

**Sveučilište u Splitu
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**Izražaj kalcij/kalmodulin-ovisne protein kinaze II (CaMKII) u putu
prijenosa boli od periferije do središnjeg živčanog sustava
u modelu šećerne bolesti**

DOKTORSKA DISERTACIJA

Split, veljača 2015.



Ova doktorska disertacija izrađena je u Laboratoriju za istraživanje boli Zavoda za anatomiju, histologiju i embriologiju Medicinskog fakulteta Sveučilišta u Splitu. Istraživanje je provedeno uz potporu projekta Hrvatske zaklade za znanost (HRZZ) broj 02.05./28.

Voditeljica rada: prof. dr. sc. Livia Puljak

ZAHVALE

Rad na ovoj disertaciji obilježili su brojni dobri ljudi i mnoga lijepa iskustva.

Stoga se želim zahvaliti osobama koje su obilježile moj dosadašnji znanstveni život.

Zahvaljujem svojoj mentorici, uzoru u znanosti i radišnosti, prof. dr. sc. Liviji Puljak, koja je nesebično i strpljivo prenosila na mene svoje znanje i mudrost. Hvala na ukazanom povjerenju, volji i strpljenju tijekom izrade i pisanja ove disertacije.

Hvala prof. dr. sc. Damiru Sapunaru na savjetima kojima me usmjeravao tijekom mog znanstveno-istraživačkog rada.

Hvala dr. sc. Antoniji Jeličić Kadić koja mi je bezuvjetno bila spremna pomoći u svakom trenutku, zajedno smo otkrivali znanost.

Hvala dr. sc. Lejli Ferhatović Hamzić na pomoći pri svladavanju laboratorijskih vještina. Činilo se nedostižno, no uz njenu pomoć je postalo stvarnost.

Hvala gđi Asji Miletić, tehničarki Zavoda za anatomiju, histologiju i embriologiju na perfekcionizmu u izradi imunohistokemijskih preparata.

Tijekom proteklih istraživanja surađivao sam sa gotovo svim kolegama na Zavodu za anatomiju, histologiju i embriologiju Medicinskog fakulteta Sveučilišta u Splitu te bih se svima želio zahvaliti na pomoći i savjetima.

Hvala obitelji i prijateljima, zlatne ste niti u tkanju životnih postignuća.

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2. POPIS KRATICA I OZNAKA

ANOVA	test jednosmjerne analize varijance (engl. <i>one-way analysis of variance</i>)
CaM	kalmodulin (engl. <i>calmodulin</i>)
CaMKII	kalcij/kalmodulin ovisna protein kinaza II
CaMKII β	kalcij/kalmodulin-ovisna protein kinaza II beta
CaMKII γ	kalcij/kalmodulin-ovisna protein kinaza II gama
CaMKII δ	kalcij/kalmodulin-ovisna protein kinaza II delta
CLSP	protein nalik na kalmodulin (engl. <i>calmodulin-like skin protein</i>)
DAPI	4',6-diamidino-2-fenilindol dihidroklorid
DM	šećerna bolest (lat. <i>diabetes mellitus</i>)
DM1	šećerna bolest tip 1
DM2	šećerna bolest tip 2
engl.	engleski jezik
HLA	humani leukocitni antigen (engl. <i>human leukocyte antigen</i>)
HFD	hrana s visokim udjelom masti (engl. <i>high fat diet</i>)
IB4	izolektin B4
i.p.	intraperitonealno
KON-DM1	kontrolna skupina štakora za šećernu bolest tipa 1
KON-DM2	kontrolna skupina štakora za šećernu bolest tipa 2
lat.	latinski jezik
NMDA	N-metil D-aspartat
PBS	fosfatna puferska otopina (engl. <i>phosphate buffered saline</i>)
pCaMKII α	fosforilirana kalcij/kalmodulin-ovisna protein kinaza II alfa
PGP 9.5	protein-genski produkt (engl. <i>protein gene product 9.5</i>)
ROS	reaktivni kisikovi spojevi (engl. <i>reactive oxygen species</i>)
STZ	streptozotocin

SŽS	središnji živčani sustav
tCaMKII	ukupna kalcij/kalmodulin-ovisna protein kinaza II
TRPA1	transmembranski receptor (engl. <i>transient receptor potential ankyrin 1</i>)

3. PREGLED OBJEDINJENIH RADOVA

Ova doktorska disertacija temelji se na objedinjenim sljedećim znanstvenim radovima:

1. Boric M, Jelicic Kadic A, Puljak L. The expression of calcium/calmodulin-dependent protein kinase II in dorsal horn of rats with type 1 and type 2 diabetes. *Neurosci Lett* 2014;579:151-6.
2. Boric M, Jelicic Kadic A, Ferhatovic L, Sapunar D, Puljak L. Calcium/calmodulin-dependent protein kinase II in dorsal horn neurons in long-term diabetes. *NeuroReport* 2013;24(17):992-6.
3. Boric M, Jelicic Kadic A, Puljak L. Cutaneous expression of calcium/calmodulin-dependent protein kinase II in rats with type 1 and type 2 diabetes. *Journal of Chemical Neuroanatomy* 2014;61-62C:140-146.

3.1. UVOD

3.1.1. Šećerna bolest

Šećerna bolest (lat. *diabetes mellitus*, DM) obuhvaća niz kroničnih metaboličkih oboljenja čije je zajedničko obilježje stanje hiperglikemije tijekom dužeg razdoblja nastalo zbog apsolutnog ili relativnog nedostatka inzulina (tip 1 DM), inzulinske rezistencije (tip 2 DM), povećanog stvaranja glukoze ili pojačanog djelovanja hormona čiji je učinak suprotan djelovanju inzulina (1, 2). Osim navedena dva osnovna tipa DM, klasifikacija iz 1999. godine, temeljena ponajprije na etiologiji, uključuje i niz drugih, specifičnih tipova te gestacijski DM (3).

Simptomi kronične hiperglikemije tipični za DM su žeđ, učestalo mokrenje, zamućen vid i gubljenje na tjelesnoj težini unatoč povećanom apetitu. Ipak, kod nekih je ljudi bolest dugo asimptomatska ili praćena blažim simptomima, pa može proći i više godina tijekom kojih bolest ostaje neprepoznata (4). U svijetu je 2011. bilo 366 milijuna oboljelih od dijabetesa (5), a pretpostavlja se da će do 2035. godine broj oboljelih doseći 592 milijuna ljudi (6, 7).

Tip 1 DM (DM1) je multifaktorijalna autoimuna bolest, nastala međudjelovanjem genetskih i okolišnih čimbenika. Obrazac nasljeđivanja tipa 1 DM je poligenski, no prema suvremenim spoznajama najveći stupanj povezanosti imaju geni regije HLA (engl. *human leukocyte*

antigen) (8). Autoimuno uništavanje β -stanica Langerhansovih otočića gušterače dovodi do smanjenog stvaranja inzulina. Uništenja 80-90% β -stanica dovodi do razvoja hiperglikemije (9). Iako je funkcija ostalih stanica Langerhansovih otočića u početku očuvana, manjak inzulina oštećuje i α -stanice koje proizvode glukagon (10).

Tip 2 DM (DM2) je najučestaliji tip šećerne bolesti koji se javlja se u 90% oboljelih (11). Glavnim okidačima odgovornima za razvoj DM2 smatraju se životni stil i pretilost, no u patogenezu bolesti je također značajno uključena i genetska podloga. U tom tipu DM postoje tri osnovna patološka poremećaja: smanjeno izlučivanje inzulina, periferna inzulinska rezistencija i povećana proizvodnja glukoze u jetri (12, 13).

Stanice koje su najviše podložne oštećenju hiperglikemijom su kapilarne endotelne stanice mrežnice oka, mezangijske stanice glomerula, neuroni i Schwannove stanice perifernih živaca. Te stanice imaju velik rizik oštećenja jer, za razliku od ostalih stanica, ne mogu kontrolirati tj. smanjiti unos (14). Glavne komplikacije šećerne bolesti su oštećenja kardiovaskularnog sustava s razvojem pratećih bolesti, retinopatija, nefropatija, neuropatija, dijabetičko stopalo i posljedične amputacije donjih ekstremiteta (14).

Šećerna bolest vodeći je uzrok periferne neuropatije u razvijenim zemljama svijeta. Neuropatija je naziv za skupinu različitih bolesti od kojih je najčešća senzorimotorička polineuropatija koja započinje u stopalima i napreduje proksimalno (15, 16). Dijabetička neuropatija najčešće pogađa donje udove, stopala i gležnjeve, a rjeđe gornje udove (17). Prema Lowu i sur., oko 50% oboljelih nakon 15 godina trajanja šećerne bolesti razvije neuropatiju, a 10% njih osjeća stalne dizestezijske i bol (18). Bol je simptom dijabetičke neuropatije koji bolesnike najviše uznemiruje (19). Bol kod dijabetičke neuropatije je značajan uzrok morbiditeta i povezana je s velikim medicinskim troškovima (20). Ipak, patofiziologija dijabetičke neuropatske boli nedovoljno je poznata i liječenje je često nezadovoljavajuće jer trenutno dostupni lijekovi često ne pružaju prikladnu analgeziju ili imaju izražene nuspojave (21). Dakle, za razvoj učinkovitih novih analgetika u liječenju neuropatske boli važno je razjasniti temeljne molekularne mehanizme njenog nastanka (22, 23).

3.1.2. Kalcij/kalmodulin-ovisna protein kinaza II (CaMKII)

Dugoročna potencijacija (engl. *long-term potentiation*), oblik sinaptičke plastičnosti, barem djelomično utječe na razvoj kronične boli (24). N-metil-D-aspartat (NMDA) receptori

posreduju ulazak kalcija iz izvanstaničnog prostora u citosol, pri čemu pokreću kaskadu događaja koja između ostalog dovodi do aktivacije enzima kalcij/kalmodulin-ovisne protein kinaze II (CaMKII) (25).

CaMKII je višestruko funkcionalna serin/treonin kinaza koja ima 4 izoforme: alfa, beta, gama i delta (26). Enzim CaMKII izražen je u mozgu, kralježničnoj moždini te u malim i srednjim neuronima u spinalnom gangliju, područjima važnim za obradu i prijenos osjeta boli (27, 28). Izoforma α (CaMKII α) čiji se izražaj prvenstveno nalazi u središnjem živčanom sustavu (SŽS) najčešće je istraživana izoforma (29).

Jedna od važnih osobina CaMKII jest da nakon fosforilacije ostaje aktivirana iako se utok kalcija vraća na svoju osnovnu razinu (30). Trajna aktivacija CaMKII predmet je istraživanja koja se bave modulacijom boli (31). CaMKII je vjerojatno presudna za izazivanje i održavanje rane faze dugoročne potencijacije u dorzalnog rogu kralježnične moždine (32).

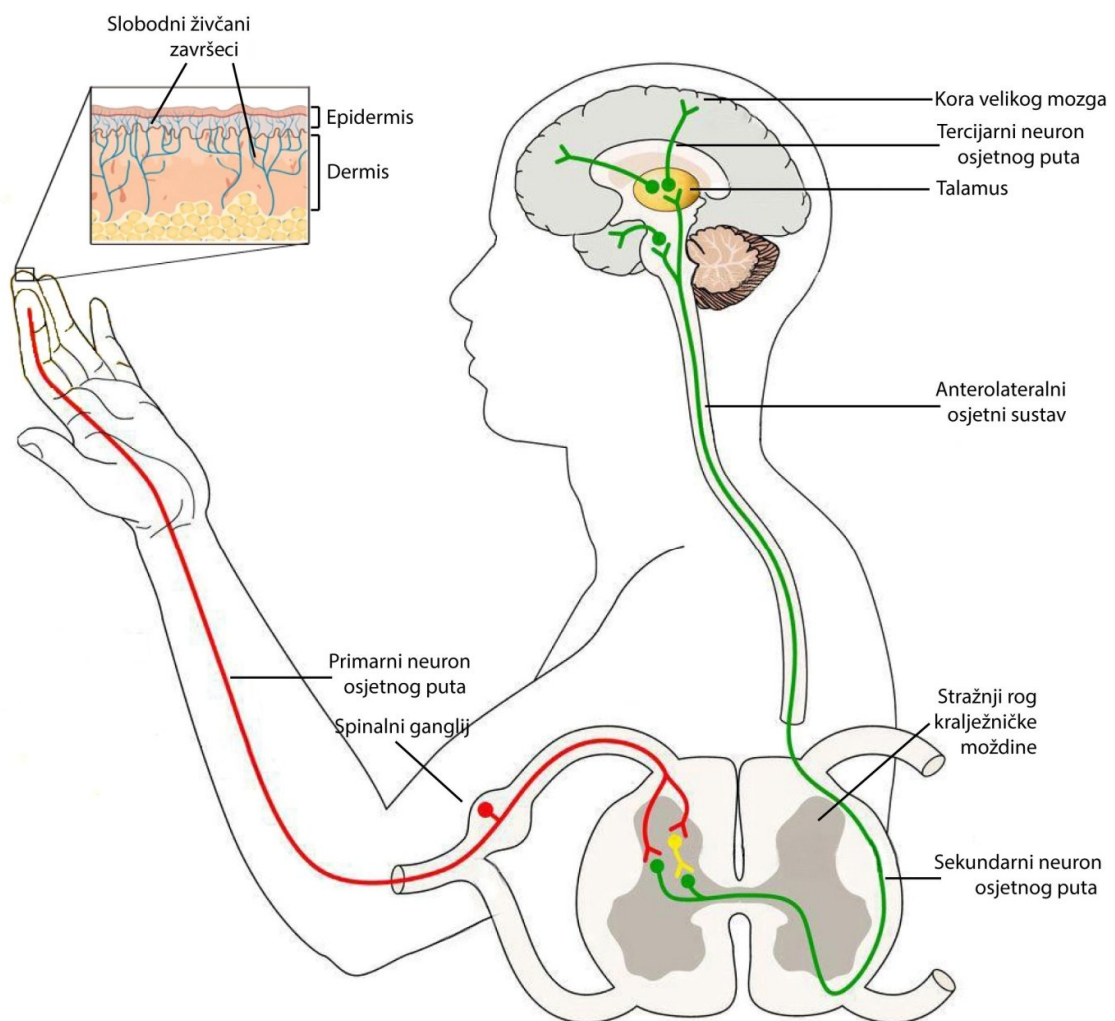
Povećan izražaj CaMKII i njena fosforilacija utvrđeni su u neuronima stražnjeg roga kralježnične moždine štakora nakon intradermalnog nanošenja kapsaicina, dobro istraženog modela bolnog ponašanja (33). Podvezivanje spinalnog živca ipsilateralno povećava aktivnost CaMKII u kralježničnoj moždini, dok snažan inhibitor CaMKII poništava djelovanje podvezivanja spinalnog živca (induciranu mehaničku alodiniju, toplinsku hiperalgeziju i aktiviranje CaMKII) bez narušavanja lokomotorne funkcije (34).

Pretpostavlja se da aktivirana CaMKII služi kao ključna komponenta unutarstaničnih signalnih putova pridonoseći razvoju neuropatske boli i trajne podraživosti neurona stražnjeg roga nakon ozljede kralježnične moždine. Liječenje inhibitorima CaMKII rezultiralo je značajnim smanjenjem mehaničke alodinije i širokog dinamičkog raspona neuralne aktivnosti potaknute raznim podražajima (31).

CaMKII nije intenzivno istraživana u živčanom tkivu dijabetičnih štakora. Dosadašnja istraživanja u Laboratoriju za istraživanje boli Medicinskog fakulteta Sveučilišta u Splitu pokazala su da se izražaj CaMKII povećava u spinalnim ganglijima štakora dva tjedna i dva mjeseca nakon indukcije šećerne bolesti streptozotocinom (STZ), te da izravno ubrizgavanje inhibitora CaMKII u spinalni ganglij dijabetičnog štakora smanjuje izražaj CaMKII u njemu što je praćeno smanjenjem bolnog ponašanja štakora (35, 36). Međutim, ne postoje studije o promjenama CaMKII u ostalim dijelovima puta prijenosa bolnog podražaja u modelu šećerne bolesti (22, 23).

3.1.3. Prijenos osjeta boli

Osjet boli nastaje podraživanjem posebnih receptora koji se nazivaju nociceptori. Nociceptori (lat. *noceo*, *nocere*=škoditi) su receptori koji reagiraju na štetne podražaje. Opisane su dvije glavne vrste nociceptora: mehanički i polimodalni. Mehanički nociceptori su slobodni završeci primarnih aferentnih neurona A δ -vlakana i njihova aktivacija uzrokuje osjet oštre, štipajuće boli. Posebno učinkovit podražaj je štipanje ili gnječenje kože. Polimodalni nociceptori (slobodni završeci C-vlakana) reagiraju na raznolike snažne mehaničke, kemijske ili termičke podražaje (37-39).

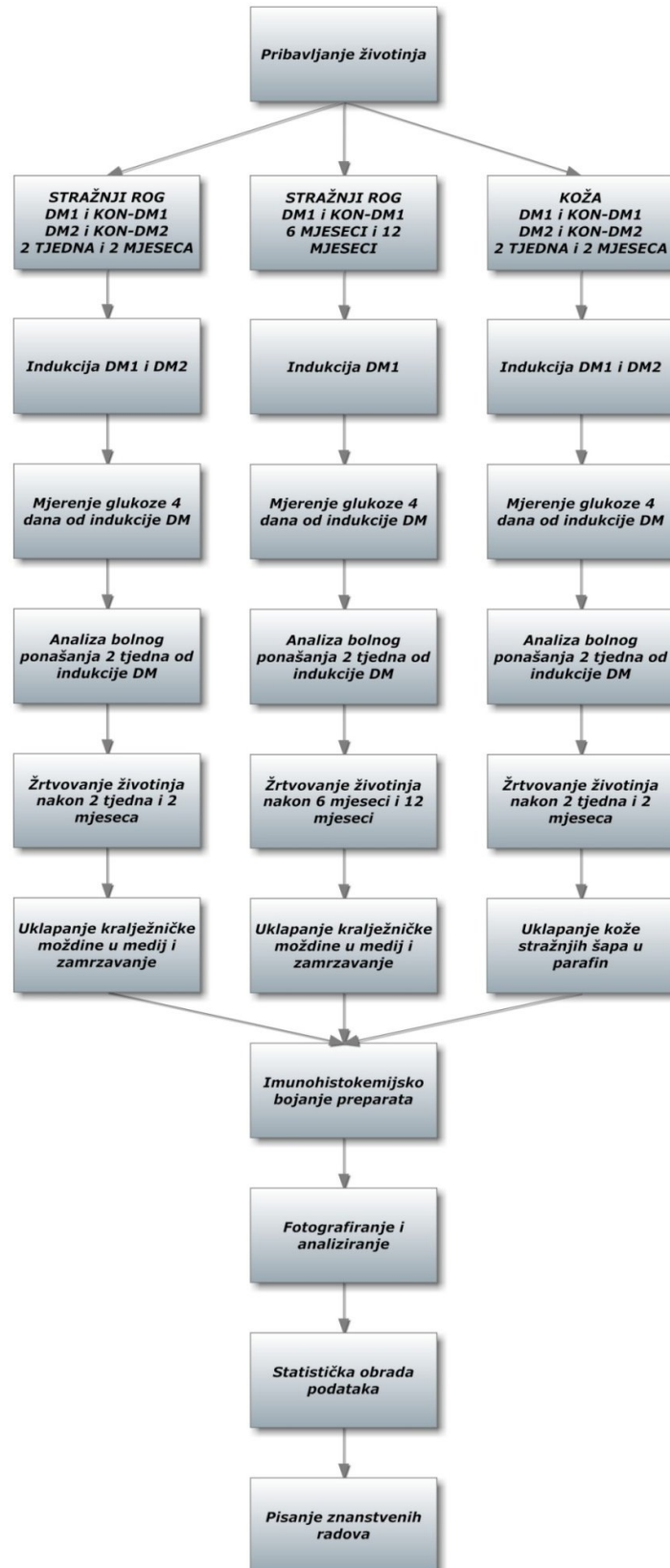


Slika 1. Put prijenosa boli od perifernih nociceptora do središnjeg živčanog sustava.

Tanka mijelinizirana (A δ) i nemijelinizirana (C) vlakna slobodno se granaju u koži, sežu u epidermis, granaju se u vezivnom tkivu dermisa te oko krvnih žila. Tijela primarnih osjetnih neurona nalaze se u spinalnim ganglijima. Primarna aferentna nocicepcijska vlakna završavaju na sekundarnim osjetnim neuronima i interneuronima smještenim u nekoliko Rexedovih slojeva stražnjeg roga kralježnične moždine. Tijela sekundarnih osjetnih neurona smještena su u stražnjem rogu kralježnične moždine (40). Aksoni sekundarnih osjetnih neurona oblikuju anterolateralni sustav za prijenos osjeta boli i temperature, sastavljen od 4 uzlazna puta: spinotalamički, spinoretikularni, spinomezencefalički i spinocervikalni, koji se nastavljaju prema subkortikalnim centrima srednjeg mozga i talamusu te tercijarnim neuronima završavaju u kortikalnim centarima za bol (41, 42).

U koži se nalaze osjetni receptori koji putem primarnih osjetnih neurona čija se tijela nalaze u spinalnim ganglijima šalju informacije u SŽS. Tijela sekundarnih osjetnih neurona smještena su u stražnjem rogu kralježnične moždine (40). Na temelju dosadašnjih spoznaja o izražaju CaMKII u neuronima spinalnog ganglija, cilj je ovog istraživanja bio na životinjskom modelu šećerne bolesti istražiti izražaj ukupne CaMKII (tCaMKII) i njezine fosforilirane alfa izoforme (pCaMKII α) u koži, mjestu perifernih završetaka primarnog osjetnog neurona, i u stražnjem rogu kralježnične moždine gdje se nalaze tijela sekundarnih osjetnih neurona.

3.2. PREGLED METODOLOGIJE OBJEDINJENIH RADOVA



Slika 2. Hodogram istraživanja.

3.2.1. Pokusne životinje

Hodogram istraživanja prikazan je na Slici 2. U istraživanju su korišteni Sprague-Dawley štakori muškog spola (mase oko 200 g) iz Nastambe za male pokusne životinje Sveučilišta u Splitu. Štakori su smješteni u plastične kaveze po skupinama, s podlogom od piljevine i kukuruzne stelje u omjeru 3:1, i uzgajani od 2 tjedna do 1 godinu, ovisno o skupinama. Tijekom trajanja istraživanja životinje su bile smještene u prostor s osiguranom stalnom temperaturom od ~22°C i automatskom izmjenom ciklusa svjetla i tame svakih 12 h.

