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Remodeling of ammon's horn during the first two weeks of experimental diabetes development

Дослідження проведено з метою вивчення просторових і часових паттернів розвитку астрогліоза та ушкодження нейронів CA1–CA3 зон на початкових стадіях індукованого стрептозотоцином діабета у щурів. Для візуалізації нейронів і гліальних клітин використовували імуногістохімічне виявлення маркерних білків NeuN та GFAP. Кількість імунопозитивних клітин була підрахована на 3, 7 та 14-ту добу розвитку діабету з застосуванням конфокального мікроскопа "Olympus FV 1000". Значне зниження кількості нейронів в зоні CA2 спостерігалося у щурів з діабетом на 3-ю добу захворювання. В зоні CA1 та CA3 кількість NeuN-позитивних клітин почала знижуватися на 7-му добу (нижче за контроль на 7 та 9 % відповідно). Це зниження розвивалося надалі, до 14-ї доби, коли кількість нейронів в зоні CA1 та CA3 була меншою за контроль на 20,3 та 18,1 % відповідно. Ці зміни супроводжувались астрогліозом: кількість астроцитів у пірамідальному шарі була вірогідно вищою за контроль у всі досліджені строки. Таким чином, наше дослідження демонструє, що індукований стрептозотоцином діабет асоційований з нейродегенерацією гіпокампа. Це дає можливо припустити, що розвиток клінічно значимих когнітивних розладів починається вже на ранніх стадіях захворювання.

INTRODUCTION

Both types of diabetes mellitus (DM) are associated with cognitive deficits and brain impairment, both presenting signs of so-called «diabetic encephalopathy» [4]. The huge body of evidence indicate structural and functional hippocampal alterations during long-term development of diabetes, however the clinical characteristics of diabetic encephalopathy still present a subject for discussion because of frequently observed inconsistences in behavioral and cognitive changes [3, 10, 16, 21] . The number of papers during the recent past had shown evidences for hippocampal dysfunction occurring already at the early stages of type I diabetes. These data include dendritic branch remodelling, decreased neurogenesis and progressive astrogliosis [13, 17]. However, there is still a lack of data on the dymanics of neuronal damage and astroglial reaction during the initiation and first weeks of DM. Thus, the present investigation was undertaken to study temporal and spatial patterns of the neurodegeneration and gliosis in hippocampal CA1-C3areas during the first two weeks of streptozotocin-induced diabetes development.

METHODS

Male Wistar rats (b.w. 190–230g) were received from the animal house Bogomoletz institute of Physiology. Animals were handled according to European Community guidelines [1]. Diabetes mellitus was induced in male Wistar rats by a single intraperitoneal injection of streptozotocin (STZ) in dose of 45 mg/kg in citrate buffer solution (pH=4.5); The same citrate buffer volume without sreptozotocin was used in control group. Diabetic rats with the glycemic level >10 mmol/l selected for further investigations. Animals were sacrificed at day 3, 7 and 14 after the STZ injec-

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tion under the calipsol (75 g/kg) anesthesia and perfused transcardially with 4% formaldehyde. 50-μM-thick brain sections were washed twice in phosphate-bufferred saline (PBS) and incubated overnight at 4 °C in the mixture of anti-GFAP polyclonal antibodies (diluted 1:500) and anti-NeuN monoclonal antibodies (diluted 1:1000). Secondary anti-mouse Alexa-488 and anti-rabbit Alexa-568 antibodies were used to detect neurons and astroglial cells. NeuN is a neuron-specific nuclear protein, playing important roles in nervous system development and function [15]. GFAP (glial acidic fibrillary protein) is an intermediate filament protein that is expressed in astrocytes only [8]. NeuN and GFAP – positive cells were identified with Olympus Fluoview FV-1000 LSM system (Japan). Optical XY sections was obtained through the full thickness of the sections using multi-line argon (488nm) and green helium neon (543nm) lasers. Spatial distribution and density of neuronal and glial cells as well as their morphological parameters were measured using image analysis suite from Altanin Tech. (Ukraine) in automatic and semi-automatic modes. Two-way ANOVA and LSD-test was used to estimate statistical significance of the data.

