# 神経修復時の神経突起、軸索ガイダンスに及ぼす
# インテグリンリンクドキナーゼの役割
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# はしがき

Integrin-linked kinase（以下ILK）は、近年β1-Integrinの細胞質内ドメインに結合し、インテグリン接着斑を構成するセリン/スレオニンキナーゼとしてヒト胎盤よりクローニングされた。ILKは、上皮系の細胞において、直接インテグリンと相互作用することで細胞内外の情報を二方向性に橋渡しするキーエンザイムと考えられており、インテグリンを介した細胞外マトリックスへの細胞接着、細胞増殖、あるいは癌細胞の転移に関与することで着目されている。 最近、研究代表者は、N1E-115細胞をはじめとする神経未分化細胞にもILKが発現していること、また、その発現量は神経細胞への分化、未分化にかかわらず恒常的で、かつ高発現していることを初めて明らかにした。神経未分化細胞の増殖と神経分化は、細胞外マトリックスの種類とそれらとの接着特性に大きく影響されることから、神経細胞におけるILKの機能はたいへん興味深い。

脳神経系は、複雑な神経回路網を形成しているが、その基本回路は発生時期において個々の神経細胞が種々の細胞外ガイダンス因子に誘導されて正しい標的細胞へ神経軸索が伸長することにより形成される。神経突起先端には成長円錐と呼ばれる神経終末前駆領域があり、そこには様々なガイダンス因子に対する受容体が存在し、軸索の運動性、方向性および標的の認識を行う探索子として機能する。成長円錐に存在するガイダンス因子に対する受容体が刺激され、成長円錐内の細胞内情報伝達系が作動することで、軸索の成長、退縮、停止の決定がなされるが、軸索成長に対して促進的

かあるいは抑制的かなどの反応性の決定は、成長円錐の側にあると考えられている。一方、成長円錐の運動とそれに伴う軸索ガイダンスにおいて、どのような細胞内シグナル分子が機能し細胞内情報伝達を行うかなどは全く理解されていないが、これら一連の反応には細胞外マトリックスとの相互作用や細胞間の接着に依存した細胞内情報伝達機構の関与が必要不可欠である。

本研究は、神経修復時に認められる神経未分化細胞の遊走、定着後の細胞増殖と神経分化において、インテグリン接着斑ならびに神経成長因子を介した細胞内情報伝達機構に着目し、インテグリン依存性の神経分化を生じるN1E-115細胞ならびに神経成長因子依存性の神経分化を生じるPC12細胞をモデル細胞として神経におけるILKの機能を調べた。すなわち、細胞内外へ情報を橋渡しするILKが、これら神経未分化細胞における神経突起伸展・退縮や細胞増殖において果たす役割を解明すると共に、神経分化時の微小管再構成や神経疾患における微小管形成異常へのILKの関与について調べた。

N1E-115細胞は、血清非存在下でラミニンをコートしたプレート上で培養する（分化条件）と神経突起を形成し神経細胞へと分化する。N1E-115細胞の神経分化は、細胞をβ１インテグリン抗体で前処置すると阻害されるので、β１インテグリンを介した細胞内情報伝達系が作用していると考えられる。近年、上皮系の細胞で、β１インテグリンの細胞内ドメインに結合し、インテグリン接着斑を形成しているILKは、インテグリンを介した細胞外マトリックスへの細胞接着の調節や、細胞増殖あるいは癌

細胞の転移に関与することで着目されているが、 N1E-115 細胞や PC12 細胞をはじめとする神経未分化細胞にも ILK が発現していること、また、その発現量は神経細胞への分化、未分化にかかわらず恒常的で、かつ高発現していることを本研究で初めて明らかにした。そこで、モルモット脳から ILK をクローニングし (GenBank accession number:AF256520)、モデル細胞に内在する ILK を阻害するために ILK の不活化変異体 (DN-ILK) を作成した。作製した DN-ILK は、哺乳類細胞への発現ベクターに構築後、N1E-115 細胞に導入し DN-ILK がステイブルで高発現した細胞株を樹立した。DN-ILK を高発現した細胞は、分化条件下においても神経突起を伸張せず未分化を維持した。一方、野生型 ILK を、トランジエントに高発現した細胞は、非分化条件下においても神経突起を形成し神経へと分化した。これらの結果は、N1E-115 細胞のインテグリン依存性の神経分化には、ILK の機能が必要不可欠であることを示している。更に、内在 ILK は、非分化条件下においても弱い活性を維持しているが、分化条件下で培養を開始すると一過性に高度に活性化されること、ILK の活性化とほぼ同様な時間経過で p38 MAP kinase が一過性に活性化されること、ILK と p38 MAP kinase の活性化は、PI3-kinase の阻害薬である LY294002 の処置や DN-ILK の強制発現により阻害されたこと、また LY294002 や p38 MAP kinase の阻害薬である SB203580 は神経突起形成を阻害する等の結果から、N1E-115 細胞の神経分化機構において ILK の活性化にはβ1インテグリンと PI3-kinase の両者の活性化が必要であり、ILK シグナルの下流に位置し ILK の活性化を介して生じる p38 MAP kinase の活性化が必要不可欠であることを明ら

かにした。

神経細胞における微小管は、神経分化時の軸索や神経突起の形成、細胞骨格として細胞形態の構築、細胞内輸送などに重要に機能しており、微小管ならびにその関連タンパク質の異常は、種々の異なる神経変性疾患において認められている。そこで、DN-ILK をステイブルに高発現させることにより、N1E-115 細胞の内在 ILK を阻害した場合の微小管ならびにその関連タンパク質であるタウタンパク質の変化について調べた。タウタンパク質は、中枢ならびに末梢神経系神経細胞の軸策や樹状突起に多く発現しており、その機能は主にチュブリンと結合することで微小管形成を促進、あるいは安定化することにある。本研究において、DN-ILK 発現細胞の神経突起形成不全を調べる過程でタウタンパク質が高度に異常リン酸化されていることを見いだした。タウタンパク質の異常リン酸化は、アルツハイマー病の病理学所見で認められている神経原線維変化の発生機構の一つとして考えられており大変興味深い。中でも、本研究で同定した $Ser^{199}$ ならびに $Ser^{202}$ のリン酸化部位は神経原線維変化の異常タウタンパク質で確認されている異常リン酸化部位と一致している。タウタンパク質の異常リン酸化は、コントロール細胞ではほとんど認められないが、DN-ILK を発現させると高度に認められることから、内在 ILK はタウタンパク質を異常リン酸化されないように保護する機能を有することが示唆された。また、共焦点レーザー顕微鏡を用いた解析では、異常リン酸されたタウタンパク質が繊維化し、それが細胞形質膜内面を覆う形で籠状のに分布していることが明らかになった。次に、DN-ILK の発現により内在 ILK

