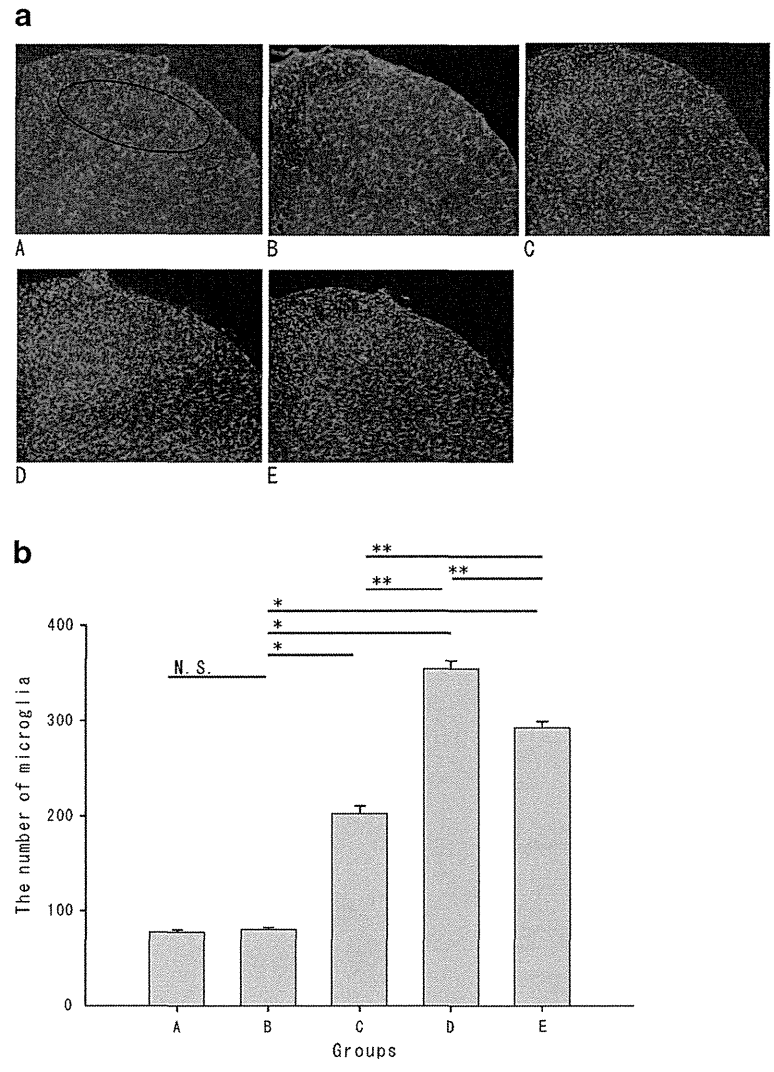

Figure 2 *A.* **Iba1 positive microglia in laminas I–II of the injured L5 segmental level: Activated microglia in the nerve injury groups (C, D, E) were observed, compared with the normal (A) and sham group (B).** *B.* The total number of microglia in laminas I–II of the injured L5 segmental level : There was no significant difference in the number of activated microglia between groups A and B. The nerve injury groups (C, D, E) were more sensitive than the sham group (*$P < 0.001$), with significant differences between C, D and E (**$P < 0.001$).

feed-forward cycle. Therefore, microglia is closely associated with the mechanisms of radiculopathy, with the number of activated microglia acting as an index for radiculopathy [13]. In this study, we show an approximate fourfold increase in microglia activation when compared with sham rats after surgery. Results consistent with previous studies, which found nerve injury to cause a two- to four-,fold increase in the number of microglia in the dorsal horn [13-17]. Interestingly, we show the number of microglia in rats injured distal to the DRG was significantly increased when compared with rats injured proximal to the DRG, consistent with the mechanical sensitivity results in the behavioral study. Together, these results indicate a difference in the degree of radiculopathy at distinct levels of nerve injury.

Microglia is also closely associated with neurons and astrocytes. Astrocytes have a more direct and active role in glutamatergic synapse function by releasing glutamate [30], releasing modulatory substances [31] and expressing functional N-Methyl D-Aspartate (NMDA) receptors [32]. NMDA receptors are activated by the wind-up phenomena, which is central pain sensitization caused by repeated stimulation of peripheral nerve fibers, leading to stimulation of C-fibers and a progressively increasing electrical response in the corresponding superficial dorsal horn. The mechanism underlying this phenomenon involves the release of glutamate by these pathologically sensitized C-fibers. The glutamate interacts with the postsynaptic NMDA receptors, which aids the sensitization of the dorsal

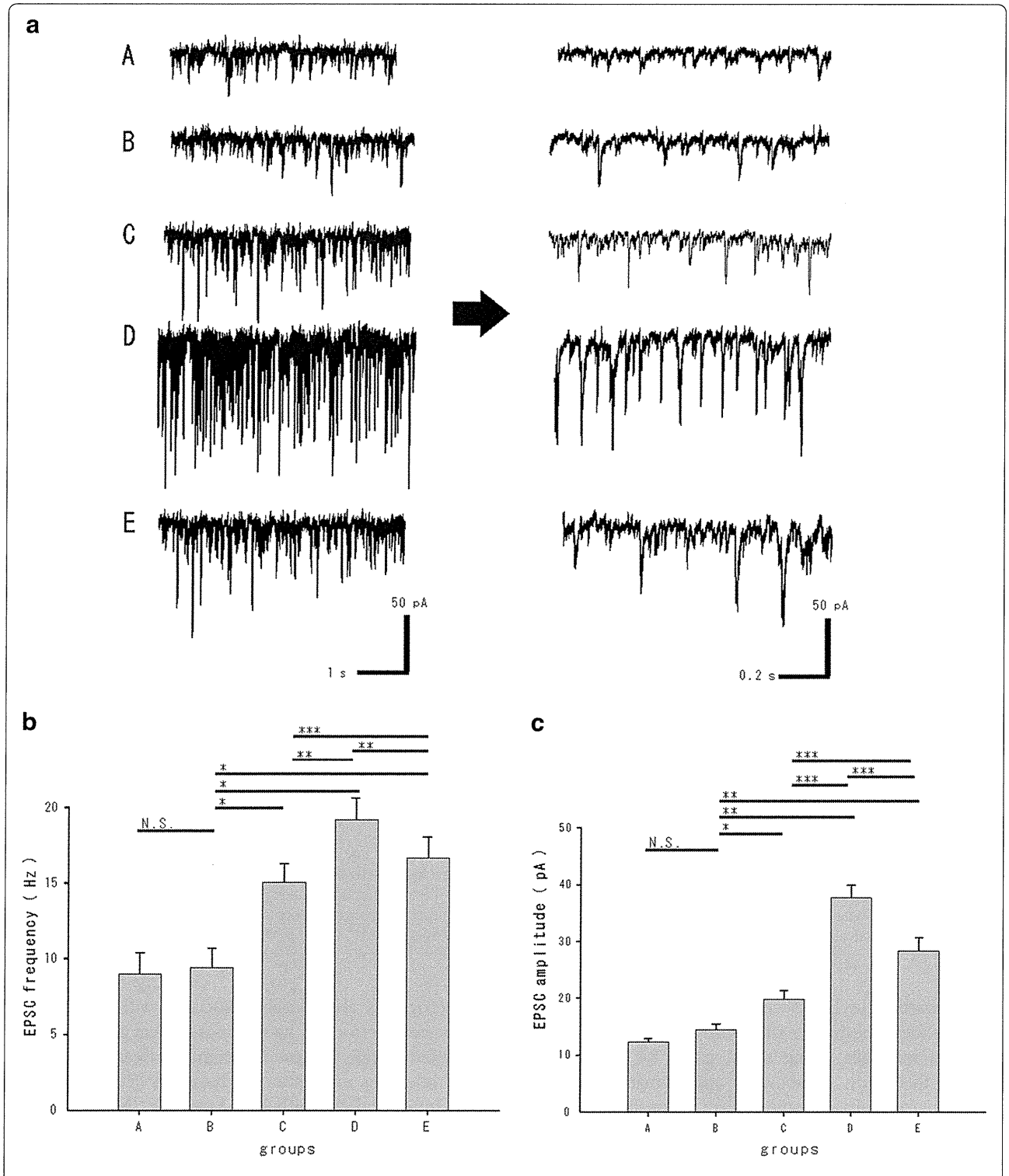

Figure 3 ***A. In vivo*** **patch clamp data: EPSC were recorded at the SG of the injured L5 segmental level.** All SG neurons were recorded at a holding potential (V_H) of −70 mV, where no IPSC were observed. *B.* The average frequencies of EPSC in laminas I–II of the L5 segmental level: No significant difference in EPSC frequency was shown between groups A and B. The frequencies of EPSC in nerve injury group (C, D, E) were significantly increased compared with the sham group (*$P < 0.001$) and interestingly there were significant differences between groups C, D and E (**$P < 0.001$ and ***$P < 0.05$, respectively). *C.* The average amplitudes of EPSC in laminas I–II of the L5 segmental level: No significant difference in the mean amplitudes of EPSC was observed between groups A and B. There were significant differences in mean amplitudes of EPSC between the nerve injury groups (C, D, E) and the sham group (B) (*$P < 0.05$ and **$P < 0.001$, respectively) as well as significant differences between groups C, D and E (**$P < 0.001$).

