厚生労働科学研究費補助金
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Fynチロシンキナーゼ・シグナリングを介した統合失調症分子病態の解析

平成２１年度　総括研究報告書

研究代表者　服部　功太郎

平成２２（２０１０）年５月

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研究要旨

本研究の目的は、抗精神病薬伝達機構を担うFynシグナルカスケードの統合失調症分子病態における役割を明らかにし、その臨床応用の可能性を検討することである。

本年度の研究により、主として(1) 統合失調症の血液においてfyn mRNAの発現が低下していること、(2) 統合失調症死後脳においてFynタンパク質の量は変化ないが、活性型Fynの量が増加していること、Fynの基質の1つであるNMDA受容体のサブユニットが減少していること、(3) fynゲノム領域の8つのSNPは、いずれの疾患とも関連がなかったが、いくつかのSNPは正常検体のWAIS-R(知能)やWCST(前頭葉機能)と関連していたことが判明した。

A. 研究目的

統合失調症の分子病態を担うシグナルカスケードを突きとめることは、本疾患の創薬やバイオマーカーの開発につながる。我々はこれまで、マウス等を用いた基礎研究からFynチロシンキナーゼカスケードが、その候補のひとつであることを明らかにしてきた。すなわち、Fynが抗精神病薬によって活性化され、ドーパミンD2受容体とNMDA受容体とを結ぶ細胞内情報伝達を担っていること、この伝達経路は少なくとも抗精神病薬による錐体外路症状発現に関わっており、Fyn欠損マウスでは錐体外路副作用が生じなくなることが判明した。さらに、Fynの発現量が統合失調症の死後脳で変化しているという報告もあり、Fynカスケードが統合失調症の分子病態に直接関与している可能性が高い。

そこで、我々は本研究において、統合失調症の分子病態に、Fynカスケードがどのように関わっているか、そして、Fynカスケードの測定がどのように臨床応用できるかを検討したいと考えている。

B. 研究方法

(1) 血液におけるfyn mRNA発現のcase-control study（１－２年目）

1. 検体：　神経研究所疾病３部の保有するDSM-IVにより診断され、PANSS（陽性・陰性症状評価尺度），BACS（認知機能評価尺度）などを評価済みの統合失調症および、うつ病、双極性障害、健常対照者を含む約700検体の凍結血液RNAサンプルを使用した。

2. 測定方法：　Pax-gene採血管を用いてRNA抽出を行い、SuperScript Viloにてc DNA合成を行った。fyn mRNAの発現解析はTaq-Man probeを用いたReal-time PCR法で行った。トリプレットの平均値を内在性コントロールのGAPDHの平均値で標準化した。各プレート間の標準化はプール検体の希釈系列を用いて行った。

(2) Fynゲノム領域の遺伝子多型関連解析(１年目、解析終了)

神経研究所疾病３部の保有する統合失調症497例および、うつ病約528例、双極性障害200例、健常対照者932例のゲノムDNAを用い、国際HapMapプロジェクトにおいて見出されたfynゲノム上の8個のTag SNPについてTaqMan assayによるタイピングを行った。健常者のうち166例においては、ウィスコンシン・カード・ソーティングテスト(WCST)とIQの評価もされている。

(3) 死後脳におけるFynおよび関連タンパク質の解析（１－２年目）

スタンレー研究所の死後脳（統合失調症、躁うつ病、うつ病、健常者各15例）を用いFynおよび関連分子の解析を行った。解析には独自に確立したFynのSandwich ELISAとドットブロットを用い、スタンレーコンソーシアムに定められた盲検方式で行った。

C. 研究結果

(1)　血液における fyn mRNA 発現の case-control study

統合失調症においてfyn発現の有意な減少が認められた(ANCOVA, age, sex)。双極性障害でも減少傾向があったが有意な差はみられなかった。うつ病では変化がみられなかった。

(2)　Fyn ゲノム領域の遺伝子多型関連解析

いずれの SNP も統合失調症やうつ病、双極性障害との関連は認められなかった。

Fyn の翻訳開始地点近傍の SNP、および同じハプロタイプに存在するもう１つの SNP（両者とも intron）

が健常対象者におけるWCSTやIQの成績と有意に関連していた。

(3) 死後脳における Fyn および関連分子の解析

いずれの疾患の死後脳においても、Fyn タンパク質の量は変化していなかったが、統合失調症では活性型 Fyn の量が有意に亢進していた。また、Fyn 関連分子のうち NMDA 受容体サブユニットである NR2B の量が統合失調症において有意に減少していた。

D. 考察

(1) 血液中のfyn mRNA減少は、我々が前回見出したFynタンパク質の減少に関わっていると考えられた。今後、治療前後や薬剤投与との関連を検討したい。

(2) Fynの遺伝子多型が統合失調症やうつ病などとの病因に関与している可能性は低いことが判明したが、統合失調症の中間表現型の１つである認知機能に関与していたことが判明した。

(3) 死後脳でみられた活性型Fyn量の亢進やNR2Bの減少は、薬剤やストレスなどによる二次性の変化とともに、疾患自体による可能性も考えられた。今後、薬剤投与動物実験などにより、この判別をすすめていきたい。

E. 結論

血液や死後脳の解析によりFynが統合失調症の分子病態に直接的に関与している可能性が示された。今後、バイオマーカーとしてあるいは、治療標的としての可能性を追求していきたい。

F. 健康危険情報

特になし

G. 研究発表

1. 論文発表

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2. 学会発表

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2. 知的財産権の出願登録状況

なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Isosaka T, Kida S, Kohno T, Hattori K, Yuasa S	Hippocampal Fyn activity regulates extinction of contextual fear	Neuroreport	20	1461-5	2009
Hattori K, Fukuzako H, Hashiguchi T, Hamada S, Murata Y, Isosaka T, Yuasa S, Yagi T	Decreased expression of Fyn protein and disbalanced alternative splicing patterns in platelets from patients with schizophrenia	Psychiatry Res	168	119-28	2009

Hippocampal Fyn activity regulates extinction of contextual fear

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Contextual fear memory is attenuated by reexposure of animals to a context alone without pairing it with an unconditioned stimulus, a phenomenon referred to as fear extinction. Here, we report that Fyn tyrosine kinase in the hippocampus is involved in the extinction of contextual fear. We inhibited Src-family tyrosine kinases in the dorsal hippocampus by stereotaxic injection of an inhibitor, PP2, and observed facilitation of extinction. We then biochemically analyzed dorsal hippocampal tissue during extinction training, and found that activated Fyn was significantly downregulated among the Src-family tyrosine kinases examined. These findings suggest that downregulation of Fyn activity facilitates the extinction of contextual fear. *NeuroReport* **20:1461–1465 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Contextual fear conditioning is modeled experimentally by pairing placement of an animal in a training cage with a mild foot shock, so that the animal associates the context with the shock. Contextual memory can be measured by the increased freezing behavior, that is, elicited by reexposure to the context [1]. In contrast, if the animal is reexposed to the same context without pairing with foot shock, the freezing behavior eventually declines, a phenomenon referred to as 'extinction' of contextual fear memory [2,3].

A number of studies have shown that some, but not all, components of the molecular system that is required for the initial encoding of fear memory also regulate extinction [4,5]. We have previously reported finding that a member of the Src family of tyrosine kinases (SFKs), Fyn, plays a crucial role in memory formation in contextual fear conditioning, and that activation of the Fyn signaling pathway in the hippocampus is involved in the formation of contextual fear memory [6]. SFKs in the hippocampus have been reported to be involved in the extinction of fear-motivated memory based on a study that used the passive avoidance paradigm [7]. However, the significance of Fyn in fear memory extinction based on contextual fear conditioning remained to be elucidated.

In this study, we examined the effect of injection of PP2 (an SFK inhibitor [8]) into the hippocampus of mice on extinction of contextual fear memory, and we also examined the profile of hippocampal Fyn activation during the course of extinction training.

