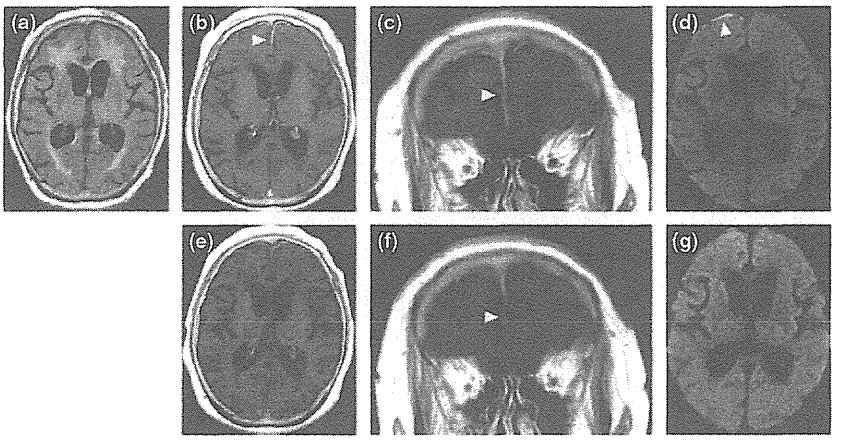

Figure 1 Serial brain magnetic resonance images before (top) and after (bottom) treatment with methylprednisolone from a patient with relapsing polychondritis complicated by both hypertrophic pachymeningitis and encephalitis. (a) Axial slice of fluid-attenuated inversion recovery imaging. (b, e) Axial slices of T_1-weighted imaging with contrast enhancement. (c,f) Coronal slices of T_1-weighted imaging with contrast enhancement. (d,g) Axial slices of diffusion-weighted imaging.

deterioration. Because of severe dementia, neuropsychological testing could not be carried out; however, she showed increased spontaneous speech after treatment.

Discussion

The present patient was diagnosed with hypertrophic pachymeningitis on the basis of dura mater enhancement on T_1-weighted images and the steroid responsiveness of this condition. Furthermore, a high signal intensity area was observed in the right frontal dura mater on diffusion-weighted images. Differential diagnoses include subdural empyema or subdural hematoma; both could be excluded in our patient because of the clinical course and steroid responsiveness. Therefore, this high signal intensity might have been associated with hypertrophic pachymeningitis.

Although the present patient was ANCA-negative, hypertrophic pachymeningitis is frequently associated with systemic vasculitis, such as Wegener's granulomatosis, particularly in ANCA-positive patients, and immunoglobulin G4-related disease.[3,4] All the three previously reported RP patients complicated by hypertrophic pachymeningitis showed ANCA-positive vasculitis, with an implication that ANCA-related vasculitis might be the cause of hypertrophic pachymeningitis in these patients.[4] We also excluded recognizable autoimmune diseases and paraneoplastic syndrome according to the clinical presentation and laboratory examinations. A post-mortem pathological study of RP reported diffuse vasculitis of the brain, indicating that it extended to the intracranial dura mater.[5]

In addition to hypertrophic pachymeningitis, the patient presented with progressive cognitive impairment, involuntary movements, cerebrospinal fluid abnormality and non-specific deep white matter changes on magnetic resonance imaging. These findings have been previously reported in RP complicated by encephalitis.[2,6] We diagnosed the patient with encephalitis associated with RP on the basis of the negative viral screen and the absence of malignancy.

The present patient also showed serum antiglucosylceramide antibody positivity. Previously, this antibody was only detected in patients with RP and encephalitis, not in patients with RP alone or other neurological disorders.[7] Therefore, these antibodies might be associated with RP and associated encephalitis.

In conclusion, we reported the first case, as per our knowledge, of a RP patient who developed both hypertrophic pachymeningitis and encephalitis, thereby expanding the RP-associated clinical spectrum.

Acknowledgment

The authors declare no conflict of interest.

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原　著

亜急性軸索型ニューロパチー症例に認められた抗Trk抗体の意義*

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要旨　我々は、亜急性軸索障害型ニューロパチーの一部の患者の血清中にNGFの高親和性受容体であるTrk蛋白に対する抗体が出現してくる事を報告した。現在まで、当科で経験した自験2例は、共に非Hodgkin B細胞リンパ腫の既往があり、亜急性にの感覚障害を中核症状とする軸索型ニューロパチーが出現し、IVIg療法に程度の差はあるものの一定の効果を認めた。抗Trk抗体の検出は、PC12細胞にTrkを高発現させたPCtrk細胞のcell-free lysateを市販の抗Trk抗体で免疫沈降し、患者血清でWestern　blot（WB）し陽性バンドを検出した。また、細胞のcell-free lysateを治療前後の患者血清で免疫沈降し、その免疫沈降物を市販の抗Trk抗体でWBしで陽性バンドを検出した。

亜急性軸索障害型ニューロパチーで原因が明らかでない場合、特に非Hodgkin B細胞リンパ腫の既往のある場合には、抗Trk抗体の検索も考慮すべきと考えられた。

Key Words：Trk，抗Trk抗体，ニューロ パチー，lymphoma，神経成長因子

Peripheral Nerve 2014; 25(1): 100–106

はじめに

神経免疫学の進歩により、神経細胞やグリア細胞の細胞膜上や細胞内蛋白に対する種々の抗体が、次々と明らかにされてきた。我々は、亜急性軸索型ニューロパチー症例の血清中に世界で初めて抗Trk抗体を見出し報告した[1)]。一方、これら蛋白に対してばかりではなく、細胞膜上に存在する糖脂質に対しても生じる事が知られるようになってきた。最近、我々は脳脊髄根末梢神経炎患者（EMRN：encephalomyeloradiculoneuropathy）の髄液脳脊髄液及び血清中に、抗中性糖脂質抗体というこれまで未知の抗体を発見し、それらが病態発現に関与している可能性を報告した[2)]。

TrkにはTrkA、TrkB（BDNF：brain derived neurotrophic factor, neurotrophin-4/5の受容体）、TrkC（neurotrophin-3の受容体）の3種類の高親和性受容体が存在する。TrkAは神経栄養因子類の神経成長因子NGF（nerve growth factor）の高親和性受容体型チロシンキナーゼであり、細胞膜上のシグナル伝達の窓口として重要な役割を担う脂質ラフト（lipid rafts）に局在している[3)]（以下特に断らない限りTrkAをTrkと記載する）。NGFの作用により、Trkは二量体化し、それぞれのチロシンキナーゼドメインにあるチロシンキナーゼが互いをチロシン自己リン酸化することによって、Trkを始点とするキナーゼカスケードが活性化し、神経細胞の分化、生存維持に必須な情報伝達系を司っている[4)]。

本稿では、抗Trk抗体陽性のニューロパチー患者についてその臨床像を提示し、本抗体の培養神経細胞系への影響を明確にする事から、本ニューロパチーに於ける抗Trk抗体の病因論的意義を考察する。

対象・方法

【症例】

症例1（文献1と同一症例）：86歳、男性。75歳時に、びまん性B細胞性リンパ腫と診断され治療により完全寛解していた。当院受診の

* Significance of anti-Trk neurotrophin receptor antibody for the patients with subacute axonal neuropathy.

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[2)]Youko INAGUMA and Nobuhiko EMI：藤田保健衛生大学血液内科学［〒470-1192 豊明市沓掛町田楽ヶ窪1-98］；Department of Hematology, Fujita Health University School of Medicine, Aichi

（受付日 2014.1.15）

5ヵ月前から次第に進行する四肢のしびれ感と灼熱痛を主訴に当院へ紹介受診した。神経学的診察では、脳神経には異常なし。運動系では筋力はMMT5で正常、腱反射は減弱、病的反射は認めなかった。感覚系では、四肢遠位部に自覚的なしびれ感と灼熱痛を認めた。他覚的には四肢遠位部で温痛覚・触覚・振動覚の鈍麻を認めた。自律神経系では、両下肢に発汗障害による皮膚の乾燥と魚鱗癬様の皮膚を認め、排尿障害を認めた。関節痛や眼や口の乾燥症状は認めなかった。脳脊髄液検査では、蛋白60mg/dlと軽度の上昇を認めたが、細胞数は正常であった。血算、生化学検査で異常なし。抗核抗体陰性、リウマチ因子陰性。血清中抗Hu抗体と抗Yo抗体は陰性。末梢神経伝導検査では、MCV (motor nerve conduction velocity)、CMAP (compound muscle action potentials) 異常なし。SCV (Sensory Nerve Conduction Velocity) 異常なし、SNAP (sensory nerve action potential) の低下を認めた。^{18}F-FDG-PET (positron emission tomography with [18F] fluoro-2-deoxy-D-glucose) と骨髄穿刺を含む各種検査ではリンパ腫の再発は明らかではなかった。また、各種血液検査などで末梢神経障害をきたす他疾患は否定された。

大量免疫グロブリン療法 (IVIg：intravenous immunoglobulin) 施行後、四肢の異常感覚や皮膚の乾燥は改善し、電気生理学的にも、SNAPの改善が認められた（文献1、table参照)。その後、他院でリンパ腫の再発によって死亡した。