horn. Presynaptic neuronal voltage-gated sodium calcium channels are largely responsible for the release of this glutamate as well as the neuropeptide substance P [33]. Microglia also express α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate (AMPA/KA) receptors that mediate the release of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) [34]. AMPA receptors are glutamate receptors that are integral to plasticity and synaptic transmission at many postsynaptic membranes. One of the investigated forms of plasticity in the nervous system is long-term potentiation LTP. LTP explained that glutamate was bound to postsynaptic AMPA receptors. Ligand binding causes AMPA receptors to open and for sodium ions to flow into the postsynaptic cells, resulting in a depolarization. Therefore, we assumed that microglia participated in the changes of glutamatergic synaptic transmission observed at the nerve injury. Receptors expressed on microglial membranes are thought to allow sensing of neuronal activity and/or communication with astrocytes [35]. Glutamate neurotransmission has been associated with pain processing at multiple levels of the neuroaxis [36]. The connections between the C-fibers and the SG neurons play a critical role in pain sensation through an action of the neuropeptides. In the spinal dorsal horn neurons, activation of fine-afferent fibers produce a variety of synaptic events which are likely to be mediated by a number of neurotransmitters, including excitatory amino acids and neuropeptides. We examined the synaptic activities in the superficial dorsal horn neurons response to nerve injury using *in vivo* patch clamp methods. We show EPSC amplitudes were significantly larger following injury distal to the DRG compared with injury proximal to the DRG. In a previous study, EPSC amplitudes obtained from SG neurons at the L5 segmental level of the spinal cord after nerve injury was larger than the sham-operated animals [37]. Interestingly, this is the first study to show significant differences in excitatory synaptic activities in a nerve injury proximal and distal to the DRG. This result also indicates a marked difference in the degree of radiculopathy at distinct points of nerve injuries.

In general, the direction of information flow from the periphery to the DRG to the spinal cord itself is a main factor in the distal lesion giving rise to stronger neuropathic signs. Thus the changes from peripheral inputs are more critical for neuropathy. It has been demonstrated that radiculopathy is caused by changes in the expression and function of receptors and voltage-dependent sodium channels in peripheral nerves and DRG neurons, as well as at synapses in the nociceptive pathway in the CNS [38,39]. In particular, tetrodotoxin resistant (TTX-r) sodium channels are closely related to radiculopathy. The NaV 1.8, which is one of TTX-r sodium channel, is important in the neurophysiological and behavioral effects [40,41]. The NaV 1.8 sodium channels are expressed in both A- and C-fiber populations [42,43] and differentially regulated after peripheral nerve injury, and selectively distributed in peripheral sensory neurons [44]. Sodium channels are involved in the propagation of action potentials, while glutamate receptors expressed on presynaptic terminals of primary afferents in the dorsal horn regulate the release of neurotransmitters. After peripheral nerve injury, injured and uninjured DRG neurons become excitable and exhibit ectopic firing [45,46]. When the spinal nerve is injured distal to the DRG, the DRG neurons become excitable and exhibit ectopic firing, resulting in intense radiculopathy. In this study, there were differences between the degrees of radiculopathy injured proximal or distal to the DRG. This difference of the nerve injury level might have affected the activation and number of sodium channels to which the dorsal root projects. This may have led to activation of glutamatergic transmission, resulting in activation of AMPA receptors and NMDA receptors. As described above, there were differences in the degrees of radiculopathy.

One possible reason for the difference in the degree of radiculopathy at distinct nerve injury points may be due to changes in blood flow. The blood flow in the nerve root is affected by root constriction [47]. The blood flow supply in the nerve root proximal to the DRG is greater compared with blood flow distal to the DRG [48]. Nerve roots are surrounded by cerebrospinal fluid but not the spinal or peripheral nerves. Spinal nerve roots receive 58% of their nutritional supply from cerebrospinal fluid and 38% from intramural blood vessels, whereas peripheral nerves receive 95% of their nutritional supply from intramural blood vessels [49]. Another possible reason for the difference in the degree of radiculopathy may be the number of apoptotic neurons in the spinal cord. Expression of apoptosis in the spinal cord was found to be associated with radiculopathy after spinal nerve or nerve root injuries [50]. The percentage of apoptosis in the DRG was significantly increased in the distal crush group compared with the proximal crush group. This difference in response was because the DRG was not injured directly, resulting in apoptosis secondary to the nerve injuries [51].

Conclusion

We investigated the degree of radiculopathy using different points of mechanical sensitivity, immunohistochemistry and *in vivo* patch-clamp recordings. Our study indicated that there was a more intense radiculopathy following injury distal to the DRG rather than proximal to the DRG. Indicating the degree of radiculopathy is dependent on the level of nerve injury.

Materials and methods

A total of 125 adult male Sprague–Dawley rats weighing 180–210 g were used in this study. Animals were housed in plastic cages at room temperature on a 12-h light/dark

cycle with free access to food and water. All animal experimental procedures were approved by the Ethics Committee on Animal Experiments, Wakayama Medical University and were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986 and associated guidelines.

Surgical protocol for the radiculopathy model

The surgical protocol was modified from the lumbar radiculopathy model, as previously reported [52-54]. Rats were divided into five groups at random: Normal (group A), sham (group B), ligated proximal to the DRG (group C), ligated at the DRG (group D), ligated distal to the DRG (group E). The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), placed into a prone position, and received an incision to the middle of the spine at the L4-L6 level. The paraspinal muscles were retracted to expose the right laminas and facet joints between the L4 and L6 vertebrae. A right L5 hemilaminectomy and foraminotomy was performed. The right L5 nerve and DRG were carefully exposed not to influence the electrical properties. The injury was created by ligating the nerve root 2 mm proximal to the DRG (group C), at the DRG (group D) and at the spinal nerve 2 mm distal to the DRG (group E) using 6.0 silk sutures. [Figure 1A] Great care was taken to ligate such that the diameter of the nerve was seen to be just ligated by a microscope at 5× magnification. The desired degree of constriction retarded, but did not arrest, circulation through the superficial epineural vasculature and sometimes produced a small, brief twitch in the muscle surrounding the exposure [54]. After enough washing, the incision was closed in layers. Rats were allowed to recover in their normal environment. Two groups of control rats were used; One was not operated on (group A), the other received sham procedures, a right L5 nerve root exposure without ligation (group B).

Mechanical sensitivity

The hind paw withdrawal threshold was measured as the frequency of foot withdrawals elicited by a defined mechanical stimulus using a 10 g von Frey filament [55,56]. The rats were placed in a chamber, measuring 18 × 25 × 18 cm above a wire mesh floor. They were acclimatized to the environment and investigator for at least 20 minutes before the test. A evaluation was performed approximately 7 days (7.8 ± 2.1 days, 220.5 ± 21.6 g, n = 50) after surgery.

The mechanical stimulus was applied to the middle area between the footpads on the plantar surface of the ipsilateral (nerve root injury site) hind paw and maintained for approximately 2 seconds. A withdrawal response was considered valid only if the hind paw was removed completely from the platform. If a rat walked immediately after stimulation of a hair instead of lifting the paw, the hair was reapplied. A trial consisted of application of a von Frey hair to the hind paw five times at 5-second intervals. The hind paw was probed consecutively with 10 stimulations. The trial was repeated 3 times with at least a 10-minute interval. Mechanical sensitivity was evaluated as the frequency of withdrawal responses, expressed as the mean frequency of responses.

Differences between groups were compared using Student's *t* test or a one-way analysis of variance (ANOVA). Data were presented as mean ± SEM. When ANOVA showed a significant difference, pair-wise comparisons between means were tested by the *post-hoc* Tukey method. Hind paw withdrawal threshold values between groups were considered significantly different with a P value < 0.05.

Immunohistochemistry

Spinal sections were processed for immunohistochemistry using the immunofluorescence [57,58].

