Histol Histopathol (2014) 29: 649-658
DOI: 10.14670/HH-29.10.649

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Changes of calcium/calmodulin-dependent protein kinase II expression in dorsal root ganglia during maturation in long-term diabetes

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Sumamry. Calcium/calmodulin-dependent protein kinase II (CaMKII) is considered one of the key intracellular signaling proteins for development of neuropathy. We analyzed the expression of total CaMKII (tCaMKII) and its alpha, beta, gamma and delta isoforms in dorsal root ganglia (DRG) in a rat model of *Diabetes mellitus* type I (DM1), 6 months and 1 year after diabetes induction. Diabetes was induced with streptozotocin and confirmed by measuring glucose levels and weight increase. Immunohistochemistry was performed for detection of tCaMKII and its isoforms in L4 and L5 DRGs. A significant decrease of CaMKII alpha and beta isoforms was noted 6 months after diabetes induction, while CaMKII gamma and delta were significantly decreased after 12 months in diabetic rats compared to controls. Analysis of neuronal subgroups based on the neuronal diameter revealed that the expression of alpha, beta and delta isoforms decreased only in small-diameter neurons. In conclusion, a significant decrease of specific CaMKII isoforms in small-diameter DRG neurons may suggest involvement of CaMKII alpha, beta and delta in the development of complex events responsible for the development of neuropathy in long-term diabetes during maturation. CaMKII is a part of the neuronal pathway that regulates the firing properties of excitable cells, especially neurons, and decreased CaMKII activity may be responsible for generation of aberrant signals, hyperalgesia and neuropathic pain.

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Key words: Diabetes, CaMKII, Rats, Neuropathic pain, Dorsal root ganglion.

Introduction

Proper functioning of the nervous system relies on efficient transmission of impulses from neurons to their target cells. There are many receptors and membrane-bound proteins that regulate binding of transmitters, but there are also other proteins in neurons, particularly at synaptic junctions, which serve as critical regulators of neuronal transmission (Liu and Murray, 2012). Neuronal protein calcium/calmodulin protein-kinase II (CaMKII) has emerged as one of the key regulators of synaptic function, critical for regulating forms of synaptic plasticity, including long-term potentiation (Lisman, 2003).

CaMKII is a multifunctional serine/threonine kinase which is regulated by intracellular calcium concentrations. CaMKII has four distinct isoforms (alpha, beta, gamma and delta), encoded by four separate genes (Tobimatsu and Fujisawa, 1989). The concentration of free intracellular calcium is finely regulated by a large variety of different cellular mechanisms. Since prolonged calcium elevation generally promotes cell death, increased intracellular calcium is generally transient, encoding a variety of

Abbreviations: CaMKII, calcium/calmodulin-dependent protein kinase II; CaM, calmodulin; DM, *Diabetes mellitus*; IASP, International Association for the Study of Pain; tCaMKII, total calcium/calmodulin-dependent protein kinase II; CaM, calmodulin; STZ, streptozotocin; PBS, phosphate-buffered saline.

messages that are deciphered by a number of calcium-binding proteins such as calmodulin (CaM). The calcium/CaM complex binds to numerous target proteins, including CaMKII. When activated, CaMKII autophosphorylates and thus generates autonomous or calcium-independent activity that persists even after dissociation of calcium/CaM. In this way, a transient increase of calcium enables prolonged CaMKII activation (Wayman et al., 2011).

A growing number of studies demonstrate that CaMKII plays a key role in nociceptive transmission. CaMKII alpha is a major CaMKII isoform expressed in the brain, and it is preferentially located in pain-processing regions in the central nervous system, such as lamina II of the dorsal horn and the dorsal root ganglion (DRG) (Bruggemann et al., 2000). After inflammation of injuries to peripheral tissues, CaMKII is upregulated in the superficial laminae of the dorsal horn and DRG (Carlton, 2002; Fang et al., 2002). In the DRG, CaMKII alpha was more abundant in small DRG neurons, indicating that this subpopulation of neurons may be involved in the processing of nociceptive information (Carlton, 2002). It has been suggested that CaMKII modulates nociceptor activity and plasticity of primary sensory neurons, and participates in the development and maintenance of nociceptive hypersensitivity. Thus, it has been suggested as a novel target for treatment of chronic pain (Petrenko et al., 2003).

While CaMKII expression in DRG and its relation to increased nociceptive sensitivity was studied in several models of neuropathic pain, caused by injury (Kawano et al., 2009; Kojundzic et al., 2010) and inflammation (Carlton, 2002), its potential role in diabetic neuropathy has been suggested only recently (Ferhatovic et al., 2013a,b). Diabetic neuropathy is a common, debilitating disorder with prevalence of about 30% in patients with *Diabetes mellitus* (DM). Up to 50% of diabetic patients will eventually develop neuropathy. Neuropathic pain is one of the most important complications of diabetes that causes disabling symptoms in diabetic patients and severely affects their quality of life (Callaghan et al., 2012). Pain in diabetic neuropathy is characterized by burning, electric and stabbing sensations with or without numbness. Patients frequently develop painful sensations to innocuous stimuli (allodynia) or increased sensitivity to painful stimuli (hyperalgesia) (Daousi et al., 2004). However, little is known about the mechanisms involved in the development of neuropathic pain in diabetes and therefore treatment of diabetic neuropathy is unsatisfactory, including glucose control and non-specific medications such as anticonvulsants and antidepressants (Callaghan et al., 2012). It has been proposed that drugs modulating CaMKII could be utilized as analgesics (Kawano et al., 2009). For example, we already know that antidepressants activate CaMKII in neuron cell bodies, which could be one of the reasons why antidepressants may be partly effective for the treatment of neuropathic pain (Tiraboschi et al., 2004).