U prvoj studiji, u kojoj su ispitivane dvotjedne i dvomjesečne promjene CaMKII u stražnjem rogu kralježnične moždine kod STZ-dijabetičkih modela DM1 i DM2 korišteno je ukupno 45 štakora koji su podijeljeni u sljedeće skupine:

- 1) štakori sa šećernom bolesti tip 1 žrtvovani nakon 2 tjedna (DM1-2TJ) i kontrolni štakori za dvotjedni model šećerne bolesti tipa 1 (KON-DM1-2TJ);
- 2) štakori sa šećernom bolesti tipa 2 žrtvovani nakon 2 tjedna (DM2-2TJ) i kontrolni štakori za dvotjedni model šećerne bolesti tipa 2 (KON-DM2-2TJ);
- 3) štakori sa šećernom bolesti tipa 1 žrtvovani nakon 2 mjeseca (DM1-2MJ) i kontrolni štakori za dvomjesečni model šećerne bolesti tipa 1 (KON-DM1-2MJ);
- 4) štakori sa šećernom bolesti tipa 2 žrtvovani nakon 2 mjeseca (DM2-2MJ) i kontrolni štakori za dvomjesečni model šećerne bolesti tipa 2 (KON-DM2-2MJ).

U drugoj studiji, u kojoj su ispitivane šestomjesečne i jednogodišnje promjene CaMKII u STZ-modelu šećerne bolesti tipa 1 korištena su 24 štakora koji su podijeljeni u sljedeće skupine:

- 1) štakori sa šećernom bolesti tip 1 žrtvovani nakon 6 mjeseci (DM1-6MJ) i kontrolni štakori za šestomjesečni model šećerne bolesti tipa 1 (KON-DM1-6MJ);
- 2) štakori sa šećernom bolesti tip 1 žrtvovani nakon 12 mjeseci (DM1-12MJ) i kontrolni štakori za dvanaestomjesečni model šećerne bolesti tipa 1 (KON-DM1-12MJ).

U trećoj studiji, u kojoj su ispitivane dvotjedne i dvomjesečne promjene CaMKII u koži tabana stražnjih šapa kod STZ-modela šećerne bolesti tipa 1 i 2 korištena su ukupno 42 štakora koji su podijeljeni u sljedeće skupine:

- 1) štakori sa šećernom bolesti tip 1 žrtvovani nakon 2 tjedna (DM1-2TJ) i kontrolni štakori za dvotjedni model šećerne bolesti tipa 1 (KON-DM1-2TJ);

- 2) štakori sa šećernom bolesti tipa 2 žrtvovani nakon 2 tjedna (DM2-2TJ) i kontrolni štakori za dvotjedni model šećerne bolesti tipa 2 (KON-DM2-2TJ);
- 3) štakori sa šećernom bolesti tipa 1 žrtvovani nakon 2 mjeseca (DM1-2MJ) i kontrolni štakori za dvomjesečni model šećerne bolesti tipa 1 (KON-DM1-2MJ);
- 4) štakori sa šećernom bolesti tipa 2 žrtvovani nakon 2 mjeseca (DM2-2MJ) i kontrolni štakori za dvomjesečni model šećerne bolesti tipa 2 (KON-DM2-2MJ).

3.2.2. Štakorski modeli šećerne bolesti

DM1 model induciran je intraperitonealnim (i.p.) ubrizgavanjem STZ-a otopljenog u citratnom puferu (pH=4,5) u dozi od 55 mg/kg. STZ djeluje toksično na beta stanice Langerhansovih otočića u gušterači te se 2 do 4 dana nakon ubrizgavanja STZ-a razvija hiperglikemija (35). Pokusne životinje korištene za model šećerne bolesti tipa 1 tijekom cijelog pokusa su hranjene *ad libitum* uobičajenom hranom za pokusne štakore koja se sastoji od 27% proteina, 9% masti i 64% ugljikohidrata (4RF21 GLP, Mucedola srl, Settimo Milanese, Italija). Kontrolnoj skupini za model šećerne bolesti tipa 1 (KON-DM1) i.p. se ubrizgao citratni pufer bez STZ-a. Prehrana KON-DM1 skupine bila je ista kao i kod DM1 skupine.

DM2 model je induciran prema modelu koji su prethodno opisali Srinivasan i suradnici (43). Životinje su hranjene *ad libitum* posebnom masnom hranom koja se sastoji od 58% masti, 25% proteina i 17% ugljikohidrata u ukupnom kalorijskom unosu (PF, 4269, Mucedola srl, Settimo Milanese, Italija). Nakon dva tjedna štakorima je i.p. ubrizgana niža doza STZ-a od 35 mg/kg otopljenog u citratnom puferu (pH=4,5). Životinje su se nastavile hraniti istom prehranom bogatom visokim udjelom masti sve do žrtvovanja. Kontrolna se skupina za model šećerne bolesti tipa 2 (KON-DM2) hranila istom prehranom kao skupina DM2, ali im se dva tjedna od početka pokusa i.p. ubrizgao citratni pufer bez STZ-a.

Termalni i mehanički podražaji (testiranje hladnoćom, testiranje toplinom, testiranje tupom iglom i testiranje Von Freyevim vlaknima) korišteni su da se potvrdi da su životinje razvile bolno ponašanje.

Štakori s DM1 koji su bili žrtvovani nakon 2 i 6 mjeseci te godinu dana primali su jednu jedinicu dugodjelujućeg inzulina (Lantus Solostar, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Njemačka) tjedno kako se ne bi razvila ketoacidoza i smanjenje tjelesne mase koji bi mogli dovesti do lošeg općeg stanja pokusne životinje. Prema dosada

provedenim istraživanjima dijabetičke neuropatije na STZ-modelu štakora utvrđeno je ta doza inzulina održava dobro stanje štakora, a ne utječe na istraživane varijable (35).

3.2.3. Validacija indukcije šećerne bolesti

Pokusnim životinjama je pomoću glukometra One Touch Vita (LifeScan, High Wycombe, Velika Britanija) izmjerena koncentracija glukoze u plazmi četiri dana nakon indukcije dijabetesa analizom kapljice krvi s vrha repa (35). U nastavak istraživanja uključeni su samo DM1 štakori koji su natašte imali koncentraciju glukoze u plazmi iznad 300 mg/dL i DM2 štakori čija je razina glukoze u plazmi bila veća od 200 mg/dL.

3.2.4. Priprema tkiva za imunofluorescenciju

Na kraju svakog istraživanja štakori su anestetizirani 5%-tnim izofluranom u struji zraka (Forane, Abbot Laboratories Ltd., Queenborough, Velika Britanija) te perfundirani kroz uzlaznu aortu preko lijeve klijetke s 300 mL fiziološke otopine i istim volumenom Zambonijevog fiksativa (4% paraformaldehid i 15% pikrinska kiselina u 0,1M fosfatnom puferu – PBS), pH 7,4. U prvoj i drugoj studiji pažljivo im je izvađen dio kralježnične moždine povezan sa spinalnim ganglijima L4 i L5, te je fiksiran 48 h u Zambonijevu fiksativu, ispran u PBS-u a zatim krioprotektiran preko noći u otopini 30% saharoze, zamrznut i rezan na kriotomu pri temperaturi od -22°C na rezove debljine 8 µm. U trećoj studiji je odstranjena koža s tabana stražnje šape, razvučena na parafinskoj pločici i fiksirana 48 h u Zambonijevu fiksativu te potom isprana u destiliranoj vodi. Nakon 24 h, tkivo je prebačeno u formalin, potom dehidrirano u alkoholu, prosvijetljeno u ksilolu i uklopljeno u parafin. Koža je rezana na mikrotomu na rezove debljine 5 µm. U svim je studijama bojan svako peti rez. Koža uklopljena u parafin prije bojanja morala se deparafinirati, isprati u destiliranoj vodi i kuhati u citratnom puferu (pH 6.0) 12 minuta na 95°C. Nakon hlađenja na sobnoj temperaturi nanese se primarno protutijelo.

Imunohistokemijskim je metodama u stražnjem rogu kralježnične moždine i u koži analizirana tCaMKII i pCaMKII α . Primarna poliklonalna protutijela za analizu tCaMKII (1:100, sc-9035, lot no. F0304, Santa Cruz Biotechnology, Santa Cruz, CA, SAD) i pCaMKII α (1:100, sc-12886-R, lot. no. K2305, Santa Cruz Biotechnology, Santa Cruz, CA, SAD) podrijetlom su iz kunića. Za sekundarnu detekciju tCaMKII i pCaMKII α koristilo se Rhodamin red X-konjugirano sekundarno protutijelo (1:300, Donkey Anti-rabbit IgG (H+L)

Jackson ImmunoResearch, Lot No 106114). Za biljeg nociceptora koristio se konjugirani oblik aglutinina izolektin B4 (IB4) koji na sebi ima vezan fluorescein (1:50; 2895, IB4 labeled FITC, Sigma-Aldrich, St. Louis, MO, SAD) tako da su pozitivni neuroni fluorescirali zelenom bojom. Za detekciju intraepidermalnih vlakana u koži rabilo se mišje primarno protutijelo PGP 9.5 (engl. *protein gene product 9.5*) (1:1000, cat. no. 480012, Invitrogen Corporation, Camarillo, CA, SAD), dok je za sekundarnu detekciju korišteno biotinizirano kozje protutijelo (1:100, Biotinylated goat anti-mouse IgG-B, cat. no. sc-2039, Santa Cruz Biotechnology, Santa Cruz, CA, SAD), a za vizualizaciju je upotrijebljen konjugat Streptavidin Alexa Fluor 488 (1:500; S-32354, lot no. 508205, Molecular Probes, Eugene, OR, SAD). Bojanje jezgre stanica kože provelo se pomoću 4',6-diamidino-2-fenilindol dihidroklorida (DAPI).

Nakon ponovljenog ispiranja u PBS-u, rezovi su osušeni i prekriveni pokrovnim stakalcem.

3.2.5. Analiza slika i kvantifikacija

Svako peti rez kralježnične moždine i kože proučen je mikroskopom (BX61, Olympus, Tokyo, Japan) i fotografiran pomoću hladene digitalne kamere (DP71, Olympus, Tokyo, Japan) pri istom povećanju (40x) i pri jednakoj vremenskoj ekspoziciji za svako pojedino bojanje. Slike su analizirane kao monokromne mikrofotografije (2040x1536 piksela, 12 bitova, 0-4096 razina sive boje). Intenzitet fluorescencije u stražnjem rogu izračunat je duž linije postavljene između ulaznog mjesta stražnjeg korijena u dorzalni rog i centralnog kanala (opcija skeniranja linije širine 15 piksela) u računalnom programu Metamorph (Molecular Devices, Sunnyvale, CA, SAD), a u koži je analiza rađena programom Image J (National Institutes of Health, Bethesda, MD, SAD) korištenjem alata *Frehand selection* tako da se označilo područja imunofluorescencije i mjerio se prosjek intenziteta fluorescencije tog područja.

3.2.6. Izračun veličine uzorka

Izračun potrebne veličine uzorka proveden je korištenjem programa koji se nalazi na mrežnoj stranici <http://www.stat.ubc.ca/~rollin/stats/ssize/>. Za izračun veličine uzorka korišteni su podatci dobiveni pilot istraživanjem na 10 štakora, gdje je uspoređen izražaj tCaMKII između skupina DM1 i KON-DM1 te utvrđena srednja vrijednost izražaja totalne CaMKII za DM1= 49,49, a za KON-DM1= 30,81; standardna devijacija=10,1. Izračunom je dobiveno da je uz $\alpha=0,05$ i statističku snagu od 90% potrebno najmanje 5 štakora u svakoj skupini.

3.2.7. Statistički testovi

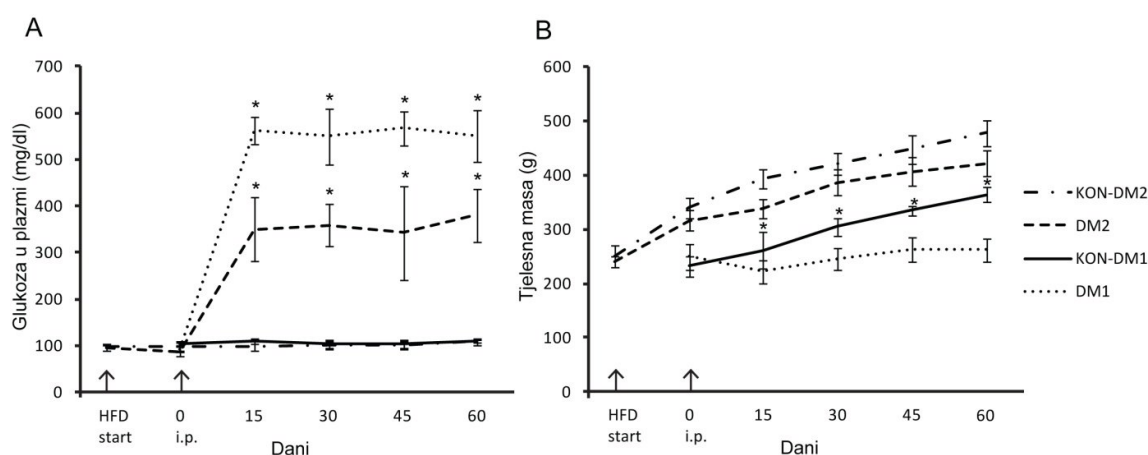
Razina glukoze u plazmi i masa kontrolnih i dijabetičnih štakora analizirana je ANOVA testom za ponovljena mjerenja i jednosmjernom ANOVA-om nakon koje je uslijedio *post hoc* test. Usporedba kontrolnih i dijabetičnih tkiva analizirana je Student t-testom. Za statističke postupke korišten je računalni program Statistica 7.0 (StatSoft, Tulsa, OK, SAD). Razina značajnosti bila je postavljena na $p < 0,05$.

3.3. SAŽETI PREGLED REZULTATA OBJEDINJENIH RADOVA

3.3.1. Rad 1

Validacija životinjskih modela šećerne bolesti tipa 1 i 2

Nakon indukcije šećerne bolesti, koncentracija glukoze u plazmi mjerena 15., 30., 45. i 60. dan istraživanja značajno se povećala u DM1 ($F(4)=105,1$, $p<0,001$) i DM2 skupini ($F(4)=42,7$, $p<0,001$). Petnaest dana nakon i.p. injekcije STZ-a zabilježeno je značajno povećanje glukoze u plazmi kod DM1 štakora u odnosu na KON-DM1 štakore ($t(8)=33,6$, $p<0,001$) kao i kod DM2 štakora u odnosu na njihove kontrole ($t(8)=8,3$, $p<0,001$). Mjerenja 30., 45. i 60. dan su pokazala isti trend (Slika 3A).

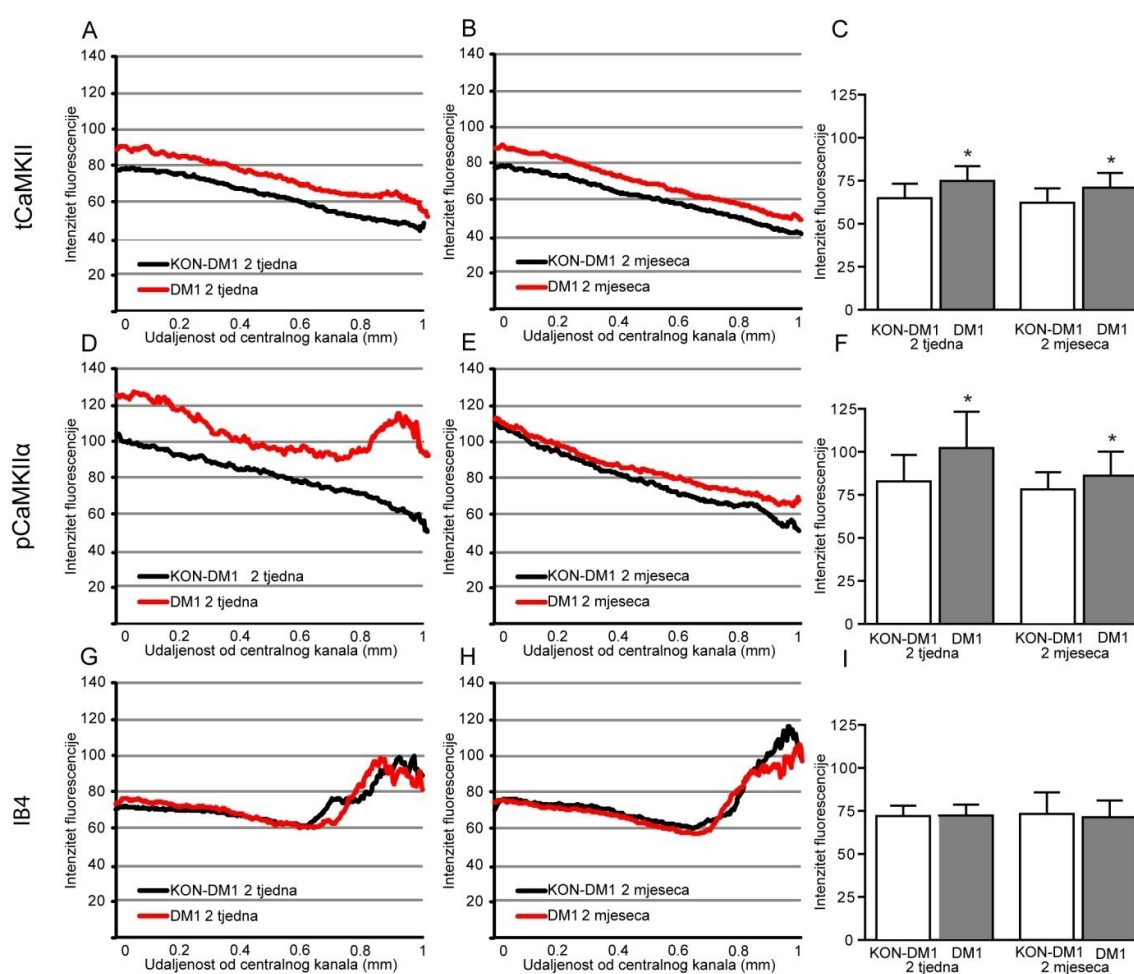


Slika 3. A) koncentracija glukoze u plazmi i B) tjelesna masa pokusnih životinja. Podatci su prikazani kao aritmetička sredina±standardna devijacija. Zvjezdica * označava statistički značajnu razliku ($p<0.05$) u usporedbi s kontrolnom skupinom (t-test). Legenda: KON-DM1 = kontrolna skupina za šećernu bolest tipa 1, DM1 = skupina sa šećernom bolesti tipa 1, KON-DM2 = kontrolna skupina za šećernu bolest tipa 2, DM2 = skupina sa šećernom bolesti tipa 2, i.p. = intraperitonealna injekcija (Dan 0), HFD = hrana s visokim udjelom masti (engl. high fat diet) kojom su hranjeni DM2 i KON-DM2 štakori dva tjedna prije i.p. injekcije.

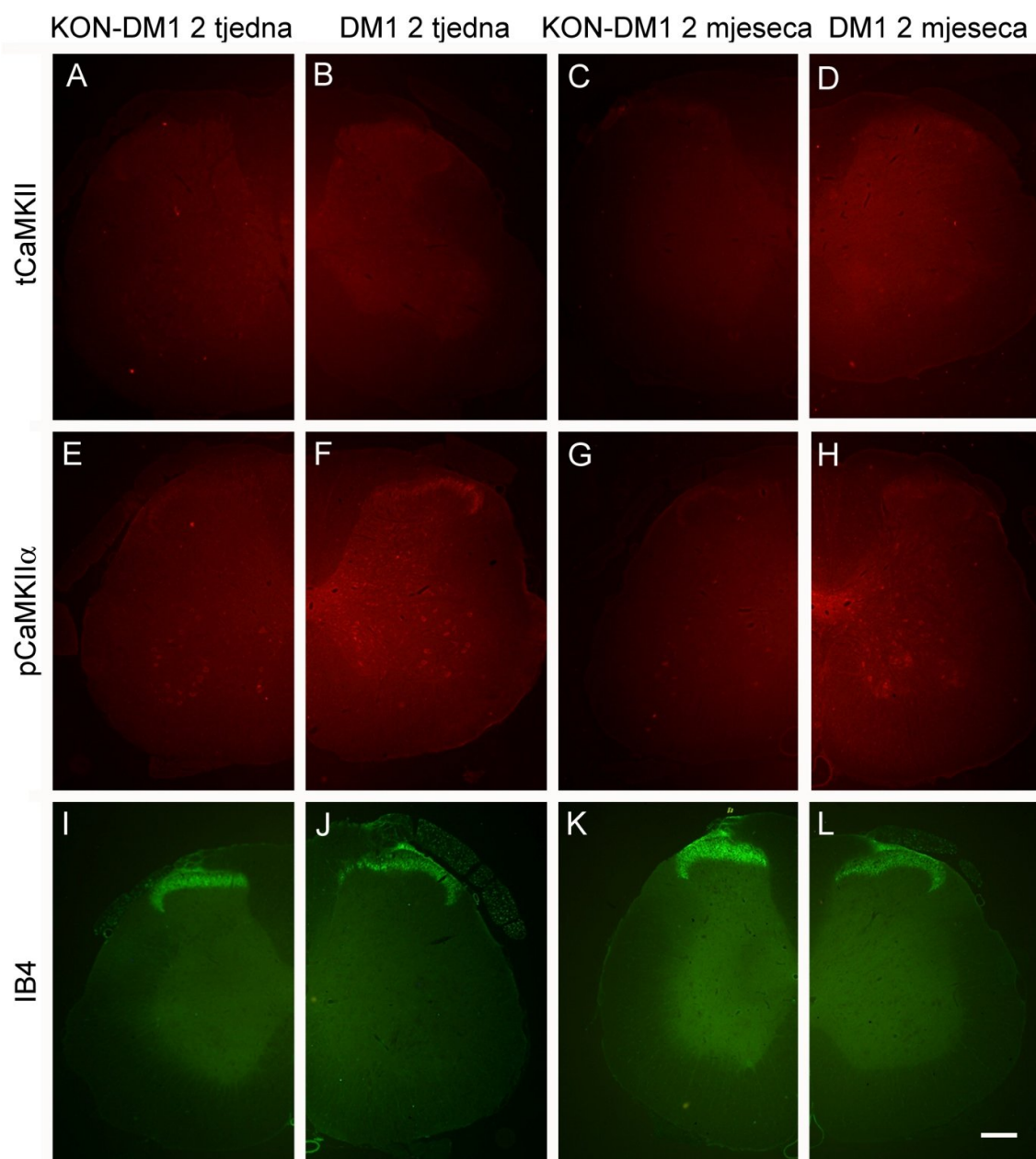
Petnaest dana nakon indukcije šećerne bolesti su DM1 štakori imali značajno manje povećanje tjelesne mase nego KON-DM1 štakori ($t(8)=-3.5$, $p=0,008$). Kontrolnim se životinjama tjelesna masa nastavila povećavati, dok je masa DM1 skupine ostala na početnim vrijednostima. Tjelesna masa značajno se povećala u DM2 skupini ($F(4)=207,1$; $p<0,001$) i KON-DM2 skupini ($F(4)=201,6$, $p<0,001$) nakon i.p. injekcije (Slika 3B).

Izražaj CaMKII u stražnjem rogu kralježnične moždine dva tjedna i dva mjeseca nakon indukcije šećerne bolesti tipa 1 i 2

Dva tjedna nakon indukcije šećerne bolesti, izražaj tCaMKII bio je značajno veći u DM1 štakora nego u kontrolnih ($t(58)=4,7$, $p<0.001$; Slike 4A, C i 5A, B). Izražaj pCaMKII α pokazao je značajnu razliku između DM1 i KON-DM1 štakora ($t(52)=4.2$, $p<0,001$; Slike 4D, F i 5E, F). Nadalje, u istom vremenskom razdoblju su i DM2 štakori imali značajno veću razliku izražaja tCaMKII ($t(70)=5.3$, $p<0.001$; Slike 6A, C i 7A, B) i pCaMKII α ($t(48)=5.7$, $p<0.001$; Slike 6D, F i 7E, F) u odnosu na KON-DM2 štakore.