At 7 days post-surgery, rats (208.4 ± 21.7 g, n = 25) were perfused through the ascending aorta with saline followed by 4% paraformaldehyde with 1.5% picric acid in 0.16 M phosphate buffer, pH7.2–7.4 (4°C). After perfusion, spinal cords were removed and the L4-L5 spinal cord segments were dissected, post fixed in the same perfusion fixative for 4 hours and then 15% sucrose overnight. All of the spinal cords for each experiment were arranged on the same blocks with optimal cutting temperature embedding medium and mounted on the same slides after sectioning. Transverse spinal sections (15 μm) were cut on a cryostat and processed for immunostaining. Spinal sections were blocked with 2% goat serum in 0.3% Triton X-100 for 1 hour at room temperature and incubated overnight at 4°C with rabbit (polyclonal) antiserum directed against the ionized calcium binding adapter molecule 1 (Iba1; 1:1000; Wako Chemicals, Tokyo, Japan), a marker of microglia [59].

After washing, the sections were then incubated with fluorescent–conjugated secondary antibody (1:1000 Alexa Fluor 594 goat anti-rabbit; Invitrogen, San Diego, CA) for 90 minutes at room temperature.

The immunostained sections were examined and photographed with an Olympus (FSX100, Japan) fluorescence microscope at 40× magnification. Quantification of microglia activation was assessed by the number of Iba1-positive microglia in the lamina I–II of the L5 segment.

Differences between groups were compared using Student's *t* test or a one-way ANOVA. Data were presented as mean ± SEM. When ANOVA showed a significant difference, pair-wise comparisons between means were tested by the *post-hoc* Tukey method. Values were considered statistically significant with a P value < 0.05.

In vivo patch-clamp recordings

The methods used for the *in vivo* patch-clamp recordings were as described previously [6,7,60,61]. Rats (218.4 ± 23.5 g, n = 45) were assessed approximately 7 days (7.6 ± 2.4 days) post- surgery when mechanical hypersensitivity had developed fully. Rats were anesthetized with urethane (1.2 g/kg, i.p.). Artificial ventilation of the pneumothorax was not performed as the rats could be maintained in good condition by supplying oxygen through a nose cone [60]. If a withdrawal reflex appeared, a supplemental dose of urethane was given during surgery and the data collection period. A heating pad was placed beneath the rat to maintain its body temperature at 37–38°C. The rat was placed in a stereotaxic apparatus (Model STS-B&SR-5R-HT, Narishige, Tokyo, Japan) and lumbar spinal cord at the L5 segmental area was exposed by a thoracolumbar laminectomy at the level from Th12 to L2. The dura was cut under a microscope with 40× magnification and a recording electrode was advanced into the SG from the surface of the spinal cord. The pia-arachnoid membrane of the right L5 dorsal root entry zone was removed allowing the patch electrode to enter the spinal cord. The surface of the spinal cord was irrigated with Krebs solution (10–15 ml/min) and equilibrated with a 95% O_2, 5% CO_2 gas mixture (117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 11 mM glucose and 25 mM $NaHCO_3$) through glass pipettes at 36.5 ± 0.5°C. At the end of the experiments, the rats were given an overdose of urethane and killed by exsanguinations.

The patch electrodes were pulled from thin-walled borosilicate glass capillaries (outer diameter of 1.5 mm, TW150F-4, World Precision Instruments, Sarasota, FL, USA) using a p-97 puller (Sutter Instrument, Novato, CA, USA) and filled with a patch-pipette solution that contained: 135 mM K-gluconate, 5 mM KCl, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM ATP-Mg and 5 mM Hepes-KOH; pH7.2 for excitatory postsynaptic current (EPSC) recordings. The recording electrode with a resistance of 8-12MΩwas advanced at an angle of 30 degrees into the SG through the pia-arachnoid membrane by a micromanipulator (Model MWS-32 S, Narishige, Tokyo, Japan). A gigaohm seal (resistance of 10GΩ) was formed with neurons at a depth of 30–150 μm from the surface of the spinal cord, the membrane patch ruptured because of a period of negative pressure and the whole-cell patch-clamp recording was initiated. Holding potential was set to −70 mV in voltage-clamp mode and recordings were collected using an Axopatch 200B amplifier in conjunction with a Digidata 1440A A/D converter (Molecular Devices, Sunnyvale, CA, USA) and stored on a computer using pCLAMP 10 data acquisition program (Molecular Devices, Sunnyvale, CA, USA).

Differences between groups were compared using Student's *t* test or a one-way ANOVA. Data were presented as mean ± SEM. When ANOVA showed a significant difference, pair-wise comparisons between means were tested by the *post-hoc* Tukey method. Values were considered statistically significant with a P value < 0.05.

Abbreviations

DRG: Dorsal root ganglia; SG: The substantia gelatinosa;.LTP: Long term potential; CNS: Central nervous system; EPSC: Excitatory postsynaptic currents; IPSC: Inhibitory postsynaptic currents; NMDA receptor: N-methyl D-aspartate receptor; AMPA receptor: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; Iba 1: Anti-ionized calcium binding adapter molecule 1; TTX-r sodium channel: Tetrodotoxin resistant sodium channel.

Competing interests

The authors declare that they have no competing interests.

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Author details

[1]Department of Orthopaedic Surgery, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, Japan. [2]Pain Research Center, Kansai University of Health Sciences, 2-11-1 Wakaba Kumatori Sennan, Osaka 590-0482, Japan.

Authors' contributions

All authors read and approved the final manuscript. NT performed or contributed to all experiments, analyzed data and drafted the paper. WT and HH contributed to experiments and analysis. NN contributed to experiments. NM and HY participated in the design of the studies. TN and MY conceived and supervised the project and edited the manuscript.

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Original Article

Reactive oxygen species actions on excitatory synaptic transmission in spinal substantia gelatinosa neurons

Naoko Nishio[1], Wataru Taniguchi[2], Yae Sugimura[1], Noboru Takiguchi[2]
Yasukuni Kiyoyuki[1], Sumi Matsukawa[1], Yuuka Sakurai[1]
Yasuhiko Kawasaki[1], and Terumasa Nakatsuka[1]

[1]Pain Research Center, Kansai University of Health Sciences
[2]Department of Orthopaedic Surgery, Wakayama Medical University

Abstract

Reactive oxygen species (ROS) are a molecular species which come from O_2 generated in intravital energy metabolism and the phylaxis process, and are highly-reactive. Two common examples of ROS are super-oxide (O_2^-) and hydrogen peroxide (H_2O_2). ROS are absolutely necessary to maintain life, but it is thought that surplus ROS which cannot be fully degraded by intracellular enzymes cause various disorders such as cancer or arteriosclerosis. Recent studies have suggested that ROS are involved in chronic pain such as neuropathic pain or inflammatory pain in the spinal cord. For instance, it has been reported that ROS participate in long-term potentiation in the dorsal horn of the spinal cord and is a contributing factor of secondary hyperalgesia. However, the cellular mechanism of ROS is still unclear in the dorsal horn of the spinal cord. To address this issue, we investigated the effect of ROS on glutamatergic excitatory synaptic transmission in substantia gelatinosa (SG) neurons of adult rat spinal cord slices by using the whole-cell patch-clamp technique. A ROS donor, tert-butyl hydroperoxde (t-BOOH), superfused for 5 min markedly increased the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs). The t-BOOH-induced increases in sEPSC frequency were resistant to tetrodotoxin. However, in the presence of a non-NMDA receptor antagonist, CNQX, t-BOOH did not generate any sEPSC. Furthermore, the t-BOOH-induced increases in sEPSC frequency were inhibited by the scavenger N-tert-butyl-α-phenyl nitrone. In the presence of a TRPA1 channel antagonist, HC-030031, t-BOOH-induced increases in sEPSC frequency were inhibited. On the other hand, in the presence of a TRPV1 channel antagonist, capsazepine, t-BOOH-induced increases in sEPSC frequency and amplitude were not affected. These results indicate that ROS enhances the spontaneous release of L-glutamate from presynaptic terminals onto the SG neurons through TRPA1 channel activation.

