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ORIGINAL PAPER

Expression Changes of NMDA and AMPA Receptor Subunits in the Hippocampus in rats with Diabetes Induced by Streptozotocin Coupled with Memory Impairment

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Abstract

Cognitive impairment in diabetes (CID) is a severe chronic complication of diabetes mellitus (DM). It has been hypothesized that diabetes can lead to cognitive dysfunction due to expression changes of excitatory neurotransmission mediated by *N*-methyl-D-aspartate receptors (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR); however, the pathogenesis involved in this has not been fully understood, especially at early phase of DM. Here, we sought to determine the cognitive changes and aim to correlate this with the expression changes of NMDAR and AMPAR of glutamate signaling pathways in the rat hippocampus from early phase of DM and in the course of the disease progression. By Western blot analysis and immunofluorescence labeling, the hippocampus in diabetic rats showed a significant increase in protein expression NMDAR subunits NR1, NR2A and NR2B and AMPAR subunit GluR1. Along with this, behavioral test by Morris water maze showed a significant decline in their performance when compared with the control rats. It is suggested that NR1, NR2A, NR2B and GluR1are involved in learning and memory and that their expression alterations maybe correlated with the occurrence and development of CID in diabetic rats induced by streptozotocin.

Keywords CID · Diabetic rat · Glutamate receptors · Hippocampus · Neuronal apoptosis

Abbreviations

CID	Cognitive impairment in diabetes
Glu	Glutamate (glutamic acid)

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GluR	Glu receptor
mGluR	Metabotropic glutamate receptors
iGluR	Ionotropic glutamate receptor
NMDA	*N*-methyl-D-aspartate
NMDAR	NMDA glutamate receptor
NR1,	NMDAR subunits 1
NR2A	NMDAR subunits 2A
NR2B	NMDAR subunits 2B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA glutamate receptor
DM	Diabetes mellitus
LTP	Long-term potentiation
LTD	Long-term depression
STZ	Streptozotocin
H&E	Hematoxylin-eosin
AD	Alzheimer's disease

Background

Diabetes mellitus (DM) is a leading cause of the neurodegenerative disease known as diabetic encephalopathy [1], such as cognitive dysfunction, dementia, mental illness and other chronic brain symptoms. Increasing evidence has indicated that diabetes is a risk factor for cognitive impairment in diabetes (CID), and the risk of CID patients is 1.5 times higher than that in normal subjects [2]. CID is one of the chronic complications of DM, and the main clinical manifestations of mild to moderate cognitive dysfunction, learning and memory capacity decline, and brain aging and Alzheimer`s disease (AD) are closely linked in humans and experimental models [3]. Lower scores in global cognitive function have been recognized as a severe complication of DM in patients [4]. The precise in vivo intracellular molecular mechanisms underlying the diabetes mediated deficits in learning and memory have not been fully elucidated. *N*-methyl-D- aspartate (NMDA) glutamate receptors (AMPARs/NMDARs) are the predominant receptors in mediating advanced neural activities [5]. Molecular alterations in NMDAR subunits can affect physiological and pathological processes in the hippocampus. NMDARs are involved in numerous physiological processes, including basic neuronal communication, axonal pathfinding, mood regulation and memory formation [6]. Hyperactivity of NMDARs has been implicated in a variety of neurodegenerative disorders, such as Parkinson's disease and AD [5]. NMDARs are involved in the formation of cognitive functions. NMDAR dependent long-term potentiation (LTP) is the major cellular mechanism thought to underlie spatial learning and memory in the hippocampus [7]. NMDAR activation is obligatory for the induction of diverse forms of synaptic plasticity. The molecular composition and function of NMDARs are themselves modified by synaptic activity, which, in turn, alters the ability of synapses to undergo subsequent plastic modification. This homeostatic control of synaptic plasticity is well-known for the experience-dependent development of sensory cortices [8]. However, it is now becoming clear that NMDAR properties may not only be altered at juvenile, but also at mature synapses [9, 10].NMDAR is a heteromeric complex that interacts with multiple intracellular proteins by three different classes of subunits: NR1, NR2 and NR3 [11]. Multiple receptor isoforms with distinct brain distributions and functional properties arise by selective splicing of the NR1 transcripts and differential expression of the NR2 subunits. NMDAR subunits especially NR2A and NR2B are essential for LTP induction and maintenance and are required for hippocampal synaptic plasticity [12]. Diverse types of behavioral manipulation such as sensory experience, learning and sleep deprivation alter the NR2A/NR2B ratio of hippocampal NMDARs [13]. As an additional facet to the dynamics of NMDAR function, NMDAR trafficking is regulated by G-protein-coupled neurotransmitter receptors implicated in learning and arousal, such as orexin and dopamine [13]. These findings suggest that mature glutamatergic synapses may be modified by recent activity via alterations in synaptic NMDAR function. Rapid forms of NMDAR trafficking, perhaps controlled by the neurochemical environment featuring specific states of arousal and learning, may regulate plasticity and modulate cognitive abilities in adulthood [13]. However, the molecular changes contributing to its effects in DM are not fully understood.

AMPA has been traditionally classified as a non-NMDA-type receptor, AMPAR consists of different subunits (GluR1–GluR4) assembled in various combinations. Several studies have shown that GluR1—containing AMPA receptors are critically involved in working memory [14]. Mice lacking the GluR1 subunit show impaired spatial working memory and genetic rescue of the GluR1 deficiency leads to restoration of working memory [15, 16]. GluR1 activity is essential for both LTP induction and maintenance [17]. Pharmacological and electrophysiological studies have shown that several neuronal functions, including LTP and long-term depression (LTD), specifically require PKC-γ [18]. However, the molecular changes contributing to its effects in DM are uncertain. Relevant to this is the activated microglia which are known to release large amounts of pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), nitric oxide (NO), reactive oxygen species (ROS)etc, which can cause neuronal damage or apoptosis [19]. It has been reported that hippocampal neuron apoptosis and caspase-3 activity were increased in diabetic rats [20]. The relationship between the memory ability changes and neuronal apoptosis accompanied invariably by

microglia activation in the hippocampus following the DM and in the course of the disease progression has not been fully explored.

In view of the importance of CID and its possible relationship with the above factors related to neuronal apoptosis, this study was undertaken to ascertain the link between DM and changes in cognition. Additionally, this study was aimed to determine on how alterations in glutamate signaling pathways might affect the cognitive function in the early phase of DM. We report here that the expression changes of NR2A and NR2B in the hippocampus in streptozotocin induced diabetic rats may contribute to the development and progression of diabetes-related cognitive dysfunction. A better understanding of the regulation and action of NMDAR at the central synapses would undoubtedly provide further insight and clues for designing of more effective therapeutic strategies for treating memory disorders in DM.

Methods

Rats and Diabetes Induction

One hundred male Sprague–Dawley (SD) rats weighing 260–280 g were obtained from Chengdu Biological Technology firm (Sichuan, China). Rats were housed under climate-controlled conditions and provided with standard food and water, with five rats per cage. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. An acclimation period of at least 3 weeks was allowed before the rats were subjected to Morris water maze test for assessment of cognitive dysfunction. When the rats reaching their body weight of about 300 g, they were randomly divided into two groups: 15 rats were used as the normal control group (NC), 75 rats were used as experimental groups at different time points after DM at 3/6/9 weeks (DM3/6/9w). Type 1 diabetes was induced by a single intraperitoneal injection of freshly prepared streptozotocin [21, 22] (STZ, 63 mg/kg; Sigma S0130, Chemicals, St. Louis, MO, USA). Before diabetes induction, the rats were weighed and fasted for 12 h. In the control, the STZ in lemon acid buffer was administered by intraperitoneal injection. The concentration of blood glucose was repeatedly monitored by test strips (Johnson, IN, CHINA). Three days after STZ injection, blood glucose levels were measured; only subjects with glucose levels > 16.67 mmol/L were considered diabetic [23] and were used for experimental analysis. There were 68 rats to be diabetic and used in the present study. These rats were randomly divided into three groups: DM 3w, (n = 21), DM6w (n = 23) and DM6w (n = 24).