症例2：54歳、男性。49歳時に左腎から脾臓にかけてを認められた腫瘤からの生検で、高度異型性細胞のびまん性かつ密な髄様増殖がみられ、腫瘍細胞はクロマチンに富んだ不整形の核と乏しい胞体を有するN/C比の高い形態をとっており、分裂像も多数認められた。免疫染色で腫瘍細胞はL-26、CD79a等のB-cell系マーカーが陽性であった。これらの結果から、大細胞型びまん性B細胞性リンパ腫（stage Ⅳb）と診断され、R-CHOP-14療法を6コース＋IFRT (Involved Field Radiation) 40 Gy施 行、CCR (complete clinical remission) と判断され退院後は無治療で経過観察となっていた。52歳時に骨髄に再発し、化学療法を施行するも、頭蓋内（左側尾状核～視床～第3脳室左側）再発も認めたため、放射線療法（全脳照射）も追加された。頭蓋内病変改善後の53歳時に、自己末梢血幹細胞移植も実施された。54歳時に四肢遠位のしびれ感を自覚し次第に増悪したため、神経内科初診となった。神経学的には、脳神経には異常なし。運動系では、両上肢下肢近位筋MMT5であったが、両上肢遠位筋MMT4、両下肢遠位筋MMT3程度の筋力低下を認めた。腱反射は下肢で減弱していた。病的反射は認めなかった。歩行はふらつきながらも可能であった。感覚系では、四肢遠位部に自覚的なしびれ感と時に疼痛を認めた。他覚的には、四肢遠位部で温痛覚・触覚・振動覚の鈍麻を認めた。自律神経系では、両上肢遠位部と両下肢に発汗障害を認め皮膚は乾燥し、両側下腿に魚鱗癬様変化を認めた。脳脊髄液検査では、蛋白46mg/dl、細胞数32/3 (mono/poly：30/2) と軽度上昇していたが、細胞診は陰性であった。末梢神経伝導検査では、四肢の運動神経・感覚神経共に軸索障害の所見を認めた (Median MCV 49.2m/s, CMAP 8.5mV, SCV 44.0m/s, SNAP 4μV. Ulnar MCV 40.0m/s, CMAP 4.4mV, SCV not evoked. Tibial CMAP not evoked. Sural SANP not evoked.)。抗Trk抗体を血清で認めたことからIVIgを施行したところ、軽度の筋力の改善と、自覚的なしびれ感、疼痛の消失・両側下腿の魚鱗癬様変化の改善を認めた。しかし、効果は一時的で、末梢神経伝導検査では明瞭な改善は認められなかった。2ヵ月後には全身状態の悪化などにより四肢の筋力低下が進行し、2クール目のIVIgを行うもはっきりした効果が認められず、末梢神経障害が出現して9ヵ月後、リンパ腫の再発・増悪により死亡した。

【対象・方法】前述の患者血清2例と、正常コントロール血清6例、疾患コントロールとして神経疾患患者血清13例 (chronic inflammatory demyelinating polyneuropathy 10例、autoimmune autonomic ganglionopathy 1例、optic neuritis 1例、acute disseminated encephalomyelitis 1例) を用いた。

ラット褐色細胞腫由来のPC12細胞に、ヒト*trk*-complimentary DNAをトランスフェクタン

トしTrkを高発現するPCtrk細胞を作製した。PCtrk細胞をコラーゲンとpoly-L-lysineでコートした24穴プレートに播き、5% CO_2濃度、37℃の条件下においてovernightで培養した（serum free Dulbecco's modified Eagle medium containing 7.5% FBS, horse serum, 100 U/ml penicillin and 100μg/ml streptomycin)。Serum-free mediumに患者血清と対照血清を最終濃度0.5%になるように加え、NGF 50ng/ml（mouse nerve growth factor 2.5S, Merck Millipore Co., Billerica, MA, USA）存在下で48時間培養を行い細胞の形態変化を位相差観察法（Culture Microscope CK40, Olympus Co., Tokyo, Japan）で観察した[1,5,6]。

また、一部の血清を用いて、PCtrk細胞を0.1%患者血清と0.1%対照血清でそれぞれ1時間前処理し、NGF（50ng/ml）で5分間刺激後Trkの自己チロシンリン酸化状態を抗リン酸化チロシン抗体（α-PY, 4G10, Upstate Biotechnology Inc., Lake Placid, NY, USA）を用いて調べた[1]。

病理解剖で得られたヒト前頭葉脳組織、脳基底核、神経根、脾臓のホモジェネートを用いて、0.5%の患者血清と対照血清を用いてWBを行った[1]。

PCtrk細胞のcell-free lysateから、一次抗体として患者血清と対照血清、二次抗体としてprotein L-agarose（Protein L-Agarose Immunoprecipitation Reagent：sc-2336, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA）で免疫沈降（IP：immunoprecipitation）を行い、免疫沈降物を作成した。この免疫沈降物を市販の抗Trk抗　体（Trk C-14：sc-11, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA）を一次抗体としてWBし、陽性バンドの検出を行った[1]。

結　果

PCTrk細胞に患者血清を加えて培養すると、NGFによる神経突起伸長反応が明瞭に阻害された（図1-2と3を比較)。しかし、患者血清単独では明らかな形態学的変化を来さなかった（図1-1と6を比較)。また、治療前患者血清は、治療後血清よりもNGFによる神経突起伸張をより強く阻害していた（図1-3と4を比較)。一方、患者血清で前処理したPCtrk細胞では、NGFによるTrkのチロシン自己リン酸化反応が抑制された（文献1、Figure 1参照)。

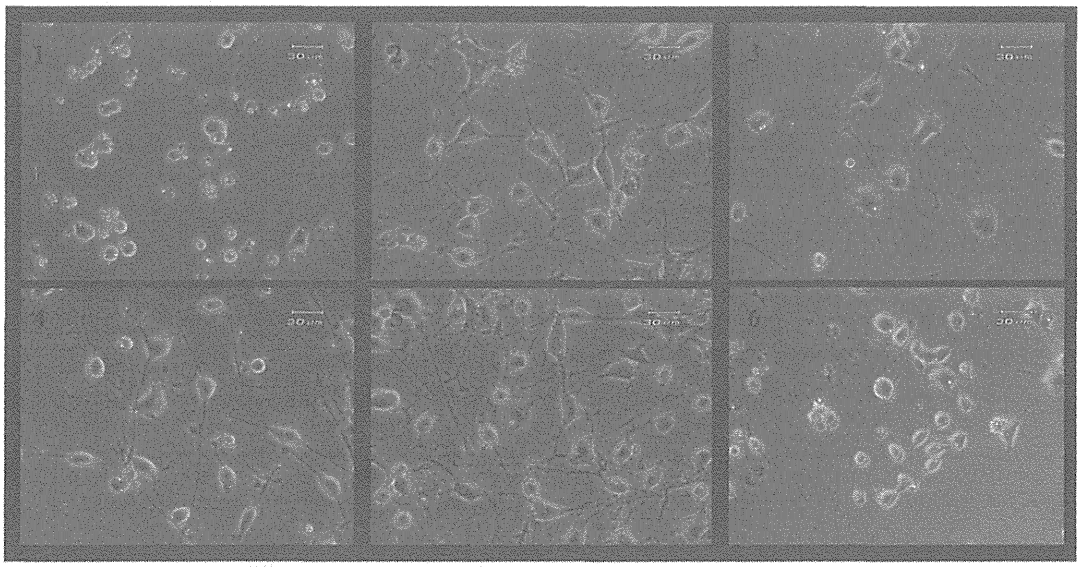

図1 NGFによる神経突起伸張反応を及ぼす患者血清の影響
PCtrk細胞を24穴のプレートに播きovernightで培養．Serum-free mediumに患者血清を加えNGF 50 ng/ml存在下で48時間培養を行った。1. Serum-free mediumのみ；2. NGF単独；3. 治療前症例2血清（0.5%）+ NGF；4. 治療後症例2血清（0.5%）+ NGF；5. コントロール血清（0.5%）+ NGF；6. 治療前症例2血清（0.5%）単独。最も代表的な部分を位相差顕微鏡で撮影した。患者血清は明らかにNGFによる神経突起伸張を阻害している。

剖検で得られたヒト前頭葉脳組織、脳基底核、神経根、脾臓の組織のホモジェネートより作成したサンプルを電気泳動しPVDF膜（Immobilon-P membrane, PVDF, 0.45μm, Merck Millipore Co., Billerica, MA, USA）に転写後、患者血清と対照血清でWBしたところ対照血清では認められないTrkの分子量である140kDaの位置に患者血清で陽性バンドを認めた（文献1、Figure 2参照）。

PCtrk細胞のcell-free lysateから市販の抗Trk抗体で免疫沈降してきた免疫沈降物（Trk蛋白）を、患者血清と対照血清でWBしたところ、患者血清を一次抗体として用いた場合のみにTrkの分子量である140kDaの位置に陽性バンドを認めた。対照血清の場合には陽性バンドは認められず、患者血清中に抗Trk抗体が存在することが示唆された（図2）。

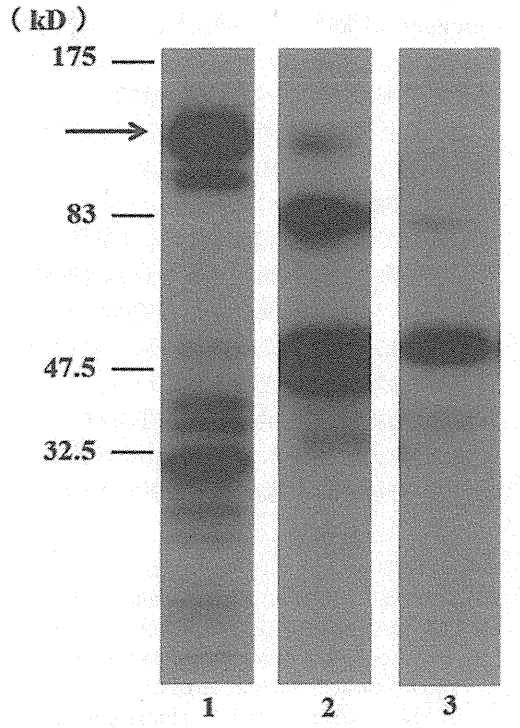

図2 患者血清中の抗Trk抗体
1. PCtrk細胞のcell-free lysateから市販の抗Trk抗体でIP、2. PCtrk細胞のcell-free lysateから症例2患者血清でIP、3. PCtrk細胞のcell-free lysateから対照血清でIPした免疫沈降物（血清中に抗Trk抗体が存在すれば免疫沈降物にTrk蛋白が含まれている）をSDS-PAGEし、市販の抗Trk抗体でWBした結果を示す。抗Trk抗体でWBすると140kDaに陽性バンドを認める（矢印）。症例2患者血清でIPした場合にも陽性バンドを認め、患者血清中に抗Trk抗体の存在を示している。対照血清では陽性バンドは認めていない。