RESULTS

NeuN-immunoreactive neurons in intact rat hippocampus are located in several rows forming pyramidal cell layer (*stratum pyramidale*). This layer in CA1 and CA2 areas usually has three row of neurons with cell bodies in close contact with each other. Hippocampal astrocytes have moderate branching and short processes and are located mainly in *stratum radiatum* and *stratum oriens*, were moderate branched astrocytes with short processes rarely penetrating pyramidal layer (Fig. 3A).

DM at day 3 led to the significant decrease in neuronal count (NeuN-positive cells) in CA2 area but not in CA1 and CA3 (Fig. 1). Later on, at day 7 and day 14 no significant differences were observed in CA2 area as compared to day 3, while in CA1 area at day 7 a significant neuronal loss was found. This neurodegeneration continued furthermore to the day 14, when the number of neurons in CA1 area decreased to 20.3% of the control values. Neuronal loss in CA3 area was statistically insignificant during the first three days of diabetes, but at the end of the second week the number of neurons was decreased by 18 % as compared to control.

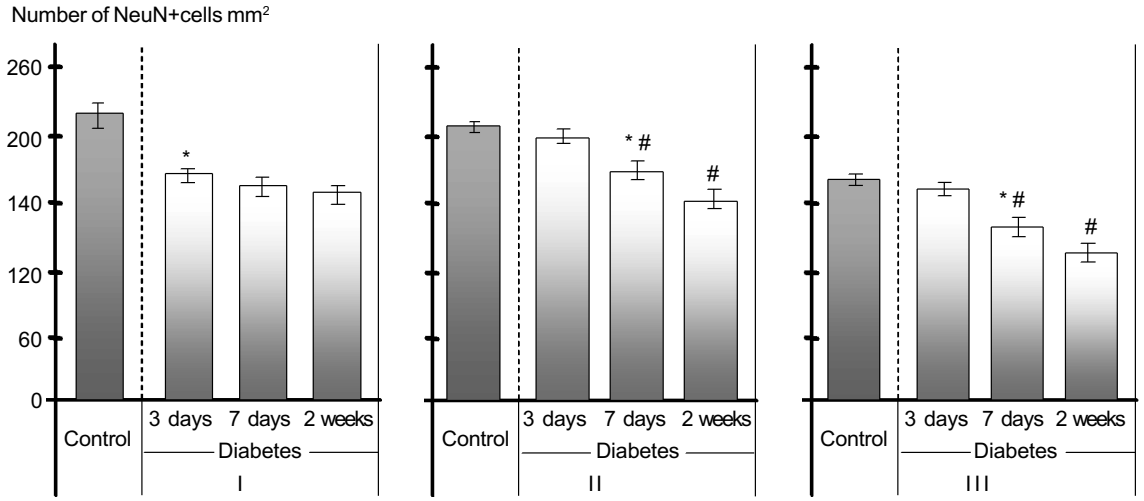

Fig. 1. The number of NeuN-positive neurons in the hippocampal CA1 (I), CA2 (II) and CA3 (III) pyramidal layers at day 3, 7, 14 after the DM induction. There is a significant decrease in neuronal density in CA2 area at day 3 *vs.* control. At the day 7 there is a marked reduction in CA1 and CA3 areas. Data are presented as mean ± SEM; * $p < 0.05$ *vs.* control; # $p < 0.05$ (t-test) *vs.* preceding time-point

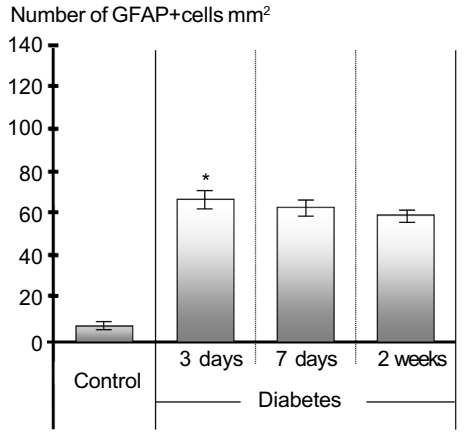

Fig. 2. The number of GFAP-positive astrocytes in hippocampal stratum pyramidale at day 3, 7 and 14 after the diabetes onset. There is a significant increase in astrocyte number in stratum pyramidale at day 3.. Data are presented as mean ± SEM; * $p < 0.05$ vs. control

The number of GFAP-positive cells (astrocytes) was increased dramatically through the period of observation with the most substantial changes taking place during the first three days (Fig. 2). The most prominent increase in the number of astrocytes was observed in stratum pyramidale at day 3.

