

イオン強度を、ClinProt システム (ブルカー・ダルトニクス社) を用いて数値化する。この数値を用いて、AD 群と健常群間で t 検定を行い、群間で量に有意差を認めるペプチドを選定する。さらに、SIMCA-P+ソフト (ユーメトリクス社) により多変量解析を行い、疾患判別モデルを作製する。

なお、この研究は臨床試験として聖マリアンナ医科大学生命倫理委員会にて承認された後に、各被験者の文書による同意を得て行った。

### C. 研究結果

AD 群および健常群の血清由来ペプチドを質量分析により網羅的に解析した結果、1 症例あたり 100 本のペプチドイオンピークを認められた。検出された全 100 個のペプチドイオン強度について、AD 群と健常群の間で t 検定を行ったところ、両群間で有意差のあるペプチドを 18 個検出した。

全 100 個のペプチドイオン強度の数値を用いた主成分分析により AD 群と健常群を判別するモデルの作製を試みたところ、 $R^2=0.999$ 、 $Q^2=0.324$  の判別モデルを作製することができた。(  $R^2$  : 説明変数、 $Q^2$  : 予測変数。 $Q^2$  は全体の 6/7 の症例を用いてモデル作製を行い、残り 1/7 の症例をどれだけ予測できるかの施行を 7 通り行った結果である。) 全 100 個のペプチドのうち当判別モデルの作製において寄与度の高かったペプチドを 16 個検出した。この 16 個を t 検定の結果と比較した結果、10 個のペプチドが重複した。また寄与度 0.2 以上、信頼性 0.3 以上の条件を満たすペプチドを 5 個検出し、それらの質量電荷比は 1614.36m/z、4054.90m/z、4211.19m/z、4965.90m/z、5907.63m/z であった。1614.36m/z、4211.19m/z、5907.63m/z のペプチドは AD 群で、4054.90m/z、4965.90m/z のペプチドは健常群で、イオン強度が有意に高値を示した。

### D. 考察

AD 患者および健常人の血清ペプチドを網羅的に解析した結果、1 症例あたり 100 本と以降の研究に十分な数のペプチドイオンピークを認めた。AD 群と健常群のペプチドイオンピークのスペクトルパターンは酷似しており、ほぼ共通したペプチドプロファイルを持つと考えられた。両群間でイオン強度に有意差を認めるペプチドを 18 個検出し、これらは AD の診断マーカーとなる可能性が示唆された。今後 MS/MS 法によりこれらペプチドのアミノ酸配列の解析を予定している。

また全 100 個のペプチドイオン強度より AD 群と健常群を判別する疾患判別モデルを作製することができ、これは非常に高い  $R^2$  値を示した ( $R^2=0.999$ )。生物学的な研究においては、 $Q^2$  値が 0.4 以上となることが望ましいため、今後症例数を増やして、より予測度の高い判別モデルを作製すると共に、新規症例においても当該モデルの有用性を検討していく予定である。

今後 AD の重症度とペプチド量の変化の相関を解析し、病状評価に有用なペプチドの選定を試みる。さらに、AD のハイリスク群である軽度認知機能障害患者血清を含めた検討も予定している。

### E. 結論

AD 患者および健常人血清ペプチドの網羅的探索を行い、AD で特異的に量変化を示すペプチドを検出した。また AD の疾患判別モデルを作製した。今後 AD の診断および病状評価のマーカーとなる、或いは病因に関与する血清ペプチドを同定できる可能性が示された。

### F. 健康危険情報

特記事項なし。

## G. 研究発表

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Identification of autoantigens specific for systemic lupus erythematosus with central nerve system involvement. American College of Rheumatology 71th scientific meeting, Oct., 2009.

H. 知的財産権の出願、登録状況

1. 特許取得  
なし。
2. 実用新案登録  
なし。
3. その他  
特になし。

厚生労働科学研究費補助金（認知症対策総合研究事業）  
分担研究報告書

タウオパチーに起因する認知症に随伴するパーキンソニズムに関する研究

研究分担者 千葉 俊明 聖マリアンナ医科大学医学部免疫学・病害動物学教室

研究要旨

タウ蛋白異常症（タウオパチー）に起因する認知症では症状として性格変化とパーキンソニズムを高頻度に伴う。また、他のパーキンソニズムとは異なる L-dopa 抵抗性を示すことが特徴である。同じ原因による、他の疾患と異なる特徴的な病態を詳細に解析することは、認知症を示すタウオパチーの病態を側面からアプローチする上で非常に有用であることが予想される。昨年度の結果から線条体におけるドーパミン受容体細胞がヒトタウ異常蛋白の蓄積によって病的な細胞死をきたすことが解明されたので、本年度はそのパーキンソニズムがヒトにおける特徴を示すかどうか、また、受容体細胞を保護するような薬物療法で改善が可能であるかどうかを解析した。結果として L-dopa 抵抗性であり、ヒト疾患モデルに値するが、薬物療法では一時的な改善のみであり、本研究主題である細胞置換療法が必要である事が示唆された。

A. 研究目的

パーキンソニズムがヒトにおける特徴を示すかどうか検討するため、L-dopa投与下での、行動学的評価を行い、さらに、カフェインを使った薬物治療効果を検討した。

B. 研究方法

1. 4ヶ月齢を発症前、5ヶ月齢を発症初期と仮定して、ヒト異常（N279K）タウ遺伝子改変マウスであるSJLBマウス、非遺伝子改変マウス（nonTG）を用いてパーキンソニズムにおける行動学的評価をL-dopa投与下にてFootprintテストおよびRotarodテストにて2Wおきに経時的に解析、比較した。

2. 1と同様に、非選択性アデノシン受容体拮抗薬であるカフェインの連日投与による、パーキンソニズムにおける行動学的評価をFootprintテストおよびRotarodテストにて2Wおきに経時的に解析、比較した。

3. ドーパミン受容体細胞がヒトタウ異常蛋白の蓄積によって病的な細胞死をきたすことを、統計学的にも確固たる証拠として報告すべく、昨年度と同様に、12ヶ月齢SJLBマウス、

nonTGマウスおよびヒトタウ遺伝子改変マウスUBJAP（変異なし）の脳サンプル（それぞれ、n>4）を追加し、Western blots法によるドーパミンシステムにおける、TH,DARPP-32の定量を行い、統計学的処理を行った。また、6ヶ月齢においても同様にサンプルを用意し、検討した。

（倫理面への配慮）

動物実験に関しては当大学実験動物取り扱い規約に則り、また苦痛軽減措置も適切に行った。

C. 研究結果

1. 行動学的評価において4ヶ月齢以降のSJLBはL-dopaに対して抵抗性を示す事が確認され、ヒトタウ病（FTDP-17）におけるパーキンソニズムの特徴を示す事を確認した。

（図1）

2. パーキンソン病でも報告のある神経保護作用をもつカフェインの投与においては投与後2wの時点では投与群で優位な歩行の改善（後ろ足一歩あたりの間隔の延長）がみられたが、それ以降は非投与群との間に治療による有意差はみられなかった。

3. サンプル数を増加でも昨年度結果と同様に 12 ヶ月齢の TH および DARPP-32 の両方が SJLB において統計学的に優位に減少していた。また 6 ヶ月齢では組織染色によってドーパミン受容体細胞のみが優位に減少している結果を以前報告したが、蛋白定量における統計学的有意差がみとめられ、裏付けがなされた。