Morris Water Maze Test

Normal control rats (n = 15), and rats of the diabetes group (n = 25, each at 3, 6 and 9 w after diabetes mellitus) were evaluated for changes of learning and memory ability by using Morris water maze (Shanghai XinRuan Information Technology Co Ltd).The Morris Water Maze (MWM) test, which is a hippocampal dependent test of spatial learning and memory for rodents, was performed as described previously with minor modifications [24]. The MWM test setup was designed with reference to Ma et al [25]. In our study, the cognitive ability of rats was assessed with MWM test before being sacrificed by decapitation at 3, 6 and 9 weeks after the induction of diabetes. In this test, the rats relied on distal cues to navigate from start locations around the perimeter of an open swimming arena (diameter, 190 cm; water temperature, 22 ± 1 °C; the color of the water, black) to locate a submerged escape platform (diameter, 15 cm). Spatial learning was assessed across repeated trials for 7 days. The pool was situated in a room with visual cues. The animals' movements were recorded with a video camera attached to the ceiling. On the first day, rats were released to the water for 2 min. On the second day, the platform was placed in the pool and the water level was 2 cm higher than it. The rats were released on the platform for 30 s and then guided into the water. If the rats found the platform within 30 s, they were allowed to stay for 30 s. If the rats did not find the platform in 30 s, they were guided onto the platform and were allowed to stay on it for 30 s. After that the rats were air dried under a heater and returned to the cage. All rats were trained similarly until all of them had completed the training. After this, the rats were released into the water facing the wall of the pool from one of four separate quadrants, one by one. If a rat failed to find the platform within 30 s, it was guided onto the platform for 30 s. This rotation was repeated until all animals were completely trained. From the third day to the sixth day, the platform was placed in the pool and the water level was 2 cm lower than it. The rats were released into the water facing the wall of the pool from one of four separate quadrants, one by one. If the rats found the platform within 120 s, it was allowed to stay for 30 s. If a rat failed to find the platform within 120 s, it was guided on the platform for 30 s. The rotation was repeated until all animals completed the training. On the last day, the platform was removed and the rats were released into the water facing the wall of the pool from the farthest point of the platform position. In this test, if the rat recalled the platform position, it would swim along a shorter path to the goal on the second trial or test. On the last day, because the platform was moved away, the rats which were not trained to learn the platform position from the previous days would not be able to locate the platform position easily. We compared the differences in all groups of rats to evaluate their memory ability.

Paraffin Embedding and Sectioning

For histology and double immunofluorescence labeling, four rats in each group were anesthetized with 10% chloral hydrate (4 mL/kg body weight) and perfused with filtered saline (150 mL, 12 mL/min), followed by 4% paraformaldehyde in phosphate-buffered saline, pH7.4 (500 mL, 12 mL/min). The whole brain 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 and Eosin (H&E) Staining

Hippocampus sections stained with hematoxylin and eosin were used for assessment of overall hippocampal morphology according to methods described previously (Wu et al., 2005) [26]. In brief, the rats were anesthetized with pentobarbital sodium, and the brain was removed and immersed in a fixative mixture (37.5% ethanol, 9.3% formaldehyde, 12.5% acetic acid, and 3% glutaraldehyde) for 12 h at room temperature. The fixed brain containing the hippocampus was then embedded in paraffin. Coronal sections were cut at 5 μm thickness and were stained with H&E.

Double Immunofluorescence Labeling

Normal control rats and rats of the diabetes groups (DM 3, 6 and 9w) were used for immunofluorescence studies. Paraffin coronal sections of the brain derived from different time points were incubated in primary antibodies (see Table 1) including anti-NMDAR2A (1:250, Cell Signaling), anti-NMDAR2B (1:250, Cell Signaling) and GluR1(1:250, Bioss) overnight at 4 °C. NeuN (1:250, Abcam/Millipore) was used for neuronal labeling. After incubation and washing, FITC-conjugated and Cy3-conjugated secondary antibodies were added. After mounting, images representing at least one brain section each from four rats at different time points were captured under a confocal microscope (Leica, TCS SP5). Immunofluorescence labeling for the various antibodies directed against the respective cell types was consistent and reproducible across different rats.

Western Blotting Analysis

The rats in each group were anesthetized with 10% chloral hydrate. The hippocampus tissue samples were removed, kept in liquid nitrogen immediately, and stored in the refrigerator (− 80 °C) for further analysis. Hippocampal tissue samples were homogenized in RIPA containing PMSF at − 20 °C.After this, they were centrifuged at 12000 r/min to collect the supernatant. Protein was quantified in tissue extracts by a modified Lowry procedure with bicinchoninic acid (BCA Protein Assay Kit,Beyotime). After the determination of its protein concentration with enzyme standard instrument, partial tissue samples were stored under − 80 °C. Before the analysis of the target proteins, loading buffer was added. Samples containing equivalent amounts of protein were separated by SDS–polyacryl-amide gel electrophoresis (PAGE). Proteins were transferred to an immobilon PVDF membrane and then blocked with TBST solution (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1%Tween 20) containing 5% w/v nonfat dry milk for 1 h at room temperature. The membranes were incubated overnight at 4 °C with anti-NMDAR2A (1:1500, Cell Signaling), anti-NMDAR1 (1:1000, Cell Signaling), anti-NMDAR2B (1:2000, Cell Signaling) and GluR1 (1:250, Bioss). β-actin (1:500, Santa Cruz) was used as an internal control. The primary antibodies were listed in Table 2. After washing, membranes were incubated with

Table 1 Primary antibodies used in immunofluorescence studies

Name	Dilution	Catalog number	Company
NMDAR2A	1:250	4205	Cell Signaling Technology,Inc, MA, USA
NMDAR2B	1:250	5580	Cell Signaling Technology,Inc, MA, USA
GluR1	1:250	bs-10042R	Bioss,Beijing Biosynthesis Biotechnology co.,ltd,China
NeuN	1:250	ab177487	Abcam, Cambridge, MA, USA
NeuN	1:250	MAB377	Millipore Corporation, Bioscience, Billerica, MA, USA

Table 2 Primary antibodies used in Western blotting analysis

Name	Dilution	Catalog number	Company
NMDAR1	1:1000	5704	Cell Signaling Technology, Inc, MA, USA
NMDAR2A	1:1500	4205	Cell Signaling Technology, Inc, MA, USA
NMDAR2B	1:2000	5580	Cell Signaling Technology, Inc, MA, USA
GluR1	1:250	bs-10042R	Bioss,Beijing Biosynthesis Biotechnology co.,ltd,China
β-actin	1:500	sc-47778	Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA

peroxidase-conjugated antirabbit IgG or antimouse IgG for 2 h and then washed 3 times with TBST. Immunoreactive proteins were visualized by chemiluminescence.

Statistical Analysis

For Morris Water Maze tests, Western blots and Immunofluorescence, data are reported as mean ± SD. The integrated density of Western blot bands and Immunofluorescence were measured by Image J (version 1.50i software; National Institutes of Health, USA). Data were analyzed by one-way ANOVA (spss version 16.0 software; SPSS, Chicago, IL, USA) to determine the statistical significance of differences between normal control rats, rats of DM 3, 6 and 9w. Significance is accepted as P-value < 0.05 and is denoted by asterisks ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

Results

H & E Staining

In the CA1 region of hippocampus, H&E staining showed that the hippocampus of diabetic rats did not exhibit noticeable structural changes at 3w after diabetes induction (Fig. 1a–f). However, pathological changes in the hippocampus became evident in in experimental rats with prolonged diabetes. Thus, compared with NC and DM 3w groups, the hippocampal pyramidal neurons in DM 6w group were loosely arranged and disorganized. There were signs of karyopycnosis along with neuronal shrinkage. In DM 9w group, the CA1 of hippocampus was more severely affected with drastic pathological changes. The pyramidal neurons were sparsely arranged, indeed in a haphazard manner admixed with a variable number of neurons undergoing degeneration or necrosis.