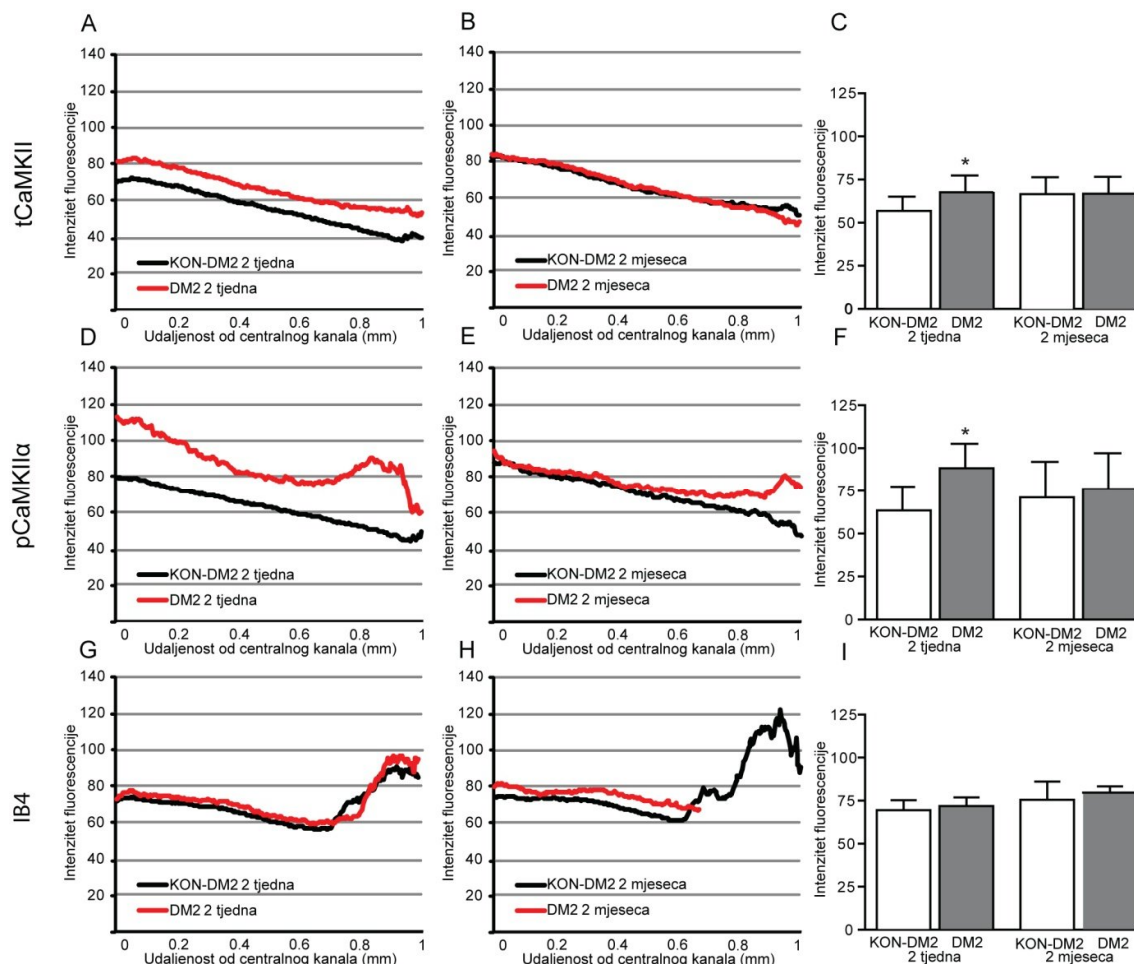
Slika 4. Izražaj tCaMKII (A-C), pCaMKII α (D-F) i IB4 (G-I) u stražnjem rogu DM1 štakora 2 tjedna i 2 mjeseca nakon indukcije šećerne bolesti. Podatci su prikazani kao aritmetička sredina \pm standardna devijacija. Zvijezdica * označava statistički značajnu razliku (Student t-test) u usporedbi s odgovarajućim kontrolnim životinjama bez šećerne bolesti tipa 1 ($p<0.05$).



Slika 5. Reprezentativni primjer imunofluorescencijskog bojanja tCaMKII (A-D), pCaMKII α (E-H) i IB4 (I-L) u stražnjem rogu DM1 štakora i kontrolnih životinja bez šećerne bolesti tipa 1, 2 tjedna i 2 mjeseca nakon indukcije šećerne bolesti. Povećanje: 4x. Mjerilo je dugo 100 μ m i odnosi se na sve slike.

Dok je izražaj tCaMKII i njene alfa izoforme pokazivao isti trend kako kod DM1 tako i kod DM2 štakora dva tjedna nakon indukcije, dva mjeseca nakon indukcije zabilježene su razlike među tipovima šećerne bolesti. Značajno povećanje izražaja tCaMKII ($t(72)=4,1$, $p<0,001$;

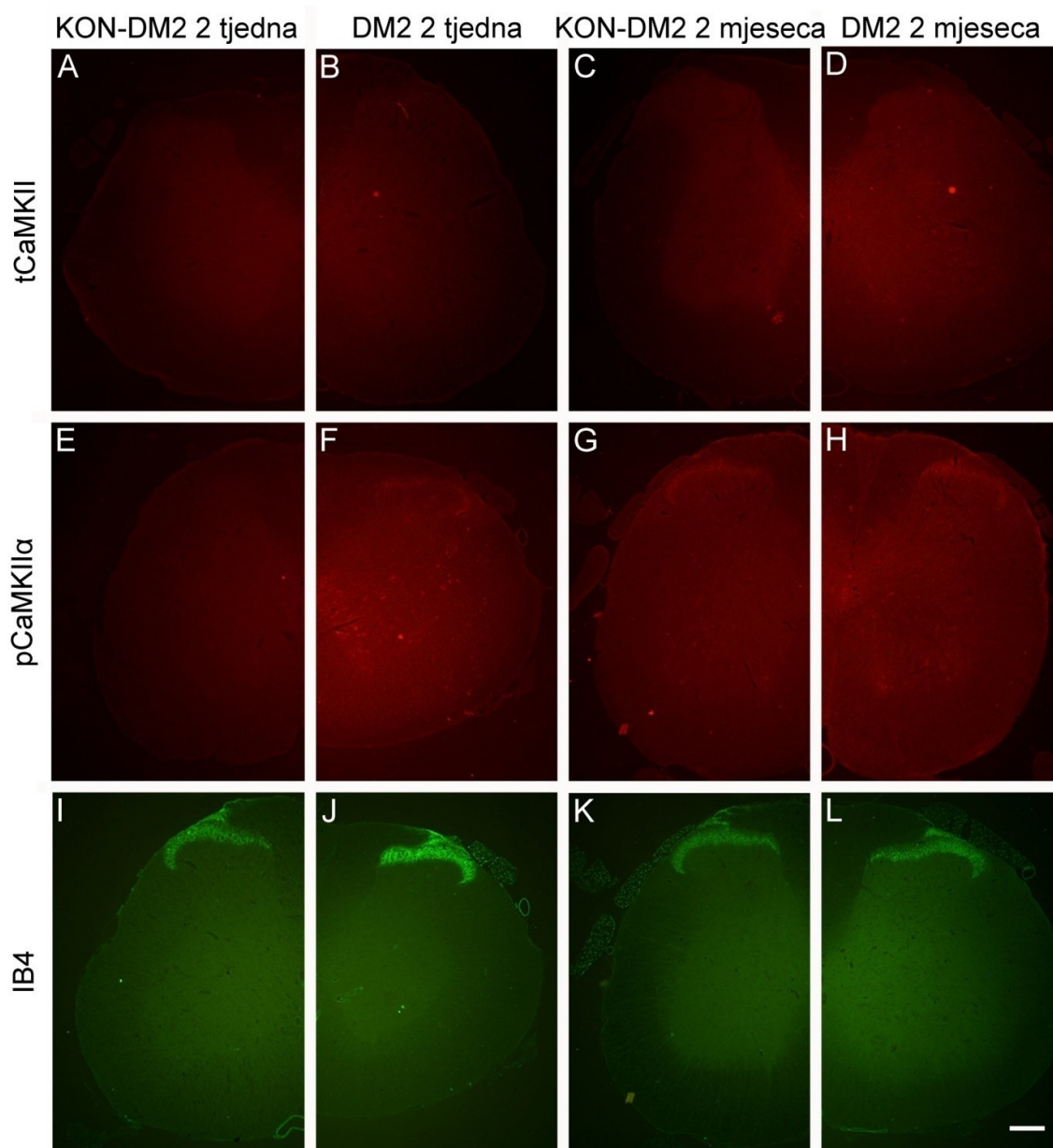
Slike 4B, C i 5C, D) imali su DM1 štakori u odnosu na KON-DM1. Ovaj porast bio je popraćen većim izražajem pCaMKII α ($t(30)=2,6$, $p<0.001$; Slike 4E, F i 5G, H). Međutim, DM2 štakori se nisu razlikovali od kontrolne skupine nakon dva mjeseca niti u izražaju tCaMKII ($t(73)=0,1$, $p=0,921$; Slike 4B, C i 5C, D) niti pCaMKII α ($t(49)=0,9$, $p=0,388$; Slike 6E, F i 7G, H).



Slika 6. Izražaj tCaMKII (A-C), pCaMKII α (D-F) i IB4 (G-I) u stražnjem rogu DM1 štakora 2 tjedna i 2 mjeseca nakon indukcije dijabetesa. Podatci su prikazani kao aritmetička sredina \pm standardna devijacija. Zvezdica * označava statistički značajnu razliku (Student t-test) u usporedbi s odgovarajućim kontrolnim životinjama bez šećerne bolesti tipa 2 ($p<0.05$).

Fluorescencija tCaMKII bila je ravnomjerno izražena duž linije mjerenja postavljene od ulazne zone stražnjeg korijena i centralnog kanala kralježnične moždine (Slike 4A-B, 6A-B), a fluorescencija pCaMKII α je prevladavala u laminama I-III (Slike 4D-E, 6D-E). Slično alfa

izoformi, IB4 se predominantno izrazio u laminama I-III stražnjeg roga (podatci nisu prikazani) te nije bilo razlike u izražaju među skupinama (Slike 4G-I, 6G-I, 5I-L, 7I-L).

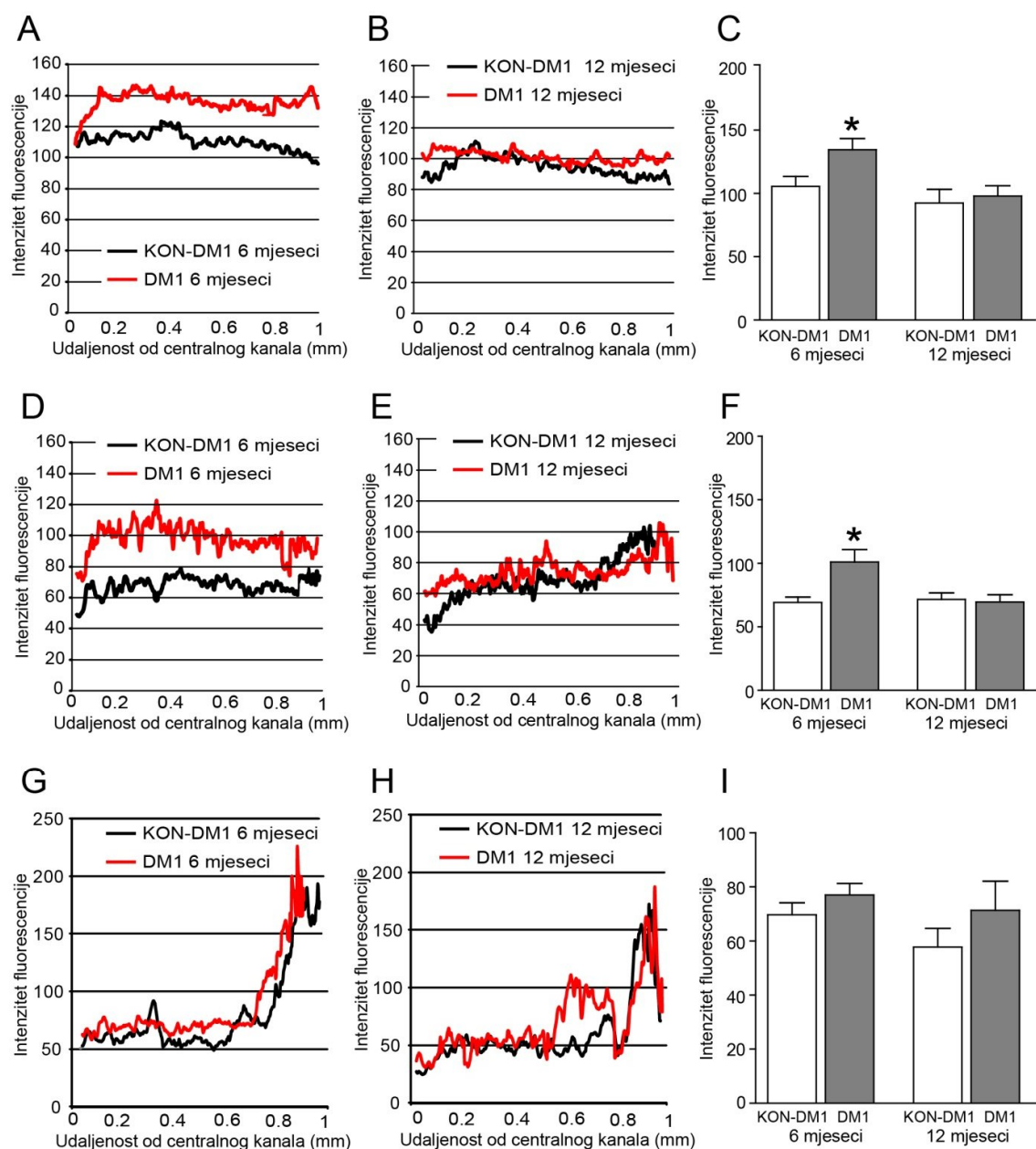


Slika 7. Reprezentativni primjer imunofluorescencijskog bojanja tCaMKII (A- D), pCaMKIIα (E-H) i IB4 (I-L) u stražnjem rogu DM2 štakora i kontrolnih životinja bez šećerne bolesti tipa 2, 2 tjedna i 2 mjeseca nakon indukcije šećerne bolesti. Povećanje: 4x. Mjerilo je dugo 100 μm i odnosi se na sve slike.

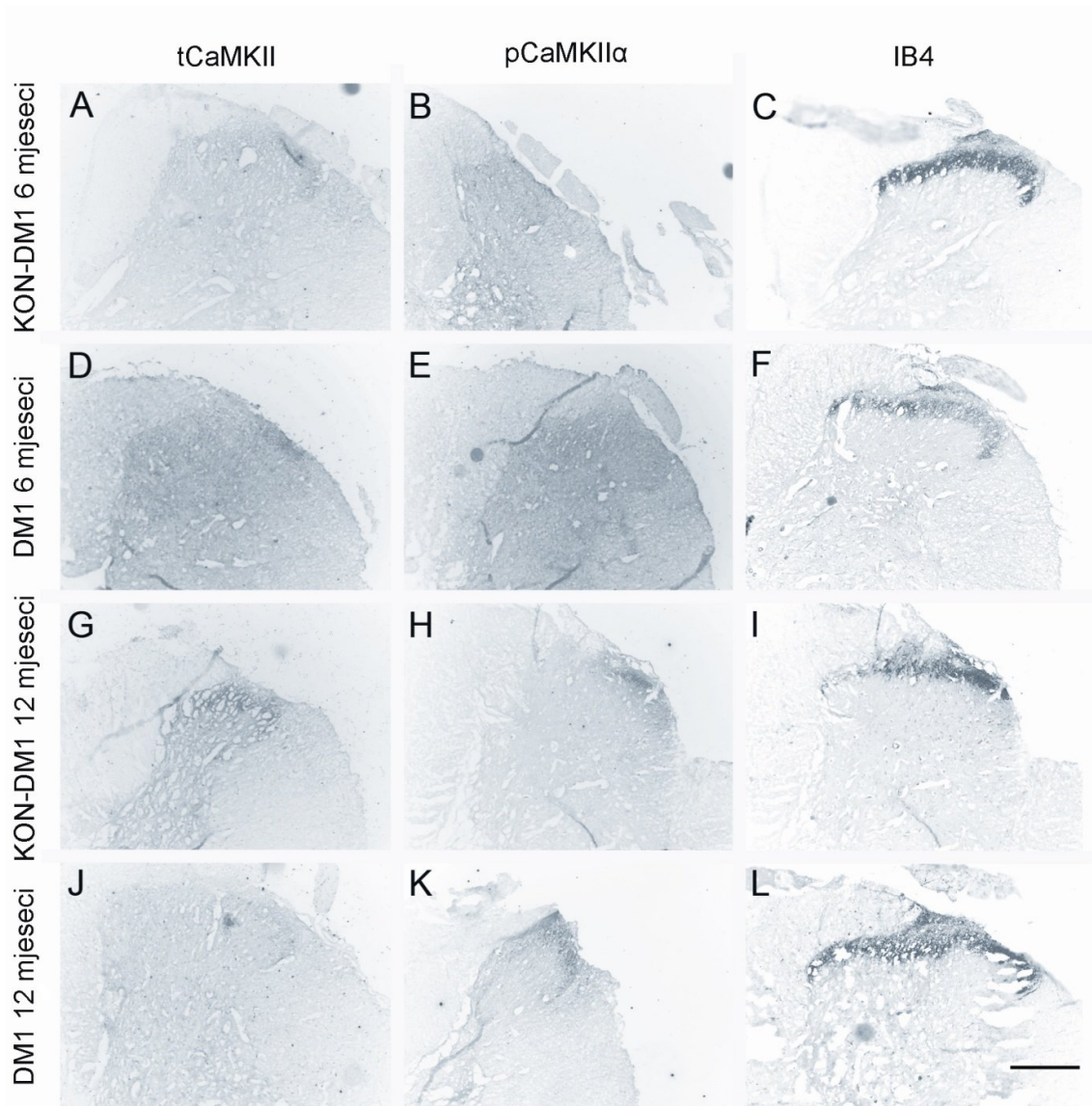
3.3.2. Rad 2

Izražaj CaMKII u stražnjem rogu kralježnične moždine šest mjeseci i godinu dana nakon indukcije šećerne bolesti tipa 1

Šest mjeseci nakon indukcije šećerne bolesti tipa 1 uočeno je značajno povećanje izražaja tCaMKII u stražnjem rogu kod DM1 štakora u usporedbi s kontrolama (Slika 8A, C). Ovo povećanje bilo je posredovano povećanim izražajem pCaMKII α (Slika 8D, F). Godinu dana nakon indukcije šećerne bolesti nije bilo značajne razlike u izražaju tCaMKII (Slika 6B, F) i pCaMKII α u stražnjem rogu (Slika 8E, F). Fluorescencija tCaMKII bila je ravnomjerno raspoređena duž linije mjerenja postavljene od ulazne zone stražnjeg korijena i centralnog kanala kralježnične moždine šest mjeseci i godinu dana nakon i.p. injekcije kod DM1 štakora i njihovih kontrola (Slika 8A-B) te pCaMKII α nakon 12 mjeseci (Slika 8D). Povećan izražaj IB4 uočen je u stražnjem rogu dijabetičnih štakora nakon šest mjeseci i godine dana, no ta razlika nije bila značajna. IB4 je bio najjače izražen u laminama I-III stražnjeg roga (Slika 8G-I), dok je tCaMKII i pCaMKII α uočena difuzno u lamina I-VI (Slika 8A, D). Reprezentativni primjer imunofluorescencijskog bojanja prikazan je na Slici 9.



Slika 8. Izražaj tCaMKII (A-C), pCaMKII α (D-F) i IB4 (G-I) u stražnjem rogu DM1 štakora 6 i 12 mjeseci nakon indukcije šećerne bolesti. Podatci su prikazani kao aritmetička sredina \pm standardna devijacija. Zvezdica * označava statistički značajnu razliku (Student t-test) u usporedbi s odgovarajućim kontrolnim životinjama bez šećerne bolesti tipa 1 ($p < 0.05$).



Slika 9. Reprezentativni primjer imunofluorescencijskog bojanja tCaMKII (A, D, G, J), pCaMKII α (B, E, H, K) i IB4 (C, F, I, L) u stražnjem rogu DM1 štakora 6 i 12 mjeseci nakon indukcije šećerne bolesti. Povećanje: 10x. Mjerilo je dugo 100 μ m i odnosi se na sve slike.