#### Functional analyses of the parkinsonism in mutant tau transgenic mice with L-dopa treatment

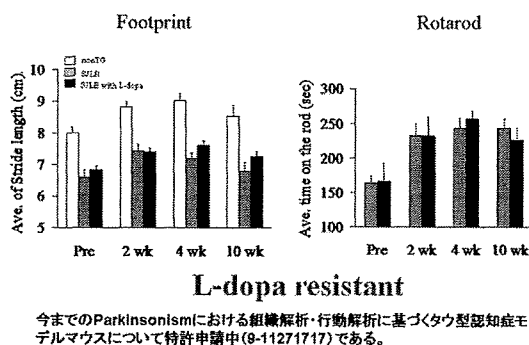


図 1. L-dopa 投与下でのパーキンソニズムの行動機能評価

#### A nonselective antagonist of adenosine receptors, Caffeine partially improved the parkinsonism in mutant tau transgenic mice

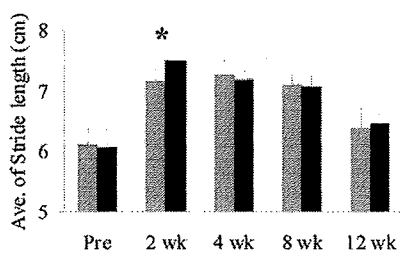


図 2. カフェイン投与による効果

#### D. 考察

以上の結果からヒト異常 (N279K) タウ蛋白による選択的なドーパミン受容体細胞障害がパーキンソニズムの主因であることが統計学的にも確認された。ドーパミン受容体細胞の障害による L-dopa 抵抗性は進行性核上性

麻痺、および多系統萎縮症などでも報告されており、同様の機序による L-dopa 抵抗性が本疾患モデルマウスにも存在し、病的な機構の解明と共に、受容体細胞へ限局した治療標的の選択が可能となった。

#### E. 結論

一時的ではあるが、神経保護を目的とした薬物療法の効果がみられ、さらに選択性の高いアデノシン受容体拮抗薬の選択、もしくは細胞置換療法などによって、パーキンソニズムのみならず、同様の原因でおこる認知症の治療においての可能性が示唆された。

#### F. 健康危険情報

特記事項なし。

#### G. 研究発表

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Shunmei Chiba, Erika Takada, Mamoru Tadokoro, Taizo Taniguchi, Keiichi Kadoyama, Noboru Suzuki: Loss of Dopaminoreceptive Neurons causes L-dopa Resistant Parkinsonism in Tauopathy (Brain, submitted)

##### 2. 学会発表

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千葉 俊明、谷口 泰造、鈴木 登: 認知症モデルマウスにおける移植治療への検討。第 9 回日本再生医療学会、2010 年 3 月

#### H. 知的財産権の出願、登録状況

【発明の名称】 非ヒトモデル動物を用いたパーキンソン症候群の検査方法

【整理番号】 9-11271717

2009年8月21日 特許出願中

### Ⅲ. 研究成果の刊行に関する一覧表

## 1) 雑誌

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Kumagai Y, <u>Kurokawa MS</u> , Ueno H, Kayama M, Tsubota K, Nakatsuji N, Kondo Y, Ueno S, <u>Suzuki N</u> .	Induction of corneal epithelium-like cells from cynomolgus monkey embryonic stem cells and their experimental transplantation to damaged cornea.	Cornea			in press
Shimizu J, Yoshikawa H, Takada E, Hiroso C, <u>Suzuki N</u> .	Skewed helper T cell function in Behcet's disease.	Inflammation and Regeneration			in press
<u>Kurokawa MS</u> , <u>Suzuki N</u> .	Behcet's Disease.	Current Research in Immunology			in press
Hazama Y, <u>Kurokawa MS</u> , <u>Chiba S</u> , Tadokoro M, Imai T, Kondo Y, Nakatsuji N, Suzuki T, Hashimoto T, <u>Suzuki N</u> .	SDF1/CXCR4 Contributes to Neural Regeneration in Hemiplegic Mice with a Monkey ES-cell-derived Neural Graft.	Inflammation and Regeneration			in press
<u>Kurokawa MS</u> , <u>Suzuki N</u> .	Effect of nocotine on differentiation of vascular endothelial cells and mural cells derived from mouse embryonic stem cells.	St. Marianna Medical journal			in press
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#### IV. 研究成果の刊行物・別刷



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## Fluvoxamine and sigma-1 receptor agonists dehydroepiandrosterone (DHEA)-sulfate induces the Ser<sup>473</sup>-phosphorylation of Akt-1 in PC12 cells

Miho Nakano <sup>a</sup>, Kenichi Osada <sup>a,\*</sup>, Atsushi Misonoo <sup>b</sup>, Keisuke Fujiwara <sup>a</sup>, Miho Takahashi <sup>c</sup>, Yuriko Ogawa <sup>a</sup>, Toshiaki Haga <sup>a</sup>, Shigeto Kanai <sup>a</sup>, Daisuke Tanaka <sup>a</sup>, Yasuo Sasuga <sup>a</sup>, Takuyoh Yanagida <sup>a</sup>, Mikio Asakura <sup>a</sup>, Noboru Yamaguchi <sup>a</sup>

<sup>a</sup> Department of Neuropsychiatry, St. Marianna University School of Medicine, 2-16-1 Sugao Miyamae-ku Kawasaki, Kanagawa 216-8511, Japan

<sup>b</sup> Department of Neuropsychiatry, Oofuji Hospital, 249-2 Nakano Fuji, Shizuoka 417-0809, Japan

<sup>c</sup> HBC Co., Ltd, 2-2-3 Nishishinbashi Minato-ku, Tokyo 105-0003, Japan

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### ABSTRACT

**Aims:** The expression of brain-derived neurotrophic factor (BDNF) may be a downstream target of a variety of antidepressant treatments, and selective serotonin reuptake inhibitors (SSRIs) are used clinically for the treatment of depression. BDNF binds to and activates tyrosine kinases receptor (TrkB) to exert its effects. TrkB, after activation by ligands, stimulates phosphoinositide 3-kinase (PI3K). The downstream target of PI3K is Akt-1, a serine-threonine kinase. BDNF has signaling through the PLC-IP<sub>3</sub>/Ca<sup>2+</sup> pathway. Furthermore, the PLC-γ/IP<sub>3</sub>/Ca<sup>2+</sup> pathway is regulated by the sigma-1 receptors. Here, we examined whether fluvoxamine (FLV) activated Akt-1 and increased phosphorylation of Akt-1 via sigma-1 receptor in PC12 cells.

**Main methods:** We examined the effect of the SSRI, FLV and BDNF on the phosphorylation levels of serine-threonine kinase Akt-1 in PC12 cells using immunoblotting techniques.

**Key findings:** Treatment with 10 μM and 100 μM FLV of PC12 cells stimulated a 2.4- and 3.8-fold maximal increase in Ser<sup>473</sup>-phosphorylated Akt-1 levels at 40 min, respectively. Treatment with 50 ng/ml BDNF also stimulated Ser<sup>473</sup>-phosphorylated Akt-1 by 2.6-fold with a maximal increase at 5 min. In addition, the phosphorylation induced by FLV and BDNF was blocked by LY294002, a selective inhibitor of PI3K. The sigma-1 receptor agonists dehydroepiandrosterone (DHEA)-sulfate also stimulated a 2.1-fold increase in the level of Ser<sup>473</sup>-phosphorylated Akt-1.

