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Increased expression of vanilloid receptor 1 (VR1) on myelinated primary afferent neurons contributes to the antihyperalgesic effect of capsaicin cream in diabetic neuropathic pain in mice

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a) Running title: Increased expression of VR1 in DRG neurons of diabetic mice

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d) Abbreviations: VR1, vanilloid receptor 1; STZ, streptozotocin; SP, substance P; ATP, adenosine triphosphate; PGI_2, prostaglandin I_2; i.pl., intraplantar; PBS, phosphate-buffered saline; DRG, dorsal root ganglion; ANF, algogenic-induced nociceptive flexion; Neocap, neonatal capsaicin-treated; CPZ, capsazepine; ONO-54918-07, 15-cis-(4-n-propylcyclohexyl)-16,17,18,19,20-pentanor-9-deoxy-6,9 alpha-nitriloprostaglandin F1.

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Abstract:

Topical capsaicin is believed to alleviate pain by desensitizing the vanilloid receptor 1 (VR1) at the peripheral nerve endings. Here, we report that an upregulation of VR1 expression on myelinated fibers contributes to the antihyperalgesic effect of capsaicin cream in streptozotocin (STZ)-induced diabetic neuropathic pain. Intravenous injection of STZ (200 mg/kg) in mice caused rapid onset of diabetes within 24 h. Thermal and mechanical hyperalgesia developed by 3 days after STZ injection and persisted at all time points tested until 28 days. There was also hyperalgesic response to intraplantar (i.pl.) prostaglandin I_2 (PGI_2) agonist-induced nociception in such mice. Application of capsaicin cream dose-dependently reversed the thermal, mechanical and PGI_2 agonist-induced hyperalgesia observed in the diabetic mice. The i.pl. injection of capsaicin solution (0.4 μg/20μl) produced nociceptive biting-licking responses in control mice, and these responses were significantly increased in STZ-induced diabetic mice. After neonatal capsaicin-treatment, which destroys most unmyelinated C-fibers, the i.pl. capsaicin-induced biting-licking responses were almost abolished. However, in neonatal capsaicin-treated diabetic mice, the i.pl. capsaicin-induced biting-licking responses reappeared. The i.pl. capsaicin-induced biting-licking responses were blocked by the competitive VR1 antagonist capsazepine. All these results suggest an increase in capsaicin receptor on myelinated fibers due to diabetes. Finally, we confirmed the upregulation of VR1 expression on myelinated primary afferent neurons of diabetic mice by immunohistochemistry. Altogether our results suggest that increased expression of VR1 on myelinated fibers might contribute to the antihyperalgesic effect of topical capsaicin in diabetic neuropathic pain.

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Painful peripheral neuropathy is one of the most common complications in early stages of diabetes mellitus. The underlying mechanisms for the development of painful peripheral neuropathy in diabetic patients are poorly understood. Hyperglycemia is considered as a major pathogenic factor in the development of peripheral diabetic neuropathy. In experimental animals, local infusion of glucose into dorsal root ganglion (DRG) or sciatic nerve induced profound and rapid mechanical hyperalgesia (Dobretsov et al., 2001). It is not clear which types of primary afferents are involved in mediating the diabetic neuropathic pain. Hyperactivity of small diameter C-fibers has been suggested in the development of diabetic neuropathic pain (Chen and Levine 2001). However, in a recent study the development of hyperalgesia could not be prevented in STZ-induced diabetic rats after the systemic pretreatment with resiniferatoxin, which produces long-lasting desensitization of unmyelinated nociceptive C-fibers (Khan et al., 2002). Moreover, ectopic discharges and spontaneous activity were mainly confined to the myelinated A-δ and A-β fibers, but not the C-fibers, in the diabetic rats (Khan et al., 2002). Thus, the myelinated primary afferent neurons may play an important role in the development of diabetic neuropathic pain.

The vanilloid receptor 1 (VR1) is a ligand-gated cation channel that can be activated by heat, decreased pH or exogenous ligand such as capsaicin (Caterina et al., 1997 Tominaga et al., 1998). In addition, VR1 can be activated by endogenous fatty acid-derived mediators such as anandamide and N-arachidonyl-dopamine (NADA) (Di Marzo et al., 2002). The VR1 protein has attracted tremendous attention since it can serve as a molecular integrator of painful stimuli on the primary sensory neurons. Recent findings also suggest its presence in various brain regions including hippocampus, hypothalamus and locus coeruleus (Mezey et al., 2000). VR1 has also been found in the

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spinal cord post-synaptic neuronal dendrites (Valtschanoff et al., 2001). The functional differences between the VR1 in central nervous system (CNS) and in the periphery are not yet known. While neonatal capsaicin treatment kills most VR1-expressing neurons in the sensory ganglia (Jancso et al., 1977), those in the CNS are not affected by neonatal capsaicin injection (Mezey et al., 2000). Although poorly known, the neurotoxic effect of capsaicin is reported due to depletion of nerve growth factors (Otten et al., 1983). It has been speculated that neonatal capsaicin treatment does not kill VR1-expressing neurons in the brain because these cells do not depend on any neurotrophic factor for survival that capsaicin may deplete (Mezey et al., 2000). In the periphery, VR1 is mainly expressed on unmyelinated C-fibers with very little presence on the thinly myelinated Aδ-fibers (Caterina et al., 1997). Nevertheless, VR1 has been recognized as a marker of the nociceptive polymodal C-fibers in the sensory ganglia (Caterina et al., 1997).

