

Original Article

POSSIBLE INVOLVEMENT OF SPINAL GLIAL CELLS AND GLUTAMATERGIC TRANSMISSION IN STREPTOZOTOCIN-INDUCED NEUROPATHIC PAIN IN RATS

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Summary

Recently, evidence has accumulated that supports a role of spinal microglia in chronic pain, especially the facilitation of neuropathic pain. Astrocytes activate neuronal sensitization not only through inflammatory cytokines, but also more directly by release of glutamate, suggesting this target for new pain therapies. Therefore, in this study we tried to examine the astroglia in the spinal cord of streptozotocin-induced diabetic rats and the ability of NMDA-receptor blockers to suppress allodynia and hyperalgesia in them. Diabetes was induced in adult male Wistar rats by a single i.p. injection of streptozotocin (70 mg/kg; plasma glucose concentrations  $\geq 15$  mmol/l). The acute analgesic effects of ketamine (5, 10 and 30 mg/kg, i.p.), MK-801 (0.1 mg/kg, i.p.) were examined. Paw pressure and von Frey tests were used to measure nociceptive thresholds. Light and electron microscopy were applied to reveal the glia in spinal cord. Ketamine was effective against mechanical allodynia. Thermal and mechanical hyperalgesia were reversed in dose-dependent manner. MK-801 possessed a pronounced activity against mechanical allodynia. Astrocytes were found to be more numerous than in controls both electron microscopically and by immunofluorescent histochemistry. Possibly astroglia is involved in the neuropathic pain and NMDA-receptor blockers alleviated it in streptozotocin-induced diabetic rats.

Key words: streptozotocin-induced diabetes, spinal cord, glial cells, NMDA-receptor blockers, rat.

Introduction

The mechanisms of neuropathic pain remain poorly understood and the current therapies have limited efficacy. Painful neuropathy is one of the most common complication of diabetes. Chronically elevated blood glucose in diabetes is associated with degenerative abnormalities in axonal nerve fibers [1]. A growing body of evidence indicates that spinal microglia has causal roles in pain hypersensitivity following nerve injury [2] and neuropathic pain behavior [3]. However, the role of glia in the cellular mechanisms underlying the symptoms of neuropathic pain, such as hyperalgesia or allodynia, is not clear [4]. Astrocytes represent the most abundant cell type in the central nervous system. They provide structural, trophic, and metabolic support to neurons and modulate synaptic activity. Astrocyte functions that are known to influence neuronal survival include glutamate uptake, glutamate release, free

radical scavenging, and the production of cytokines and nitric oxide [5]. However, whether prolonged reactive astrocytic response is beneficial to neuronal recovery remains controversial [6]. Recent studies have found functional NMDA receptors in brain macroglia, in astrocytes, and oligodendrocytes. Glial and neuronal NMDA receptors are functionally and structurally different. The discovery of glial NMDA receptors further indicates the complex nature of intercellular signaling mechanisms in the brain, which involve all types of neural cells, connected through diverse types of chemical and electrical synapses [7]. Astrocytes activate neuronal sensitization not only through inflammatory cytokines, but also more directly by release of glutamate, suggesting this target for new pain therapies [8]. It has been suggested that NMDA-channel blockers could function as “gatekeepers” and effectively suppress the otherwise highly treatment-resistant neuropathic pain resulting from diabetic neuropathy [9, 10].

We examined the effects of streptozotocin (STZ)-induced diabetes in rats on the level of the glial fibrillary acidic protein (GFAP), number of astrocytes in the spinal cord and ability of NMDA-channel blockers ketamine and dizocilpine (MK-801) to suppress allodynia and hyperalgesia.

## Materials and Methods

Adult male Wistar rats (250-300 g) were used in all experiments. Rats were housed in standard laboratory condition with free access to food and water. Behavioral testing and injections were performed during the light cycle.

All procedures were approved by the Ethics Committee of Medical University, Sofia. Diabetes was induced with an intraperitoneal (i.p.) injection of 70 mg/kg STZ (Sigma, UK). Blood glucose levels were measured 5 days after STZ injection and levels higher than 15 mM/l were considered to be diabetic.