の活性を阻害した場合のタウリン酸化酵素の活性について調べた。最近、神経原線維変化病変におけるタウタンパク質の $Ser^{199}$ ならびに $Ser^{202}$ のリン酸化に関与する酵素の候補として、glycogen synthase kinase-3β（GSK-3β）が注目されている。そこで、GSK-3βの活性状態について調べた。GSK-3βの活性は、$Ser^{9}$ と $Tyr^{216}$ のリン酸化により調節されている。すなわち、$Ser^{9}$ のリン酸化は、GSK-3βの活性を不活化し、$Tyr^{216}$ のリン酸化は逆に GSK-3βを活性化する。そこで、DN-ILK 発現細胞ならびにコントロール細胞の GSK-3β リン酸化状態について分化ならびに非分化条件下で調べた。その結果、分化、非分化条件にかかわらず、DN-ILK 発現細胞は、コントロール細胞に比べて GSK-3βの $Tyr^{216}$ がリン酸化されている割合の高いことが明らかになった。一方、$Ser^{9}$ のリン酸化は分化、非分化条件にかかわらずコントロール細胞においてのみ認められた。また、実際、DN-ILK 発現細胞の GSK-3βの酵素活性を測定した結果、コントロール細胞に比べて約３倍程度の高い活性を示した。なを、GSK-3βタンパク質発現量は、分化、非分化条件にかかわらず、DN-ILK 発現細胞とコントロール細胞間で違いが認められなかった。ILK は直接あるいは protein kinase B (PKB/AKT)を介して間接的に GSK-3βの $Ser^{9}$ をリン酸化することが知られているので、神経未分化細胞においても内在 ILK は、GSK-3βの $Ser^{9}$ をリン酸化することでそれを不活化し、タウタンパク質が異常リン酸化されないように保護していると考えられる。事実、ILK は、非分化条件下のレスティング状態においても、弱いながら活性を維持していた。一方、DN-ILK を導入発現させたことによる内在 ILK の不活化は、$Tyr^{216}$ にリン酸化を受けた

活性型 GSK-3β の割合が増加し、GSK-3β を高い活性化状態に導く結果となった。現在、$Tyr^{216}$ のリン酸化に寄与する tyrosine kinase 種は不明であり、今後、この tyrosine kinase の同定と ILK の不活性化がどのように tyrosine kinase を活性型に導くのか調べる必要がある。次に、GSK-3β がタウタンパク質の $Ser^{199}$ ならびに $Ser^{202}$ をリン酸化している直接的な証拠を得るために、GSK-3β の特異的阻害薬である LiCl 処置がタウタンパク質のリン酸化に及ぼす効果について調べた。LiCl 処置は、コントロール細胞におけるタウタンパク質のリン酸化には何ら影響を与えなかったが、DN-ILK 発現細胞における $Ser^{199}$ ならびに $Ser^{202}$ のリン酸を含めたタウタンパク質の高度異常リン酸化を用量依存性に阻害した。これらの結果は GSK-3β がタウタンパク質の高度異常リン酸化に直接関与していることを示唆している。さらに、タウタンパク質の高度異常リン酸化が神経突起形成に及ぼす影響について調べた。コントロール細胞の LiCl 処置は、インテグリン依存性の神経突起形成に影響を与えないが、DN-ILK 細胞における神経突起形成の阻害を部分的に回復したことから、内在 ILK を阻害したことに起因する神経突起形成の阻害に、一部ではあるが GSK-3β を介したタウタンパク質の $Ser^{199}$ ならびに $Ser^{202}$ の異常リン酸化が関与していることが明らかになった。

次に、神経成長因子依存性の神経突起形成における ILK の機能を調べるために、神経成長因子(NGF)依存性の神経突起形成を生じる PC12 細胞を用いて解析した。PC12 細胞内在の ILK は、NGF 処置後、PI3-kinase 活性依存的に一過性に活性化された。さらに、PC12 細胞の NGF 依存性の神経突起形成は、DN-ILK の導入発現により阻害され

たことから、神経成長因子依存性の神経突起形成においてもILKが機能していることが示唆された。現在、ILKのシグナル伝達機構に関する詳細な解析を行っている。

以上の結果から、ILKは神経系の細胞においてインテグリン依存性の神経分化機構のみならず、成長因子依存性の分化機構においても関与していることが明らかとなった。N1E-115細胞を用いたインテグリン依存性の神経突起形成は、ILKシグナルの下流に位置し、ILKの高度活性化に伴い活性化されるp38 MAP kinaseが必要であるが、ILKは非分化条件下のレスティングレベルにおいても低レベルの活性を維持し、タウのリン酸化を制御することで微小管の重合と解離の定常状態を調節していると理解できる。アルツハイマー病などの神経変性疾患で認められる神経原線維変化は、タウタンパク質の高度異常リン酸化が原因する可能性が示唆されているが、本研究で得られた結果は、ILKが神経原線維変化における病態形成に関与している可能性を強く示唆している。本研究で得られた成果が、神経原線維変化の予防・治療薬の開発に寄与できることを期待する。

研究組織

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研究発表

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Full Paper

Categories: Intracellular signaling, Biochemical pharmacology

# Calcineurin contributes to the enhancing effect of adenosine on NGF-induced neurite outgrowth via the decreased duration of p38 MAP kinase phosphorylation.

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Running title: Calcineurin regulates p38 MAP kinase

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Adenosine enhances NGF-induced neurite outgrowth in PC12 cells. We found that adenosine increases NGF-induced phosphorylation of extracellular signal-regulated kinase (ERK) but decreases the duration of phosphorylation of p38 MAP kinase. Therefore, we further examined the involvement of protein phosphatase in these effects of adenosine. FK506, a specific calcineurin inhibitor, inhibited the enhancing effect of adenosine on the NGF-induced neurite outgrowth and increased the duration of p38 MAP kinase phosphorylation without affecting ERK phosphorylation. These results suggest that adenosine decreases the duration of p38 MAP kinase via calcineurin activation, which contributes to the enhancement of NGF-induced neurite outgrowth.

Key words: PC12 cell; NGF; p38 MAP kinase; neurite outgrowth; calcineurin

The abbreviations used are: NGF, nerve growth factor; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase.

## Introduction

Nerve growth factor-induced neuronal differentiation of the phaeochromocytoma cell (PC)-12 cell line is well-studied [1,2]. NGF-induced activation of mitogen-activated protein (MAP) kinase pathways is critical for neuronal differentiation of PC12 cells [3,4]. Previously, Huffaker et al. [5] demonstrated that low concentrations of adenosine (10-50 μM) enhance NGF-induced neurite outgrowth of PC12 cells. Moreover, adenosine enhances NGF-induced activation of extracellular signal-regulated kinase (ERK) via adenosine $A_{2A}$ receptor-mediated increases in cyclic adenosine monophosphate (cAMP) [6]. These studies indicate a synergistic coupling between adenosine and NGF signaling pathways to potentiate neurite outgrowth in PC12 cells. The present study investigated the effect of adenosine on NGF-induced activation of p38 MAP kinase, because the p38 MAP kinase pathway is also critically involved in NGF-induced neuronal differentiation in PC12 cells [4]. We demonstrated that adenosine decreases the duration of NGF-induced activation of p38 MAP kinase via calcineurin activation, which contributes to the enhancement of NGF-induced neurite outgrowth.