Topical capsaicin is widely used in the clinic to alleviate various painful conditions including diabetic neuropathic pain (The capsaicin Study Group 1991, Low et al., 1995). Capsaicin stimulates the VR1 and initiates a complex cascade of events including neuronal excitation and release of proinflammatory mediators as well as desensitization of the receptor (Caterina et al., 1997; Holzer 1991). The analgesic action of topical capsaicin in painful diseases is believed to occur through desensitization of the capsaicin receptor VR1 (Jancsó and Jancsó 1949; Holzer 1991; Szallasi and Blumberg 1999). Thus, it might be speculated that upregulated VR1 expression could contribute to neuropathic pain and hyperalgesia. Indeed, recent works indicate the involvement of vanilloid receptors in the development and maintenance of inflammatory and neuropathic pain (Di Marzo et al., 2002). Upregulation of VR1 has been indicated for the development of nerve injury-induced neuropathic pain in the rats (Hudson et al., 2001). Recently, we have also

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reported that increased expression of VR1 on myelinated, neonatal capsaicin-insensitive fibers accounts for the antihyperalgesic action of topical capsaicin cream in nerve injury-induced neuropathic pain in mice (Rashid et al., 2003). However, it is not yet known whether an upregulation of VR1 might contribute to the neuropathic pain in diabetes. Kamei et al. (2001) showed that intrathecal injection of anti-VR1 serum blocked the thermal and mechanical hyperalgesia observed in diabetic mice, suggesting the involvement of this receptor in diabetic neuropathic pain. In the present study, for the first time, we reported an upregulation of VR1 expression on myelinated primary afferent neurons of STZ-induced diabetic mice. We also showed that this upregulated VR1 on myelinated fibers might contribute to the antihyperalgesic action of topical capsaicin cream in diabetic neuropathic pain.

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Materials and Methods

Experimental animals: Male ddY mice were used throughout the experiments. They were housed in the animal facility of the University, which had been always maintained at 21 $\pm$ 2 ˚C, 55 $\pm$ 5 % relative humidity and an automatic 12-h light/dark cycle. The animals received standard laboratory diet (Oriental Yeast Co. Ltd., Japan) and tap water *ad libitum*. The animals were adapted to the testing environment (maintained at 21 $\pm$ 2 ˚C, 55 $\pm$ 5 % relative humidity and 12-h light/dark cycle) by keeping them in the testing room 24 h before the experiments. Experiments were performed during the light phase of the cycle (10:00 – 17:00). All procedures were approved by Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain (Zimmerman 1983).

Drugs: The following drugs were purchased, Substance P (SP; Peptide Institute, Osaka, Japan), adenosine triphosphate (ATP; Nacalai Tesque, Kyoto, Japan), capsaicin (Nacalai Tesque, Kyoto, Japan) and capsazepine (CPZ; Sigma, St. Louis, MO). ONO-54918-07 (a stable prostaglandin I_2/PGI_2 agonist; Iguchi et al., 1989) was a kind gift from Ono Pharmaceutical Co. Ltd., Osaka, Japan. Capsaicin cream and base cream were prepared at the Central Research Laboratories of the Maruishi Pharmaceutical Co., Ltd., Osaka, Japan. The capsaicin cream labeled 0.01%, 0.025%, 0.05% and 0.1% contained 0.1, 0.25, 0.5 and 1 mg of capsaicin in 1 g of hydrophilic cream base respectively. The base cream contained 18% polyoxy-ethylated castor oil, 17% liquid paraffin, 5% white Vaseline, 4% 1-hexadecanol, 0.1% EDTA disodium salt, and 0.75% triethanolamine (Minami et al., 2001). All drugs except capsaicin and capsazepine were dissolved in physiological saline. Capsaicin and capsazepine were dissolved in 10 % ethanol, 10 % Tween 80 and 80 %

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physiological saline (5 mg/ml stock solution), which were then diluted with physiological saline before injection. This vehicle was found to be innocuous. The cream was applied in a volume of 0.1 ml /10 g and then gently rubbed over the mouse footpad skin 3 h before the behavioral test. The footpad was covered with adhesive tape to prevent the mice from licking up the cream.

STZ-induced diabetes: The pancreatic β-cell cytotoxic agent streptozotocin (STZ) is widely used to induce diabetes in rodents. The glucosamine-nitrosourea compound STZ is taken up into the insulin-producing β-cells of the islets of Langerhan's via the GLUT-2 glucose transporter. The cytotoxic effect of STZ is mediated through a decrease in NAD levels, and the formation of intracellular free radicals leading to various toxic effects including DNA-strand breaks (Schnedl et al., 1994). The STZ-induced diabetic rodents are hypoinsulinemic, but generally do not require exogenous insulin treatment to survive. STZ-induced diabetic rodents show common features of human diabetes that include damage to the eye, kidney, blood vessels and nervous system. Diabetic neuropathic pain occurs mainly due to the damage in the nervous system (Sima and Sugimoto 1999). In the present study, diabetes was induced in mice by a single intravenous (i.v.) injection of STZ (200 mg/kg, Wako Pure Chemicals, Richmond, VA) as reported previously (Kamei et al., 1991; Rashid and Ueda 2002). Mice weighing ~30 g were injected i.v. with STZ in the tail vein. STZ solution was prepared freshly by dissolving it in saline adjusted to pH 4.5 in 0.1 N citrate buffer. Age-matched non-diabetic control mice were injected with the vehicle alone. Due to frequent urination (polyuria) in the diabetic mice, special care is needed for these animals. The STZ-injected mice were kept in a group of 4 per cage. The bed of the cage was changed daily and special attention was paid for food and water supplement.

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The plasma glucose level in the mice was measured using the 'glucose test kit' (Wako Pure Chemicals, Osaka, Japan) in blood samples obtained from tail vein. Only mice with a plasma glucose concentration greater than 300 mg/dl (16.7 mmol/L) were considered as diabetic. All efforts were made to minimize both the sufferings and number of animals used.

Thermal and mechanical nociception tests: In the thermal paw withdrawal test, antinociception or analgesia was measured from the latency to withdrawal evoked by exposing the right hind paw to a thermal stimulus (Hargreaves et al., 1988). Unanesthetized animals were placed in Plexiglas cages on top of a glass sheet and an adaptation period of one hour was allowed. The thermal stimulus (IITC Inc., Woodland Hills, CA, USA) was positioned under the glass sheet to focus the projection bulb exactly on the middle of plantar surface of the animals. A mirror attached to the stimulus permitted visualization of the undersurface of the paw. A cut-off time of 20 seconds was set in order to prevent tissue damage. The paw pressure test was performed as described previously (Rashid and Ueda 2002). Briefly, mice were placed into a Plexiglas chamber on a 6×6 mm wire mesh grid floor and were allowed to accommodate for a period of one hour. The mechanical stimulus was then delivered onto the middle of the plantar surface of the right hind-paw using a Transducer Indicator (Model 1601, IITC Inc., Woodland Hills, USA). With this apparatus, a control response of 10 g was earlier adjusted for naïve mice. A cut-off pressure of 20 g was set to avoid tissue damage.