### *Behavioural testing*

Tactile allodynia was measured by assessing rat hind paw withdrawal thresholds in response to mechanical stimulation (von Frey test), using a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). Briefly, each animal was placed in a clear

acrylic cubicle (22 x 16.5 x 14 cm) with a metal grid floor giving access to the underside of their paws and allowed to acclimate for 10 min before testing. A mechanical stimulus was applied to the plantar surface of one hind paw by a stainless steel filament (0.5mm diameter) exerting a linearly increasing force (2.5g/s). Each rat paw withdrawal threshold was calculated as the average of three consecutive tests performed at 5 min intervals. A cut-off of 50g was imposed.

Mechanical hyperalgesia was measured as the threshold of response to increasing pressure by the paw-pressure test, using an analgesimeter (Ugo Basile, Italy). A constantly increasing pressure was applied to the dorsal surface of right hind paw of the rat to determine the minimum stimulus necessary to evoke an obvious nociceptive response (sharp paw withdrawal). The nociceptive threshold is determined as the force, in grams. A 500-g cut off value was used to prevent tissue damage.

### *Pharmacological treatment*

The effects of ketamine (5, 10 and 30 mg/kg, i.p.), MK-801 (Sigma; 0.1 mg/kg, i.p.) were examined.

### *Light and electron microscopy*

Rats were deeply anesthetized with intraperitoneal injection of thiopental (40 mg/kg) and transcardially perfused through the ascending aorta by a freshly prepared fixative containing 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4. Subsequently the vertebral canal was opened and the spinal cord was thoroughly exposed. Small tissue pieces from its lumbar segments were postfixed overnight in the same fixative. They were then osmicated with 1% OsO<sub>4</sub> in phosphate buffer for 1 hour, dehydrated in graded series of ethanol and embedded in Durcupan (Fluka, Buchs, Switzerland). Thin sections were cut with an ultramicrotome (LKB, Stockholm-Bromma, Sweden), counterstained with uranyl acetate and lead citrate and examined with a Hitachi H-500 electron microscope (Hitachi, Tokyo, Japan). Additionally, semithin sections were cut and stained with methylene blue. Their observation was carried out under a light microscope (Nikon, Tokyo, Japan). Glial cells were identified according their morphology [11] and counted by using an Olympus image analyzer (Olympus, Tokyo, Japan) and a 40x objective.

Transversal sections of the lumbar segments of the spinal cord were immunohistochemically

stained to determine glial fibrillary acidic protein (GFAP) expression. Spinal cords slides (30 μm) were incubated overnight at 4°C with a GFAP (astrocyte marker, rabbit polyclonal, 1:200; Santa Cruz Biotechnology) antibody, followed by a goat anti-rabbit IgG-FITC secondary antibody (Santa Cruz Biotechnology) for 1 h at RT. Nonspecific staining was determined by excluding the primary antibodies. Images were captured using a microscope (Nikon, Tokyo, Japan) equipped for epifluorescence.

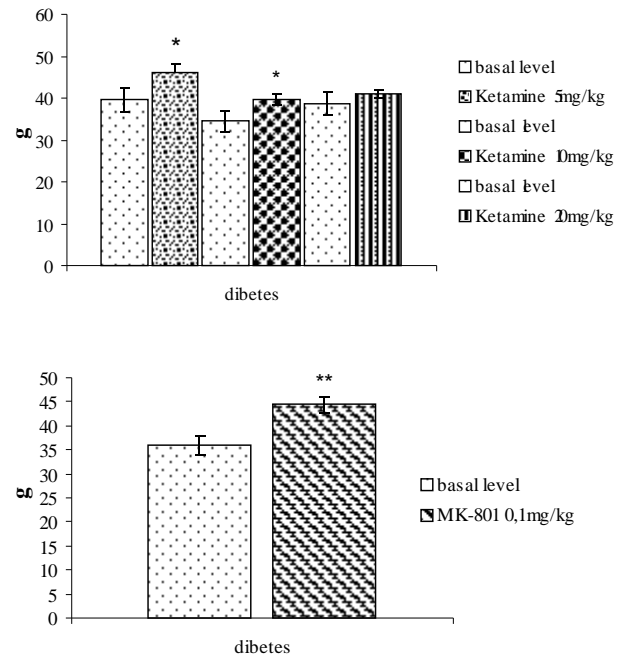
**Statistical analysis**

The data are expressed as means ± SEM. The obtained data were evaluated by the one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test; p < 0.05 was considered significant.