Keywords
ROS ; Spinal cord ; Patch-clamp

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脊髄膠様質細胞の興奮性シナプス伝達に対する活性酸素の作用

西尾 尚子[1]／谷口 亘[2]／杉村 弥恵[1]／瀧口 登[2]
清行 康邦[1]／松川 澄[1]／櫻井 悠加[1]
川崎 康彦[1]／中塚 映政[1]

[1]関西医療大学 保健医療学部 疼痛医学分野
[2]和歌山県立医科大学 整形外科学教室

はじめに

活性酸素種(reactive oxygen species: ROS)とは生体内のエネルギー代謝や感染防御過程において発生する，酸素分子（O_2）に由来し反応性に富む一群の分子種を指し，その代表的なものとしてスーパーオキシド（O_2^-），過酸化水素(H_2O_2)などが挙げられる。ROSは，癌，動脈硬化，リウマチ，老化および神経疾患といったさまざまな疾病との関連が報告されている。ROSは種々のfree radicalとH_2O_2などの分子種からなり，正常では細胞内でその強力な酸化作用により生体防御に利用されている。しかし，脊髄損傷や一過性脊髄虚血後の再灌流障害などによりフリーラジカルが細胞内から放出されると二次損傷を引き起こすことや，筋萎縮性側索硬化症などの神経変性疾患においても細胞内でのROSの増加が関与していることが報告されており，本来の生体防御から逸脱した作用を有することがわかっている[10]。また，神経障害性疼痛や炎症性疼痛を含む慢性疼痛においても脊髄レベルでのROSの関与が近年報告されている。例えば，神経因性疼痛モデルなどにおいてROS Scavenger（消去剤）の髄腔内投与，腹腔内投与，静注投与などの研究において動物行動学的に鎮痛作用があることや炎症モデルにおける二次性疼痛や長期増強（Long Term Potentiation: LTP)にROSが関与していると報告されており，ROSそのものが脊髄レベルでの疼痛情報伝達の修飾やシナプス伝達の可塑性に関与していることが示唆されている[4,5,9]。脊髄後角の膠様質（Rexedの分類：脊髄第II層）は，皮膚末梢からの痛み情報が入力するところで，痛み情報の伝達や修飾が行われる重要な領域である[12]。脊髄後角の膠様質における単一細胞レベルでのROSの作用の有無，ならびにその作用機序はいまだ不明であることから，成熟ラットから作製した脊髄横断スライス標本の膠様質細胞にブラインド・ホールセル・パッチクランプ法を適用し，ROSドナーであるtert-butylhydroperoxide(t-BOOH)が脊髄後角感覚細胞の興奮性シナプス伝達にどのような作用を及ぼすかを検討した。

方　法

本実験計画は関西医療大学動物実験委員会で承認された。

1. 脊髄横断スライス標本の作製

脊髄横断スライス標本の作製は，Nakatsukaらの方法に従って行った[8]。5〜6週齢のSprague-Dawley系成熟雄性ラットにウレタン（腹腔内投与：1.2〜1.5 g/kg）で深麻酔後，背側の胸腰椎部に皮切を行った。エピネフリン添加0.5%キシロカイン液を棘突起の両側に局注後，傍脊柱筋群を切離し脊椎を露出した。中位胸椎から下位腰椎まで椎弓切除を行った。神経根を切離しながら脊髄を摘出し，酸素負荷した2〜4℃の人工脳脊髄液に浸した。摘出した脊髄を実体顕微鏡下に硬膜，前根，後根，クモ膜および軟膜を除去し，溝を設けた寒天ブロックに設置した。マイクロスライサー（堂阪イーエム社DTK-1000）を用いて厚さ約650 μmの脊髄横断スライス標本を作製した。腰膨大部の脊髄スライスを記録用チャンバーに移し，グリッドにて上方から軽く固定した後，酸素負荷した人工脳脊髄液で還流した。人工脳脊髄液の組成は，NaCl 117 mM，KCl 3.6 mM，$CaCl_2$ 2.5 mM，$MgCl_2$ 1.2 mM，NaH_2PO_4 1.2 mM，glucose 11 mM，$NaHCO_3$ 25 mMであった。

2. 脊髄膠様質細胞からのパッチクランプ記録

膠様質は，脊髄スライスに下方から透過光を当て，実体顕微鏡下(20〜40倍)において観察すると後角の灰白質部表層に位置する半透明なバンド状として視認できる。膠様質細胞からブラインド・ホールセル・パッチクランプ法により膜電位固定下で膜電流記録を行った。ガラス電極は，入力抵抗が8〜15 MΩのものを用い，その内液組成はpotassium gluconate 135 mM，KCl 5 mM，$CaCl_2$ 0.5 mM，$MgCl_2$ 2 mM，EGTA 5 mM，ATP-Mg 5 mM，Hepes-KOH 5 mMであった。薬液の灌流は人工脳脊髄液と同ラインを用いて行った。興奮性シナプス後電流（excitatory postsynaptic currents: EPSC）は，−70 mVの保持膜電位で記録した。得られた応答はパッチクランプ用増幅器（Molecular Devices社；Axopatch200B）により増幅され，A/D変換（Molecular Devices社；Digidata 1440A）後，データ記録および解析用ソフトウェア(Molecular Devices社；pClamp10, Synaptosoft社；Mini Analysis 6.0)を用いてコンピュータにより記録・解析した。実験結果は平均±標準誤差で表し，検定は2群間の場合はpaired *t*-testを用い，多群間の場合には，まずOne way analysis of varianceにて比較検定を行い，有意差を認めた場合は多重比較検定（Games/Howel法）を行った。また危険率5%（$p<0.05$）をもって有意と判定した。括弧内のnの値は記録した細胞の数である。使用した薬物について，t-BOOH，N-tert-Butyl-α-phenylnitrone（PBN），HC-030031はSigma-Aldrich，tetrodotoxin（TTX）はLatoxan，6-cyano-7-nitroquinoxaline-2,3-dione(CNQX)，Capsazepineは和光純薬から購入した。

結　果

膜電位固定下（−70 mV）では，記録した全ての細胞において自発性興奮性シナプス後電流（spontaneous excitatory postsynaptic current:

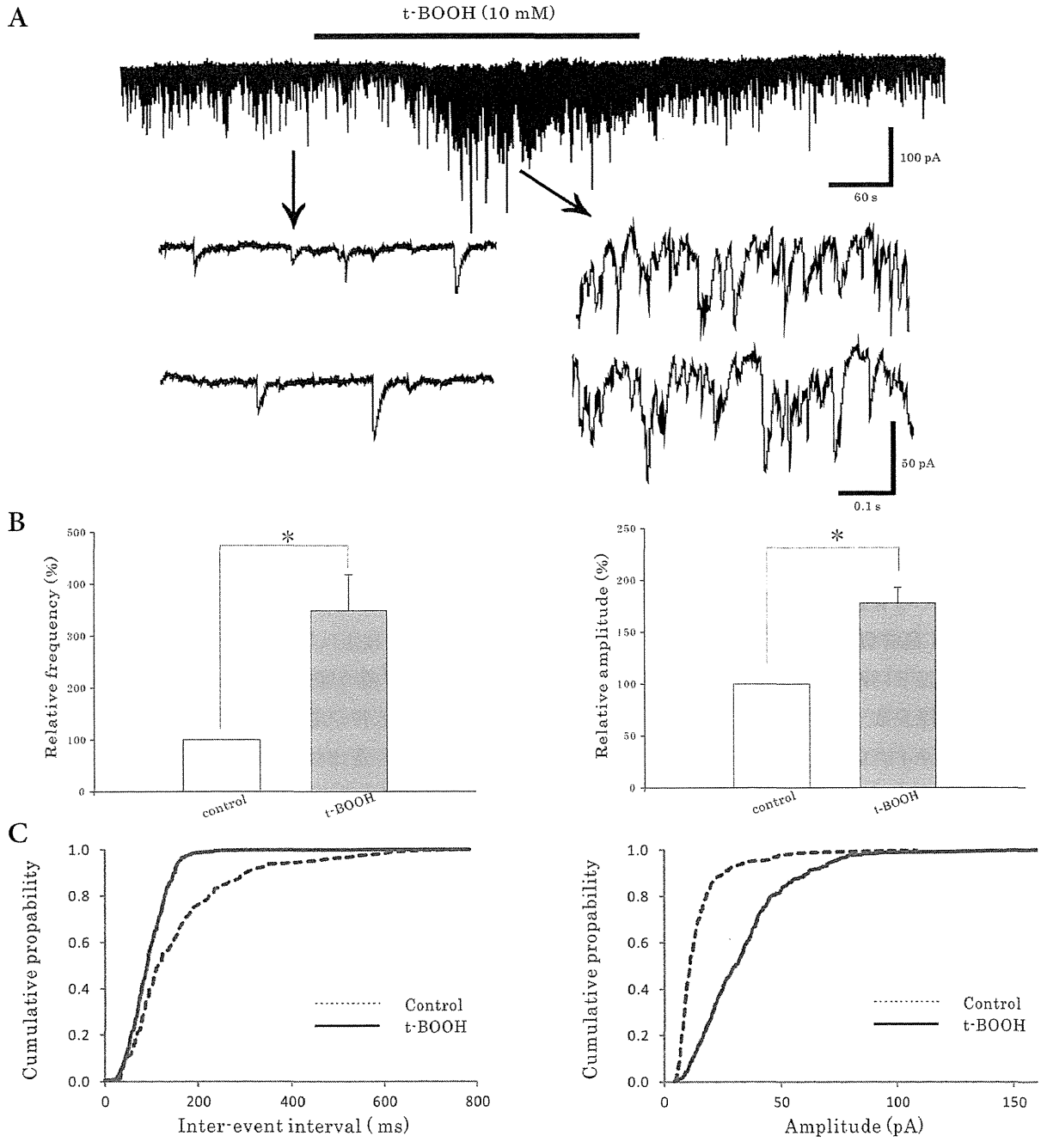

Fig.1

A: A ROS donor, tert-butyl hydroperoxde (t-BOOH), superfused for 5 min markedly increased the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSC). The two traces, shown in an expanded scale in time, indicate that the frequency and amplitude of EPSC are clearly increased during t-BOOH perfusion compared with those of controls. B: Summary of sEPSC frequency and amplitude. Vertical lines accompanied by bars show SEM. Statistical significance between data shown by bars is indicated by an asterisk; $^{*}p<0.05$. C: Cumulative distributions of the interevent interval and amplitude of sEPSCs, before (dotted line) and during (continuous line) the action of t-BOOH. T-BOOH shifted the interevent interval and amplitude to a shorter and a larger one, respectively.