**Significance:** This study demonstrates that fluvoxamine treatment rapidly increased phosphorylation of Akt-1. And BDNF activated Akt-1 phosphorylation by the TrkB/PI3K/Akt-1 pathway. We conclude that the phosphorylation of Akt-1, downstream of PI3K, was the key to their antidepressant effects.

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### Introduction

Mood disorders are among the most prevalent, recurrent and disabling of all mental illnesses (American Psychiatric Association 1994). Selective serotonin reuptake inhibitors (SSRIs) are representative antidepressant agents. These drugs increase the levels of serotonin, norepinephrine, glutamate, and brain-derived neurotrophic factor (BDNF) by blocking the reuptake of these neurotransmitters at presynaptic terminals (Duman et al. 1997; Skolnick 1999). Although an increase in the monoamine levels occurs soon after drug administration, the efficacy of antidepressants only emerges gradually after several weeks of continuous administration (Nestler et al.

2002a). Therefore, in recent years, research directed at sites beyond the level of the monoamines and receptors has examined the potential post-receptor mechanisms involved in the actions of antidepressant treatments.

Several lines of evidence suggest that the expression of BDNF may be a downstream target of a variety of antidepressant treatments (Altar 1999; Coyle and Duman 2003; Duman et al. 2000; Nestler et al. 2002b). BDNF is a 27-kDa polypeptide that binds to and activates tyrosine kinase receptor (TrkB) to exert their effects (Schlessinger and Ullrich 1992; Barbacid 1995; Weiss et al. 1998). TrkB, once activated by ligands, stimulates the expression of PI3K (Araki et al. 2000). The downstream target of phosphoinositide 3-kinase (PI3K) is Akt-1, a serine-threonine kinase, also referred to as protein kinase B (PKB). Akt-1 has a pleckstrin homology domain in its N-terminus. Activation of Akt-1 by PI3K is induced when the homology domain binds to phosphatidylinositol-3,4,5-trisphosphate and phosphatidylinositol-3,4-bisphosphate, which leads to the translocation of Akt-1 to the

\* Corresponding author. Department of Neuropsychiatry, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki, 216-8511, Japan. Tel.: +81 44 977 8111; fax: +81 44 976 3341.

E-mail address: [k2osada@marianna-u.ac.jp](mailto:k2osada@marianna-u.ac.jp) (K. Osada).

plasma membrane (Franke et al. 1995, 1997a,b). The phosphorylation of Akt-1 on Thr<sup>308</sup> and Ser<sup>473</sup> by the PI-dependent kinases PDK1 and PDK2 is also important for the activation of Akt-1 (Marte and Downward 1997). The expression of BDNF is decreased by exposure to stress (Smith et al. 1995a,b; Nibuya et al. 1999), suggesting that BDNF might be involved in the pathophysiology of stress-related mood disorders.

Several animal and human studies suggest that BDNF may be involved in the actions of antidepressants. For example, infusions of BDNF directly into the rat midbrain area or hippocampus mimics the effects of antidepressants in behavioral models of depression, such as learned helplessness and the forced swim test (Siuciak et al. 1997; Shirayama et al. 2002; Hashimoto et al. 2004). These results suggest that antidepressant drugs might partially exert their effects through an up-regulation of BDNF, and that BDNF is a key molecule involved both in the pathophysiology of depressive disorders and perhaps in the pharmacological action of antidepressants as well (Shirayama et al. 2002).

BDNF has signaling through the PLC- $\gamma$ /IP<sub>3</sub>/Ca<sup>2+</sup> pathway. Furthermore, the PLC- $\gamma$ /IP<sub>3</sub>/Ca<sup>2+</sup> pathway is regulated by sigma-1 receptors (Hayashi et al. 2001). Yagasaki et al. reported that BDNF-stimulated PLC- $\gamma$  activation and the intracellular Ca<sup>2+</sup> and BDNF signaling is modulated by sigma-1 receptors (Yagasaki et al. 2006). Nishimura T. et al. reported that the FLV, with sigma-1 receptor agonist, and sigma-1 receptor agonists DHEA-sulfate were shown to potentiated nerve-growth factor (NGF)-induced neurite outgrowth in PC 12 cells (Nishimura et al. 2008).

Here, we examined the activation of the PI3K/Akt-1 signaling pathway with FLV, BDNF and DHEA-sulfate through the protein and phosphorylation levels of Akt-1 in PC12 cells using immunoblotting techniques.

## Materials and methods

### Drugs

Recombinant human BDNF and dehydroepiandrosterone (DHEA)-sulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA), and LY294002 was obtained from Cell Signaling Technology (Beverly, MA, USA).

### Cell culture

PC12 cells were maintained in 75-cm<sup>2</sup> flasks in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100  $\mu$ g of streptomycin/ml, 100 U of penicillin/ml and 250  $\mu$ g/ml of amphotericin B, and incubated at 37 °C with a 5% CO<sub>2</sub> humidified atmosphere. The culture medium was replaced twice a week with fresh medium. The stock culture was routinely sub-cultured at a ratio of 1:5 at weekly intervals.

### Treatments

Before each experiment, the cells were detached using 5 mM EDTA in Hank's balanced buffer (HBS) and seeded in 12- or 6-well plates (coated with poly-D-Lysine, 10  $\mu$ g/ml) at a density of 3–6  $\times$  10<sup>5</sup> cells/well in 2% serum medium for 24 h. Thereafter, to ensure a low degree of phosphorylation of Akt-1 in the controls all experiments were serum starved for 2 h before the desired reagents were added. The cells were treated to study the effects of FLV and BDNF on the Ser<sup>473</sup>-phosphorylation of Akt-1. Alternatively, the cells were pretreated with LY294002 (50  $\mu$ M, 30 min) followed by stimulation with FLV (100  $\mu$ M, 10–80 min) and BDNF (50 ng/ml, 5 min). PC 12 cells were treated with sigma-1 receptor agonists DHEA-sulfate (10  $\mu$ M, 24 h) in free-serum medium. LY294002 and DHEA-sulfate were dissolved in 0.2% dimethyl sulfoxide.

### Western blotting

PC12 cells were washed with ice-cold PBS three times and then the protein was extracted with RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.1  $\mu$ g/ml DTT, 0.2 nM okadaic acid (OA), and 10  $\mu$ g/ml each of PMSF, calyculin A, and NaVO<sub>3</sub>), and placed on ice for 30 min. Thereafter, the extracts were centrifuged at 15,000 rpm at 4 °C for 15 min. Aliquots containing 20  $\mu$ g of protein from each sample, as determined extemporarily following instructions for the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc, CA, USA), were mixed with Laemmli sample buffer (2% SDS), placed in a boiling water bath for 10 min, and processed for SDS-PAGE. Proteins were separated in 10% SDS polyacrylamide gels (Perfect NT Gel; DRC, JAPAN) and the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with 50 mM Tris-HCl buffer (pH 7.5) containing 10% skim milk for 1 h. Immunoblots were probed with antibodies to anti-phospho-Akt-1 (Ser<sup>473</sup>) at a dilution of 1:500 (Cell Signaling Technology, Beverly, MA, USA), anti-Akt 1 at a dilution of 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti- $\beta$ -Actin at a dilution of 1:1000 (Cell Signaling Technology, Beverly, MA, USA) overnight at 4 °C in the same buffer; and then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) at a dilution of 1:1000 for 1 h. Proteins were detected using the Enhanced Luminol Chemiluminescence system (GE Healthcare, Bio-Science, NJ, USA) and direct exposure to the LAS-3000 imaging system with a full-frame-capture CCD camera (FUJIFILM Corporation, Tokyo, Japan). The immunoblots were quantified using a densitometric analyzer (ATTO CS Analyzer 2.0, Tokyo, Japan).