An alteration in the pyramidal CA1 layer morphology was observed at day 7 of DM development: neurons irregularly placed and intermixed with astrocytes (Fig. 3). GFAP-positive cells were increased in volume and had ramified dendrites. Neuronal loss was associated with increase in astrocyte number and extension of GFAP-positive astrocyte processes (Fig. 3B). Taken together, these data suggest reactive astrogliosis accured already at day 7 of diabetes progression.

DISCUSSION

The present study had shown that the early stages of DM are associated with significant decrease in the number of NeuN-positive cells. It is important to indicate that a decrease in NeuN labeling is not necessarily related to neuronal death. Probable reasons for the decrease in stained cells number are reduction in protein expression level and loss of antigenicity [5]. In view of this data, the decrease of NeuN-positive cell count in hippocampus is not direct evidence for neuronal loss. On the other hand, the decline of NeuN immunoreactivity indicates in alteration of NeuN antigenic sites [5] and can be consider as a marker of neuronal damage. Thus, the decline of NeuN-positive cell number in hippocampal pyramidal layer during two weeks after STZ injection is an evidence for neuronal damage and may be related to neuronal death.

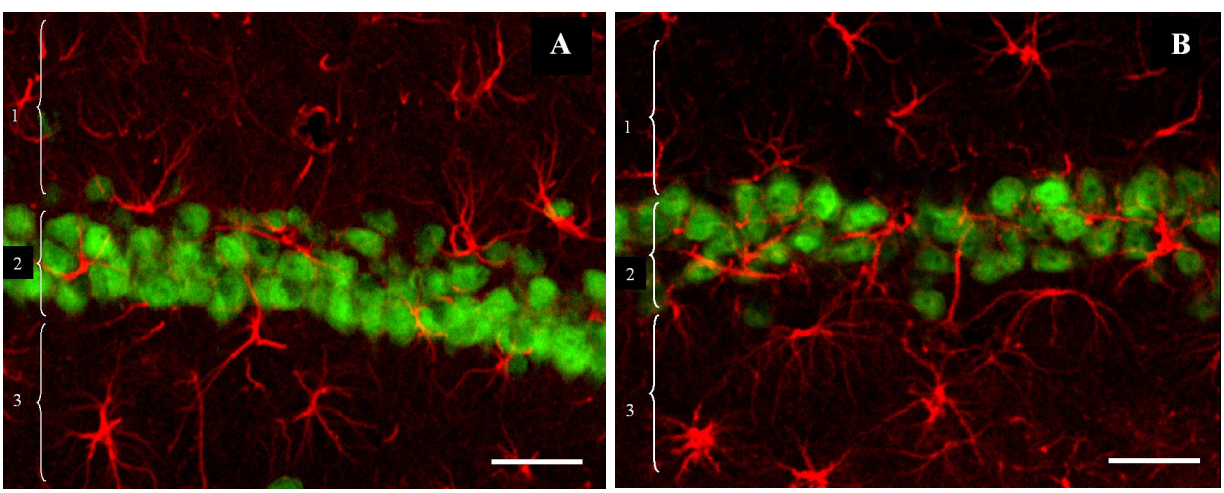

Fig.3. Microphotograph of hippocampal CA1 neurons and astrocytes labeled with anti-NeuN and anti-GFAP antibodies in control (A) and streptosotocine-treated (B) rats (day 14). Scale bar: 50 μm

In our study we investigated early signs that point at the degenerative changes. Diabetes was provoked by streptozotocin injection and the important question was: whether this substance can lead to neuronal damage. To investigate the toxic effect on neuronal cells we incubated cultured hippocampal slices in 0.2 μM/ml streptozotocin in culture medium for 30 min. The necrotic cells were identified with propidium iodide. We hadn't found necrotic cells in cultured hippocampal slices. That methodical approach allows us to suppose, that changes in hippocampal structure during early stages of diabetes development emerges due to specific diabetes-related neuroendocrine changes, but the question about indirect toxic effect remains unsettled.