考　察

我々は、2005年に非Hodgkin B細胞性リンパ腫の完全完解中に亜急性の経過で軸索型感覚神経ニューロパチーを呈した症例を経験し、急性期血清中に抗Trk抗体が存在することを見出した[1)]。当該症例では、IVIg治療後にその抗体価が明瞭に減少していたことから、同症例で見られた亜急性軸索型ニューロパチーの成因に、抗Trk抗体が関与している可能性を想定した。事実、患者血清中の抗-Trk抗体は、*in-vitro*でNGFによるPC12細胞からの神経突起伸長反応を抑制し、NGFによるTrk自己リン酸化反応も抑制した。従って、今後同様な症例では本抗体のチェックが重要であることを提言した[1)]。さらにその後見出した1例の臨床像・所見を交えて抗Trk抗体の病因論的意義について考察を加えたい。

感覚神経細胞、交感神経節神経細胞の神経突起伸張や生存維持には、NGFによるTrkを介した細胞内情報伝達系の情報が必要である[7)]。この事実は、先天性無痛無汗症患者を見れば明らかである。先天性無痛無汗症は、無痛により自傷や怪我を繰り返すが、先天的に無痛であるためにそれらを回避することが難しい。また、無汗であるために体温上昇をきたしやすく、一方運動機能は保たれる事が多いとされるが、運動の継続は体温上昇のため困難である。また、軽度の知的障害を伴う場合もある。1996年Indoらにより、この先天性無痛無汗症患者の*trk*遺伝子に変異があることが報告をされ、NGFによるTrkを始点とするキナーゼカスケードが、特に末梢感覚神経や自律神経の機能維持に重要な役割を果たしていることが明らかにされた[8)]。

症例1は、その主要症候が亜急性に経過した末梢神経障害で、特に感覚神経系と自律神経系の障害を強く認めた。前述の先天性無痛無汗症の臨床特徴との類似性から想定して、患者血清中の抗Trk抗体が、これら神経系でのTrkの機能を阻害し症候を惹起したことが推測される。これは、*in-vitro*の培養神経細胞系でも患者血

清がNGFの作用に対して抑制的に作用したこと、神経症候に免疫療法が奏功したことからも支持される。

一方、症例2でも、四肢の疼痛を伴う感覚障害が症候の中心であることは共通するが、運動系の症候を認め症例1に比しより急性の経過を辿るなど、必ずしも臨床像は一致していない。症例2は、末梢神経障害出現後9ヵ月で死亡しており、全身状態が症例1より明らかに不良であった点や、seonsory axonopathy自体も症例1より重篤であった可能性が高いことなどが、症例1との臨床像の違いに影響した可能性がある。今後、より多くの抗Trk抗体陽性のニューロパチー症例の蓄積と詳細な解析を待たなければ、この臨床像の多様性の分子基盤は議論できないと考えられる。

現在、我々はこの問題に対しての解答を得るには、以下に述べる3点を先ず検討しなければならないと考えている。即ち、第一に、これら患者で検出した抗Trk抗体の標的抗原部位の特定が重要で、これら患者中の抗体がTrk分子の同一部位をepitopeとしているのか否かの決定は一つの解答を与えてくれると予想される。

第二に、Trk分子の細胞内局在部位に関する考察も極めて重要ではないかと考えられる。これまでの神経糖鎖生物学的研究よりTrk分子は細胞膜lipid raftsという糖脂質、スフィンゴミエリン、コレステロールおよびシグナル伝達分子例えば受容体型チロシンキナーゼやチャネル蛋白で形成される特別なドメインに局在していることが明らかとなっている。最近、我々は軸索障害型ギラン-バレー症候群患者血清中に見出される抗GM1抗体（標的抗原であるGM1はlipid raftsを構成する主要な酸性糖脂質）はPCtrk細胞のlipid rafts上に局在するTrkをlipid rafts外へ移動させ、Trkを介したシグナル伝達系を阻害させる事実を見出した[9]。さらに、XuらはHIVのgp120蛋白が初代培養神経細胞のlipid raftsの大きさを変化させること、NMDA（N-methyl-D-aspartate）受容体の必須のsubunitであるNR1をlipid rafts上にリクルートし機能変化を惹起させることを見出している[10]。さらに、先述したように最近我々が見出した抗中性糖脂質抗体[2]は、抗原分子である中性糖脂質が局在するlipid raftsに共局在するTrk分子の機能に重大な影響を与えることを見いだしている（未発表データ）。これらの事実はから、共にlipid rafts上の物質に作用する抗体や蛋白が存在すれば、それらが直接に作用する物質、例えば「抗Trk抗体が作用する抗原であるTrk蛋白」のみに影響を与えるだけでなく、足場であるlipid raftsそのものにも影響を与え、lipid raftsの構造や組成を変化させ同部位に局在している他の重要な分子の働きにも影響を与える可能性があると考えられる。

第三に、どうして抗Trk抗体が産生されたのか？ という疑問に答える必要がある。我々が抗Trk抗体を検出した患者は、両者とも非Hodgkin B細胞性リンパ腫であったこと、非Hodgkin B細胞性リンパ腫細胞にTrkが発現していることも報告されており[11]、何れの例も最終的には原病のリンパ腫の再発で死亡していることに注目すべきと考えられる。この事実は症例1のように血液学的に完全完解状態であってもリンパ腫細胞はゼロにはなっていない可能性を示唆していると解釈出来よう。視点を変えれば、傍腫瘍神経症候群に見出される抗神経抗体のように、極めて特異的かつ高感度の悪性細胞の検出法にもなり得る可能性がある事も指摘しておかねばならないと考える。現在、こうした観点から血液内科と共同して抗Trk抗体がリンパ腫の新たなバイオマーカーになり得るか否かをリンパ腫患者を対象に研究を進めている。

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Significance of anti-Trk neurotrophin receptor antibody for the patients with subacute axonal neuropathy.

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Here, we report two patients with a previous history of non-Hodgkin lymphoma who presented subacute sensory dominant axonal neuropathy. The neuropathy responded to intravenous immunoglobulin therapy. Anti-Trk high affinity nerve growth factor receptor antibody was detected in sera of the above cases and its titers were correlated with disease activity. Rat pheochromocytoma-derived PC12 cells and their stable transfectants of human trk complimentary DNA (PCtrk cells) were cultured as described. The Trk protein was immunoprecipitated with commercial anti-Trk antibody and subjected to immunoblot analysis. They were probed with patients' sera. To test whether sera can immunoprecipitate Trk, they were used for immunoprecipitation as the first antibody with a recovery by protein L-agarose. These immunopreciptiates were probed with commercial anti-Trk antibody. All of these experiments strongly suggested the presence of anti Trk antibody in their sera. The present antibody exhibits inhibitory action on the Trk-initiated neurotrophic signal transduction pathway and NGF-induced morphological differentiation *in vitro*.

We should check this autoantibody for further patients who exhibit subacute sensory dominant axonopathy exhibiting the previous history of non-Hodgkin lymphoma.

Key Words: Trk, anti-Trk antibody, neuropathy, lymphoma, nerve growth factor

Austin Journal of Clinical Neurology

Open Access

Austin Publishing Group

Case Report

Deep Neck Inflammatory Diseases: Implication of Cervical Magnetic Resonance Imaging for Early Diagnosis

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Received: January 30, 2015; **Accepted:** February 18, 2015; **Published:** February 20, 2015

Abstract

Deep neck inflammatory disorders such as retropharyngeal abscess and pyogenic cervical spondylitis are potentially life-threatening disorders and are quite rare in healthy individuals. With abnormal findings on emergency cervical magnetic resonance imaging (MRI), we succeeded in making prompt diagnoses and initiate appropriate treatments. Both cases recovered almost fully without any orthopedic intervention. Especially in the case of pyogenic cervical spondylitis, we could detect the very early stage of the disease by cervical MRI, i.e., the inflammations were confined to the vertebra without affecting adjacent tissues. Thus, emergency MRI of cervical spine would offer reliable methods for diagnosis and speedy treatments of deep neck inflammatory diseases.

Keywords: Retropharyngeal abscess; Pyogenic cervical spondylitis; Deep neck inflammatory disorder

Introduction

Deep neck infectious disorders in healthy adults (without vertebral surgical history) are very rare but may lead to potentially life-threatening complications [1]. Here, we report two cases, i.e., a case of fulminant retropharyngeal abscess and a case of acute pyogenic cervical spondylitis, both of them showed favorable outcomes. The present cases well illustrated the usefulness and diagnostic importance of emergency MRI examination of the spine.

Case Presentation

Case 1

A 39-year-old man had fever and occipital pain. On the next day, he visited a local practice and was prescribed a NSAID and antibiotics. High fever continued following day and occipital pain got worse. He could not turn his neck in any direction. Then, he visited another hospital and was transferred to our hospital. On admission, he was alert. He had a difficulty to open his mouth and had hoarseness. Although other cranial nerves were not involved, nuchal rigidity was observed. Other neurological findings were all normal. Blood examination showed that white blood cell count was 11500 (4000 - 9000/μm^3) and CRP was 30.3mg/dl (< 0.3 mg/dl). Cerebrospinal fluid examination disclosed pleocytosis (106 leucocytes/mm3, 82% of lymphocytes) with extremely elevated protein (392 mg/dl; normal range, 10-40mg/dl) and IgG concentrations (69 mg/dl; normal range, < 4 mg/dl). Blood culture was negative. PCR examination for tuberculosis in cerebrospinal fluid was negative. The cervical MRI revealed widening of the prevertebral space along C1 to C5 (Figure 1). Under the diagnosis of retropharyngeal abscess, puncture of the abscess and drainage was performed immediately by otolaryngologists. Culture of the abscess was negative, which may be due to the prior administration of antibiotics at other hospital. Previous study has shown that retropharyngeal abscess tends to occur mostly in children and microbiological review of children indicated that anaerobic organisms are predominantly isolated [2]. In adults, aerobic organisms were also isolated [2]. Therefore, a broad spectrum antibiotic (MEMP) was administered intravenously. Eventually, neck pain was alleviated. On 7 days after the diagnosis, however, the patient complained sensory disturbances in his left hand and forearm. Cervical MRI showed the extradural (epidural) abscess in C3 and C4. Therefore, the antibiotic was changed to another broad spectrum antibiotics (PAMP/BP). At 15days after hospitalization, MRI showed prominent shrinkage of extradural abscess. He was discharged at 24 days after hospitalization without orthopedic surgery.