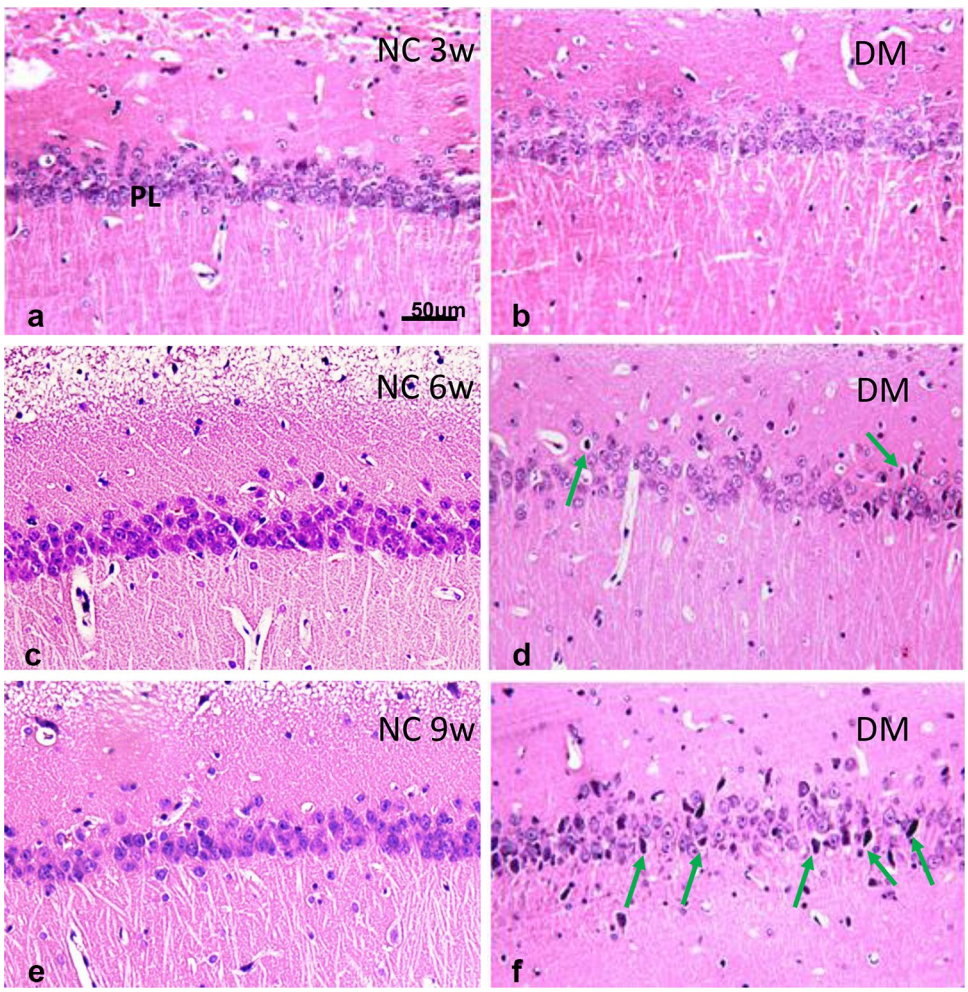

Fig. 1 H & E staining of hippocampus. H & E staining of CA1 of hippocampus in normal control and induced diabetes (400 × Bar = 50 μm). The normal control group (**a, c, e**) showed full integrity of cellular layers and highly organized arrangement of neurons in pyramidal layer (PL). Showing also photo-images of diabetes groups at 3 weeks (**b**), 6 weeks (**d**) and 9 weeks (**f**). In DM3 group (**b**), the pyramidal layer did not exhibit obvious structural changes. In DM6 group (**d**), the pyramidal neurons underwent pathological changes as indicated by the disordered arrangement, shrinkage of some neurons, and nuclear pyknosis. In DM9 group (**f**), the morphological alteration of the pyramidal neurons was more severe when compared with the DM6 group. Microscopic evaluation revealed nuclear pyknosis (green arrows). (Color figure online)

Morris Water Maze

Morris Water Maze of learning scores (escape latency) (Fig. 2):The escape latency was increased gradually in the course of development of DM. The escape latency showed a significant increase at DM 6 and 9w ($P<0.01$) in comparison to the normal control (NC); it showed a significant increase at DM 6w ($P<0.01$ or $P<0.05$) compared to DM 3 and 9w; At DM 9w, it was decreased to some extent compared to the DM 6w. Morris Water Maze of learning scores (the frequency in crossing the platform) (Fig. 3): the frequency in crossing the platform was reduced gradually following the course of development of DM. The frequency in crossing the platform was significantly decreased ($P<0.01$) at DM 3, 6 and 9w in comparison to the NC; it was moderately increased at DM 6w ($P>0.05$) in comparison to the DM 3w; there was no significant change in the number of crossing the platform at DM 3, 6 and 9w ($P>0.05$) through pairwise comparison. Morris Water Maze of learning scores (the time of staying at the target area) (Fig. 4): the time of staying at the target area was shortened gradually through the course of development of DM. It was not reduced significantly at DM 3w ($P>0.05$) compared with that of NC. The duration of staying at the target area was not reduced significantly at DM 6w ($P>0.05$) compared with that of DM 9w. The time of staying at the target area was reduced significantly at DM 6 and 9w ($P<0.01$ or $P<0.05$) compared with that of NC and DM 3w.

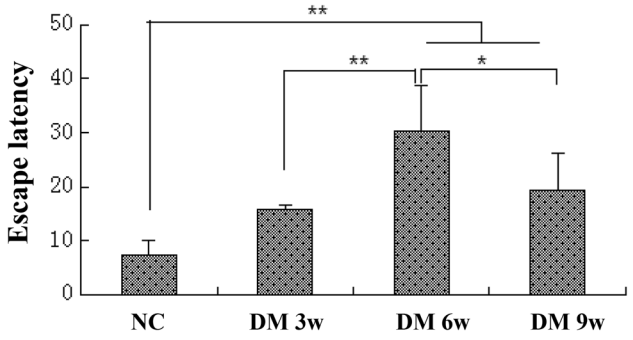

Fig. 2 Morris Water Maze test of learning scores (escape latency) in the normal control rats and diabetic rats. The escape latency is increased gradually through the time course of DM. The escape latency shows a significant increase in diabetic group at DM 6w and DM 9w. Compared DM 6w group with the control (P=0.0016, **<0.01) and DM 3w ($P=0.0060$, **<0.01). Compared DM 9w group with the control ($P=0.0091$, **<0.01) and DM 6w (P=0.0179, *<0.05)

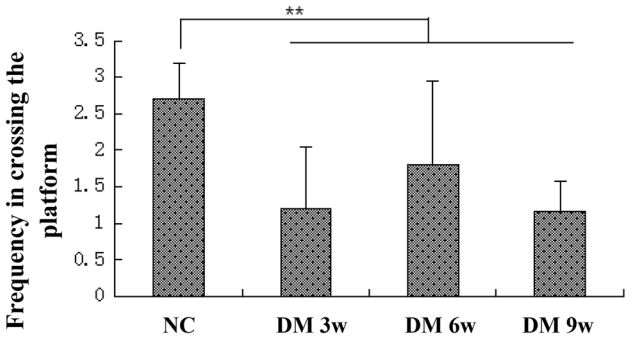

Fig. 3 Morris Water Maze test of learning scores (the frequency in crossing the platform) in the normal control rats and diabetic rats. The frequency in crossing the platform is reduced gradually with the progression of DM. The frequency in crossing the platform shows a significant decrease in diabetic group at DM3, 6 and 9w. Compared normal control group with other groups (DM 3w, $P=0.0014$, **<0.01; DM 6w, $P=0.0057$, **<0.01; DM 9w group, $P=0.0032$, **<0.01)

Double Immunofluorescence Labeling

Marked Increase in NR2A + /NeuN + Pyramidal Neurons in Hippocampus STZ-Induced Diabetes

NR2A expression was observed in stratum pyramidale in the CA1 (Fig. 5a), CA3 (Fig. 5b) and dentate gyrus (Fig. 5c) in the hippocampus. The widely distributed NR2A expressing cells were identified as the pyramidal cells as evident by the colocalization of NR2A with NeuN in the double immunofluorescence labeling (Fig. 5a–c A–L). In the normal control, DM 3and6w groups, NR2A immunoreactivity was hardly detected (Fig. 5a–c A–I),

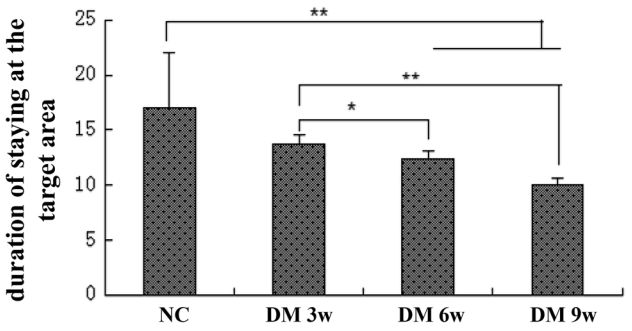

Fig. 4 Morris Water Maze test of learning scores (the duration of staying at the target area) in the normal control rats and diabetic rats. The time of staying at the target area is shortened gradually through the time course DM. The duration of staying at the target area shows a significant decrease in diabetic group at DM 6 and 9w. Compared DM 6w group with the control ($P=0.0016$, **<0.01) and DM 3w ($P=0.0060$, **<0.01). Compared DM 6w group with the control ($P=0.0085$, **<0.01) and DM 3w ($P=0.0394$, *<0.05). Compared DM 9w group with the control ($P=0.0066$, **<0.01) and DM 3w ($P=0.0051$, *<0.01)