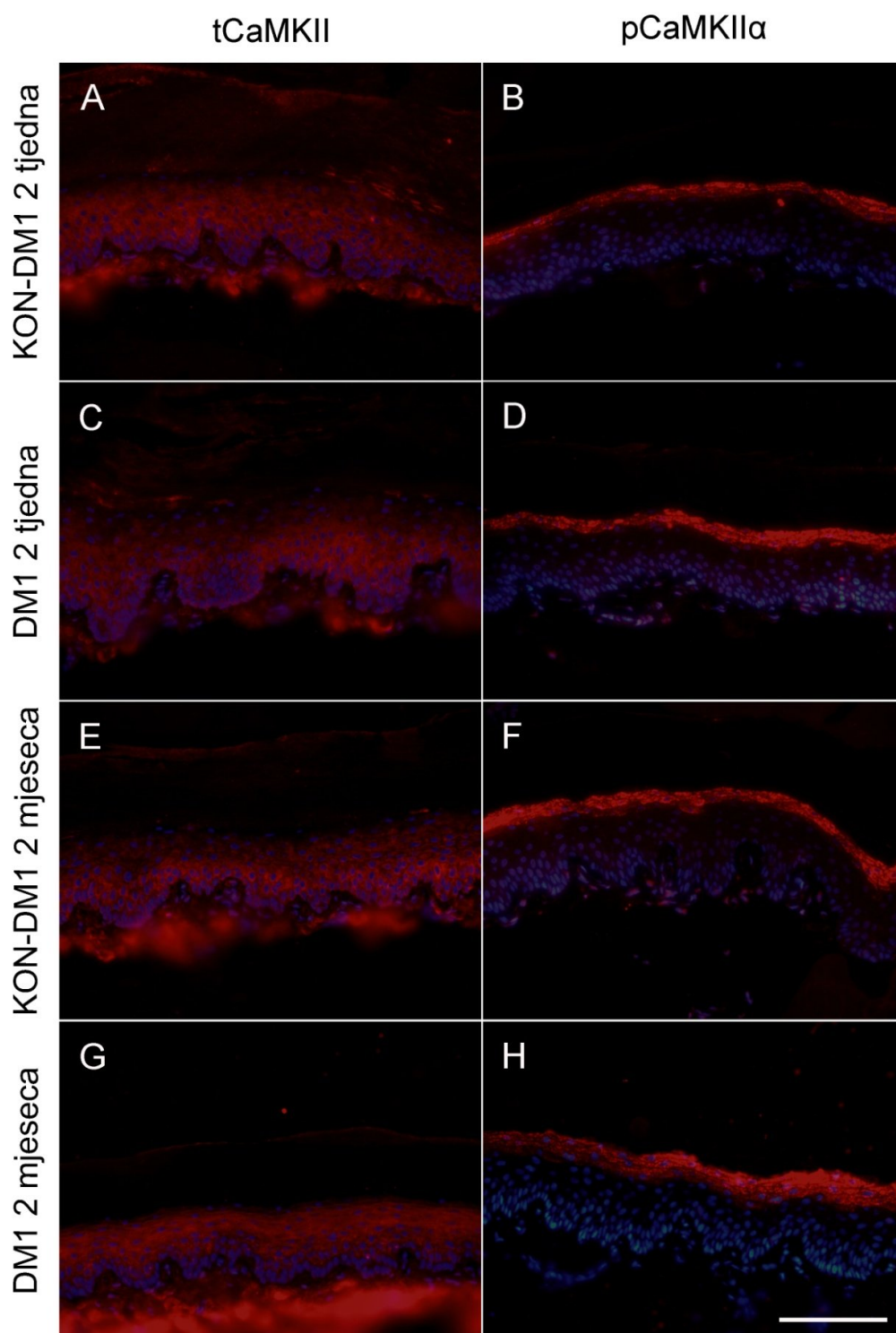
3.3.3. Rad 3

Validacija šećerne bolesti

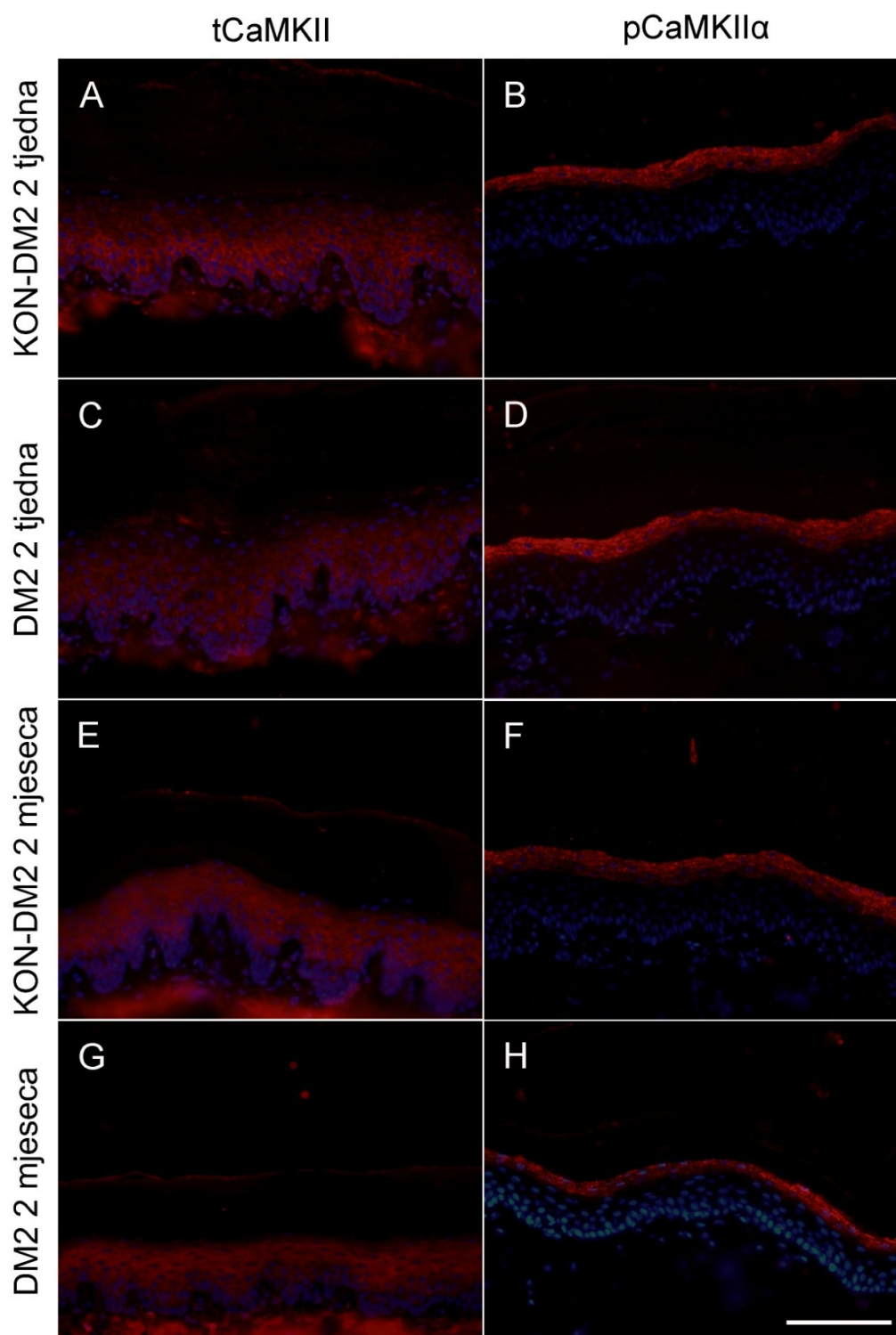
Četiri dana nakon i.p. injekcije STZ-a ili čistog citratnog pufera, koncentracija glukoze u plazmi DM1 štakora bila je značajno veća u usporedbi s kontrolnim štakorima ($533,0 \pm 46,6$ mg/dl vs. $90,9 \pm 4,4$ mg/dl, $p < 0,001$). DM2 štakori su također imali značajno veću razinu glukoze od kontrola ($342,3 \pm 42,3$ mg/dl vs. $93,0 \pm 8,3$ mg/dl, $p < 0,001$).

Utvrđivanje mjesta izražaja CaMKII u koži

Imunohistokemijske analize dokazale su postojanje CaMKII u koži. Mjesto izražaja enzima bilo je isto u kontrolnih i dijabetičnih štakora dva tjedna i dva mjeseca nakon indukcije. Ukupna CaMKII izražena je u svim slojevima epidermisa, dok je pCaMKII α ograničena na zrnati sloj epidermisa (lat. *stratum granulosum*) (Slike 10 i 11).

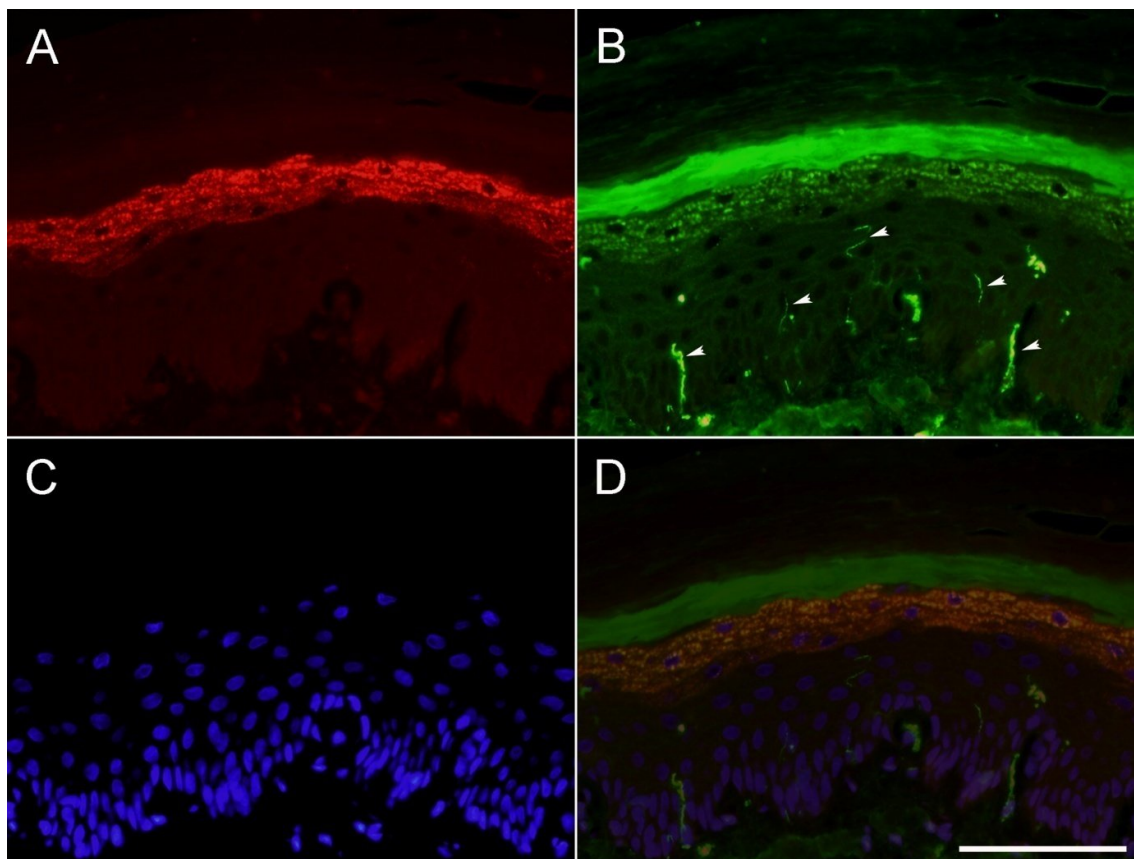


Slika 10. Reprezentativni primjer imunofluorescencijskog bojanja tCaMKII (A, C, E, G) i pCaMKII α (B, D, F, H) preklapljeno s DAPI bojanjem u koži DM1 štakora i kontrolnih životinja bez šećerne bolesti tipa 1, 2 tjedna i 2 mjeseca nakon indukcije šećerne bolesti. Povećanje: 40x. Mjerilo je dugo 100 μ m i odnosi se na sve slike.



Slika 11. Reprezentativni primjer imunofluorescencijskog bojanja tCaMKII (A, C, E, G) i pCaMKII α (B, D, F, H) preklopljeno s DAPI bojanjem u koži DM2 štakora i kontrolnih životinja bez šećerne bolesti tipa 2, 2 tjedna i 2 mjeseca nakon indukcije šećerne bolesti. Povećanje: 40x. Mjerilo je dugo 100 μ m i odnosi se na sve slike.

Preklopljena fotografija dvostrukog bojanja protutijelima PGP 9.5 i CaMKII pokazuje da nema izražaja CaMKII u PGP 9.5 pozitivnim intraepidermalnim živčanim vlaknima (Slika 12).

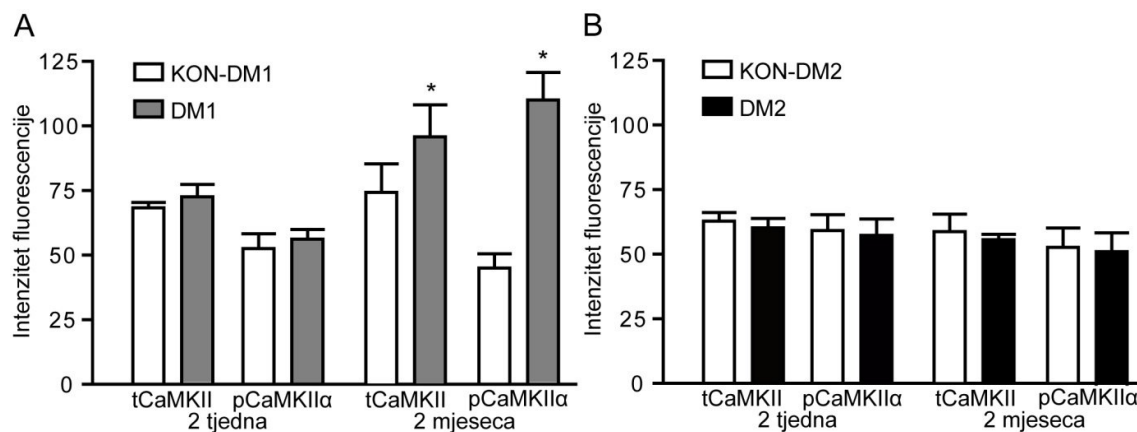


Slika 12. Smještaj pCaMKII α u koži (A), PGP9.5 pozitivna vlakna (B), DAPI obojane jezgre (C) i preklopljena fotografija pCaMKII α , PGP-9.5 i DAPI bojanja (D). Strelice označavaju intraepidermalna živčana vlakna. Povećanje: 40x. Mjerilo je dugo 100 μ m i odnosi se na sve slike.

Izražaj CaMKII u koži dva tjedna i dva mjeseca nakon indukcije šećerne bolesti tipa 1 i 2

Dva tjedna nakon indukcije šećerne bolesti, analizom je utvrđeno da ne postoji značajna razlika između DM1 štakora i njihovih kontrola u izražaju tCaMKII (71.7 ± 4.8 vs. 67.5 ± 2.1 ; $p=0.167$) i pCaMKII α (55.5 ± 3.8 vs. 51.9 ± 5.7 ; $p=0.348$) u koži (Slike 10A-D, 13A). Nadalje, nije bilo značajne razlike u izražaju tCaMKII (59.4 ± 3.6 vs. 61.9 ± 3.3 , $p=0.315$) i pCaMKII α (56.5 ± 6.3 vs. 58.3 ± 6.0 , $p=0.595$) između DM2 štakora i njihovih kontrola u analiziranoj koži (Slike 11A-D, 13B). Dva mjeseca nakon indukcije šećerne bolesti izražaj tCaMKII bio je

značajno veći u koži DM1 životinja u odnosu na KON-DM1 štakora ($65,7 \pm 12,5$ vs. $74,1 \pm 11,1$, $p=0,026$). Isto tako, izražaj pCaMKII α bio je značajno veći u koži DM1 životinja u odnosu na KON-DM1 životinje ($109,1 \pm 10,7$ vs. $44,1 \pm 5,5$, $p<0,001$) (Slike 10E-H, 13A). Naprotiv, DM2 štakori nisu u usporedbi s kontrolnim životinjama pokazali značajnu razliku u izražaju tCaMKII ($55,0 \pm 2,1$ vs. $58,2 \pm 6,7$, $p=0,763$) ili pCaMKII α ($50,6 \pm 7,3$ vs. $52,2 \pm 7,5$, $p=0,274$) u koži nakon dva mjeseca (slika 11E-H, 13B).



Slika 13. Izražaj tCaMKII i pCaMKII α u DM1 (A) i DM2 štakora (B) 2 tjedna i 2 mjeseca nakon indukcije šećerne bolesti. Podatci su prikazani kao aritmetička sredina \pm standardna devijacija. Zvezdica * označava statistički značajnu razliku (Student t-test) u usporedbi s odgovarajućima kontrolnim životinjama bez šećerne bolesti tipa 2 ($p<0.05$).

3.4. RASPRAVA

Rezultati znanstvenih radova broj 1 i 2 su pokazali promjene izražaja enzima CaMKII u akutnom i kroničnom stadiju šećerne bolesti. Uočen je povećan izražaj tCaMKII i pCaMKII α u stražnjem rogu kralježnične moždine u DM1 štakora 2 tjedna i 2 mjeseca nakon indukcije šećerne bolesti tipa 1, dok je značajno povećanje izražaja tCaMKII i pCaMKII α DM2 štakora zapaženo nakon 2 tjedna, ali ne i nakon 2 mjeseca. Ukupna tCaMKII je izražena ravnomjerno u laminama I-VI, dok su pCaMKII α i IB4 najviše izraženi u laminama I-III. Nije bilo razlike u izražaju IB4 između skupina u ranom stadiju obaju tipova šećerne bolesti. Nastavak istraživanja kroničnih promjena CaMKII 6 mjeseci i 1 godine nakon indukcije šećerne bolesti tipa 1 pokazao je značajno veći izražaj tCaMKII i pCaMKII α u DM1 štakora nakon 6 mjeseci, ali te promjene nisu bile uočljive nakon 1 godine. Nakon 1 godine tCaMKII i pCaMKII α su bile ravnomjerno izražene u laminama I-VI, dok je IB4 ostao izražen u laminama I-III kao u ranijem razdoblju.

Promjene CaMKII istražene su u raznim regijama za obradu boli, poput spinalnih ganglija stražnjih korjenova, sjela primarnih osjetnih neurona (44, 45). Ujedno je izražaj enzima CaMKII u kralježničnoj moždini proučavan na različitim modelima boli poput modela štakorske mononeuropatije i ozljede perifernog živca (34, 46-51).

Najranija i najozbiljnija patofiziološka zbivanja u dijabetičkoj senzornoj polineuropatiji zbivaju se u neuronima s najdužim aksonima (52). Zbog toga je u prvom i drugom radu imunohistokemijska analiza bila usredotočena na lumbalne djelove kralježnične moždine.

Opisane studije su prve koje prikazuju promjene izražaja CaMKII na modelu šećerne bolesti tipa 1 i 2. U prvom radu o akutnim promjenama opisano je različito mjesto izražaja CaMKII od onog u drugom radu o kroničnim promjenama šećerne bolesti. Naime, pokazano je da se izražaj pCaMKII α u ranom razdoblju najveći u laminama I-III stražnjeg roga kralježnične moždine, dok je u kasnom razdoblju pCaMKII α bila ravnomjerna u laminama I-VI. Ti rezultati ukazuju da promjene izražaja CaMKII prvo pogađaju regije uključene u obradu boli, a potom se ravnomjerno povećavaju, sugerirajući da povećan izražaj CaMKII s vremenom zahvaća cijeli osjetni prijenos. Promjene u izražaju IB4 nisu uočene u ranom stadiju šećerne bolesti tipa 1 i 2, niti u modelu kasne šećerne bolesti tipa 1. IB4 se veže na male nepeptidergičke neurone (53). Brzo smanjenje udjela neurona bojanih IB4 protutijelom primijećen je kod drugih modela boli (54). Međutim, takve promjene nisu uočene na modelu šećerne bolesti koji je proučavan u opisanim radovima, što sugerira da različiti modeli neuropatske boli imaju različite patofiziološke mehanizme.

Patofiziološki mehanizam sudjelovanja CaMKII u nocicepciji na razini kralježnične moždine može uključivati centralnu senzitivaciju kao jedan od mehanizama odgovornih za nastanak kronične neuropatske boli. Centralna senzitivacija je povećana aktivnost koja nastaje zbog sinaptičke plastičnosti u somatosenzornim neuronima stražnjeg roga kralježnične moždine nastale uslijed perifernog štetnog podražaja (55). Nakon senzitivacije, povećana sinaptička plastičnost smanjuje prag boli šireći sinaptički prijenos boli i bolne osjetljivosti na neozlijeđena područja (56).

Trajni aktivacijski signal može inducirati autofosforilaciju enzima omogućavajući tako aktivnost enzima čak i kada aktivacijski signal prestane. S obzirom da je povećanje tCaMKII i pCaMKII α prisutno i 60 dana nakon indukcije šećerne bolesti tipa 1, može se pretpostaviti da je CaMKII uključena u stvaranje i/ili održavanje Ca²⁺-posredovane središnje senzitivacije neurona stražnjeg roga kralježnične moždine. Naime, stražnji se rog kralježnične moždine odlikuje sporijom eliminacijom kalcija iz citoplazme preko endoplazmatskog retikuluma (57). Nadalje, trajno produljenje faze usporenja kalcijevog toka primijećeno je u hiperglikemijskim uvjetima, dajući dodatan dokaz da promjene u kalcijevom signaliziranju mogu doprinijeti razvoju neuropatije i njenih simptoma u ranom stadiju šećerne bolesti (58). Ciljano istraživanje mogućih promjena homeostaze kalcija u sekundarnim osjetnim neuronima moglo bi dodatno razjasniti patofiziologiju dijabetičke neuropatije. Naš nalaz povećanog izražaja CaMKII u stražnjem rogu dijabetičnih štakora daljnji je dokaz da CaMKII može biti uključena u patofiziologiju odraza šećerne bolesti na središnji živčani sustav. Sve do sad provedene studije o CaMKII su bile kratkoročne i ispitivale su promjene izražaja CaMKII kroz nekoliko dana ili tjedana. Budući da je DM doživotna, kronična bolest, u daljnjem smo istraživanju pokazali da je povećanje CaMKII prisutno i u zreloj dobi štakora. Smanjenje izražaja tCaMKII i pCaMKII α nakon godine dana pokazuje da dolazi do prilagođanih dinamičkih promjenama u regijama središnjeg živčanog sustava uključenim u obradu boli.

Šećerna bolest inducirana streptozotocinom uzrokuje uočljive neuronalne promjene homeostaze kalcija (57) što bi mogao biti rani molekularni znak razvoja dijabetičke osjetne neuropatije (52). Iako su istraživanja o specifičnoj ulozi CaMKII u dijabetičkoj neuropatiji novijega datuma (35), poznato je da je poremećeno kalcijско signaliziranje obilježje dijabetičke neuropatije. Oslabljena funkcija inhibitornih G-proteina doprinosi povećanoj struji kalcija u štakorskom modelu dijabetičke neuropatije (59). Nedavna istraživanja usredotočila su se na ulogu T-tipa kalcijских kanala u nociceptivnom prijenosu. Promjene T-tipa kalcijevih kanala u modelu bolne dijabetičke neuropatije povećavaju podražljivost senzornih neurona

uzrokujući i fiziološku (nociceptivnu) i patološku (neuropatsku) bol (15, 60). Inhibiranje CaMKII intratekalnom injekcijom njenih dvaju različitih inhibitora dovodi do značajnog smanjenja izražaja ukupne CaMKII i njene alfa izoforme (61).

Upravo zbog spoznaja proizašlih iz prvog i drugog rada, a i prethodnih istraživanja na spinalnim ganglijima provedenim u Laboratoriju za istraživanje boli Medicinskog fakulteta u Splitu, pretpostavljeno je da CaMKII može biti izražena i u koži. U koži se nalaze osjetni receptori koji putem primarnih osjetnih neurona čija se tijela nalaze u spinalnim ganglijima šalju informacije u SŽS. Tijela sekundarnih osjetnih neurona smještena su u stražnjem rogu kralježnične moždine (40).

U trećem radu je dokazano da se CaMKII izražava u koži. Ukupna CaMKII ravnomjerno je izražena u svim slojevima epidermisa, a pCaMKII α je ograničena na zrnati sloj. Niti tCaMKII niti pCaMKII α nije bila izražena u intraepidermalnim živčanim vlaknima. Dva mjeseca nakon indukcije šećerne bolesti kod štakora, značajno povećanje epidermalnog izražaja tCaMKII i pCaMKII α zabilježeno je u DM1 štakora u usporedbi s njihovim kontrolama. DM2 štakori nisu pokazali nikakve promjene u odnosu na kontrolnu skupinu.

