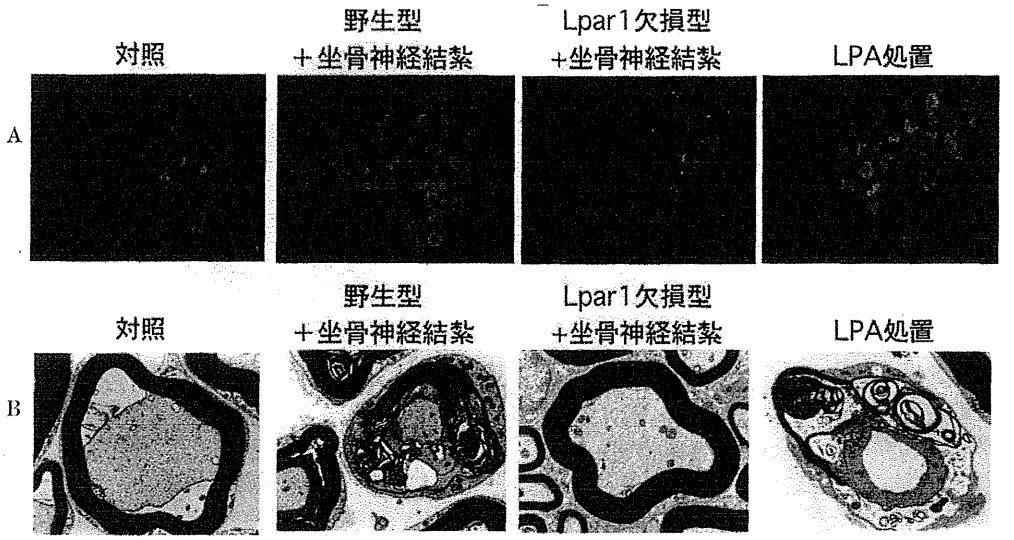

図1 (A)脊髄後根神経節(DRG)における Ca$\alpha_2\delta$-1 発現. (B)脊髄後根における脱髄現象

疾患として知られる多発性硬化症やギラン・バレー症候群，また糖尿病性ニューロパチーなどにおいて確認されており，同時にこれらの疾患は著しい知覚過敏現象を伴うことから，疼痛と密接に関連することが示唆されている．著者らは，先に述べた坐骨神経部分結紮性神経因性疼痛モデルにおいて，脊髄入力側末端に近い後根部位に脱髄が生じていることを，神経束断面像の電子顕微鏡観察により見出した[2]．図1-Bに示すように，正常マウスにおける有髄A線維(Aδ，Aβ線維)の軸索は，シュワン細胞により幾重にも巻かれており，こうして形成されたミエリン鞘が絶縁体となって，有髄A線維の特性である速い神経伝導を可能としている．これに対して，神経因性疼痛モデルの後根組織では，このコンパクトに巻かれたミエリン鞘が崩壊し，剝離しているような像が観察される(図1-B)．この脱髄現象は，隣接する線維との間に電気的な混線(エファプス)を生じることで，侵害情報の拡散・過興奮が起こり，神経因性疼痛の特徴的症状であるアロディニアを招くと考えられる[7]．また，脱髄により，シュワン細胞による軸索突起伸展抑制作用から逃れた知覚神経細胞が，異常突起伸展(スプラウティング)を生じ，脊髄入力側末端においてのシナプス入力に誤入力を招き，知覚異常に至ることも推測される[7]．実際に，脱髄の観察される後根部位において，軸索伸展に関わるgrowth-associated protein (GAP43)蛋白質の発現増加を認めており(未発表データ)，脱髄が神経機能に影響を与えている可能性が示唆される．2004年，著者らはこの脱髄誘発機構を含む神経因性疼痛の発症メカニズムに，リゾホスファチジン酸(lysophosphatidic acid; LPA)-LPA_1受容体-低分子量GTP結合蛋白質であるRhoA活性化が必須であることを同定した[2]．

神経因性疼痛におけるLPA研究の最前線

LPAは，細胞増殖，分化抑制，ストレスファイバー形成作用など多岐の生物活性を有する脂質メディエーターである．これまでに，疼痛との関連は報告されていなかったが，われわれはLPA 1 nmolを脊髄くも膜下腔内に投与すると，神経因性疼痛と酷似する熱性刺激過敏応答，アロディニア現象，無髄C線維機能低下，有髄A線維機能亢進，Ca$\alpha_2\delta$-1発現増加(図1-A)，後根部位における脱髄現象(図1-B)が惹起されることを見出した[2]．さらに重要なことに，坐骨神経部分結紮により生じる神経因性疼痛症状はLPA_1受容体遺伝子(*lpar1*)欠損マウスにおいて完全に抑制された(図1-A, B)．また，LPA投与と神経傷害による一連の神経変調は，Rhoキナーゼの阻害剤であるY-27632および，RhoA阻害剤であるボツリヌス毒素Cサブユニット(BoNT/C3)によって完全に抑制された．特に，BoNT/C3は傷害1時間前に処置することで抑制効果を示し，傷害6時間後の処置では効果は観察されなかったことから，LPAが神経因性疼痛の発症機序を担う初発分子であることが明らかになった[2,7-9]．さらに，

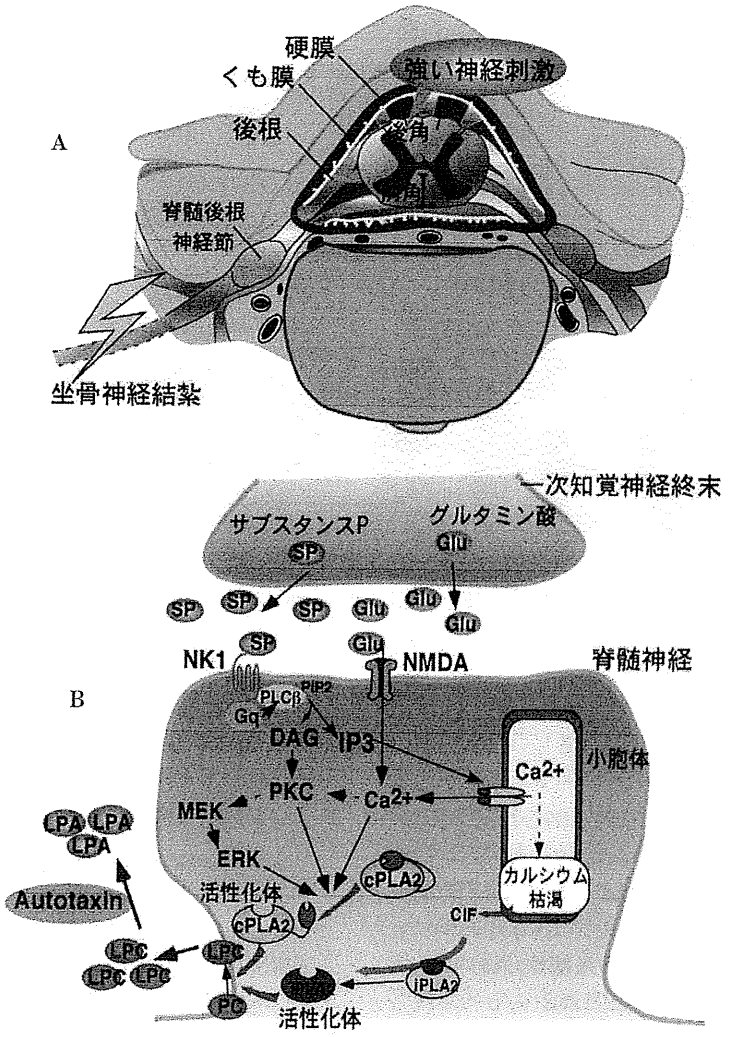

図2　(A)末梢神経傷害に伴う脊髄後角の活性化．(B) LPA の生合成経路

最近の研究から，神経傷害後早期における LPA の *de novo* 合成を支持する知見を得ている．第1に，LPA の合成酵素である Autotaxin 遺伝子(*atx*)欠損ヘテロ型マウス(ホモ型は胎生致死)において坐骨神経部分結紮による疼痛過敏が部分的に抑制された[10]．この結果から，神経因性疼痛における内在性 Autotaxin の関与が証明された．実際に，Autotaxin は脳脊髄液中に豊富に存在することを，特異的抗体を用いたウエスタンブロット法によって確認した[11]．第2に，LPA の前駆体であり Autotaxin の基質であるリゾホスファチジルコリン(lysophosphatidylcholine, LPC)を脊髄くも膜下腔内に投与すると，疼痛過敏が惹起された[12]．さらに，LPC によって惹起される疼痛過敏は，atx 欠損ヘテロ型マウス，および *lpar1* 欠損マウスにおいて顕著に抑制された[12]．第3に，脊髄スライス標本を用いた実験により，サブスタンス P 受容体 (NK1)，および NMDA 型グルタミン酸受容体を介する脊髄刺激が LPC の産生に必要であることが明らかになった[11]．以上の結果から，神経傷害に伴う一次知覚神経の活性化によって，脊髄実質における LPC 産生とそれに続く Autotaxin による代謝，すなわち LPA 産生が生じていることが明らかになった(図2-A, B)．言い換えれば，「強力な痛み刺激が LPA 産生を引き起こす」ことが証明されたことになる．