Our preliminary results have shown that CaMKII expression increased in diabetic rats 2 weeks and 2 months after induction of diabetes, and that the changes in CaMKII expression were associated with pain-related behavior (Ferhatovic et al., 2013a,b). Since diabetes is a chronic disease, the aim of this study was to analyze the expression of CaMKII in diabetic rats 6 months and 1 year after diabetes induction.

Materials and methods

Animals

A total of 45 male Sprague-Dawley rats (160-200 g) were included in the study. All rats were raised under controlled conditions (temperature 22±1°C, light schedule: 12 h light and 12 h dark) at the University of Split Animal Facility. Animals were reared for 6 and 12 months in pairs in plastic cages with sawdust and corn flooring in the ratio 3:1 and wire mesh floor. One rat was considered non-diabetic because of a glucose level lower than 300 mg/dl. Two diabetic rats died before the end of the experiment. The number of remaining rats was 26 in the diabetic group and 16 in the control group. Animals were studied for 6 and 12 months.

Rats were assigned into four experimental groups: 6-months DM (N=13) and its control group (N=8), 1-year DM (N=13) and its control group (N=8). Diabetic rats received one unit of long-acting insulin (Lantus Solostar, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany) weekly to prevent ketoacidosis.

Ethics

The Ethical Committee of the University of Split, School of Medicine approved the study. Experimental procedures and protocols followed the International Association for the Study of Pain (IASP) Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals and the European Communities Council Directive of 24 November 1968 (86/609/EEC).

Diabetes induction and validation

Rats were injected with 55 mg/kg of streptozotocin (STZ) dissolved in freshly prepared citrate buffer (pH=4.5) after an overnight fasting. Control group was injected i.p. with pure citrate buffer solution. Both groups were fed *ad libitum* with normal laboratory chow (4RF21 GLP, Mucedola srl, Settimo Milanese, Italy). Plasma glucose was measured with a glucometer (OneTouchVITa, LifeScan, High Wycombe, UK).

Tissue collection and immunohistochemistry

Rats were sacrificed six and twelve months after diabetes induction under anesthetic exposure (Isoflurane; Forane, Abbott Laboratories Ltd, Maidenhead, Berks., UK). The left and right L4 and L5 ganglia were removed

and postfixed for two hours in Zamboni's fixative (4% paraformaldehyde and 15% picric acid in 0.1 M phosphate-buffered saline (PBS)) at pH 7.4. Following overnight cryoprotection in 30% sucrose, ganglia were embedded in Optimal Cutting Temperature freezing medium (Tissue Tek, Tokyo, Japan). Sections 7 μm thick were cut parallel along the long axis of the ganglia on a cryostat (Thermo Shandon Cryotome, Pittsburgh, PA, USA) and placed on glass slides.

The rat hind paw is mainly innervated by the sciatic nerve and usualy serves as a model system for investigations of changes that affect peripheral and autonomic nerves. The sciatic nerve has major contribution from L4 and L5 (Asato et al., 2000).

Immunohistochemical analysis was performed for detection of total CaMKII (tCaMKII) and its alpha, beta, gamma and delta isoforms. Primary rabbit polyclonal antibodies were used in dilution of 1:100 for detection of tCaMKII (sc-9035, lot# F0304, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphorylated CaMKII alpha isoform (sc-12886-R, lot# K2305, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and for CaMKII beta (ab34703, Abcam, Cambridge, UK) at a dilution of 1:300. Antibodies raised in goat were used at dilution of 1:100 for detection of CaMKII gamma (sc-1541, lot# G2208, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and CaMKII delta (sc-5392, lot# H1408, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Incubation was done overnight at room temperature and 0.3% Triton X-100 (648466, lot# B65241, Merck KGaA, Darmstadt, Germany) in PBS was used for rinsing.

Secondary detection of total and alpha and beta isoform was performed using secondary antibody with Texas red (Goat Anti-Rabbit IgG-B (Santa Cruz, sc-2780) (dilution 1:100). Primary antibodies for CaMKII gamma and CaMKII delta were detected with secondary Alexa Fluor labeled donkey anti-goat polyclonal secondary antibody (ab150139, Abcam, Cambridge, UK, wavelength 647 nm), diluted 1:300. The incubation period for secondary antibodies was 90 minutes. After final rinsing in PBS, all slides were mounted, air-dried, and cover slipped (Immu-Mount, Shandon, Pittsburgh, PA, USA).