Do sada je postojalo samo jedno istraživanje o postojanju CaMKII u koži. Ichikawa i sur. su opisali CaMKII u subepitelnom i intraepitelnom živčanim vlaknima kože lica, sluznice nosa i nepca (62). Ponovljena imunohistokemijska bojanja pokazala su da se CaMKII (tCaMKII i pCaMKII α) nije obojala u živčanim vlaknima kože s tabana stražnje šape štakora pozitivno obojanog s PGP 9.5 protutijelom. Obrazac bojanja pCaMKII α sličan je bojanju kožnog proteina nalik na kalmodulin (engl. *calmodulin-like skin protein* – CLSP), dok tCaMKII boja slična područja kao kalmodulin (engl. *calmodulin* – CaM). Ranije je opisana prisutnost CLSP u koži ograničena na zrnati, dok je CaM bio izražen u svim slojevima epidermisa (63, 64). Kalmodulin je proučavan u mnogim kožnim poremećajima s epidermalnom hiperproliferacijom ili diskeratinizacijom (65, 66). Nedavno otkriveni CLSP uključen je u diferencijaciju keratinocita i pretpostavlja se da obavlja svoju biološku aktivnost nakon formiranja kompleksa s kalcijem (64, 67, 68). Aktivacija CaMKII posredovana je nizom događaja u kojima je Ca²⁺/kalmodulinski kompleks ključna karika (69).

Spinalni gangliji sadrže tijela primarnih osjetnih neurona s perifernim završecima u koži (40). Iako je ovo istraživanje potvrdilo da je CaMKII izražena u koži, daljnja je analiza pokazala da enzim nije izražen u živčanim vlaknima.

Sve je više dokaza da se živčani sustav odražava na upalna, proliferativna ili reparativna zbivanja u tkivima (70). Različiti neuropeptidi su izraženi u koži izravno s osjetnih neurona ili s različitih stanica kože (71). Isto ektodermalno podrijetlo kože i živčanog sustava moglo bi objasniti epidermalni izražaj CaMKII, enzima široko rasprostranjenog u središnjem živčanom sustavu.

Nakon dva mjeseca, DM2 štakori su imali niže vrijednosti koncentracije glukoze u plazmi nego DM1 štakori. Niska doza STZ-a uzrokovala je blago smanjenje izlučivanja inzulina (43). Istraživanje na STZ-om induciranoj šećernoj bolesti pokazalo je da liječenje inzulinom dovodi do vraćanja vrijednosti izražaja CaMKII u mozgu štakoru na početnu vrijednost (72). Smanjeno, ali postojano izlučivanje inzulina moglo bi objasniti zbog čega u DM2 štakora nije bilo razlike u izražaju CaMKII nakon dva mjeseca.

Novi rezultati ovdje prikazani podupiru teoriju da su komplikacije šećerne bolesti uzrokovane međudjelovanjem različitih produkata glikoziliranja. Jedan takav glikotoksin, metilgliksal, potiče TRPA1 kanale što dovodi do razvoja boli i bolnih metaboličkih neuropatija (73, 74) te je pokazano da blokiranje TRPA1 smanjuje mehaničku hipersenzitivnost prisutnu u šećernoj bolesti (75). Sličan produkt glikoziliranja, 4-hidroksinonenal također uzrokuje bol aktivacijom TRPA1 receptora (76). Reaktivni kisikovi spojevi (engl. *reactive oxygen species* – ROS), često povišeni u šećernoj bolesti, doprinose razvoju neuropatske boli aktiviranjem CaMKII u neuronima stražnjeg roga kralježnične moždine (77).

U ovoj su disertaciji opisane prve studije o izražaju enzima CaMKII u kralježničnoj moždini i koži istraženoj na modelu šećerne bolesti tipa 1 i 2. Rezultati podupiru teoriju o ulozi CaMKII u razvoju bolnih neuropatskih promjena u šećernoj bolesti s promjenama koje započinju u koži kao mjestu prvog kontakta s bolnim podražajem te putem primarnih odnosno sekundarnih osjetnih neurona putuju prema regijama za obradu boli. U budućim istraživanjima CaMKII se može nastaviti proučavati kao potencijalna meta farmakoloških intervencija s ciljem ublažavanja neuropatskih simptoma nastalih kao posljedica šećerne bolesti.

3.5. ZAKLJUČCI

1. Indukcija šećerne bolesti tipa 1 uzrokovala je povećanje izražaja CaMKII u ranom razdoblju šećerne bolesti, što navodi na zaključak da CaMKII sudjeluje u putu prijenosa bolnog podražaja.
2. Dvanaest mjeseci od indukcije šećerne bolesti smanjuje se izražaj CaMKII što uzrokuje kompenzatorne promjene u regijama za obradu boli središnjeg živčanog sustava.
3. Enzim CaMKII izražen je u regiji osjetnih završetaka primarnog neurona u koži, no ne u intraepidermalnim živčanim vlaknima već difuzno u epidermisu.
4. Razlike u izražaju CaMKII upućuju na drugačije patofiziološke mehanizme razvoja dijabetičke neuropatije ovisno o tipu 1 ili 2 šećerne bolesti.

3.6. SAŽETAK

Uvod: Šećerna bolesti (DM) jedan je od vodećih uzroka periferne neuropatije u razvijenim zemljama svijeta. Mehanizam nastanka dijabetičke neuropatije nije razjašnjen, no smatra se da enzim kalcij/kalmodulin-ovisna protein kinaza II (CaMKII) sudjeluje u njenom nastanku. CaMKII se nalazi u velikim količinama u svim stanicama živčanog sustava. Do sada je u modelu šećerne bolesti istraživana na razini spinalnog ganglija. Cilj ovih studija bio je proširiti spoznaju o prisutnosti CaMKII u koži gdje se nalaze osjetni receptori i u stražnjem rogu kralježnične moždine gdje dolazi do prijenosa signala primarnog osjetnog neurona sekundarnom.

Metode: Model šećerne bolesti tipa 1 (DM1) induciran je intraperitonealnom (i.p.) injekcijom 55 mg/kg streptozotocina (STZ), a model šećerne bolesti tipa 2 (DM2) kombinacijom masne hrane i i.p. injekcije 35 mg/kg STZ-a. U radu broj 1 i 3 štakori su žrtvovani nakon dva tjedna i dva mjeseca nakon čega je istražen izražaj tCaMKII i pCaMKII α u stražnjem rogu kralježnične moždine i u koži tabana stražnjih šapa. U radu broj 2 štakori kojima je induciran DM1 žrtvovani su nakon šest mjeseci i godinu dana te im je proučen izražaj CaMKII u stražnjem rogu kralježnične moždine.

Rezultati: Mjerenjem tjelesne mase i glukoze u plazmi potvrđena je uspješna indukcija DM1 i DM2. Dva tjedna i dva mjeseca od indukcije došlo je do povećanja izražaja tCaMKII i pCaMKII α u stražnjem rogu DM1 štakora, dok je značajan porast tCaMKII i pCaMKII α u DM2 štakora zapažen nakon 2 tjedna, ali ne i nakon 2 mjeseca. Nakon šest mjeseci DM1 štakori su i dalje imali veći izražaj tCaMKII i pCaMKII α , ali te promjene nisu bile statistički značajne nakon 1 godine. Ukupna tCaMKII je u ranom stadiju DM-a izražena ravnomjerno u laminama I-VI, dok je pCaMKII α ograničena na laminama I-III. U kasnom DM-u tCaMKII i pCaMKII α izražene su u laminama I-VI. Dva mjeseca nakon indukcije DM-a, dokazano je značajno povećanje tCaMKII i pCaMKII α u epidermisu DM1 štakora, bez prisutnosti u intaepidermalnim živčanim vlaknima. DM2 štakori nisu pokazali nikakve promjene CaMKII u koži. Ukupna CaMKII ravnomjerno je izražena u svim slojevima epidermisa, a pCaMKII α je ograničena na zrnati sloj epidermisa.

Zaključci: Dobiveni rezultati pokazuju da CaMKII sudjeluje u putu prijenosa bolnog podražaja. Razlike u izražaju CaMKII upućuju na drugačije patofiziološke mehanizme razvoja dijabetičke neuropatije između tipova 1 i 2 šećerne bolesti.

3.7. SUMMARY

The expression of calcium²⁺/calmodulin-dependent protein kinase II (CaMKII) in pain pathways from periphery to central nervous system in type 1 and 2 diabetic model

Introduction: Diabetes mellitus (DM) is the leading cause of peripheral neuropathy in developed countries of the world. Pathophysiology of diabetic neuropathy is unclear but it has been suggested that the enzyme Calcium/calmodulin-dependent protein kinase II (CaMKII) is involved in its development. CaMKII is highly abundant in the nervous system. So far, CaMKII was investigated in dorsal root ganglia of diabetic rats. The aim of this study was to analyze the expression of CaMKII in the skin, location of the sensory receptors and in the dorsal horn of spinal cord, location of primary sensory neuron synapse with secondary sensory neuron.

Methods: Diabetes mellitus type 1 (DM1) was induced with 55 mg/kg streptozotocin (STZ), and diabetes mellitus type 2 (DM2) using a combination of high-fat diet and low-dose STZ (35 mg/kg). In manuscript No 1 and 3 rats were sacrificed after two weeks and two months. The expression tCaMKII and pCaMKII α in dorsal horn and skin from plantar surface of hind paws was quantified. Manuscript No 2 explored expression of CaMKII in dorsal horn 6 and 12 months after DM1 induction.

Results: Body weight and plasma glucose measurement confirmed successful induction of both types of DM. Two weeks and two months following DM1 induction tCaMKII and pCaMKII α increased in dorsal horn, while DM2 animals showed increased expression of tCaMKII and pCaMKII α after 2 weeks but not after 2 months. Increased expression of tCaMKII and pCaMKII α was seen in dorsal horn of DM1 rats 6 months after diabetes induction. It was restored to control values after 12 months. Total tCaMKII expressed predominantly in laminae I-VI in early-phase DM while pCaMKII α was pronounced the most in laminae I-III. In late DM tCaMKII and pCaMKII α were expressed in laminae I-VI. Two months after DM induction, significant increase of tCaMKII and pCaMKII α was observed in the epidermis of DM1 rats with no presence in the intraepidermal nerve fibers. DM2 rats did not develop changes of CaMKII expression in skin. Total CaMKII was uniformly distributed throughout the epidermis and pCaMKII α was limited to *stratum granulosum*.

Conclusions: Our results indicate potential role of CaMKII in the development of painful diabetic neuropathy. Different expression patterns of CaMKII between type 1 and 2 diabetes suggest different pathophysiological mechanisms of development of diabetic neuropathy.

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Znanstveni radovi

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Kongresna priopćenja

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5. Jelacic Kadic A, **Boric M**, Kostic S, Sapunar D, Puljak L. Intrathecal and intraganglionic injection of calcium/calmodulin-dependent protein kinase II inhibitors as potential treatment of neuropathic pain-related behavior. Poster presentation. Workshop Application of biomaterials and in vivo imaging in stem cell research. Zagreb, Croatia, March 27-29, 2014
6. Jelacic Kadic A, **Boric M**, Kostic S, Sapunar D, Puljak L. The effects of intraganglionic injection of calcium/calmodulin-dependent protein kinase II inhibitors on pain-related behavior in diabetic neuropathy. Oral presentation. 4th RECOOP TriNet Meeting. Split, Croatia, October 10-13, 2013.
7. Jelacic Kadic A, **Boric M**, Ferhatovic L, Banozic A, Sapunar D, Puljak L. Intrathecal inhibition of calcium/calmodulin-dependent protein kinase II in diabetic neuropathy adversely affects pain-related behavior. Oral presentation. 4th RECOOP TriNet Meeting. Split, Croatia, October 10-13, 2013.

8. Ferhatovic L, Jelacic A, **Boric M**, Banozic A, Spunar D, Puljak L. Expression of calcium/calmodulin-dependent protein kinase II in dorsal root ganglia in diabetic rats 6 months and 1 year after diabetes induction. Poster presentation. 3rd Annual Meeting of the Scandinavian Association for the Study of Pain. Helsinki, Finland, June 13-15, 2013.

Usavršavanja

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4. RADOVI OBJEDINJENI U DISERTACIJI

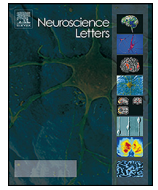
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The expression of calcium/calmodulin-dependent protein kinase II in the dorsal horns of rats with type 1 and type 2 diabetes[☆]

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HIGHLIGHTS

- tCaMKII increased in dorsal horn of DM1 animals after diabetes induction.
- pCaMKII α increased in dorsal horn of DM1 animals after diabetes induction.
- Diabetes type 2 animals did not show changes in CaMKII expression after 2 months.
- The expression of pCaMKII α was pronounced the most in laminae I–III.
- Difference in the IB4 expression was not observed between groups.

ARTICLE INFO

Article history:

Received 14 April 2014

Received in revised form 15 July 2014

Accepted 17 July 2014

Available online 24 July 2014

Keywords:

Diabetes mellitus

CaMKII

Dorsal horn

Rat

Neuropathic pain

ABSTRACT

The activation of calcium/calmodulin-dependent protein kinase II (CaMKII) has been proposed as a key factor in chronic pain development. This study therefore aimed to investigate the expression of CaMKII in the dorsal horn in a rat model of early phase diabetes mellitus (DM) types 1 and 2. Sprague–Dawley rats were used. DM1 was induced using streptozotocin (STZ) (55 mg/kg injected intraperitoneally (i.p.)). DM2 was induced using a combination of a high fat diet (HFD) and STZ (35 mg/kg i.p.). Controls received an i.p. injection of pure citrate buffer solution. DM2 animals and their controls also received HFD 2 weeks prior to the i.p. injection. Rats were sacrificed 2 weeks and 2 months after diabetes induction. The expression of tCaMKII, pCaMKII α and IB4 in the dorsal horns was quantified using immunohistochemistry. Increased expression of tCaMKII and pCaMKII α was seen in the dorsal horns of DM1 animals 2 weeks and 2 months after diabetes induction. In DM2 animals, similar changes in the expression of tCaMKII and pCaMKII α were observed after 2 weeks, but not after 2 months. The expression of pCaMKII α was most pronounced in laminae I–III. No difference in IB4 expression was observed between the groups. These results suggest a potential role for CaMKII in diabetic neuropathy development. Inhibition of CaMKII signaling pathways should be further explored as a potential treatment target in painful diabetic neuropathy.

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1. Introduction

Diabetes mellitus (DM) is the leading cause of peripheral neuropathy in developed countries [1]. Diabetic neuropathy is a common complication of diabetes and pain is one of its most disturbing symptoms [2,3]. However, little is known about the causes of diabetic neuropathic pain and treatment is often unsatisfactory [4]. Long-term potentiation (LTP), a form of synaptic plasticity successfully induced in the STZ model of DM, is known to contribute

to the development of chronic pain [5,6]. N-methyl-D-aspartate (NMDA) receptors are important in LTP and they mediate calcium influx from the extracellular space into the cytosol, triggering a cascade of events leading, among other things, to the activation of calcium–calmodulin protein kinase II (CaMKII) [7]. After its phosphorylation, the major CaMKII isoform, CaMKII α , remains activated even when calcium influx returns to baseline levels [8]. This perpetual activation of CaMKII is the focus of pain modulation studies and a possible factor in chronic pain development [9]. CaMK II is probably crucial for the induction and early-phase maintenance of LTP in the dorsal horn [10].

Our group previously described the increased expression of CaMKII in dorsal root ganglia (DRG) in rat models of early phase DM types 1 and 2, but an association with pain-related behavior was seen only in the DM1 model [11,12]. Direct injection of CaMKII

[☆] Grant support: The study was funded by the Croatian Foundation for Science (HRZZ) Grant No. 02.05./28 awarded to Livia Puljak.

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inhibitor into the DRG in early-phase diabetes significantly reduced pCaMKII expression in the DRG and attenuated pain-related behavior in a modality specific fashion [13]. Changes in CaMKII expression were also observed in the DRG and dorsal horn in late-phase DM [14,15]. However, dorsal horn expression of CaMKII in early-phase DM has not been reported. Changes in CaMKII early in DM may contribute to the development of chronic diabetic neuropathy. Therefore it would be valuable to gain more insight into expression of this enzyme in neural tissues that are crucial for nociceptive pathways. The aim of this study was to investigate the expression of CaMKII in the dorsal horn in an animal model of the early phases of diabetes types 1 and 2.

2. Methods

2.1. Ethics

The Ethics Committee of the University of Split School of Medicine approved the study. Experimental procedures and protocols followed the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Diabetes induction and validation

Forty-five, 8-week-old, male, Sprague–Dawley rats weighing ~200 g were used. The DM1 model involved intraperitoneal (i.p.) injection of 55 mg/kg of streptozotocin (STZ) freshly dissolved in citrate buffer (pH=4.5) after overnight fasting [11]. Control rats (CON-DM1) received an i.p. injection of pure citrate buffer. Rats were fed with regular laboratory chow during the experiment. The DM2 model involved a combination of high fat diet (HFD) and low dose STZ as previously described [16]. DM2 rats were fed with HFD (58% fat, 25% protein, 17% carbohydrate; PF 4269, Mucedola srl, Settimo Milanese, Italy) for 2 weeks and then i.p. injected with 35 mg/kg of STZ dissolved in citrate buffer (pH 4.5).

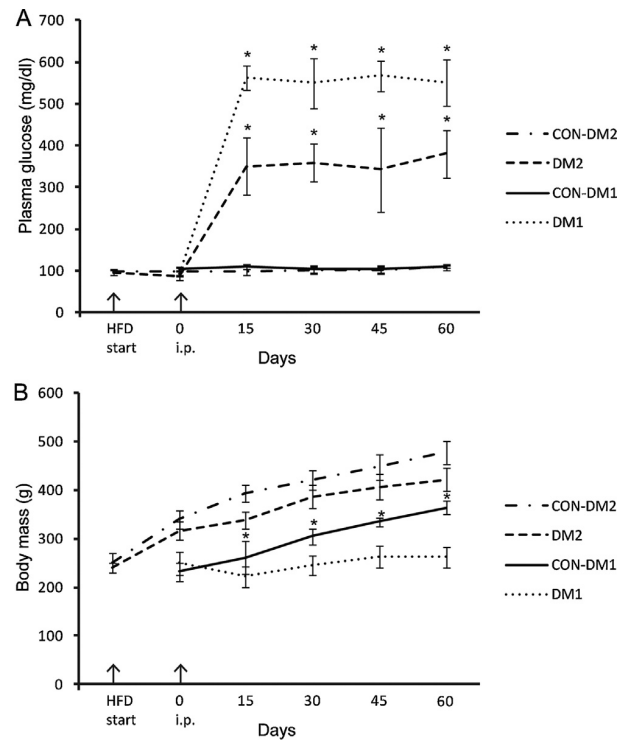


Fig. 1. Plasma glucose concentration (A) and body mass (B). Data are presented as mean ± standard deviation (SD). Asterisk * denotes significant difference ($p < 0.05$) from respective controls without diabetes (one way ANOVA followed by LSD post hoc test). Legend: CON-DM1 = control group for type 1 diabetes model, DM1 = animals with type 1 diabetes, CON-DM2 = control group for type 2 diabetes model, DM2 = animals with type 2 diabetes. i.p. = intraperitoneal injection (Day 0), HFD = high fat diet, DM2 and CON-DM2 animals were fed with HFD 2 weeks prior to i.p. injection.

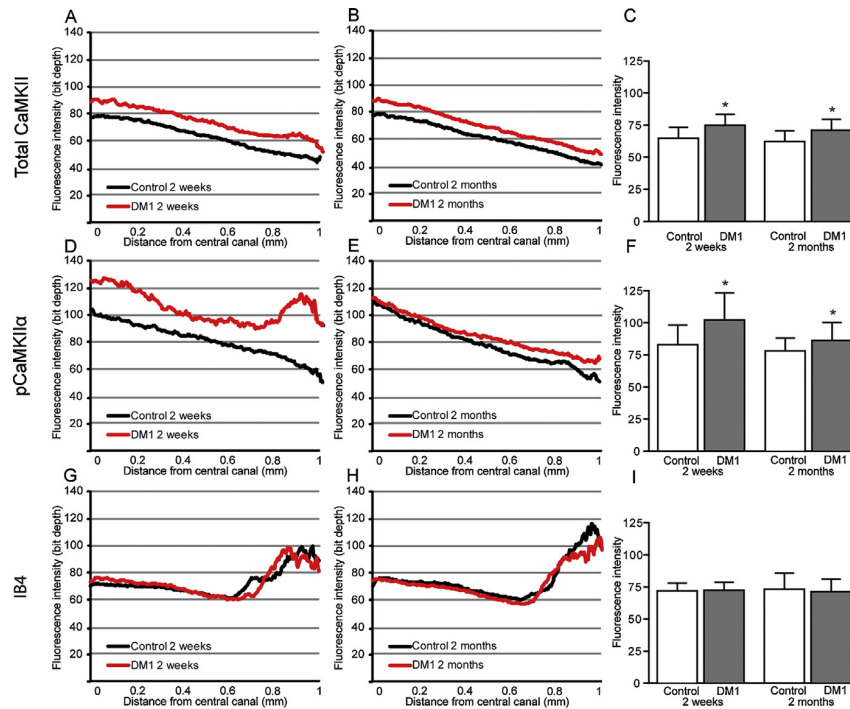


Fig. 2. DM1 dorsal horn average fluorescence intensity values of (a–c) total CaMKII, (d–f) pCaMKII α and (g–i) IB4. Data are presented as mean ± SD. Asterisk * denotes a significant difference (Student *t*-test) from respective controls without diabetes ($p < 0.05$).

DM2 control rats (CON-DM2) received pure citrate buffer solution after 2 weeks HFD. To prevent ketoacidosis in the 2-month experiment, animals received 1 unit of long-acting insulin (Lantus Solostar, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany) once a week.

Plasma glucose concentrations and body mass were measured regularly. The criteria for validating DM induction were plasma glucose >300 mg/dl for DM1 and >200 mg/dl for DM2 on the 4th day after STZ administration. Based on these criteria, one rat in the DM1 group and three in the DM2 group were excluded. One rat died during the experiment. Thermal and mechanical stimuli (acetone test, analgesia meter, pin prick test, and von Frey fibers) were used to verify that the animals had developed pain-related behavior.

Finally, there were 5 rats in each of the eight groups: DM1 and DM2 models and their respective controls for 2-week and 2-month experiments. The 15-day and 60-day time points were chosen because they represent an early stage of diabetes when changes in pain-related behavior and CaMKII expression in DRG were observed [16].

2.3. Tissue collection and immunohistochemistry

At 15 and 60 days after DM induction, rats were anesthetized with isoflurane (Forane, Abbott Laboratories Ltd., Queenborough, UK), perfused transcardially, and lumbar spinal cords from levels L5–L3 were removed, postfixed and prepared as described previously [14]. The spinal cord sections (8 μ m thick) were done

on a cryostat and placed on glass slides [11]. Immunohistochemical analysis was performed for detection of total CaMKII (tCaMKII), its alpha isoform and isolectin B4 (IB4) expression. Primary rabbit polyclonal antibodies were used in a 1:100 dilution to detect tCaMKII (sc-9035, lot# F0304, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphorylated CaMKII alpha isoform (pCaMKII α , sc-12886-R, lot# K2305, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary detection of tCaMKII and CaMKII α was performed using Rhodamin red X-conjugated secondary antibody (Donkey Anti-rabbit IgG (H+L) Jackson ImmunoResearch, Lot No. 106114, dilution 1:300). After final rinsing in PBS, all slides were mounted, air-dried and cover-slipped (Immu-Mont, Shandon, Pittsburgh, PA, USA). IB4 immunostaining was performed using fluorescein isothiocyanate-conjugated (FITC) IB4 (1:50 dilution, Sigma, St. Louis, MO, USA). Staining controls involved omission of primary antibody from the staining procedure, which resulted in no staining of analyzed spinal cord tissue.