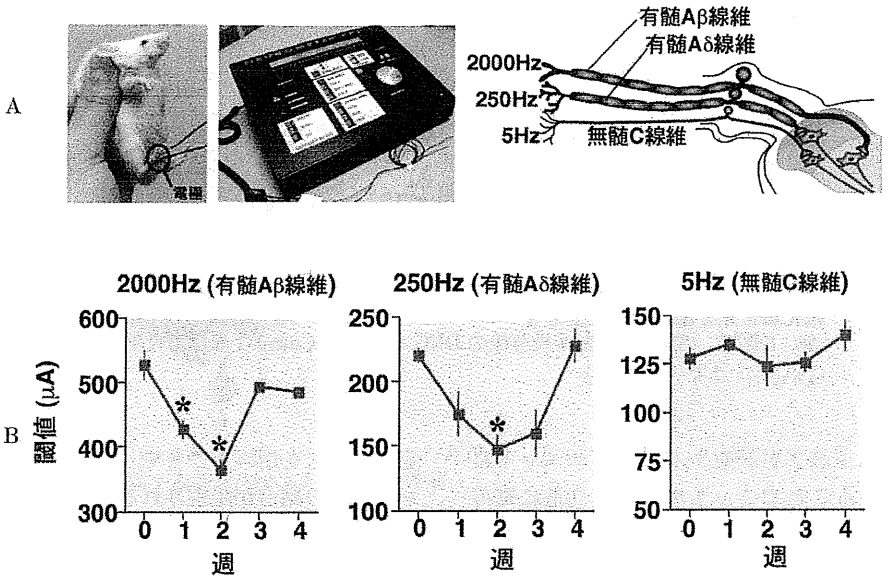

図3 (A)EPW試験法. (B)パクリタキセル投与後における有髄A線維の過敏応答

化学療法剤誘発性神経因性疼痛における有髄線維の機能変調

化学療法剤神経因性疼痛に話を移したい. 細胞内微小管ダイナミクス阻害作用をもつビンクリスチン(オンコビン®)やパクリタキセル(タキソール®), およびDNA合成阻害作用をもつシスプラチン(ランダ®, ブリプラチン®)は, 副作用として末梢性知覚神経障害を誘発することが知られている. 特に, パクリタキセルにおいては41%という高い頻度で神経障害を誘発することが報告されている[13]. パクリタキセルは, 転移性骨腫瘍に移行しやすい乳癌, 肺癌, 胃癌に適用があることから, 整形外科領域でも扱われることが多い. 神経障害は, しびれ, 刺痛, 灼熱感として表現され, 治療終了後も数カ月, ときには数年にわたって残存する症状である. 手先, 足先を好発部位として発症し, 物がうまくつかめない, 触れただけでも痛い(アロディニア現象)などという日常生活への支障がQOL (quality of life)の低下に繋がる. マッサージ等の非薬物療法や, アミトリプチン, NSAIDs(nonsteroidal anti-inflammatory durgs)などの薬物療法によって, 一部軽減される場合もあると言われているが, 神経障害の作用機序が十分に明らかにされていないことから有効な治療方法は確立されていない. 著者等は, パクリタキセル誘発性神経因性疼痛の作用機序を見出すために, 実験的モデルマウスを確立した. パクリタキセル4 mg/kgをマウス腹腔内に単回投与すると, 投与1週間後および2週間後において, 機械触覚性刺激に対する閾値の低下, すなわちアロディニア現象が認められた[14]. また, 熱性刺激に対する痛覚閾値についても, パクリタキセル投与2週間後で閾値低下が認められた. さらに, 知覚線維特異性を調べるために, EPW試験法を行ったところ, パクリタキセル処置群では有髄Aδ線維および有髄Aβ線維の閾値低下が認められ, 無髄C線維の閾値に変化は認められなかった(図3-A, B)[14]. この有髄A線維特異的な過敏応答は, 坐骨神経部分結紮性神経因性疼痛モデルと共通する現象であることから, 有髄A線維の機能亢進が神経因性疼痛の原因・実態であると考えられる. さらに, 神経因性疼痛に特徴的に有髄A線維が興奮するという事実は, 炎症性メディエーターによって感作される無髄C線維興奮が主体となる炎症性疼痛と, 本質的に異なる点であると考えられる.

化学療法剤誘発性神経因性疼痛における $Ca\alpha_2\delta$-1発現上昇と脱髄現象

パクリタキセル誘発性神経因性疼痛が, 坐骨神経結紮性モデルと同様の有髄A線維機能亢進を示すことから, 神経機能興奮に関与することの知られる $Ca\alpha_2\delta$-1の発現について解析した. このサブユニットは, カ

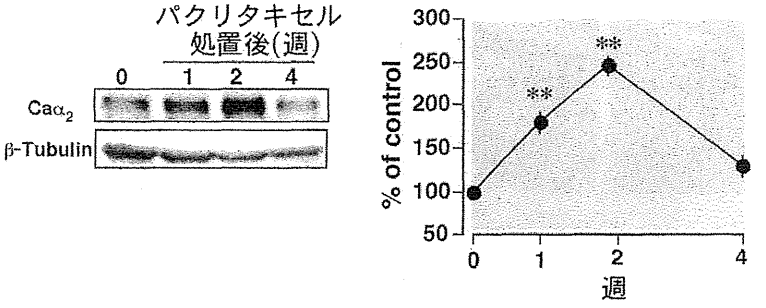

図4　パクリタキセル投与後のDRGにおけるCa$\alpha_2\delta$-1蛋白質発現

ルシウムチャネルポアを形成するα_1サブユニットに会合(図5-A)することでチャネル機能の亢進に寄与しており，特に神経終末部位においてサブスタンスP，CGRP(calcitonin gene-related peptide)，グルタミン酸などの神経伝達物質の遊離亢進に関わる[15,16]．ウエスタンブロット法による定量的解析の結果，パクリタキセル投与2週間後において脊髄後根神経節(dorsal root ganglion: DRG)におけるCa$\alpha_2\delta$-1蛋白質の2.5倍の発現上昇が認められた(図4)．また，このCa$\alpha_2\delta$-1蛋白質の発現上昇は中型から大型のDRG細胞(有髄A線維)において顕著であった[14]．同様の知見は，ラットモデルにおいても報告されている[17]．さらには，電子顕微鏡を用いた形態学的解析により，坐骨神経結紮モデルに比べて軽度ではあるが，パクリタキセル投与群に脱髄が生じていることも確認された(未発表データ)．以上のことから，パクリタキセル誘発性神経因性疼痛で認められる有髄A線維の機能変化は，Ca$\alpha_2\delta$-1蛋白質の発現上昇と脱髄という2点において，坐骨神経結紮性モデルで認められる変化と一致することが明らかになった．

ギャバペンチンの有効性

ギャバペンチン(ガバペン®)は，近年，日本で承認された神経因性疼痛治療薬であり，帯状疱疹後神経因性疼痛や糖尿病性神経因性疼痛に使用される．ギャバペンチンの作用部位については，その構造がGABA (gamma-aminobutyric acid)に類似することからGABAに関する作用であるのか，あるいはCa$\alpha_2\delta$-1に対する結合によるのか議論の的となったが，2006年，遺伝学的な実験アプローチによりCa$\alpha_2\delta$-1に結合するという事実が確認された(図5-A)[18]．この報告では，α_2サブユニット217番目アルギニンのアミノ酸変異マウスを用いることで，ギャバペンチンやプレギャバリンの結合が阻害されること，さらに，プレギャバリンの鎮痛効果が消失することを証明している．著者等は，マウスにおけるパクリタキセル誘発性モデルのDRGにおいてCa$\alpha_2\delta$-1発現上昇が認められたことから，ギャバペンチンによる鎮痛効果を解析し，このモデルにおける有意な鎮痛作用を初めて見出した(図5-B)．さらに，EPW試験法において観察された有髄A線維の過敏応答も，ほぼ正常域値レベルにまで回復した[14]．一方，正常マウスに対してギャバペンチンは痛み閾値の変化を示さないことから，ギャバペンチンは病態特異的治療法として有効であると考えられる(図5-B)．

