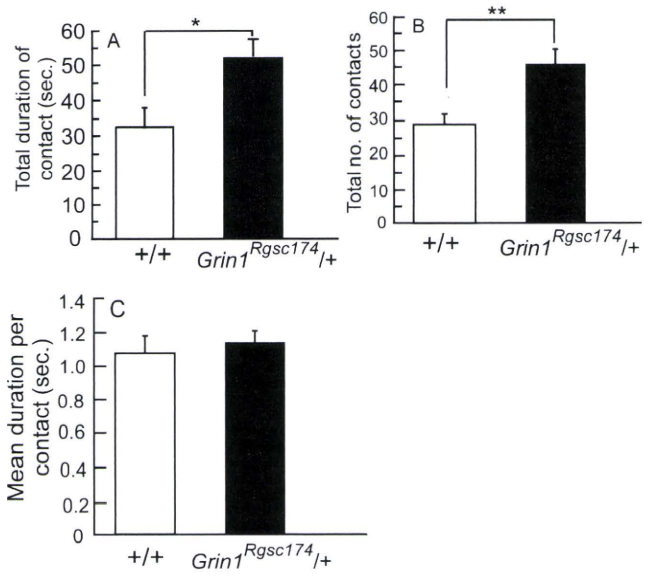

FIG. 8. Object exploration by *Grin1*^{Rgsc174}/+ and wild-type mice. (A) Total time *Grin1*^{Rgsc174}/+ and +/+ mice spent exploring the object. Student's *t*-test, $t_{18} = 2.7$, $^{*}P < 0.05$. (B) The total number of times *Grin1*^{Rgsc174}/+ and +/+ mice made contact with the novel object. Student's *t*-test, $t_{18} = -3.1$, $^{**}P < 0.001$. (C) Duration of each exploration by *Grin1*^{Rgsc174}/+ and +/+ mice. Student's *t*-test, $t_{18} = 0.6$, $P > 0.5$. Error bars represent the SEM. Male mice, $n = 10$ of each genotype at 11 weeks of age.

no significant difference was observed in the time spent in the center area of the open field (Student's *t*-test, $t_{18} = 1.9$, $P > 0.069$; Fig. 6B).

Pharmacological analysis with MPH

A significant interaction effect between genotype and drug treatment was detected in the open-field test (ANOVA, $F_{1,836} = 210.549$, $P < 0.0001$). Wild-type mice displayed increased locomotor activity following administration of MPH 30 mg/kg (Fig. 9A; Fisher's PLSD, $P < 0.0001$), whereas a sustained reduction in locomotor activity was observed in *Grin1*^{Rgsc174}/+ mice following MPH administration (Fig. 9B; Fisher's PLSD, $P < 0.0001$).

To determine how MPH alters neuronal activity, we immunohistochemically examined the expression of an immediate–early gene, c-Fos, in the brain and plotted (Fig. 10A) the c-Fos-immunoreactive (IR) cells onto a brain atlas (Paxinos & Franklin, 1997). The heterozygote exhibited a characteristic phenotype in the dorsal striatum and prelimbic cortex. In the wild type, the number of c-Fos-IR-cells significantly increased in the dorsal striatum, following MPH administration (Fig. 10Aa and Ab; supporting Fig. S6Aa and Ab), whereas the number increased to a lesser degree in the heterozygote (Fig. 10Ac and Ad; supporting Fig. S6Ac and Ad). On the other hand, although the number of c-Fos-IR cells in the prelimbic cortex of the wild type increased significantly following MPH administration (Fig. 10Aa and Ab; supporting Fig. S6Aa and Ab), the basal level of c-Fos-IR cells was much higher in the prelimbic cortex of the heterozygote and the number of c-Fos-IR cells in the prelimbic cortex were decreased following MPH administration (Fig. 10Ac and Ad; supporting Fig. S6Ac and Ad). To identify the MPH-responsive neurons we stained for c-Fos immunoreactivity and counterstained with Nissl reagent, and the results showed that c-Fos was mainly expressed in the pyramidal cells of the prelimbic cortex (supporting Fig. S6Be and Bf).