2.4. Quantitative analysis

Spinal cord photomicrographs were taken and analyzed as described previously [14]. Background subtraction was performed on all photomicrographs, including the negative controls (samples without primary antibody). Fluorescence intensity values were acquired for the dorsal horn along a line positioned between the dorsal root entry zone and the central canal (Metamorph Line scan function, scan width 15 pixels) as described previously [17].

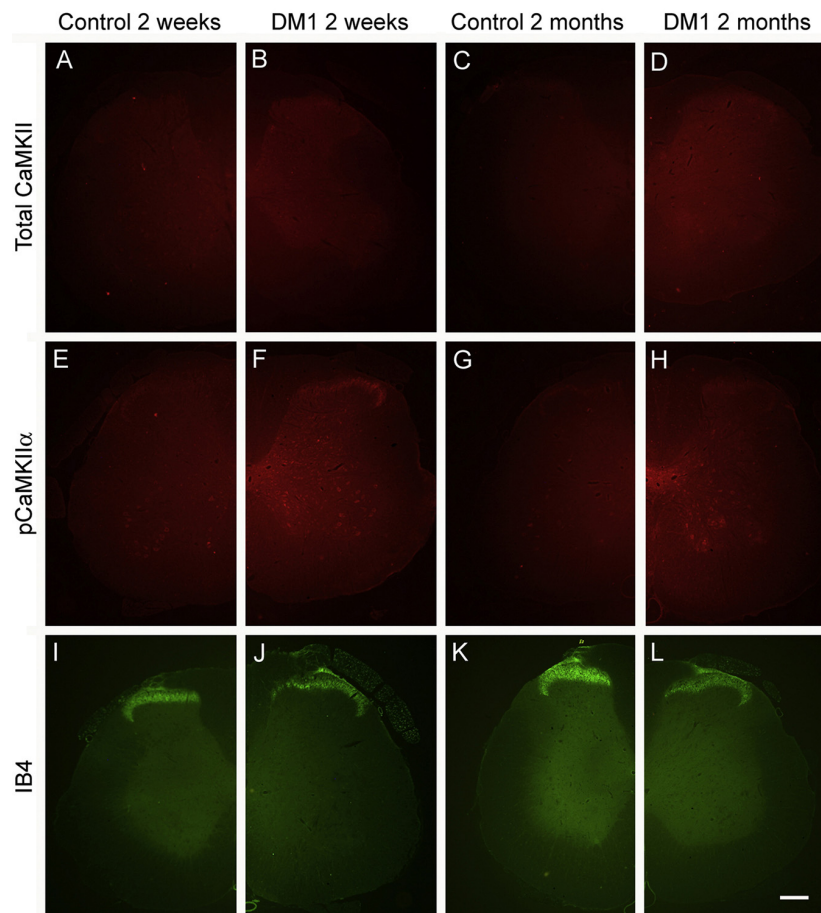


Fig. 3. Representative images of (a, b, c, d) total CaMKII, (e, f, g, h) pCaMKII α , and (i, j, k, l) IB4 staining in the dorsal horn of DM1 animals and respective controls without diabetes after 15 and 60 days. Magnification, 4 \times . Scale bar: 100 μ m, applies to all.

2.5. Statistical analysis

Comparisons of glucose plasma level and weight in control and diabetic animals were analyzed using ANOVA for repeated measurements and one way ANOVA followed by LSD post hoc test. Comparisons between control and diabetic tissue findings were analyzed using Student *t*-test (Statistica 7.0; StatSoft, Tulsa, OK, USA). Data are presented as *t*-values (*t*-test) and *F*-values (ANOVA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Validation of diabetic models

Plasma glucose concentrations increased significantly in both DM1 ($F(4, 20) = 105.1$; $p < 0.001$) and DM2 groups ($F(4, 20) = 42.7$; $p < 0.001$) following diabetes induction. On the 15th day, a significant increase in plasma glucose was observed in DM1 rats compared to CON-DM1 rats ($t(8) = 33.6$; $p < 0.001$) as well as in DM2 rats compared to their respective controls ($t(8) = 8.3$; $p < 0.001$). Measurements on the 30th, 45th and 60th day showed the same trend (data not shown) (Fig. 1A).

Fifteen days after diabetes induction, significant impairment in weight gain was observed in DM1 rats compared to CON-DM1 rats ($t(8) = -3.5$; $p = 0.008$). The CON-DM1 group continued to gain body mass while the DM1 group stayed close to the baseline body mass values. After diabetes induction, body mass increased significantly in both the DM2 ($F(4, 20) = 207.1$; $p < 0.001$) and the CON-DM2 groups ($F(4, 20) = 201.6$; $p < 0.001$) (Fig. 1B).

3.2. CaMKII expression in the dorsal horn

Two weeks after diabetes induction, the expression of tCaMKII was significantly higher in DM1 animals than in controls

($t(58) = 4.7$; $p < 0.001$; Figs. 2A, C and 3A, B). Expression of pCaMKII α showed a significant difference between DM1 and CON-DM1 ($t(52) = 4.2$; $p < 0.001$; Figs. 2D, F and 3E, F). At 2 weeks, the DM2 animals showed a significant difference in tCaMKII expression ($t(70) = 5.3$; $p < 0.001$; Figs. 4A, C and 5A, B) and pCaMKII α expression ($t(48) = 5.7$; $p < 0.001$; Figs. 4D, F and 5E, F) compared to CON-DM2 animals.

While expression of tCaMKII and its alpha isoform showed the same trend in DM1 and DM2 animals at 15 days, at 60 days there were differences between the diabetic types. DM1 animals showed a significant increase in tCaMKII expression compared with controls at 60 days ($t(72) = 4.1$; $p < 0.001$; Figs. 2B, C and 3C, D). This increase was accompanied by higher pCaMKII α expression ($t(30) = 2.6$; $p < 0.001$; Figs. 2E, F and 3G, H). However, the DM2 group did not differ from controls in the expression of tCaMKII ($t(73) = 0.1$; $p = 0.921$; Figs. 4B, C and 5C, D) or pCaMKII α in the dorsal horn at this time point ($t(49) = 0.9$; $p = 0.388$; Figs. 4E, F and 5G, H).

Fluorescence of tCaMKII was uniformly distributed along the measuring line positioned between the dorsal root entry zone and the central canal (Figs. 2A, B and 4A, B), and pCaMKII α was expressed most in laminae I–III (Figs. 2D, E and 4D, E). Similarly, IB4 was predominantly seen in laminae I–III of the dorsal horn (data not shown) but no difference in expression was observed between the groups (Figs. 2G–I, 4G–I, 3I–L, 5I–L).

4. Discussion

We observed increased expression of tCaMKII and pCaMKII α in spinal cord dorsal horn neurons of rats with STZ-induced DM1 at 2 weeks and 2 months after induction of diabetes, compared to controls. We observed a similar increase in the expression of tCaMKII and pCaMKII α in the DM2 model after 2 weeks, but not

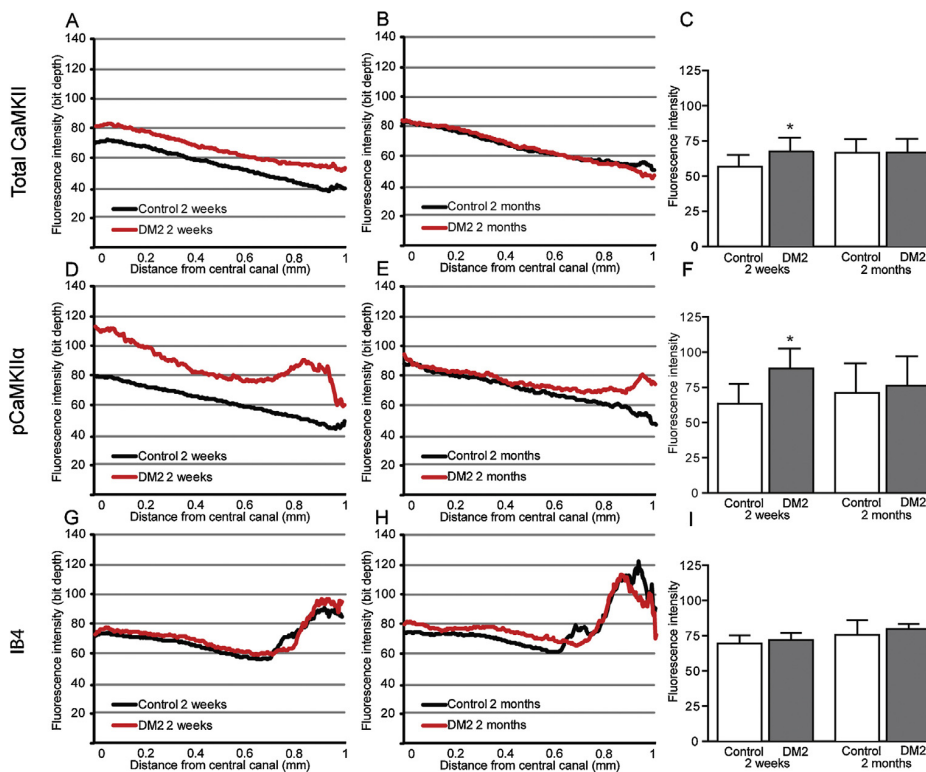


Fig. 4. DM2 dorsal horn average fluorescence intensity values of (a–c) total CaMKII, (d–f) pCaMKII α and (g–i) IB4. Data are presented as mean \pm SD. Asterisk * denotes a significant difference (Student *t*-test) from respective controls without diabetes ($p < 0.05$).

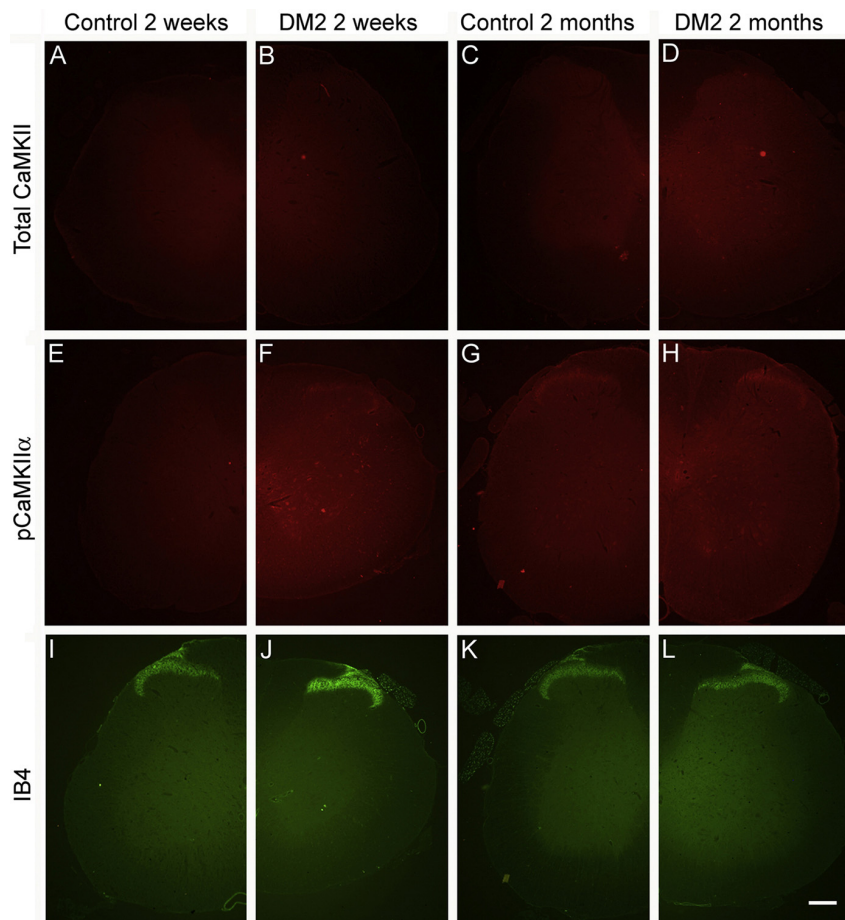


Fig. 5. Representative images of (a, b, c, d) total CaMKII, (e, f, g, h) pCaMKII α and (i, j, k, l) IB4 staining in the dorsal horn of DM2 animals and respective controls without diabetes after 15 and 60 days. Magnification, 4 \times . Scale bar: 100 μ m, applies to all.

after 2 months. The expression of pCaMKII α was most pronounced in laminae I–III. There was no difference in IB4 expression between groups. These results are consistent with our previous finding of increased expression of tCaMKII and pCaMKII α in DRG at 2 weeks and 2 months after diabetes induction [11].

Changes in CaMKII expression in the spinal cord have been observed in different pain models, such as a rat model of mononeuropathy and a nerve injury model [18,19]. However, our previous study was the first to describe changes in CaMKII expression in a model of DM1 [14]. We previously demonstrated increased expression of CaMKII, with no difference in IB4 expression, in the dorsal horn during long-term diabetes [14].

In the current study of early diabetes, we observed a different pattern of CaMKII expression to that seen in late diabetes in our previous study. Namely, we showed that pCaMKII α expression was increased only in laminae I–III of the dorsal horn, while in late-phase diabetes the expression of activated CaMKII α was greatest in laminae I–VI [14]. These results indicate that in the early phases of diabetes changes in CaMKII expression affect pain processing regions, while in the late phase its increase is diffuse, suggesting that increased CaMKII expression would affect the entire sensory input.

Changes in IB4 expression were not observed in the early phase of DM1 and DM2 models, nor previously in a model of late phase DM1 [14]. IB4 binds to small neurons that lack peptidergic transmission [20]. A rapid decrease of neurons labeled with IB4 has been observed in other pain models [21]. However, such changes

were not observed in DM models studied by our group, which may indicate that different models of neuropathic pain have different pathophysiologicals.

The present study is the first report of CaMKII expression in the dorsal horn in a DM2 model and the first report of early changes in CaMKII expression in DM1. Continuous phosphorylation of CaMKII may induce its own synthesis and subsequently result in the up-regulation of CaMKII. As the increase in tCaMKII and pCaMKII α is maintained for 60 days after induction of DM1, we suggest that CaMKII may be involved in the generation and/or maintenance of Ca²⁺-mediated central sensitization in spinal dorsal horn neurons. Our finding that CaMKII expression is increased in the dorsal horn in diabetic rats is further evidence that CaMKII may be involved in the pathophysiology of diabetes in the central nervous system.

Streptozotocin-induced diabetes causes prominent changes in neuronal calcium homeostasis [22] and this could be an early molecular marker of the onset of diabetic sensory neuropathy [23]. Recent studies have suggested a role for T-type calcium channels in nociceptive signaling causing both physiological (nociceptive) and pathological (neuropathic) pain [24]. Inhibition of CaMKII via intrathecal injection using two different CaMKII inhibitors resulted in a significant decrease in expression of total CaMKII and its alpha isoform [13].

After 2 months, DM2 animals exhibited lower plasma glucose concentrations than DM1 animals. Low-dose STZ induces a mild impairment of insulin secretion [16]. A study of STZ-induced diabetes showed that insulin treatment resulted in recovery of CaMKII

activity in rat brain close to control values [25]. The reduced but persistent insulin secretion might explain why the DM2 group did not show increased CaMKII expression at 2 months.

This novel finding supports the theory that diabetic complications are caused by the build-up of various glycation products. One such glycotoxin, methylglyoxal, stimulates TRPA1 channels leading to pain and painful metabolic neuropathies [26,27] and it has been shown that blocking TRPA1 attenuates mechanical hypersensitivity in diabetes [28]. A similar glycation product, 4-hydroxynonenal, also causes pain by activation of TRPA1 receptors [29]. Reactive oxygen species (ROS), often elevated in diabetes, contribute to neuropathic pain via activation of CamKII in the spinal dorsal horn neurons [30].

Further studies could assess the effects of CaMKII inhibitors on chronic neuropathic pain induced by diabetes.

In conclusion, our results suggest a potential role for CaMKII in the development of painful diabetic neuropathy. Inhibition of CaMKII signaling pathways should be further explored as a potential treatment target in painful diabetic neuropathy.

Acknowledgements

We are grateful to Ms. Dalibora Behmen and Dr. Liz Wager for language editing.

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DRUGI RAD

Calcium/calmodulin-dependent protein kinase II in dorsal horn neurons in long-term diabetes

Matija Boric, Antonia Jelcic Kadic, Lejla Ferhatovic, Damir Sapunar and Livia Puljak

The aim of this study was to investigate the expression of total calcium/calmodulin-dependent protein kinase II (CaMKII) and its phosphorylated α isoform in the dorsal horn of the spinal cord in an animal model of long-term diabetes. Diabetes was induced in Sprague–Dawley rats using 55 mg/kg streptozotocin, and expression of total CaMKII, the phosphorylated α -CaMKII isoform, and isolectin B4 was analyzed by immunohistochemical analysis in the dorsal horn of the spinal cord 6 and 12 months after diabetes induction. Results were compared with those for control rats of the same age. Increased expression of total CaMKII and its activated α isoform was seen in the dorsal horn of diabetic rats 6 months after diabetes induction. The increase in CaMKII fluorescence was restored to control values after 12 months. The expression of activated α -CaMKII 12 months after diabetes induction was most pronounced in laminae I–VI of the dorsal horn, not corresponding with

the highest expression of isolectin B4 in laminae I–III. Increased expression of CaMKII in the dorsal horn during long-term diabetes could be involved in the development of neuropathic symptoms in diabetes. The expression pattern of CaMKII during long-term diabetes indicates that it affects the entire sensory input. *NeuroReport* 24:992–996 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

NeuroReport 2013, 24:992–996

Keywords: Calcium/calmodulin-dependent protein kinase II, diabetes mellitus, dorsal horn, rat, streptozotocin

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Received 27 July 2013 accepted 29 August 2013

Introduction

Painful neuropathy is one of the most common complications of diabetes mellitus (DM). Various changes in the peripheral nervous system may be associated with neuropathic symptoms, and injuries of peripheral nerves can lead to extensive changes in the central transition and modulation of pain. These plastic changes may contribute to neuropathic symptoms. Treatment of neuropathic pain is often unsatisfactory, as currently available drugs often provide insufficient analgesia or are associated with adverse effects. Therefore, for the development of effective novel analgesics to treat neuropathic pain it is important to elucidate its underlying molecular mechanisms [1,2].

It has been postulated that activated neuronal calcium/calmodulin-dependent protein kinase II (CaMKII) serves as a critical component of the intracellular signaling pathways that contribute to neuropathic pain and persistent neuronal hyperexcitability of dorsal horn neurons after spinal cord injury. Further, treatment with a CaMKII inhibitor resulted in significant attenuation of mechanical allodynia and aberrant wide dynamic-range neuronal activity evoked by various stimuli [3]. An increased CaMKII expression and phosphorylation was also found in rat spinal dorsal horn neurons after applying intradermal capsaicin, a well-defined pain model [4]. It was also found that the spinal nerve ligation pain model increases the ipsilateral spinal activity of CaMKII and that a potent CaMKII inhibitor reverses spinal

nerve ligation-induced mechanical allodynia, thermal hyperalgesia, and activation of CaMKII without impairing locomotor function [5]. On the basis of these studies in different models of pain, it is suggested that persistent CaMKII activation may be involved in chronic central neuropathic pain and that by targeting this key signaling protein, novel and effective analgesics may be devised for treating chronic, central neuropathic pain [3].

CaMKII has not been extensively investigated in the neural tissues of diabetic rats. It has been shown that the expression of CaMKII increases in dorsal root ganglia of diabetic rats 2 weeks and 2 months after induction of diabetes with streptozotocin [6,7]; however, there are no studies on the effects of CaMKII in spinal dorsal horn neurons. On the basis of the findings showing increased expression of CaMKII in spinal dorsal horn neurons of other animal pain models, the aim of this study was to investigate the expression of total CaMKII and its phosphorylated α isoform in spinal dorsal horn laminae in an animal model of long-term diabetes.

Methods

Animals

Adult Sprague–Dawley rats ($N=24$) were used in the study. The animals were raised in a controlled environment (temperature: $22 \pm 1^\circ\text{C}$, light schedule: 12 h of light and 12 h of dark) and housed in pairs in plastic

cages with sawdust and corn flooring in the ratio 3:1 at the University of Split Animal Facility.

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

Diabetes type 1 was induced in rats after overnight fasting using 55 mg/kg streptozotocin that was freshly dissolved in citrate buffer (pH = 4.5). Control rats were injected intraperitoneally with pure citrate buffer solution. All rats were fed *ad libitum* with regular laboratory chow (4RF21 GLP; Mucedola srl, Settimo Milanese, Milano, Italy). Plasma glucose level was measured using a glucometer (OneTouchVITA; LifeScan, High Wycombe, Buckinghamshire, UK). Animals with a glucose level above 300 mg/dl on the fourth day after injection with streptozotocin were considered diabetic. Three rats were excluded from the experiment because their glucose level was lower than 300 mg/dl. One rat died before the end of the experiment. The number of remaining rats was 20, and they were divided into four experimental groups: diabetic animals in 6-month experiments (DM-6, $N = 5$), control animals in 6-month experiments (CON-6, $N = 5$), diabetic animals in 12-month experiments (DM-12, $N = 5$), and control animals in 12-month experiments (CON-12, $N = 5$). Body weight was measured using an electronic scale (Classe, EK3650; Zhongshan Camry Electronic Co. Ltd, Guangdong, China). Glucose levels and body weight were monitored once a month throughout the experiment. The average glucose level of control rats was 104 mg/dl and of diabetic rats was 539 mg/dl during the 12 months of observation. At 12 months, the weight of diabetic rats was 310 g and that of control rats was 553 g. Diabetic rats received 1 U of long-acting insulin (Lantus Solostar; Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany) weekly to prevent ketoacidosis.

Tissue collection and immunohistochemical analysis

Six and 12 months after induction of diabetes, rats were killed under general anesthesia (isoflurane; Forane, Abbott Laboratories Ltd, Queenborough, UK). Their lumbar spinal cords were removed and postfixed for 48 h in Zamboni's fixative (4% paraformaldehyde and 15% picric acid in 0.1 M PBS) at pH 7.4. Thereafter, the spinal cords were left for 24 h in 0.01 M PBS. The tissues were cryoprotected overnight in 30% sucrose and embedded in optimal cutting temperature freezing medium (Tissue Tek, Tokyo, Japan). Transverse, 8- μ m-thick sections of the spinal cord were cut on a cryostat (Thermo Shandon Cryotome, Pittsburgh, Pennsylvania, USA) and placed on glass slides.