今後の展望

以上のように，パクリタキセル誘発性神経因性疼痛における有髄A線維の機能亢進に，Ca$\alpha_2\delta$-1発現増加が関連しており，ギャバペンチンはCa$\alpha_2\delta$-1を介してチャネル機能を抑制し鎮痛効果を発揮することを見出した．パクリタキセルは，細胞内微小管ダイナミクス阻害作用を有するため，知覚神経およびシュワン細胞に直接的に細胞毒性を誘発すると考えられるが，知覚神経は手先や足先から長距離にわたって軸索を走行させていること，および神経における軸索輸送は代謝・生存において重要であることを踏まえると，むしろ神経側により高い薬剤感受性があると言えるかもしれない，DNA合成阻害作用を有する白金製剤(シスプラチン等)も，知覚神経における転写抑制によって神経毒性を誘発すると考えられる．しかしながら，こうした薬剤による神経代謝阻害がどのようにしてCa$\alpha_2\delta$-1発現増加に至るかは不明である．また，パクリタキセル誘発性モデルで観察された軽度の脱髄現象が，

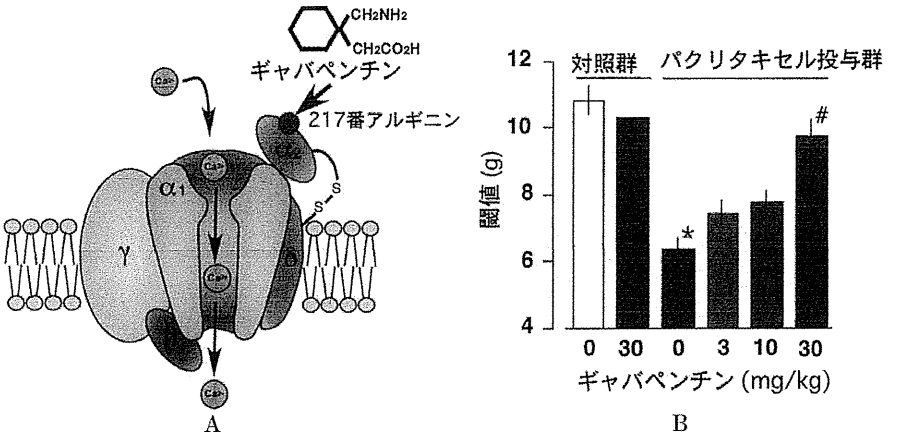

図5 (A)Ca$\alpha_2\delta$-1とギャバペンチンの作用部位. (B)パクリタキセル誘発性アロディニアに対するギャバペンチンの鎮痛効果

疼痛過敏の形成に寄与する可能性については検証されていない. こうした一連の病態解析が, 難治性とされる神経因性疼痛の新規治療法の開発に繋がることを信じ, 今後の研究に邁進したい.

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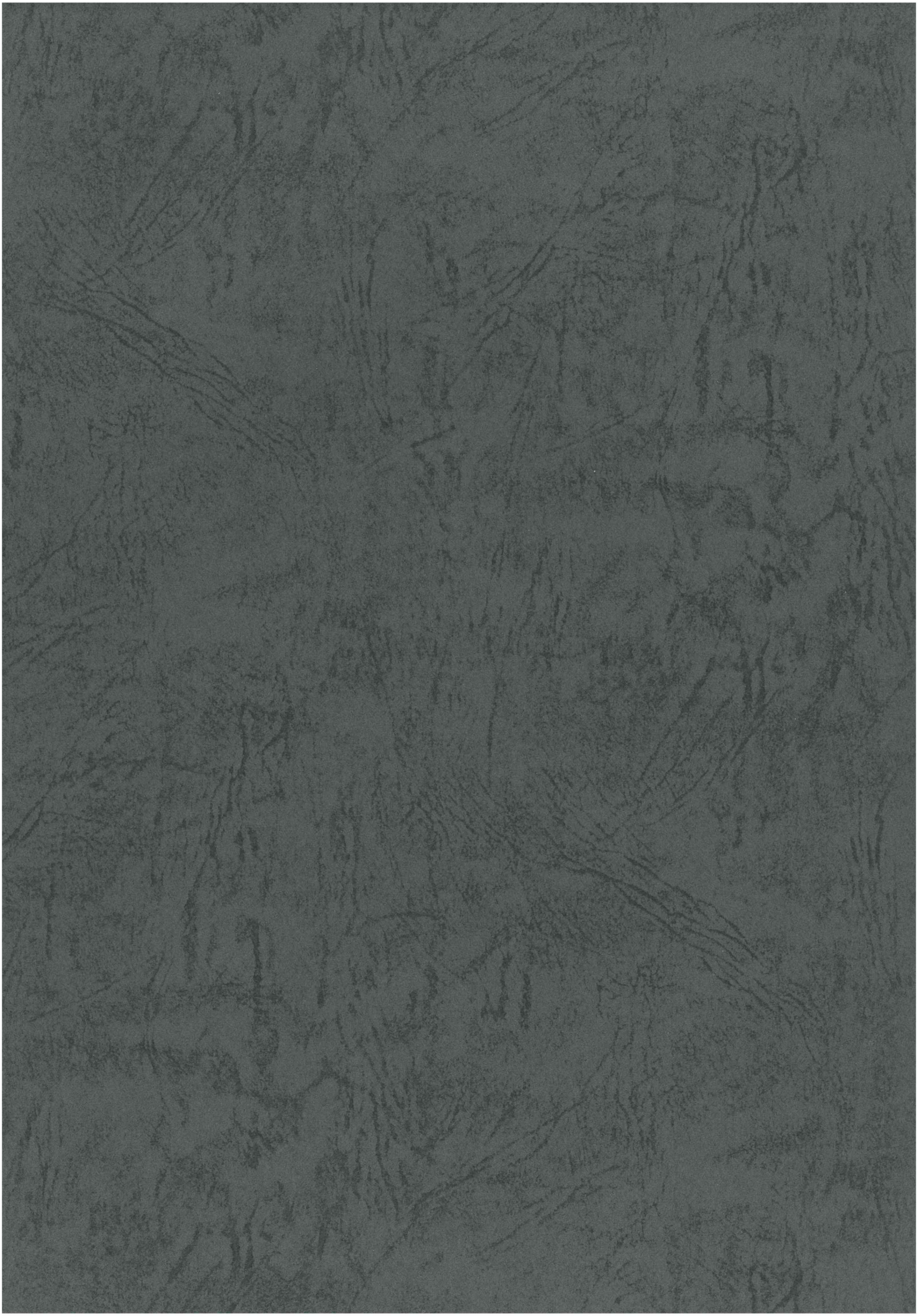

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Full Paper

Local Administration of a Synthetic Cell-Penetrating Peptide Antagonizing TrkA Function Suppresses Inflammatory Pain in Rats

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Abstract. Novel agents that inhibit nerve growth factor signaling are required for the treatment of inflammatory pain. The present study investigated the effect of local administration of inhibitory peptide of TrkA (IPTRK3), a synthetic cell-penetrating peptide that antagonizes TrkA function, in complete Freund's adjuvant (CFA)–induced hyperalgesia in rats. Three hours after subcutaneous injection of CFA into the plantar surface of the rat's left hind paw, 10 mM IPTRK3 was injected at the same site. Thermal and mechanical hyperalgesia were tested in the ipsilateral hind paw until 7 days after CFA injection. The ipsilateral dorsal root ganglion (DRG) was dissected out for immunohistochemical analysis of transient receptor potential vanilloid subfamily member 1 (TRPV1) channels and TrkA. Local injection of this peptide significantly suppressed both thermal and mechanical hyperalgesia produced by CFA and also significantly reduced TRPV1 expression at the DRG. These results suggest that local administration of IPTRK3 is likely effective in the treatment of inflammatory pain in rats.

Keywords: cell-penetrating peptide, inflammatory pain, Tat, TrkA, transient receptor potential vanilloid subfamily member 1 (TRPV1)

Introduction

As there are limited varieties of conventional therapeutics (e.g., non-steroidal anti-inflammatory drugs, opioids, local anesthetics) for the management of severe inflammatory pain, novel drugs to inhibit nerve growth factor (NGF) signaling are needed (1, 2). Increased expression of either NGF or its high-affinity receptor TrkA, which exacerbates pain states, is reported in inflammatory tissues, including burn-injured tissue (3, 4), postoperative surgical sites (5, 6), and human degenerative lumbar facet joints (7). Local injection of complete Freund's adjuvant (CFA), which is utilized for the inflammatory pain model, also increases NGF expression in the skin (8).

Local injection of NGF induces either thermal or mechanical hyperalgesia in rats (9). Several studies have revealed the mechanisms of NGF-induced hyperalgesia (1, 9, 10). NGF binds to TrkA in the cell membrane of peripheral nerves, and the activated TrkA in turn sensitizes transient receptor potential vanilloid subfamily member 1 (TRPV1) channels (1). Both NGF and TrkA are transported retrogradely to the cell bodies of the dorsal root ganglion (DRG) (10), where they enhance expression of TRPV1 with other pronociceptive proteins in the DRG (1, 2).