### MTT assay

After incubation of the PC12 cells with the experimental reagents for 24 h, cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay in which the yellow tetrazolium base compound is reduced by viable cells to a blue formazan product. The original medium was removed from the 96-well plates, and the plates were incubated at 37 °C in the presence of RPMI 1640 medium supplemented with 1% FBS containing 0.5 mg/ml MTT. A 100- $\mu$ l aliquot of acid-isopropanol (0.04 M hydrochloric acid) was then added to each well, and the plates were incubated at 37 °C overnight to dissolve the formazan that had formed in the wells. Reduced MTT was measured using a spectrophotometer (Wallac ARVO HTS 1420 Multilabel Counter, Perkin Elmer Life Sciences, Turku, Finland) at a wavelength of 570 nm. The value for each treatment group was converted to a percentage of the control.

### Statistical analysis

The results are expressed as mean  $\pm$  S.E.M. of three or more experiments performed in duplicate. One-way ANOVA with Newman-Keuls test was used to establish statistical significance, set at  $p < 0.05$ .

## Results

### Effects of FLV on MTT reduction of PC12 cells

Because the cytotoxicity of FLV in PC12 cells had not yet been established, we examined the concentration-dependent effect of FLV on the viability of cells by quantifying the reduced product of MMT in PC12 cells. One-way ANOVA revealed that the viability of the PC12 cells was reduced significantly after treatment for 24 h with 400  $\mu$ M FLV, but was not affected by lower concentrations of this drug ( $p < 0.001$ ; Fig. 1). That is, FLV is not cytotoxic at concentrations equal to or less than 200  $\mu$ M.

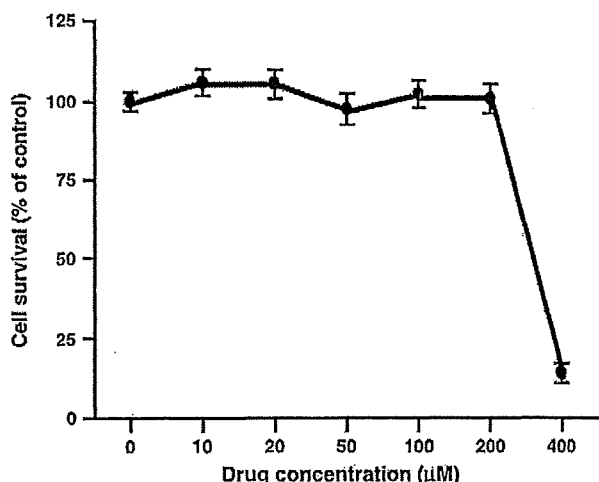


Fig. 1. Effect of FLV on MMT reduction. PC12 cells were treated with FLV for 24 h. The MTT reduction assay was carried out as described in the Methods section. Data are expressed as a percentage of the corresponding control value, which was set at 100%. The results were compared by ANOVA. ( $n=12$ ) (\*\* $p<0.001$ ).

We therefore decided to observe the phosphorylation of Akt-1 induced by 100 µM FLV.

#### FLV- and BDNF-stimulated activation of Akt-1

To examine the FLV- and BDNF-stimulated activation of Akt-1 in PC12 cells, we performed Western blotting using anti-phospho (Ser<sup>473</sup>) Akt-1 antibody. Treatment of PC12 cells with 10 µM FLV stimulated a 2.4-fold increase in the level of Ser<sup>473</sup>-phosphorylated Akt-1 at 40 min ( $p<0.05$ ) (Fig. 2). The phosphorylation of Akt-1 increased a maximum of 3.8 fold with 100 µM FLV. The phosphorylation of this kinase was observed within 20 min of treatment and reached a maximal level at 40 min ( $p<0.001$ ). The level of Ser<sup>473</sup>-

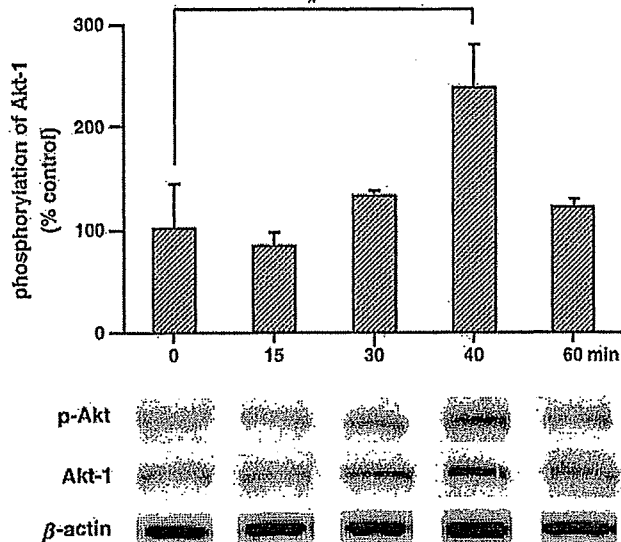


Fig. 2. Effect of 10 µM FLV on Akt-1 (Ser<sup>473</sup>) phosphorylation and total Akt-1 protein levels. 10 µM FLV stimulates the phosphorylation of Akt-1 in PC12 cells. PC12 cells were incubated with 10 µM FLV for the indicated times and lysed. The supernatant was analyzed by immunoblotting with an antibody against phospho-specific (Ser<sup>473</sup>) Akt-1, total Akt-1 and β-Actin. The densitometric data were expressed as a percentage of the corresponding control value, which was set at 100%. The results are expressed as the mean  $\pm$  S.E.M. Representative immunoblots from experiments repeated three times are shown ( $n=4$ ) (\* $p<0.05$ ).

phosphorylated Akt-1 remained elevated even at 60 min. The levels of total Akt-1 protein and β-Actin protein were unaffected (Fig. 3).

Treatment with 50 ng/ml BDNF was found to produce similar results to those produced by FLV with regard to the phosphorylation of Akt-1. This phosphorylation was observed within 2.5 min of treatment and was maximally increased at 5 min by 2.6-fold ( $p<0.01$ ). The level of phosphorylated Akt-1 remained elevated even at 40 min. The levels of total Akt-1 and β-Actin protein were unaffected (Fig. 4).

#### FLV and BDNF-induced phosphorylation of Akt-1 is mediated by the PI3K pathway

PC12 cells were then pretreated with the PI3K inhibitor, LY294002 (50 µM, 30 min), and stimulated with FLV (100 µM, 40 min) and BDNF (50 ng/ml, 5 min). The FLV-treated cells without LY294002 induced an increase in the levels of Ser<sup>473</sup>-phosphorylation of Akt-1 ( $p<0.001$ ). On the other hand, pretreatment with LY294002 blocked the FLV-induced Ser<sup>473</sup>-phosphorylation of Akt-1 ( $p<0.001$ ). Similarly, the BDNF-treated cells without LY294002 showed an increase in the Ser<sup>473</sup> phosphorylation of Akt-1 ( $p<0.001$ ), and LY294002 blocked the BDNF-induced Ser<sup>473</sup>-phosphorylation of Akt-1 ( $p<0.001$ ). No significant difference was observed between the effects of FLV and BDNF on the Ser<sup>473</sup>-phosphorylation of Akt-1 ( $p>0.05$ ). The protein level of Akt-1 and β-Actin was unchanged from the control level after treatment with FLV and BDNF (Fig. 5).