## Materiala and Method

### Materials

PC12 cells were obtained from the Riken Cell Bank. Nerve growth factor (NGF2.5S) and Dulbecco's Modified Eagle Medium (DMEM) was obtained from GIBCO BRL (Rockville, MD). Calyculin A and okadaic acid were obtained from Wako Pure Chemical Co. (Osaka Japan). Adenosine, dibutyryl adenosine-3',5'-cyclic-monophosphate (dbcAMP), 8-(3-chlorostyryl)caffeine (CSC), SQ22536, Rp-cAMPS, cyclosporin A (CsA) and SB203580 were obtained from Sigma Chemical Co. (ST. Louis, MO). FK506 was obtained from Calbiochem (San Diego, CA). Anti-phospho-ERK1/ERK2 antibody and horseradish peroxidase conjugated donkey anti-rabbit IgG were obtained from Promega (Madison, WI). Anti-phospho-p38MAP kinase was obtained from New England Biolabs (Beverly, MA). All other chemicals were of analytical grade and were obtained from Sigma Chemical Co. or Wako

Pure Chemical Co.

Cell culture

PC12 cells were maintained in DMEM containing 5% fetal bovine serum (FBS; Hyclone, Logan, UT), 5% horse serum (ICN, Costa Mesa, CA), penicillin G sodium ($1x10^5$ U/L) and streptomycin sulfate ($1x10^5$ μg/L) in 5% $CO_2$ at 37°C. For the neuronal differentiation experiments, PC12 cells were seeded on 35-mm culture dishes ($2x10^4$ cells). NGF (50 ng/ml), adenosine, and dbcAMP were added 24h after seeding, and calyculin A, okadaic acid, and FK506 were added 30 min before addition of NGF. The cells were cultured in the presence of varying concentrations of the inhibitors. Calyculin A and okadaic acid were removed by washing once with phosphate-buffered saline (PBS) 3 h after adding the inhibitors and then by changing the culture medium containing NGF. FK506 was maintained in the medium until the cells were examined. The number of cells possessing neurites greater than the diameter of a cell body was assessed 48 h after adding NGF.

Western blot analysis

The cells were solubilized in 100 μl sample buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue, and 62.5 mM Tris-HCl (pH6.8) after washing once with PBS. The solubilized materials were subjected to SDS-polyacrylamide gel electrophoresis (5-20% gradient polyacrylamide) after boiling for 3 min and transferred onto nitrocellulose membranes at 4℃ in 25 mM Tris-HCl (pH 8.4), 192 mM glycine, 20% methanol, and 0.025% SDS. After blocking, the blots were probed with anti-phospho-ERK or anti-phospho-p38 MAP kinase antibody, followed by goat anti-rabbit IgG conjugated to horseradish peroxidase. The immunoreactive proteins were visualized following the reaction with 3,3′-diaminobenzidine tetrahydrochloride.

**Results**

We first examined whether adenosine enhances NGF-induced neurite

outgrowth in PC12 cells. Adenosine alone did not have an effect on the cells. On the other hand, treatment of the cells with adenosine in the presence of NGF enhanced NGF-induced neurite outgrowth in a dose-dependent manner (Fig.1A). This enhancing effect of adenosine was completely blocked by 8-(3-chlorostyryl) caffeine (CSC), a specific adenosine $A_{2A}$ receptor antagonist (Fig.1C). We also examined the effect of SQ22536, a specific adenylate cyclase inhibitor, and Rp-cAMPS, a specific protein kinase A inhibitor, on the adenosine effect, because activation of adenosine $A_{2A}$ receptors leads to increases in cAMP via activation of adenylate cyclase in PC12 cells [7]. Both SQ22536 and Rp-cAMPS significantly inhibited the adenosine effect (Fig.1C). These results suggest that adenosine enhances the NGF-induced neurite outgrowth via activation of the cAMP-PKA pathway after specific binding to the adenosine $A_{2A}$ receptor. To obtain more direct evidence that activation of the cAMP-PKA pathway is involved in the adenosine effect, the cells were treated with varying concentrations of dbcAMP, a membrane permeable cAMP analogue, in the presence or absence of NGF. As shown in Fig.1B, treatment of the cells with dbcAMP enhanced NGF-induced neurite outgrowth in a dose-dependent manner. Only a high concentration of dbcAMP (100 μM) slightly but significantly promoted neurite outgrowth even in the absence of NGF. These results also suggest that activation of the cAMP-PKA pathway is critical for the enhancing effect of adenosine on NGF-induced neurite outgrowth.

Activation of the ERK and p38 MAP kinase pathways is required for NGF-induced neuronal differentiation in PC12 cells [3,4]. Therefore, we examined the effect of adenosine on the NGF-induced activation of ERK and p38 MAP kinase. Activation of ERK and p38 MAP kinase was assessed by measuring changes in immunoreactivity to phosphorylation-dependent antibodies. ERK and p38 MAP kinase were transiently phosphorylated soon after NGF-treatment (Fig.2Ab &Bb). The duration of p38 MAP kinase activation was longer than that of ERK activation. Thus, NGF rapidly activated both kinases. These findings are similar to those of Morooka and Nishida [4], Zhang and Jope [8], and Kao et al. [9]. On the other hand, adenosine

decreased the duration of the NGF-induced phosphorylation of p38 MAP kinase and slightly stimulated NGF-induced phosphorylation of ERK (Fig. 2A&B). The result suggests that adenosine might affect phosphorylation kinetics of p38 MAP kinase via activation of protein phosphatases.

Involvement of protein phosphatases in the regulation of many protein kinases has been suggested in many studies [10]. We, therefore, examined the involvement of protein phosphatase (PP) on the effects of adenosine. Calyculin A, an inhibitor of both type1 (PP-1) and type2A (PP-2A) protein phosphatase, inhibited NGF-induced neurite outgrowth without any effect on the phosphorylation levels of MAP kinases (Fig.3). The same results were obtained by the treatment with another phosphatase inhibitor, okadaic acid, the effect of which is less potent against PP-1 than PP-2A (Fig.3). These findings are similar to those of Chiou and Westhead [11] and Reber and Bouron [12], and suggest that PP-1 and/or PP-2A is involved in NGF-induced neurite outgrowth downstream of the signaling pathway after MAP kinase activation. On the other hand, FK506, a specific inhibitor of calcineurin (type2B protein phosphatase, PP-2B) [13], inhibited the enhancing effect of adenosine on NGF-induced neurite outgrowth but had no effect on the NGF-induced effect (Fig. 4Aa). The same results were obtained by the treatment with another calcineurin inhibitor, cyclosporin A (CsA) (Fig.4Ab). When the cells were treated with FK506 in the presence of db-cAMP, FK506 inhibited the enhancing effect of db-cAMP on NGF-induced neurite outgrowth in a dose-dependent manner (Fig. 4B). Thus, FK506 blocked the enhancing effects of both adenosine and db-cAMP on the NGF-induced neurite outgrowth. Under the same conditions, FK506, but not calyculin A and okadaic acid, recovered the duration of p38 MAP kinase phosphorylation that was decreased by adenosine to that of NGF-treatment alone (Fig. 5B). Neither the levels nor the duration of the ERK phosphorylation was affected by FK506 (Fig. 5A).