Staining controls included omission of primary antibody from the staining procedure, which resulted in staining of DRG tissue. Long-term breeding of animals resulted in accumulation of fluorescent lipid lipofuscin in neurons which was visible through all microscope filters and on non-stained sections (Sulzer et al., 2008). Because these lipids accumulate with aging, it can be assumed that rats belonging to age-matched experimental groups in this study gained a similar quantity of these lipids. The experiment was continued on the assumption that autofluorescence of sections would not interfere with the analysis of differences between age-matched experimental groups.

Further analysis was done on three separate diameter size groups: small (d≤30 μm), medium (30μm≤d≤40μm) and large (d≥40 μm) cells (Craner et al., 2002).

Quantitative analysis for immunohistochemistry

Every fourth section of each DRG was examined with a microscope (BX61, Olympus, Tokyo, Japan).We took 5 microphotographs per section and in total we analyzed 5 sections per animal. Microphotographs were taken with a cooled digital camera (DP71, Olympus, Tokyo, Japan) under the same magnification (40x objective), exposition, binning and gain for each image. Measurements were performed using Image J (National Institutes of Health, Bethesda, MD, USA) where they were examined as monochromic microphotographs (2040x1536 pixels, 12 bits, 0-4096 grey scale), following background subtraction. Only the neurons with clearly visible nuclei were analyzed. Fluorescence intensity of neuronal cytoplasm and surface area of each neuron was measured. The diameter of each DRG cell was calculated using formula: diameter = (length + width) / 2. We analyzed a minimum of 150 cells per animal in an effort to make our analysis as objective as possible.

Statistical analysis

Average values for each animal have been calculated and entered into the final analysis between groups. Comparisons between control and diabetic neuronal groups were made by Student t-test (Statistica 7.0; StatSoft, Tulsa, OK, USA). The data were presented as mean and standard deviation (M±SD). Any difference with $p<0.05$ was considered statistically significant.

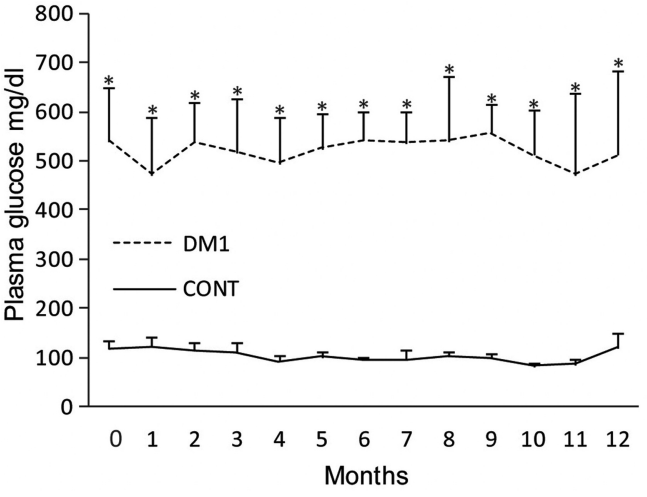

Fig. 1. Plasma glucose levels in diabetic and control rats. Data are presented as mean ± SD Asterisk denotes significant difference ($p<0.05$) from respective controls (t-test).

Results

Validation of diabetes

The concentration of plasma glucose during the 12-month follow-up was significantly higher in diabetic rats, compared to control animals, throughout the 12-month experiment (Fig. 1). The average plasma glucose levels in diabetic and control rats were 511.8±101.0 and 103.5±14.0 mg/dl, respectively, during the entire experiment.

Body mass gain in diabetic rats was significantly reduced following diabetes induction in comparison to their respective controls (Fig. 2). The average body mass increase of diabetic and control rats was 67.3±27.9 and 218.9±106.0% after 6 months, and 95.6±41.0 and 259.0±48.0% after 12 months, respectively.

CaMKII expression in DRG neurons after 6 months

Six months after diabetes induction, comparison of expression of total CaMKII (tCaMKII) and all of its analyzed isoforms in all DRG neurons of diabetic and control rats revealed that there was no significant difference between the two groups in the expression of tCaMKII (p=0.994), CaMKII gamma (p=0.309) and CaMKII delta (p=0.092). Significantly lower expression of phosphorylated CaMKII alpha (p<0.001) and CaMKII beta (p<0.001) was found in DRGs of diabetic rats compared to control rats (Figs. 3, 4). Analysis based on the diameter of DRG cells revealed no difference in tCaMKII, CaMKII gamma and CaMKII delta in small-, medium- and large-diameter neurons of diabetic and control rats. Also, there was no difference in CaMKII alpha and beta in medium- and large-diameter neurons (data not shown). However, significantly lower levels of CaMKII alpha (19.72±7.31 vs. 24.71±13.25; p<0.001) and CaMKII beta (17.72±5.89 vs. 21.37±11.82; p<0.001) were found in small-diameter neurons of diabetic rats compared to control rats.