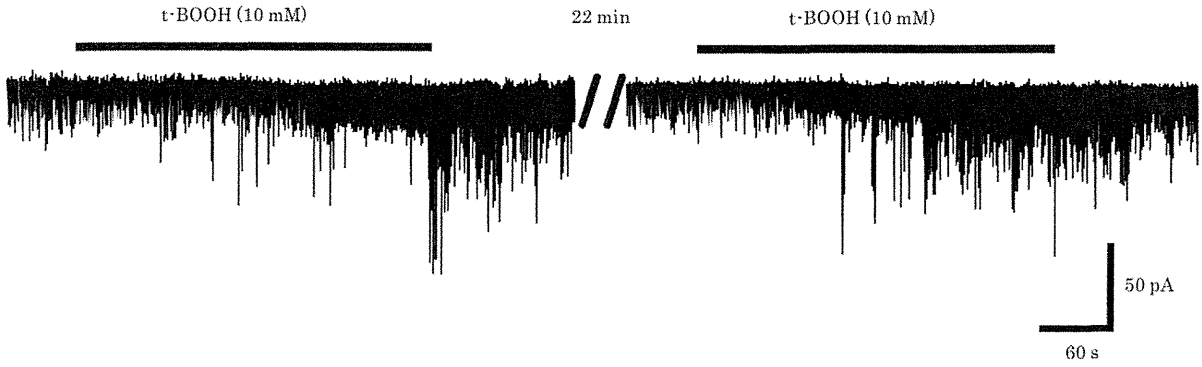

Fig.2 When t-BOOH was applied repeatedly at 25 min intervals, it produced similar increases in sEPSC frequency and amplitude.

sEPSC)が観察された。このsEPSCはグルタミン酸受容体の拮抗薬であるCNQX存在下で完全に消失することから，神経終末内のシナプス小胞から放出された興奮性神経伝達物質であるグルタミン酸に起因するものであった。また実験中のSeries Resistanceは変化がなかった。

ROSドナーであるt-BOOH(10 mM)を5分間灌流投与すると，記録した全ての膠様質細胞で，sEPSCの発生頻度ならびに振幅は増加した（Fig.1-A)。t-BOOHによるsEPSCの発生頻度ならびに振幅の程度はそれぞれコントロールの349 ± 69%（n=13, p<0.05)，178 ± 15%（n=13, p<0.05)であった(Fig.1-B)。T-BOOH存在下におけるsEPSCのinter-event intervalと振幅の累積分布を調べたところ，コントロールに比べてinter-event intervalは左方へ，振幅は右方へシフトした（Fig.1-C)。また，t-BOOHによってsEPSCの発生頻度ならびに振幅が増加した細胞に，t-BOOHを25分後反復投与したところ，初回投与と同程度の増加がsEPSCの発生頻度と振幅において観察され，脱感作はみられなかった（n=4, Fig.2)。

電位依存性ナトリウムチャネル阻害薬であるTTX存在下では微小興奮性シナプス後電流（miniature excitatory postsynaptic current: mEPSC)が観察できる。TTX(1 μM)存在下においてt-BOOHを5分間灌流投与するとmEPSCの発生頻度ならびに振幅が増加した(Fig.3-A)。その発生頻度ならびに振幅の程度はTTX単独投与時と比較してそれぞれ510 ± 222%（n=5, p<0.05)，171 ± 29%（n=5, p>0.05)であった（Fig.3-C)。T-BOOH存在下におけるmEPSCのinter-event intervalと振幅の累積分布を調べたところ，コントロールに比べてinter-event intervalは左方へ，振幅は右方へシフトした（Fig.3-D)。次にTTX存在下におけるt-BOOH投与前後でのmEPSCの発生数および振幅の分布を調べたところ，t-BOOH投与によってmEPSCの発生数が大きく増加し，全体のピークが低振幅から高振幅にシフトしたが，高振幅のmEPSCはt-BOOHによって伝達物質を含んだシナプス小胞が同期的に大量放出された結果であることによるものかと考えた(Fig.3-B)。

CNQX (20 μM)存在下ではsEPSCは完全に消失するが，t-BOOHを5分間灌流投与してもsEPSCは観察されなかった(n=4, Fig.4-A)。

次にt-BOOHによるsEPSCの発生頻度な

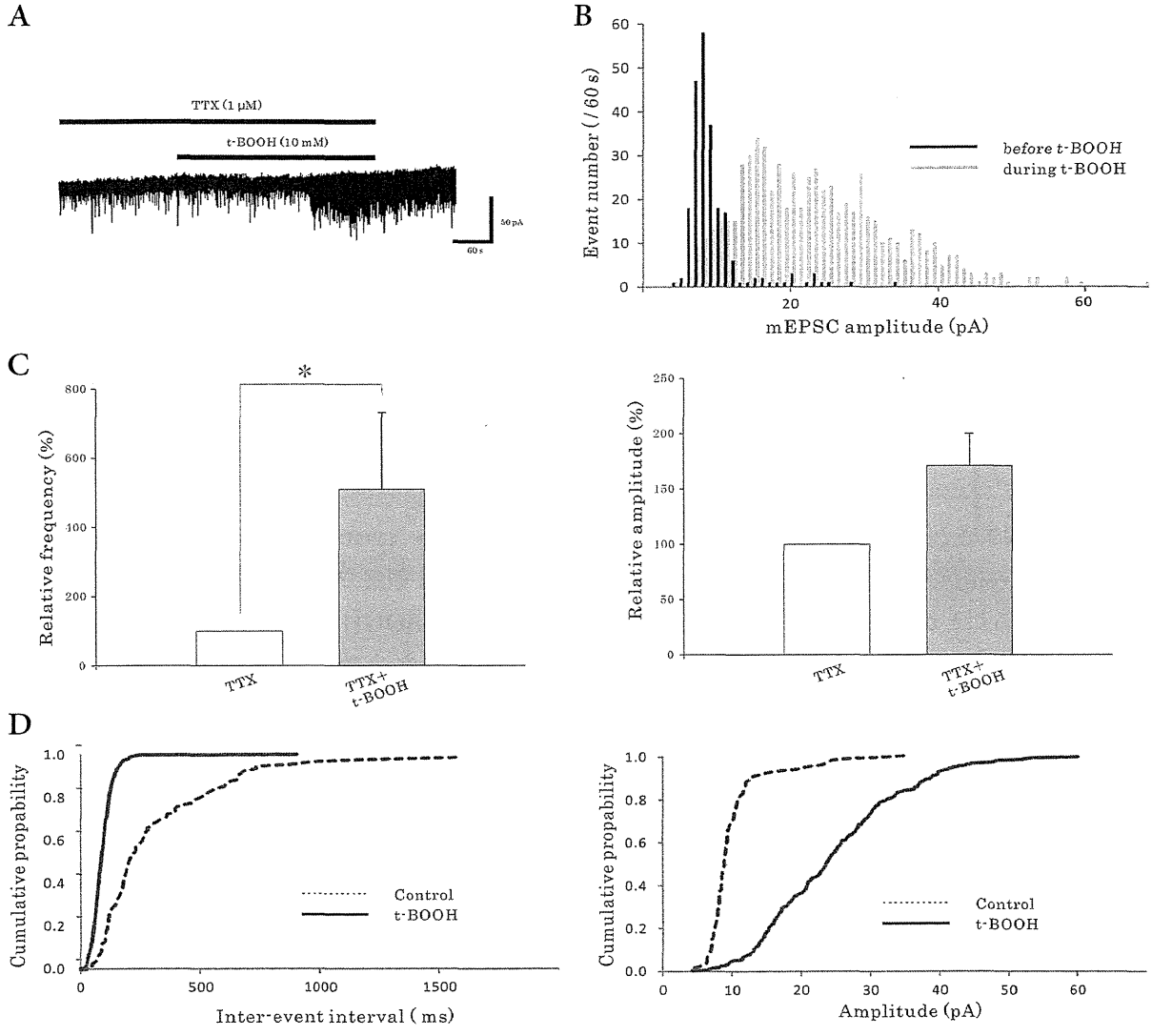

Fig.3

A: T-BOOH markedly increased mEPSC frequency in the presence of TTX. B: Distributions of mEPSC amplitude before and during (filled bar and gray bar, respectively) the action of t-BOOH. C: Summary of mEPSC frequency and amplitude. Vertical lines accompanied by bars show SEM. Statistical significance between data shown by bars is indicated by an asterisk; $^{*}p<0.05$. D: Cumulative distributions of the interevent interval and amplitude of mEPSCs, before (dotted line) and during (continuous line) the action of t-BOOH. T-BOOH shifted the interevent interval and amplitude to a shorter and a larger one, respectively.