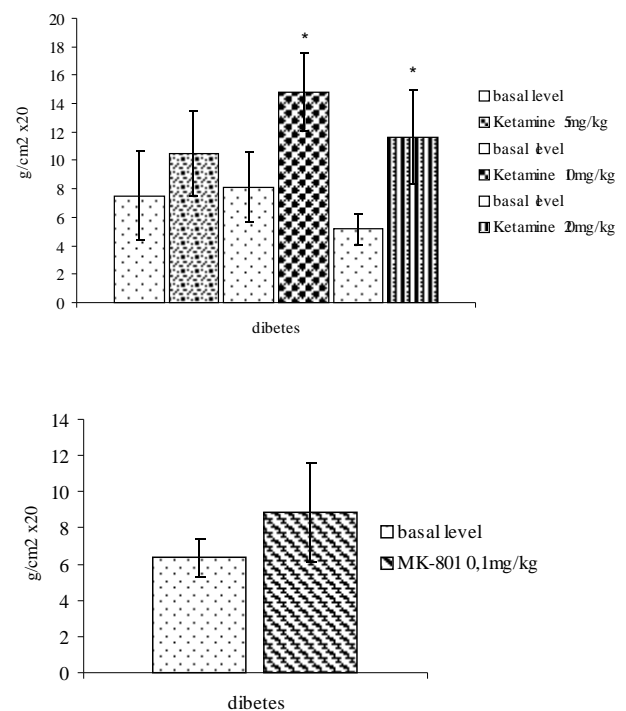
**Results**

Diabetic neuropathy developed in 3 weeks after treatment with streptozotocin (mechanical hyperalgesia and allodynia). Ketamine was effective against mechanical allodynia at 5 mg/kg (39.5±3 basal level before treatment - 46.1±1.8; p < 0.5 after treatment) and 10 mg/kg (34.5±2.6 - 39.7±1.3; p < 0.5) (Fig.1). Analgesic activity decreased with further increase of dose 20 and 30 mg/kg). MK 801 also demonstrated pronounced antialodynic effect (p < 0.01; Fig. 1). Mechanical hyperalgesia was reversed in dose-dependent manner by ketamine with significant effects at intermediate (10 mg/kg) and low (5 mg/kg) doses (Fig.2). MK 801 displayed significant activity against mechanical allodynia but no significant alleviation of mechanical hyperalgesia was found at 0.1 mg/kg (Fig.2).

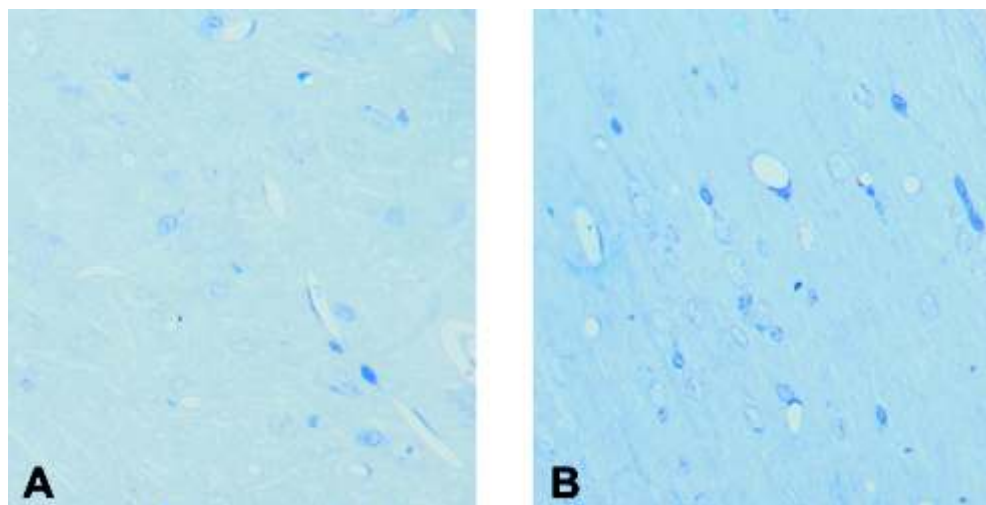
Under electron microscope spinal cords of both control and diabetic rats show the same types of glial cells astroglia, oligodendroglia and microglia. When comparing cells of each of these glial types no qualitative differences are observed between control and diabetic rats. However, the overall impression is that the astrocytes are more pronounced in diabetic spinal cord tissue than in the one of control rats (Fig. 3). To test this hypothesis semithin sections were analyzed by means of an image analyzer. The results of this quantitative analysis are shown in Fig. 4. It is evident that the number of astroglial cells in the spinal cord of diabetic rats is nearly two times increased as compared to the control rats whereas the other glial cell types do not show statistically