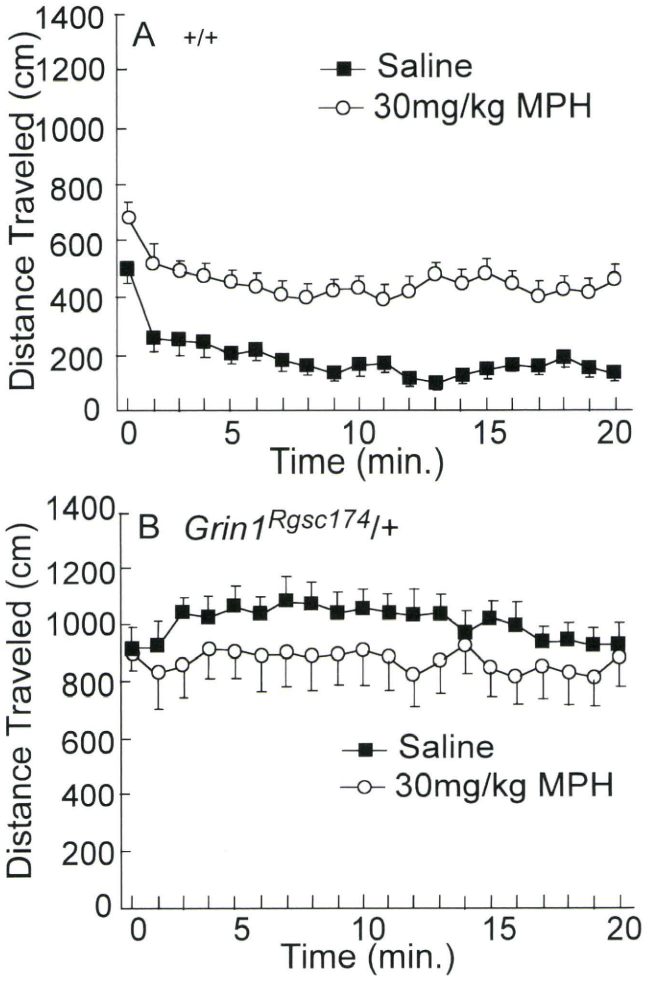

FIG. 9. Effect of MPH on the locomotor activity of *Grin1*^{Rgsc174}/+ and +/+ mice. The distance traveled by (A) wild-type and (B) *Grin1*^{Rgsc174}/+ mice in the open field 80 min after saline or MPH injection. Error bars represent the SEM. Male mice, $n = 10$ of each genotype at 11 weeks of age.

Significant effects of genotype, MPH treatment, and interaction between genotype and MPH treatment were detected in the dorsal striatum (Fig. 10B). The basal levels of c-Fos expression in the wild type and heterozygote were similar. MPH treatment increased c-Fos expression in both the wild-type and heterozygous mice. c-Fos expression in the dorsal striatum of the MPH-treated heterozygote was lower than in the MPH-treated wild type. There was a significant effect of genotype and MPH treatment on c-Fos expression in the prelimbic cortex (Fig. 10C), and a significant interaction between genotype and MPH treatment was also detected. More c-Fos-IR cells were present in the prelimbic cortex of saline-treated heterozygotes than of the wild type. MPH treatment significantly increased c-Fos expression in the wild-type prelimbic cortex but suppressed c-Fos expression in the prelimbic cortex of the heterozygote.

In the nucleus accumbens, there was a significant effect of MPH administration and a significant interaction between the MPH effect and genotype on the pERK2 level (Fig. 11). Fisher's PLSD test was used to clarify the rank relationship between the baseline group and other groups statistically. The pERK2 level in the nucleus accumbens of the MPH-treated wild type was significantly greater than baseline level. The baseline pERK level in the heterozygote was higher than in the wild type, but the pERK2 level in the MPH-treated heterozygote