Immunohistochemical analysis was carried out for detection of total CaMKII (tCaMKII) and its α isoform. Primary rabbit polyclonal antibodies were used in a 1:100 dilution for detection of tCaMKII (sc-9035, lot# F0304; Santa Cruz Biotechnology, Santa Cruz, California, USA) and the phosphorylated α -CaMKII isoform (pCaMKII α -sc-12886-R, lot# K2305; Santa Cruz Biotechnology). Secondary detection of tCaMKII and its α isoform was performed using the secondary antibody, Texas red goat anti-rabbit IgG-B (dilution 1:100, sc-2780; Santa Cruz Biotechnology). After a final rinsing in PBS, all slides were mounted, air-dried, and cover-slipped (Immu-Mont, Pittsburgh, Pennsylvania, USA). Isolectin B4 (IB4) immunostaining was performed using fluorescein isothiocyanate-conjugated IB4 (1:50 dilution; Sigma, St. Louis, Missouri, USA). Staining controls included omission of primary antibody from the staining procedure, which resulted in no staining of spinal cord tissue.

Quantitative analysis

Every fourth section of each spinal cord was examined using a microscope (BX61; Olympus, Tokyo, Japan). Photomicrographs were taken using a cooled digital camera (DP71; Olympus), with the same magnification ($\times 40$), exposure, binning, and gain for each image. Images were analyzed on Metamorph software (Molecular Devices, Sunnyvale, California, USA), in which they were examined as monochromatic photomicrographs (2040 \times 1536 pixels, 12 bits, 0–4096 gray scale), following background subtraction. Fluorescence intensity values were acquired in the dorsal horn along the line positioned between the dorsal root entry zone and the central canal (Metamorph Linescan function, scan width 15 pixels), as described previously [8].

Statistical analysis

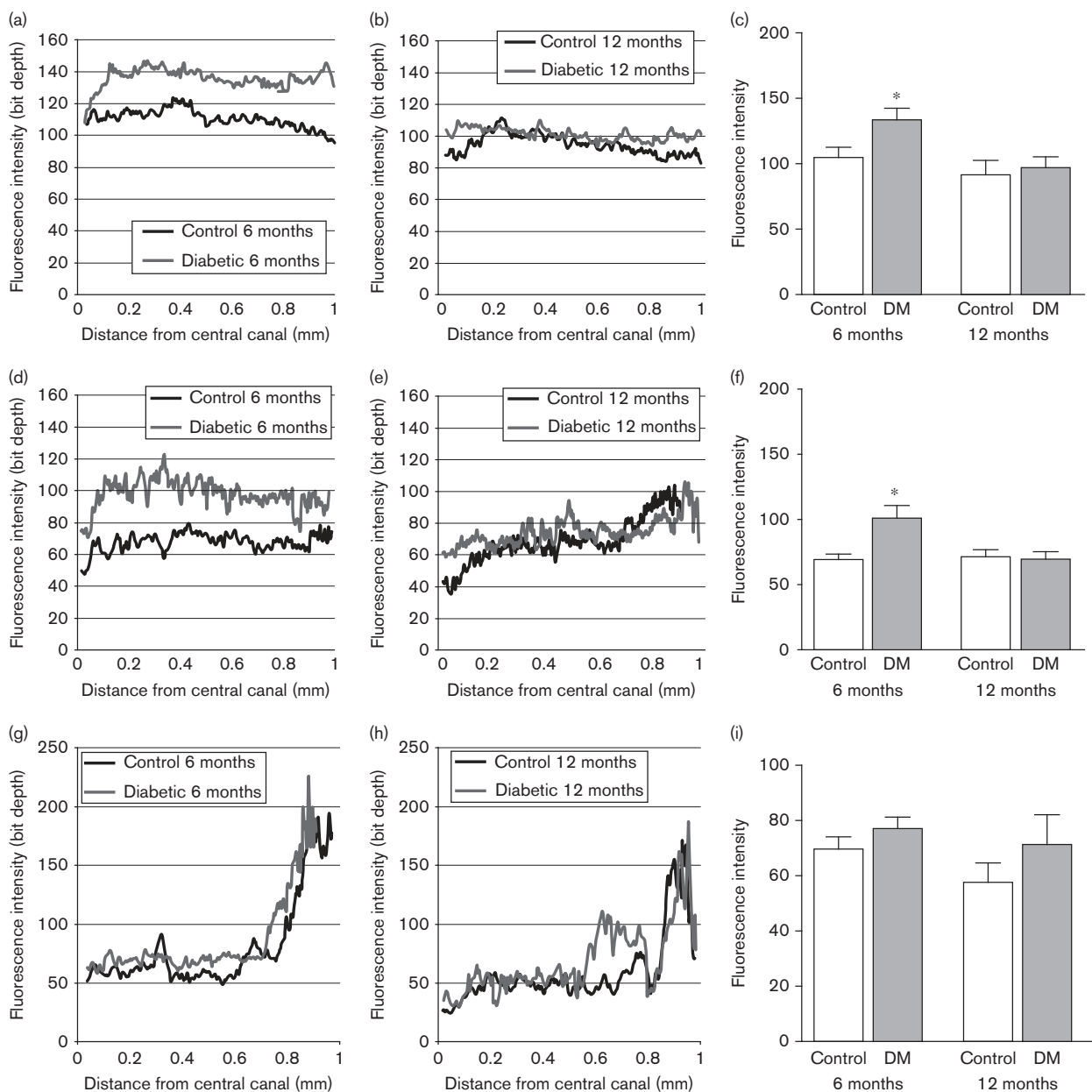
Comparisons between control and diabetic tissue findings were made using Student's *t*-test (Statistica 7.0; StatSoft, Tulsa, Oklahoma, USA). The data are presented as mean and SD. Statistical significance was set at *P* less than 0.05.

Results

Calcium/calmodulin-dependent protein kinase II expression in dorsal horn

Six months after induction of diabetes, a significant increase in tCaMKII expression was observed in the dorsal horn of diabetic rats, compared with controls (Fig. 1a and c). This increase was mediated through an increase in the expression of CaMKII α (Fig. 1d and f). Twelve months after induction of diabetes, there were no significant differences in the expression of tCaMKII (Fig. 1b and c) and pCaMKII α in the dorsal horn (Fig. 1e and f). The pattern of tCaMKII fluorescence was homogenous in the dorsal horn along the line from the central canal to the dorsal root entry zone after 6 and 12 months in diabetic and control rats (Fig. 1a and b), as well as for pCaMKII α after 12 months (Fig. 1d). Increased IB4 expression was observed in dorsal horns of diabetic rats after 6 and 12 months, although not significant. The most pronounced IB4 activity in both control and diabetic rats was

Fig. 1



Averaged fluorescence intensity values of (a–c) total CaMKII, (d–f) pCaMKII α , and (g–i) IB4 in the dorsal horns of control and diabetic rats. Data are presented as mean \pm SD. Asterisks denote a significant difference from control ($P < 0.05$). DM, diabetes mellitus; Cont., control. (a, d, and g) Data for 6-month experiments; (b, e, and h) data for 12-month experiments. CaMKII, calcium/calmodulin-dependent protein kinase II; IB4, isolectin B4; pCaMKII α , phosphorylated α -CaMKII isoform.

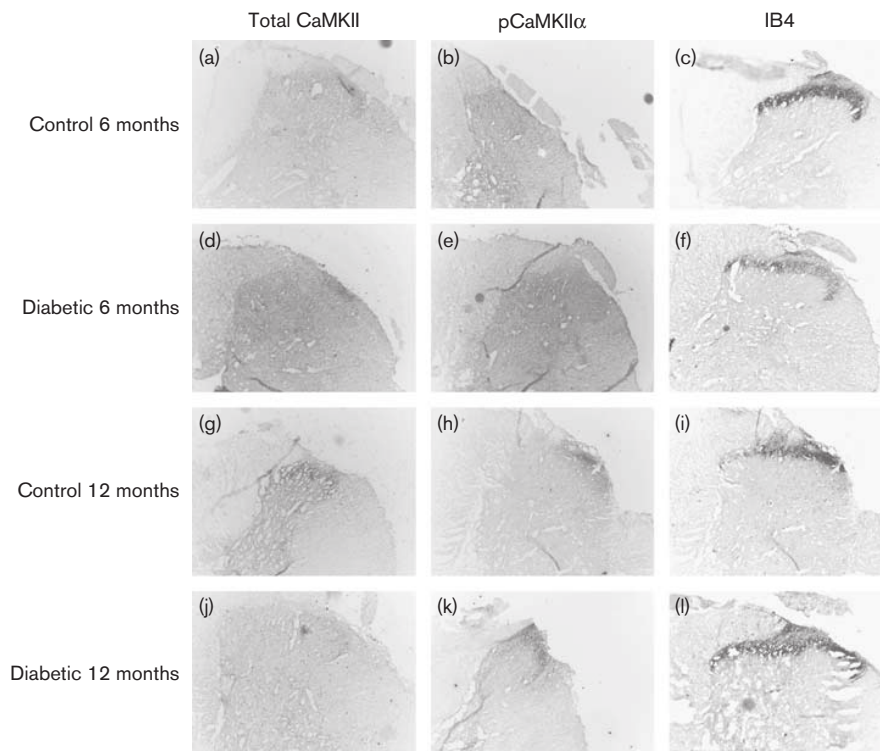
seen in laminae I–III of the dorsal horn (Fig. 1g and h). Increased expression of tCaMKII and pCaMKII α was seen diffusely in laminae I–VI (Fig. 1a and d). Representative images of tCaMKII, pCaMKII α , and IB4 staining are shown in Fig. 2.

Discussion

The present study found an increased expression of total CaMKII and its activated α isoform in the dorsal horn of

diabetic rats 6 months after diabetes induction. Although an increase in CaMKII fluorescence was observed even after 12 months, at that time point it did not reach statistical significance. Twelve months after diabetes induction, increased expression of tCaMKII and pCaMKII α was seen diffusely in laminae I–VI, whereas the highest expression of IB4 was observed in laminae I–III of the dorsal horn.

Fig. 2



Representative images of (a, d, g, j) total CaMKII, (b, e, h, k) pCaMKII α , and (c, f, i, l) IB4 staining in the dorsal horn of diabetic and control rats after 6 and 12 months. Magnification, $\times 10$. Scale bar: 100 μm , applies to all. CaMKII, calcium/calmodulin-dependent protein kinase II; IB4, isolectin B4; pCaMKII α , phosphorylated α -CaMKII isoform.

Changes in CaMKII expression have been documented in pain-processing regions such as the dorsal root ganglion, a seat of primary sensory neurons [9,10]. Increased expression of CaMKII in dorsal horn neurons after induction of neuropathy has been described previously in various pain models [5,11–16]. However, CaMKII has not been investigated in the dorsal horn of the diabetic neuropathy model.

All the studies on the expression of CaMKII that have been conducted thus far have been short-term studies examining changes in CaMKII expression within days or weeks. As diabetes is a chronic disease, the present study shows that an increase in CaMKII may be prolonged and visible well into adulthood in young rats. Our finding that the expression of tCaMKII and pCaMKII α is reduced after 12 months shows that CaMKII may be subject to dynamic and compensatory changes in pain-processing regions of the central nervous system.

Pathophysiological mechanisms of CaMKII involvement in nociception at the spinal level may include central sensitization as one of the mechanisms responsible for chronic neuropathic pain. Central sensitization is an increased activity resulting from synaptic plasticity in

somatosensory neurons in the dorsal horn of the spinal cord in response to peripheral noxious stimuli [17]. Following sensitization, the heightened synaptic plasticity reduces pain threshold and amplifies synaptic transmission of pain and a spread of pain sensitivity to noninjured areas [18].

Although studies on the specific role of CaMKII in diabetic neuropathy are very recent [6], it is already known that aberrant calcium signaling is a feature of diabetic neuropathy. It has even been suggested that altered calcium homeostasis could be an early molecular marker linked to the onset of diabetic sensory neuropathy [19]. Impaired function of an inhibitory G-protein contributes to increased calcium currents in a rat model of diabetic neuropathy [19]. In a model of painful diabetic neuropathy, changes in the T-type calcium current enhance excitability of sensory neurons [20]. Dorsal horn neurons of diabetic rats are characterized by slowdown of calcium elimination from the cytoplasm by the endoplasmic reticulum [21]. A definite prolongation of the decay phase of the calcium current transients was observed under diabetic conditions, providing further evidence that changes in calcium signaling in nociceptive

neurons may contribute to the development of neuropathy and its symptoms in the early stages of DM [22]. Therefore, direct investigation of possible changes in calcium-associated pathways in secondary nociceptive neurons could yield new insights into the pathophysiology of diabetic neuropathy.

The earliest and most severe pathophysiology in diabetic sensory polyneuropathy occurs in neurons with the longest axons [23]. Therefore, our immunohistochemical analyses were restricted to lumbar sections. Our finding that the expression of CaMKII is increased diffusely in the dorsal horn of diabetic rats is further confirmation that CaMKII may be involved in the pathophysiology of diabetes in the central nervous system, and warrants further experiments using therapies for delivery of CaMKII inhibitors through intrathecal or intraganglionic injection [8,24,25]. The pattern of expression of CaMKII shows that the enzyme is not preferentially located in certain laminae, but rather shows a diffuse increase, indicating that an increase in CaMKII affects the entire sensory input.

In this study, nociceptive behavior of animals was not studied, which can be considered a limitation. Future investigation will be carried out using a combination of pain behavioral techniques and immunohistochemical analysis to better correlate changes in activated α -CaMKII expression with the induction and maintenance of nociceptive hypersensitivity.

Conclusion

Our findings that tCaMKII and its activated α isoform are increased in the chronic diabetic state provide further support to the fact that this enzyme is involved in the development of neuropathic changes in diabetes. CaMKII could be a good candidate for pharmacological interventions aimed toward alleviation of neuropathic symptoms associated with diabetes.

Acknowledgements

This study was funded by the Croatian Foundation for Science (HRZZ) grant no. 02.05./28 awarded to Livia Puljak.

L.P. and D.S.: design and conception of the study. M.B., A.J.K., L.F.: data collection. L.P.: drafting of the manuscript. All authors: data interpretation and analysis, commenting and critically revising the manuscript, and approving the final version to be published.

Conflicts of interest

There are no conflicts of interest.

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Cutaneous expression of calcium/calmodulin-dependent protein kinase II in rats with type 1 and type 2 diabetes[☆]

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ARTICLE INFO

Article history:

Received 28 June 2014

Received in revised form 11 September 2014

Accepted 18 September 2014

Available online 26 September 2014

Keywords:

Skin

Diabetes mellitus

CaMKII

Rat

Streptozotocin

ABSTRACT

Changes in calcium–calmodulin protein kinase II (CaMKII) have been well demonstrated in nervous tissue of diabetic animal models. Skin shares the same ectodermal origin as nervous tissue and it is often affected in diabetic patients. The goal of this study was to analyze expression of CaMKII in rat foot pad 2 weeks and 2 months after induction of diabetes type 1 and 2.

Forty-two Sprague-Dawley rats were used. Diabetes mellitus type 1 (DM1) was induced with intraperitoneally (i.p.) injected 55 mg/kg of streptozotocin (STZ) and diabetes mellitus type 2 (DM2) with a combination of high-fat diet (HFD) and i.p. injection of low-dose STZ (35 mg/kg). Two weeks and two months following diabetes induction rats were sacrificed and skin samples from plantar surface of the both hind paws were removed. Immunohistochemistry was performed for detection of total CaMKII (tCaMKII) and its alpha isoform (pCaMKII α). For detection of intraepidermal nerve fibers polyclonal antiserum against protein gene product 9.5 (PGP 9.5) was used.

The results showed that CaMKII was expressed in the skin of both diabetic models. Total CaMKII was uniformly distributed throughout the epidermis and pCaMKII α was limited to stratum granulosum. The tCaMKII and pCaMKII α were not expressed in intraepidermal nerve fibers. Two weeks after induction of diabetes in rats there were no significant differences in expression of tCaMKII and pCaMKII α between DM1 and DM2 compared to respective controls. In the 2-month experiments, significant increase in epidermal expression of tCaMKII and pCaMKII α was observed in DM1 animals compared to controls, but not in DM2 animals.

This study is the first description of cutaneous CaMKII expression pattern in a diabetic model. CaMKII could play a role in transformation of skin layers and contribute to cutaneous diabetic changes. Further research on physiological role of CaMKII in skin and its role in cutaneous diabetic complications should be undertaken in order to elucidate its function in epidermis.

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Introduction

Epidermis is a highly specialized squamous epithelium consisting of basal, spinous, granular and cornified layers (Fuchs, 1990). Its differentiation and function are dependent on mechanisms which require calcium for their function (Hennings et al., 1989). Calcium is one of the most effective pro-differentiation agents of the epidermis with gradient that increases from basal to granular layers of cells (Bikle and Pillai, 1993; Pillai et al., 1993). It is believed that in the stratum granulosum calcium serves as an

inductor of keratinocyte transformation into corneocytes (Menon, 2002). In epidermis, transport and function of calcium are mediated by the calcium binding protein, calmodulin (CaM) (Fairley et al., 1985). CaM is involved in regulation of keratinocyte proliferation and differentiation. CaM is also elevated in hyperproliferative skin disease, psoriasis (Tucker et al., 1986). Calcium–calmodulin protein kinase II (CaMKII) is a major target of the CaM second messenger system (Colbran, 2004).

CaMKII is a ubiquitous multifunctional enzyme activated by increases in intracellular Ca²⁺ and it is encoded by four genes in mammals: α , β , γ and δ . Various compositions of CaMKII may explain its different cellular functions, including muscle contraction, synaptic vesicle release, proliferation and differentiation (Bruggemann et al., 2000; Colbran, 2004). Following its phosphorylation, CaMKII α remains activated and it is the key mediator of long-term potentiation which eventually at least partially contributes to development of neuropathic pain (Dai et al., 2005).

[☆] The study was funded by the Croatian Foundation for Science (HRZZ) grant no. 02.05./28 awarded to Livia Puljak.

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Like neurons, keratinocytes originate from the embryonic ectoderm and express a wide diversity of neurochemical properties (Hou et al., 2011). Lately, it has been demonstrated that keratinocytes secrete numerous neurochemical substances that modulate function of sensory nerve endings (Lumpkin and Caterina, 2007). Also, it has been implicated that changes in keratinocytes could contribute to development of chronic pain (Zhao et al., 2008).

Diabetes mellitus (DM) is a group of heterogeneous metabolic disorders characterized by hyperglycemia and glucose intolerance due to insulin deficient secretion, impaired effectiveness of insulin's action, or both (Callaghan et al., 2012). It causes various complications including neuropathic pain which is one of the most disturbing symptoms of diabetes (Backonja et al., 1998). Our previous study showed significant epidermal thinning and loss of intraepidermal nerve fibers (Boric et al., 2013b).

To date little is known about CaMKII activity in skin. Therefore, we investigated expression of CaMKII in rat foot pad after induction of diabetes type 1 and 2.

Methods

Ethics

Experimental procedures and protocols were approved by the Ethical Committee of the University of Split, School of Medicine. The animal care was in accordance to the guidelines of the institution and International Association for the Study of Pain.

Animals

Adult Male Sprague–Dawley rats ($N = 42$) weighing 160–200 g were used. Animals were raised under controlled conditions (temperature: 22 ± 1 °C; light schedule: 12 h of light and 12 h of dark) at University of Split Animal Facility. The duration of experiments was 2 months after induction of diabetes mellitus type 1 (DM1) and type 2 (DM2).

Diabetes induction

For DM1 induction, after overnight fasting, rats were i.p. injected with 55 mg/kg of streptozotocin (STZ) freshly dissolved in citrate buffer (pH = 4.5). Control rats (CON-DM1) were i.p. injected with pure citrate buffer solution. Animals in both groups were fed *ad libitum* with standard laboratory chow (4RF21 GLP, Mucedola srl, Settimo Milanese, Italy). DM2 was induced with a combination of high-fat diet (HFD) and low-dose STZ (Srinivasan et al., 2005). DM2 rats were fed *ad libitum* with HFD consisting of 58% of fat, 25% proteins and 17% carbohydrates (PF 4269, Mucedola srl, Settimo Milanese, Italy) for two weeks and then i.p. injected with 35 mg/kg of STZ dissolved in citrate buffer (pH 4.5) after an overnight fasting. DM2 control rats (CON-DM2) were also fed with HFD but after two weeks received i.p. injection of pure citrate buffer solution. Plasma glucose was measured with Single touch glucometer (OneTouch VITA, LifeScan, High Wycombe, UK) from blood collected from the tail vein of rats. In DM1 group all animals had the required glucose level higher than 300 mg/dl and in DM2 group two animals were excluded because their glucose level was lower than 200 mg/dl on the 4th day after i.p. injection. From further analysis were excluded those animals that did not develop pain-related behavior confirmed by a series of test (acetone test, analgesia meter, pin prick test and von Frey fibers).

The remaining rats ($N = 40$) were divided into eight groups: DM1 animals in 2-week experiments ($N = 5$) and its control group ($N = 5$), DM1 animals in 2-month experiments ($N = 5$) and its control group ($N = 5$), DM2 animals in 2-week experiments ($N = 5$) and its control group ($N = 5$), DM2 animals in 2-month experiments ($N = 5$) and its control group ($N = 5$).

Tissue processing and immunohistochemistry

All rats were anesthetized (Isoflurane; Forane, Abbott Laboratories Ltd., Queenborough, UK) and perfused transcardially with saline followed by 300 ml of Zamboni's fixative [2% paraformaldehyde and 15% picric acid in 0.01 M phosphate buffered saline (PBS) at pH 7.4].

Glabrous skin samples from medial plantar surface of the both hind paws were removed and placed on the paraffin block and post fixed in the Zamboni's fixative [2% paraformaldehyde and 15% picric acid in 0.01 M phosphate buffered saline (PBS) at pH 7.4] for 2 days and then washed in pure distilled water. After that tissue was transferred into formalin, dehydrated in alcohol, cleared in xylene and embedded in paraffin. The skin was sectioned on the microtome. For each sample of the skin, 5 μ m-thick sections perpendicular to the skin surface were collected and every fifth section was stained. Five sections per tissue were used in order to have same analysis regions of plantar skin. After deparaffinization, sections were

rehydrated in ethanol and water. Sections were briefly rinsed with distilled water, followed by heating in sodium citrate buffer (pH 6.0) for 12 min on 95 °C in microwave oven. After being cooled to the room temperature, sections were incubated with primary antibody.

To identify possible CaMKII expression in nerve fibers we used a double immunofluorescence method. Intraepidermal nerve fibers were detected with polyclonal antiserum against protein gene product 9.5 (PGP 9.5) and CaMKII was detected with CaMKII antibodies. The sections were incubated with mixture of PGP 9.5 primary antibodies raised in mouse (Cat. no. 480012, Invitrogen Corporation, Camarillo, CA, USA) diluted 1:1000 in PBS with either rabbit total CaMKII primary antibodies (sc-9035, lot# F0304, Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:100 or phosphorylated CaMKII alpha primary rabbit polyclonal antibodies (sc-12886-R, lot# K2305, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100. Primary PGP 9.5 antibodies were visualized with secondary biotinylated goat anti-mouse IgG-B (Cat. no. sc-2039, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 and followed by Streptavidin Alexa Fluor 488 conjugate (1:500; S-32354, lot 508205, Molecular Probes, Eugene, OR, USA) diluted 1:500. Secondary detection of tCaMKII and alpha isoform was performed using secondary antibody with Rhodamin red X-conjugated (Donkey Anti-rabbit IgG (H + L) Jackson Immuno Research, Lot No 106114, dilution 1:300). After secondary antibody incubation, the sections were washed in PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei. 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.