Case 2

A 47-year-old man gradually developed neck pain in the evening without preceded respiratory tract infection. Moreover, he had no history of vertebral surgery or injuries. Several hours later, he felt severe pain in the neck and left shoulder followed by the difficulties for extending his neck. Anterior chest pain was developed when swallowing. Next morning, he had low grade fever (37.2°C) but had no neck stiffness. Then, he visited our hospital. No muscle weakness or sensory impairment was noted. Laboratory examination revealed mild leukocytosis. CRP was abnormally high, 8.1mg/dl. Erythrocyte sedimentation rate was 72mm/1hour. Although cervical spine X-ray showed no radiographical abnormalities except comparatively hyperintensity as shown in Figure 2A, emergency cervical MRI was performed immediately and it revealed prominent low intensity signal on T1-weighted image and high intensity signal on T2-weighted image in entire C4 (Figure 2B and 2C). The patient was hospitalized and was treated with intravenous administration of antibiotics (CAZ) under the diagnosis of acute pyogenic cervical spondylitis. Neck pain disappeared within a few days. Endoscopic examination of larynx and esophagus did not reveal any abnormalities including fish bones. Blood culture was negative. QuantiFERON-TB test for tuberculosis

Citation: Hikichi C, Asakura K, Hirota S, Fukui T, Murate K, et al. Deep Neck Inflammatory Diseases: Implication of Cervical Magnetic Resonance Imaging for Early Diagnosis. Austin J Clin Neurol 2015;2(2): 1024.

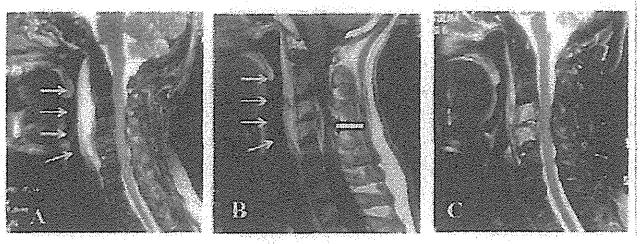

Figure 1: Sagittal cervical MRI of case 1. (A) T2-weighted image on admission. Small arrows indicate the abscess in retropharyngeal space extended from C1 to C5 level. (B) Gadrinium-enhanced T1-weighted image 7 days after admission. Epidural abscess was observed in C3 to C5 level (large arrow). (C) T2-weighted image 15days after admission. Epidural abscess was disappeared and retropharyngeal space was decreased.

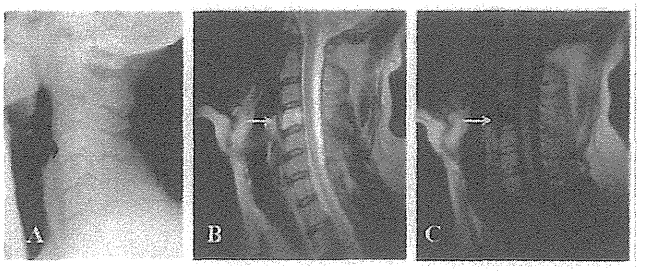

Figure 2: Cervical spine X-ray and sagittal cervical MRI of case 2. Cervical spine X-ray (A), T2-weighted (B), and T1-weighted (C) images on admission. Black arrow indicates mild hyperintensity in C4 (A). White arrows indicate prominent T2 high and T1 low intensity signals in C4 without abscess formation (B and C).

was negative. Symptoms disappeared and laboratory data were normalized within 10 days after diagnosis. He was discharged at 10 days after hospitalization. He has been followed in our department for more than 3 years and no relapse occurred.

Discussion

Despite the advancement of diagnostic procedures and antibiotics, deep neck infections occasionally cause life-threatening complications due to airway displacement, mediastinitis, spinal cord abscess, jugular vein thrombosis, and carotid artery occlusion [1]. Early diagnosis and treatment are indispensable to prevent complications for not only otolaryngologists but also neurologists.

Retropharyngeal lymph nodes usually disappear after age 4 or 5 years. Therefore, the incidence of non-traumatic retropharyngeal abscess in healthy adults is extremely rare and most of the adult cases are associated with immunocompromised condition or a foreign body complication such as fish bone [3,4]. Due to the rarity of retropharyngeal abscess in adults, the definitive diagnosis may be delayed in some adult patients [5,6]. In our case, foreign body was not identified. Though our first case showed fulminant clinical course with meningeal involvement, swift interdisciplinary treatment with otolaryngologists, i.e., transoral drainage and antibiotics administration were successful.

Pyogenic spondylitis is one of the most severe infectious diseases of the neurological system, and most of the previous cases are related to spinal surgical procedures or tuberculosis infection. Spontaneous pyogenic spondylitis in healthy individuals without any underlying disease is also extremely rare. The cervical spine is a relatively uncommon site for infection in comparison with lumbar and thoracic spine, representing less than 10% of all cases [1]. Pain of shoulder and fever occurred in most of the cases as well as our case 2. Some of them, however, were misdiagnosed initially [7]. Common MRI findings in infectious spondylitis are hypointensity of the involved tissue on T1-weighted images, hyperintensity on T2-weighted images, destruction of two or more adjacent vertebral bodies with involvement of the intervening disc, and epidural and paraspinal extension and/or abscesses [8,9]. We were able to detect the very early stage of the disease confined to C4 vertebral body without affecting adjacent tissues. To our knowledge, no case of pyogenic cervical spondylotis as mild as our case 2 has been reported in the literature.

It should be mentioned that although deep neck inflammatory disorders are quite rare in healthy individuals, detailed and emergent neuroradiological examinations such as MRI are necessary for early diagnosis and treatment of the potentially life-threatening disorders. Our cases highlight the importance of early diagnosis with cervical MRI. With timely diagnosis by cervical MRI, we can initiate the appropriate and intensive treatment of the disorders as seen in the present cases. Fortunately, both present cases recovered almost fully without any orthopedic intervention. These early diagnoses and treatments can help to improve patients' outcome.

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Austin J Clin Neurol - Volume 2 Issue 2 - 2015
ISSN : 2381-9154 | www.austinpublishinggroup.com

Citation: Hikichi C, Asakura K, Hirota S, Fukui T, Murate K, et al. Deep Neck Inflammatory Diseases: Implication of Cervical Magnetic Resonance Imaging for Early Diagnosis. Austin J Clin Neurol 2015;2(2): 1024

BRAIN RESEARCH 1596 (2015) 13–21

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Brain Research

Research Report

Neurotropin promotes NGF signaling through interaction of GM1 ganglioside with Trk neurotrophin receptor in PC12 cells

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ARTICLE INFO

Article history:
Accepted 20 November 2014
Available online 29 November 2014

Keywords:
Neurotropin
NGF signaling
GM1 ganglioside
Trk tyrosine kinase

ABSTRACT

Activation of the high-affinity nerve growth factor (NGF) receptor Trk occurs through multiple processes consisted of translocation and clustering within the plasma membrane lipid rafts, dimerization and autophosphorylation. Here we found that a nonprotein extract of inflamed rabbit skin inoculated with vaccinia virus (Neurotropin®) enhanced efficiency of NGF signaling. In rat pheochromocytoma PC12 cells overexpressing Trk (PCtrk cells), Neurotropin augmented insufficient neurite outgrowth observed at suboptimal concentration of NGF (2 ng/mL) in a manner depending on Trk kinase activity. Cellular exposure to Neurotropin resulted in an accumulation of Trk-GM1 complexes without affecting dimerization or phosphorylation states of Trk. Following NGF stimulation, Neurotropin significantly facilitated the time course of NGF-induced Trk autophosphorylation. These observations provide a unique mechanism controlling efficiency of NGF signaling, and raise the therapeutic potential of Neurotropin for various neurological conditions associated with neurotrophin dysfunction.

Abbreviations: BDNF, brain-derived neurotrophic factor; BS3, bis(succinimidyl)suberate; cAMP, cyclic AMP; CTB, choleratoxin subunit B; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; GM1, monosialotetrahexosylganglioside; HRP, horseradish peroxidase; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase; PVDF, polyvinylidene fluoride; PY, phosphotyrosine; Tris, tris (hydroxymethyl)aminomethane; Trk, tropomyosin-related kinase

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http://dx.doi.org/10.1016/j.brainres.2014.11.041
0006-8993/

1. Introduction

Nerve growth factor (NGF) stimulates survival and differentiation of sympathetic and sensory neurons (reviewed in Sofroniew et al., 2001). NGF induces morphological and biochemical differentiation of rat pheochromocytoma PC12 cells into a phenotype resembling sympathetic neurons (Greene and Tischler, 1976). NGF initiates its biological actions upon binding to the plasma membrane high-affinity NGF receptor, Trk (Kaplan et al., 1991a; 1991b), which in turn activates intracellular signaling cascades involving pathways depending on extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) (reviewed in Patapoutian and Reichardt, 2001).