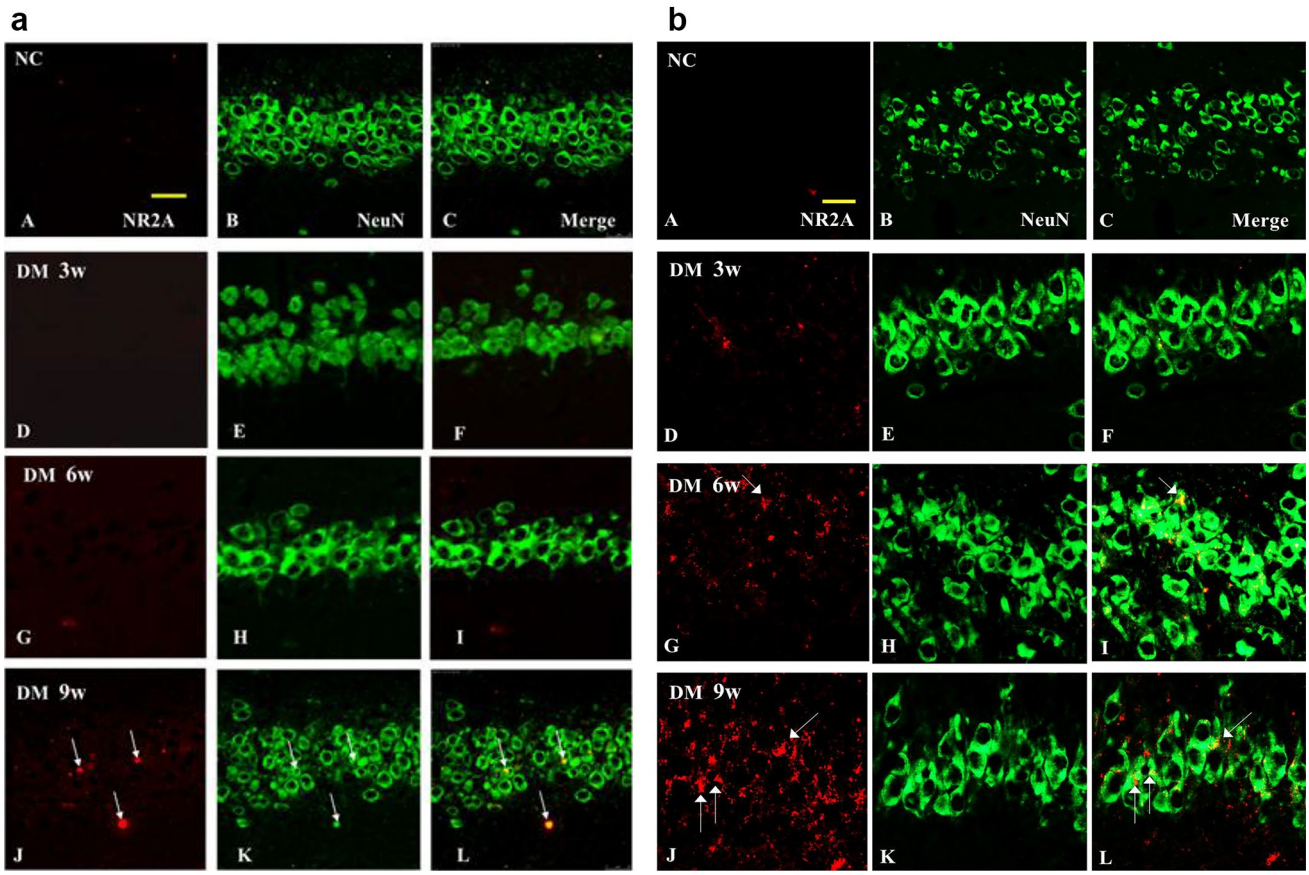

Fig. 5 Representative immunofluorescence of NR2A (red) and NeuN (green) in the hippocampus. **a** NR2A and NeuN immunofluorescence in the CA1 region in the hippocampus of normal control rats (A–C) and rats after diabetes at 3, 6 and 9w (D–L). In the normal control hippocampus and DM 3w and DM 6w, NR2A expression is hardly detected (A–C). In DM 9w (J–L), NR2A expression is increased. Note NR2A immunoreactivity is localized in NeuN-labeled neurons (white arrow) Scale bar = 20.0 μm in A (applies to A–L). **b** Expression and distribution of NR2A and NeuN immunofluorescence in the CA3 region in the hippocampus. NR2A expression in the CA3 resembles the level in the CA1. **c** NR2A and NeuN immunofluorescence in the Dentate Gyrus (DG) in the hippocampus. NR2A expression in the DG is similar to the level in the CA1 and the DG. **d** Integrated immunofluorescence density analysis. In the CA1, it shows increased NR2A-labeled cells and NR2A immunofluorescence intensity in DM 9w, $^{**}P < 0.01$ compared with the control ($P = 0.0018$,) and DM 3w group ($P = 0.0061$). In the CA3, the level of NR2A intensity is increased in DM 6w and DM 9w. Compared DM 6w group with the control ($P = 0.0242$, $^{*} < 0.05$). Compared DM 9w group with the control ($P = 0.0040$, $^{**} < 0.01$). In the DG, it shows increased NR2A intensity in DM 9w, compared with the control ($P = 0.0002$, $^{***} < 0.001$) and DM 3w group ($P = 0.0330$, $^{*} < 0.05$). (Color figure online)

but in DM 9w, the frequency of NR2A expressing neurons was increased (Fig. 5a–c J–L). Densitometry analysis showed that NR2A expression level was significantly increased in DM 6w and DM 9w groups (Fig. 5d). In the CA1, NR2A intensity was increased in DM 9w compared with the control and DM 3w group ($P < 0.01$). In the CA3, the level of NR2A intensity was increased in DM 6w ($P < 0.05$) and DM 9w ($P < 0.01$) compared with the control. In the DG, NR2A intensity was increased in DM 9w compared with the control ($P < 0.001$) and DM 3w group ($P < 0.05$).

Marked Increase in NR2B + /NeuN + Pyramidal Neurons in Hippocampus STZ-Induced Diabetes

In all groups, NR2B expression was localized in the pyramidal cell layer in the CA1 (Fig. 6a), CA3 (Fig. 6b) and dentate gyrus (Fig. 6c) in the hippocampus (Fig. 6a–c A, D, G, J). The NR2B-expressing cells were confirmed to be neurons with colocalization of NeuN by double immunofluorescence labeling (Fig. 6a–c A–L). In normal control rats, moderate NR2B immunofluorescence was detected in the soma of some neurons (Fig. 6a–c A–C). In rats of DM 3, 6

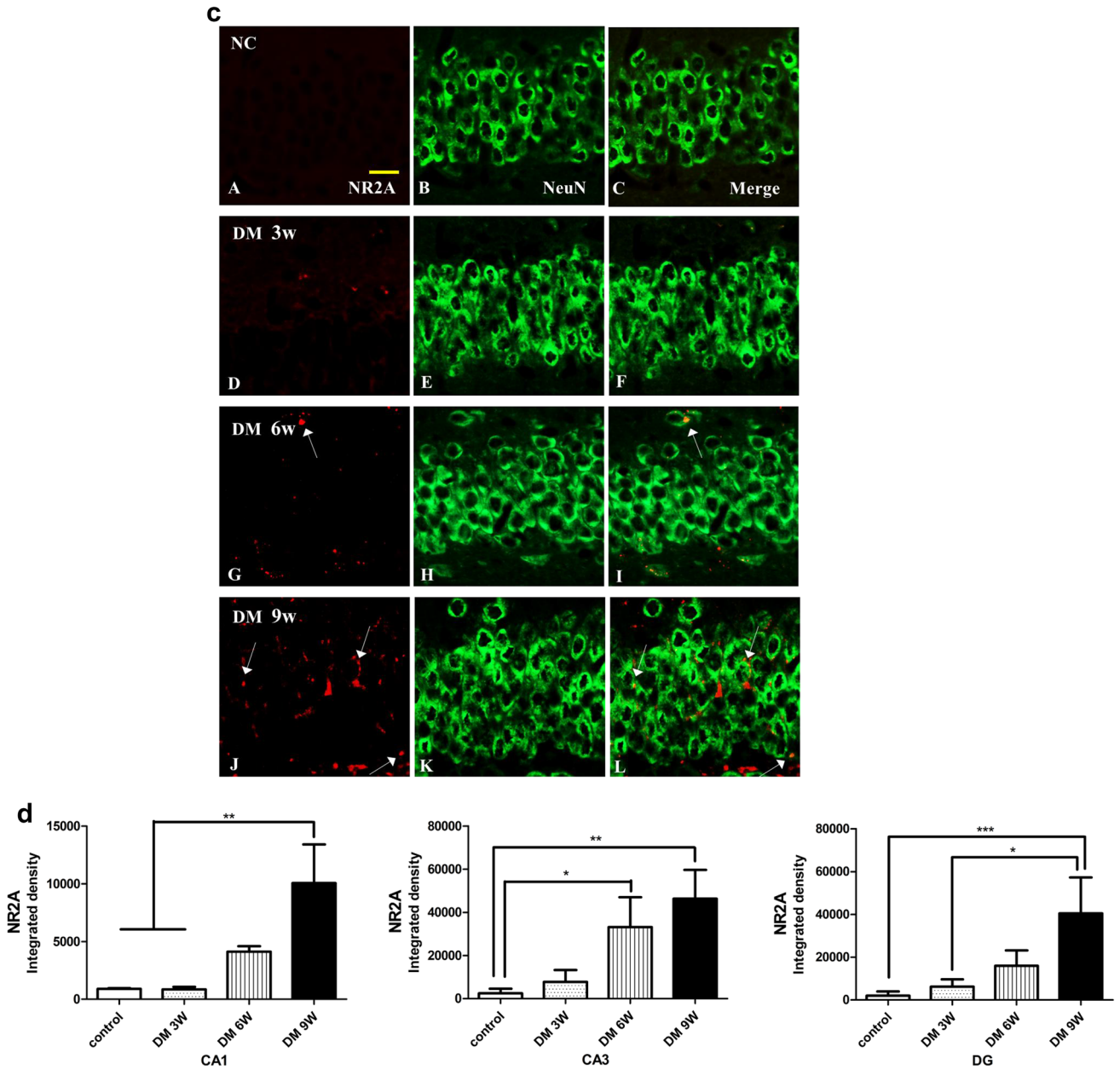

Fig. 5 (continued)

and 9w, NR2B immunoreactivity was markedly enhanced (Fig. 6a–c D–L), being more pronounced at DM 6 and 9w. Densitometry showed NR2B expression level was significantly increased in DM 6w ($P<0.05$) compared with that in the control (Fig. 6d). Furthermore, density analysis showed a marked increase in NR2B labeled neurons and immunofluorescence intensity in DM 9w ($P<0.001$) compared with the control and DM 3w groups, (or $P<0.01$) as well as DM 6w group (Fig. 6d). In the CA1, it shows increased NR2B-labeled cells and NR2B immunofluorescence intensity in DM 6w and DM 9w. In the CA3, the level of NR2B intensity was increased in DM 6w ($P<0.01$) and DM 9w ($P<0.001$) compared with the control, as well as DM 3w ($P<0.05$). In the DG, it shows increased NR2B intensity in DM 6w ($P<0.05$) and DM 9w ($P<0.001$) compared with the control, as well as DM 3w ($P<0.05$).