Algogenic-induced nociceptive flexion (ANF) test: Experiments were performed as described previously (Ueda 1999; Inoue et al., 2003 in press). Briefly, mice were lightly

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anesthetized with ether and held in a square-sized cloth sling. The cloth sling had four holes at the corners for hanging the mouse's limbs freely through the holes. After placing the mouse in the sling with four limbs hanging through the holes, two ends of the cloth sling were joined over the flanks of the mouse and the sling was suspended on a metal bar. The mouse's limbs were then tied with soft thread strings. Three limbs were fixed to the floor, while the other one (right hind-limb) was connected to an isotonic transducer and recorder. A polyethylene cannula (0.61 mm in outer diameter) filled with drug solution was connected to a microsyringe and then carefully inserted into the undersurface of the right hindpaw. All experiments were started after complete recovery from the light ether anesthesia. Nociceptive flexor responses induced by intraplantar (i.pl.) injection (2 µl) of algogenic substances (SP, ATP, ONO-54918-07) were evaluated and normalized with control saline response. The flexion responses induced by various algogenics were represented as the % of maximal reflex in each mouse as the flexion forces differ from mouse to mouse. The biggest response among the non-specific flexor responses occurred immediately following cannulation was considered as the maximal reflex. The ANF test has been found to be less stressful and more sensitive than many conventional nociception tests (Inoue et al., 2003 in press).

Capsaicin-induced biting and licking test: The biting and licking behavior after intraplantar injection of capsaicin solution (0.4 µg/20 µl) was measured as described previously by other investigators (Sakurada et al., 1992). Mice were placed in a Plexiglas cage for an hour to adapt the environment. Before the test, mice were restrained in hand and gently taken inside a hard paper tube of internal diameter 2.5 cm. The right hindpaw was taken out of the tube and capsaicin was injected under the plantar surface of right

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hindpaw in a volume of 20 µl using a 30-gauge needle fitted to a Hamilton microsyringe. Mice were immediately put back to the cage and the time spent on biting and licking of the injected paw was measured with stopwatch for a period of 10 min. In antagonism experiments, mice were treated with 1 nmol of capsazepine in association with capsaicin. Dose of capsazepine has been determined from previous similar reports in mice (Santos and Calixto 1997). Control animals received 20 µl of the vehicle used to dissolve the drugs.

Neonatal capsaicin treatment: For the degeneration of small-diameter afferent sensory neurons, capsaicin solution was injected subcutaneously into newborn (P4) ddY mice at a dose of 50 mg/kg (Hiura and Ishizuka, 1989; Inoue et al., 1999). As a control, vehicle (10 % ethanol and 10 % Tween 80 in physiological saline) was injected. No gross behavioral changes were observed in such treated mice. Induction of diabetes in neonatal capsaicin-treated mice was performed as described in the previous section.

Immunohistochemistry: For immunohistochemical experiments, control mice, diabetic mice (7, 14, 21 and 28 days after STZ injection), neonatal capsaicin-treated control mice or neonatal capsaicin-treated diabetic mice (7, 14, 21 and 28 days after STZ injection) were used. Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.v.) and perfused transcardially with 50 ml of 0.1 M potassium free phosphate-buffered saline (K^+ free PBS, pH 7.4), followed by 50 ml of 4% paraformaldehyde in K^+ free PBS. The L4-L5 DRGs were removed, postfixed and cryoprotected overnight in 25% sucrose in K^+ free PBS. The DRGs were fast frozen in cryoembedding compound on a mixture of ethanol and dry-ice and stored at –80°C until

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use. The DRGs were cut at 10 μm with a cryostat, thaw-mounted on silane-coated glass slide and air dried overnight at RT. For immunolabeling, DRG sections were first washed with K^+ free PBS 3 times 5 min each and then incubated with 50% methanol 10 min and 100% methanol 10 min, washed with K^+ free PBS and incubated with excess blocking buffer containing 2% bovine serum albumin (BSA) in PBST (2% NaCl, 0.1% Triton-X 100 in K^+ free PBS) for 60 min. The sections were then reacted overnight at 4°C with goat polyclonal antibody raised against the C-terminal of vanilloid receptor 1 (1:100; Santa Cruz Biotechnology) in blocking buffer containing 2% BSA in PBST. After three 5 min washing in K^+ free PBS, the sections were placed in Texasred-conjugated anti-goat IgG secondary antibody (1:200; Rockland, Gilbertsville, PA) for 60 min at RT. For double immunolabeling, sections were rinsed and first incubated with anti-mouse IgG (1:50; Cappel, Aurora, Ohio, USA) for 60 min and then reacted with a monoclonal antibody raised against the N52 clone of the Neurofilament 200, a marker of myelinated fibers (Franke et al., 1991) (mouse anti-N52; 1:30000; Sigma, St. Louis, MO) overnight at 4°C. The sections were then placed in fluorescein isothiocyanate-conjugated anti-mouse IgG (1:200; Cappel, Aurora, Ohio, USA) for 60 min at RT. After washing, the sections were coverslipped with Perma Fluor (Thermo Shandon, Pittsburgh, PA) and examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis: Statistical analysis of the data for the comparisons of the thermal latency or mechanical threshold at different time points after STZ injection in mice were performed by repeated measures analysis of variance (ANOVA) and bonferroni's post-hoc test. Data in the capsaicin sensitivity test for the effects of VR1 antagonist capsazepine were analyzed using a two-way ANOVA and bonferroni's post-hoc test.