To examine whether the decreased duration of p38 MAP kinase activation directly affects NGF-induced neurite outgrowth, we treated the cells with SB203580, a specific p38 MAP kinase inhibitor,

for 3 h between 90 and 270 min following the addition of NGF. This short treatment period with SB203580 significantly enhanced NGF-induced neurite outgrowth (Fig. 6A). In contrast, pretreatment of the cells with SB203580 before the addition of NGF significantly inhibited NGF-induced neurite outgrowth without affecting ERK phosphorylation (Fig.6B). Thus, p38 MAP kinase activation is necessary for NGF-induced neurite outgrowth, and the duration time of p38 MAP kinase activation importantly affects NGF-induced neurite outgrowth. These results suggest that adenosine activates calcineurin via activation of the cAMP-PKA pathway leading to a short duration of p38 MAP kinase activation, which results in enhanced NGF-induced neurite outgrowth.

## Discussion

The present study demonstrated that the duration of p38 MAP kinase activation importantly influences NGF-induced neuronal differentiation of PC12 cells. Also, adenosine activates calcineurin (PP-2B) to decrease that duration-time, which results in the enhancement of the NGF-induced neurite outgrowth.

Treatment of PC12 cells with NGF induces activation of both ERK and p38 MAP kinase and leads to growth arrest and neuronal differentiation [3,4]. Although epidermal growth factor (EGF) induces activation of both ERK and p38 MAP kinase, EGF stimulates the proliferation of cells instead of neuronal differentiation [2, 9]. These studies also indicate that there are marked differences in the kinetics of ERK and p38 MAP kinase activation between EGF and NGF treatments: EGF-induced activation of those MAP kinases is more transient than NGF-induced activation [4,9]. Therefore, such different cellular responses between NGF and EGF might derive from the different duration of ERK and p38 MAP kinase activation induced by those growth factors. Indeed, Traverse et al. [14] demonstrated that overexpression of EGF receptors in PC12 cells induces a sustained activation of MAP kinases and leads to neuronal differentiation in response to EGF. Furthermore, Morooka and Nishida [4] demonstrated that sustained activation of p38 MAP kinase in combination with EGF treatment induces neurite outgrowth.

In the present study, a short treatment with SB203580 decreased the duration of p38 MAP kinase activation and significantly enhanced NGF-induced neurite outgrowth (Fig. 6A). These results suggest that not only the extent but also the duration of both ERK and p38 MAP kinase activation is important for determining the cellular response.

Transient activation of p38 MAP kinase is critical for both growth factor-mediated and integrin-mediated neurite outgrowth [4,15,16]. p38 MAP kinase, however, is also implicated in the mediation of apoptosis in PC12 cells besides NGF-induced neuronal differentiation [17-19]. Indeed, NGF withdrawal from PC12 cells leads to sustained activation of p38 MAP kinase and then results in apoptosis of the cells [17,18]. Thus, p38 MAP kinase is activated not only after NGF addition to but also after NGF withdrawal from the cells and regulates the two different signal pathways in PC12 cells. On the other hand, ERK is activated after NGF treatment, whereas NGF withdrawal results in a reduction in ERK activity in PC12 cells [17]. Direct and selective activation of ERK pathway not only leads to stimulation of neurite outgrowth but also prevents apoptosis and promotes the survival of PC12 cells [17]. These reports suggest that simultaneous activation of p38 MAP kinase together with activation of ERK leads to neuronal differentiation but that sole sustained activation of p38 MAP kinase might result in induction of apoptosis. Indeed, transient activation of p38 MAP kinase is required for NGF-induced neuronal differentiation in PC12 cells, but its activation occurs simultaneously with ERK activation ([4] and also Fig.2). In the present study, we found that the duration of p38 MAP kinase activation is longer than that of ERK activation (Fig.2). When the duration of p38 MAP kinase activation was brought to shorter period by treatment with adenosine (Fig.2) and/or by a short treatment with SB203580 (Fig.6A), NGF-induced neurite outgrowth was enhanced. Thus, NGF-induced neurite outgrowth can be stimulated more efficiently when the duration of p38 MAP kinase activation is shorter than that of ERK activation. On the other hand, a longer duration of p38 MAP kinase activation than that of ERK activation tends to lead to suppression of

NGF-induced neurite outgrowth, which might be partly due to apoptosis signaling pathways activated after p38 MAP kinase activation, because NGF-induced increase in ERK activity, which prevents apoptosis and promotes the survival, will have been already reduced to the basal level at a later stage of p38 MAP kinase activation.

Both FK506 and CsA prevented only the enhancing effect of adenosine on the NGF-induced neurite outgrowth but not the effect of NGF alone (Fig. 4A). Under the same conditions, FK506 recovered the duration of p38 MAP kinase phosphorylation that had been reduced by adenosine-treatment to that of NGF-treatment (Fig.5B). These results suggest that adenosine activates calcineurin, which can dephosphorylate the active form of p38 MAP kinase and shift the equilibrium state between phosphorylation and dephosphorylation to favor that of dephosphorylation, resulting in a decreased duration of p38 MAP kinase phosphorylation.

The present study suggests that the decreased duration of p38 MAP kinase via adenosine-induced activation of calcineurin contributes to the enhancing effect of adenosine on NGF-induced neurite outgrowth. The mechanism by which adenosine activates calcineurin, however, remains unknown. Calcineurin is a $Ca^{2+}$- and calmodulin- dependent protein phosphatase [20]. Treatment of PC12 cells with NGF increases in inositol phosphates [21], which results in a transient increase in intracellular $Ca^{2+}$ levels. Therefore, the NGF-induced transient increase in intracellular $Ca^{2+}$ levels might be required for calcineurin activation. The present study, however, demonstrated that the enhancing effect of adenosine on NGF-induced neurite outgrowth, but not the effect of NGF alone, is blocked by Rp-cAMPS, FK506 and CsA, suggesting that adenosine-induced PKA activation is involved in calcineurin activation. These results suggest that both the NGF-induced increase in intracellular $Ca^{2+}$ and adenosine-induced PKA activation might be required for calcineurin activation to regulate p38 MAP kinase phosphorylation in PC12 cells.

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## Figure legends

Figure 1. Effects of adenosine (A) and dbcAMP (B) on NGF-induced neurite outgrowth in PC12 cells. Cells were treated with varying concentrations of adenosine (A) or dbcAMP (B) in the presence or absence of 50 ng/ml NGF. (C) shows the effects of 1 μM CSC, 100 μM SQ22536, and 30 μM Rp-cAMPS on the enhancing effect of 10 μM adenosine. The number of cells possessing neurites greater than the diameter of a cell body was assessed 2 d after the addition of the agents. Numbers of neurite-bearing cells are shown as a percentage of the 400 cells counted for each plate. A total of four independent plates were examined. Values are the means ± SD of four separate experiments.

Figure 2. Effect of adenosine on NGF-induced phosphorylation of ERK (A) and p38 MAP kinase (B) in PC12 cells. Cells were treated with none (a), 50 ng/ml NGF (b), or 50 ng/ml NGF plus 1 μM adenosine (c) for the indicated times. The phosphorylation of ERK and p38 MAP kinase was determined by immunoblotting with anti-phospho ERK antibody and anti-phospho p38 MAP kinase antibody, respectively. The visualized bands on the membranes were analyzed by Image Scanner (Epson, GT-9700F), and the band densities were quantified with image analysis software (NIH, Image). Values are the means ± SD of four separate experiments.