らびに振幅の増加が抗酸化剤である PBN（10 mM）によって影響を受けるか検討した。PBN 存在下では，t-BOOH 投与による sEPSC の発生頻度ならびに振幅の程度は PBN 単独投与時と比較してそれぞれ 141 ± 12%（n=7），128 ± 11%（n=7）であった（Fig.4-B）。T-BOOH による sEPSC の発生頻度ならびに振幅の増加の程度を PBN 存在下と非存在下において比較したところ，PBN 存在下では PBN 非存在下と比較して sEPSC の発生頻度が有意に減少した。

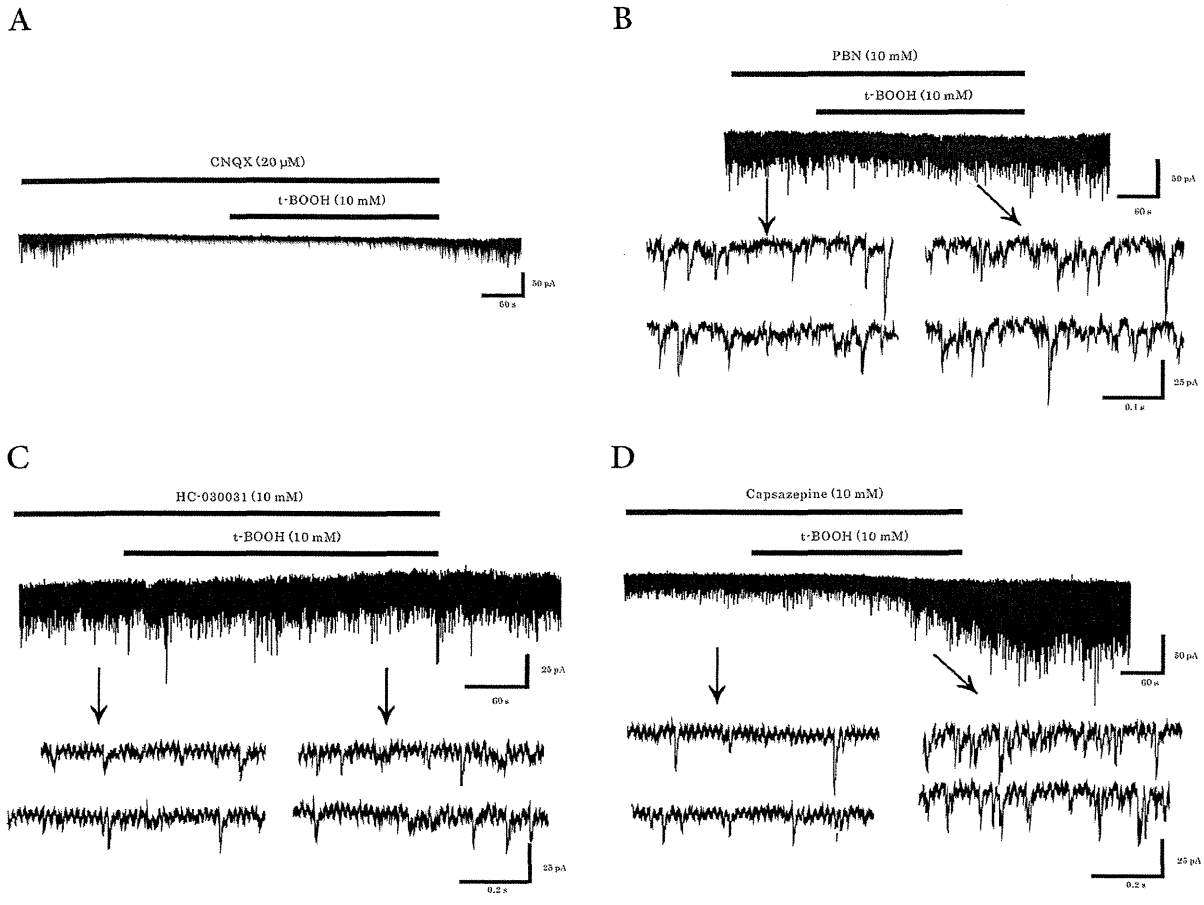

Fig.4

A: CNQX blocked sEPSCs not only in the absence of t-BOOH but also under its action. B: The scavenger PBN suppressed the t-BOOH-induced increases in sEPSC frequency. C: TRPA1 channel antagonist significantly suppressed the t-BOOH-induced in sEPSC frequency. D: TRPV1 channel antagonist did not affect the t-BOOH-induced increases in sEPSC frequency and amplitude.

さらにTRPチャネルのアンタゴニストによってt-BOOHによるsEPSCの増強作用が影響を受けるか検討した。TRPA1チャネルのアンタゴニストであるHC-030031の存在下では，t-BOOH投与によるsEPSCの発生頻度ならびに振幅の程度はHC-030031単独投与時と比較してそれぞれ132±9%(*n*=8)，138±20%(*n*=8)であった（Fig.4-C)。T-BOOHによるsEPSCの発生頻度ならびに振幅の増加の程度をHC-030031存在下と非存在下において比較したところ，HC-030031存在下ではsEPSCの発生頻度は有意に減少した。またTRPV1チャネルのアンタゴニストであるCapsazepineの存在下では，t-BOOH投与によるsEPSCの発生頻度ならびに振幅の程度はCapsazepine単独投与時と比較してそれぞれ178±27%(*n*=7)，223±59%(*n*=7)であった（Fig.4-D)。T-BOOHによるsEPSCの発生頻度ならびに振幅の増加の程度をCapsazepine存在下と非存在下において比較したところ，sEPSCの発生頻度ならびに振幅に有意な差がみられなかった（Fig.5)。

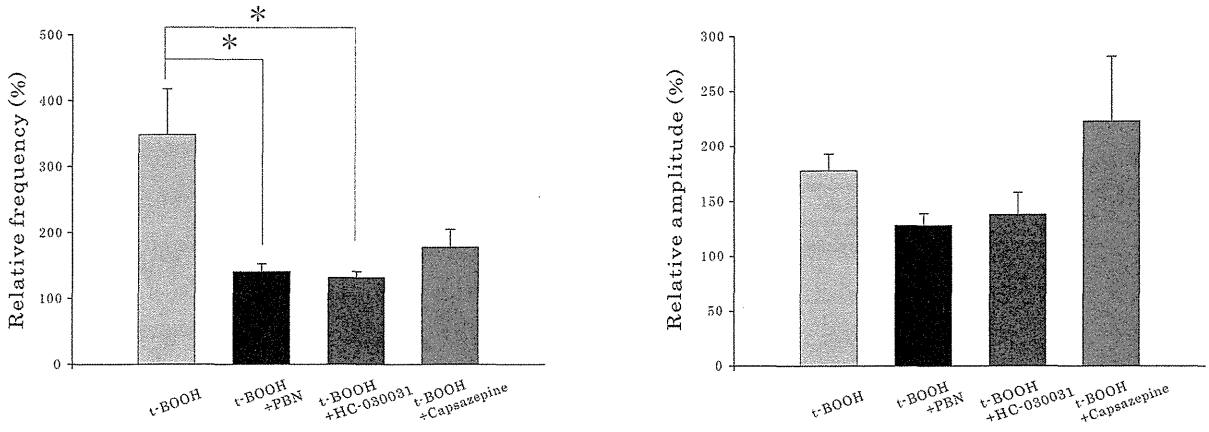

Fig.5 Summary of sEPSC frequency and amplitude.

Vertical lines accompanied by bars show SEM. Statistical significance between data shown by bars is indicated by an asterisk; $^{*}p<0.05$.

