant change was observed in cortical mRNA levels of NR2B subunit. *N* of 4 per group and $P < 0.05$.

et al. showing insulin-like growth factor 1 (IGF-1) induces enhancement of PRNP mRNA and protein levels by activation of the PI3K-Akt signaling pathway and negative regulation of PRPN gene suppressor, the forkhead box O3a (FOXO3a) [44]. This is most likely, in the insulin resistant brain, due to impairment in PI3K activity and its downstream effectors; the so called IGF-1-PI3K-Akt-FOXO3a pathway may cause a profound reduction in PrP^C expression.

Since interaction between PrP^C and NMDAR has been implicated in the neuroprotective function of PrP^C, we also examined the effect of brain insulin resistance on the expression levels of NMDAR subunits. Brain insulin resistance did not alter NR1, NR2A, and NR2D subunits expression levels, while increasing NR2B expression. NR2B plays an important role in excitotoxicity-induced neuronal apoptosis and pathogenesis of stroke. Indeed, our recent studies using MCAO rat model of stroke clearly demonstrated NR2B's role and its downstream signaling pathways in stroke-related neuronal apoptosis [15,16]. Despite increased NR2B protein expression in FF rat brain, mRNA levels was not altered compared with control rat brains. We therefore cannot rule out the possibility of enhanced NR2B protein stability in the insulin resistant brains. Almost all of the research on the effects of diabetes on the glutamate receptors expressions have been done using streptozotocin induced diabetic rodents (a model of type 1 diabetes); however, those results appear to be conflicting most likely due to different study design [45–48]. The present study differs from the above reports because our model is diet-induced brain insulin resistance reflecting the more prevalent type 2 diabetes.

Although both diabetes and stroke are multifactorial pathologies, our findings however, suggest the diet-induced brain insulin resistance may promote more brain susceptibility to stroke at least partly due to suppression of neuroprotective PrP^C and increased expression of NR2B subunit of NMDA glutamate receptor.

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