Materials and methods

Animals

Male C57BL/6J mice were purchased from Clea (Tokyo, Japan) and housed in the animal center of the institute under standard laboratory conditions as described earlier [9]. One week before the experiments, mice (12 to 13-week old) were housed individually and handled daily. All experiments were carried out in accordance with the guidelines of the United States National Institutes of Health (1996) and were approved by the Animal Care Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry.

Drugs

4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo (3,4–D) pyrimidine (PP2) and 4-amino-7-phenylpyrazol (3,4–D) pyrimidine (PP3; inactive analog of PP2) were purchased from Calbiochem (San Diego, California, USA), dissolved in dimethyl sulfoxide, and brought to a final concentration of 25 μM with saline. The doses of PP2 and PP3 administered were based on the results of a pilot study and an earlier report [7].

Surgery and intrahippocampal injection

Mice were anesthetized with pentobarbital (50 mg/kg, intraperitoneally), and mounted on a stereotaxic apparatus (Narishige, Tokyo, Japan). Bilateral guide cannulae (Eicom, Kyoto, Japan) were implanted with their tips aimed at the dorsal hippocampus (anterior–posterior −2 mm, lateral ± 1.5 mm, depth 1.5 mm from the bregma). The guide cannulae were attached to the skull with dental cement, and dummy cannulae (Eicom) were inserted into the

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guide cannulae during the experiments. PP2, PP3, or vehicle (0.25% dimethyl sulfoxide in saline), 0.8 µl per side was microinjected 1 week after surgery. The microinjections were performed at a rate of 0.4 µl/min through an injection cannula (Eicom) inserted through the guide cannula and connected through Teflon tubing to a microsyringe (Hamilton, Reno, Nevada, USA) driven by a microinfusion pump (Eicom). Cannula placement was verified by sectioning the brains (150 µM thick) coronally on a vibratome and visualizing the position of the track.

Contextual fear conditioning and extinction

The configuration of the context was as described earlier [6]. Contextual fear conditioning and extinction were carried out according to the procedure reported elsewhere [10], with slight modifications, as shown in Fig. 1a. On the day of the conditioning, the mouse was placed in the context and allowed 3 min 30 s to explore. A series of three foot shocks (0.6 mA, 125 V, 2 s) at 28 s intervals was then delivered, and 28 s later, the mouse was returned to its home cage. Extinction training was carried out 24 h after the conditioning. The hippocampus of the mice was injected with PP2, PP3, or vehicle and 10 min later the mice were reexposed to the context for 15 min without foot-shock pairing. One day after the extinction training, fear memory was assessed by measuring freezing time in the context for 5 min (Test). Freezing was defined as lack of any visible movement except respiration [12], and it was monitored by visual inspection of the video images without knowledge of the substance administered.

For the biochemical experiments, mice were conditioned as described above, and 24 h after conditioning, they were reexposed to the context alone for different times (0, 3, 15, or 30 min), and then immediately killed. The mice were designated Ext0, 3, 15, or 30, according to the duration of reexposure to the context alone. As shown in Fig. 2a, in the experiment to confirm the dependence of biochemical changes on extinction, a 'Context' mouse was placed in the context for 5 min without receiving any shocks, and 24 h later, the mouse was reexposed to the context for 30 min, and then immediately killed. A 'Shock' mouse received a series of three foot shocks at 28 s intervals immediately after being placed in the context, and was immediately returned to its home cage. Then, 24 h later, the mouse was reexposed to the context for 30 min, and immediately killed.

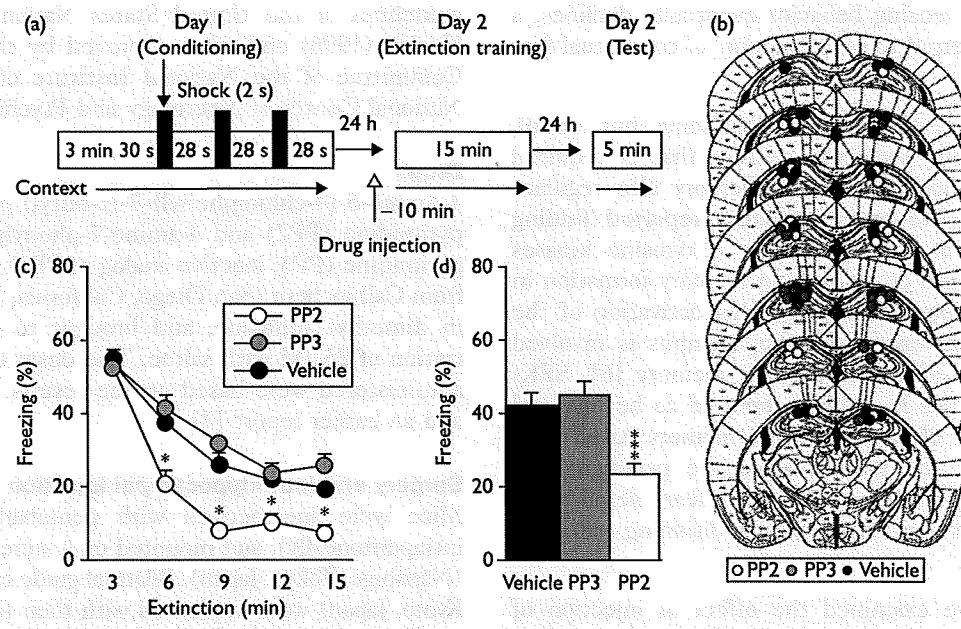
Antibodies

Antibodies against the Tyr418 of SFKs (pSrc418), Src, Yes, and Fyn were used in this study as described earlier [6].

Sample preparation and immunoblot analysis

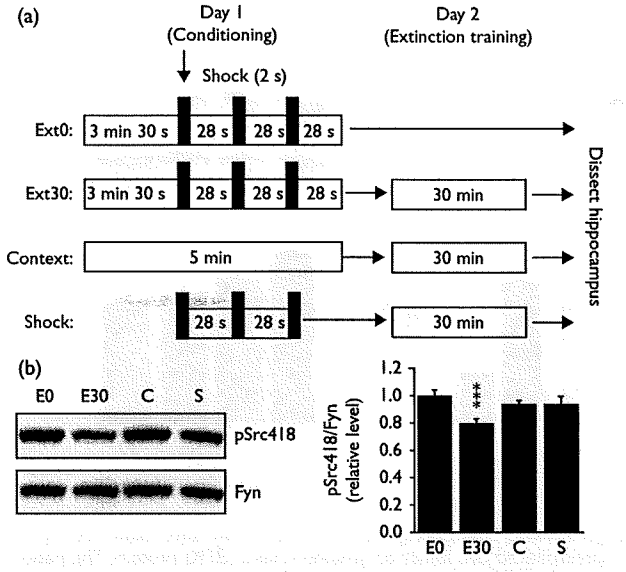
For the immunoblotting studies, mice were killed by cervical dislocation, and hippocampal tissue, mainly consisting of the dorsal hippocampal area, was dissected out as described earlier [6]. The hippocampal tissue was then sonicated in sample buffer containing 1% SDS, 10 mM Tris pH 7.4, 1 mM sodium orthovanadate, and a protease inhibitor cocktail (Roche, Mannheim, Germany), and boiled at 95°C for 3 min. After centrifugation (4000*g*,

Fig. 1



Effect of intrahippocampal injection of PP2 on contextual fear extinction. (a) Behavioral procedure. (b) Position of the tips of the guide cannulae in the hippocampus. Atlas templates were adapted from Paxinos and Franklin [11]. (c) Mean percentage freezing time averaged every 3 min during the extinction training. (d) Mean percentage freezing time during the 5 min test. $*P<0.05$, $***P<0.001$.