考　察

本研究では，脊髄膠様質細胞にパッチクランプ法を適用して，単一細胞レベルでのt-BOOHの薬理学的作用について解析を行った。ROSドナーであるt-BOOHを灌流投与すると脊髄膠様質細胞におけるsEPSCの発生頻度ならびに振幅は有意に増加した。一方，抗酸化剤であるPBN存在下でt-BOOHの作用について検討したところ，PBN存在下ではt-BOOHによるsEPSCの増強作用は頻度において抑制された。また，t-BOOHの作用はCNQX存在下では完全に抑制されたが，TTXにより殆ど影響を受けなかった。以上の結果から，ROSは脊髄膠様質細胞に入力するシナプス前終末に作用してグルタミン酸の過剰放出を惹起することが明らかとなった。ROSはTRP（transient receptor potential）チャネルを活性化し，細胞内にカルシウムの流入を惹起し，細胞内シグナル伝達を修飾することが報告されている[1]。TRPチャネルは痛みを伝える一次感覚神経の末梢端に発現しており，皮膚末梢において痛みや温度情報を受容し中枢神経系に伝えることが知られているが，我々は，TRPチャネルが痛みを伝える一次感覚神経の中枢端にも存在し，痛み情報伝達の増強に関与していることを明らかにしてきた[3]。TRPA1チャネルのアンタゴニストであるHC-030031の存在下で，t-BOOHによるsEPSCの増強作用が発生頻度において抑制された。一方TRPV1チャネルのアンタゴニストであるCapsazepineの存在下では，t-BOOHの頻度ならびに振幅の増加は抑制されなかった。以上から，ROSは脊髄膠様質細胞に入力している一次感覚神経の中枢端に発現するTRPA1チャネルを活性化し，グルタミン酸の過剰放出を惹起して中枢性の痛覚過敏を引き起こす可能性が示唆された。今回は，脊髄後角の神経回路におけるt-BOOHの役割を明らかにするためmEPSCではなくsEPSCの応答を記録したが，t-BOOHがmIPSCに作用を及ぼすという報告がある[13]。この報告は低濃度（2 mM）のt-BOOHは脊髄膠様質細胞の興奮性シナプス伝達に影響を与えないもの

の，GABA の遊離抑制を示唆している。今回高濃度の t-BOOH によって興奮性シナプス伝達が増強されたが，抑制性応答ならびに濃度依存性の検討はしておらず，これらは今後の課題としたい。

脊髄損傷や一過性脊髄虚血後の再灌流障害などによりフリーラジカルが細胞内から放出されると二次損傷を引き起こすことが知られている[2,6,7]。脊髄損傷は後遺症として損傷レベル以下の運動神経麻痺と感覚障害により ADL を著しく制限する。一方，脊髄損傷後疼痛は一般的にあまり注目されていないが，運動神経麻痺や感覚障害といった後遺症に匹敵するあるいはそれ以上の ADL 制限をもたらす。海外においては全脊損患者の約 65～80%に疼痛の程度の差はあるものの脊髄損傷後疼痛が発生しているとの報告があり[11]，我が国においても統計学的調査がないものの脊髄損傷後疼痛に困窮している患者は多数存在する。脊髄損傷後疼痛は神経障害性難治性疼痛であり，程度の強いものはアロディニア様の異常知覚を生じる。一般的な NSAIDs やオピオイドといった鎮痛薬は効果が薄く，新たな作用機序の治療薬や治療法の開発が待たれている。しかしながら，脊髄損傷後疼痛が発生するメカニズムはいまだ不明な点が多く，その解明が待ち望まれている。近年，神経障害性疼痛に ROS が関与しているとの報告が散見されるようになり，脊髄損傷後疼痛の発生機序にも ROS が関与し，神経系の可塑的変化を惹起している可能性がある。今後，脊髄損傷後疼痛の発症メカニズムに ROS が関与することが証明できれば，脊髄損傷後疼痛の予防や治療に光を当てることが可能になるかもしれない。すなわち，ROS Scavenger や ROS の興奮性シグナル伝達に関与する TRP チャネル拮抗薬の髄腔内投与や静脈内注射などによって脊髄損傷後疼痛が克服できる可能性が高まる。したがって，これらの薬剤を用いることによって，脊髄損傷後疼痛を未然に予防することが可能になるだけでなく，すでに慢性疼痛化した患者にも治療薬となり得る可能性を秘めており，脊髄損傷後疼痛の有効な薬剤が存在しない現状を劇的に変える可能性がある。

結　語

ROS は脊髄膠様質細胞に入力する一次感覚神経終末に発現する TRPA1 チャネルに作用し，グルタミン酸の過剰放出を惹起することによって痛み情報伝達を増強することが明らかとなった。

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Address for correspondence: Naoko Nishio
Pain Research Center,
Kansai University of Health Sciences
2–11–1 Wakaba, Kumatori, Sennan,
Osaka 590–0482, Japan

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ORIGINAL INVESTIGATION

The *KCNH2* gene is associated with neurocognition and the risk of schizophrenia

RYOTA HASHIMOTO[1–3], KAZUTAKA OHI[2,3], YUKA YASUDA[2,3], MOTOYUKI FUKUMOTO[2,3], HIDENAGA YAMAMORI[2–4], KOUZIN KAMINO[2,5], TAKASHI MORIHARA[2], MASAO IWASE[2], HIROAKI KAZUI[2] & MASATOSHI TAKEDA[1,2]

[1]*Molecular Research Center for Children's Mental Development, United Graduate School of Child Development, Osaka University, Kanazawa University and Hamamatsu University School of Medicine, Osaka, Japan,* [2]*Department of Psychiatry, Osaka University Graduate School of Medicine, Osaka, Japan,* [3]*CREST (Core Research for Evolutionary Science and Technology) of JST (Japan Science and Technology Agency), Saitama, Japan,* [4]*Department of Molecular Neuropsychiatry, Osaka University Graduate School of Medicine, Osaka, Japan, and* [5]*Shoraiso National Hospital, Yamatokoriyama, Nara, Japan.*

Abstract

Objectives. A genetic variant (rs3800779; M30) in the *KCNH2* gene has been associated with schizophrenia, a lower intelligence quotient (IQ) and processing speed scores, altered brain functions and increased *KCNH2-3.1.* mRNA levels in the hippocampus. The aims of this study were to investigate whether the *KCNH2* polymorphism is associated with schizophrenia-related neurocognitive deficits and to confirm the association between the variant and schizophrenia. *Methods.* The effects of the risk genotype on IQ and seven neurocognitive batteries were examined by the analysis of covariance in 191 healthy subjects. We performed a meta-analysis of the association between M30 and schizophrenia using five independent ethnic groups (1,720 cases; 2,418 controls). *Results.* Consistent with the previous study, we provided evidence that subjects with the risk T carriers had significantly lower IQ scores than those with the G/G genotype ($P = 0.048$). Of the seven neurocognitive batteries, subjects with the risk genotype demonstrated lower performances on attention/vigilance ($P = 0.0079$) and working memory ($P = 0.0066$) relative to subjects with the G/G genotype. Meta-analysis demonstrated evidence for an association between M30 and schizophrenia without showing heterogeneity across studies (odds ratio = 1.18; $P = 0.0017$). *Conclusions.* These data suggest that the *KCNH2* polymorphism could be associated with schizophrenia-related neuropsychological deficits and the risk of developing schizophrenia.

Key words: *schizophrenia, KCNH2 (potassium channel, voltage-gated subfamily H, member 2), intelligence quotient (IQ), single nucleotide polymorphism (SNP), meta-analysis, neurocognition*

Introduction

Schizophrenia is a common, complex psychiatric disease characterized by both clinical and genetic heterogeneity. There are strong genetic components of the disease with an estimated heritability of approximately 80% (Cardno and Gottesman 2000; Tsuang 2000). Attempts have been made to minimize this heterogeneity and to clarify the genetic architecture. One strategy for gene discovery proposes using quantitative neurobiological traits as intermediate phenotypes instead of relying on the diagnosis of schizophrenia alone to identify cases for investigation (Meyer-Lindenberg and Weinberger 2006; Tan et al. 2008a). This strategy has the potential to reduce clinical and genetic heterogeneity by applying alternative phenotypes that better reflect the underlying genetic vulnerability than does diagnostic categorization. Neurocognitive deficits, a core component of schizophrenia (Green 2006), are considered promising intermediate phenotypes for gene

Correspondence: Ryota Hashimoto, Molecular Research Center for Children's Mental Development, United Graduate School of Child Development, Osaka University, Kanazawa University and Hamamatsu University School of Medicine, D3, 2-2, Yamadaoka, Suita, Osaka, 565-0871, Japan. Tel: +81 6 6879 3074. Fax: +81 6 6879 3059. E-mail: hashimor@psy.med.osaka-u.ac.jp

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discovery in schizophrenia (Snitz et al. 2006; Husted et al. 2009). There is substantial evidence suggesting that most cognitive abilities have a genetic basis (Chen et al. 1998; Posthuma et al. 2001; Berrettini 2005; Husted et al. 2009). The estimated heritabilities of processing speed, attention/vigilance, verbal intelligence quotient (IQ) and performance IQ are 33–48, 48–62, 85 and 69%, respectively.