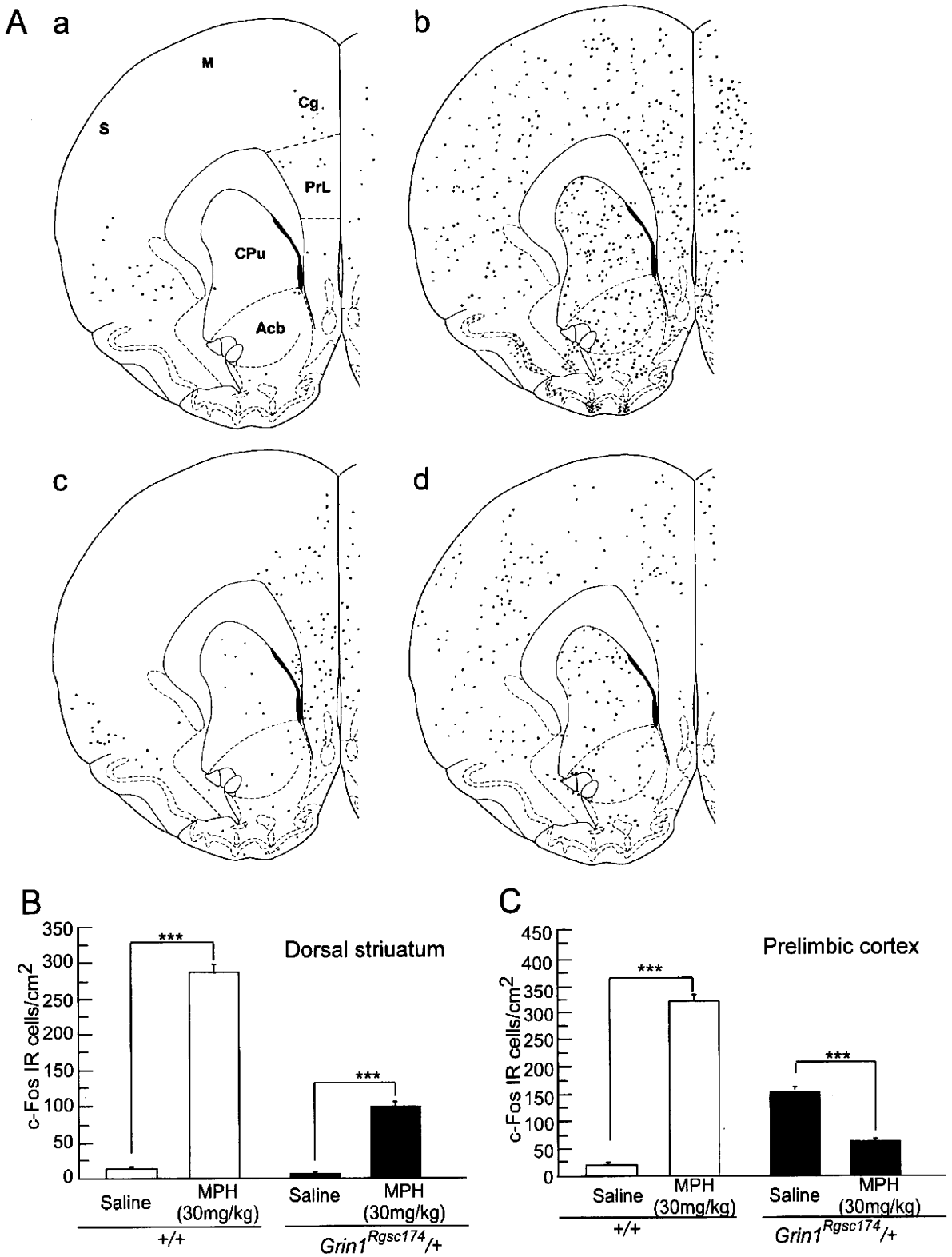

FIG. 10. Effect of MPH on c-Fos expression in *Grin1*^{Rgsc174}/+ and +/+ mice. (A) Diagram of the distribution of c-Fos-IR cells and regions selected for analysis. Dots indicate c-Fos-IR cells 2 h after saline or MPH 30 mg/kg administration. Brain regions are abbreviated as follows: Acb, accumbens nucleus; Cg, cingulate cortex; CPu, caudate-putamen (striatum); M, motor cortex; PrL, prelimbic cortex; S, somatosensory cortex. The diagram was modified from the brain atlas of Paxinos & Franklin (1997). (a) Saline-treated wild type, (b) MPH-treated wild type, (c) saline-treated heterozygote, and (d) MPH-treated heterozygote. (B and C) Quantification of c-Fos-IR cells in the dorsal striatum and prelimbic cortex (E). Error bars represent the SEM. ***$P < 0.0001$, Fisher's PLSD test. Male mice, $n = 7$ in each group at 11 weeks age. (B) ANOVA, effect of genotype $F_{1,24} = 206.7$, $P < 0.0001$; effect of MPH treatment, $F_{1,24} = 735.543$, $P < 0.0001$; interaction between genotype and MPH treatment, $F_{1,24} = 180.89$, $P < 0.0001$. (C) ANOVA, effect of genotype, $F_{1,24} = 76.32$, $P < 0.0001$, effect of MPH treatment, ANOVA, $F_{1,24} = 222.584$, $P < 0.0001$, interaction between genotype and MPH treatment, $F_{1,24} = 761.468$, $P < 0.0001$. Scale bar in A, 500 μm.