Marked Increase in GluR1 + /NeuN +

GluR1 expression was observed in stratum pyramidale in the CA1 region in the hippocampus (Fig. 7). In the control rats, GluR1 expression was hardly detected in the CA1 region (Fig. 7a A). In the course of diabetes at 3, 6 and 9w, GluR1 expression was augmented (Fig. 7a G–L). Densitometry

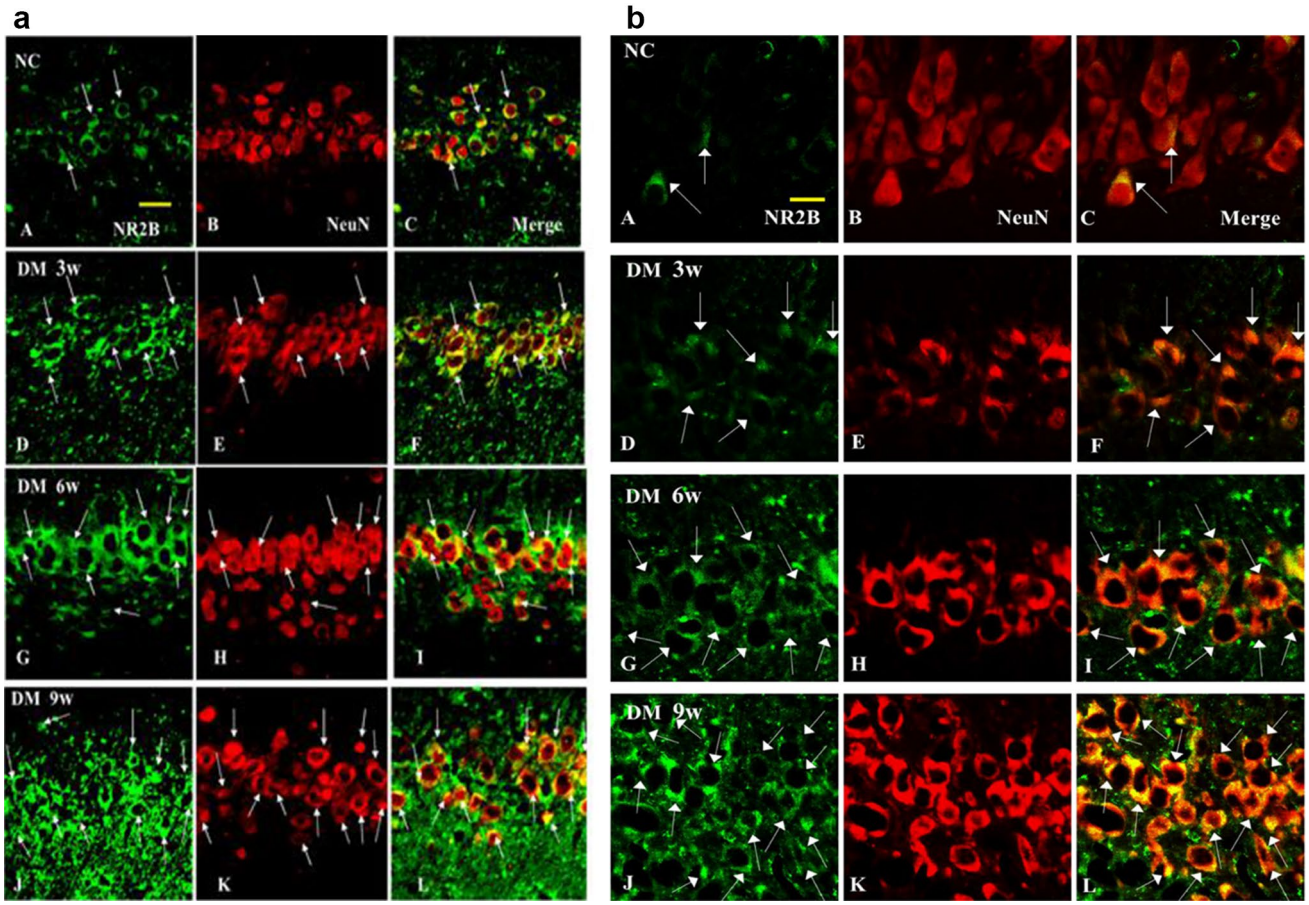

Fig. 6 Representative immunofluorescence of NR2B (green) and NeuN (red) in the hippocampus. **a** Expression and distribution of NR2B(green) and NeuN(red) in hippocampal CA1 region of normal control (NC) rats (A–C) and rats after diabetes at 3, 6 and 9w (D–L). Confocal microscopy shows NR2B colocalization of some neurons (white arrows). Scale bar = 20.0 μm in A (applies to A–L). **b** Expression and distribution of NR2B and NeuN immunofluorescence in the CA3 region in the hippocampus. NR2B expression in the CA3 resembles the level in the CA1. **c** NR2B and NeuN immunofluorescence in the Dentate Gyrus (DG) in the hippocampus. NR2B expression in the DG is similar to the level in the CA1 and the DG. **d** Integrated immunofluorescence density analysis. In the CA1, it shows increased NR2B-labeled cells and NR2B immunofluorescence intensity in DM 6w and DM 9w. Compared DM 6w group with the control ($P = 0.0318$, $*< 0.05$). Compared DM 9w group with other groups (the control, $P = 0.0001$, $***< 0.001$; DM 3w, $P = 0.0001$, $***< 0.001$; DM 6w, $P = 0.0079$, $**< 0.01$). In the CA3, the level of NR2B intensity is increased in DM 6w and DM 9w. Compared DM 6w group with the control ($P = 0.0183$, $**< 0.01$). Compared DM 9w group with the control ($P = 0.0002$, $***< 0.001$) and DM 3w ($P = 0.0313$, $*< 0.05$). In the DG, it shows increased NR2B intensity in DM 6w and DM 9w. Compared DM 6w group with the control ($P = 0.0105$, $*< 0.05$). Compared DM 9w group with the control ($P = 0.0001$, $***< 0.001$) and DM 3w ($P = 0.0409$, $*< 0.05$). (Color figure online)

showed GluR1 expression level was markedly increased in DM 9w ($P < 0.05$) compared with the normal control level (Fig. 7b).

Western Blot Analysis

By western blot analysis, the hippocampal tissue showed a steady and significant increase in protein levels of NR1, NR2A, NR2B and GluR1 with time after diabetes induction. (Fig. 8).

NR1 immunoreactive bands, with a molecular weight of approximately 120 kDa respectively, increased significantly in integrated optical density at 6w ($P < 0.01$) and 9w ($p < 0.01$) after diabetes induction compared with the control levels. Moreover, NR1 expression level was higher in DM 9w group than that in DM 3w ($P < 0.01$) group and DM 6w group ($P < 0.01$) (Fig. 8a, b). Densitometry of the NR2A and NR2B immunoreactive band was approximately 180 kDa.Western blotting showed that the protein expression levels of NR2A and NR2B were weak in the hippocampal tissue of normal control rats. The expression levels of these markers were progressively increased following the course development of DM (Fig. 8a, c, d). The protein expression of NR2A and