Figure 3. Effects of calyculin A (A) and okadaic acid (B) on NGF-induced neurite outgrowth in PC12 cells. Cells were treated with varying concentrations of calyculin A (A) or okadaic acid (B) in the presence of 50 ng/ml NGF. The number of cells possessing neurites greater than the diameter of a cell body was assessed 2 d after adding the reagents. Numbers of neurite-bearing cells are shown as a percentage of the 400 cells counted for each plate. A total of four independent plates were examined. Values are the means ± SD of four separate experiments. The phosphorylation

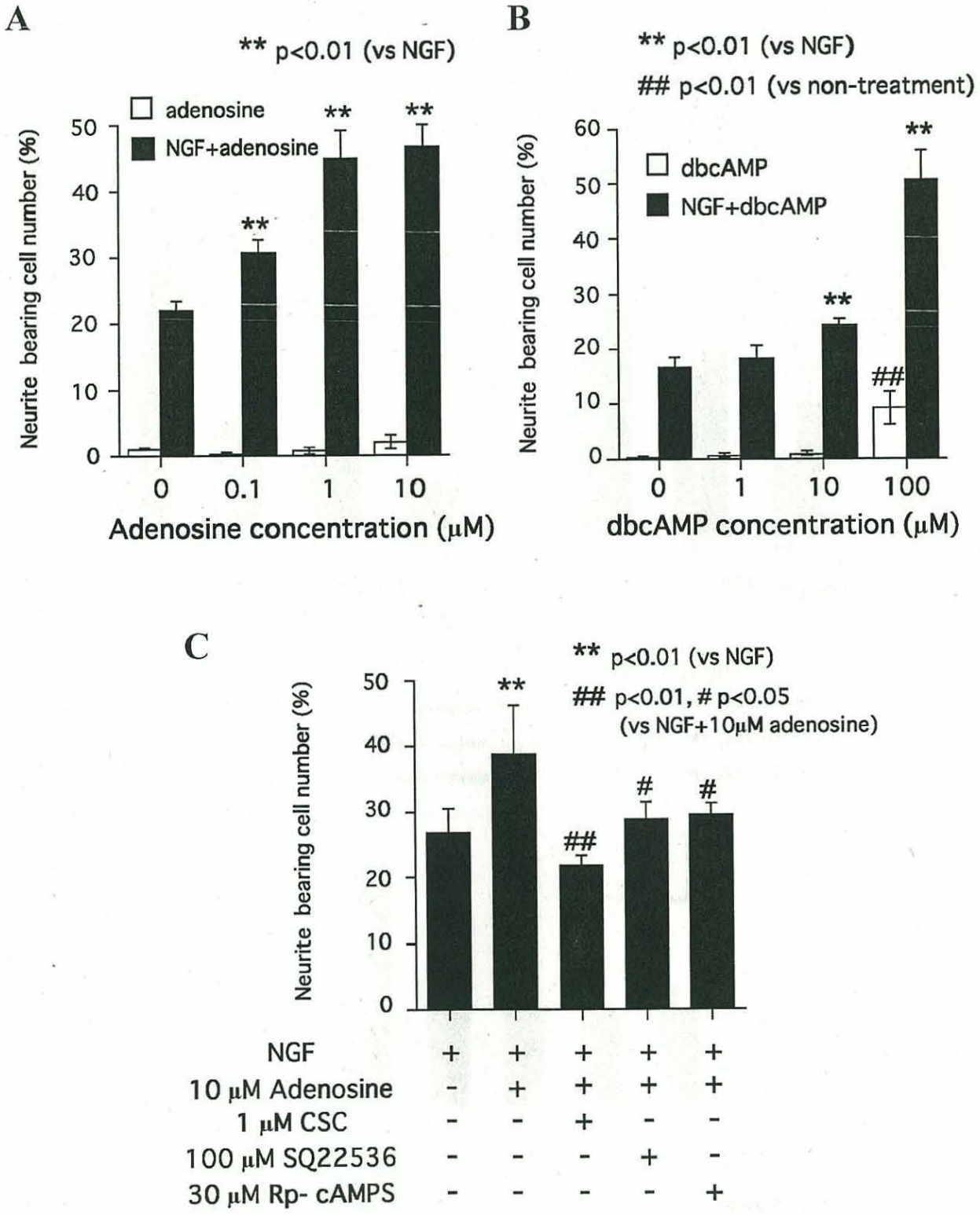
levels of ERK and p38 MAP kinase (C) after treatments of cells with 50 ng/ml NGF (a), 50 ng/ml NGF plus 500 pM calyculin A (b), or 50 ng/ml NGF plus 100 nM okadaic acid (c) for the indicated times. For treatments with calyculin A or okadaic acid, the phosphatase inhibitors were added to the cells 30 min before addition of NGF. The phosphorylation of ERK and p38 MAP kinase was determined by immunoblotting with anti-phospho ERK antibody and anti-phospho p38 MAP kinase antibody, respectively.

Figure 4. Inhibition of calcineurin prevented the enhancing effects of adenosine (A) and dbcAMP (B) on the NGF-induced neurite outgrowth in PC12 cells. Cells were treated with varying concentrations of FK506 (a) or CsA (b) in the presence of 50 ng/ml NGF and 1 μM adenosine (A). Cells were treated with varying concentrations of FK506 in the presence of 50 ng/ml NGF and 10 μM dbcAMP (B). For FK506 and CsA treatment, these inhibitors were added to the cells 30 min before addition of NGF plus adenosine or NGF plus dbcAMP. The number of cells possessing neurites greater than the diameter of a cell body was assessed 2 d after the addition of the agents. Numbers of neurite-bearing cells are shown as a percentage of the 400 cells counted for each plate. A total of four independent plates were examined. Values are the means ± SD of four separate experiments.