Previously, we developed a new inhibitor of NGF signaling, an inhibitory peptide of TrkA (IPTRK) activity with both the cell-penetrating peptide and the amino acid sequence corresponding to the activation loop of TrkA (YGRKKRRQRRR-acp-SRDIYSTDYYR-NH_2, acp = epsilon-aminocaproic acid) (11). We named this peptide IPTRK3, since it was the third peptide we tested. When IPTRK3 is injected locally, it is expected to sup-

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press intracellular TrkA activity after penetrating the cell membrane of peripheral nerves, from which it would be transported retrogradely to the DRG, where it may cause suppression of the expression of TRPV1 in the DRG in vivo (11). In the present study, we investigated the effect of local administration of IPTRK3 at the site of inflammation in a CFA-induced hyperalgesia model and examined its effect on TRPV1 expression in rats.

Materials and Methods

Experimental animals

The study protocol was approved by the Institutional Animal Research Committee and was performed in accordance with the Ethical Guidelines of the International Association for the Study of Pain (12). Adult male Sprague-Dawley rats (weighing 200 – 250 g) were used for this study. Rats were housed in a room maintained at 25°C and illuminated in a 12:12 h cycle. Rats were provided with ad libitum access to standard rodent chow and water.

Synthetic cell-penetrating peptide antagonizing TrkA function

IPTRK3 has two components: one is the cell-penetrating peptide sequence based on the human immunodeficiency virus type1 Tat (transactivator of transcription)–derived peptide (47-YGRKKRRQRRR-57), and the other is the amino-acid sequence of the activation loop of TrkA (666 -SRDIYSTDYYR- 676), which inhibits TrkA activity (11). Acp was inserted as a highly flexible spacer between these two amino acid sequences. IPTRK3, designed as YGRKKRRQRRR-acp-SRDIYSTDYYR, was synthesized and purified by HPLC (Peptide Institute, Osaka).

IPTRK3 was dissolved in phosphate-buffered saline (PBS) to the concentration of 10 mM/L and then stored at −30°C. We selected this concentration of 10 mM/L of IPTRK3, which is over a hundred times the concentration required for the inhibitory effects of TrkA activity both in vitro and in cell cultures (30 – 60 μM), as shown in our previous study (11), because local anesthetics are reported to gradually diffuse into surrounding tissues after local injection and get diluted to approximately a hundredth or less of the original concentration in the peripheral nerve (13).

Induction of hyperalgesia

Hyperalgesia was induced by subcutaneously injecting 50 μL of CFA (Sigma-Aldrich, St. Louis, MO, USA) into the plantar surface of the left hind paw of the rats using a 30-gauge hypodermic needle under sevoflurane anesthesia. Classical signs of inflammation, including edema and redness, were observed for at least 7 days.

Behavioral assessments

The mid-plantar area of the left hind paw was tested for thermal hyperalgesia (14). The light heat source, applied for a maximum of 20 s, was adjusted to produce withdrawal latencies of approximately 10 s (Model 7370 Plantar Test; Ugo Basile, Milan, Italy). Rats were acclimatized to the testing environment, and paw withdrawal latencies of the left hind paws, as the noxious heat threshold, were measured.

Mechanical hyperalgesia was also tested at the mid-plantar area of the left hind paw, using an automated version of the von Frey hair assessment (Model 37400 Dynamic Plantar Aesthesiometer, Ugo Basile). After rats were acclimatized to the testing environment, a mechanical stimulus, using a pointed metallic filament, was applied to the plantar surface of the hind paw through a wire mesh–bottomed cage until the paw was withdrawn or the preset cut-off was reached (50 g). The mechanical threshold was defined as the force in grams at which the rat withdrew its paw.

The noxious heat and mechanical thresholds were separately measured in each group of rats. The threshold was measured five times in each rat and then averaged. Stimulus interval was 5 min. All measurements were performed in a blinded fashion, with the investigator unaware of the injected agent, whether IPTRK3 or PBS.

The effect of IPTRK3 on CFA-induced inflammatory pain

To assess the effect of IPTRK3 on CFA-induced inflammatory pain, we once again anesthetized the rats with sevoflurane 3 h after CFA injection and then injected 50 μL of either 10 mM IPTRK3 (n = 5) or PBS (n = 6) subcutaneously into the same site as the CFA injection, using a 30-gauge hypodermic needle under sevoflurane anesthesia. Paw withdrawal latencies were measured before and at 2, 4, and 6 h and 2, 4, and 7 days after CFA injection. Mechanical thresholds were also measured in the same way in other rats injected with either IPTRK3 (n = 5) or PBS (n = 5).

We also evaluated whether IPTRK3 itself affects the noxious heat threshold or not. Either 50 μL of 10 mM IPTRK3 alone (n = 6) or PBS (n = 6) alone, without prior CFA administration, was injected subcutaneously into the plantar surface of the left hind paw of the rats, using a 30-gauge hypodermic needle under sevoflurane anesthesia. Paw withdrawal latencies were measured before injection as control values and at 2 h and 2 and 7 days after injection.

Immunohistochemistry

Three hours after CFA injection, rats were injected locally with either IPTRK3 (n = 4) or PBS (n = 4). Then, 1 h after local administration of peptide/PBS, all rats were over-anesthetized with sevoflurane and perfused transcardially with saline, followed by 4% paraformaldehyde. The left L4/5 DRGs were dissected out, post-fixed in 4% paraformaldehyde, and transferred to 20% sucrose overnight for cryoprotection. Ten-micron-thick sections of the DRG were cut on a cryostat and processed for TRPV1 and TrkA immunohistochemistry. Sections were incubated overnight in rabbit anti-TRPV1 antibody (1:3000; Neuromics, Edina, MN, USA) or rabbit anti-TrkA antibody (1:200; Millipore, Billerica, MA, USA) in 10 mM PBS at 4°C. This was followed by incubation in biotinylated anti-rabbit IgG (1:50; Vector Labs, Burlingame, CA, USA) for 1 h and in avidin-biotin complex (Vector Labs) for 1 h at room temperature. Sections were exposed to 3,3′-diamino-benzidine-4HCl (Wako, Osaka) in 50 mM Tris buffer, pH 7.4, containing 0.2% H_2O_2.

The stained sections of the DRG were analyzed under a light microscope for TRPV1-positive neuron distribution. Every fourth section was picked from a series of consecutive DRG sections, ten sections being counted for each DRG. Results of microscopy were expressed as the percentage of TRPV1-positive neurons (15).

Statistical analyses

Data were analyzed using one-way analysis of variance with Bonferroni *post hoc* analysis. Statistical significance was established at the $P < 0.05$ level. All values are reported as the mean ± S.D.

Results

IPTRK3 suppresses CFA-induced hyperalgesia

Paw withdrawal latencies after CFA injection in the PBS group decreased significantly from 2 h to 7 days (Fig. 1). IPTRK3 showed significant suppression of these decreases in paw withdrawal latencies compared to the PBS-injection group from 4 h to 4 days. Mechanical thresholds after CFA injection in the PBS group decreased significantly from 4 h to 4 days (Fig. 2). The CFA-induced significant reduction of mechanical threshold, however, was only observed at 4 and 6 h in the IPTRK3 group. There was a significant difference in mechanical thresholds between IPTRK3 and PBS groups at 6 h. These results suggest that IPTRK3 likely suppresses both CFA-induced thermal and mechanical hyperalgesia.

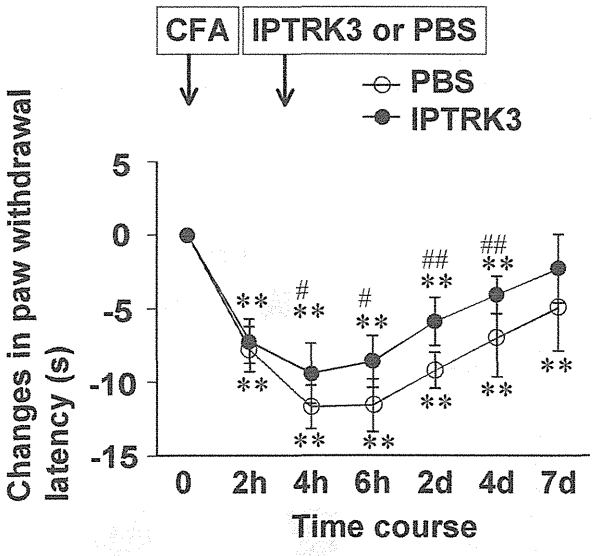

Fig. 1. CFA-induced thermal hyperalgesia in rats. Time course of changes in paw withdrawal latency were investigated starting before subcutaneous injection of CFA (base line value, time point 0 h) and at the time points of 2, 4, and 6 h and 2, 4, and 7 days after CFA injection. Either PBS (n = 6) or IPTRK3 (n = 5) was injected subcutaneously 3 h after CFA injection. $^{**}P < 0.01$, compared with the baseline value at 0 h in each group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, with comparison between values in the two groups at the same time point. Results represent means ± S.D.