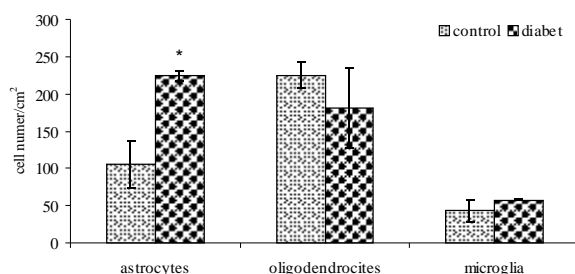
**Fig.1.** Effects of ketamine and MK-801 on tactile allodynia (von Frey test): \*p < 0.5; \*\*p < 0.01 vs. basal level; n=6-8.



**Fig.2.** Effects of ketamine and MK-801 on mechanical hyperalgesia (Paw pressure test): \*P < 0.5 vs. basal level; n=6-8.



**Fig. 3.** Semithin sections of the spinal cord of control (A) and diabetic (B) rats showing the increased number of astrocytes in the latter. x 40.



**Fig. 4.** Cell density of different glial cell types in the spinal cord of control and diabetic rats: \*  $P < 0.5$  vs. Control.

## Discussion

Notwithstanding that diabetes mellitus is the leading cause of peripheral neuropathy, the underlying mechanisms of development of the concomitant neuropathic pain are still poorly understood. Some evidence suggests the role of glial cells in neuropathic pain states [12]. Therefore, different investigations were centered on this topic. However, controversial results have been collected while some authors have found an enlarged number of astrocytes in diabetic brain structures [13, 14], others claim a reduction of this number [12, 15]. On the other hand, increased [14] or decreased [15] levels of GFAP in the astrocytes was pointed out in different papers. This is the reason why in the present study

we analyze quantitatively the number of astrocytes not on the basis of GFAP immunohistochemistry but on the basis of pure morphological criteria. Maybe this is a difficult task and very time consuming, but in our opinion such an approach can eliminate the errors based on different GFAP equipment of the astrocytes at different conditions. One of the advantages of the present study is the quantitative analysis of the density of different glial cell types in the spinal cord tissue of both diabetic and control rats. A principal finding of this work is the established statistically significant increase of the astrocyte density in spinal cord of diabetic rats as compared to control ones. Additionally, an increased content of GFAP in astrocytes of diabetic spinal cord was shown by immunofluorescence. The density of the other major glial cell types oligodendrocytes and microglia in the spinal cord does not show remarkable difference between diabetic and control animals.

In our study increased number of activated astrocytes correlates with behavioral manifestation of neuropathic pain. NMDA-receptor blockers (ketamine and MK 801) alleviate mechanical allodynia and hyperalgesia in streptozotocin-induced diabetes. NMDA receptor mediates central sensitization and neuropathic pain. A reversal of central sensitization by NMDA antagonists such as ketamine is believed to reduce pain. The discovery of glial NMDA receptors further indicates the complex nature of intercellular



signaling mechanisms in the brain, which involve all types of neural cells, connected through diverse types of chemical and electrical synapses [7].

## Conclusion

Our results demonstrate that STZ-induced diabetic neuropathy in rats is associated with increased number of astrocytes and activation of GFAP in the grey substance in spinal cord in comparison to non diabetic rats. Astrocyte activation could bidirectionally modulate synaptic transmission through activation of different glutamate receptors [16]. The data show that diabetes associated mechanical hyperalgesia and allodynia was alleviated by NMDA-receptor antagonists. They proved to be active against diabetes-induced neuropathic pain in streptozotocin-induced diabetes in rats.

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