Fig. 2



Downregulation of activated Fyn depends on extinction. (a) Behavioral procedure. (b) Western blot analysis of hippocampal tissue obtained from 'Ext0 (E0)','Ext30 (E30)','Context', and 'Shock' mice. The level of pSrc418 is shown as the density of pSrc418 normalized to the density of Fyn. The 'Ext0' level was set to 1.0. ***$P<0.001$. C, Context; S, Shock.

10 min), the supernatants were added to 4 × SDS sample buffer (50% glycerol, 125 mM Tris–HCl pH 6.8, 4% SDS, 0.08% bromophenol blue; Kinexus/*www.kinexus.ca*) and 40 mM dithiothreitol (Sigma, St. Louis, Missouri, USA), and boiled at 95°C for 2 min. An equal amount of lysate (10 µl per lane) was separated by SDS–polyacrylamide gel electrophoresis. The separated proteins were subsequently blotted onto Immobilon membranes (Millipore Co., Billerica, Massachusetts, USA), probed with each antibody, and visualized as described earlier [6].

Immunoprecipitation

Immunoprecipitation was performed as reported earlier [6] with slight modification. The sample buffer used in this study contained 50 mM Tris–HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM sodium orthovanadate, and a protease inhibitor cocktail (Roche).

Statistical analysis

All data are reported as means ± SEM. Student's *t*-test was used for pair-wise comparisons. One-way analysis of variance (ANOVA) or two-way repeated-measure ANOVA, followed by Tukey–Kramer test was used for biochemical or behavioral analysis as appropriate. *P* values less than 0.05 were considered statistically significant.

Results

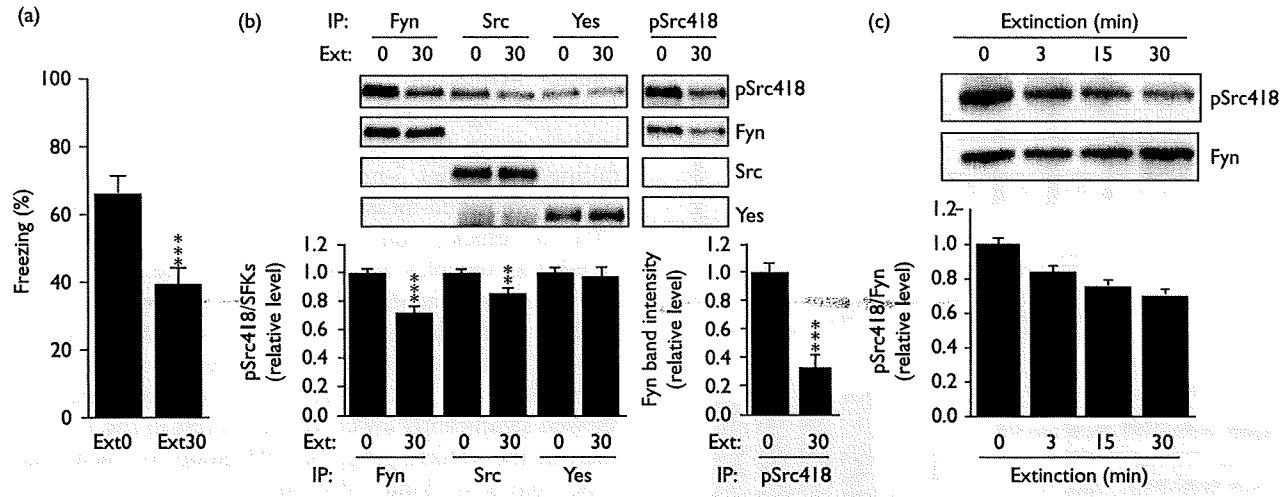
To examine the effect of the SFK inhibitor on contextual fear extinction, the hippocampus of mice was implanted with a microcannula, and the mice were trained according to the behavioral schedule shown in Fig. 1a. Placement of the cannula tip for microinjection was almost identical across all groups of mice included in the analysis (Fig. 1b). During extinction training, the PP2 group ($n = 11$) exhibited significantly lower freezing levels than the vehicle group ($n = 12$) or PP3 group ($n = 11$), except during the initial 3-min extinction period (Fig. 1c). A two-way repeated-measure ANOVA with drug group (PP2, PP3, or vehicle) and extinction time (3, 6, 9, 12, and 15 min) as variables was performed on the freezing scores for extinction training, and the results showed a main effect of drug group [$F(2,31) = 12.773$, $P < 0.0001$], extinction time [$F(4,124) = 160.63$, $P < 0.0001$], and interaction [$F(8,124) = 5.5$, $P < 0.0001$]. Post-hoc Tukey–Kramer test showed significantly lower freezing levels in the PP2 group than in the vehicle group or PP3 group for each training time from 6 to 15 min ($P < 0.05$).

Next, we examined whether the extinction facilitated by the injection of PP2 before the extinction training was maintained for 24 h later. As shown in Fig. 1d, the freezing time during the test (5 min) was significantly lower in the PP2 group ($n = 11$) than in the vehicle group ($n = 12$) ($P < 0.001$). The freezing time in the PP3 group ($n = 11$) was unchanged in comparison with the vehicle group.

As shown in Fig. 1, inhibition of SFKs facilitated the extinction of contextual fear. However, as PP2 is not selective for any individual SFK, next we attempted to biochemically identify the SFK that was involved in the extinction. On the basis of the pilot study, we conducted stronger extinction training consisting of a 30 min reexposure to the context to identify clear differences between the SFKs. As shown in Fig. 3a, the mice reexposed to the context for 30 min (Ext30, $n = 12$) exhibited significantly lower freezing levels than the control mice (0 min reexposure; Ext0, $n = 12$) during the test ($P < 0.001$).

Next, we attempted to identify the SFK that exhibited a significant change after the extinction training. Hippocampal tissue was dissected immediately after training, and immunoprecipitation was performed with antibody against individual SFKs (Fyn, Src, and Yes). Thirteen to 14 animals were used in each group. Each immunoprecipitate was probed with the antibody against the phosphorylated Tyr418 residue of SFKs (pSrc418), because SFKs are activated by phosphorylation at Tyr418. As shown in Fig. 3b, the pSrc418 level in the immunoprecipitated Fyn ($P < 0.001$) and Src ($P < 0.01$) of the 'Ext30' group was significantly lower than in the 'Ext0' group. The immunoprecipitate with anti-pSrc418 was then probed with each SFK antibody. The amount of Fyn in the 'Ext30' immunoprecipitate was significantly lower than in the 'Ext0' immunoprecipitate ($P < 0.001$), and either Yes or Src was hardly detected in the anti-pSrc418 immunoprecipitate. On the basis of these

Fig. 3



Activated Fyn in the hippocampus after extinction training. (a) Mean percentage freezing time during the 5 min test 1 day after extinction by reexposure to the context for 30 min. (b) Western blot analysis of each immunoprecipitated Src family of tyrosine kinase (SFK) protein. The panel on the left shows the density of pSrc418 normalized to that of immunoprecipitated Fyn, Src, and Yes, respectively. The panel on the right shows the density of Fyn in the immunoprecipitate by anti-pSrc418. (c) Western blot analysis of the hippocampal tissue during the course of extinction training. The level of pSrc418 is shown as the density of pSrc418 normalized to the density of Fyn. The level at 'Ext0' was set to 1.0. $^{**}P<0.01$, $^{***}P<0.001$. IP, immunoprecipitation.

findings, activated Fyn accounted for most of the activated SFK in the hippocampus, and for the most significant decrease after extinction training.

We then investigated the changes in activated Fyn during the course of extinction training by performing Western blotting of hippocampal tissue dissected immediately after extinction training for different times (3, 15, 30 min). Thirteen to 14 animals were used in each group. As shown in Fig. 3c, the pSrc418 level decreased during the course of extinction training. A one-way ANOVA of the pSrc418 level during extinction training showed a main effect of extinction time [$F(3,50) = 14.752$, $P < 0.0001$].