Trk-mediated NGF signaling is initiated by well-coordinated, seamless processes occurred on Trk molecules, which are composed of formation of homodimers (Jing et al., 1992), autophosphorylation of tyrosine residues (Kaplan et al., 1991a) and spatial assemblies with downstream signaling effectors such as Shc and phospholipase C (reviewed in Huang and Reichardt, 2003). Moreover, it has recently become appreciated that molecules requisite for initiating NGF signaling cascade colocalize into certain membrane subdomains, often referred to as lipid rafts, that contain cholesterol and glycosphingolipids (Limpert et al., 2007; reviewed in Hakomori, 2000). While the exact structure and function of neuronal lipid rafts are currently under debate, initiation of NGF signaling is known to be controlled in the lipid rafts by recruitment of signaling molecules and their interactions with lipid components (reviewed in Pike, 2003). As a prime example, it had been shown that monosialoganglioside GM1, a major lipid constituent of the lipid rafts, enhances NGF-dependent homodimerization (Farooqui et al., 1997) and autophosphorylation (Mutoh et al., 1995) of Trk. In addition, GM1 depletion by inhibiting glucosylceramide synthase abolished the NGF response in PC12 cells (Mutoh et al., 1998). These observations clearly indicate structural and functional modulation of Trk by this lipid molecule. Thus, the lipid rafts serve as a highly organized, regulatory core requisite for initiating Trk-mediated NGF signaling.

A non-protein extract of inflamed rabbit skin inoculated with vaccinia virus, designated Neurotropin®, has been widely distributed in Japan and China for the treatment of chronic pain conditions and other various neurologic symptoms. However, precise molecular mechanisms underlying in these pharmacological actions are not fully understood. An active ingredient(s) of this multi-component drug remains yet to be elucidated, in spite of substantial efforts supported by current fractionation technologies such as ultra performance liquid chromatography and capillary electrophoresis. Recently, screening of active ingredients has been started in vitro based on our findings that brain-derived neurotrophic factor (BDNF) expression was augmented in human neuroblastoma SH-SY5Y cells by this drug (Fukuda et al., 2010). Because the BDNF induction by Neurotropin was abolished by co-treatment of the cells with anti-Trk antibody or K252a, a selective inhibitor of Trk tyrosine kinase, it was suggested that targeting and activation of Trk are pivotal for Neurotropin action. In order to test this assumption, here we examined NGF-induced Trk activation in PC12 cells overexpressing Trk (PCtrk cells) (Mutoh et al., 2000). Although Neurotropin itself lacked ability to induce Trk autophosphorylation, it largely facilitated the time course of Trk autophosphorylation in response to NGF. In PCtrk cells exposed to Neurotropin, association of Trk with GM1 ganglioside was found to occur without affecting Trk dimerization and autophosphorylation states. These data implicate that Neurotropin controls the efficiency of Trk-mediated NGF signaling pathway through a novel mechanism associating with ligand-independent interaction of Trk and GM1.

2. Results

2.1. *Trk-dependent promotion of neuritogenesis by Neurotropin*

PC12 cells differentiate into a neuron-like morphology through high-affinity NGF receptor, Trk (Hemstead et al., 1992). In order to evaluate Trk-dependent cellular processes in a steady and sensitive manner, we employed PC12 cells overexpressing human Trk (PCtrk cells) (Mutoh et al., 2000). As expected, NGF (50 ng/mL) dramatically promoted neurite extension, a marker of cellular differentiation, within 24 h (Fig. 1A, Panel c). Neurite extension was not evident at low concentration of NGF (2 ng/mL; Fig. 1A, Panel b), but was enhanced by Neurotropin in a dose-dependent manner (Fig. 1A, Panels d–f; Fig. 1C). Maximal neurite extension was observed at 20 mNU/mL of dosage, and the effect was partly reversed at a higher dosage (100 mNU/mL; Fig. 1C; $P<0.05$ vs. 20 mNU/mL, ANOVA). We also observed a similar biphasic dose-dependency in neurite extension of the parental PC12 cells treated with Neurotropin, confirming that the action was not strain-specific (data not shown). Interestingly, enhancement of neuritogenesis by Neurotropin was not definite in the absence of NGF (Fig. 1B), indicating that Neurotropin may assist the action of NGF in the cells. In addition to such morphological observations, intracellular expression of neurofilament M (NF-M), a major component of the neuronal cytoskeleton supporting axonal construction, was evaluated by Western blot analysis. Expression of NF-M (160 kDa) was significantly augmented by Neurotropin at a dosage of 20 mNU/mL (Fig. 1D). Thus, Neurotropin was shown to enhance neuritogenesis at suboptimal NGF concentration.

We next examined the role for Trk in Neurotropin action by using a selective inhibitor of Trk tyrosine kinase activity, K252a. An enhanced neurite extension by Neurotropin (20 mNU/mL) was drastically prevented by K252a at the concentration effective for inhibition of NGF-induced neuritogenesis (500 nM; Fig. 2A and B). In addition, K252a prevented phosphorylation of intracellular signaling molecules ERK1/2 and Akt induced by NGF or Neurotropin (Fig. 2C). These observations suggest that Neurotropin stimulates neuritogenesis through activations of Trk and downstream signaling molecules.

2.2. *Neurotropin pretreatment facilitates time course of NGF signaling*

In order to characterize the Neurotropin effect on Trk, we next examined the time course of NGF-induced Trk autophosphorylation. Undifferentiated PCtrk cells pretreated for 3 h with

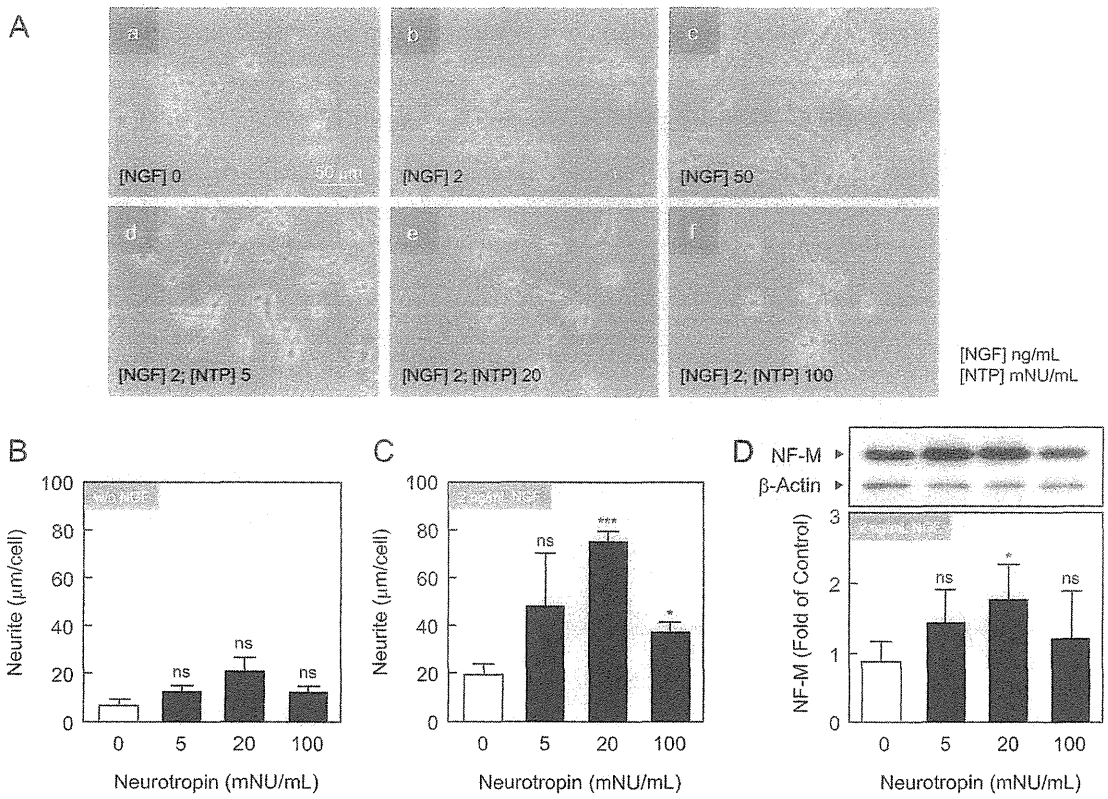

Fig. 1 – Neurotropin promotes neurite outgrowth in PCtrk cells treated at suboptimal concentration of NGF. (A) Neurite extension by Neurotropin. PCtrk cells (1000 cells per well, 6-well plastic plate) were treated for 24 h in the absence (*a*) or the presence of NGF (*b*, *d–f*, 2 ng; *c*, 50 ng/mL) and Neurotropin (NTP; *d*, 5 mNU; *e*, 20 mNU; *f*, 100 mNU/mL). Phase-contrast micrographs taken for typical areas of cultures were shown. (Bar = 50 μm in length) (B) and (C). Effect by Neurotropin on neurite length. PCtrk cells were cultured for 24 h in the absence (B) or the presence of NGF (2 ng/mL, C) and indicated concentrations of Neurotropin (0, 5, 20, or 100 mNU/mL). Neurite length was measured by Image J as described in Section 4. Data represents the mean and standard deviations (SD) of neurite length in three independent cultures. (D) Neurofilament M (NF-M) expression. Cell lysates of PCtrk cells (1×10^5 cells, 26 h) were subjected to Western blotting against NF-M and β-actin (an internal control) as described in Section 4 (*upper panel*, typical blotting images). Data represents the mean and SD of the intensity ratio of NF-M to β-actin in three independent cultures.*$P < 0.05$, **$P < 0.001$ vs. saline-treated controls (*open bars*); ns, not significant (two-sided t-test).

saline (control) or Neurotropin at 20 mNU/mL, an effective concentration for neuritogenesis (see Fig. 1), were incubated with NGF (50 ng/mL) for various time periods (0.5–20 min), and tyrosine phosphorylation of Trk was examined by a double-antibody ELISA using antibodies against Trk (α-Trk) and phosphotyrosine (α-PY) for cell lysates (Fig. 3A), or by Western blot analysis against phosphotyrosine (PY) in α-Trk immunoprecipitates (Fig. 3B). NGF-induced Trk autophosphorylation was observed in a time-dependent fashion, peaking at 5 min (Fig. 3A, open symbols). To our surprise, pre-exposure of PCtrk cells to Neurotropin (20 mNU/mL, 37 °C, 3 h) significantly accelerated the time course of NGF-induced Trk autophosphorylation, although total and peak intensities of phosphorylated Trk were virtually unaffected (Fig. 3A, closed symbols). Since basal levels of phosphorylated Trk without NGF stimulation were equivalent between treatments (Fig. 3A and B, time "0"), Neurotropin seemed not to enhance Trk phosphorylation directly, but rather to improve the efficiency of Trk-mediated NGF signaling.