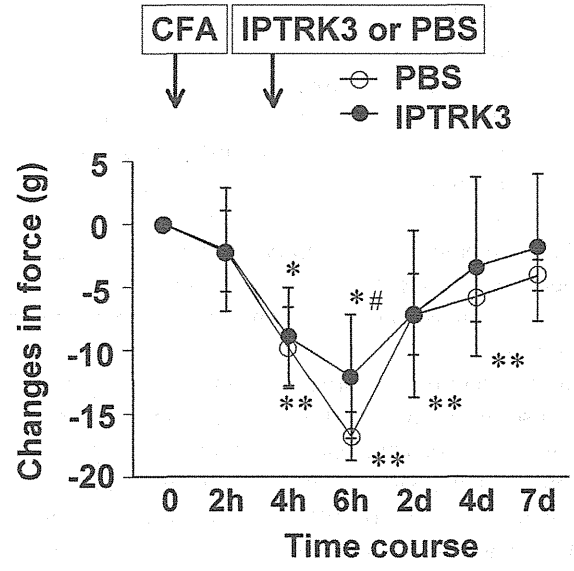

Fig. 2. CFA-induced mechanical hyperalgesia in rats. The time course of changes in the mechanical threshold was investigated starting before subcutaneous injection of CFA (baseline value, time point 0 h) and at the time points of 2, 4, and 6 h and 2, 4, and 7 days after CFA injection. Either PBS (n = 5) or IPTRK3 (n = 5) was injected subcutaneously 3 h after CFA injection. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the baseline value at 0 h in each group. $^{\#}P < 0.05$, comparison between values in the two groups at the same time point. Results represent means ± S.D.

We also examined the changes in paw withdrawal latencies after subcutaneous injection of IPTRK3 or PBS without CFA. There were no significant differences in noxious heat thresholds between and within groups of rats injected with IPTRK3 or PBS alone at 2 h and 2 and 7 days. These results suggest that IPTRK3 itself induces neither thermal hypoalgesia nor hyperalgesia.

IPTRK3 suppresses the expression of TRPV1 at the DRG

Protein expressions of TRPV1 and TrkA at the DRG are shown in Figs. 3 and 4, respectively. Expression of TRPV1 at the DRG after CFA injection was significantly suppressed by IPTRK3 (Fig. 5). On the other hand, there was no significant difference in TrkA expression in the DRG between PBS and IPTRK3 groups (Fig. 5).

Discussion

IPTRK3, a cell-penetrating peptide with TrkA inhibitory activity, suppressed CFA-induced thermal and mechanical hyperalgesia after local administration at the site of CFA injection, together with reduction of protein expression of TRPV in the ipsilateral DRG.

NGF antagonists can be categorized as NGF-capturing agents, antagonists at the NGF-binding site, and antagonists of TrkA function (1). K252a, an antagonist of TrkA function, is reportedly effective for inflammatory pain in rats (16, 17). However, K252a likely has many adverse effects because of lack of specificity for TrkA (1). IPTRK3 also belongs to the class of antagonists of TrkA function. In our previous study (11), this peptide showed no effect on tyrosine kinase activities of the insulin receptor and epidermal growth factor receptor in vitro. Further studies are needed to investigate the effect of IPTRK3 on other pronociceptive protein kinases, such as mitogen-activated protein kinase, protein kinase C, c-Jun N-terminal kinase, and Ca^{2+}/calmodulin-dependent protein kinase.

Single injection of a drug at the inflammatory area is a fairly useful regimen for the clinical management of severe inflammatory pain states, especially if it provides a prolonged analgesic effect. A single injection of local anesthetic at the site of inflammation, however, induces a transient analgesic effect (18 – 21). Therefore, continuous infusion of local anesthetics, either locally or systemically, is needed for prolonged analgesia (20, 22, 23). As single injection of IPTRK3 showed sufficient inhibitory effects on thermal hyperalgesia lasting for at least 4 days in the present study, a single local injection of IPTRK3 at the site of inflammation would be a favorable regime for severe inflammatory pain states, for which conventional therapy does not work.

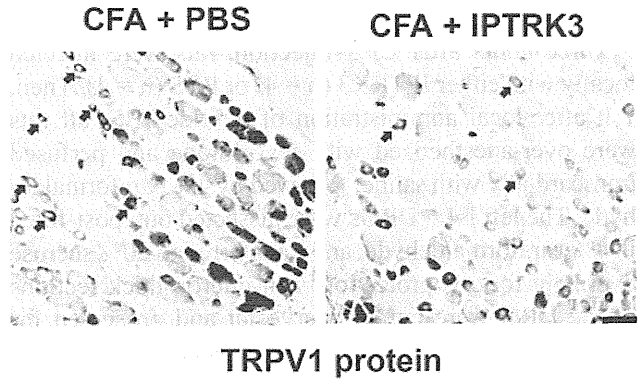

Fig. 3. Immunohistochemistry for TRPV1 channels in the dorsal root ganglion 4 h after subcutaneous injection of CFA. Either PBS or IPTRK3 was injected subcutaneously 3 h after CFA injection. Arrows indicate typical TRPV1 protein. Scale bar, 50 μm.

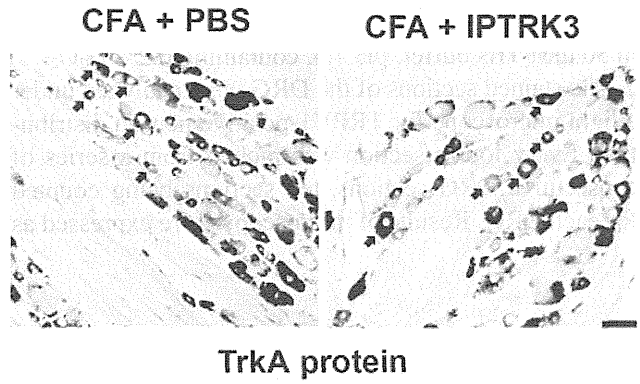

Fig. 4. Immunohistochemistry for TrkA in the dorsal root ganglion 4 h after subcutaneous injection of CFA. Either PBS or IPTRK3 was injected subcutaneously 3 h after CFA injection. Arrows indicate typical TrkA protein. Scale bar, 50 μm.

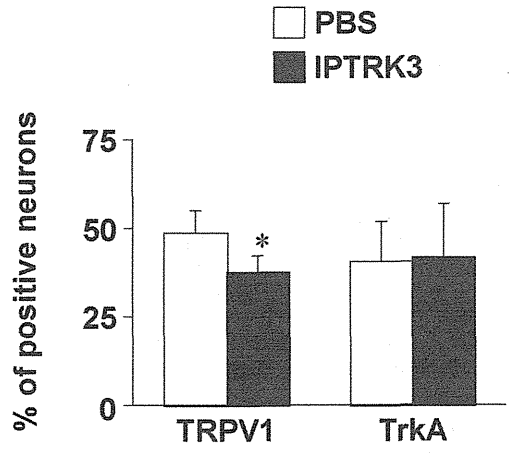

Fig. 5. Quantification of protein levels, as shown in Figs. 3 and 4, by the percentage of either TRPV1- or TrkA-positive neurons. $^*P < 0.05$, comparison between PBS and IPTRK3 groups. Results represent means ± S.D. of four separate experiments in each group.

The suppressive effect of IPTRK3 on CFA-induced mechanical hyperalgesia was smaller than that on CFA-induced thermal hyperalgesia in the present study. An electrophysiological study reported that the mechanism causing mechanical sensitization of cutaneous nociceptors after CFA injection into the rat's hind paw is different from that causing thermal sensitization (24). Other investigators reported that a central mechanism causes NGF-induced mechanical hyperalgesia, whereas both peripheral and central mechanisms cause NGF-induced thermal hyperalgesia (9, 25). Although these different mechanisms inducing mechanical and thermal hyperalgesia might explain the distinction between suppressive effects of IPTRK3 on each hyperalgesia, the precise reason is unclear.

The present study showed that IPTRK3 suppressed protein expression of TRPV1 in the DRG during inflammatory pain. NGF signaling upregulates the expression of pronociceptive proteins, such as TRPV1, the brain-derived neurotrophic factor, substance P, calcitonin gene-related peptide, and the $Na_v1.8$ sodium channel in sensory neurons, that mediate nociception via peripheral and central sensitization (1, 2). TRPV1 is essential for thermal hyperalgesia induced by inflammation (15, 26). Therefore, the suppression of TRPV1 expression is one possible mechanism of the inhibitory effect of IPTRK3 on CFA-induced thermal hyperalgesia.