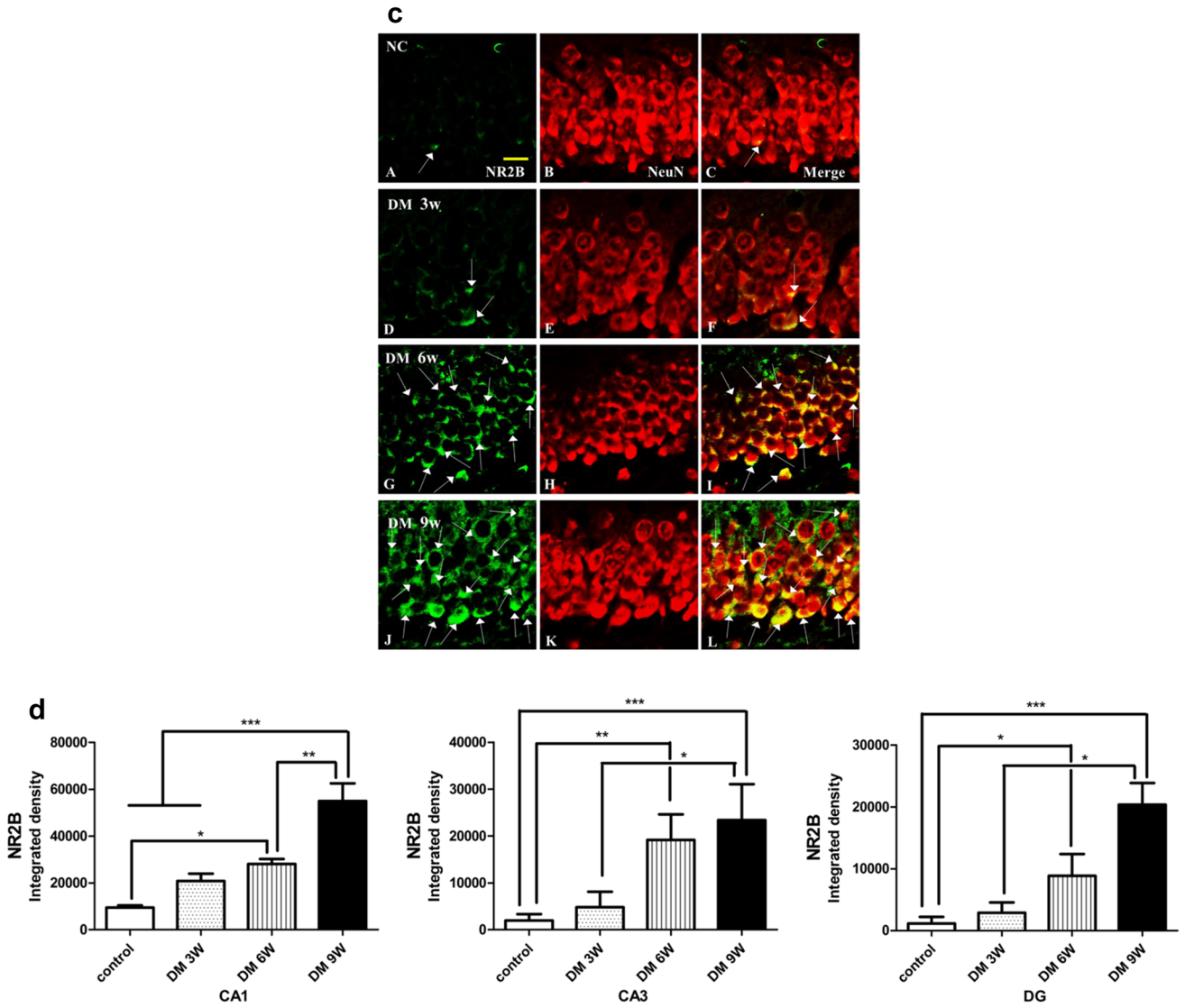

Fig. 6 (continued)

NR2B showed a significant increase in integrated optical density at DM 3W ($P < 0.05$) compared to normal control level (Fig. 8c); NR2A and NR2B protein expression showed a significant increase in integrated optical density at DM 6 and 9w ($P < 0.01$ or $P < 0.05$) compared to NC level (Fig. 8c, d); NR2A and NR2B protein expression showed significant changes in integrated optical density at DM3, 6 and 9w ($P < 0.01$ or $P < 0.05$) through pairwise comparison (Fig. 8c, d).

GluR1immunoreactive band, with a molecular weight of approximately 97 kDa, increased significantly in integrated optical density at 6w ($P < 0.05$) and 9w ($P < 0.01$) after diabetes induction compared with the control levels. (Fig. 8e).

Discussion

Relationship Between CID and Hippocampus

Over the past decade, the incidence of diabetes has shown explosive growth and the global disease rate is projected to double by 2030 [1, 27]. Increasing evidence has indicated that the effect of diabetes is associated with cognitive dysfunction and an increased risk of dementia [2]. The molecular mechanism of cognitive impairment is multifactorial, although dysfunction in each interconnecting pathway ultimately leads to discordance in metabolic signaling. McCrimmonet al.[28] reported that chronic

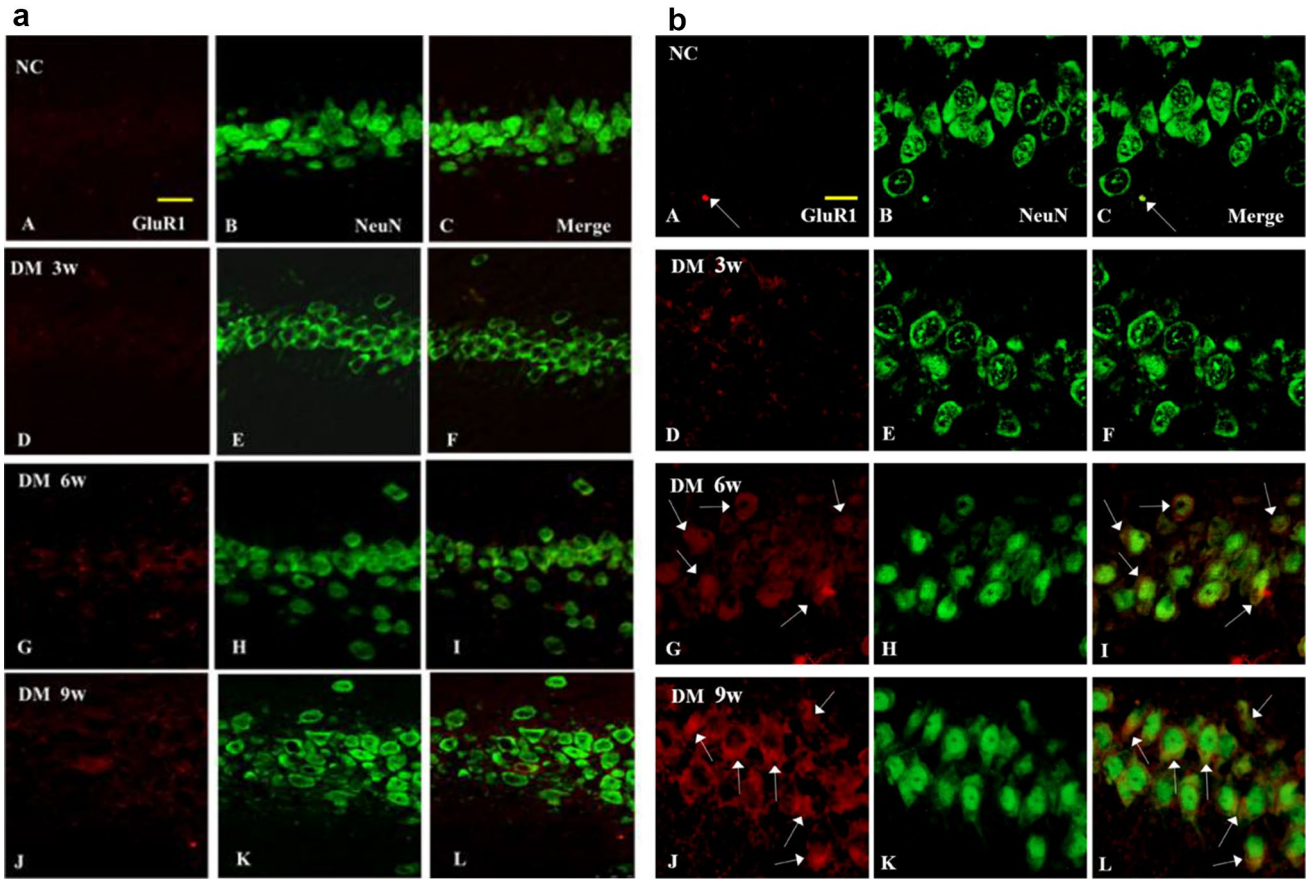

Fig. 7 Representative immunofluorescence of GluR1 (red) and NeuN (green) in the CA1 region in the hippocampus. **a** Expression and distribution of GluR1 (red) and NeuN (green) in hippocampal CA1 region of normal control (NC) rats (A–C) and rats after diabetes at 3, 6 and 9w (D–L). The normal control (A) and DM 3W groups (D) present very weak GluR1 immunofluorescence which is hardly detected (A, D). In the DM 6w and 9w group, it is mildly enhanced in the neurons (G–L). Scale bar = 20.0 μm in A (applies to A–L). **b** Expression and distribution of GluR1 and NeuN immunofluorescence in the CA3 region in the hippocampus. GluR1 expression in the CA3 resembles the level in the CA1. **c** GluR1 and NeuN immunofluorescence in the Dentate Gyrus (DG) in the hippocampus. GluR1 expression in the DG is similar to the level in the CA1 and the DG. **d** Integrated immunofluorescence density analysis. In the CA1, it shows increased GluR1-labeled cells and GluR1 immunofluorescence intensity in DM 9w, compared with the control (*P = 0.0204*, ** < 0.05). In the CA3, the level of GluR1 intensity is increased in DM 9w, compared with the control ($P = 0.0003$, *** < 0.001). In the DG, it shows increased GluR1 intensity in DM 6w and DM 9w. Compared DM 6w group with the control ($P = 0.0405$, * < 0.05). Compared DM 9w group with the control ($P = 0.0001$, *** < 0.001) and DM 3w ($P = 0.0199$, * < 0.05). (Color figure online)

hyperglycaemia and microvascular disease contributed to cognitive dysfunction in both type 1 and type 2 diabetes. Additionally, both diabetes types are characterized by neural slowing, increased cortical atrophy, microstructural abnormalities in white matter tracts, and similar, but not identical, changes in concentrations of brain neurometabolites. Meanwhile, Zillioxet al. [29] reported that cognitive deficits may occur at the very early stages of diabetes which may be further exacerbated by the metabolic syndrome. Animal models of insulin resistance help to bridge CID and point to hippocampal insulin resistance as a potential mediator of cognitive dysfunction in type 2 diabetes as well as in Alzheimer disease (AD) [30].CID is closely related to the changes of synaptic plasticity in the hippocampus. Hence, the effect of cognitive function due to diabetes on the central nervous system was investigated in this study, focusing specifically on the hippocampus. Hippocampal damage has been confirmed in many studies investigating different types of diabetes [27, 31]. The present results are in general agreement with observations made by other studies. In the latter, a salient feature across the different studies was the occurrence of pathological changes notably in CA1region of the hippocampus. By H&E staining, the present results have shown that the hippocampal neurons in diabetic rats were reduced in numbers. The neurons were disorderly arranged or in disarray. Many of them appeared hyperchromatic with some showing pyknotic nucleus compared to the normal control rats. There was no noticeable structural changes in the hippocampus at 3w after diabetes induction; however,