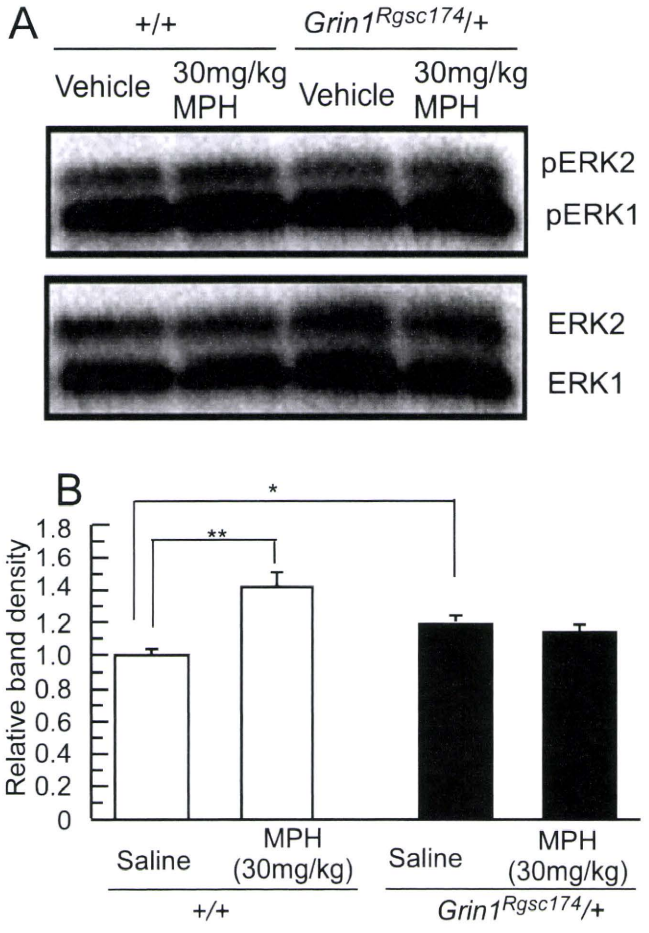

FIG. 11. Phospho-ERK levels after intraperitoneal administration of MPH 30 mg/kg to wild-type and heterozygous mice. (A) Immunoblotting of pERK1 and 2 in nucleus accumbens extracts following MPH administration. (B) Quantitative analysis of the relative band density of pERK2. The baseline level of pERK2 was calculated as the average ratio pERK2/ERK2 prepared from saline-treated wild-type mice, and the data were normalized by using the following formula: pERK level = (band density of pERK2)/(band density of ERK2)/(baseline level). Male mice, $n = 5$ in each group at 12 weeks age. Error bars represent the SEM. ANOVA, effect of genotype, $F_{1,32} = 0.271$, $P > 0.6$; effect of MPH administration, $F_{1,32} = 9.8$, $P < 0.004$; interaction between the MPH effect and genotype, $F_{1,32} = 16.9$, $P < 0.0004$. $^*P < 0.05$ and $^{**}P < 0.01$, Fisher's PLSD test.

was not upregulated in comparison with the saline-treated heterozygote (Fig. 11).

Discussion

Phenotype comparison between Grin1^{Rgsc174} and other Grin1 *mutants*

The following *Grin1* mutant mice have been reported previously: *Grin1* null mutants (Forrest *et al.*, 1994; Li *et al.*, 1994), *Grin1^{tm2Blt}* with reduced glycine affinity (Kew *et al.*, 2000; Ballard *et al.*, 2002), *Grin1$^{tm1.1Phs}$* with reduced single-channel conductance (Single *et al.*, 2000), and *Grin1* knockdown mutants (Mohn *et al.*, 1999; Duncan *et al.*, 2006). We observed common phenotypes, including fearfulness, embryonic lethality of homozygote, and social avoidance, in *Grin1^{Rgsc174}/+* and known *Grin1* mutants (Forrest *et al.*, 1994; Li *et al.*, 1994; Mohn *et al.*, 1999; Kew *et al.*, 2000; Single *et al.*, 2000; Ballard *et al.*, 2002; Duncan *et al.*, 2006). Increased anxiety is a common finding in known *Grin1* mutant mice (Kew *et al.*, 2000; Labrie *et al.*, 2009), but measurements of time spent in the center area of the open field and the results of the light–dark transition test revealed no increase in anxiety in *Grin1^{Rgsc174}/+* mice. The increased novelty-seeking behavior and the absence of increased anxiety are phenotypes unique to the *Grin1^{Rgsc174}* mutant.

Functional change in GRIN1 *protein*

The missense mutation R844C is located in the intracellular C-terminal domain of NMDAR1, which is referred to the C0 domain. The C0 domain spans amino acid residues 834–863 (Akyol *et al.*, 2004). Previous reports have indicated that the C0 domain is an important regulatory domain of *GRIN1* protein (Holmes *et al.*, 2002; Leonard *et al.*, 2002). The cystein in the wild-type allele that is replaced by arginine in the mutant is very hydrophobic, whereas arginine is a positively charged hydrophilic amino acid and binds to negatively charged amino acid groups. This change in the C0 domain should produce an alteration in the conformation and function of the C0 domain in *GRIN1* protein and, in fact, we observed that after NMDA stimulation calcium influx was increased and prolonged in cortical neurons from the *Grin1^{Rgsc174}* mutant.

Altered interaction between NMDARs and dopamine receptors may be responsible for the phenotypes of the Grin1^{Rgsc174} mutant

NMDAR and dopamine (DA) receptor functions are co-regulated by direct (Lee *et al.*, 2002) and indirect (Cepeda & Levine, 2006) interactions. Morphological evidence suggests that glutamate receptors and DA receptors interact in synaptic complexes or triads in cortical pyramidal neurons (Goldman- Rakic *et al.*, 1989). This type of arrangement is found in striatal neurons (Smith & Bolam, 1990) and provides a morphological basis for close DA receptor–glutamate receptor interaction. As MPH has been reported to be involved in activation of DA signaling, we used MPH to compare the altered DA signaling in the wild-type and *Grin1^{Rgsc174}/+* mice. The basal level of c-Fos expression in the prelimbic cortex and striatum was very low in the wild type, and MPH administration significantly increased c-Fos expression in both areas. Increased c-Fos expression was observed in the prelimbic cortex of *Grin1^{Rgsc174}/+* mice at the basal level, and MPH paradoxically reduced c-Fos expression in the prelimbic cortex. Phosphorylation of ERK2, a DA signaling-related protein, was increased in the nucleus accumbens of *Grin1^{Rgsc174}/+* mice at the basal level, and little change was observed even after the high dose of MPH (30 mg/kg). Thus, NMDAR dysfunction in these regions should underlie the aberrant DA signaling and result in the behavioral phenotypes of *Grin1^{Rgsc174}/+* mice. In the present study, the difference in MPH-induced behavioral difference between the wild type and *Grin1* mutant was detected only at the high dose of MPH (108 μmol/kg). Taking into consideration that K_I of MPH for the mouse dopamine transporter is < 0.3 μM (Chen *et al.*, 2005), the effect of the high dose of MPH in the present study is considered to be due not to the specific action on the DA transporter but to the effect on any other receptors or transporters that crosstalk with DA signaling system.