A limitation of this study is that the reason why a single local injection of IPTRK3 induced a prolonged inhibitory effect on CFA-induced hyperalgesia is unknown. Further studies are needed to investigate the time-course of expression of several pronociceptive proteins in peripheral and central neurons and also to examine the half-life of this peptide in vivo.

In summary, local administration of IPTRK3, a cell-penetrating peptide having direct inhibitory effects on TrkA activity, suppresses hyperalgesia induced by inflammation in rats. This peptide would be a new candidate as a viable therapeutic drug for inflammatory pain.

Acknowledgments

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Full Paper

A Synthetic Cell-Penetrating Peptide Antagonizing TrkA Function Suppresses Neuropathic Pain in Mice

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Abstract. Nerve growth factor (NGF) and its high-affinity receptor, TrkA, are one of the targets in the production of new drugs for the treatment of neuropathic pain. NGF contributes to both the initiation and maintenance of sensory abnormalities after peripheral nerve injury. This study examined the effects of IPTRK3, a new synthetic cell-penetrating peptide that antagonizes TrkA function, on neuropathic pain in mice. Partial sciatic nerve ligation (PSNL) was used to generate neuropathic pain, and we injected IPTRK3 (2 or 10 mg/kg) intraperitoneally on day 7 after PSNL. Effects of the peptide on hyperalgesia, allodynia, and expression of Fos in the spinal cord were examined. Single administration of the peptide on day 7 significantly suppressed both thermal hyperalgesia and mechanical allodynia. Gentle touch stimuli–evoked Fos expression in the lumbar spinal cord was also significantly reduced. Intraperitoneal injection of a cell-penetrating peptide antagonizing TrkA function appears effective for treatment of neuropathic pain in a mouse pain model.

Keywords: cell-penetrating peptide, neuropathic pain, Tat, TrkA

Introduction

Chronic pain is classified into 3 categories: nociceptive pain, neuropathic pain, and pain without a known somatic background (1). Neuropathic pain is a common symptom, defined as pain resulting from lesions or diseases of the sensory transmission pathways in the peripheral or central nervous system (2, 3). Neuropathic pain disrupts the lives of patients, in the form of paresthesia, allodynia, or hyperesthesia, and treatment remains a major challenge in modern medicine (4, 5).

As nerve growth factor (NGF) and TrkA, the high-affinity receptor for NGF, play pivotal roles in the pathophysiology of peripheral neuropathic pain (6), these proteins represent molecular targets for potential treatment of neuropathic pain in the future (7). Our previous studies described the development of a new synthetic cell-penetrating peptide (IPTRK3) to directly inhibit tyrosine kinase activity of TrkA (8) and successful suppression of nociceptive pain using IPTRK3 in rats (9). As pathophysiological changes in the peripheral nervous system are involved in mechanisms of nociceptive pain, induced by subcutaneous injection of complete Freund's adjuvant into the hind paw of rats at the initial several hours, we injected IPTRK3 subcutaneously at the same site in the previous study (9). On the other hand, the present study was designed to evaluate the effects of IPTRK3 on neuropathic pain, elicited by partial sciatic nerve ligation (PSNL) in mice (10). As pathophysiological changes in both the peripheral and central nervous systems are crucial for mechanisms of neuropathic pain, we decided to inject IPTRK3 intraperitoneally in the present study.

In animal models of neuropathic pain, the expression of the proto-oncogene product Fos is reportedly evoked

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in the spinal cord by repetitive gentle touch, which normally does not elicit Fos expression (11). The evoked expression of Fos indicates a state of spinal sensitization. We also investigated the effects of IPTRK3 on gentle touch–evoked Fos expression in the spinal cord.

Materials and Methods

Animals

All the experimental protocols were approved by the Animal Investigation Committee at our institution and adhered to the Ethical Guidelines of the International Association for the Study of Pain (12). Male ddY mice (Japan SLC, Hamamatsu) weighing 30 – 40 g at the time of experiments were maintained in wire-mesh cages in a temperature-controlled room, on a 12-h light/dark cycle. Food and water were available ad libitum.

Peptide synthesis

IPTRK3 contains both the amino-acid sequence of a cell-penetrating peptide and that of the activation loop of TrkA. The former sequence is based on the human immunodeficiency virus type 1 Tat (transactivator of transcription)–derived peptide (8, 9). IPTRK3 was synthesized and purified by HPLC (Peptide Institute, Osaka).

Neuropathic pain models

The PSNL model of neuropathic pain was used as described for mice (10). Under sevoflurane anesthesia and aseptic conditions, the left sciatic nerve was carefully freed from surrounding connective tissues at a site near the trochanter. The nerve was fixed in place by pinching the epineurium on the dorsal aspect by using small forceps, and then one-third to one-half of the nerve thickness was tightly ligated with a 8-0 silk suture. The wound was closed with 1 muscle suture and 2 skin staples. In sham-operated mice, the nerve was exposed, but not ligated.

Behavioral testing

Thermal hyperalgesia in the hind paw was measured using a plantar test (Ugo Basile, Comerio, Italy) (13). Mice were placed in plastic cages with a glass plate surface, and then a heat stimulus was applied from beneath to the middle of the left plantar surface by means of a radiant heat source. When the mouse withdrew its paw, the sudden drop in reflected energy stopped the timer. A cut-off time of 30 s was imposed to prevent tissue damage. Thermal paw withdraw threshold (PWT) was recorded once per trial as the withdrawal latency in seconds.

Mechanical allodynia of the hind paw was assessed using a dynamic plantar anesthesiometer (Ugo Basile). Mice were placed in a test box with a wire mesh floor. Under visual guidance with an attached angled mirror, the thin metal blunt rod of this device was positioned under the lateral region of the left plantar hindpaw. A trigger was depressed, initiating contact of the rod to the footpad. The applied force increased at a rate of 2.5 g/s with a cut-off of 25 g at 10 s. The mechanical PWT was measured once per trial and expressed as the tolerance level in grams.

Thermal and mechanical PWTs were separately measured in each group of mice. Animals were habituated to the testing environment daily for at least 5 days before baseline testing. Prior to the behavioral test, mice were acclimated to the testing boxes for 30 min. The threshold was measured five times in each rat and then averaged. The stimulus interval was 10 min.

Immunohistochemistry

Mice were put under deep anesthesia with sevoflurane and intracardially perfused with 50 ml of 0.9% sodium chloride, followed by 30 ml of 4% paraformaldehyde in 0.01 M phosphate–buffered saline (PBS). The spinal cord was carefully transected in the upper sacral and lower thoracic regions and post-fixed for 4 – 16 h in paraformaldehyde followed by incubation overnight in 20% sucrose at 4°C. Transverse sections of the L4-5 spinal cord were identified by identification of the lumbar enlargements and nerve roots. The L4-L5 segment was sliced into 40-μm-thick sections on a cryostat and collected in groups of 12 sections.

All reactions were performed at room temperature on floating sections agitated on a shaker (except for reactions with the avidin-biotin-peroxidase complex). After washing twice for 10 min each time in 0.01 M PBS and blocking for 90 min in 5% normal goat serum, the tissue sections were incubated with a rabbit polyclonal anti-c-fos antibody (1:20,000, anti-c-Fos Ab-5 Rabbit pAb; Calbiochem, Darmstadt, Germany) at room temperature for 16 – 18 h. These sections were washed again with 0.01 M PBST (0.01 M PBS and 0.3% Triton X-100) and incubated for 1 h in biotinylated goat anti-rabbit secondary antibody (1:600; Vector Laboratories, Burlingame, CA, USA). Sections were left at room temperature for 1 – 2 h in the presence of avidin-biotin-peroxidase complex (Vector Laboratories) and then incubated in diaminobenzidine (Vector Laboratories). Sections were mounted on gelatin-coated slides and cover-slipped.

To count Fos-labeled cells, sections from each mouse were coded and 12 sections were selected at random from among the L4-L5 spinal segments. The average numbers of Fos cells either at laminae I-II (superficial laminae) or laminae III-IV (deep laminae) were counted. Sections were first examined under dark-field illumina-

tion to determine gray matter landmarks, and then numbers were counted under bright-field illumination. Fos cells were counted using a 10× objectives lens. A higher-magnification 40× objective lens was used if labeled cells were not clearly resolved.

Study design

To examine the effects of IPTRK3 itself on basal thermal and mechanical PWTs, we measured them in sham-operated mice. Thermal PWTs were measured 1 and 2 days before sham operation and also measured at 2, 6, 10, and 14 days after the operation. On day 7 after the sham operation, PBS alone (0 mg/kg group) or IPTRK3 dissolved in PBS (10 mg/kg group) were injected intraperitoneally. Body weight was also measured on each day. Mechanical PWTs were measured in the same way as thermal PWTs in other mice.