CaMKII expression in DRG neurons after 12 months

After 12 months of diabetes, analysis of all DRG neurons revealed that there was no significant difference in the expression of tCaMKII (p=0.47), phosphorylated CaMKII alpha (p=0.120) and CaMKII beta (p=0.620) between diabetic and control rats. However, the expression of CaMKII gamma (p=0.048) and CaMKII delta (p=0.026) was significantly lower in diabetic DRGs compared to primary sensory neurons of control rats (Figs. 5 and 6). When the expression of CaMKII and its isoforms was analyzed in different subsets of neurons, the only difference was found for CaMKII delta in small-diameter neurons (26.15±12.76 vs. 22.40±10.52; p=0.018), while the expression of tCaMKII and all the other isoforms did not differ in DRGs of diabetic and control rats.

To consider age-dependence, data from 6 and 12 months were compared. A significant increase was observed in tCaMKII, CaMKII beta and delta isoforms in both control and diabetic rats (p<0.05 for all). A significant decrease was observed in CaMKII alpha and gamma isoforms in both control and diabetic rats (p<0.01 for all).

Discussion

In this study we found temporal differences in the expression of alpha, beta, gamma and delta isoforms of

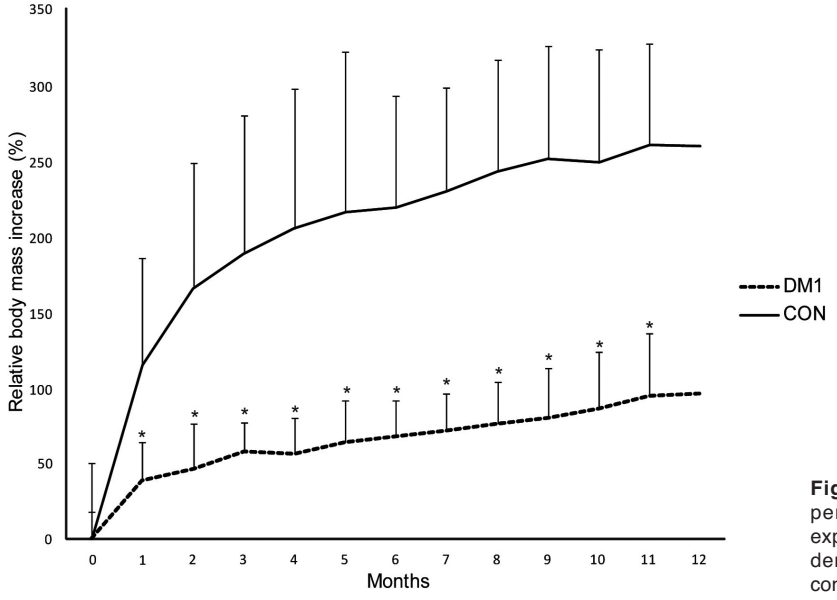

Fig. 2. Relative body mass increase (expressed as a percentage of the body mass at the beginning of the experiment). Data are presented as mean ± SD. Asterisk denotes significant difference (p<0.05) from respective controls (t-test).

CaMKII during maturation in long-term diabetes, indicating that all CaMKII isoforms may be involved in the development of diabetic neuropathy.

Chronic neuropathic pain remains refractory for therapeutic interventions and the underlying mechanisms have remained largely unknown. It has been suggested that a novel approach should include targeting key intracellular signaling proteins that are known to contribute to continued activation by phosphorylation of kinases, transcription factors and receptors that contribute to changes in membrane excitability (Crown et al., 2012). CaMKII is one such signaling kinase, shown to be critical in maintaining aberrant dorsal horn hyperexcitability. Crown et al suggested that persistent CaMKII activation contributed to chronic central neuropathic pain by mechanisms that involve maintained hyperexcitability of wide dynamic range dorsal horn neurons. Experiments in their study were conducted 35 days after spinal cord injury (Crown et al., 2012). Taking the data on age rate conversion from the age of rats to age of humans into consideration, 35 days would be approximately 3 human years, which would indeed signify chronic pain (Sengupta, 2011).

In the present study, STZ-diabetic rats were followed for 6 and 12 months after diabetes induction. In our previous study, 2 weeks after diabetes induction the expression of total CaMKII increased in DRGs of rats with diabetes type 1, compared to control rats. After 2 months of diabetes, a significant difference between total CaMKII expression was still present, but the values of tCaMKII expression were reduced tri-fold, compared to the 2-week experiment (Ferhatovic et al., 2013a). Therefore, the results in the present study are not surprising, considering the decreasing trend of tCaMKII expression in our previous experiments.

CaMKII has been extensively studied in the spinal cord dorsal horn of animal pain models, but there are few reports about its expression in DRGs of animals with neuropathic pain. DRG contains primary sensory neurons and initial pathological changes in the DRG are the main triggers for neuropathic pain (Sapunar et al., 2012), and it is an important target for studying neuropathies and developing targeted therapies (Puljak et al., 2009; Sapunar et al., 2011).