Grin1^{Rgsc174} mutant as an animal model of psychiatric disorders

The implication of mutations in NMDAR has been suggested in schizophrenia by human association study (Georgi *et al.*, 2007; Galehdari, 2009). The increased locomotor activity observed in the *Grin1^{Rgsc174}/+* mice may represent fearfulness, and the mutant also

exhibited social isolation in the social interaction test. Thus, the phenotype's fearfulness and social isolation observed in a schizophrenia model (Mohn *et al.*, 1999) were also exhibited by this mutant. MPH is one of the main therapeutic agents used to treat attention deficit hyperactivity disorder (AD/HD) and narcolepsy patients. However, the mechanism of action of MPH is still unclear. According to previous reports, spontaneously hypertensive rat (SHR), a well validated animal model of AD/HD, was found to exhibit the three major characteristics of AD/HD (hyperactivity, impulsivity and poor sustained attention) in a comparison with their progenitor Wistar–Kyoto rat strain, and SHR has been shown to lack responsiveness to MPH in several behavioral tests (Van den Bergh *et al.*, 2006). The *Grin1* mutant mouse described here also exhibited altered pharmacological reactions to MPH. In view of the fact that SHR also exhibits altered glutamatergic functions (Jensen *et al.*, 2009), *Grin1*^{Rgsc174} mice may be a useful model for gaining insight into the mechanism of action of MPH on behavioral disorders in regard to DA receptor-glutamate receptor interactions.

These phenotypes of *Grin1*^{Rgsc174} indicate that this mutant displays some of the signs and symptoms of psychiatric disorders and may be a useful tool for elucidating the molecular mechanisms of abnormal behaviors and the actions of therapeutic agents.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Strategy of animal production for phenotypic screen, genetic analyses, and detailed phenotypic analyses.

Fig. S2. Results of pilot study for pharmacological analysis using MPH Wild-type male mice.

Fig. S3. Brain histology of *Grin1*^{Rgsc174}/+ and wild-type mice.

Fig. S4. Result of social interaction test.

Fig. S5. Light/dark transition test results.

Fig. S6. Effect of MPH on c-Fos expression in *Grin1*^{Rgsc174}/+ and +/+ mice.

Table S1. SNP markers chosen for genome-wide scanning.

Table S2. Results of the resident intruder test.

Appendix S1. Procedure of pilot study for pharmacological analysis of *Grin1*^{Rgsc174}/+ using MPH.

Appendix S2. Preparation of cells for measurement of intracellular calcium levels.

Appendix S3. Preparation of samples for immunoblotting of NMDAR subunits.

Appendix S4. Preparation of samples for immunoblotting of extracellular signal-regulated kinase2 (ERK2) and phospho-ERK2 (pERK2) proteins.

Appendix S5. Procedure of social interaction test.

Appendix S6. Procedure of resident intruder paradigm.

Appendix S7. Procedure of light/dark transition test.

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Abbreviations

B6, C57BL/6J; D2, DBA/2J; DA, dopamine; ENU, *N*-ethyl-*N*-nitrosourea; ERK2, extracellular signal-regulated kinase; *Grin1*, glutamate receptor, ionotropic, NMDA1; i.p., intraperitoneal; IR, immunoreactive; MPH, D,L-methylphenidate hydrochloride; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptors; pERK2, phospho-ERK2.

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Van den Bergh, F.S., Bloemarts, E., Chan, J.S., Groenink, L., Olivier, B. & Oosting, R.S. (2006) Spontaneously hypertensive rats do not predict symptoms of attention-deficit hyperactivity disorder. *Pharmacol. Biochem. Behav.*, **83**, 380–390.

Yano, M. & Steiner, H. (2005) Topography of methylphenidate (regulation, ritalin)-induced gene in the striatum: effects, differential on c-Fos, substance, P and peptides, opioid. *Neuropsychopharmacology*, **30**, 901–915.

Analyses of fyn-tyrosine kinase in schizophrenia

Hattori K, Tanaka H, Uchiyama H, Yamamoto N, Iijima Y, Fujii T, Hori H, Teraishi T, Tatsumi M, Omori M, Okamoto N, Arima K, Kinoshita Y, Matsuo J, Kawamoto Y, Kunugi H

Dept Mental Disorder Research, National Inst Neurosci, Nat Center Neurol & Psychiatry, Japan

Summary

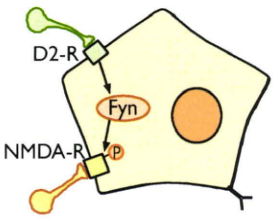
Fyn's function: learning, emotion, dopamine signaling

Blood	fyn mRNA ↓ in schizophrenia
SNP (fyn)	No association with schizophrenia Association with IQ/WCST in control
Brain	fyn mRNA/protein→ in schizophrenia Active-form Fyn ↑ in schizophrenia NMDA-R ↓ in schizophrenia

Blood Fyn: Biomarker?
Brain Fyn: Drug target? (to rescue NMDA-R ↓)

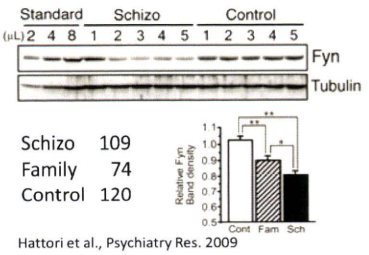
Introduction

Fyn kinase is a key mediator of the crosstalk between D2-R and NMDA-R



Hattori et al., JBC, 2006

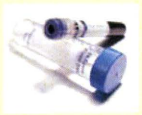
Fyn protein levels were decreased in schizophrenic platelets.