We also investigated whether the decrease in activated Fyn was dependent on the extinction of contextual fear memory by exposing mice to the behavioral procedure shown in Fig. 2a. Ten to 12 mice were used in each group. In this experiment, the 'Context' group was exposed to the context alone without pairing with a foot shock. The 'Shock' group received foot shocks immediately after being placed in the context. This procedure causes a defective association between the context and the shock, called 'immediate shock deficit' [13,14]. As shown in Fig. 2b, the pSrc418 level decreased significantly in the 'Ext30' group in comparison with the 'Ext0' group ($P < 0.001$). In contrast, the differences in pSrc418 levels in the 'Context' and 'Shock' group were not statistically significant from the level in the 'Ext0'group. This finding clearly confirms that the downregulation of activated SFKs, mostly activated Fyn, depends on fear memory extinction.

Discussion

The results of this study show that inhibition of hippocampal SFKs activity by PP2 facilitates extinction of contextual fear. In contrast, the level of activated Fyn in the hippocampus decreased significantly during the course of extinction training, and the decrease was dependent on the extinction of contextual fear memory. These results strongly suggest that downregulation of hippocampal Fyn activity facilitates the extinction of contextual fear memory.

Downregulation of cyclin-dependent kinase 5 (Cdk5) in the hippocampus has also been reported to facilitate extinction of contextual fear [15]. Fyn regulates Cdk5 activity through Tyr15 phosphorylation, and Fyn and Cdk5 are involved in the regulation of dendritic growth through the control of cytoskeletal reorganization [16]. Actin rearrangement and the subsequent alteration of synaptic structures in the hippocampus are now considered to be one of the mechanisms that underlie fear memory extinction [17]. Accordingly, both Fyn and Cdk5 in the hippocampus are presumed to be involved in the regulation of contextual fear extinction by crosstalk for synaptic remodeling through the cytoskeletal rearrangement.

We have reported earlier that activation of the Fyn signaling pathway in the hippocampus is required for acquisition of contextual fear memory [6], and the results of this study suggest that contextual fear extinction is regulated by counteracting the Fyn signaling pathway, and that mechanisms that downregulate activated Fyn might be involved in the process of extinction. The phosphatase activity of calcineurin in the amygdala has been reported

to be upregulated during extinction, and Akt, which is involved in fear memory acquisition, has been reported to be dephosphorylated [18]. Accordingly, any tyrosine phosphatase that dephosphorylates the activated Fyn may be an upstream regulator of Fyn in fear memory extinction. Further study will be necessary to identify the phosphatase that acts on the Fyn signaling pathway in extinction of contextual fear.

Conclusion

We showed that inhibition of SFK activity in the hippocampus facilitates extinction of contextual fear. Furthermore, among the SFKs investigated only activated Fyn was downregulated after extinction learning. These results strongly suggest that Fyn regulates extinction of contextual fear memory.

Acknowledgements

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Decreased expression of Fyn protein and disbalanced alternative splicing patterns in platelets from patients with schizophrenia☆

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Abstract

Fyn, a Src-family kinase, is highly expressed in brain tissue and blood cells. In the mouse brain, Fyn participates in brain development, synaptic transmission through the phosphorylation of *N*-methyl-D-aspartate (NMDA) receptor subunits, and the regulation of emotional behavior. Recently, we found that Fyn is required for the signal transduction in striatal neurons that is initiated by haloperidol, an antipsychotic drug. To determine whether Fyn abnormalities are present in patients with schizophrenia, we analyzed Fyn expression in platelet samples from 110 patients with schizophrenia, 75 of the patients' first-degree relatives, and 130 control subjects. A Western blot analysis revealed significantly lower levels of Fyn protein among the patients with schizophrenia and their relatives, compared with the level in the control group. At the mRNA level, the splicing patterns of *fyn* were altered in the patients and their relatives; specifically, the ratio of *fyn*Δ7, in which exon 7 is absent, was elevated. An expression study in HEK293T cells revealed that FynΔ7 had a dominant-negative effect on the phosphorylation of Fyn's substrate. These results suggest novel deficits in Fyn function, manifested as the downregulation of Fyn protein or the altered transcription of the *fyn* gene, in patients with schizophrenia.

Keywords: Schizophrenia; Fyn tyrosine kinase; Platelet; Alternative splicing; Dominant-negative

1. Introduction

Fyn (OMIM # 137025), a member of the Src-family tyrosine kinases, is highly expressed in brain tissue and blood cells. In the mouse brain, Fyn participates in brain development as well as adult neuronal functions. The

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disruption of the *fyn* gene in mice results in the disarray of hippocampal cell orientation (Grant et al., 1992), defective cortical upper layer formation (Yuasa et al., 2004), and myelin deficits in the forebrain (Sperber et al., 2001). Interestingly, hippocampal pyramidal cell disarray (Kovelman and Scheibel, 1986; Conrad et al., 1991; Casanova and Rothberg, 2002), abnormal cortical upper layer formation (Arnold et al., 1991; Selemon et al., 1998; Benes et al., 2001) and deficits in myelination (Davis et al., 2003; Tkachev et al., 2003) have also been reported in brain tissue from patients with schizophrenia.

In postnatal brains, Fyn is localized at postsynaptic densities and regulates NMDA receptor activity by phosphorylating its NR2B subunits (Salter and Kalia, 2004). This Fyn-mediated phosphorylation of NR2B is involved in learning (Grant et al., 1992), ethanol tolerance (Miyakawa et al., 1997), and susceptibility to seizures (Kojima et al., 1998). Fyn also regulates emotional behavior in mice. Fyn-deficient mice exhibit increased fearfulness and defective maternal behavior (Yagi, 1999).

Recently, we found that Fyn is also involved in dopamine D2 receptor (D2-R) signaling (Hattori et al., 2006). Fyn-deficient mice do not manifest the extrapyramidal symptoms induced by haloperidol. Haloperidol or a D2-R-selective antagonist, L741,626, induced Fyn activation, and subsequently NR2B phosphorylation, in the striatum of wild-type mice but not in that of Fyn-deficient mice. Using calcium imaging, we also found that Fyn is required for the D2-R-mediated regulation of NMDA receptors in striatal neurons. Clozapine was also reported to upregulate NMDA receptor function in the nucleus accumbens of mouse brain; this effect was inhibited by a Src-family-tyrosine-kinase inhibitor (Wittmann et al., 2005). Thus, Fyn participates in the molecular pharmacology of antipsychotics.

Platelets are membrane-encapsulated fragments of megakaryocytes. Although platelets have no nucleus, they are metabolically active and are able to express functional membrane receptors such as 5HT-2A receptors (Plein and Berk, 2001), NMDA receptors (Hitchcock et al., 2003) and D2-R (Martini et al., 2006). Since platelets have several properties in common with monoaminergic neurons, both of which can transport, metabolize, store and release monoamines — especially for 5-HT- they are often used as a peripheral marker in psychiatric illnesses, including major depression, panic disorder and schizophrenia (Plein and Berk, 2001). Among hematopoietic cells, platelets contain particularly high levels of Fyn (Brickell, 1992), which participates in platelet aggregation (Crosby and Poole, 2003).

Taking these findings into account, we used Western blotting to evaluate the expression of Fyn protein in platelets from patients with schizophrenia, first-degree relatives of the patients, and healthy controls. Furthermore, we semi-quantitatively examined the *fyn* transcripts (*fyn*B, *fyn*T and *fyn*Δ7) in platelets from the patients and their relatives. Consequently, we found a reduction in Fyn protein and a relative increase in *fyn*Δ7 in the patients with schizophrenia and their relatives. We further analyzed the function of FynΔ7 protein using over-expression studies in HEK293T cells and observed a dominant-negative effect on the phosphorylation of Fyn's substrate. The significance of the altered expression of *fyn* to the pathogenesis of schizophrenia is also discussed in the present report.