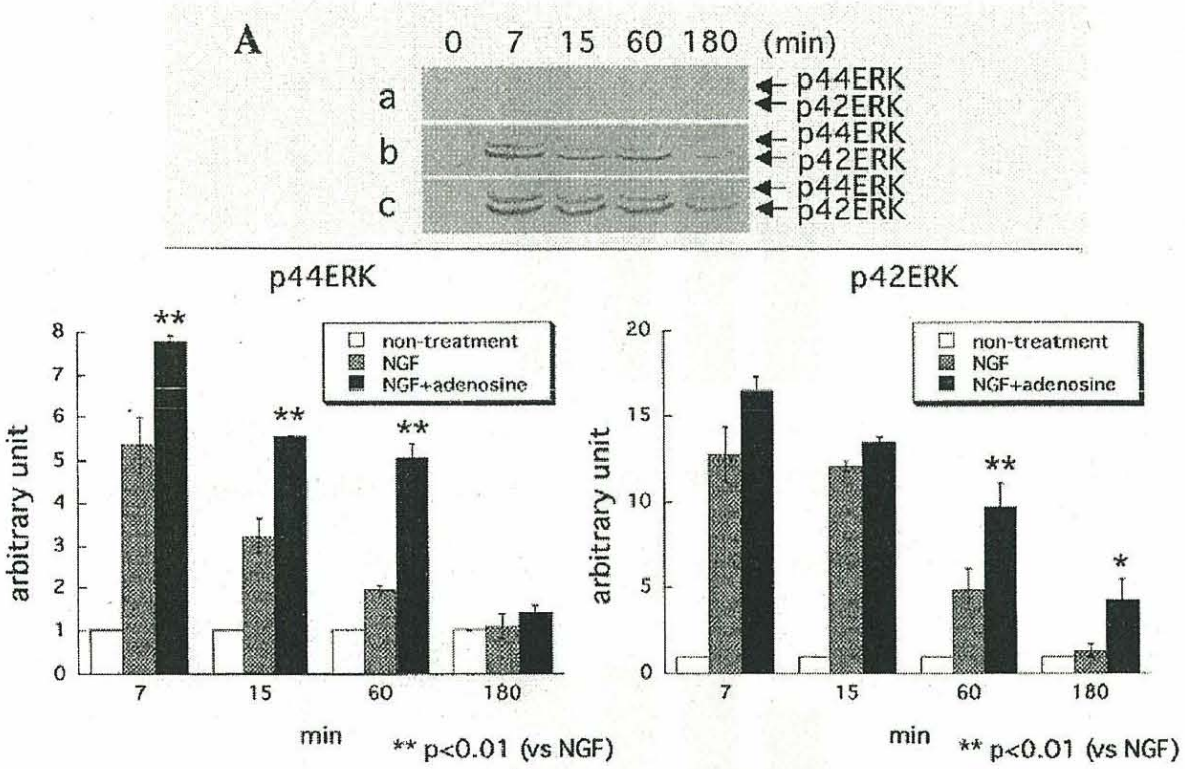
Figure 5. Inhibition of calcineurin recovered the decreased duration of p38 MAP kinase phosphorylation induced by adenosine. Cells were treated with 50 ng/ml NGF (a), 50 ng/ml NGF plus 1 μM adenosine (b), or 50 ng/ml NGF plus 1 μM adenosine in the presence of 1 μM FK506 (c) for the indicated times. The phosphorylation of ERK (A) and p38 MAP kinase (B) was determined by immunoblotting with anti-phospho ERK antibody and anti-phospho p38 MAP kinase antibody, respectively. The visualized bands on the membranes were analyzed by Image Scanner (Epson, GT-9700F), and the band densities were quantified with image analysis software (NIH, Image). Values

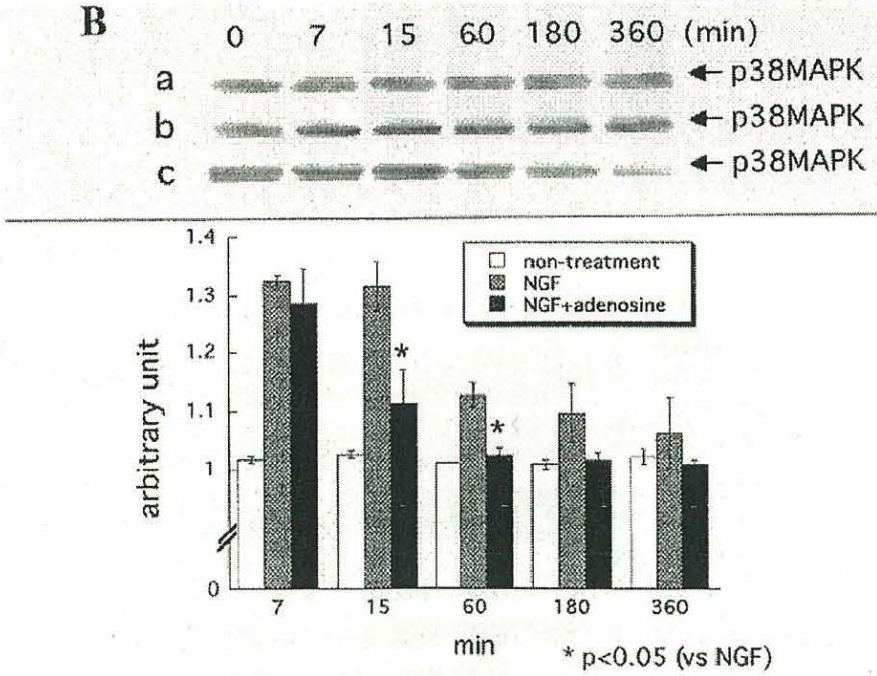
are the means ± SD of three separate experiments.

Figure 6. Short-duration treatment with SB203580 significantly enhanced NGF-induced neurite outgrowth. A: Cells were treated with SB203580 for 3 h between 90 and 270 min following the addition of NGF. B: Pretreatment of cells with SB203580 before the addition of NGF partly blocked NGF-induced neurite outgrowth. *Inset* shows the phosphorylation levels of p44/p42 ERK in the presence and the absence of SB203580. SB203580 did not affect ERK phosphorylation. The number of cells possessing neurites greater than the diameter of a cell body was assessed 2 d after the addition of the agents. Numbers of neurite-bearing cells are shown as a percentage of the 400 cells counted for each plate. A total of four independent plates were examined. Values are the means ± SD of four separate experiments.




Muroi et al. Figure.1

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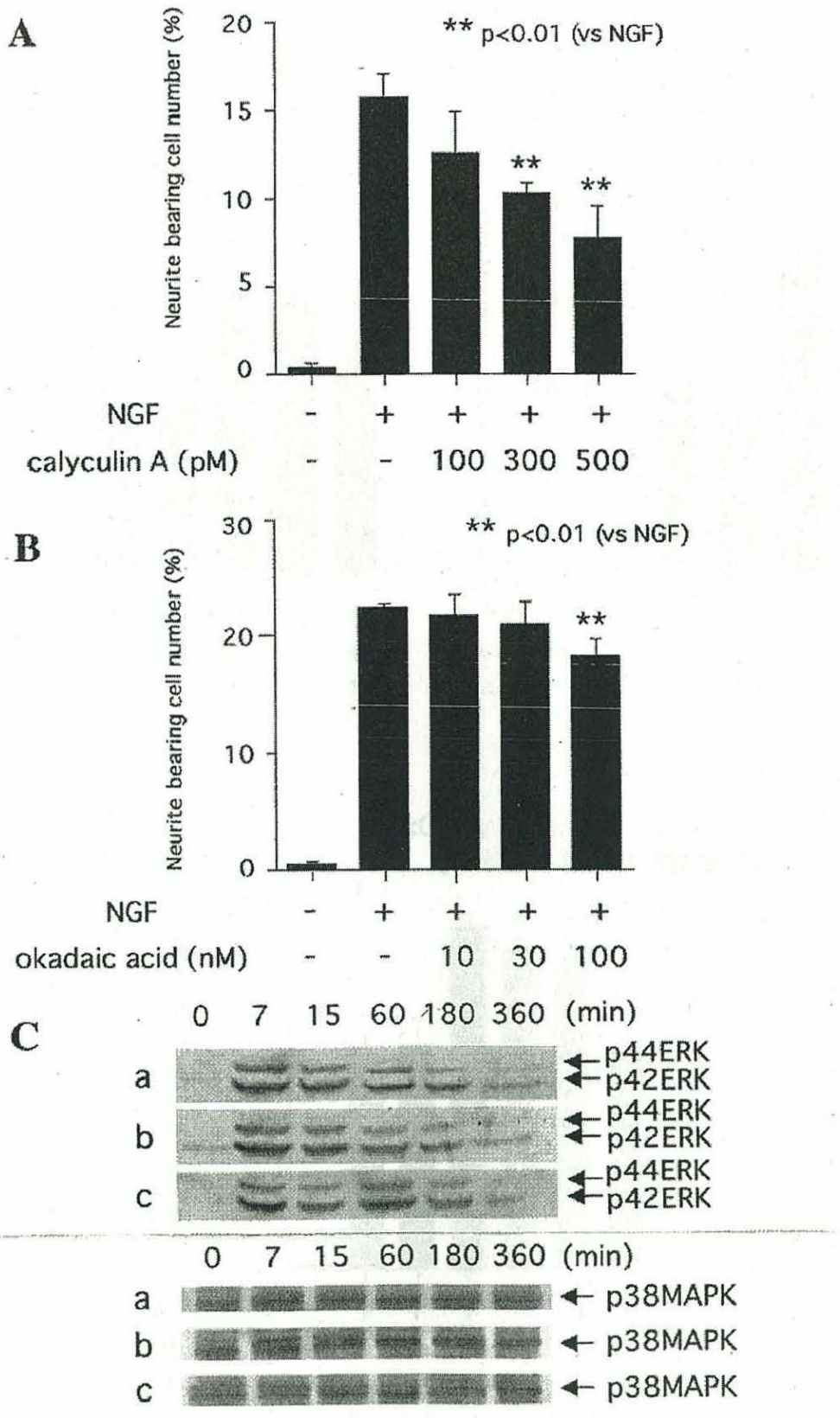