#### Sigma-1 receptor agonists DHEA-sulfate stimulated activation of Akt-1

PC12 cells were then pretreated with the sigma-1 receptor agonists DHEA-sulfate (10 µM, 24 hr). DHEA-sulfate stimulated a 2.1-fold increase in the level of Ser<sup>473</sup>-phosphorylated Akt-1

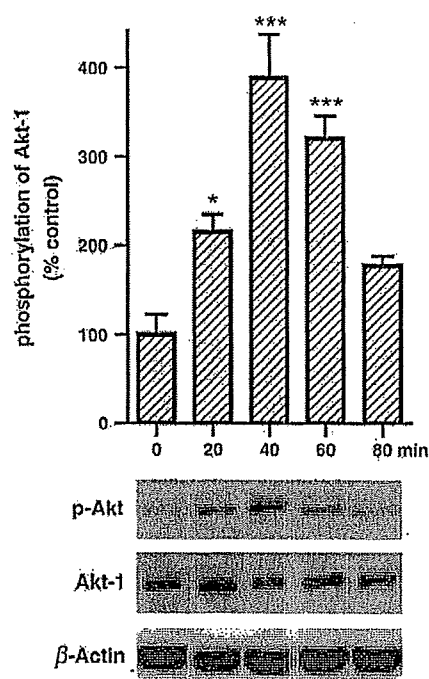
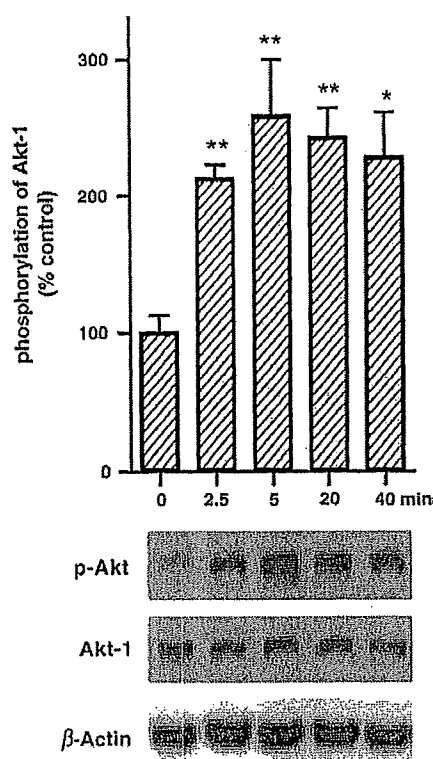


Fig. 3. Effect of 100 µM FLV on Akt-1 (Ser<sup>473</sup>) phosphorylation and the total Akt-1 protein levels. PC12 cells were incubated with 100 µM FLV for the indicated times and lysed. The supernatant was analyzed by immunoblotting with an antibody against phospho-specific (Ser<sup>473</sup>) Akt-1, total Akt-1 and β-Actin. The densitometric data were expressed as a percentage of the corresponding control value, which was set at 100%. The results are expressed as the means  $\pm$  S.E.M. Representative immunoblots from experiments repeated three times are shown ( $n=7$ ) (\* $p<0.05$ ; \*\* $p<0.001$ ).



**Fig. 4.** Effect of BDNF on Akt-1 (Ser<sup>473</sup>) phosphorylation and the total Akt-1 protein levels. BDNF stimulated the phosphorylation of Akt-1 in PC12 cells. PC12 cells were incubated with 50 ng/ml BDNF for the indicated times and lysed. The supernatant was analyzed by immunoblotting with antibodies against phospho-specific (Ser<sup>473</sup>) Akt-1, total Akt-1 and  $\beta$ -Actin. The densitometric data were expressed as a percentage of the corresponding control value, which was set at 100%. The results are expressed as the mean  $\pm$  S.E.M. Representative immunoblots from experiments repeated three times are shown ( $n=6$ ) (\* $p<0.05$ ; \*\* $p<0.01$ ).

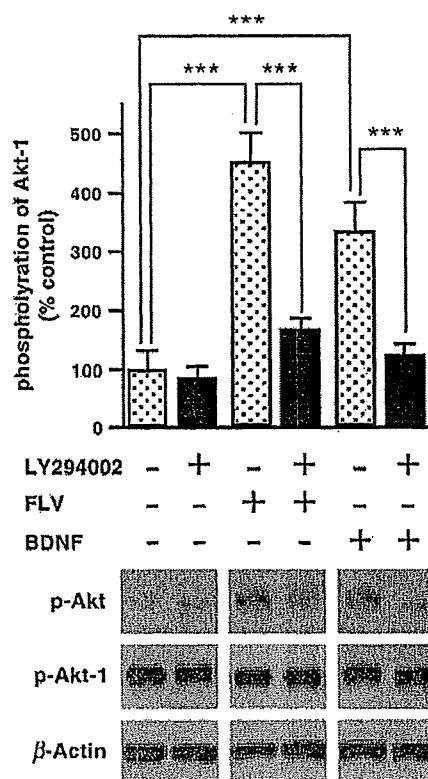
( $p<0.001$ ); however, the levels of total Akt-1 and  $\beta$ -Actin protein were unaffected (Fig. 6).

## Discussion

Our results demonstrate that the administration of both the antidepressant FLV and BDNF in PC12 cells rapidly increased Akt-1 phosphorylation in a time-dependent manner, as shown by the immunoblots.

The phosphorylation of Akt-1 has been reported to be critically dependent on PI3K activity. High levels of basal Akt-1 activity are most likely caused by the presence of growth factors in the culture medium, and the removal of serum induced a rapid depletion of Akt-1 activity (Elzbieta and De-Maw 1999). We measured the phosphorylation of Akt-1 in PC12 cells incubated with 2% serum medium for 24 h, after which the serum was removed for 2 h, 4 h, 12 h, and 24 h. The degree of phosphorylation of Akt-1 was not changed from 2 h to 24 h (data not shown) and we concluded that the phosphorylation of Akt-1 induced by serum was not affected by removal of the serum for 2 h. PC12 cells were under the same physiologic conditions as those observed at 2 h. Therefore, all of our experiments were performed after 2 h of serum starvation. Because phosphorylation of Akt-1 is crucial to the activity of this kinase, our results demonstrate that FLV and BDNF affected the phosphorylation state of Akt-1 in PC12 cells incubated with serum-free medium.