The effects of IPTRK3 on thermal and mechanical PWTs were examined in PSNL mice. Thermal PWTs were measured 1 and 2 days before surgery for the PSNL and also measured at 2 and 6 days after PSNL. On day 7 after PSNL, PBS alone (0 mg/kg group) or IPTRK3 dissolved in PBS (2 and 10 mg/kg group) were injected intraperitoneally. Thermal PWT was then evaluated at 1, 4, and 8 h after injection on day 7 and also evaluated on days 8, 9, 10, and 14 after PSNL. Mechanical PWTs were also measured in the same way as thermal PWTs in other mice.

To induce Fos expression on day 7 after PSNL, we used gentle touch stimuli, which were applied manually with the flat surface of the experimenter's thumb to the left plantar surface of the mouse, as previously described (14). Mice received intraperitoneal injection of PBS alone or 10 mg/kg of IPTRK3 on day 7 after PSNL or sham operation, and then gentle touch stimuli were applied 1 h after injection. Each touch lasted 2 s and moved from the middle position of the foot to the distal foot pad, applied once every 4 s for 10 min without anesthesia. Mice were divided into six groups: sham-operated mice injected PBS alone with or without stimulation; PSNL mice injected PBS alone with or without stimulation; and PSNL mice injected IPTRK3 with or without stimulation. Mice were deeply anesthetized at 2 h after stimulation, and then perfused and processed for immunohistochemical analysis of Fos expression in the spinal cord.

Statistical analyses

Data were analyzed using one-way analysis of variance with Bonferroni post hoc analysis. Statistical significance was established at the $P < 0.05$ level. All values are reported as the mean ± S.D.

Results

Intraperitoneal IPTRK3 showed no effects on thermal hyperalgesia and mechanical allodynia in sham-operated mice

Administration of IPTRK3 (10 mg/kg) to sham-operated mice caused no significant changes in thermal and mechanical PWTs, compared to that of PBS (0 mg/kg). Body weight also showed no significant difference between IPTRK3 and PBS (Fig. 1).

Intraperitoneal IPTRK3 suppressed thermal hyperalgesia on day 7 in PSNL mice

Figure 2A shows the results for IPTRK3 in terms of thermal hyperalgesia. In all mice, thermal PWT showed a large decrease on day 6 after PSNL. IPTRK3 administered intraperitoneally on day 7 resulted in a significant increase in thermal PWT for 2 days after injection. The 10-mg/kg dose significantly attenuated thermal hyperalgesia and PWT at 1 h after injection, almost reaching preligation values (returning to 96% of preligation val-

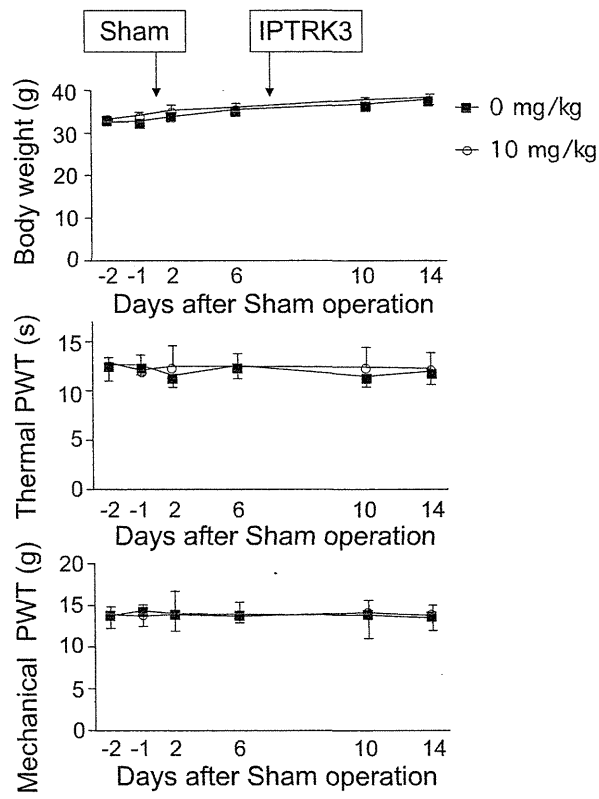

Fig. 1. Effects of IPTRK3 on body weight, thermal hyperalgesia, and mechanical allodynia in mice on day 7 after sham operation. Sham operation was performed on day 0. Body weight, thermal paw withdrawal threshold (PWT), and mechanical PWT were measured. Phosphate-buffered saline (PBS) alone (0 mg/kg, n = 6) or IPTRK3 (10 mg/kg, n = 6) was administered intraperitoneally on day 7 after sham operation. Results represent means ± S.D.

ues). The 2-mg/kg dose also increased thermal PWT at 1 h after injection, returning to 67% of preligation values.

Intraperitoneal IPTRK3 suppressed mechanical allodynia on day 7 in PSNL mice

Figure 2B shows results for IPTRK3 on mechanical allodynia. In all mice, mechanical PWT showed a large decrease on day 6 after PSNL. On day 7, IPTRK3 increased mechanical PWT, which peaked at 1 h and lasted for 1 – 2 days after intraperitoneal injection of the peptide. The 10-mg/kg dose significantly attenuated mechanical allodynia and PWT returned to 88% of preligation values at 1 h after injection. The 2-mg/kg dose also increased PWT at 1 h after injection, returning to 76% of preligation values.

IPTRK3 reduced evoked-Fos expression on day 7 after PSNL

To investigate the effect of tactile stimulation on spinal cord Fos expression on day 7 after PSNL, gentle touch stimuli were applied at the hind paw. The number of Fos-positive cells in the spinal cord dorsal horn was assessed after gentle touch stimuli in sham-operated and PSNL mice injected with either PBS alone (0 mg/kg) or IPTRK3 (10 mg/kg). Micrographs (Fig. 3) qualitatively demonstrated the patterns of ipsilateral dorsal horn Fos expression in these three groups with or without tactile stimulation.

Quantitative data for Fos expression in superficial and deep laminae are presented in Fig. 4. Without stimulation, the number of Fos-positive cells in L4-L5 lumbar segments was very low (0 – 3 Fos-positive neurons/section), and no significant differences were seen among the three groups. With stimulation, a small number of Fos-positive cells were observed in L4-L5 lumbar segments in sham-operated mice with PBS alone (sham group). In PSNL mice with PBS alone (0 mg/kg group), numbers of Fos-positive cells were increased significantly in both superficial and deep laminae on day 7 after PSNL, compared to numbers in sham-operated mice. Administration of IPTRK3 (10 mg/kg group), however, suppressed the number of Fos-positive cells significantly in both super-

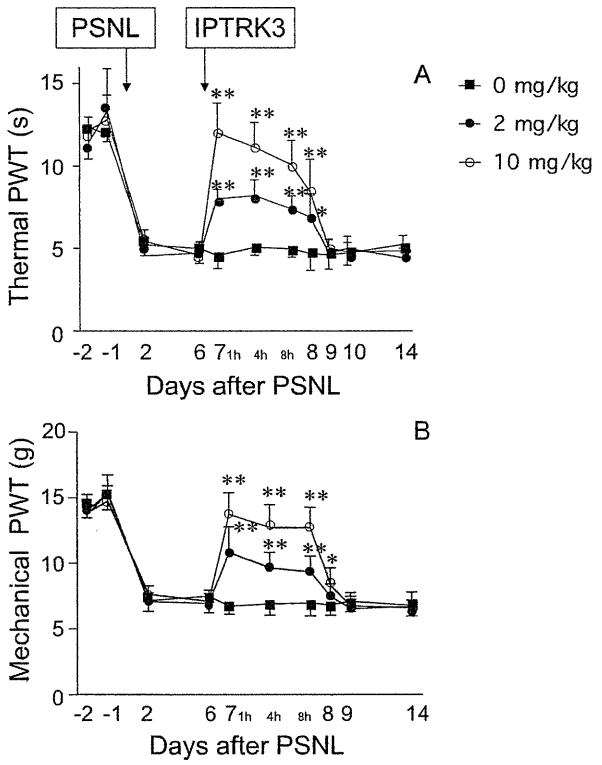

Fig. 2. Effect of IPTRK3 on thermal hyperalgesia and mechanical allodynia in mice on day 7 after partial sciatic nerve ligation (PSNL). A) PSNL was performed on day 0, and thermal paw withdrawal threshold (PWT) was measured. Phosphate-buffered saline (PBS) alone (0 mg/kg, n = 6) or IPTRK3 (2 mg/kg, n = 6; 10 mg/kg, n = 6) was administered intraperitoneally on day 7 after PSNL. B) PSNL was performed on day 0, and mechanical PWT was measured. PBS alone (0 mg/kg, n = 6) or IPTRK3 (2 mg/kg, n = 6; 10 mg/kg, n = 6) was administered intraperitoneally on day 7 after PSNL. $*P < 0.05$, $**P < 0.01$, compared with values in the PBS-alone group (0 mg/kg) at the same time point. Results represent means ± S.D.