Previous findings of the expression of CaMKII in DRGs after the onset of neuropathic pain are conflicting. Two groups have independently described a decrease of CaMKII expression after injury-induced neuropathic pain. In the first of those studies, CaMKII was studied in large-diameter DRG neurons between 17 and 28 days after spinal nerve ligation (SNL) surgery, and pCaMKII expression was lower in SNL animals. Additionally, activation of ATP-sensitive potassium (KATP) channels was suppressed by axotomy that results in hyperexcitability (Kawano et al., 2009). K-ATP channels regulate firing properties in excitable cells, such as neurons. CaMKII enhances basal K-ATP channel opening, as well as the channel activation evoked by calcium. Specifically, inhibition of K-ATP channels in DRG neurons leads to depolarization, aberrant firing, and increased neurotransmission. Because calcium/calmodulin/CaMKII signaling regulates K-ATP channel opening, deficiency of this pathway may contribute to hyperalgesia and neuropathic pain (Kawano et al., 2009).

In the second study, an SNL model was also used, and it was found that reduction of phosphorylated alpha CaMKII isoform 2 weeks after surgery is due to the loss of IB4-positive neurons, indicating that reduced afferent activity after axotomy may lead to decreased phosphorylation of CaMKII (Kojundzic et al., 2010).

These studies indicate that changes in the calcium/calmodulin/CaMKII signaling are induced with axotomy. The changes may be mediated by interruption of sensory activity, loss of normal afferent neuronal traffic, loss of depolarization events that decreases influx of calcium through voltage-gated calcium channels and diminished release from intracellular stores (Fuchs et al., 2005, 2007). The reduction of basal calcium currents and diminished release of calcium ions from intracellular stores after nerve injury resulted in the loss of CaMKII activity (Griffith, 2004).

Conversely, in a model of inflammatory pain, a significant increase of CaMKII alpha in unmyelinated and myelinated axons of DRG neurons was reported within 48 h after inflammation. The same study demonstrated that CaMKII is transported from the neuron body into both the peripheral and central processes of DRG cells (Carlton, 2002).

Since CaMKII has been implicated as a possible target for treatment of neuropathic pain, inhibition of CaMKII was tried in multiple studies, also with

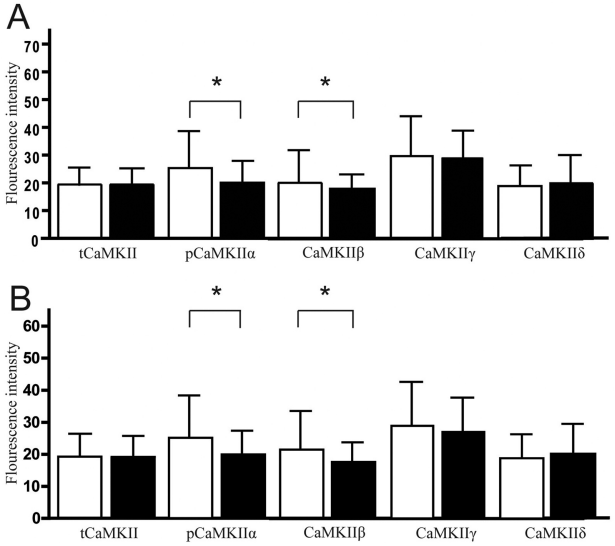

Fig. 3. Fluorescence intensity of CaMKII and its isoforms in 6-month rats for all neurons **(A)** and small-diameter neurons **(B)**. Diabetic rats are represented with gray bars and controls with white bars. Data are presented as mean ± SD. Asterisk denotes significant difference ($p<0.05$) from respective controls (t-test).