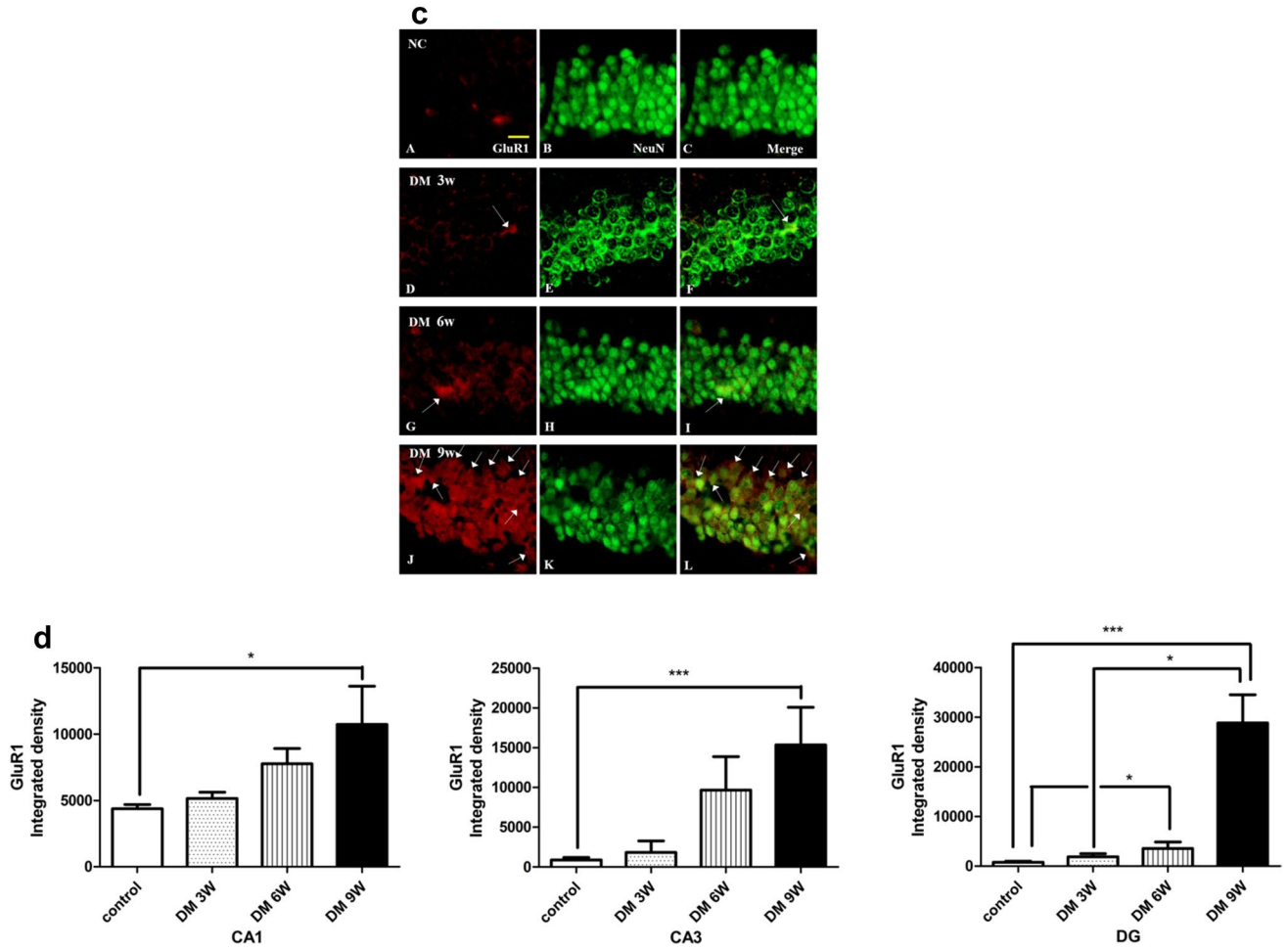

Fig. 7 (continued)

in prolonged diabetes such as at 6 and 9w, pathological changes were evident and indeed with time they were aggravated. Hippocampal pyramidal neurons in DM 6w group were loosely and disorderly arrangement with some of them showing signs of karyopycnosis and shrinkage of the soma. The CA1 in DM 9w group showed a sparsity of pyramidal neurons; many of them appeared to have degenerated or necrotic. It has been reported that in the CID, learning and memory behaviors displayed different degrees of reduction with the development of diabetes and over a protracted duration. The hippocampus is sensitive to chronic hyperglycemia in diabetes mellitus and this is manifested by a reduction in hippocampal neuron number, abnormal morphology, learning and cognitive dysfunction, delayed responses to surroundings, and cognitive impairment characterized by acquired cognitive and behavioral deficiency [32, 33]. It is therefore suggested that the drastic pathological changes affecting the CA1 hippocampal neurons as observed in the present study may have accounted for the cognitive impairment in the diabetic rats as shown by the MWM test.

Effects of Cognitive Dysfunction by MWM Test

MWM has been shown to be the method of choice to evaluate the cognitive function in hippocampal lesions models [34]. Hence, MWM test has been adopted in this study to determine the effects of diabetes on learning and memory. The present results have shown that the escape latency of STZ-induced diabetic rats was increased gradually in the course of development of DM. On the contrary, the frequency in crossing the platform was reduced gradually in the course of development of DM. Moreover, the time of staying at the target area was shortened gradually through the course of development of DM. The prolonged escape latencies in the acquisition phase indicated a slowing down of learning in STZ-induced rats. Because of the poor performance of spatial reference learning, the rats