Hattori et al., Psychiatry Res. 2009

Method I

Blood mRNA



Q-PCR → fyn

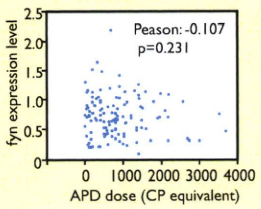
TaqMan® Probes

Results I

Decreased fyn mRNA in schizophrenia

	fyn		
Dx	n	Mean	p
Schizo	158	0.753	0.027
Dep	49	1.05	1
Bipolar	31	0.735	0.24
Control	409	0.907	

ANCOVA (age, sex), bonferroni



Method II

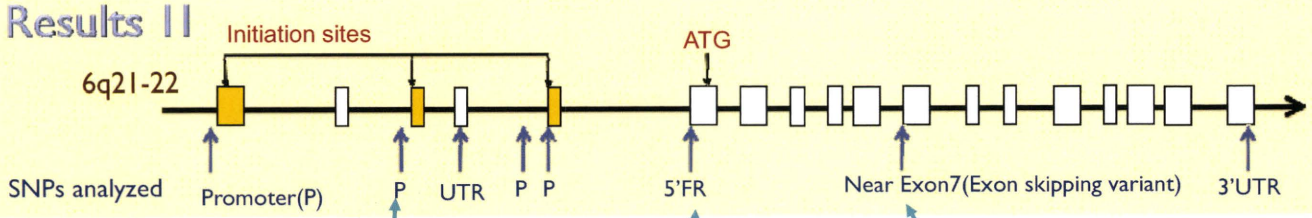
Genomic DNA

Sample	Nos
Schizo	497
Dep	528
Bipolar	204
Control	932

8 TagSNPs

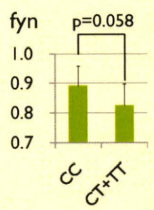
Genotyped TaqMan PCR

Results II

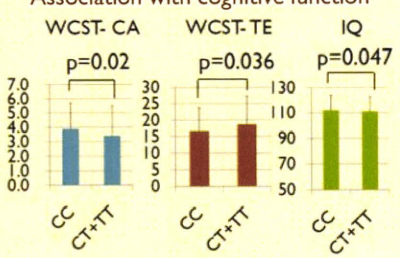


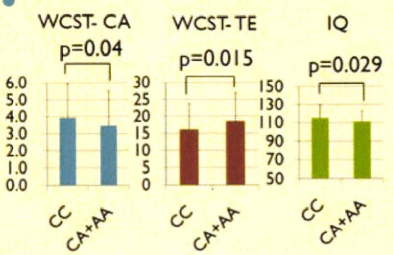
No association between 8 SNPs and Schizo / Dep / Bipolar.

Association with fyn expression levels



Association with cognitive function





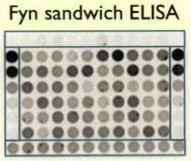
Method III

Postmortem brain BA6
Stanley Medical Research Institute Neuropathology Consortium
Age, Sex, pH etc matched

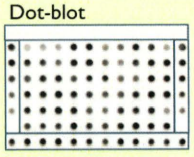
Sampl	Nos
Schizo	15
Dep	15
Bipolar	15
Control	15

Blind analyses → Un code after data submission

Fyn sandwich ELISA



Dot-blot



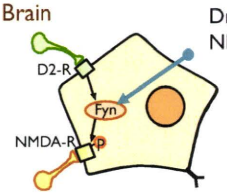
Results III

Main findings

- No change in Fyn mRNA/protein level
- Increase in active form Fyn
- Decrease in fyn's substarate, NR2B (NMDA-R subunit)

Discussion

Brain



Drug target to rescue NMDA-R reduction?

Blood

Bio marker? (trait or state)

Genome

Fyn participates in executive function in humans

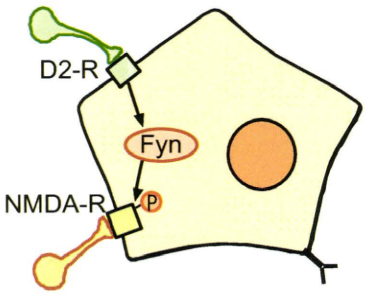
- Emotional deficits
- Cognitive deficits