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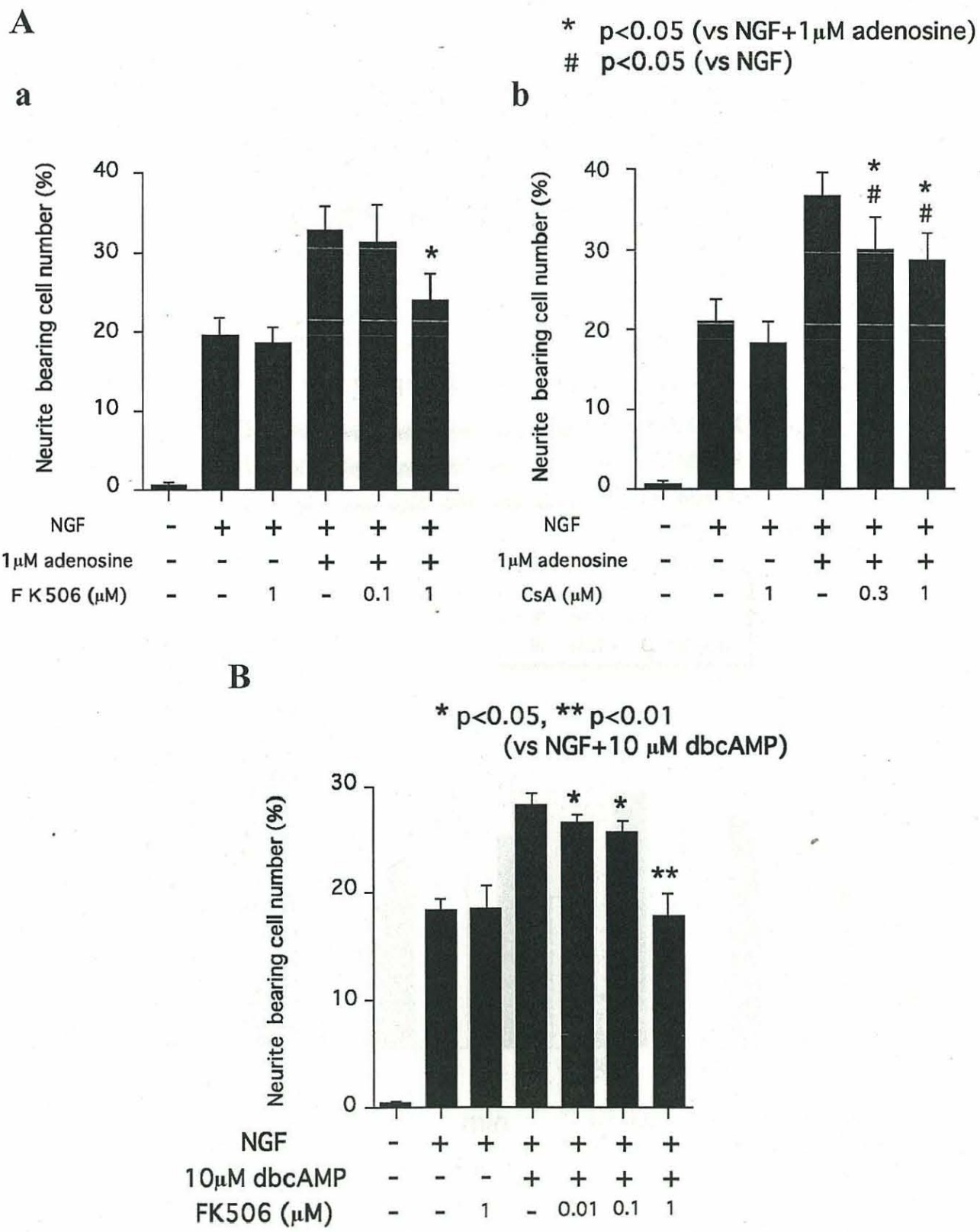