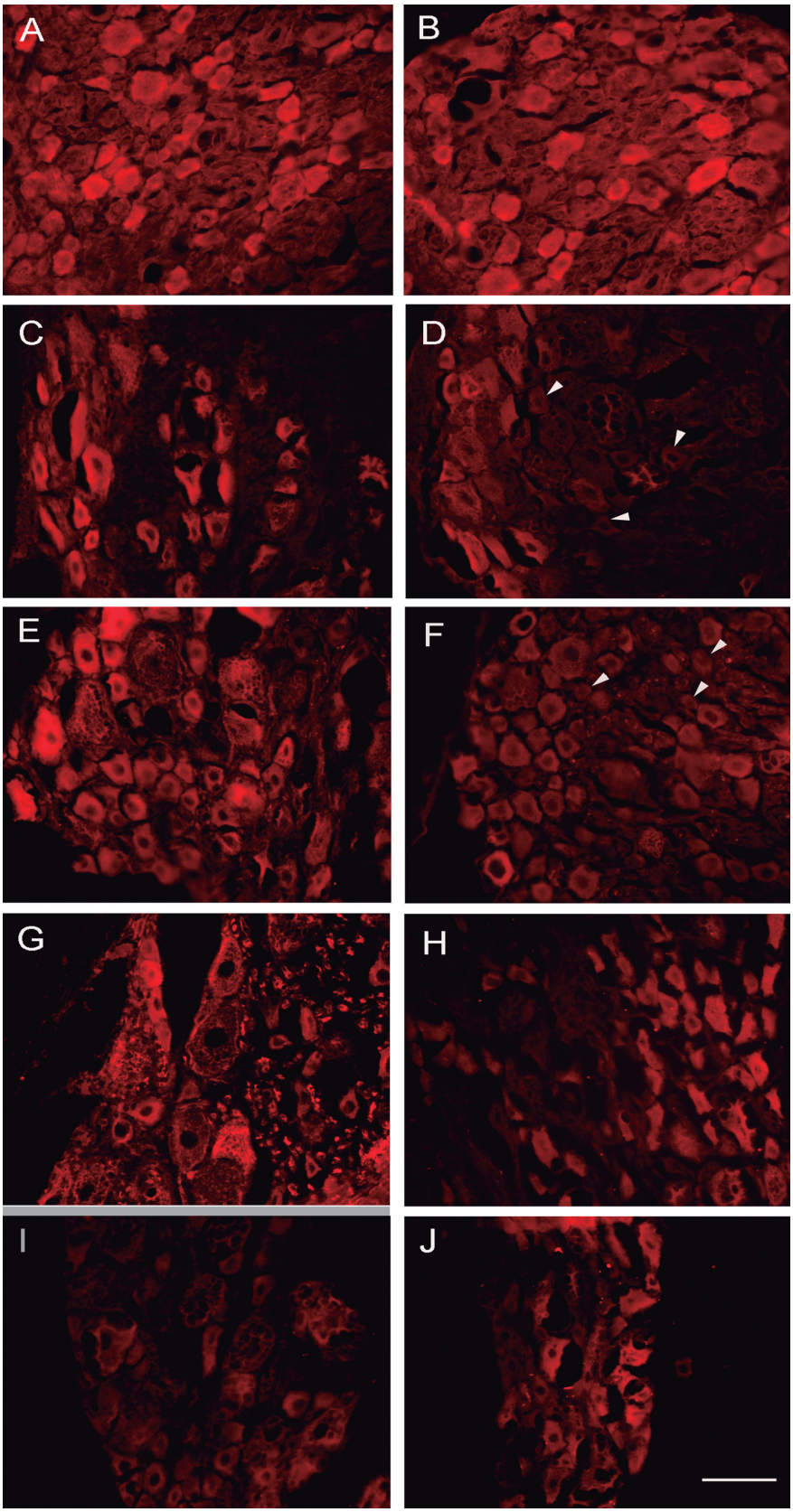

Fig. 4. Representative images of staining for CaMKII and its isoforms in diabetic and control rats at 6 months: tCaMKII in control **(A)** and diabetic rats **(B)**, alpha CaMKII in control **(C)** and diabetic rats **(D)**, beta CaMKII in control **(E)** and diabetic rats **(F)**, gamma CaMKII in control **(G)** and diabetic rats **(H)** and delta CaMKII in control **(I)** and diabetic rats **(J)**. White arrows point to the neurons where significant decrease of fluorescence was observed compared to controls. Scale bar: 100 μm (applies to all).

conflicting results. When CaMKII inhibitor KN93 was given to animals with chronic constriction injury (CCI), it was found that CaMKII inhibitor may reduce pain-related behavior given before the induction of neuropathic pain, but not if it is applied after the onset of pain-related behavior (Dai et al., 2005). Another study showed that CaMKII inhibitor can reverse already induced pain with CCI, but the exact details were not provided (Garry et al., 2003). A subsequent study showed that KN93 was effective in reversing established inflammatory pain in mice (Luo et al., 2008). Acute administration of KN-93 strongly reversed the already established oxaliplatin-induced mechanical allodynia in von Frey test, while it did not affect the oxaliplatin-induced cold hyperalgesia in acetone test (Shirahama et al., 2012). Therefore, although many studies implicate CaMKII in the development of neuropathic pain, its exact role and the value of CaMKII inhibitors as therapeutic agents is still unclear. These seemingly contradictory results could be explained with differential effects of CaMKII if activated in the peripheral versus central nervous system. It has been suggested that CaMKII has multiple roles, by acting directly on effector ion channels, such as TRPV1, and it also regulates the sensitization signaling network, which is involved in the computation of sensitizing inputs and which is initiated mainly by G-protein coupled receptors (Hucho et al., 2012).

Neuropathic pain, by definition, involves damage of the nervous system (Jensen et al., 2011). Peripheral sensitization, observed after nerve injury, stems from reduced threshold for activation of transducer channels, and the best characterized among them is the non-selective cation channel vanilloid receptor transient receptor potential vanilloid subtype 1 (TRPV1), with a well established role in peripheral inflammatory pain (Huang et al., 2006). TRPV1 activation induces influx of calcium ions into neurons, leading to activation of Ca^{2+}-mediated signal transduction, including activation of CaMKII (Means, 2000). CaMK activates the transcription factor cAMP response element-binding protein (CREB) via phosphorylation of Ser-133 (Mayr and Montminy, 2001). Activated CREB leads to target gene expression and regulates various neuronal functions (Lonze and Ginty, 2002).