統合失調症・気分障害のゲノム・死後脳を用いたFynチロシンキナーゼの解析

国立精神・神経センター
神経研究所　疾病研究第３部

○服部功太郎、田中治子、内山博文、山本宜子、飯嶋良味、藤井崇、堀弘明、寺石俊也、木下裕紀子、松尾淳子、川本由実子、有馬邦正、功刀浩

FynはドーパミンD2受容体とNMDA受容体の橋渡しをする鍵分子

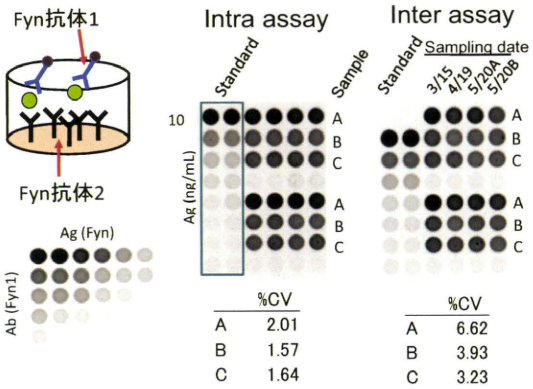


Hattori et al., JBC, 2006

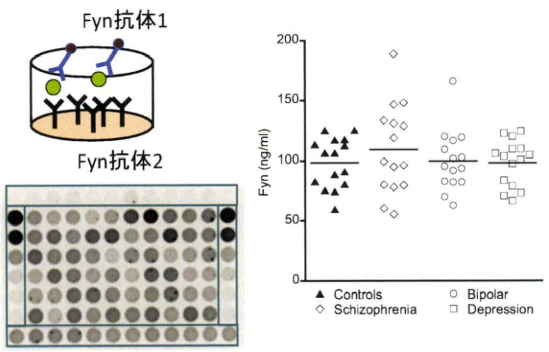
スタンレー死後脳(BA6)の解析

- 60検体：Sz, BD, Dep, Cont各15例
- 年齢、性別、pH等をマッチ
- ブラインドで測定
- 測定結果を登録後、対応表が送られる

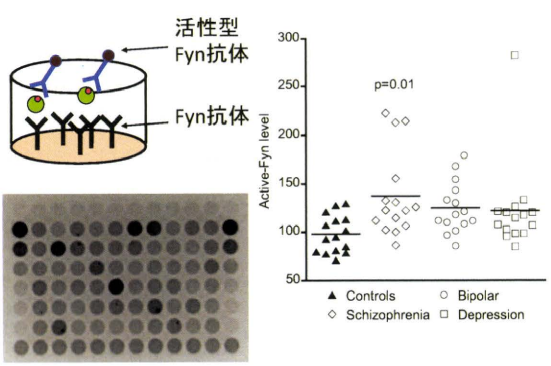
Fyn-ELISAの作製



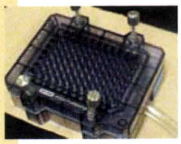
ELISAによる解析- Fyn



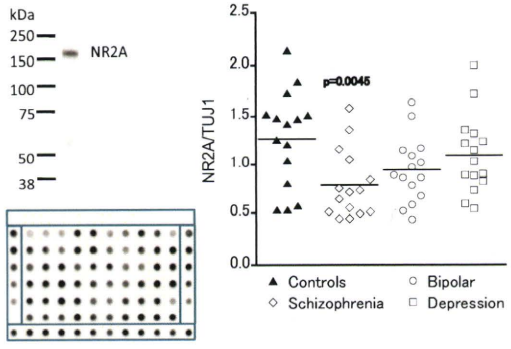
ELISAによる解析- 活性型Fyn



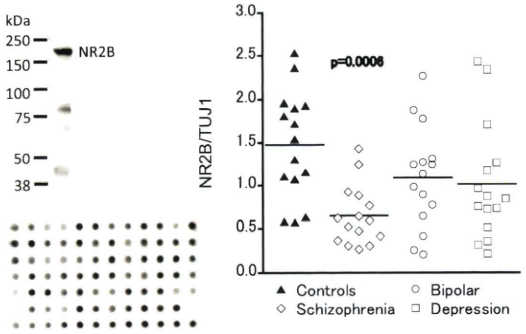
Dot-blotによる解析-NR1



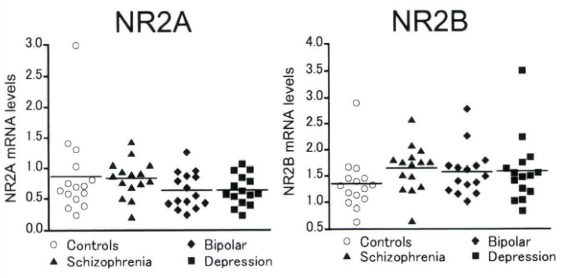
Dot-blotによる解析-NR2A



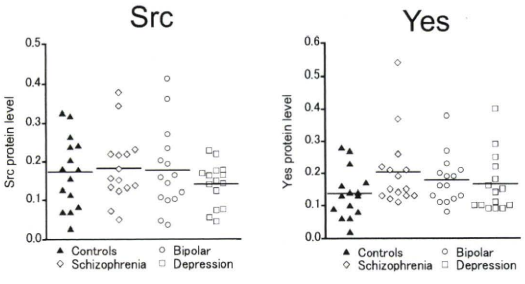
Dot-blotによる解析-NR2B



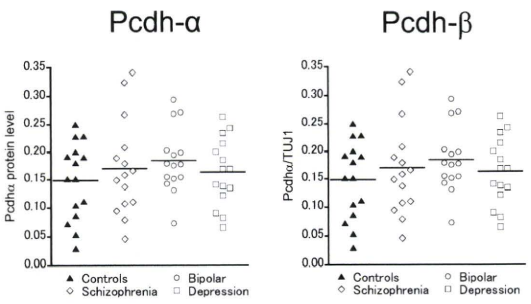
mRNA発現の解析 (TaqMan)