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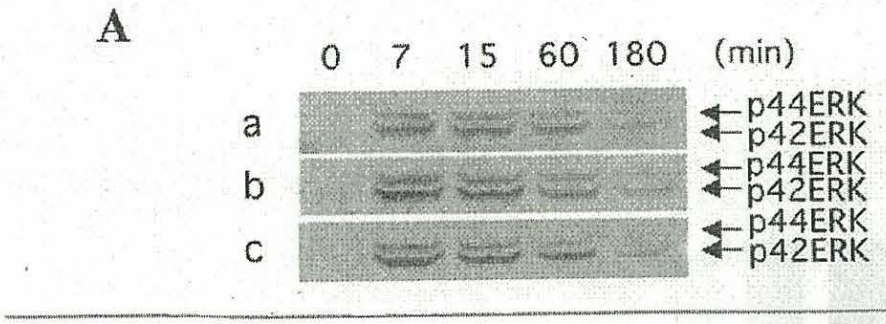
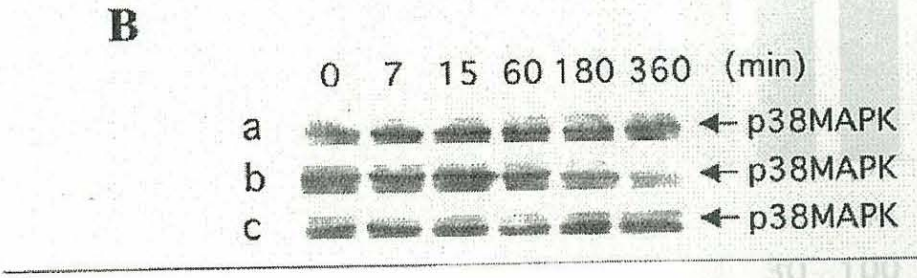
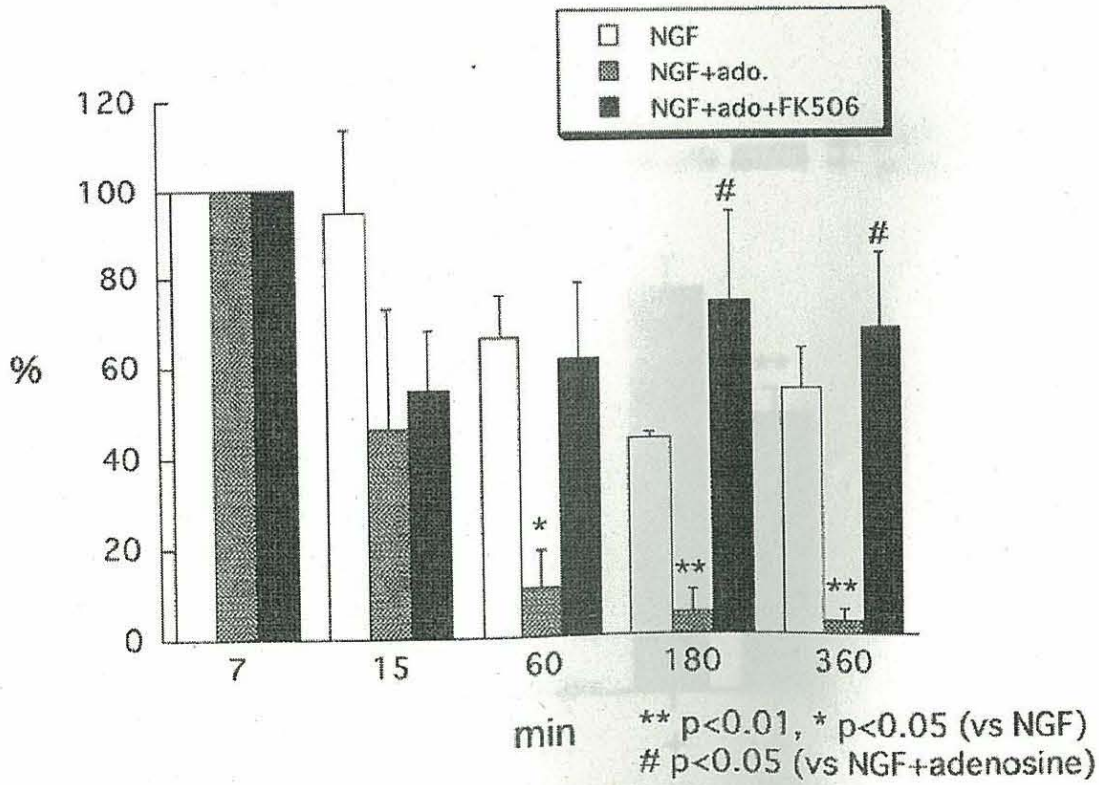
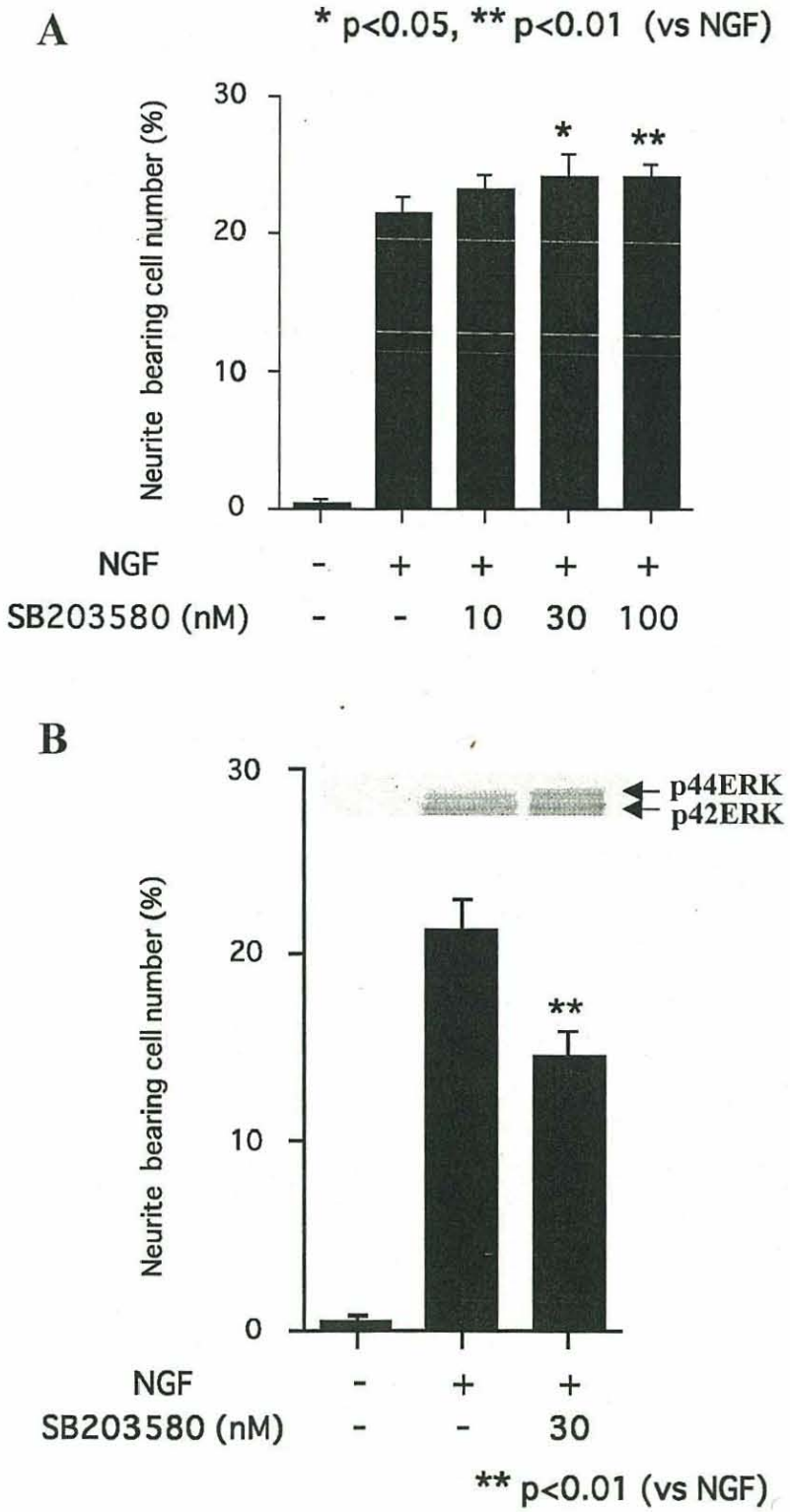



** p<0.01, * p<0.05 (vs NGF)
# p<0.05 (vs NGF+adenosine)

Muroi et al. Figure.5




Muroi et al. Figure.6