2. Methods

2.1. Clinical subjects

Participants comprised 110 patients with schizophrenia (60 men and 50 women, mean age 31.4 ± 10.6 years) who had been diagnosed and subtyped according to DSM-IV criteria by two well-trained psychiatrists (H.F. and T.H.) Antipsychotic medications at clinically determined dosages were being administered to 102 patients. Psychiatric symptoms were assessed using the Brief Psychiatric Rating Scale (BPRS) (Ventura et al., 1993) by the same psychiatrists. Seventy-five first-degree relatives of the patients (40 men and 35 women, mean age 54.7 ± 11.4 years) who were ascertained and interviewed by the same psychiatrists also participated in this study. Relatives with a psychiatric history were excluded. For non-patient controls, 130 volunteers who did not have a psychiatric history (67 men and 63 women, mean age, 27.8 ± 6.5 years) were recruited from among both the hospital staffs and the students at Kagoshima University. None of the participants showed evidence of acute infectious, inflammatory, or neurological diseases. Informed consent was obtained from all the individuals. The procedures used in this study were approved by the ethics committees of both Osaka University and Kagoshima University.

2.2. Sample preparation

Peripheral blood samples were collected between 2 pm and 3 pm and were processed for platelet preparation within 2 h. Platelets were obtained by differential centrifugation (Lingjaerde and Kildemo, 1981). Briefly, samples containing 30 ml of heparinized peripheral

blood were spun at 50×g for 15min; the supernatant was then spun at 1000×g for another 15min. The resulting precipitate was dissolved in 1 ml of phosphate- buffered saline (PBS), divided into three tubes, and centrifuged at 1000×g for 15 min; the precipitates were then frozen and stored in liquid nitrogen until use.

2.3. Western blotting

The Western blotting method used in this study was described previously (Hattori et al., 2006). Briefly, samples were solubilized in SDS sample buffer (50 mM Tris–HCl [pH 6.8], 5% SDS, 10% glycerol, 1 mM sodium orthovanadate), sonicated, and spun at 20,000×g for 15 min. The supernatant was collected, and the protein concentration was measured using a BCA Protein Assay (Pierce). After the addition of 40 mM of dithiothreitol, the samples were boiled for 5 min, and an equal amount of protein (20 μg) from each sample was subjected to SDS-polyacrylamide (10%) gel electrophoresis. The proteins were then transferred to PVDF membranes. The membranes were blocked with 10% skim milk, probed with a rat monoclonal anti-Fyn antibody (γC3, raised by Dr. Masahiro Yasuda (Yasunaga et al., 1996)), and further probed with a peroxidase-conjugated anti-rat IgG antibody (diluted 1:20,000; Jackson). Immunoreactive proteins were visualized using ECL-plus (Amersham) and an ATTO Cool Saver (ATTO). The membranes were then stripped with stripping buffer (100 mM β-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris–HCl [pH 6.7]) at 50 °C for 30 min. After being washed and blocked, the membranes were reprobed with an anti-β-tubulin antibody (H-235, diluted 1:4000; Santa Cruz) followed by a peroxidase-conjugated anti-rabbit IgG antibody (diluted 1:20,000; Jackson). As a pilot study, we evaluated the Fyn concentrations in platelet samples obtained from five control subjects at six time points during a 3-week time period. A two-way analysis of variance (ANOVA) detected no significant differences in the Fyn band densities measured at the various time points in each subject, but significant differences among the control subjects were detected (Fig. S1).

2.4. Chronic antipsychotic treatment in mice

Thirty 10-week-old C57BL/6J male mice were purchased from Clea Japan (Tokyo, Japan). Haloperidol decanoate (Janssen Pharmaceutical) was diluted with sesame oil (The Japanese Pharmacopoeia) and was injected intramuscularly (in the quadriceps muscle) using an insulin syringe (Terumo 0.5mL, 29G × 1/2) at a dose of 20 μl/10 g body weight for 3 weeks (one injection per week) under anesthesia (pentobarbital, 50 mg/kg). One week after the third injection, blood was collected under anesthesia by cardiac puncture using syringes containing 10 U of heparin. Platelets were prepared as described above with appropriate modifications for the mouse samples (Schraw et al., 2003). In brief, blood was diluted with an equal volume of saline, and 0.5 ml of sample was spun at 100×g for 10 min; the supernatant was then spun at 5000×g for 2 min. The samples were then analyzed as described above. All experimental procedures were performed in accordance with the guidelines of the United States' National Institutes of Health (1996) and were approved by the Animal Care Committee of the National Institute of Neuroscience, NCNP.

2.5. RNA extraction, RT-PCR, and cloning

Total RNA was isolated from samples using TRIzol reagent (Invitrogen) and reverse transcribed using Super-Script II (Invitrogen) and an oligo-dT primer. As a result of alternative splicing, the *fyn* gene encodes three isoforms: *fyn*B, which includes exon 7A; *fyn*T, which includes exon 7B; and *fyn*Δ7, which lacks exon 7 (Goldsmith et al., 2002). The *fyn*B, *fyn*T, or *fyn*Δ7 cDNA region, spanning a region from nucleotide 38 upstream of the start codon through to nucleotide 64 downstream of the stop codon, was amplified using LA-Taq polymerase (Takara) and the primers hfyn-30F (5′-AGTTTGT-GATCGTTGGCG-3′) and hfyn+50R (5′-TGAAAGC-TAATGGGGAGG-3′). The PCR products were then ligated into a pBluescript SK-vector (Stratagene). The nucleotide sequences of *fyn*B and *fyn*T were based on GenBank NM_002037 and S74774, respectively.

2.6. Competitive RT-PCR

Modifications of the PCR and runoff procedures (Weil et al., 1999) were used. Briefly, cDNA was amplified using Ex-Taq polymerase (Takara) with the primers hfyn390F (5′-ACAGGTTACATTCCCAGC-3′) and FAM-hfyn850R (5′-FAM-GTGTTTCCATAC-CAGGTACC-3′). The PCR fragment was then purified using Suprec-PCR (Takara) and analyzed using an ABI Prism 3100 Genetic Analyzer.

2.7. Real-time RT-PCR

The methods for Real-time PCR have been described previously (Isosaka et al., 2006) using SYBR Green Mix and an ABI PRISM 7900 HT (Applied Biosystems).

The primer combinations were hGAPDH20F (5′-GTCAACGGATTTGGTCGTATTGG-3′) and hGAPD-Hex3RV (5′-GACAAGCTTCCCGTTCTCAG-3′) for GAPDH, and hfyn610F (5′-AAAATTCGCAAACTT-GACAATGGT-3′) and hfynT719R (5′-AAACA-CAAACCGTCAGCTTTCTCT-3′) for *fyn*T. *fyn*T- and GAPDH-containing plasmids were prepared, and serial dilutions of the plasmids were used as standards. The number of molecules of each cDNA in a given sample was normalized to the number of GAPDH cDNA molecules in the same sample. Samples that contained less than 1000 GAPDH molecules or less than 10 *fyn*T molecules per test tube (1 μl cDNA) were omitted. Expression levels (*fyn*T / GAPDH × 1000) within the mean ± 2 × S.D. were included in further analyses.