2.3. *Neurotropin promotes Trk-GM1 association*

We have previously reported that GM1 associates with Trk and enhances NGF signaling in PC12 cells (Mutoh et al., 1995). It is believed that this process involves translocation of Trk molecules into compartmentalized microdomains in the plasma membrane, so-called lipid rafts, where GM1 and other glycosphingolipids reside. Accordingly, we next examined the effect of Neurotropin on the receptor-lipid association. Immunoprecipitates with α-Trk from PCtrk cells incubated with NGF (1 min) and/or Neurotropin (1 min or 3 h) were subjected to SDS-PAGE for Western blot analysis against GM1. Multiple bands were probed by anti-GM1 antibody (α-GM1) as shown in Fig. 4 (Panel C). NGF treatment gave a strongly immunoreactive band approximately at 140 kDa (lane 2, *asterisk*), corresponding to the molecular size of Trk. This observation indicates NGF-dependent formation of the Trk–GM1 complex that is resistant to sample treatment with SDS as previously reported (Mutoh et al., 1995). Both the receptor-lipid association (Panels C and E) and Trk autophosphorylation (Panels A, B and

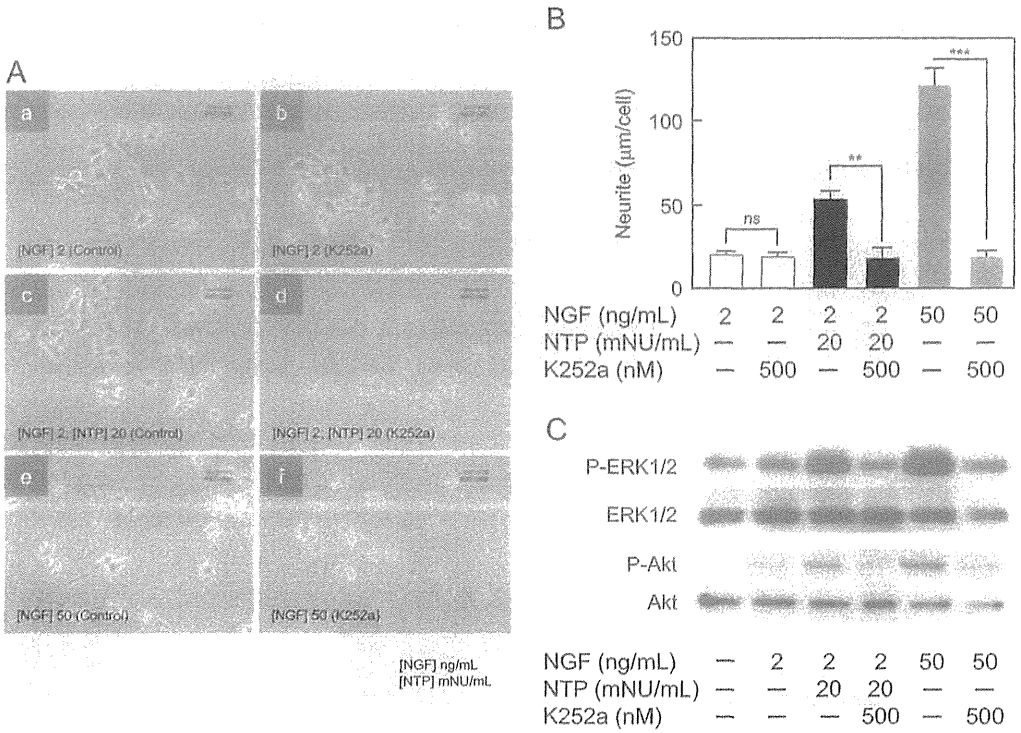

Fig. 2 – Role of Trk in Neurotropin action. (A) Neurite extension by Neurotropin was blocked by K252a. PCtrk cells (1000 cells per well) cultured in serum-free DMEM in the presence of NGF (*a–d*, 2 ng/mL; *e*, *f*, 50 ng/mL) and/or Neurotropin (*c*, *d*, 20 mNU/mL) were treated with K252a (500 nM; *b*, *d*, *f*) for 6 h prior termination of the culture for 18 h. Phase-contrast micrographs were taken for typical areas of cultures. (Bar=30 μm in length) From three independent cultures, neurite length was quantified by Image J software as described in Section 4. (B) Data represents the mean and SD. ns, not significant; $^{**}P<0.01$, $^{***}P<0.001$ vs. controls in the absence of K252a (*t*-test). (C) Involvement of ERK1/2 and Akt in Neurotropin action. PCtrk cells (1×10^5 cells) were treated for 24 h in serum-free DMEM containing NGF and/or Neurotropin as indicated. K252a (500 nM) was added at 30 min prior termination of the treatment. Cells were lysed in SDS sample buffer, and the lysate was subjected to Western blot analysis detecting phosphorylated and total forms of ERK1/2 (P-ERK1/2 and ERK1/2, respectively, *upper panels*) or Akt (P-Akt and Akt, respectively, *lower panels*) as described in Section 4.

D) by NGF was enhanced by prolonged incubation with Neurotropin in the presence of Neurotropin (Panels A–C, lanes 4 and 6). Intriguingly, even in the absence of NGF, a 3-h exposure to Neurotropin at 20 mNU/mL stimulated association of Trk and GM1, without affecting Trk autophosphorylation (Panels A–C, lane 5). Thus, Neurotropin induced association of Trk and GM1, which may determine response acquisition of the cells to NGF stimulation. In addition, these observations strongly suggest that such receptor–lipid complex can be constructed independently of Trk autophosphorylation.

2.4. *Effect on Trk homodimerization*

Since dimer formation of Trk promotes efficient autophosphorylation by NGF (Jing et al., 1992), we next tested the effect of Neurotropin on the receptor homodimerization (Fig. 5). Cell surface molecules in PCtrk cells treated with NGF (50 ng/mL, 5-min stimulation) or Neurotropin (20 mNU/mL, 3-h exposure) were crosslinked with a membrane impermeable, bifunctional crosslinker BS3, followed by immunoprecipitation of the cell lysates with α-Trk antibody. Western blotting showed that Trk homodimer (approximately 300 kDa in size) was significantly formed by NGF (lane 2; $P=0.043$ vs. untreated control), but not by Neurotropin alone (lane 3; $P>0.05$). These data suggest that formation of the receptor dimer depends on the presence of NGF, but is not stimulated by Neurotropin.

3. Discussion

In this study, neuritogenic action of Neurotropin was found to accompany interaction of Trk and GM1 in PCtrk cells. In NGF signaling, GM1 potentiates Trk responsiveness at least in the steps of homodimerization (Farooqui et al., 1997) and autophosphorylation (Rabin and Mocchetti, 1995) presumably by a direct association with each other to form Trk–GM1 complex (Mutoh et al., 1995). However, little is known about the precise mechanisms of functional modulation of Trk by GM1. In our experiments, since 3-h exposure to Neurotropin stimulated endogenous GM1 association with Trk without affecting dimerization and autophosphorylation (Figs. 4 and 5), the association of endogenous GM1 is confirmed to be an antecedent event which is dissociable from the subsequent dimerization and phosphorylation processes. Oppositely, a brief Neurotropin treatment failed to induce apparent complex formation

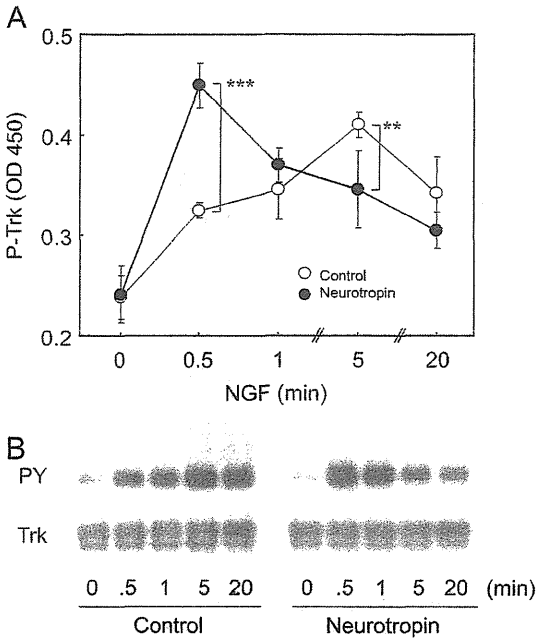

Fig. 3 – Facilitation of NGF signaling by Neurotropin. Serum-free culture of PCtrk cells (5×10^5 cells) was pretreated with 20 mNU/mL of Neurotropin for 3 h, and stimulated with 50 ng/mL of NGF for indicated time period (0, 0.5, 1, 5, or 20 min). The cells were immediately lysed at 4 °C, and the lysate was subjected to ELISA assay (A) and Western blot analysis (B) for determination of Trk autophosphorylation. (A) The cell lysates containing equal amount of proteins were directly assayed by ELISA assisted by α-Trk and α-PY as described in Section 4. Phosphorylated Trk levels (P-Trk) were expressed as OD at 450 nm of the reaction mixture. Data represents the mean and SD of 4 independent experiments. Open circles, saline-treated controls; closed circles, Neurotropin pretreatment (20 mNU/mL, 3 h). Statistically significant induction of Trk phosphorylation was observed after treatment both in control and neurotropin-treated cultures ($P<0.001$ vs. 0 min; Dunnett-type comparison). $^{***}P<0.001$, $^{**}P<0.01$ vs. saline-treated controls (ANOVA). (B) The lysates were immunoprecipitated by α-Trk, and subjected to SDS-5–20% PAGE, followed by Western blot analysis against total (Trk) or phosphorylated (PY) forms of Trk as described in Section 4. Each lane contained equal amount of proteins. Representative images are shown.

between Trk and GM1 (1 min, Fig. 4C, lane 3). A similar time-requiring process was also reported for exogenously supplied GM1 in the induction of Trk autophosphorylation with an interval of 1 h or 6 h (Rabin and Mocchetti, 1995). Neurotropin action may involve such time-consuming events as redistribution and assembly of Trk molecules into GM1-rich environment like lipid rafts, leading an efficient NGF signaling (Limpert et al., 2007). In agreement with this notion, PCtrk cells with prolonged Neurotropin treatment (3 h) showed a significantly accelerated time frame of Trk autophosphorylation response by NGF (Fig. 3). These observations suggest that Neurotropin enabled rapid cellular responses to NGF probably through the formation of Trk–GM1 complexes, although underlying precise mechanism by which Trk–GM1 complexes are formed remains to be elucidated.