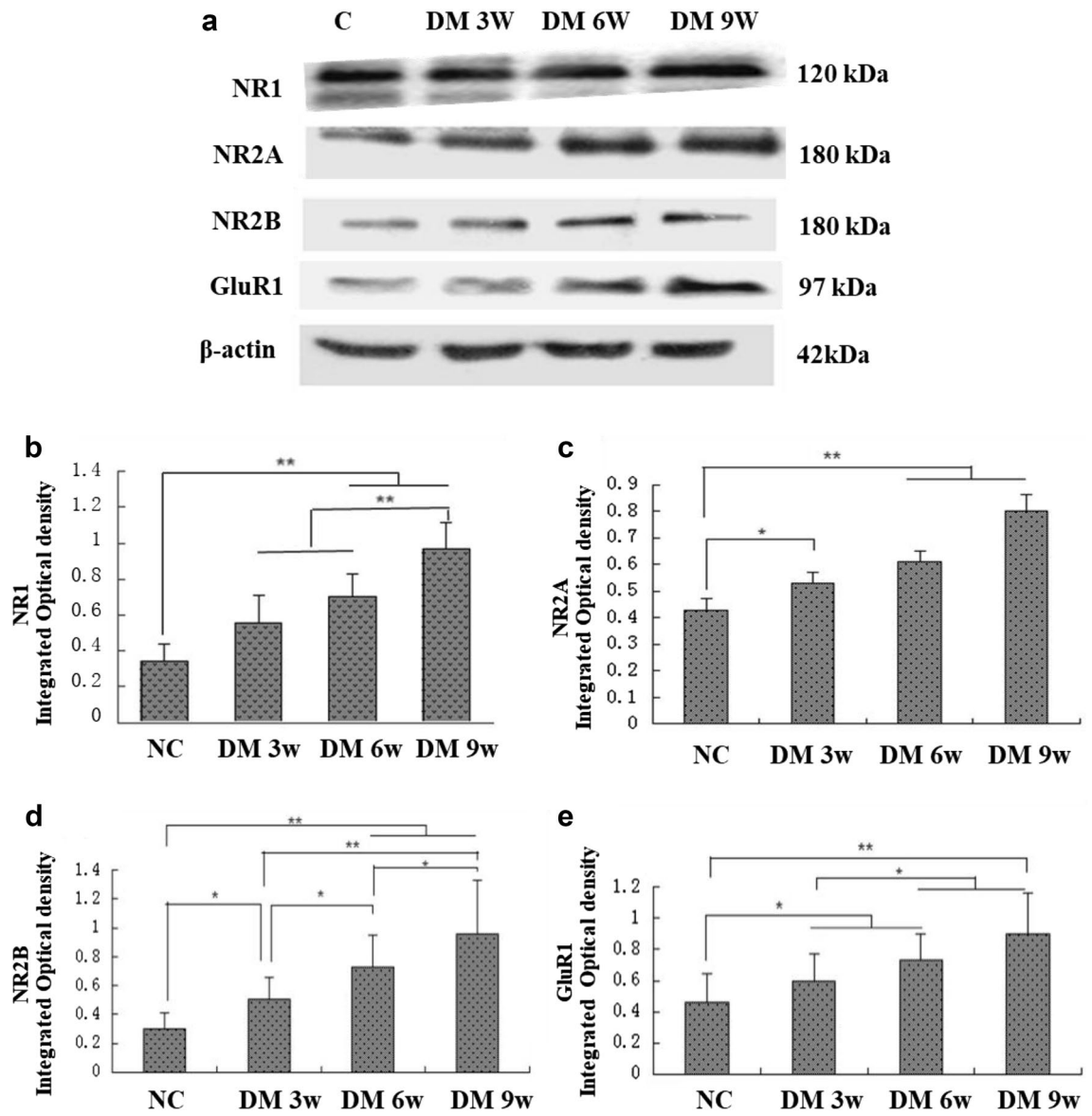

Fig. 8 Western blotting analysis of NR1, NR2A, NR2B and GluR1 protein expression levels in the hippocampus. Expression of NR1, NR2A, NR2B and GluR1 in the hippocampus of normal control group rats (Lane 1); expression of the various markers in the diabetic rats for 3, 6 and 9 w of protein change is shown in Lane 2, Lane 3, Lane 4. **a** NR1(120 kDa), NR2A(180 kDa), NR2B (180 kDa), GluR1(97 kDa) and β-actin (42 kDa) immunoreactive bands. Bar graphs representing integrated optical density (mean ± SD) of NR1 (**b**), NR2A (**c**), NR2B (**d**) and GluR1(**e**) normalized to β-actin for control, DM 3w, 6w and 9w (*$P<0.05$; **$P<0.01$). **b** NR1 shows an obvious increase at 9w in the DM group, as compared with control and two other groups of diabetic rats (NC vs DM 6w, $P=0.0044$; NC vs. DM 9w, $P=0.0020$; DM 3w vs. DM 6w, $P=0.0028$; DM 6w vs. DM 9w, $P=0.0030$). **c** NR2A shows a significant increase at 3, 6 and 9w in the DM group, as compared with the control (NC vs. DM 3w, $P=0.0378$; NC vs. DM 6w, $P=0.0019$; NC vs. DM 9w, $P=0.0063$). **d** NR2B shows a steady increase at 3, 6 and 9w in the DM group, as compared with the control. (NC vs. DM 3w, $P=0.0407$; NC vs. DM 6w, $P=0.0041$; NC vs. DM 9w, $P=0.0067$; DM 3w vs. DM 6w, $P=0.0270$; DM 3w vs. DM 9w, $P=0.0047$; DM 6w vs. DM 9w, $P=0.0141$) **e** GluR1 shows a progressive increase at 3, 6 and 9w in the DM group, as compared with the control (NC vs. DM 3w, $P=0.0179$; NC vs. DM 6w, $P=0.0410$; NC vs. DM 9w, $P=0.0057$; DM 3w vs. DM 6w, $P=0.0400$; DM 3w vs. DM 9w, $P=0.0209$)

had to search for the hidden platform around the whole pool, which led them to spend more time in the non-target quadrant [35]. Therefore, deficit learning and hindered orientation might have contributed to the prolonged escape

latencies and resulted in a poor performance in the times of crossings of the hidden platform.

CID Affects NMDA Receptor Subunit Levels in Hippocampus

Glutamate is a critical excitatory neurotransmitter in the central nervous system of mammals and humans. It acts through the excitatory GluR which mediates a series of higher neural activities [11]. GluR comprises 2 types: first, the NMDAR which is a "molecular switch" that triggers synaptic plasticity and contributes to the induction of LTP postsynaptic AMPAR quantity and function [36]; secondly, the non-NMDAR, which is divided into AMPAR and KAR. The AMPAR mainly mediates fast synaptic transmission; it also participates in and sustains LTP expression [37]. NMDA receptor is an ionotropic glutamate receptor. It is known to have important implications for the origin of cell injury and death. NMDAR is very important for controlling synaptic plasticity and memory function through allowing positively charged ions to flow through the cell membrane [38].

NMDA receptors contain primarily three subunits NR1, NR2A, and NR2B. Each receptor is composed of two obligatory NR1 subunits and two regionally localized NR2 subunits. NR1 subunits bind the co-agonist glycine and NR2 subunits bind the neurotransmitter glutamate. NR1-NR2B receptors exhibit much slower decay kinetics than NR1-NR2A receptors [39]. We found expression of NR1, NR2A and NR2B was increased in the course of DM progression. It has been reported that expression ofNR1-NR2A and NR1-NR2B receptors was increased in damaged regions of epileptic brain [40]. Because responses mediated by NR1-NR2B receptors decay more slowly than those mediated by NR1-NR2A receptors, the associated Ca2 + influx would be enhanced. A large influx of Ca2 + through NMDA channels may facilitate seizure generation, and influx through extrasynaptic NR1-NR2B receptors can precipitate excitoxicity [39]. Moreover, Sanz-Clemente et al. reported receptors conformed by NR1/NR2A and NR1/NR2B subunits have different electrophysiological properties and synaptic location [41].

NR2 subunits are confined to specific nuclei defined within the CNS. There are 4 isoforms in NR2 family: NR2A, NR2B, NR2C and NR2D, with the NR2A and NR2B subunits being expressed mainly in the hippocampus [42]. NR2A and NR2B play a role in neuronal calcium overload caused by ischemia and hypoxia. Increase in NR2A and NR2B expression and opening of L type Ca2 + channels play a synergistic role [43]. The common mediated extracellular Ca2 + influx leads to neuronal damage, and ultimately a decline in cognitive abilities [44]. The view on expression of NR2A and NR2B and the involvement of these receptors in cognitive dysfunction has not been conclusive. In chronic stress model of depression rats, the mRNA content of NMDAR2B in the hippocampus and left prefrontal cortex layer was found to be significantly higher than in the normal control group [45]. Arising from the above studies, we hypothesized that during acute lesions such as repeated persistent seizure, the expression of NR2A and NR2B would be increased and ultimately cause cognitive impairment. Matsunaga, Y [10] indicated that early alterations in insulin receptor signaling in the GK rat hippocampus may affect cognitive performance by suppressing synaptic maturation via inhibition of NR2.In our study, the expression ofNR1, NR2A and NR2Bwas increased in the course of DM progression. The results of immunofluorescence and immunohistochemistry showed that the expression of NR2A and NR2B protein in the neurons in the normal control group was weak, but with the progression of DM, NR2A and NR2B expression was enhanced. Western blot results also showed a similar trend of changes in NR1, NR2A and NR2B expression. The possible explanation for this is thatNR1, NR2A and NR2B activity is regulated by some endogenous factors. In acute neuronal excitation, the activation of NR2A and NR2B limits the excessive stimulation and prevents neuronal injury [46]. Therefore, we speculate that up-regulation of NR2A and NR2B may be induced by nerve cells responding to sustained excitement cell injury. It is suggested that chronic lesions, e.g. DM, chronic cerebral ischemia and hypoxia depression and Alzheimer's disease, may exert chronic stress on the nervous system causing the neural activity changes. The nerve endings may release excessive amounts of neurotransmitters, and inducedNR2A and NR2B over expression thus causing cognitive impairment.

It has been reported that synaptic plasticity is important in memory and learning. GluR1 plays an important role in the formation process of LTP [47]. It has also been reported that GluRl knockout mice fail to induce LTP [17]. AMPAR is not only involved in fast excitatory synaptic transmission, but it can also regulate the release of neurotransmitters, inducing and maintaining the LTP, LTD etc; it is closely related to learning and memory behavior [48]. We showed that GluRl protein expression in the control group was weak, but was enhanced with DM, which was paralleled by western blot results. It is therefore suggested that enhanced GluR1 expression in DM may be related to the decline in learning and memory.

Conclusion

We have shown in this study that the protein expression of NR1, NR2A, NR2B and GluR1 in the hippocampal tissue was increased gradually in the course of DM development. The induced type 1 diabetic rats also presented different degrees of cognitive dysfunction with the progression of

DM. Along with this, we also found neuronal apoptosis. It is suggested that all the markers have a close relationship or are linked to the occurrence and development process of CID. They would be useful molecular biomarkers and could be used, whether collectively, individually or separately for the early prediction and diagnosis of CID. In other words, the present results suggest that all the factors which are augmented in DM as demonstrated in this study have a close relationship or are linked to the occurrence and development process of CID.

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Compliance with Ethical Standards

Competing interests The authors declare that they have no competing interests.

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