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Statistical analyses of all other data were performed using one-way ANOVA followed by a two-tailed Student's *t*-test. All data were presented as mean $\pm$ SEM. P values less than 0.05 were considered to indicate statistical significance.

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Results

Rapid onset of diabetes and thermal and mechanical hyperalgesia in mice by intravenous injection of streptozotocin: Diabetes was induced in mice by intravenous (i.v.) injection of streptozotocin (STZ). Intravenous (i.v.) injection of STZ is reported to induce rapid onset of diabetes and hyperalgesia symptoms in rats (Aley and Levine 2001). In the present study, a series of parameters including body weights, blood glucose levels, thermal latencies and mechanical thresholds were measured at different time points after a single i.v. injection of STZ (200 mg/kg) into the tail vein of mice. A rapid onset of diabetes was observed in the STZ-treated mice within 24 h (blood glucose level, 402.5 $\pm$ 22.7 mg/dl). Thermal and mechanical hyperalgesia was detectable by 3 days after STZ administration. Blood glucose levels in the STZ-injected mice were almost similar at all later time points tested (7, 14, 21, and 28 days after STZ injection). Similarly, thermal and mechanical hyperalgesia persisted in the diabetic animals at all these time points (Table 1). The blood glucose level, thermal latency and mechanical threshold did not differ significantly in the vehicle-treated control mice at all time points tested (data not shown). The rate of increase in the body weight of STZ-treated mice was much slower than the vehicle-treated control mice. The body weight of control non-diabetic mice at 7, 14, 21 and 28 days were 108.8%, 123.7%, 130.8% and 135.9% of the initial weight respectively while they were 104.4%, 106.4%, 111.6% and 107.5% of initial weight respectively in case of diabetic mice. The body weight of the STZ-treated mice started to decline at 28 days after STZ injection. In an effort to minimize animal sufferings, we used mice at 7 days post-STZ injection in the following behavioral experiments.

Reversal of thermal and mechanical hyperalgesia in diabetic mice by capsaicin

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cream: The effect of the capsaicin cream was evaluated on the thermal and mechanical hyperalgesia observed in diabetic mice. The cream was applied 3 h before examining the thermal latency or pressure threshold where maximal analgesic effect was observed in our previous study (Rashid et al., 2003). As shown in Fig. 1A, application of capsaicin cream onto the footpad of diabetic mice concentration-dependently reversed the thermal hyperalgesia from 0.01% to 0.1% concentration in the thermal paw withdrawal test. The cream also concentration-dependently reversed the mechanical hyperalgesia in diabetic mice (Fig. 1B). The EC_{50} values of the capsaicin cream were 0.064 % and 0.07 % in the thermal and mechanical tests, respectively. Consistent with our previous report (Rashid et al., 2003), capsaicin cream (0.1%) did not significantly change the thermal latency or mechanical threshold in control mice.

Phenotypic changes in the peripheral receptor ligand-induced nociceptive flexion responses in diabetic mice and effects of capsaicin cream thereon: Using the algogenic-induced nociceptive flexion (ANF) test in mice, previously we proposed the presence of three distinct types of nociceptors depending on their stimulation by specific receptor ligands. The nociceptors called neonatal capsaicin-sensitive type I, were stimulated by intraplantar (i.pl.) injection of substance P, bradykinin, nociceptin/orphanin FQ; the nociceptors called neonatal capsaicin-sensitive type II were stimulated by i.pl. $P2X_3$ receptor agonists; the nociceptors called neonatal capsaicin-insensitive type III were stimulated by i.pl. prostaglandin I_2 (PGI_2) agonist, ONO-54918-07 (Ueda et al., 2000). Very recently, we reported the peripheral nerve injury-induced phenotypic changes in the above three types of nociceptors and the effects of capsaicin cream thereon (Rashid et al., 2003). In the present study, we also found phenotypic changes in these

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three types of fibers in diabetic mice. As shown in Fig. 2A and B, substance P (SP) and ATP produced dose-dependent nociceptive flexion responses in the control non-diabetic and STZ-induced diabetic mice through capsaicin-sensitive type I and type II nociceptive fibers respectively. There was no significant difference in the responses of SP and ATP in STZ-induced diabetic mice compared with the vehicle-treated control mice. On the other hand, similar to the case with nerve injury model, the capsaicin-insensitive type III fiber-mediated nociceptive responses of the PGI_2 agonist, ONO-54918-07 were sensitized in diabetic mice giving nociceptive flexion responses at much lower doses compared with the control mice (Fig. 2C). Application of capsaicin cream concentration-dependently blocked the PGI_2 agonist-induced hyperalgesic responses in diabetic mice suggesting an increase in capsaicin-sensitive sites on neonatal capsaicin-insensitive type III fibers due to diabetes (Fig. 3).

Capsaicin-induced pain sensitivity in STZ-induced diabetic mice: Intraplantar (i.pl.) injection capsaicin solution (0.4 μg/20μl) induced nociceptive biting-licking response in control mice. The i.pl. capsaicin-induced biting-licking responses were significantly increased after STZ treatment (Fig. 4A). The competitive VR1 antagonist, capsazepine (1 nmol or 0.377 μg) blocked the capsaicin-induced biting-licking response both in control and diabetic mice (Fig. 4A). After neonatal capsaicin treatment in mice, which destroys most unmyelinated C-fibers, the i.pl. capsaicin-induced biting-licking responses almost completely disappeared (Fig. 4B). However, STZ-induced diabetes in the neonatal capsaicin-treated mice caused reappearance of the i.pl. capsaicin-induced biting-licking behaviors and these newly induced responses were blocked by the VR1 antagonist capsazepine (Fig. 4B). Capsazepine alone did not produce any biting-licking

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behavior in mice (data not shown).