The activity of BDNF is also directly influenced by antidepressant treatment. For example, chronic, but not acute, antidepressant treatment up-regulates BDNF mRNA in the rat brain (Nibuya et al. 1995; Russo-Neustadt et al. 2000). Chen et al. reported that the levels



**Fig. 5.** Effect of FLV and BDNF on the inhibition of Akt-1 (Ser<sup>473</sup>) phosphorylation and the total Akt-1 protein levels treated with or without the PI3K inhibitor, LY294002. The cultures were pretreated for 30 min, where indicated with 50  $\mu$ M LY294002, and then 100  $\mu$ M FLV was administered for 40 min and 50 ng/ml BDNF was administered for 5 min. The cultures not treated with LY294002 were treated with vehicle (0.2% dimethyl sulfoxide). The supernatant was analyzed by immunoblotting with antibodies against phospho-specific (Ser<sup>473</sup>) Akt-1, total Akt-1 and  $\beta$ -Actin. The densitometric data were expressed as a percentage of the corresponding control value, which was set at 100%. The results are expressed as the mean  $\pm$  S.E.M. The representative immunoblots from experiments repeated three times are shown ( $n=6$ ) (\*\* $p<0.001$ ).

of BDNF were higher in post-mortem hippocampi obtained from antidepressant-treated subjects than in those from untreated subjects (Chen et al. 2001). Saarelainen et al. showed that antidepressants (imipramine and fluoxetine) acutely increase TrkB signaling in the cerebral cortex and that this signaling is required to determine the behavioral effects caused by antidepressants. They suggested that antidepressants exert their effects, at least in part, through the actions of endogenous BDNF (Saarelainen et al. 2003).

SSRIs appear to affect the activity of serotonin transporters (SERTs), but this has not yet been definitively demonstrated. Recently, the effects of citalopram, one of the SSRIs, have been assessed in a forced swimming test using mice in which SERTs were knocked down via siRNA. In that report, the effects of the SSRI were not changed after SERT knockdown (Thakker et al. 2005). Therefore, SSRIs may not act via SERTs. Furthermore, Nishimura et al. showed that, in an experiment with PC12 cells, the activity of neurite outgrowth due to SSRIs (FLV, sertraline and paroxetine) occurs because SSRIs activate IP3 receptors via sigma-1 receptors (Nishimura et al. 2008). Moreover, Zhou et al. reported that SSRIs such as FLV exhibit a suppressive activity on plasma membrane monoamine transporters (PMATs), which are not SERTs, but are also important sites of action for SSRIs (Zhou et al. 2006). Therefore, it is currently difficult to conclude that SSRIs act only on SERT. Some studies have reported that antidepressants have a similar effect to BDNF, and both antidepressants and BDNF produced antidepressant effects. Nibuya et al. reported that when K252a, an inhibitor of TrkB receptors, is



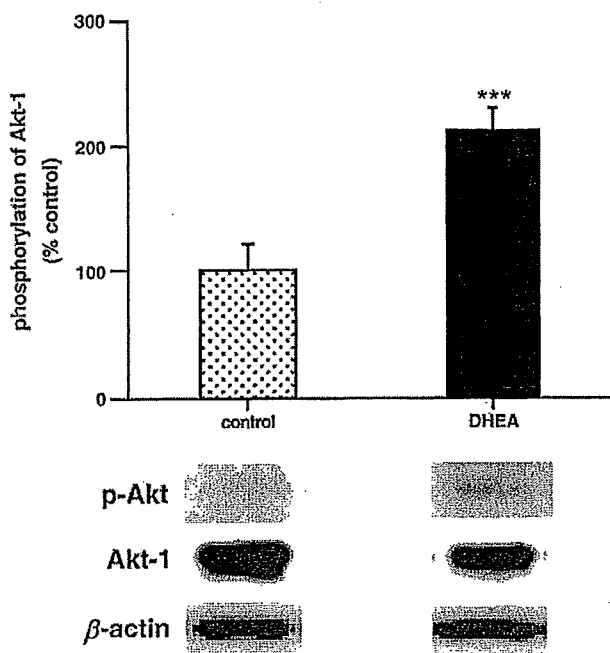


Fig. 6. Effect of the increase in the Ser<sup>473</sup>-phosphorylation of Akt-1 by sigma-1 receptor agonists DHEA-sulfate. The cultures were treated for 24 hr with 10  $\mu$ M DHEA-sulfate or vehicle (0.2% dimethyl sulfoxide) in serum-free medium. The supernatant was analyzed by immunoblotting with antibodies against phospho-specific (Ser<sup>473</sup>) Akt-1, total Akt-1 and  $\beta$ -Actin. The densitometric data were expressed as a percentage of the corresponding control value, which was set at 100%. The results are expressed as the mean  $\pm$  S.E.M. The representative immunoblots from experiments repeated three times are shown ( $n=6$ ) (\*\*\*) $p<0.001$ .

administered with BDNF concurrently, the effects of BDNF were abolished, suggesting that the antidepressant activity of BDNF exerts its effects through TrkB receptors (Nibuya et al. 1999). However, no reports have yet described antidepressants involved after the activation of TrkB.

BDNF activated Akt-1 phosphorylation by the TrkB/PI3K/Akt-1 pathway. We confirmed the present results with BDNF-induced activation of Ser<sup>473</sup>-phosphorylated Akt-1 by PI3K. Furthermore, BDNF signals through the PLC- $\gamma$ /IP<sub>3</sub>/Ca<sup>2+</sup> pathway regulated by sigma-1 receptors (Yagasaki et al. 2006). Y. Yagasaki et al. reported that BDNF-stimulated PLC- $\gamma$  activation and the intracellular Ca<sup>2+</sup> and BDNF signaling are modulated by sigma-1 receptors; FLV, with sigma-1 receptor agonism, enhanced BDNF-induced glutamate release and increased intracellular Ca<sup>2+</sup> in cultured cortical neurons (Yagasaki et al. 2006). Nishimura T. et al. reported that the FLV and sigma-1 receptor agonists DHEA-sulfate were shown to potentiate NGF-induced neurite outgrowth by the PLC- $\gamma$ /IP<sub>3</sub>/Ca<sup>2+</sup> pathway in PC 12 cells (Nishimura, T. PLoS ONE 3(7), 1–9, 2008). In the present study, FLV and DHEA-sulfate increased in the level of Ser<sup>473</sup>-phosphorylated Akt-1. Therefore, we considered that FLV activated Akt-1 and increased phosphorylation of Akt-1 via the sigma-1 receptor in a manner similar to that shown by DHEA-sulfate in PC12 cells.

Up-regulation of BDNF has been suggested to contribute to the action of antidepressants. BDNF activated Akt-1 by the TrkB/PI3K/Akt-1 pathway, and FLV activated Akt-1 by the PLC- $\gamma$ /IP<sub>3</sub>/Ca<sup>2+</sup>/Akt-1 pathway via the sigma-1 receptor. In the present study, Akt-1 phosphorylation induced by FLV was blocked by pretreatment with LY294002, a highly selective inhibitor of PI3K as shown in the Fig. 5. Therefore, we have shown firstly presented that FLV activated Ser<sup>473</sup>-phosphorylated Akt-1 by PI3K in PC12 cells. Because BDNF activated Akt-1 phosphorylation by the TrkB/PI3K/Akt-1 pathway and FLV activated Akt-1 phosphorylation by the PLC- $\gamma$ /IP<sub>3</sub>/Ca<sup>2+</sup>/Akt-1 pathway via the sigma-1 receptor, we conclude that the phosphorylation

of Akt-1, the downstream of PI3K, was the key enzyme to their antidepressant effects.