2.8. In vitro expression

Full-length cDNAs for *fyn*B, *fyn*T, and *fyn*Δ7 in pBlueScript SK-vectors described above were transferred into the pcDNA 3.1 (Invitrogen) expression vector. The expression plasmid for the Fyn substrate, CD8-ζ chimera (Ghosh et al., 2004) was a kind gift from Dr. Sachiko Miyake (National Institute of Neuroscience, NCNP, Japan), with the permission of Dr. Hamid Band (Northwestern University). HEK293T cells were transfected with the constructs using Lipofectamine 2000 reagent (Invitrogen). After 24 h, the cell samples were harvested in the sample buffer and Western blotted, as described above. The immunoprecipitation methods were described previously (Hattori et al., 2006). Briefly, cells were lysed in lysis buffer (10 mM Tris–HCl [pH 7.5], 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 1 mM Na_3VO_4), sonicated and spun down to remove insoluble material. The samples were then precleared with protein G-sepharose (Amersham), incubated for 1 h at 4 °C with 1 μg of anti-Fyn antibody (γC3), and then incubated with 10 μl of protein G-sepharose. After three washes with lysis buffer, the pelleted protein G-sepharose was boiled for 5 min in 30 μl of SDS sample buffer. The separated proteins were then Western blotted, as described above. To evaluate tyrosine phosphorylation, the blot was probed with an anti-phosphotyrosine antibody, pY100 (Cell Signaling Technology), followed by peroxidase-conjugated anti-mouse IgG. To evaluate CD8-ζ the blot was probed with anti-CD3-ζ antibody (6B10.2; Santa Cruz), followed by peroxidase-conjugated anti-mouse IgG.

2.9. Kinase assay

HEK293T cells transfected with *fyn*B, *fyn*T, or *fyn*Δ7 in 60-mm dishes were eluted in 1 ml of TNE buffer (10 mM Tris–HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, 1 mM Na_3VO_4, 1% Nonidet P-40). The eluate was then immunoprecipitated, as described above, with 2 μg of anti-Fyn antibody (γC3). After being adsorbed to protein G-sepharose, the immunoprecipitates were washed three times with TNE buffer and twice with kinase assay buffer (50 mM Tris–HCl [pH 7.4], 3 mM $MnCl_2$, and 0.1 mM Na_3VO_4). For the kinase assay, the immunoprecipitate, 2.5 μg of acid-treated enolase, and 10 μCi of γ ^{32}P-ATP were added to 25 μl of kinase assay buffer. Phosphorylation was allowed to proceed at 30°C for 10 min, and the phosphoproteins were resolved by SDS-polyacrylamide (10%) gel electrophoresis, followed by autoradiography.

2.10. Statistical analyses

Unless specified, Bartlett's test was used to analyze the homogeneity of variance, and the differences between the means were compared using a one-way ANOVA, followed by a post-hoc Dunnett's test (StatView-J 5.0).

3. Results

3.1. Fyn protein concentration is lower in platelets from patients with schizophrenia

To survey the patients with schizophrenia for Fyn protein abnormalities, we examined platelets from the

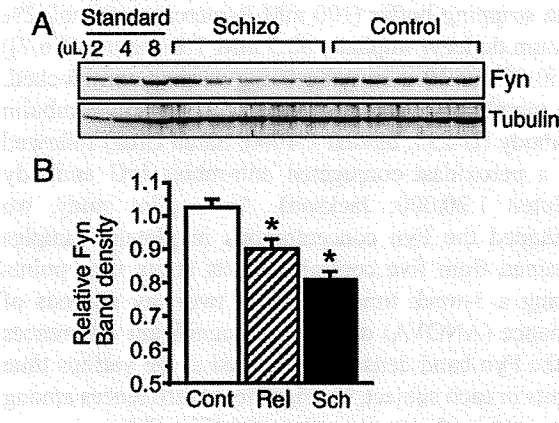

Fig. 1. Decreased Fyn protein in platelets from patients with schizophrenia. (A) Representative blot of the Fyn protein analysis. Many samples from patients with schizophrenia (Schizo) exhibited weaker Fyn immunoreactivity than the control samples. (B) The mean intensity of the Fyn band was significantly lower in the patient group – and to a lesser degree in the relative group – than in the control group. The density of each 59-kD band was measured and compared with that of a standard sample (4 μl lane, density=1.0) that was run on every blot. Control (Cont, n =110); relative (Rel, n =71); schizophrenia (Sch, n=100). Each bar represents the mean±SEM. *, $P<0.01$; one-way ANOVA followed by Dunnett's test.

patients, their first-degree relatives, and healthy control subjects using Western blotting for Fyn protein. The Fyn-immunoreactive bands from the healthy control subjects were consistent and strong. In contrast, many samples from the patients with schizophrenia showed weak Fyn immunoreactivity (Fig. 1A). To further examine the relationship between Fyn immunoreactivity and schizophrenia, we measured the density of each band and standardized the densities by comparing them with the densities of the standard lanes (a mixture of 10 control samples with volumes of 2, 4 or 8 μl and a band density of 1.0 for the 4-μl sample) on each membrane. The mean intensity was significantly lower among the patients with schizophrenia, and to lesser extent among their relatives (Figs. 1B and S2A), than among the control group. Age, schizophrenia subtypes (Fig. S3A), BPRS scores and antipsychotic dose had no effects on the Fyn band densities. We also found that chronic haloperidol treatments (haloperidol decanoate, 0–25 mg/kg, single injection per week for 3weeks) did not change the platelet Fyn concentration in mice (Fig. S4).

3.2. Detection of an alternative splice form of the *fyn* transcript in samples from patients with schizophrenia

To determine whether the above-mentioned reduction in Fyn protein originated because of an alteration in the *fyn* transcript, we performed RT-PCR for mRNA from each platelet sample. After amplifying the full-length *fyn* cDNA, we found an extra fragment that was ~150 bp shorter than the normal *fyn* transcripts, *fyn*T and *fyn*B (Fig. 2A). The intensity of the extra band was stronger in the samples from the patients with schizophrenia (Fig. 2A) and their relatives (Fig. S2B) than in the control samples. The extra band was cloned and sequenced, and the product was identified as a *fyn* splice variant lacking exon 7, previously described as *fyn*Δ7 (Goldsmith et al., 2002) (Fig. 2B).

3.3. *fyn*Δ7 mRNA is preferentially transcribed in samples from patients with schizophrenia

Because the result of the above-mentioned PCR study suggested that the expression of *fyn*Δ7 was higher in the samples from patients with schizophrenia, we performed semi-quantitative RT-PCR (Weil et al., 1999) to evaluate the relative amounts of each *fyn* isoform. mRNA from each sample was amplified with primers that hybridized to regions upstream and downstream of exon 7 (Fig. 2B). Electropherograms of the PCR products obtained using an automated sequencer revealed that these PCR products contained *fyn*B, *fyn*T, and *fyn*Δ7; *fyn*B differed from *fyn*T by 9bp, and *fyn*Δ7 was 156bp shorter than *fyn*T (Fig. 2C). The