In the present study, Neurotropin was demonstrated to induce neurite extension in the presence of suboptimal concentration of NGF (2 ng/mL; Fig. 1). Neuritogenic actions by Neurotropin had been reported for the first time by Morita et al. (1998), where Neurotropin enhanced neurite outgrowth of PC12h cells, a subclone of PC12 cells which also responds to NGF (Hatanaka, 1981), independently of cAMP-driven pathways. Recently, neuroprotective actions of Neurotropin had been demonstrated in PC12 cells and primary dorsal ganglion neurons manifested by neurite degeneration induced by anticancer agents such as paclitaxel (Kawashiri et al., 2009) and oxaliplatin (Kawashiri et al., 2011). In addition, a cytoprotective action of Neurotropin has been reported on oxidant-exposed lung A549 cells by inducing a redox-regulating molecule, thioredoxin-1 (Hoshino et al., 2007). Thioredoxin-1 is recognized as a neurotrophic co-factor having a regulatory role in NGF-mediated signal transduction in PC12 cells (Bai et al., 2003). Furthermore, our recent approaches employing human neuroblastoma SH-SY5Y cells revealed that Neurotropin activates the biosynthesis of brain-derived neurotrophic factor (BDNF) (Fukuda et al., 2010). The effect involved activation of PI3K, ERK and cAMP-responsive element binding protein. Since these intracellular signaling pathways in neurons are known to be initiated by Trk receptors (reviewed in Huang and Reichardt, 2003), these independent observations may share the molecular mechanism as described in this report. Consistently, effective concentrations of Neurotropin needed for all these phenomena observed in vitro were between 10 and 100 mNU/mL.

Association of abnormalities in Trk-mediated intracellular signaling has been implicated in numerous disorders such as Alzheimer's disease, stroke, amytrophic lateral sclerosis (ALS) and diabetic neuropathy (see review by Chao et al., 2006). Mutations in the tyrosine kinase domain of Trk have been reported in patients with congenital insensitivity to pain with anhidrosis (CIPA), an autosomal-recessive disorder characterized by recurrent episodes of unexplained fever, absence of sweating, absence of response to noxious stimuli, self-mutilating behavior and mental retardation (Indo et al., 1996). In addition, we found α-Trk autoantibodies in patients with subacute sensory neuropathy, which provoked a functional disturbance of the Trk-mediated signaling in PCtrk cells (Mutoh et al., 2005). Moreover, we recently documented that clioquinol, a causative agent of subacute myelo-optico neuropathy (SMON), interrupted NGF-induced Trk signaling and neurite outgrowth in PC12 cells (Asakura et al., 2009). These observations suggest the importance of Trk-mediated signaling in the maintenance of the autonomic, peripheral and central nervous systems. Based on clinical experiences over a half century in Japan, Neurotropin had been noted to possess therapeutic potential for various neurological disorders associated with neurotrophin signaling dysfunction, such as ischemic brain infarction (De Reuck et al., 1994), senile dementia (Kimura et al., 1987), SMON-associated dysesthesia (Sobue et al., 1992), and chemotherapy-induced neuropathy (Zhang et al., 2012). Translational studies using corresponding animal models are now conducted to evaluate the contribution of Trk-mediated NGF signaling in clinical effectiveness of Neurotropin.

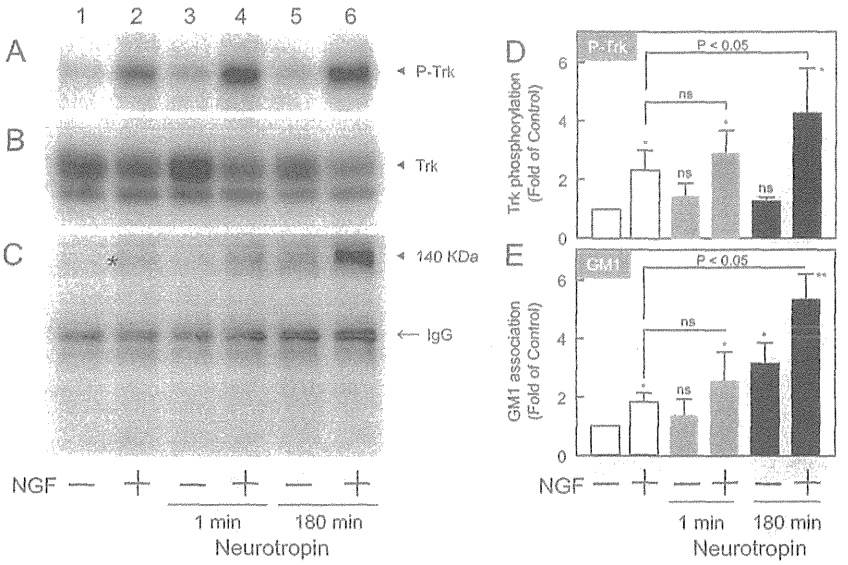

Fig. 4 – NGF-independent association of Trk and GM1 in PCtrk cells treated with Neurotropin. (A–C) Serum-free culture of PCtrk cells (5×10^5 cells) was pretreated with saline (*lanes 1 to 4*) or 20 mNU/mL of Neurotropin (*lanes 5 and 6*) for 3 h, and stimulated with 50 ng/mL of NGF for 1 min at 37 °C (*lanes 2, 4, and 6*). For *lanes 3 and 4*, Neurotropin (20 mNU/mL) was added simultaneously with NGF and treated only for 1 min. Western blot analyses of α-Trk immunoprecipitates against phosphorylated ((A) P-Trk), total ((B) Trk) forms of Trk, or GM1 ganglioside (C) were performed as described in Section 4. Anti-GM1 antibody primary recognized a broad band around 140 kDa (corresponding to the size of Trk), whereas secondary antibody recognized α-Trk IgGs used for immunoprecipitation (*arrow*, ca. 50 kDa). When focused on the 140-kDa band, Neurotropin alone stimulated an association of GM1 immunoreactivity in α-Trk immunoprecipitates even in the absence of NGF ((C), *lane 5*). The mean and SD of activated Trk ((D) PY in panel A) and Trk-associated GM1 ((E) a 140-kDa band in panel C) were summarized by three independent experiments. Data was represented as fold induction of saline-treated control (1.0). *P < 0.05; **P < 0.01 vs. saline-treated controls without NGF stimulation; ns, not significant (Student t-test).

Neurotrophins have potential for the treatment of neurological diseases. However, their therapeutic application has been largely limited because of their poor pharmacological properties, such as low stability in serum, restricted penetration across blood–brain barrier, minimal diffusion in central nervous system and, more importantly, the pleiotropic actions triggered by their ability to bind multiple receptors (Longo and Massa, 2013 for review). In order to overcome such disadvantages of native neurotrophins, substantial efforts have been made to discover small molecules mimicking NGF actions with a better pharmacokinetics and receptor selectivity (Lee and Chao, 2001; Jang et al., 2007; Yamada et al., 2008; Scarpi et al., 2012). Most of these compounds act as robust Trk agonists that induce Trk signals even in the absence of NGF. Therefore, there still remains a concern about unexpected adverse on-target effects associated with highly activated Trk signaling. For example, early clinical trials that investigated the therapeutic efficacy of exogenously administered NGF had revealed unaccepted incidents of pain (Dyck et al., 1997; Eriksdotter Jonhagen et al., 1998; McArthur et al., 2000). Ironically, such clinical observations have largely provided a biological basis for the recent understandings that Trk-mediated NGF signaling play a key role in the peripheral sensitization process establishing chronic pain (see reviews by Bennett, 2001; Sah et al., 2003). Antagonism of NGF can prevent many of sensory abnormalities that develop in a number of animal models of inflammatory pain, further confirming the role of NGF in pain progression (Woolf et al., 1994; McMahon et al., 1995; Koltzenburg et al., 1999). In long-term clinical experiences in Japan, Neurotropin has never been reported to accentuate pain or other sensory abnormalities. This might be related to our present observations that Neurotropin, unlike other Trk agonists, demonstrated neuroprotection only when cells were lacking adequate trophic support.

4. Experimental procedure

4.1. Chemicals

All reagents were purchased from Sigma (MO, USA) unless stated otherwise. Neurotropin was provided from Nippon Zoki Pharmaceutical Co., Ltd. (Osaka, Japan). The analgesic activity of Neurotropin (expressed in Neurotropin unit, NU) is standardized by a behavioral testing in rodents loaded with the "stress alteration of rhythm in environmental temperature" (SART), a repeated cold stress by which hypersensitivity to a noxious stimulus is produced (Kita et al., 1979). Neurotropin does not contain detectable known proteins such as neurotrophins (HPLC). Neurotropin was diluted with saline (Otsuka, Tokushima, Japan) as a vehicle.