## Conclusions

Treatment with fluvoxamine increased in Ser<sup>473</sup>-phosphorylated Akt-1 levels on PC12 cells. Treatment with BDNF also stimulated Ser<sup>473</sup>-phosphorylated Akt-1. In addition, the phosphorylation induced by fluvoxamine and BDNF was blocked by LY294002, a selective inhibitor of PI3K. Sigma-1 receptor agonists DHEA-sulfate also increased Ser<sup>473</sup>-phosphorylated Akt-1. This study is the first to demonstrate that fluvoxamine treatment rapidly increases the phosphorylation of Akt-1.

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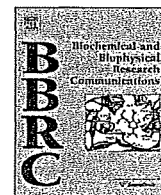
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## Suppression of dynamin GTPase activity by sertraline leads to inhibition of dynamin-dependent endocytosis

Kiyofumi Takahashi<sup>a,1</sup>, Hiroshi Miyoshi<sup>b,1,\*</sup>, Masahiro Otomo<sup>a,1</sup>, Kenichi Osada<sup>a</sup>, Noboru Yamaguchi<sup>a</sup>, Hideki Nakashima<sup>b</sup>

<sup>a</sup> Department of Neuropsychiatry, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki 216-8511, Japan

<sup>b</sup> Department of Microbiology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki 216-8511, Japan

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### ABSTRACT

Dynamin (Dyn) 1 plays a role in recycling of synaptic vesicles, and thus in nervous system function. We previously showed that sertraline, a selective serotonin reuptake inhibitor (SSRI), is a mixed-type inhibitor of Dyn 1 with respect to both GTP and L- $\alpha$ -phosphatidyl-L-serine (PS) *in vitro*, and we suggested that it may regulate the neurotransmitter transport by modulating synaptic vesicle endocytosis *via* inhibition of Dyn 1 GTPase. Here, we investigated the effect of sertraline on endocytosis of marker proteins in human neuroblastoma SH-Sy5Y cells and HeLa cells. Sertraline inhibited endocytosis in both cell lines. Western blotting showed that SH-Sy5Y expresses Dyn 1 and Dyn 2, while HeLa expresses only Dyn 2. GTPase assay showed that sertraline inhibited Dyn 2 as well as Dyn 1. Therefore, the effect of sertraline on endocytosis was mediated by Dyn 2, at least in HeLa cells, as well as by Dyn 1 in cell lines that express it. Moreover, the inhibition mechanism of transferrin (Tf) uptake by sertraline differed from that in cells expressing Dyn 1 K44A, a GTP binding-defective variant, and sertraline did not interfere with the interaction between Dyn 1 and PS-liposomes.

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### Introduction

Dynamin (Dyn) has GTPase activity and plays a critical role in clathrin- and caveolae-dependent endocytosis [1,2]. A major role of Dyn GTPase activity in endocytosis is to produce a mechanical force for membrane fission during clathrin-coated vesicle budding, either by constriction or expansion of the collar surrounding the neck of the invaginated vesicle [3].

Mammals have three Dyn isoforms with different tissue distributions [4,5]. Dyn 1 is only expressed in neurons and has been implicated in presynaptic vesicle recycling [4]. It is thought to play a role in the clathrin-dependent endocytotic pathway at neuronal synapses [6,7]. Dyn 1 has four functional domains: an N-terminal GTPase domain, a pleckstrin homology domain (PHD), a proline/arginine-rich domain (PRD), and a GTPase effector domain

**Abbreviations:** SSRI, selective serotonin reuptake inhibitor; His<sub>6</sub>, polyhistidine segment 6 residues in length; Dyn, dynamin; Dyn-His<sub>6</sub>, dynamin 1 with a His<sub>6</sub> tag fused to the C-terminus; PHD, pleckstrin homology domain; PRD, proline/arginine-rich domain; SH3, Src homology 3; PS, L- $\alpha$ -phosphatidyl-L-serine; Pi, orthophosphate; SPR, surface plasmon resonance; CTB, cholera toxin subunit B; Tf, transferrin; CPZ, chlorpromazine; DAPI, 4',6-diamidino-2-phenylindole; CHC, clathrin heavy chain

\* Corresponding author. Fax: +81 44 977 7818.

E-mail address: [hmiyoshi@marianna-u.ac.jp](mailto:hmiyoshi@marianna-u.ac.jp) (H. Miyoshi).

<sup>1</sup> These authors contributed equally to this work.

[3,8–11]. L- $\alpha$ -Phosphatidyl-L-serine (PS) or phosphatidylinositol-4,5-bisphosphate binds the PHD of Dyn [8,9], stimulates the GTPase activity, and induces cooperative helix assembly [10,12]. Moreover, after deletion of the PHD, Dyn shows elevated GTPase activity independently of those lipids [8,9]. PRD of Dyn binds to Src homology 3 (SH3) domain-containing proteins, such as amphiphysin [13], and the complex is necessary for adaptation of clathrin [14]. PRD peptide disrupts the interaction between Dyn and amphiphysin and inhibits endocytosis [15].

The K44A variant of Dyn lacks GTPase activity owing to defects in both GTP binding and hydrolytic activity [2,16]. As a result, overexpression of the K44A variant inhibits clathrin-dependent endocytosis in neuronal cells [2,16,17].

The GTPase activities of Dyn 1 and 2 are inhibited by cationic surfactants such as myristyl trimethyl ammonium bromide [18], and by 3-(2-chloro-10H-phenothiazin-10-yl)-N,N-dimethylpropan-1-amine (chlorpromazine), which is an antipsychotic [19]. We previously reported that (1S)-cis-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-N-methyl-1-naphthalenamine (sertraline) inhibits the GTPase activity of Dyn 1 [20]. Our previous results indicated that the inhibition of Dyn 1 GTPase by sertraline may regulate the endocytic pathway at neuronal synapses.

Sertraline is a selective serotonin reuptake inhibitor (SSRI) [21]. The serotonin transporter is proposed to modulate a variety of brain functions, including mood, anxiety and sleep, by the

elimination of the neurotransmitter serotonin from the synaptic cleft [22]. Depressive disorder has been postulated to be associated with continuously low levels of serotonin [23]. However, our finding that some SSRIs inhibit Dyn 1 GTPase [20] was unexpected, and furthermore, the relationship between inhibition of Dyn 1 GTPase and regulation of endocytosis by sertraline is still unknown. In this study, we further examined the mechanism of sertraline's inhibitory action on endocytosis.

## Materials and methods

**Materials.** Sources of materials were: restriction enzymes (Takara Bio); pEGFP-C1 (Clontech); pET 21a (Merck); Alexa Fluor<sup>®</sup>555 conjugate-CTB, Alexa Fluor<sup>®</sup>633 conjugate-Tf, Prolong Gold and Lipofectamin<sup>™</sup>2000 (Invitrogen); L- $\alpha$ -phosphatidyl-L-serine (Sigma Aldrich); Dyn 1 antibody (Epitomics); Dyn 2 (C-18) antibody, clathrin HC (C-20) antibody, rabbit anti-goat antibody and goat anti-mouse antibody (Santa Cruz Biotechnology);  $\beta$ -actin rabbit antibody (Cell Signaling); sertraline, citalopram and chlorpromazine (Sigma); ECL Western blotting detection reagents (GE Healthcare).