It is considered that the main causal factor for neuronal injury can be the high level of glucocorticoids. The level of these hormones is controlled by hypothalamo-pituitary-adrenal axis which is chronically hyperactivated in diabetic patients. Circulating glucocorticoids can interact with hippocampal glucocorticoid receptors in CA2 and CA1 areas leading to neuronal injury and death [20]. The most early changes of CA2 neurons as compared to CA1 probably can be explained by a constitutive deficiency of NO synthesis at CA2 area [12] responsible for increasing neuronal vulnerability.

The analysis of the number of astrocytes had shown the early appearance of hippocampal astrocyte reaction what can be possible related to a hyperglycemia and neurodegenerative events [9]. As previously described, hyperglycemia exerts a powerful stimulatory effect on hippocampal astrocytes [18]. We found, that the number of astrocytes is highly increased with prominent changes happening in pyramidal cell layer. The astrocytes around this area were hypertrophic, had many branched processes. They were tightly interwoven, contacted one another and surrounded pyramidal neurons. Composing the cellular web, activated astrocytes form glial scar which is considered as an ambiguous event [7]. Beneficial role of glial scar was supported by the data illustrating that reactive astrocytes had a neuroprotective role during metabolic insults, stress and injury by secreting growth factors and removing neurotoxins [11]. Nevertheless, adverse effect of glial scar is rather potent: astrocytes produce substancies that have been shown to be inhibitory to axon regeneration [2, 14, 19]. Dense web, formed by astrocytic prosesses, inhibits the migration of olygodendrocyte precursors and Schwann cells and restricts their myelinating activity [5]. The exact role of glial scarring depends on the size of the lesion and intensity of the astrocytes activation [6]. In our study we showed, that the astrocytes count increased considerably and morphology of these cells changed significantly in a hypertrophic manner. It allows us to suggest, that glial activation in hippocampus during the first two weeks of diabetes development has an inhibitory properties.

On the basis of the obtained data two main conclusions can be made: (1) the early stages of STZ diabetes development (starting from day 3) are associated with significant neuronal loss and gliosis; (2) neuronal and glial alterations during first two weeks of diabetes development are characterized by a diverse dynamics in separate hippocampal fields with most early and profound changes taking place in CA2 area; astroglial reaction seemingly display regeneration-inhibiting properties.

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REMODELING OF AMMON'S HORN DURING THE FIRST TWO WEEKS OF EXPERIMENTAL DIABETES DEVELOPMENT

It is known that long-term diabetes mellitus causes hippocampal dysfunction, however, early events leading to diabetes-related impairments of hippocampal tissue remain obscure. The present study was performed to examine temporal and spatial patterns of neuronal damage and astrogliosis in hippocampal CA1-C3 areas during the early stage of streptozotocin-induced diabetes in rats. NeuN and GFAP immunohistochemistry was used to visualize neurons and glial cells. Immunopositive cells were counted in hippocampal CA1-CA3 areas at days 3, 7 and 14 of diabetes development using confocal Olympus FV1000 microscope. Significant decrease in the number of neurons in CA2 area was observed in diabetic rats at day 3. In contrast, in CA1 and CA3 areas NeuN-positive cell count started to decrease later being at day 7, correspondingly, by 7 and 9 % lower than that in the control. This trend developed further till day 14, when the number of neurons in CA1 and CA3 areas was, respectively, 20.3 and 18.1 % smaller as compared with the control. These changes were accompanied by astrogliosis: the number of astrocytes in pyramidal cell layer was increased significantly in all examined time-points. Thus, our study demonstrates that streptozotocin-induced diabetes is associated with early neurodegeneration in Ammon's horn. It suggests that clinically relevant cognitive deficits development in diabetic patients starting from the early stage of the disease.

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