Recently, Huffaker et al. identified a potential schizophrenia susceptibility (human ether-a-go-go-related) gene, *KCNH2,* which encodes a voltage-activated potassium channel (Huffaker et al. 2009). The *KCNH2* gene contains 15 exons spanning 33 kb on chromosome 7q35–q36. A genetic variant (rs3800779; M30) in the *KCNH2* gene predicts lower IQ and processing speed scores, decreased hippocampal volume, altered memory-linked hippocampal functions and working memory-linked prefrontal functions. It also predicts increased expression levels of a primate- and brain-specific *KCNH2*-3.1 isoform in the hippocampus (Huffaker et al. 2009). Expression of *KCNH2*-3.1 in rodent cortical neurons causes a marked alteration in KCNH2 channel physiology resulting in high-frequency, nonadapting neuronal firing patterns (Huffaker et al. 2009). In this study, we examined the effects of the M30 genotype on IQ and seven neurocognitive functions shown to be associated with genetic liability in schizophrenia. We then conducted a meta-analysis of M30 in previously reported samples added to a Japanese sample to establish further evidence for an association between the *KCNH2* gene and schizophrenia.

Methods and materials

Subjects

Neurocognitive test data were available for 191 Japanese healthy individuals (49.2% males (94/97); mean age ± SD: 36.0 ± 11.5 years; years of education ± SD: 15.5 ± 2.4 years). Data from different number of subjects were available in each test (general IQ 143 subjects, speed of processing 188, attention/vigilance 191, working memory 190, Verbal Learning and Memory 190, Visual Learning and Memory 190, Reasoning and problem solving 150, and Social cognition 86). Demographic variables for subjects included in each cognitive test are shown in Supplementary Table I (available online). Although we attempted to examine all neurocognitive tests from all subjects as much as we could, all tests data were available for 83 subjects. Because an association between an SNP in the *KCNH2* gene and cognitive function was observed in healthy controls, we attempted to replicate the previous association finding in healthy controls (Huffaker et al. 2009). The use of healthy subjects to investigate an association between a genetic variant and neurocognitive function avoids the potential confounders related to the duration of illness and medical treatment. Healthy controls were recruited by local advertisements in Osaka, Japan. Psychiatrically, medically and neurologically healthy controls were evaluated using the Structured Clinical Interview for DSM-IV-Non-Patient Edition (SCID-I/NP) to exclude individuals who had received psychiatric medications. Subjects were also excluded from this study if they had neurological or medical conditions that could potentially affect the central nervous system, such as atypical headaches, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, active cancer, cerebrovascular disease, epilepsy, seizures, substance-related disorders or mental retardation. We excluded any control subjects with neurological disorders or first- or second-degree relatives with psychiatric disorders using an unstructured interview. All subjects were biologically unrelated Japanese individuals.

The subjects for the genetic association study consisted of 478 unrelated patients with schizophrenia (48.3% males (231/247); mean age ± SD: 48.4 ± 15.7 years) and 640 unrelated healthy controls (46.3% males (296/344); mean age ± SD: 58.9 ± 21.4 years). All subjects used in this analysis are unrelated Japanese, as described previously (Ohi et al. 2009b, 2010). Cases were recruited from both outpatients and inpatients at Osaka University Hospital and the psychiatric hospitals. Each subject with schizophrenia had been diagnosed by at least two trained psychiatrists based on an unstructured clinical interview; diagnoses were made based on the criteria of the DSM-IV. Controls were recruited through local advertisements. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals who had current or past contact with psychiatric services. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and was approved by the Research Ethical Committee of Osaka University.

SNP selection and SNP genotyping

We selected rs3800779 (M30) in the *KCNH2* gene because this SNP has been associated with schizophrenia, as described in the introduction (Huffaker et al. 2009). Venous blood was collected from the subjects, and genomic DNA was extracted from whole blood according to standard procedures. The SNP was genotyped using the custom-designed

TaqMan 5'-exonuclease allelic discrimination assay (Applied Biosystems, Foster City, CA, USA), as described previously (Hashimoto et al. 2007). No deviation from Hardy–Weinberg equilibrium in the examined SNP was detected in patients with schizophrenia or in controls ($P > 0.05$).

Neurocognitive testing

General intellectual function was derived from the Full Scale IQ portion of the Wechsler Adult Intelligence Scale-Third Edition (WAIS-III) (Wechsler 1997). The Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) Neurocognition Committee selected seven neurocognitive domains from all available factor-analytic studies of cognitive performance in schizophrenia patients (Green et al. 2004; Nuechterlein et al. 2004). Seven neurocognitive batteries were selected based upon previous studies to assess the following seven domains (Nuechterlein et al. 2004, 2008): (1) speed of processing, (2) attention/vigilance, (3) working memory, (4) verbal learning and memory, (5) visual learning and memory, (6) reasoning and problem solving, and (7) social cognition. The speed of processing was assessed using the Category Fluency Test (total number of animals named in 60 s) (Sumiyoshi et al. 2004). Attention/vigilance was evaluated using the Continuous Performance Test-Identical Pairs version (*d'*) (Cornblatt et al. 1988). Working memory was measured using the Wechsler Memory Scale-Revised (WMS-R) digit span subtest (number of correct trials) (Sugishita 2001). Verbal learning and memory was assessed using the immediate recall portion of the Rey Auditory Verbal Learning Test (Lezak 1995) in which the participants were asked to recall a list of 15 words spoken by a tester. The procedure was repeated five times (sessions 1–5), and the sum of the recalled words from sessions 1 to 5 was used for the analysis. If the participants scored 15/15, we treated the scores of the participant as 15 after the session; possible scores range from 0 to 75. Visual learning and memory was evaluated using the visual reproduction I subtest of the WMS-R (number of correct trials) (Sugishita 2001). Reasoning and problem solving was measured using the tower of Hanoi task (number of correct trials) (Ohi et al. 2009a). Social cognition was assessed using the Emotion Recognition test (correct rate of the Facial Emotion Labeling Test (FELT)) (Sekiyama et al. 2008). The subjects included in this analysis were assessed by trained clinical psychologists to obtain scores on the WAIS-III Full Scale IQ and the seven schizophrenia-related neurocognitive batteries.

Meta-analysis

The studies included in the meta-analysis were obtained using PubMed using the search terms "KCNH2" and "schizophrenia". The analyzed data encompassed all publications up to October 2010. Additionally, references cited in the publications obtained were examined to identify additional potentially relevant studies that might not be listed in PubMed. Studies were included in the meta-analysis if they met the following criteria: (1) published in a peer-reviewed journal in English and (2) included a genetic association study between the *KCNH2* gene and schizophrenia. Our meta-analysis included allele frequency data from all available case–control studies only and did not include the original family-based dataset that provided strong evidence for the positive association in the original report by Huffaker et al. (Huffaker et al. 2009). We calculated each number of alleles from the allele frequency and the odds ratio data for each study.

Statistical analyses

Statistical analyses were performed using the PASW Statistics 18.0 software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls or between genotypes were analyzed using χ^2-tests for categorical variables and the Mann–Whitney *U*-test for continuous variables. Based on the assumption that demographic variables such as age and education years might not be fitted to a normal distribution, we used the nonparametric Mann–Whitney test arbitrary to assess the demographic variables. The presence of Hardy–Weinberg equilibrium was examined using the χ^2-test for goodness of fit. To control for confounding factors such as age, sex and years of education, we used a one-way analysis of covariance (ANCOVA) for neurocognitive tests, based on the assumption that the neurocognitive variables could be fitted to a normal distribution. The effect of the *KCNH2* genotype on IQ was analyzed by a one-way ANCOVA with sex and years of education as covariates because the IQ scores were already corrected for age. The effects on the seven neurocognitive domains were analyzed by a one-way ANCOVA with age, sex and years of education as covariates. Bonferroni correction was applied for multiple testing on seven domains to avoid type I errors. Standardized effect sizes were indicated using Cohen's d and η^2.

The meta-analysis was performed using the Comprehensive Meta-Analysis software (Version 2.0, BIOSTAT, Englewood Cliffs, NJ, USA). Cochran's χ^2-based Q-statistical test was performed to assess possible heterogeneity among studies. The fixed-effect