Changes in the expression of TRPV1 correlate with the development of thermal hyper- and hypoalgesia in early diabetic neuropathy, while altered TRPV1 expression and function contribute to diabetes-induced changes in thermal perception (Hong and Wiley, 2005; Pabbidi et al., 2008). Intraperitoneal treatment with TRPV1 blocker capsaicin reduced glycemia in type 1 diabetic mice (Radu et al., 2013). Also, the reappearance of the capsaicin-induced biting and licking behaviors was reported in diabetic mice after neonatal capsaicin treatment, suggesting the restoration of TRPV1 function (Rashid et al., 2003). All this information provides support for the hypothesis that CaMKII could be involved in the mechanisms of diabetes-induced neuropathic pain, but they also show the complexity of plastic changes of the nervous system affected by neuropathic changes. Pathogenesis of neuropathic pain is not only confined to the changes in neurons, but also depends on interactions between neurons and immune cells, such as resident macrophages, which exhibit different activation mechanisms, depending on the type of DRG lesion (Ton et al., 2013). Alterations of acid-sensing ion channels (ASICs) from DRGs, such as proton sensors during ischemia and inflammation, have also been implicated in the genesis of neuropathic pain in a DM1 model (Radu et al., 2014).

While there are numerous studies in which mechanisms of neuropathic pain have been studied in early diabetic neuropathy, long-term studies in animal models of diabetes are rare. Since diabetes is a chronic disease, short-term studies may not reveal a clear picture and provide guidance for pharmacological interventions. While our earlier study has shown a significant increase of CaMKII expression in animals with diabetes type 1 and control animals (Ferhatovic et al., 2013a), such a difference was not seen 6 months or 12 months after induction of diabetes in the present study. Furthermore, a decrease of alpha and beta CaMKII isoforms was observed after 6 months. It is possible that the mechanisms of chronic pain in the diabetic neuropathic model have something in common with acute changes observed after injury-induced neuropathic pain in rodents (Kawano et al., 2009; Kojundzic et al., 2010).

Moreover, we investigated the expression of all four isoforms of CaMKII, together with the expression of total CaMKII. Mostly, in the context of neuropathic pain, only the role of tCaMKII and alpha CaMKII

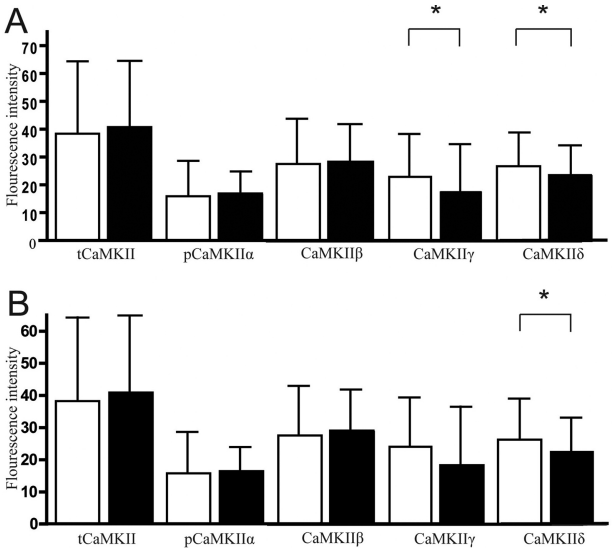

Fig. 5. Fluorescence intensity of CaMKII and its isoforms in 12-month rats for all neurons **(A)** and small-diameter neurons **(B)**. Diabetic rats are represented with gray bars and controls with white bars. Data are presented as mean ± SD. Asterisk denotes significant difference ($p<0.05$) from respective controls (t-test).