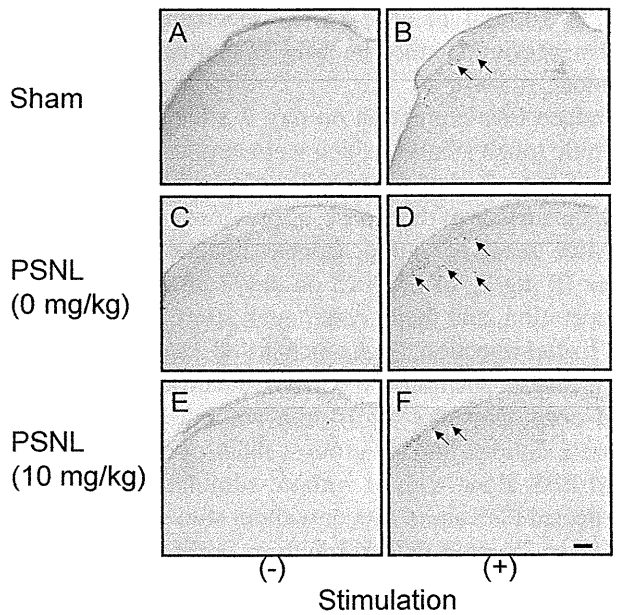

Fig. 3. Photomicrographs illustrating expression of Fos in the L5 segment of the spinal dorsal horn in sham-operated mice and mice that received partial sciatic nerve ligation (PSNL) on day 7 after operation with or without gentle touch stimulation. Photomicrographs in (A) and (B) were from sham-operated mice with or without stimulation, respectively. Photomicrographs in (C) and (D) were from PSNL mice given PBS alone (0 mg/kg) with or without stimulation, respectively. Photomicrographs in (E) and (F) were from PSNL mice given IPTRK3 (10 mg/kg) with or without stimulation, respectively. All photomicrographs were obtained using ×10 magnification. Arrows indicate typical Fos protein. Scale bar, 100 μm.

ficial and deep laminae in PSNL mice (Fig. 4).

Discussion

As many patients with neuropathic pain cannot obtain sufficient pain relief with available drugs, new analgesic drugs are urgently needed for the treatment of neuropathic pain (7, 15). Close attention has been paid to NGF and TrkA, which are prospective targets for the development of such drugs (7). Peripheral nerve injury causing neuropathic pain increases NGF levels at peripheral sites (16, 17). Intraperitoneal injection of either anti-NGF antibody or anti-TrkA antibody after peripheral nerve injury reportedly suppresses neuropathic pain in rodents (18, 19). The present study showed that IPTRK3, a cell-penetrating peptide that inhibits TrkA activity, produced anti-allodynic and anti-hyperalgesia effects on neuropathic pain in mice.

IPTRK3 suppressed both thermal hyperalgesia and mechanical allodynia on day 7 after PSNL. Wild et al. reported that a single dose of anti-NGF antibody, injected intraperitoneally 7 days after spinal nerve ligation in rats, started to suppress allodynia on day 2 after injection, and this persisted to day 7 after injection (18). Conversely, a single dose of IPTRK3, injected intraperitoneally 7 days after the PSNL, started to suppress both thermal hyperalgesia and mechanical allodynia at 1 h after injection, and this persisted to day 2 after injection in the present study. IPTRK3 is thus likely to have characteristics of more rapid onset and shorter duration compared to anti-NGF antibody. Although anti-NGF antibody can not penetrate into the central nervous system, IPTRK3 could penetrate into it. As TrkA-immunoreactive neurons were observed in the rat spinal cord (20) and intrathecal administration of the TrkA kinase inhibitor K252a reportedly suppressed neuropathic pain induced by chronic constriction injury in rats (21), TrkA kinase activity would play a role in mechanisms of neuropathic pain. Therefore the difference of effect sites between the peripheral nervous system for anti-NGF antibody and both peripheral and central nervous systems for IPTRK3 might explain the difference of the time of onset for both agents. On the other hand, as peptides made of L-amino acids, like IPTRK3, are degraded by peptidases, and also because the half-life of the cell-penetrating peptide Tat does not exceed 48 h (22), degradation of IPTRK3 might be one of the mechanisms responsible for its short duration. Therefore an analogue of IPTRK3 made by substituting L-amino acids for the D-amino acids would be resistant against peptidases and might have a long-lasting effect (22). Further study is required to investigate the effects of repetitive or continuous administration of IPTRK3 or single administration of D-IPTRK3 after nerve injury on neuropathic pain.

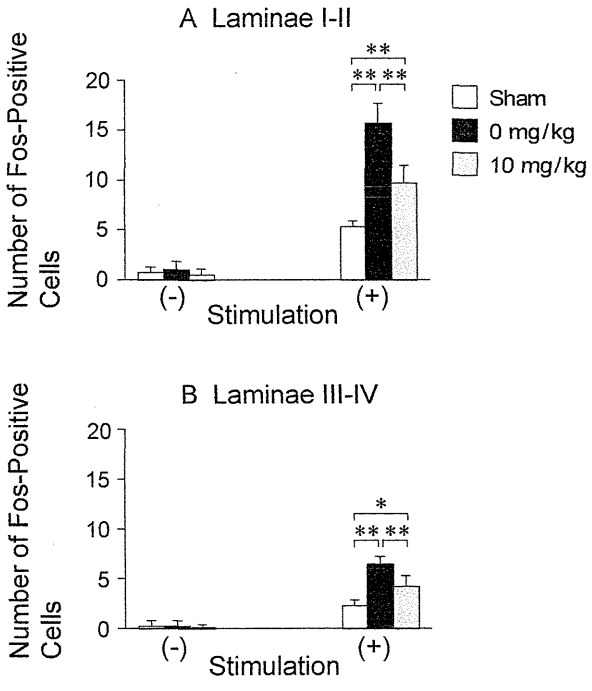

Fig. 4. Histograms showing the distribution of Fos-positive cells in L4-L5 segments in laminae I-II (A) and III-IV (B) on day 7 after sham operation or partial sciatic nerve ligation (PSNL). One hour after sham-operated or PSNL mice received either PBS alone (0 mg/kg) or IPTRK3 (10 mg/kg) intraperitoneally, gentle touch stimuli were applied to half of the mice. Each group contained 4 mice. $*P < 0.05$, $**P < 0.01$, comparing values between the two groups. Results represent means ± S.D.

Fos, the protein of the immediate early gene c-fos, has been extensively used as a marker for studying neural correlates of nociception and as a neuronal marker for testing the efficacy of analgesic compounds (23). In the spinal cord of animal models for neuropathic pain, however, non-nociceptive stimuli, like repetitive gentle touch, increase Fos level, which corresponds to mechanical allodynia (11). IPTRK3 reduced gentle touch stimuli–evoked Fos expression in both superficial and deep laminae on day 7 after PSNL. The superficial laminae are the sites of termination of afferents responding to noxious stimulation (C and Aδ), and the deep laminae are the sites of termination of non-nociceptive afferents responding to gentle touch (Aβ) (24). Mechanisms of the inhibitory effects of IPTRK3 on evoked Fos expression and mechanical allodynia might involve the suppression of the interaction between nociceptive and non-nociceptive afferents through TrkA (18).

IPTRK3 involves both the amino acid sequence of transactivator of transcription-derived peptide, Tat (YGRKKRRQRRR), which facilitates penetration into the cell membrane, and the amino acid sequence of the

activation loop of TrkA (666-SRDIYSTDYYR-676), which directly suppresses TrkA function (8). In our previous in vitro and cell culture studies, IPTRK3 (30 – 60 μM) inhibited TrkA activity (8). In our other study using the complete-Freund's adjuvant–induced nociceptive pain model of rats, we selected the concentration of 10 mM peptide for local administration at the inflammatory site, as we expected that this would gradually diffuse into surrounding tissues after local injection and be diluted to approximately one-hundredth or less of the original concentration in the peripheral nerve (9). Therefore, in our previous study we injected 50 μl of 10 mM peptide into the hind paw of rats weighing around 200 g, corresponding to approximately 6 mg/kg (9). In the present study, however, intraperitoneal injection of IPTRK3 at 2 mg/kg, a concentration smaller than that used for local injection, showed effectiveness against neuropathic pain in mice. This distinction can be explained by differences in site of injection (e.g., inflammatory site vs. normal site) and differences in the animal used (e.g., rats vs. mice). Moreover, not only the inhibitory effect of IPTRK3 on TrkA, but also some unknown effects might be involved in the suppression of neuropathic pain in the present study.

In summary, single intraperitoneal injection of a synthetic cell-penetrating peptide that directly inhibits TrkA activity has anti-hyperalgesic and anti-allodynic effects after peripheral nerve injury in mice. Synthetic peptides targeting TrkA could be candidates for new therapeutic agents for neuropathic pain.

Acknowledgments

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