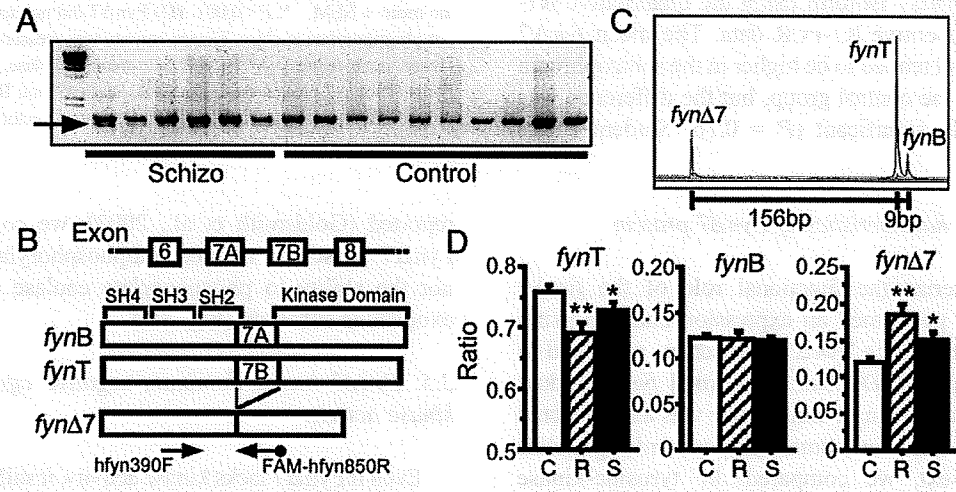

Fig. 2. Splicing patterns of *fyn* in samples from patients with schizophrenia. (A) Electrophoresis of *fyn* PCR products shows that in addition to the main band (~1600 bp), a smaller band (~1450 bp) was more obvious in the samples from the patients with schizophrenia (Schizo). (B) Schematic representation of the splice variants of *fyn*. As a result of alternative splicing, *fyn* produces 3 isoforms: *fyn*B, which includes exon 7A; *fyn*T, which includes exon 7B; and *fyn*Δ7, which lacks exon 7. Cloning and sequencing revealed the extra band (~1450 bp) to be *fyn*Δ7. (C) mRNA from each sample was amplified using semi-quantitative RT-PCR and primers that hybridized to either side of exon 7. An electropherogram created using an automated sequencer revealed that the PCR products were *fyn*B, *fyn*T, and *fyn*Δ7. The lengths and fluorescence intensities of the products were measured and compared. (D) The mean ratio of the *fyn*T intensity divided by the sum of the intensities of all 3 isoforms was significantly lower in the patient and relative groups, compared with that in the control group. The mean ratio of *fyn*B was similar for each group. The mean ratio of *fyn*Δ7 was significantly higher in the patient and relative groups, compared with that in the control group. Control (C, n =127); relative (R, n =55); schizophrenia (S, n =100). One-way ANOVA followed by Dunnett's test. Each bar represents the mean±SEM. *, $P<0.05$; **, $P<0.01$.

fluorescence intensities of each product were also measured and compared. As shown in Fig. 2D, the mean ratio of the *fyn*B intensity divided by the sum of the intensities of all three isoforms was similar for each group. On the other hand, the mean intensity of *fyn*T was significantly lower while that of *fyn*Δ7 was significantly higher in the patients with schizophrenia and their relatives, compared with that in the control group (Fig. 2D). The extent of the increased *fyn*Δ7 ratio tended to be greater among the relatives than among the patients with schizophrenia. A correlation analysis showed no significant relationship between the level of the Fyn protein density and the *fyn*Δ7 expression ratio. Age, schizophrenia subtypes (Fig. S3B), BPRS scores and antipsychotic dose had no significant effects on the *fyn*Δ7 expression ratios. To clarify whether the *fyn* mRNA expression level had changed in the patients with schizophrenia, we analyzed the amount of one of the splice variants, *fyn*T, using quantitative RT-PCR in the patient and control samples. Samples with less than 1000 GAPDH molecules or less than 10 *fyn*T molecules per test tube (1 μl cDNA) were omitted, and samples with expression levels (*fyn*T / GAPDH × 1000) that fell within the mean ± 2 S.D. were included in subsequent analyses. The mean expression levels of *fyn*T in the patient and control groups were not significantly different (Fig. S5A). Then, we calculated the expression levels of the *fyn*Δ7 isoform using the quantitative RT-PCR and competitive RT-PCR data. The mean *fyn*Δ7 expression level tended to be higher in the schizophrenia group than in the control group, but the difference was not statistically significant ($P = 0.16$, Student *t*-test) (Fig. S5B).

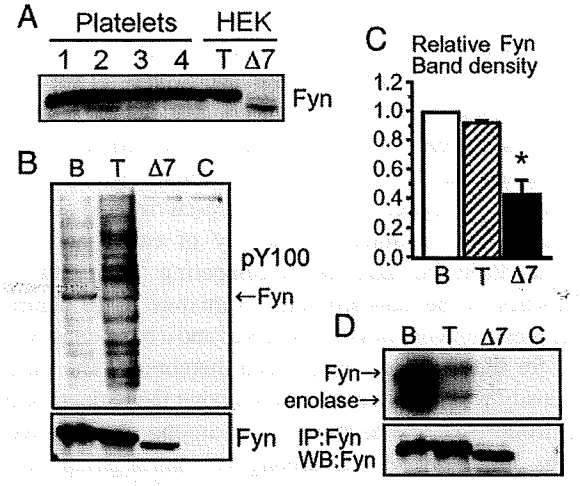

Fig. 3. General characteristics of FynΔ7 protein. (A) In addition to normal Fyn products (59 kDa), extra Fyn bands with sizes similar to that of the FynΔ7 (53-kDa) protein produced in HEK293T cells were observed in the samples from patients with schizophrenia. Platelets (1, control; 2–4, schizophrenia). (B) The *fyn*Δ7 vector did not produce kinase activity and produced a lower amount of Fyn protein. While transfection with *fyn*B or *fyn*T vectors enhanced tyrosine phosphorylation in HEK293T cells, as detected using an anti-phosphotyrosine antibody (pY100), such enhancement was not detected after *fyn*Δ7 transfection. In addition, the Fyn band density of FynΔ7 was lower than that of FynB or FynT. Equal amounts (20 μg) of total cell lysate protein from each sample were analyzed using western blotting. (C) One-way ANOVA followed by the Bonferroni/Dunn test revealed that the mean band density of FynΔ7 was significantly lower than that of FynB of FynT. Each bar represents the mean ± SEM. *, $P<0.001$. (D) FynΔ7 did not autophosphorylate nor phosphorylate enolase. Kinase activity was measured by an *in vitro* kinase assay using [γ- ^{32}P]ATP, acid-treated enolase, and one of the FynB, T, or Δ7 protein isoforms. WB, Western blot; IP, immunoprecipitation; B, *fyn*B; T, *fyn*T; Δ7, *fyn*Δ7; C, control vector (pcDNA 3.1).

3.4. General characteristics of FynΔ7 protein

To characterize the functional role of the *fyn*Δ7 transcript, we performed an expression analysis using HEK293T cells. The *fyn*Δ7 transcript produced a smaller protein (53kD) than the normal *fyn*T product (57kDa), and a protein of similar size was also detected as an extra band in some schizophrenia platelets (Fig. 3A). Next, we compared the tyrosine-kinase activity of each *fyn* isoform in HEK293T cells. While transfection with vectors containing *fyn*B or *fyn*T enhanced the protein tyrosine phosphorylation, such enhancement was not detected after *fyn*Δ7 transfection (Fig. 3B). We also found that transfection with the *fyn*Δ7 vector produced a smaller amount of Fyn protein, compared with the *fyn*B and *fyn*T vectors (Fig. 3B, C). To further characterize the kinase activity of FynΔ7, we performed an *in vitro* kinase assay. As previously reported (Goldsmith et al., 2002), we confirmed that FynΔ7 possessed neither autophosphorylation activity nor the ability to phosphorylate enolase added as an exogenous substrate (Fig. 3D).

3.5. FynΔ7 has a dominant-negative effect on Fyn's kinase activity

Even if FynΔ7 lacks kinase activity, it still has SH3 and SH2 domains that are important for substrate binding or ligand recognition (Li, 2005). Therefore, we supposed that FynΔ7 may have a dominant-negative effect on Fyn's kinase activity. To test this possibility, HEK293T cells were transfected with plasmids encoding FynB protein and/or FynΔ7 protein plus a Fyn substrate, CD8-ζ chimeric protein (Ghosh et al., 2004); CD8-ζ phosphorylation was then analyzed using Western blotting (Fig. 4A). The FynB-induced phosphorylation of CD8-ζ was attenuated by the