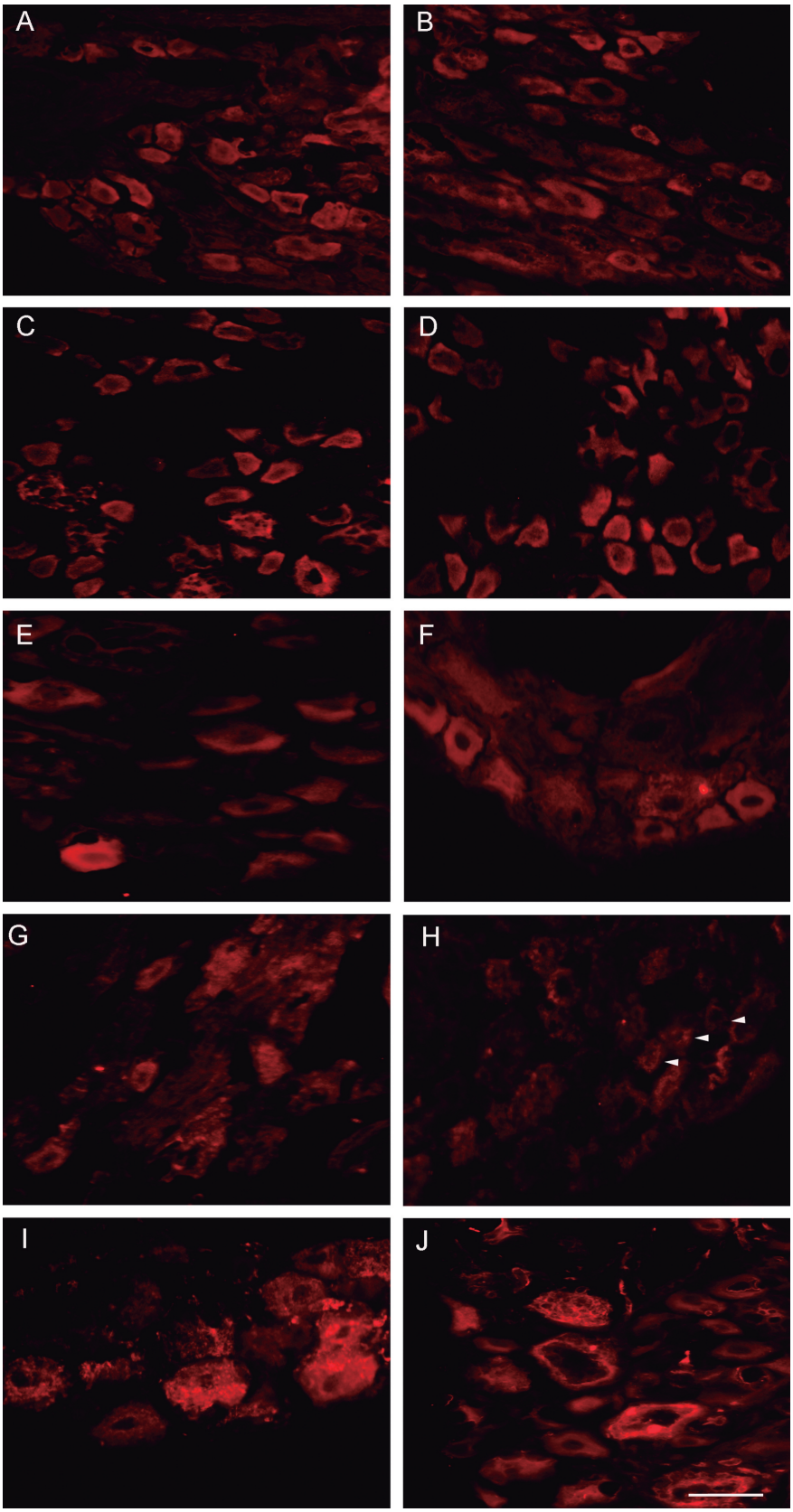

Fig. 6. Representative images of staining for CaMKII and its isoforms in diabetic and control rats at 12 months: tCaMKII in control **(A)** and diabetic rats **(B)**, alpha CaMKII in control **(C)** and diabetic rats **(D)**, beta CaMKII in control **(E)** and diabetic rats **(F)**, gamma CaMKII in control **(G)** and diabetic rats **(H)** and delta CaMKII in control **(I)** and diabetic rats **(J)**. White arrows point to the neurons where significant decrease of fluorescence was observed compared to controls. Scale bar: 100 μm (applies to all).

isoform is studied, and here we show that other CaMKII isoforms may be involved in the mechanisms of chronic neuropathic pain. Apart from the alpha isoform, the beta isoform was also significantly decreased after 6 months, while delta and gamma isoforms were significantly lower in diabetic animals, compared to controls, after 12 months of diabetes in all DRG neurons.

Significant changes in alpha, beta, gamma and delta CaMKII isoforms were observed in small-diameter neurons, which are considered to be nociceptors. The changing expression of CaMKII alpha was already described in nociceptive small diameter primary sensory neurons (Carlton, 2002; Carlton and Hargett, 2002; Kojundzic et al., 2010). Changes of the expression of different CaMKII isoforms in small-diameter DRG neurons could be related to nociception, and point to the conclusion that CaMKII isoforms beta, gamma and delta are also important in the pathogenesis of chronic neuropathy in long-term diabetes, together with the CaMKII alpha isoform. Our study about CaMKII expression in dorsal horn of 6-month and 12-month diabetic rats showed that the expression of total CaMKII and its activated alpha isoform decreases by about one third from 6 to 12 months, while such changes were not observed in control animals (Boric et al., 2013). Our findings suggest that CaMKII may be subject to dynamic and compensatory changes in pain-processing regions of the central nervous system. Reversible abnormalities in other tissues affected with diabetes have been described by others, before irreversible structural changes become apparent.

Diabetes is a major factor contributing to accelerated aging (Sakul et al., 2013), and older age is an independent risk factors associated with diabetes-related complications (Chew et al., 2013). Therefore, studies contributing to understanding of changes associated with diabetes and its complications during maturation may help us find new therapeutic targets and improve patients' quality of life. CaMKII is, at the moment, the most likely therapeutic target for diabetic neuropathy.

A limitation of this study is that results were obtained using exclusively immunohistochemical staining technique. Future studies should include additional lines of evidence, including behavioral or pathophysiological data.

Conclusion

A significant decrease of specific CaMKII isoforms was observed 6 and 12 months after diabetes induction, suggesting involvement of CaMKII alpha, beta, gamma and delta isoforms in the development of complex events responsible for the development of neuropathy in long-term diabetes.

Acknowledgements. The study was funded by the Croatian Foundation for Science (HRZZ) grant no. 02.05./28 awarded to Livia Puljak. We are grateful to Ms. Dalibora Behmen for language editing.

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Accepted November 21, 2013