Increased expression of VR1 on myelinated, capsaicin-insensitive type III fibers in the STZ-induced diabetic mice: In order to confirm our speculation that STZ-induced diabetes in mice caused upregulation of capsaicin receptors on myelinated, neonatal capsaicin-insensitive type III fibers, immunohistochemical double-labeling was performed on DRG neurons with antibody to VR1, the putative capsaicin receptor and antibody to N-52 clone of Neurofilament 200, a marker of the myelinated A-fiber. As shown in Fig. 5A, in DRG of control mice VR1-immunoreactive neurons (red) were not colocalized with N-52-immunoreactive neurons (green) indicating the presence VR1 mostly on unmyelinated C-fibers in naïve condition. In the DRG of STZ-induced diabetic mice, there was a visible increase in VR1 expression on myelinated A-fibers which was revealed by a colocalization of VR1-immunoreactive and N-52-immunoreactive neurons observed as yellow (Fig. 5B,C). The level of increase in VR1 expression on myelinated fibers was almost similar in the DRGs of diabetic mice at 7, 14, 21 and 28 days after STZ injection (Fig. 5B,C,G; 14 and 21 days data are not shown). Moreover, VR1 expression in unmyelinated C-fibers was not significantly increased in the diabetic mice (Fig. 5B,C). In neonatal capsaicin-treated control mice, the VR1-immunoreactive neurons almost completely disappeared (Fig. 5D). However, in neonatal capsaicin-treated diabetic mice, large numbers of VR1-immunoreactive neurons were observed in the DRGs which were colocalized with N-52 confirming the upregulation of VR1 expression on myelinated, neonatal capsaicin-insensitive fibers due to diabetes (Fig. 5E,F; 14 and 21 days data are not shown). When the numbers of VR1-immunoreactive cells were plotted in a bar graph as percent of total cells, a significant increase in the numbers of cells that were

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colocalized with N-52 was observed in the diabetic mice (Fig. 5G).

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Discussion

In the present report, we attempted to identify whether an upregulation of vanilloid receptor subtype 1 (VR1) expression on myelinated fibers contributed to the antihyperalgesic effect of capsaicin cream in diabetic neuropathic pain in mice. Intravenous (i.v.) injection of streptozotocin (STZ) in the tail vein of mice induced a rapid onset of hyperglycemia within 24 h, and significant thermal and mechanical hyperalgesia was detectable by 3 days after STZ injection (Table 1). Our results are consistent with previous reports in rat where i.v. STZ induced hyperglycemia by 24 h and thermal and mechanical hyperalgesia and tactile allodynia within 48 h after injection (Aley and Levine 2001). The rapid elevation of blood glucose level by i.v. STZ might contribute to the rapid induction of thermal and mechanical hyperalgesia as already suggested in the study of Aley and Levine (2001) where pretreatment with insulin prevented the development of hyperalgesia in STZ-treated rat. The thermal and mechanical hyperalgesia observed in the diabetic mice were concentration-dependently reversed by topical application of capsaicin cream onto mouse's footpad (Fig. 1A,B). Capsaicin, the active ingredient of capsaicin cream, gives its analgesic effect by desensitizing the capsaicin receptor (Jancsó and Jancsó 1949; Holzer 1991; Szallasi and Blumberg 1999). Thus an upregulation and/or sensitization of the capsaicin receptor could be speculated in the STZ-induced diabetic mice.

With the algogenic-induced nociceptive flexion (ANF) test, we recently reported that capsaicin cream could block the nociceptive responses mediated through neonatal capsaicin-sensitive type I and type II, but not neonatal capsaicin-insensitive type III, fibers in naïve mice (Rashid et al., 2003). After partial sciatic nerve injury, the type I fiber-mediated responses were lost, type II fiber-mediated responses remained unchanged

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and the type III fiber-mediated responses were hypersensitized, and capsaicin cream reversed the type III fiber-mediated hyperalgesia in injured mice (Rashid et al., 2003). In the present study, the substance P-induced nociceptive response, which is mediated through type I fibers, and the ATP-induced nociceptive response, which is mediated through type II fibers, did not differ between the non-diabetic and diabetic mice (Fig. 2A,B). However, the PGI_2 agonist-induced nociceptive response, which is mediated through type III fibers, was hypersensitized in the diabetic mice compared with the control mice (Fig. 2C). The contrasting phenotypic change in type I responses in partial sciatic nerve-injured and diabetic mice might be due to the intense mechanical injury to the sciatic nerve and consequent drastic changes including decrease in SP-immunoreactivity in DRG and spinal cord with the injury model (Malmberg and Basbaum 1998; Lee et al., 2001). On the other hand, similar to the case with nerve injury model, capsaicin cream concentration-dependently reversed the PGI_2 agonist-induced type III fiber-mediated hyperalgesia in diabetic mice (Fig. 3). Thus, the induction of PGI_2 agonist-induced hyperalgesia in diabetic mice could be due to an upregulated capsaicin receptor on neonatal capsaicin-insensitive type III fibers. PGI_2 agonist produces nociceptive responses through activation of G_s-coupled prostaglandin I_2 receptor. The hyperalgesic responses of PGI_2 agonist in diabetic mice might be due to a protein kinase A-mediated transactivation of the newly expressed VR1 receptors as reported elsewhere (De Petrocellis et al., 2001).

To further investigate whether capsaicin receptor expression is increased in STZ-induced diabetic mice, we performed tests for capsaicin-induced pain sensitivity in control and diabetic mice. Intraplantar (i.pl.) injection capsaicin solution-induced nociceptive biting-licking responses were significantly increased in the diabetic mice

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indicating an increase in capsaicin-sensitive sites due to diabetes (Fig. 4A). Moreover, after neonatal capsaicin treatment in mice, which destroys most unmyelinated C-fibers, the i.pl. capsaicin-induced biting-licking responses almost completely disappeared indicating that capsaicin-induced biting-licking responses in control mice were mainly mediated through the C-fibers (Fig. 4B). This finding is consistent with the fact that capsaicin receptor VR1 is mainly expressed in the polymodal nociceptive C-fibers (Caterina et al., 1997). In the neonatal capsaicin-treated diabetic mice, however, the i.pl. capsaicin-induced biting-licking responses surprisingly reappeared (Fig. 4B). This finding clearly indicates that STZ-induced diabetes in mice caused an upregulation of the capsaicin receptor on myelinated, neonatal capsaicin-insensitive type III fibers. Furthermore, involvement of capsaicin receptor in the increased i.pl. capsaicin solution-induced responses in the diabetic mice was revealed by the fact that the competitive VR1 antagonist capsazepine completely blocked these responses (Fig. 4A,B). All these results suggest an increased expression of capsaicin receptor VR1 on previously capsaicin-insensitive type III fibers due to diabetes.