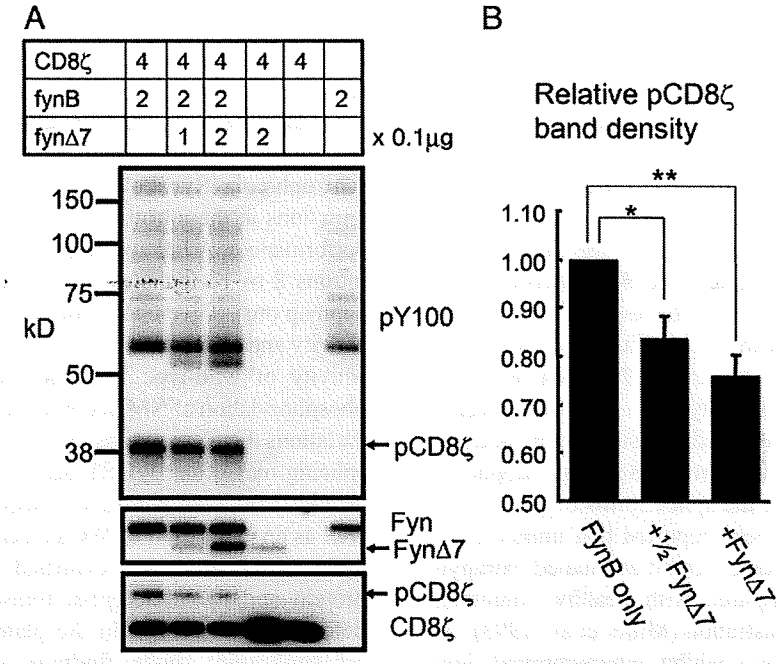

Fig. 4. Expression of FynΔ 7 attenuates the tyrosine phosphorylation of Fyn's substrate, CD8-ζ chimera. (A) HEK293T cells were transfected with plasmids encoding FynB protein and/or FynΔ 7 protein and a CD8-ζ chimeric protein as an internal indicator of tyrosine phosphorylation activity. The total amount of plasmid DNA was adjusted to 0.8 μg using pcDNA3.1. Twenty-four hours after transfection, the cell lysate proteins were resolved by SDS-PAGE, transferred, and probed with anti-phosphotyrosine, pY100, anti-Fyn and anti-CD3-ζ (also recognizes CD8-ζ) antibodies. The addition of FynΔ 7 attenuated the phosphorylation of CD8-ζ. (B) Densitometric analysis of the Fyn-mediated phosphorylation of CD8-ζ. The band densities of phospho-CD8-ζ (38 kD) on the pY100 immunoblots were measured and normalized by the FynB (59 kD) band densities. FynΔ7 dose-dependently attenuated CD8-ζ phosphorylation. Each bar represents the mean±SEM. *, $P<0.01$; **, $P<0.001$. One-way ANOVA followed by the Bonferroni/Dunn test.

coexpression of FynΔ7 (Fig. 4B). FynΔ7 had the same effect on the FynT-mediated phosphorylation of CD8-ζ (data not shown). Therefore, FynΔ7 had a dominant-negative effect on wild-type Fyn kinase activity.

4. Discussion

In the present study, we revealed a decrease in Fyn protein levels and an increase in *fyn*Δ7 mRNA ratio, a defective *fyn* isoform, in platelets from patients with schizophrenia. We also found that FynΔ7 induces a dominant-negative effect on the phosphorylation of Fyn's substrate.

Altered expression levels of several molecules have been reported in platelets from patients with schizophrenia. These alterations include increased levels of the 5-HT_{2A} receptor (Arranz et al., 2003), the integrin $\alpha_{IIb}\beta_{IIIa}$ receptor (Thakore et al., 2002), and mitochondrial complex I (Dror et al., 2002), and a decrease in the cAMP-dependent protein kinase (PKA) regulatory subunit (Tardito et al., 2000). Interestingly, the integrins and PKA are functionally related to Fyn. Src-family kinases, including Fyn, regulates the binding activity of integrin $\alpha_{IIb}\beta_{III}$ receptors of platelets (Bauer et al., 2001). PKA is a major modulator of Fyn activity in the neurons (Salter and Kalia, 2004), though it is unclear if platelets also have this pathway. Thus, the decrease in Fyn expression may represent one aspect of a defective molecular pathway in platelets from patients with schizophrenia.

Ohnuma et al. described an increase in *fyn* mRNA and Fyn protein levels in postmortem brains from patients with schizophrenia (Ohnuma et al., 2003a). Six samples of prefrontal cortex from patients with schizophrenia were studied using *in situ* hybridization and immunoautoradiography; the signal intensity was stronger in the samples from the patients than in the samples from the controls. The increase in the *fyn* mRNA level was not incompatible with our findings because it might have been due to the increase in *fyn*Δ7. On the other hand, the Fyn protein level was increased, rather than decreased, in the prefrontal cortex of these patients. Various factors, such as differences in the tissues that were analyzed (brain vs. platelets), the method of analysis, and disease heterogeneity probably underlie the discrepancy between our study and this previous

study. Schizophrenia is not a uniform disease caused by a single gene defect, but a heterogeneous disorder resulting from multiple genetic, epigenetic and non-genetic factors. Recently, some components or endophenotypes of schizophrenia have been suggested to be more closely linked to specific genes that contribute to a vulnerability to this disorder (Braff et al., 2007). Though our demographic data, including the age of onset, the dosages of antipsychotics, and the BPRS scores, were not statistically correlated with the levels of Fyn mRNA and protein, patients with the residual type of schizophrenia tended to have a lower *fyn*Δ7 mRNA ratio than patients with the other types of schizophrenia (Fig. S3B). Thus, further studies using brain tissue or blood should include an evaluation of the subphenotypes as well as each of the splice forms of *fyn*.

Interestingly, it has been reported that unmedicated patients with schizophrenia exhibit attenuated extrapyramidal symptoms compared with healthy volunteers after haloperidol administration (Miller et al., 1993). As Fyn-deficient mice do not exhibit extrapyramidal signs after haloperidol injection (Hattori et al., 2006), the alterations in Fyn expression in human brains may also reduce sensitivity to antipsychotics.

A tendency towards an increased *fyn*Δ7 among the schizophrenia patients was also found using quantitative PCR in a subset of samples (Fig. S5B). However, no significant correlation between the levels of Fyn protein and the *fyn*Δ7 mRNA ratios was observed. Therefore, the decreased protein levels in the schizophrenia patients cannot be simply explained by a change in the mRNA level. The reduction in Fyn protein may have been caused by other mechanisms, such as a reduction in synthesis and mis-sorting, the destabilization of the Fyn complex, or an increase in degradation. *In vitro* studies have suggested that *fyn*Δ7 produces a lower amount of Fyn protein and exerts a dominant-negative effect on the activity of wild-type Fyn. Thus, the alterations in Fyn protein and *fyn* mRNA may contribute to the depression of Fyn function through different mechanisms.

The mechanism responsible for the increased ratio of *fyn*Δ7 in patients with schizophrenia is unclear. It may be due to alterations in splicing, mRNA stability, or degradation. Variations in exon and intron sequences are thought to alter the balance in the ratio of alternatively spliced isoforms (Pagani and Baralle, 2004). Interestingly, the *fyn* gene is located on 6q21; this locus was included in a positive area identified by a schizophrenia linkage analysis (Cao et al., 1997), which was supported by several large-scale studies (Martinez et al., 1999; Levinson et al., 2000; Lewis et al., 2003). Two previous studies evaluated the association between *fyn* genomic variations (three SNPs) and schizophrenia (Ishiguro et al., 2000; Ohnuma et al., 2003b) and did not find any association. Recently, Rybakowski et al. examined an association between these variations and performance on the Wisconsin Card Sorting Test (WCST) in patients with schizophrenia (Rybakowski et al., 2007). They found two variations in the introns that were associated with better performances. A linkage disequilibrium existing between introns 8 to 12 includes one of these variations (www.hapmap.org). Given that *fyn*Δ7 does not exert kinase activity and even exerts a dominant-negative effect on the activity of wild-type Fyn and that Fyn mediates the phosphorylation of NMDA-R to increase channel activity, variations between exons 6 to 8 that regulate exon 7 splicing may affect WCST performance. Further studies should include an analysis of sequences in that area as well as an evaluation of WCST performance.

In conclusion, we identified a reduction in Fyn protein and the preferential transcription of *fyn*Δ7, a defective *fyn* isoform, in the platelets of patients with schizophrenia; these findings suggest a defective molecular pathology for this disorder.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2008.04.014.

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