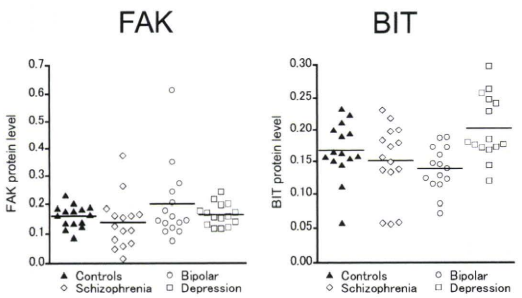
他の分子 – Src family Kinases



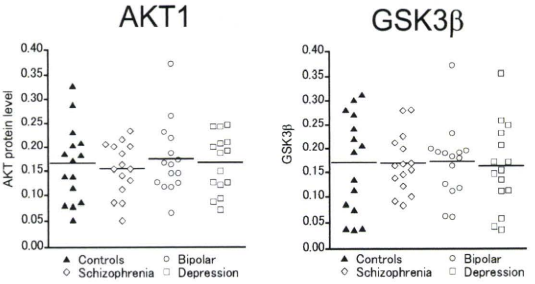
他の分子 – 膜受容体



他の分子 – Fynの基質

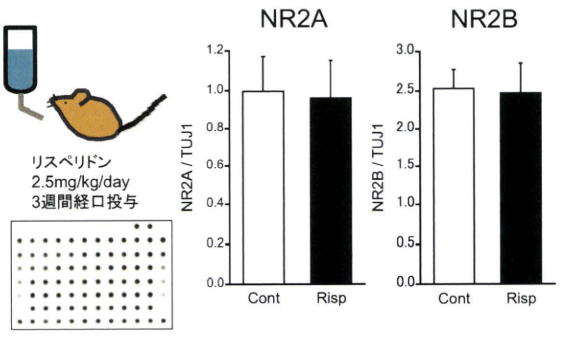


他の分子 – AKT1- GSK3β

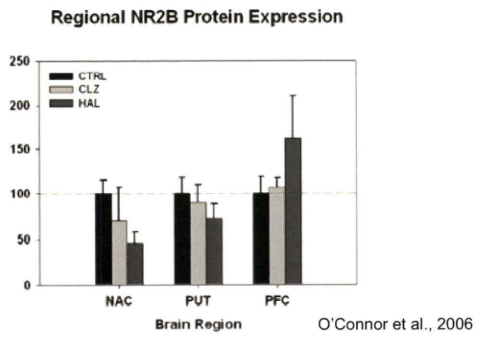


抗精神病薬の影響

抗精神病薬はNR2A, NR2B量に影響しない

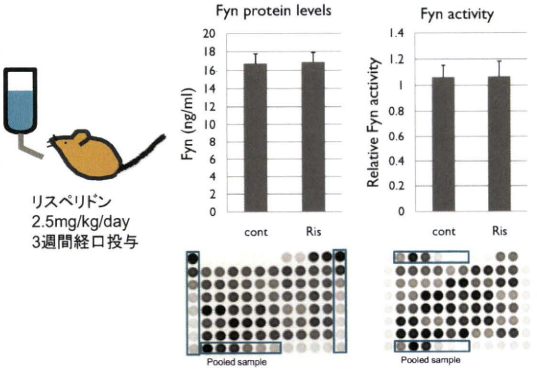


抗精神病薬はNR2B量に影響しない

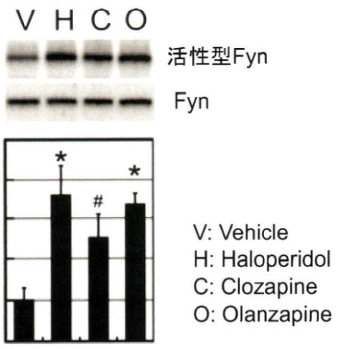


O'Connor et al., 2006

抗精神病薬はFyn量・活性化に影響しない



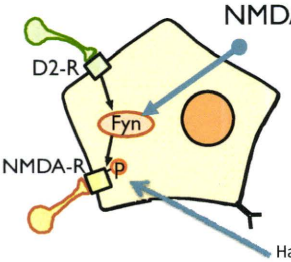
抗精神病薬はFynを活性化する



V: Vehicle
H: Haloperidol
C: Clozapine
O: Olanzapine

Discussion

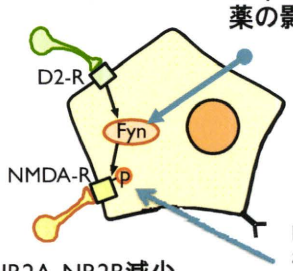
Drug target to rescue
NMDA-R reduction?



Hattori et al., JBC, 2006
Hayashi et al., Neuron 2009

Discussion

②Fyn活性亢進
薬の影響? 代償作用?



Fynによるリン酸化
機能上昇・安定化

①NR2A, NR2B減少

Hattori et al., JBC, 2006
Hayashi et al., Neuron 2009