We next confirmed the upregulation of VR1 expression on myelinated, neonatal capsaicin-insensitive type III fibers due to diabetes by immunohistochemistry. Consistent with our previous report (Rashid et al., 2003), almost all of the VR1-immunoreactive neurons in the DRG of control mice were not colocalized with the A-fiber marker N-52, indicating their presence on unmyelinated C-fibers in naïve state. In the STZ-induced diabetic mice, the VR1 expression significantly increased only on the myelinated A-fibers (Fig. 5B,C,G). VR1-immunoreactive neurons in the DRGs of neonatal capsaicin treated mice almost completely disappeared, which is consistent with previous reports (Rashid et al., 2003; Mezey et al., 2000). However, STZ-induced diabetes in neonatal

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capsaicin-treated mice caused an increased expression of VR1 which were colocalized with A-fiber marker N-52 (Fig. 5E,F). These results confirmed our speculation that capsaicin cream reversed the hyperalgesia in diabetic mice (Fig. 1A,B and Fig. 3) by desensitizing the newly expressed VR1 receptors mainly located on myelinated, neonatal capsaicin-insensitive type III fibers. Our findings of the upregulated VR1 expression on myelinated fibers in diabetic mice would be both timely and pertinent in view of the recent indications that endogenous vanilloid receptor agonists such as anandamide, N-arachidonoyl-dopamine (NADA) might play a crucial role in the maintenance of neuropathic pain (Di Marzo et al., 2002). Moreover, phosphorylation of VR1 by protein kinase A and protein kinase C, which are easily produced by pro-inflammatory mediators such as bradykinin and prostaglandins, has been well known (Premkumar and Ahern 2000; De Petrocellis et al., 2001). Such phosphorylation increases the probability of channel gating by agonists such as heat, proton and endovanilloids (Vellani et al., 2001). Direct activation of VR1 channel by protein kinase C has also been reported (Premkumar and Ahern 2000). Thus, the upregulation of VR1 expression on myelinated fibers may contribute to the altered activities of these fibers as well to the maintenance of peripheral and central sensitization in neuropathy states.

In conclusion, we demonstrate that the thermal, mechanical and chemical hyperalgesia observed in the STZ-induced diabetic mice might be due to the upregulation of VR1 expression on neonatal capsaicin-insensitive, myelinated A-fibers. Our results also indicate that this upregulated VR1 on myelinated fibers may account for the antihyperalgesic action of capsaicin cream in diabetic neuropathic pain.

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Figure legends:

Fig. 1: Effects of capsaicin cream on the thermal and mechanical hyperalgesia in the STZ-induced diabetic mice. *A:* Concentration-dependent reversal of thermal hyperalgesia in diabetic mice by capsaicin cream with the thermal paw withdrawal test. *B:* Concentration-dependent reversal of mechanical hyperalgesia in diabetic mice by topical application of capsaicin cream with the mechanical paw pressure test. Capsaicin cream labeled 0.01%, 0.025%, 0.05% and 0.1% or base cream (0%) was applied onto the mouse's footpad 3 h before the test. * indicates significantly different compared with the base cream (0%)-treated diabetic mice at $p<0.05$. Each data point represents mean $\pm$ SEM of 6-8 separate experiments. The vertical bars represent the standard error of the means. 'Control' is the paw withdrawal latency or threshold in vehicle-treated control non-diabetic mice.

Fig. 2: Phenotypic changes in the peripheral receptor ligand-induced nociceptive flexion responses in the STZ-induced diabetic mice. *A, B:* In the algogenic-induced nociceptive flexion (ANF) test, the flexion responses induced by substance P (SP) and ATP mediated through neonatal capsaicin-sensitive type I and type II fibers respectively, remained unchanged in the STZ-induced diabetic mice. It was revealed by no significant difference in the dose-response curves between control non-diabetic and STZ-induced diabetic mice. *C:* Dose-response curves of the neonatal capsaicin-insensitive type III fibers stimulant PGI_2 agonist, ONO-54918-07 in control non-diabetic and STZ-induced diabetic mice with the ANF test. The dose-response curve for ONO-54918-07 was shifted leftward in diabetic mice giving hyperalgesic responses. The results are represented as the % of maximal reflex. Details are described the 'Materials and Methods' section. Each data point represents mean $\pm$ SEM of 6 separate experiments. The vertical bars represent

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the standard error of the means.

Fig. 3: Reversal of PGI_2 agonist-induced hyperalgesia in diabetic mice by topical application of capsaicin cream. Dose-response curves of the PGI_2 agonist, ONO-54918-07-induced nociceptive flexion responses in the ANF test in diabetic mice after application of capsaicin cream (0.025%, 0.05% and 0.1%) or base cream onto the mouse's footpad 3 h before the test. Prior topical application of capsaicin cream concentration-dependently reversed the type III fiber-mediated hyperalgesic responses. The symbols ◆, ▼, ▲ and ■ represent the effects of base cream and 0.025%, 0.05%, 0.1% capsaicin cream on the ONO-54918-07-induced responses in diabetic mice respectively; the symbols ❍ and ● represent the ONO-54918-07-induced responses in control and diabetic mice respectively. The results are represented as the % of maximal reflex. Details are described the 'Materials and Methods' section. Each data point represents mean $\pm$ SEM of 6 separate experiments. The vertical bars represent the standard error of the means.