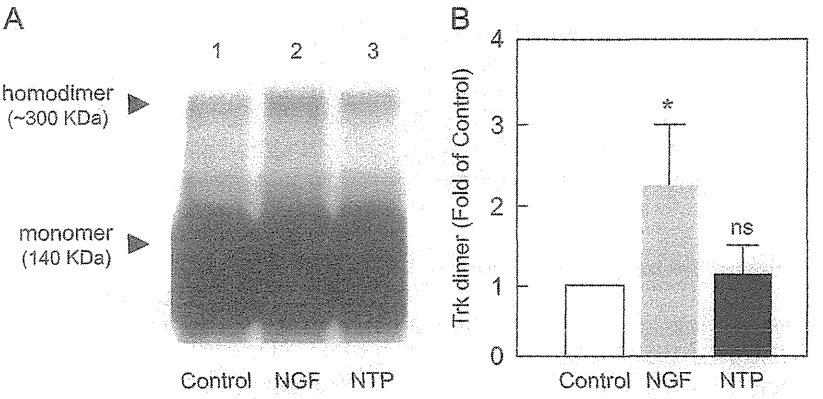

Fig. 5 – Effect of Neurotropin on dimerization of Trk in PCtrk cells. (A) PCtrk cells (5×10^5 cells) were incubated at 37 °C in serum-free DMEM with saline (Control; *lane 1*), NGF (50 ng/mL; *lane 2*), or Neurotropin (NTP; 20 mNU/mL; *lane 3*). Cell surface molecules were crosslinked by membrane-impermeable bifunctional crosslinker, BS3, at 4°C for 30 min. α-Trk immunoprecipitates of the cell lysates were subjected to SDS-7.5% PAGE for the detection of monomeric (approximately 140 kDa) and dimeric (approximately 300 kDa) forms of Trk by Western blot analysis. Each lane contained equal protein amount of lysates. Representative data was shown. (B) Intensity of Western blot for dimerized Trk derived from same protein amount of cell lysates was calculated by Image J as described in Section 4. Data represents the mean and SD of three independent experiments. *$P < 0.05$ vs. saline-treated controls; ns, not significant (Student t-test).

4.2. *Cell cultures*

PC12 cells overexpressing Trk (PCtrk cells) were constructed as described elsewhere (Mutoh et al., 2000). The cells were grown in DMEM (Invitrogen, CA, USA) supplemented with 2 mM L-glutamine, 5% horse serum and 5% fetal bovine serum (Biowhittaker, MD, USA) in polystyrene culture flasks or dishes (Becton Dickinson, NJ, USA) at 37 °C in an humidified chamber supplied with 5% CO_2. Expression of Trk in PCtrk cells is almost 10 fold greater than the parental PC12 cells cultured in normal DMEM. Viability of the cells was always more than 90% when assessed by staining dead cells with 0.4% Trypan Blue dye.

4.3. *Evaluations of cell differentiation*

Undifferentiated PCtrk cells (approx. 1000 cells per well) were allowed to adhere on 6-well plate surface, and stimulated with 2.5S NGF (Millipore, MA, USA) and/or Neurotropin for 18–24 h. For quantitative analysis, neurite length was measured under microscope by using Image J software (ver. 1.44; NIH, USA). Several typical fields containing at least 100 cells were randomly chosen to obtain total neurite length and the number of cell bodies. Total neurite length divided by the total numbers of cell bodies was defined as averaged neurite length (μm per cell). Three independent cultures were analyzed to calculate the mean and standard deviations (SD) of neurite length for each condition. In inhibition assay, K252a (500 nM; Biomol, PA, USA) was added simultaneously with NGF. For quantitative analysis, typical images of three independent cultures were captured under microscopy and analyzed as described above.

In addition to the morphological evaluation, expression of neurofilament, a major axonal constituent, was quantified. PCtrk cells (1×10^5) stimulated with or without NGF and/or Neurotropin were lysed and homogenized at 4 °C in SDS sample buffer (58.3 mM Tris–HCl, pH 6.8, 1.7% SDS, 5% glycerol, 3.3% 2-mercaptoethanol, 0.002% bromophenol blue). The lysate was boiled and stored at −80 °C for Western blot analysis as described below. Blot intensities by Western analysis against neurofilament M (NF-M) and β-actin were determined by Image J software to calculate relative NF-M expression per β-actin.

4.4. *Immunoprecipitation and immunoblotting*

PCtrk cells were stimulated with NGF following medium replacement by serum-free DMEM at least for 1 h. After NGF treatment, medium was removed and cells were immediately washed with ice-cold phosphate-buffered saline (PBS, pH 7.4), followed by solubilization in SDS sample buffer for whole-cell analysis, or in lysis buffer (20 mM HEPES, pH 7.2, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 10 μg/mL leupeptin). After centrifugation at 12,000 rpm for 2 min at 4 °C, the lysates were subjected to immunoprecipitation with an antibody against Trk (clone C-14; Santa Cruz Biotechnology, Santa Cruz, CA, USA, α-Trk) and protein A-Sepharose conjugate (Sigma, USA) at 4 °C overnight. After washing extensively, the precipitates were eluted from the Sepharose beads by boiling in SDS sample buffer for 5 min. The eluates were separated on SDS-5–20% PAGE (ePA-GEL; Atto Chemicals, Tokyo, Japan), and blotted onto PVDF membrane (Immobilon-P; Millipore, USA). The membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) with 3% nonfat milk. Incubations with the primary, as well as with the HRP-coupled secondary, antibodies were performed for 1 h at room temperature (RT) in TBS-T. Immunoreactive bands were visualized by an ECL detection system (ECL Plus; GE Healthcare, Buckinghamshire, UK). Antibodies used in this study were as follows: α-Trk (clone C-14), anti-phosphotyrosine monoclonal antibody (clone 4G10; Upstate, NY, USA; α-PY), anti-GM1 antisera (EMD Bioscience,

CA, USA; α-GM1), anti-neurofillament M antibody (NA1216; Affiniti Research, Devon, UK; α-NFM), and antibodies against ERK1/2, phospho-ERK1/2, Akt, and phospho-Akt (Cell Signaling Technologies, MA, USA) for primary antibodies; anti-rabbit IgG (AP132P; Chemicon International, CA, USA) and anti-mouse IgG (Amersham Bioscience, Buckinghamshire, UK) for secondary antibodies.

4.5. *Quantitative analysis for Trk autophosphorylation*

For quantitation of phosphorylated Trk, a double-antibody ELISA system was constructed. Briefly, a 96-well microplate (Immulon® 4 HBX; Thermo Electron, MA, USA) coated with 100 μL/well of α-Trk (1:1000 dilution) was blocked by 200 μL of TBS containing 3% nonfat milk. Lysates prepared from confluent cultures (1×10^5 cells) in 3-cm dishes were incubated in the wells for 2 h at RT. Each well contained an equal protein amount of lysate, determined by BCA protocol (Thermo Fisher Scientific, IL, USA). After washing with TBS-T, wells were incubated sequentially for 2 h with α-PY (1:1000 dilution, 100 μL) and anti-mouse IgG conjugated with HRP (1:2000 dilution, 100 μL). Bound enzyme activity was assessed by chromogenic substrate (TMB One; Promega, CA, USA) with optical density at 450 nm (OD 450) employing a microplate reader (Benchmark microplate reader; BioRad). In a separate experiment, OD 450 values in the assay were confirmed to be linearly related to the band intensities of Western blot analysis against phosphotyrosines in a-Trk immunoprecipitates.

4.6. *Trk homodimerization*

Trk dimerization was carried out as described by Fukumoto et al. (2000). Cells (5×10^6) were plated on 10-cm dishes and treated with NGF (50 ng/mL, 5 min) or Neurotropin (20 mNU/mL, 3 h) in serum-free DMEM. The medium was removed, and the cells were washed twice with ice-cold PBS, and cross-linked in a buffer (25 mM HEPES, pH 8.5, 120 mM NaCl, 6 mM KCl, 1 mM MgCl2, 10 mM EGTA) containing 1 mM bis (sulfosuccinimidyl) suberate (BS3) at 4 °C for 30 min. The reaction was terminated by adding 1 M Tris–HCl (pH 7.4) to a final concentration at 50 mM. Cells were then washed twice with TBS and lysed in a lysis buffer as described above. Cell lysates were subjected to SDS-7.5% PAGE, followed by Western blot analysis with α-Trk. Intensities of bands corresponding to the size of Trk monomer (140 kDa) and dimer (ca. 300 kDa) were quantified by Image J software. Each lane contained an equal amount of proteins.

4.7. *Statistics*

All data were analyzed after the completion of experiments by SAS system (version 8.2; SAS Institute, Japan). All significance tests used a level <0.05.

Conflict of interest

The affiliations of each author are noted in the citation appended to the list of authors. The authors have disclosed the following industry relationships: YF, TA, HI and KF are full-time employees of Nippon Zoki Pharmaceutical. Till 2008, APK had been a scientific advisor for Nippon Zoki Pharmaceutical. Other authors have no disclosure to report. All the experimental works included in this article was performed by YF with technical assistance by TF, CH, TI, and KM. TA, KF and HI did statistic analyses and quality assessment of the data. TM, AU, HI, KF and APK contributed to the article preparation.

Acknowledgments

We are grateful to Dr. Irwin J. Kopin (National Institutes of Health, USA) for helpful discussions. We also thank Ms. Chieko Nishikawa for her excellent technical assistance. This work was supported by the MUSC Foundation for Research Development (Charleston, SC, USA) to A.P.K., and by MEXT-Supported Program for the Strategic Research Foundation at Private Universities 2010–2014 and a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan to T.M.

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