**Cloning and construction of expression plasmids.** Dyn 2 gene (gi:87299636) was amplified by PCR using appropriate primers. The amplified gene was ligated into the NdeI and SalI sites in pET 21a to create an expression vector for Dyn 2 *wt* bearing a polyhistidine segment 6 residues in length (His<sub>6</sub> tag) fused to the C-terminus (Dyn2-His<sub>6</sub>). Cloning and construction of pETDyn1, an expression vector for Dyn 1 *wt* bearing a His<sub>6</sub> tag fused to the C-terminus (Dyn-His<sub>6</sub>), were reported previously [20]. Dyn 1 *wt* gene was subcloned into pBluescript2SK(+) from pETDyn1 to reconstruct expression plasmids for mammalian cells. The subcloned vector was named pBlueDyn1*wt*. Site-directed mutagenesis was performed by PCR to create an expression vector for Dyn K44A bearing a His<sub>6</sub> tag fused to the C-terminus (Dyn K44A-His<sub>6</sub>). This was digested with NdeI and XhoI, and ligated into the same sites in both pET 21a and pBlueDyn1*wt*, affording pETDyn1K44A and pBlueDyn1K44A, respectively. A pETDyn1 plasmid was digested with BstBI and BspEI, blunt-ended and ligated to delete 530–550 amino acid residues (Dyn  $\Delta$ PHD-His<sub>6</sub>), affording pETDyn1 $\Delta$ PHD. pBlueDyn1 plasmid was digested with BglII and EcoRI, and ligated into the same sites in pEGFP-C1 to create an expression vector for Dyn 1 linked at the N-terminus to GFP, designated pEGFP-Dyn1. pEGFP-Dyn1K44A plasmid was constructed similarly to pEGFP-Dyn1.

**Expression and purification of Dyn.** Expression and purification of Dyn-His<sub>6</sub> were reported previously [20]. DynK44A-His<sub>6</sub>, Dyn $\Delta$ PHD-His<sub>6</sub>, and Dyn2-His<sub>6</sub> were expressed and purified similarly.

**GTPase assay and preparation of PS-liposomes.** The Malachite Green GTPase assay of Dyn was performed as described previously [20]. A solution of PS in chloroform/methanol (95:5) (10 mg ml<sup>-1</sup>, 40  $\mu$ l) was evaporated to about 5  $\mu$ l, resuspended in 1 ml of 30 mM Tris-HCl pH 7.4, and sonicated for 2 min on ice to afford a working solution of 400  $\mu$ g ml<sup>-1</sup>.

**Surface plasmon resonance (SPR).** SPR analyses were essentially performed as described previously [9]. SPR analyses were performed on a Biacore 3000 with a Sensorchip NTA (Biacore K.K.), using Buffer A (10 mM Tris-HCl, 10 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.05% Tween 80, pH 7.4) as the eluent (20  $\mu$ l min<sup>-1</sup>), at 25 °C. All four sensor sites were treated with 20  $\mu$ l of 100  $\mu$ M NiSO<sub>4</sub>, then Dyn-His<sub>6</sub>, DynK44A-His<sub>6</sub>, and Dyn $\Delta$ PHD-His<sub>6</sub> (50  $\mu$ l, 10  $\mu$ g ml<sup>-1</sup>) were trapped via the His<sub>6</sub> tag on sites 1, 2, and 3, respectively. Site 4 was the blank control (Buffer A only). PS-liposomes (100  $\mu$ l; 100  $\mu$ g ml<sup>-1</sup>) were injected simultaneously over all four sites, with or without 50  $\mu$ M sertraline. Sensorgrams for specific interactions

were obtained by subtracting the sensorgram for Buffer A from those for Dyn-His<sub>6</sub>, DynK44A-His<sub>6</sub>, and Dyn $\Delta$ PHD-His<sub>6</sub> with BIA Evaluation Software.

**Cell culture and fluorescence imaging.** HeLa and SH-Sy5Y cells were cultured with 5% or 10% fetal bovine serum in DMEM, seeded onto collagen IV-coated coverslips, and transfected with pEGFP-Dyn1 or pEGFP-Dyn1K44A using Lipofectamin<sup>™</sup>2000. Adhering cells were treated with drugs (30 min, 37 °C) and with 1  $\mu$ g ml<sup>-1</sup> Alexa-CTB (30 min, on ice), then incubated with 5  $\mu$ g ml<sup>-1</sup> Alexa-Tf (5 min, 37 °C). The cells were washed with ice-cold 150 mM glycine (pH 2.0), fixed with 4% paraformaldehyde, rinsed with PBS, air-dried, mounted on Prolong Gold antifade reagent with DAPI, and observed with a Carl Zeiss LSM510 confocal microscope (63 $\times$ /1.4 oil immersion objective). Excitation wavelengths of DAPI, GFP, Alexa-CTB, and Alexa-Tf were 405, 488, 543 and 633 nm, respectively.

**Image analysis.** Microscopic images were analyzed using ImageJ software version 1.42q (NIH, USA: <http://rsb.info.nih.gov/ij/index.html>) [24]. Outlines of cells were traced using the polygon selection tool, and fluorescence intensity was obtained for each cell.

**Western blotting.** Cells were lysed with SDS-PAGE sample buffer and lysates were subjected to 8% SDS-PAGE. Gels were blotted on PVDF membrane, which was incubated in 5% non-fat dried milk in TBST, then with anti-Dyn 1 (1:250), anti-Dyn 2 (1:250), anti-clathrin HC (1:250), or anti- $\beta$ -actin (1:1000), followed by HRP-conjugated secondary antibody. Bands were evaluated with the ECL Western Blotting Detection System using a LAS-3000 (Fuji Photo Film).

## Results

### Sertraline preferentially and reversibly inhibits endocytosis of Tf

Chlorpromazine (CPZ) is well known, not only as an antipsychotic [19], but also as an inhibitor of clathrin-dependent endocytosis [25]. Citalopram is a SSRI, like sertraline [26]. Moreover, we previously reported that sertraline potently inhibited Dyn1 GTPase activity *in vitro* and that sertraline, CPZ and citalopram inhibited Dyn 1 GTPase with IC<sub>50</sub> values of 7.3  $\pm$  1.0, 47.2  $\pm$  23.1 and >100  $\mu$ M, respectively [20]. Therefore, we first compared the effects of sertraline, CPZ and citalopram on endocytosis of marker proteins in HeLa cells (Fig. 1). Sertraline and CPZ inhibited uptake of transferrin (Tf) and cholera toxin subunit B (CTB) into HeLa cells, while citalopram was ineffective (Fig. 1A and B). Interestingly, sertraline was strongly Tf-selective, while CPZ was less so (Fig. 1B). These results suggested that Dyn-dependent endocytosis of Tf was blocked *via* inhibition of Dyn GTPase by sertraline.

Time course analyses showed that most of the internalization of Tf was blocked within 5 min after addition of sertraline (Fig. 2A), while the Tf uptake was unaffected by addition of citalopram (Fig. 2B). At 20 min after washout of sertraline, endocytosis was found to have returned to control levels (Fig. 2C), suggesting that sertraline inhibition is rapidly reversible.

### Sertraline also inhibits endocytosis of Tf in neuronal cells

We next examined the effect of sertraline on endocytosis in human neuroblastoma SH-Sy5Y cells. Internalization of Tf, but not CTB, was strongly inhibited by 20  $\mu$ M sertraline in SH-Sy5Y cells (Fig. 3A). These results are consistent with the suppression of endocytosis observed in HeLa cells, as shown in Fig. 1A.

### Sertraline inhibits Dyn 2 GTPase

Endogenous expression of Dyn isoforms in both HeLa and SH-Sy5Y cells was investigated by Western blotting in order to identify