Fig. 4: Capsaicin-induced biting and licking responses and their blockade by capsazepine in the STZ-treated diabetic mice. *A:* Increase in the intraplantar (i.pl.) capsaicin-induced nociceptive biting-licking responses in STZ-treated diabetic mice and their blockade by the competitive VR1 antagonist capsazepine. Capsazepine (1 nmol or 0.377 μg) was injected in association with capsaicin (0.4 μg) in a volume of 20 μl. The symbol * indicates significant difference in the i.pl. capsaicin-induced responses between the control and STZ-treated diabetic mice. # indicates significant difference in the biting-licking responses between i.pl. capsaicin treated (Cap) and i.pl. 'capsaicin + capsazepine' (Cap + CPZ) treatment group. *B:* Reappearance of capsaicin-induced biting-licking responses in neonatal capsaicin-treated (Neocap) diabetic mice. * indicates

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significant difference in the i.pl. capsaicin-induced responses between the 'Neocap control' and 'Neocap diabetic' mice. # indicates significant difference in the biting-licking responses between i.pl. capsaicin (Cap) and i.pl. 'capsaicin + capsazepine' (Cap + CPZ) treatment group. Results are represented as the time (sec) spent in biting and licking of the injected paw for a period of 10 min after i.pl. injection of drug substances. 'Veh' is vehicle-induced response. Each data point represents mean $\pm$ SEM of 6-8 separate experiments. The vertical bars represent the standard error of the means.

Fig. 5 Upregulation of VR1 expression on myelinated, neonatal capsaicin-insensitive primary afferent neurons after induction of diabetes in mice. *A:* VR1 expression in DRG neurons of control non-diabetic mouse. Almost all of the VR1-immunoreactive neurons (red) were not colabeled with A-fiber marker N-52 (green). *B,C:* VR1 expression in DRG of diabetic mice at 7 and 28 days after STZ injection. In both groups, many VR1-immunoreactive neurons were colocalized with A-fiber marker N-52 (observed as yellow). *D:* VR1 expression in the DRG of neonatal capsaicin-treated control mouse. Almost complete absence of VR1-immunoreactive neurons indicates loss of VR1-containing primary afferents due to neonatal capsaicin treatment. Most of the DRG neurons were labeled by N-52. *E, F:* Upregulation of VR1 expression on myelinated fibers in neonatal capsaicin-treated diabetic mice at day 7 and 28 after STZ injection. In neonatal capsaicin-treated diabetic mice, many VR1-immunoreactive neurons were observed which were colabeled with A-fiber marker N-52 (observed as yellow). G: Bar graph showing the percent of VR1-immunoreactive (VR1-IR) neurons which were colabeled with N-52 in control, diabetic (7 and 28 days after STZ injection), neonatal capsaicin-treated control (Neocap control) and neonatal capsaicin-treated diabetic (Neocap diabetic, 7 and 28 days after STZ injection) mice from three separate

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experiments. *p<0.05. Scale bars, 20 µm.

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Table 1:

Changes in body weight, blood glucose level, thermal latency and mechanical threshold in mice at different time points after i.v. injection of STZ.

Before the injection of streptozotocin (STZ, 200 mg/kg, i.v.), the body weight (g), plasma glucose level (mg/dl), thermal paw withdrawal latency (sec) and mechanical paw withdrawal threshold (g) of the mice were taken. The same parameters were then measured at day 1, 3, 7, 14, 21 and 28 after the STZ injection. The symbol * indicates significantly different compared with the thermal latency and paw withdrawal threshold measured before STZ administration (day 0) at $p<0.05$. Each data point represents mean $\pm$ SEM of 6-8 separate experiments.

Days after i.v. STZ	Body weight (g)	Blood glucose level (mg/dl)	Paw withdrawal latency (sec)	Paw withdrawal threshold (g)
0	29.3 $\pm$ 1.1	151.7 $\pm$ 13.4	10.15 $\pm$ 0.80	9.6 $\pm$ 0.6
1	29.7 $\pm$ 1.2	402.5 $\pm$ 22.7	9.08 $\pm$ 0.65	8.0 $\pm$ 0.6
3	30.1 $\pm$ 1.3	562.7 $\pm$ 18.2	7.03 $\pm$ 0.60*	6.1 $\pm$ 0.3*
7	30.6 $\pm$ 1.0	603.3 $\pm$ 26.3	5.91 $\pm$ 0.52*	5.9 $\pm$ 0.4*
14	31.2 $\pm$ 1.7	566.3 $\pm$ 20.2	6.26 $\pm$ 0.55*	5.7 $\pm$ 0.2*
21	32.7 $\pm$ 1.5	582.2 $\pm$ 32.4	5.63 $\pm$ 0.59*	5.9 $\pm$ 0.5*
28	31.5 $\pm$ 1.7	599.6 $\pm$ 54.1	6.13 $\pm$ 0.53*	5.3 $\pm$ 0.5*

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Figure 1

Thermal paw withdrawal test

A.

Control

Diabetic

Paw withdrawal latency (sec)

0 2 4 6 8 10 12

0 0.1 0 0.01 0.025 0.05 0.1

Capsaicin cream (%)

Paw pressure test

B.

Control

Diabetic

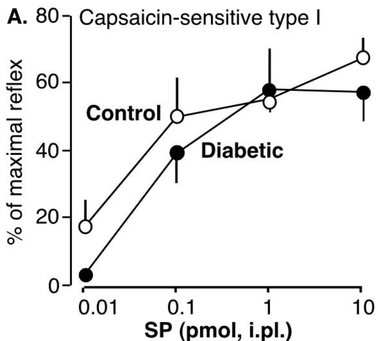
Paw withdrawal threshold (g)

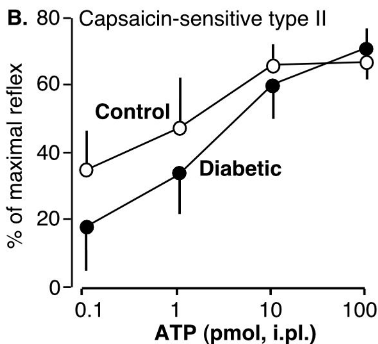
0 2 4 6 8 10 12

0 0.1 0 0.01 0.025 0.05 0.1

Capsaicin cream (%)