Qualitative and quantitative analysis for immunofluorescence

Skin sections were examined under a microscope (BX61, Olympus, Tokyo, Japan) and microphotographs were captured at 40 \times magnification using a digital camera (DP71, Olympus, Tokyo, Japan) always under same exposition, binning and gain. Image analysis was performed using Image J (National Institutes of Health, Bethesda, MD, USA) by digitizing microphotographs of either tCaMKII or pCaMKII α into monochrome microphotographs (2040 \times 1536 pixels, 12 bits, 0–4096 gray scale) which accurately delineate stained tissue from background. Background subtraction was performed on all microphotographs, including the negative control ones (samples without primary antibody). Using *Freehand selection* tool, areas of immunofluorescence were then selected, and the total average intensity of selected areas was measured. The average values of the intensity of each animal in control group was calculated, and compared with diabetic animals. The analysis was blind performed on at least five images per one animal in the group with and maximally variability in the group was ± 21.90 .

Statistical analysis

Comparisons between control and diabetic tissue findings were analyzed using Student's *t*-test. The data were presented as mean and standard deviation ($M \pm SD$). Any difference with $p < 0.05$ was considered statistically significant.

Results

Validation of diabetes

Four days after injection of STZ or pure citrate buffer solution, glucose level in plasma of DM1 animals was significantly higher compared to control animals (533.0 ± 46.6 mg/dl vs. 90.9 ± 4.4 mg/dl; $p < 0.001$). DM2 animals also had significantly higher glucose levels then controls (342.3 ± 42.3 mg/dl vs. 93.0 ± 8.3 mg/dl; $p < 0.001$).

CaMKII expression pattern in skin

Immunohistochemical analyses revealed that CaMKII was expressed in the skin. The expression pattern was the same in diabetic models two months after diabetes induction and control animals. Total CaMKII was expressed throughout all the layers of epidermis, while the pCaMKII α expression was restricted to the stratum granulosum of epidermis. Cell nuclei are stained with DAPI. Merging of tCaMKII/pCaMKII α with DAPI nuclear staining shows intracellular distribution of tCaMKII/pCaMKII α expression (Figs. 1 and 2).

Merged photographs of PGP 9.5, CaMKII and DAPI staining revealed no co-localization of CaMKII in PGP 9.5 positive neurofibers, indicating that CaMKII was not present in intraepidermal nerve fibers (Fig. 3).

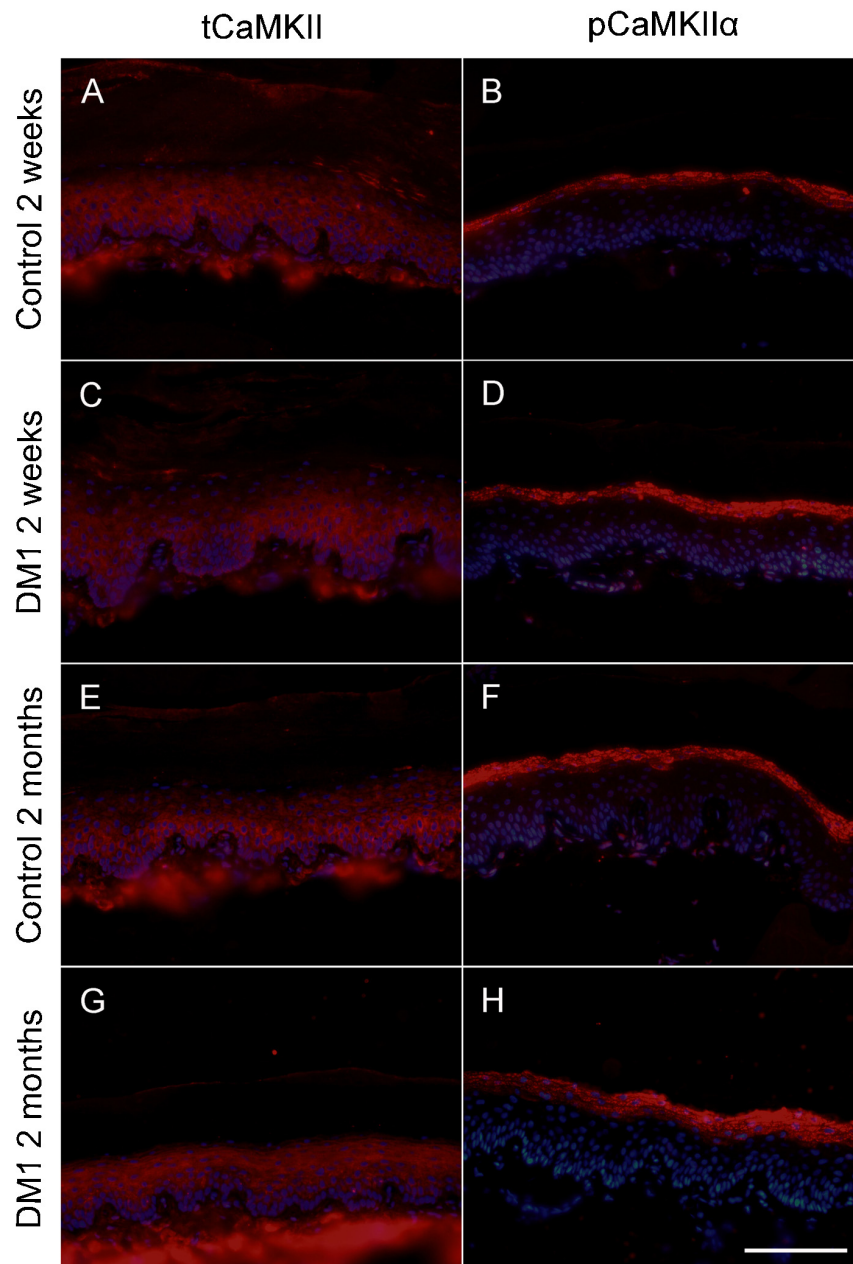


Fig. 1. Representative images of total CaMKII (A, C, E, G) and pCaMKII α (B, D, F, H) merged with DAPI staining in the skin of DM1 animals and respective controls without diabetes in the 2-week and 2-month experiment. Magnification, 40 \times . Scale bar: 100 μ m, applies to all.

CaMKII expression in diabetes type 1 and type 2 model

Two weeks after diabetes induction, comparison of CaMKII expression revealed that there was no significant difference between DM1 animals and their respective controls (CON-DM1) in the expression of tCaMKII (71.7 ± 4.8 vs. 67.5 ± 2.1 ; $p = 0.167$) and pCaMKII α (55.5 ± 3.8 vs. 51.9 ± 5.7 ; $p = 0.348$) in the analyzed skin (Figs. 1A–D and 4A).

Furthermore, there was no significant difference in the expression of tCaMKII (59.4 ± 3.6 vs. 61.9 ± 3.3 ; $p = 0.315$) and pCaMKII α (56.5 ± 6.3 vs. 58.3 ± 6.0 ; $p = 0.595$) between DM2 and CON-DM2 animals in the analyzed skin (Figs. 2A–D and 4B).

Two months after diabetes induction, tCaMKII expression was significantly higher in the skin of DM1 animals compared to CON-DM1 rats (95.7 ± 12.5 vs. 74.1 ± 11.1 ; $p = 0.026$). Likewise, the

expression of the phosphorylated alpha isoform of CaMKII (pCaMKII α) in the skin was significantly higher in DM1 animals compared to the CON-DM1 animals (109.1 ± 10.7 vs. 44.1 ± 5.5 ; $p < 0.001$) (Figs. 1E–H and 4A).

On the contrary, DM2 animals, when compared to their respective controls (CON-DM2), did not show significant difference in the expression of tCaMKII (55.0 ± 2.1 vs. 58.2 ± 6.7 ; $p = 0.763$) or pCaMKII α (50.6 ± 7.3 vs. 52.2 ± 7.5 ; $p = 0.274$) in the skin (Figs. 2E–H and 4B).

Discussion

This study showed that CaMKII was expressed in the skin. Total CaMKII was uniformly distributed throughout the epidermis and pCaMKII α was limited to stratum granulosum. Both tCaMKII and

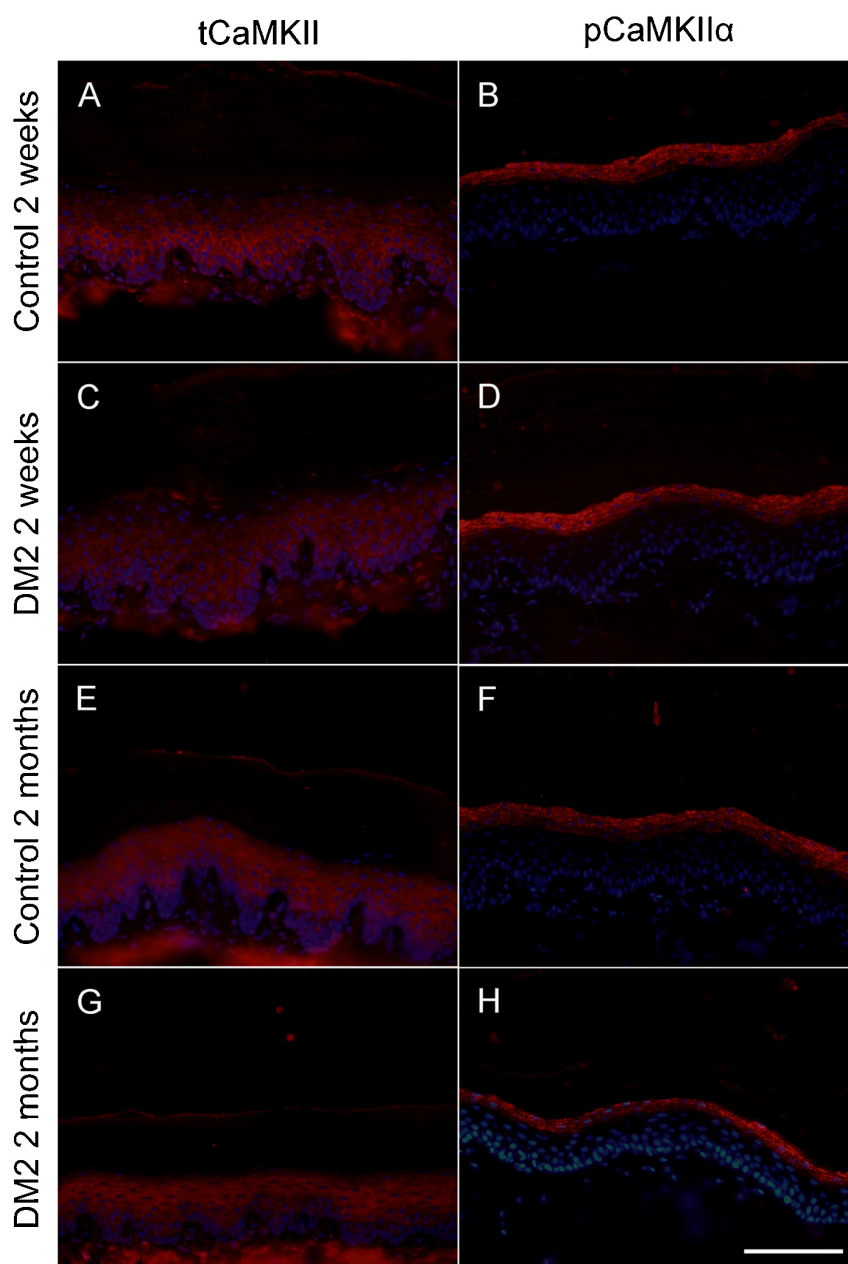


Fig. 2. Representative images of total CaMKII (A, C, E, G) and pCaMKII α (B, D, F, H) merged with DAPI staining in the skin of DM2 animals and respective controls without diabetes in the 2-week and 2-month experiment. Magnification, 40 \times . Scale bar: 100 μ m, applies to all.

pCaMKII α were not visible in intraepidermal nerve fibers. Two months after induction of diabetes in rats, significant increase in epidermal expression of tCaMKII and pCaMKII α was observed in DM1 animals compared to its controls while DM2 animals did not show any changes compared to controls.

Up to date, there has been only one report of CaMKII existence in skin. Ichikawa et al. described CaMKII in subepithelial and intraepithelial nerve fibers in facial skin, nasal mucosa and palate (Ichikawa et al., 2004). Our repeated double staining revealed that CaMKII (tCaMKII or pCaMKII α) was not expressed in rat foot pad nerve fibers visualized with PGP 9.5. Judged by the data available in the literature, immunofluorescent-labeling pattern of pCaMKII α is close to that of calmodulin-like skin protein (CLSP) while tCaMKII stained similar areas as CaM (Mehul et al., 2001). Previous studies have indicated that presence of CLSP in skin was restricted to

stratum granulosum while CaM was expressed throughout all the layers of the epidermis (Mehul et al., 2001; Wollina et al., 1991). Calmodulin was observed in many skin disorders with epidermal hyperproliferation or dyskeratinization (Tucker et al., 1984; van Erp and van de Kerkhof, 1987). Recently discovered CLSP is involved in keratinocyte differentiation and it probably performs its biological activity after the formation of complex with calcium (Crivici and Ikura, 1995; Mehul et al., 2001, 2000). Therefore, overlapping pattern of CaMKII and CLSP expression in skin indicates that the role CaMKII in skin should be further explored.

Activation of CaMKII is mediated through series of events with Ca²⁺/calmodulin complex as the key player (Colbran, 2004). The CaMKII expression has already been explored in different regions of the nervous system such as dorsal root ganglion (DRG), spinal cord or brain. Our studies involving diabetic rats showed that 2

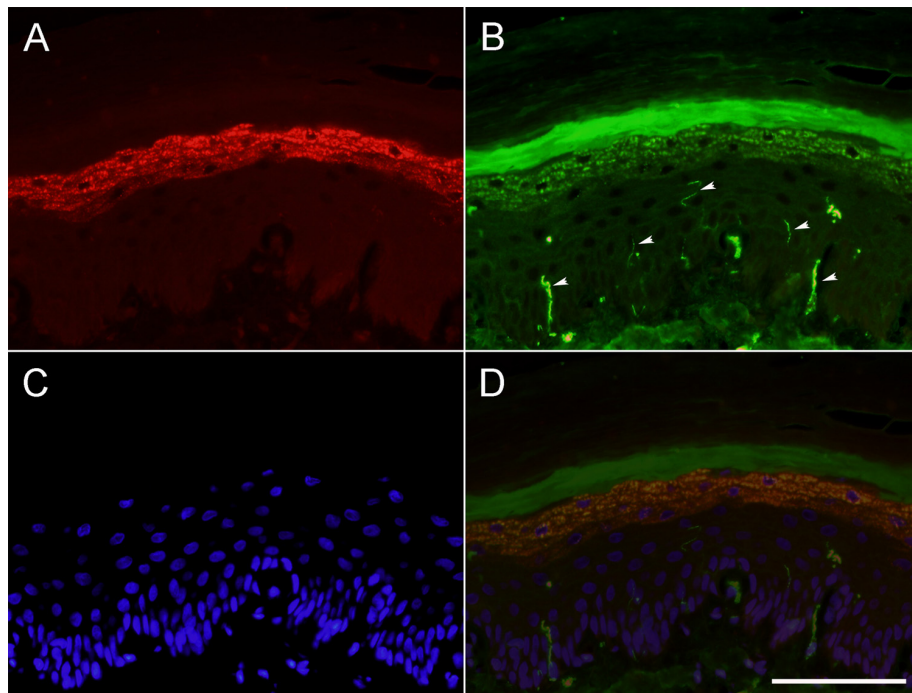


Fig. 3. Cutaneous localization of pCaMKII α (A), PGP9.5-staining shows epidermal innervation in skin (B), DAPI nuclear staining (C) and merged image of pCaMKII α , PGP-9.5 nad DAPI staining (D). Arrows indicate intraepidermal nerve fibers. Magnification, 40 \times . Scale bar 100 μ m (applies to all).

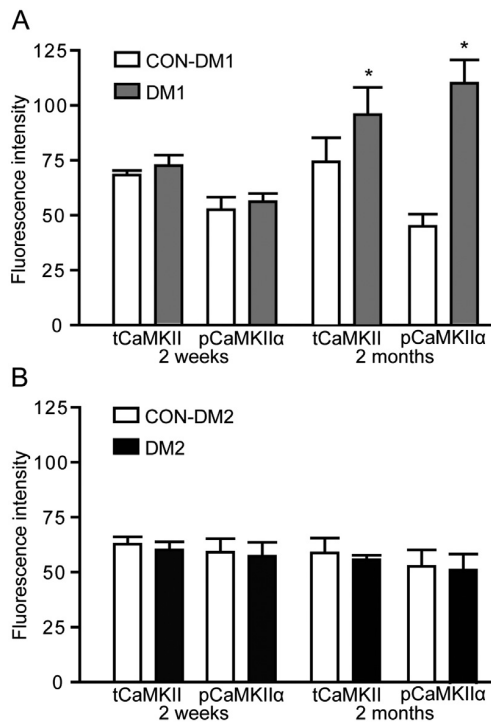


Fig. 4. Average fluorescence intensity values of tCaMKII and pCaMKII α in DM1 (A) and DM2 animals (B) 2 weeks and 2 months after induction of diabetes. Data are presented as $M \pm SD$. Asterisk * denotes significant difference ($p < 0.05$) from respective controls without diabetes (t -test). Legend: CON-DM1 = control group for diabetes type 1 model, DM1 = animals with diabetes type 1, CON-DM2 = control group for diabetes type 2 model, DM2 = animals with diabetes type 2.

weeks and 2 months after induction of DM1 tCaMKII and pCaMKII α were significantly increased in DRG and dorsal horn neurons. However, difference in CaMKII expression in these tissues was not apparent 2 weeks and 2 months after DM2 induction (Ferhatovic et al., 2013a,b). Our further studies in long-term diabetes indicated that CaMKII expression changes after 6 months and 12 months in DRG and dorsal horn neurons (Boric et al., 2013a; Ferhatovic et al., 2014). Ferhatovic et al. in their studies on DRG also analyzed expression of other CaMKII isoforms (β , γ , δ). They found changes of other isoforms only in late diabetes while in the early diabetes showed difference only in total CaMKII and its alpha isoform (Ferhatovic et al., 2013a, 2014). Therefore, total CaMKII and alpha isoform were analyzed in this study of early model of diabetes.

Dorsal root ganglia contain bodies of primary sensory neurons with peripheral terminals within skin (Perl, 1992). Peripheral nerve fibers visualized with PGP 9.5 and epidermal thickness studied on DM1 and DM2 animal models showed significant loss of intraepidermal nerve fibers and epidermal thinning two months following diabetes induction. Both changes were more pronounced in DM1 model (Boric et al., 2013b).

We hypothesized that CaMKII may be changed in diabetic skin as well. While this study confirmed that CaMKII is expressed in the skin, further analyses revealed that the enzyme was not expressed in neural fibers. We believe that different structural and functional environment of ganglia and dorsal horn compared to free nerve terminals, alongside with complex role of CaMKII in LTP in central nervous system can explain its presence in DRG and DH and its absence in free nerve terminals in skin.

There is increasing evidence that nervous system reflects on inflammatory, proliferative or reparative processes in tissues (Ansel et al., 1996). Various neuropeptides are expressed in normal skin directly from sensory neurons or from different skin cells (Scholzen et al., 1998). Various neuropeptides are known to be involved in pain transmission and hyperalgesia, such as substance

P, calcitonin-gene related peptide, IB4, vasoactive intestinal peptide and neuropeptide Y following spinal nerve ligation (Shehab, 2014). Abnormalities of similar neuropeptides were observed in skin biopsy specimens from diabetic patients (Levy et al., 1989).

The same ectodermal origin of skin and nervous system could possibly explain epidermal expression of CaMKII, enzyme that is highly abundant in the nervous system. Moreover, free nerve endings in skin have contacts and cross-talk with other skin cells, such as keratinocytes. This enables sensory nerves to function in not only afferent, but also in efferent system secreting many neuropeptides (Koizumi et al., 2004). It is possible that one of those cross-link products in keratinocytes is CaMKII, and its increased expression in skin layers of diabetic animals could contribute to altered pain perception.

Increased expression in DRG and dorsal horn neurons was associated with increased pain-related behavior in animal models of diabetes type 1 (Boric et al., 2013a; Ferhatovic et al., 2013a). Hereby we observed comparable difference between CaMKII expression in DM1 and DM2 rat models – changes in CaMKII expression were not observed between DM2 and their control animals throughout the experiment, while changes in CaMKII expression between DM1 and its control animals after two months. It is possible that different plasma glucose levels contribute to these differences in CaMKII expression in skin of two diabetic models. While DM1 animals are characterized by a lack of insulin secretion, DM2 animals are characterized by the lack of insulin effect which can explain difference in CaMKII expression between two types of diabetes (Srinivasan et al., 2005). Furthermore, some potent glycation agents as glyoxal, methylglyoxal or 3-deoxyglucosone react with proteins to form advanced glycation end-product. Its accumulation alongside with effect of oxidative stress and glucose levels are leading to development of diabetic neuropathy (Gwak et al., 2013; Meerwaldt et al., 2005). Although DM1 and DM2 have very similar phenotypes, they are not identical. Possible reasons for those varieties could be addressed in multifactorial pathogenesis; disease duration, glycaemic control, dyslipidemia and possibly patient age (Day and Ranum, 2005). Ozay et al. postulated that oxidative stress and inflammatory response may play an important role in the pathogenesis of high-fat diet induced neuropathy described in DM2 animal model that could explain different pain pathway from those in DM1 animals (Ozay et al., 2014). This study, like the previous one from our group, could contribute to elucidation of neural phenotypic differences between different types of diabetes.

Changes of CaMKII expression in neural tissues and its relationship with pain-related behavior have been well documented and it has been suggested that treatment with chemicals regulating levels of CaMKII could alleviate complications of diabetes (Jelicic Kadic et al., 2013, 2014). It has recently been suggested that activation of a mitochondrial/ox-CaMKII pathway contributes to increased sudden death in diabetic patients after myocardial infarction (Luo et al., 2013). CaMKII pathway has been implicated in diabetic retinopathy development, diabetic vascular dysfunction and renal dysfunction in a model of insulin-dependent diabetes (Benter et al., 2005; Kato et al., 2008; Kim et al., 2010). Our results are the first report indicating that CaMKII pathway should be further explored in the context of diabetic complications. Skin is easily accessible and potential effect of CaMKII-targeted therapies could be studied easier than in dorsal root ganglion or dorsal horn.

A limitation of this study is that results were obtained using exclusively immunohistochemical staining method. Future studies should include additional lines of evidence, including Western blot or RNA analysis.

To conclude, this is the first description of the CaMKII expression pattern in the skin of diabetic animal models. Different

cutaneous expression of CaMKII between diabetes type 1 and type 2 model could indicate that CaMKII may be involved in cutaneous diabetic pathology. Further research on the role of CaMKII in healthy and diabetic skin should elucidate its function in epidermis.

Ethical approval

All experimental procedures and protocols were approved by the Ethical Committee of the University of Split School of Medicine. The work described has been conducted according to the International Association for the Study of Pain (IASP) Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals, Directive 2010/63/EU for animal experiments and Uniform requirements for manuscripts submitted to biomedical journals.

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