Figure 2





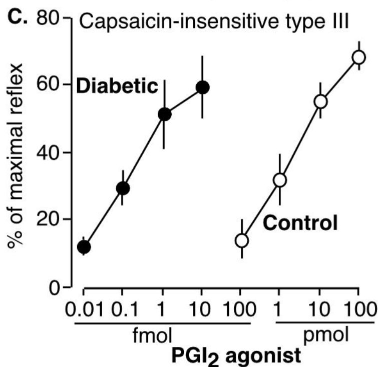


Figure 3

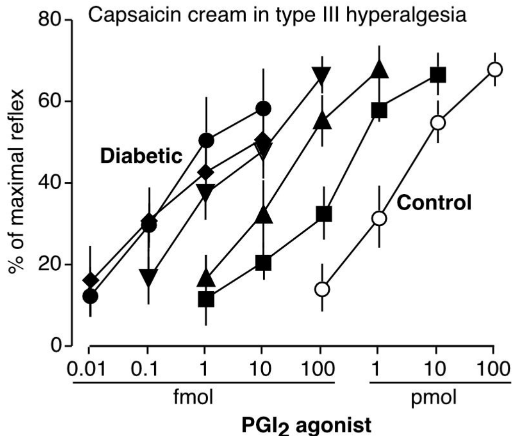


Figure 4

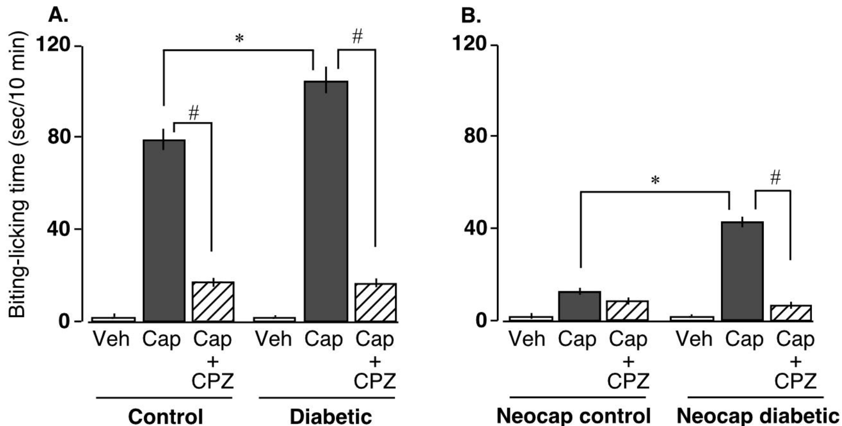
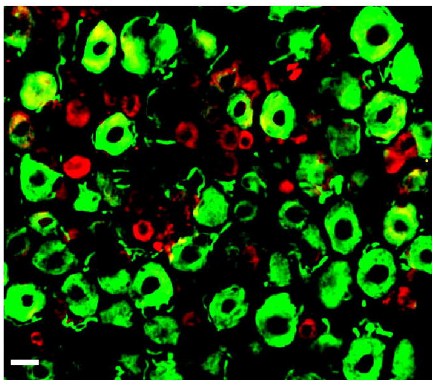
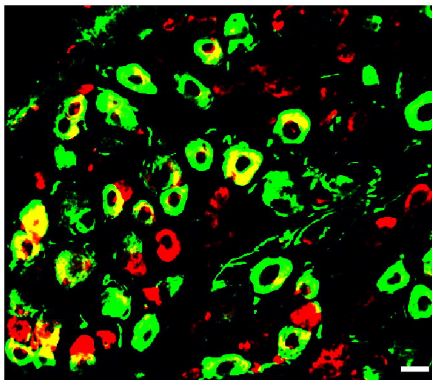


Figure 5

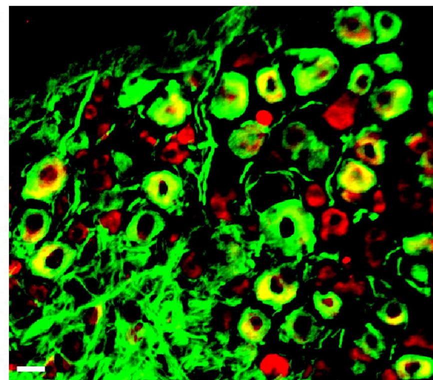
A. Control



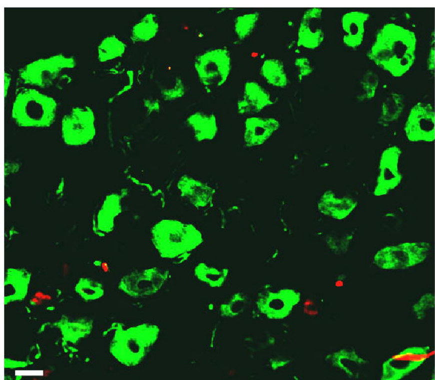
B. Diabetic - 7 d



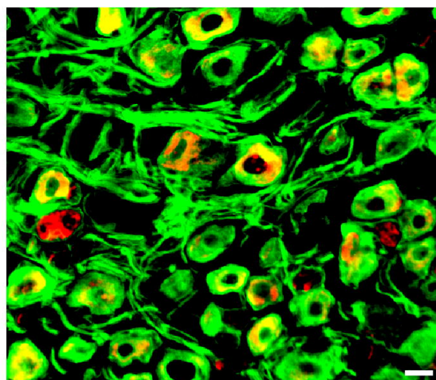
C. Diabetic - 28 d



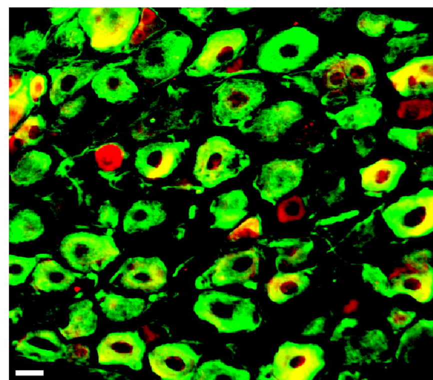
D. Neo-cap control



E. Neo-cap diabetic - 7 d



F. Neo-cap diabetic - 28 d



G.