表4　認知症を伴うパーキンソン病に特徴的な運動障害

対称性（左右差の目立たない）の錐体外路障害
L-dopaに対する反応性が不十分
ジスキネジア
（振戦よりも）姿勢歩行障害が目立つタイプ
UPDRS IIIスコアの急峻な低下
易転倒性
レム睡眠行動障害などの睡眠障害
嚥下機能障害

た視覚空間機能の評価では，PDDとDLBは同様でアルツハイマー型認知症より強く障害されている。

言語に関しては，注意力低下に伴う障害を認めるが中核機能は保たれている。単語リスト産生は，パーキンソン病よりアルツハイマー型認知症でより強く障害され，会話のメロディはアルツハイマー型認知症よりパーキンソン病でより強く障害されていた[21]。

Beattyらは，認知機能障害の検査スコアをパターンで分けた場合，視覚空間機能や構成機能，注意などの“皮質下スコア”はPDDでアルツハイマー型認知症に比してより障害が強く，言語や遅延再生記憶といった“皮質スコア”はアルツハイマー型認知症でPDDに比してより障害が強いことを示した[22]。

PDDにおける行動心理症状（Behavioral and psychological symptoms of dementia：BPSD）の特徴として，無関心，無感動，無気力といったアパシー（Apathy）や，感情の変化・うつ状態，人格変化，幻覚，妄想，日中過眠，レム睡眠行動異常があげられる[1]。本稿では，他稿と重なるため，PDDの幻覚について詳細に述べる。

The Neuropsychiatric Inventory（NPI）を用いた検討によると，地域住民調査ではパーキンソン病の25%[23]，医療機関調査ではパーキンソン病の40%で幻覚を認めている[24]。PDDでは45-65%[24-27]，DLBでは60-80%に認める[25, 28, 29]のに対して，ADでは4-8%であった[26, 28, 30]。認知障害を伴うパーキンソン病では，幻覚は比較的ありふれた症状であるといえることから，幻覚は，後に出現する認知症の主要予測因子[31]，あるいは剖検で認めるレビー小体のマーカーであるとの示唆もある[32]。

われわれの教室による検討では，Kitayamaらが運動症状の発症年齢と認知症発症までの期間を検討した結果，両者に負の相関を認め，多くのPDD患者が幻視から1年以内に認知症と診断されていることを報告した[33]。Imamuraらは，認知症のないパーキンソン病，鮮明な夢を見るパーキンソン病，幻覚を伴うパーキンソン

る。MIBG心筋シンチグラフィにより，レビー小体関連の認知症を他の認知症と鑑別することには有用であるが，PDDとDLBの鑑別は困難である。

2. 治療

PDDの治療について，簡略に述べる[37, 38]。現在は，PDDの根本的治療法はなく，主として薬物治療と非薬物治療による対症的治療が行われている。

薬物治療としては，コリンエステラーゼ阻害薬のドネペジル，リバスチグミン，ガランタミンおよびNMDA受容体拮抗薬のメマンチンが認知機能障害やBPSDに対して用いられる。また，漢方薬の抑肝散もBPSDに対して用いられるが，いずれも保険適用外である。

非薬物療法としては，家族や介護者と症状の変動などの情報を共有し，連携したケア体制の構築，便秘予防を配慮した食事，リハビリテーションによるADL維持向上や，家族の介護負担を考慮した医療・介護保健サービスの利用などが薦められている。

1つの症状を改善させる治療は，他の症状を悪化させる場合もありうるため，有害事象に十分留意し，患者ごとに治療の主要な標的とすべき臨床症状を見定めて対応する必要がある。

おわりに

パーキンソン病における認知障害について，疫学や臨床的検討に関する既報告をレビューして考察した。社会の高齢化に向けて，認知障害に関する注目度は今後ますます高まることが予想される。パーキンソン病のみならずPDDの病態解明や治療の進展とともに，PDD発症の予測法や予防法の開発も望まれる。

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脆弱X症候群の分子機構と治療

Molecular mechanism and treatment of fragile X syndrome

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脆弱X症候群(FXS)はX染色体上に位置するFMR1遺伝子の異常によって発症し，知的障害，巨大睾丸，細長い顔などを主症状とする．日本人での頻度は欧米よりやや低く，男性で10,000人に1人と考えられる．本疾患では代謝型グルタミン酸受容体(mGluR)のシグナルが異常に亢進し，そのためにシナプスの可塑性が変化し，シナプス樹状突起棘の形態に異常をもたらすことが明らかにされてきている．この異常の機構が詳細に研究され，mGluR理論が確立され，それに基づいた治療法が開発されてきている．動物実験のみならず，ヒトでの臨床治験も行われており，近い将来治療法が確立されることが期待されている．さらに，これらの治療法は他の知的障害や自閉症にも応用できる可能性があり，注目される．日本でも，この治療研究を推進する体制を充実させていくことが重要である．

Key word：脆弱X症候群(FXS)，FMR1遺伝子，CGG繰返し配列延長，グルタミン酸受容体

脆弱X症候群(fragile X syndrome：FXS)は1943年にX連鎖性遺伝形式をもつ知的障害として報告され，Martin-Bell症候群ともよばれた．1969年にX染色体上の脆弱部位が明らかにされ，1991年に原因遺伝子が解明された[1]．遺伝性の知的障害としてはもっとも研究が進んでいる．FXSは巨大睾丸，長い顔などを特徴とし，てんかんや睡眠障害などを合併することも多い．男性患者は重度の知的障害を呈するが，女性では軽度や中等度の場合も多い．まれにfragile X mental retardation(FMR)2遺伝子が原因となるが，そのほとんどはFMR1遺伝子の異常である[2]．FXSの頻度は，男性の4,000人に1人，女性の8,000人に1人と報告されているが，民族によって差がある．日本人では男性の10,000人に1人と推定されている[3]．FXSは知的障害のなかで研究がもっとも進んでおり，病態解明から治療法の開発が行われ，近年では臨床治療研究に到達している．さらに，このFXSで明らかにされてきた脳の病態は，他の原因による知的障害や自閉症とも共通していると考えられ，FXSの研究はひとつの遺伝性疾患の研究にとどまらない．

本稿ではFMR1異常によるFXSの病態と，それに基づく治療法開発の現状を中心に解説する．

FXSのCGG繰返し配列異常

FXSではX染色体の脆弱部位であるXq27.1に存在するFMR1遺伝子の5′非翻訳領域あるCGG繰返し配列が異常に延長している[1]．このCGG繰返し配列は正常では54以内であるが，患者では200を超える．FXS患者の母親は50～200の繰返し配列(前変異)をもつ保因者である．FXSは，母親の不安定なCGG繰返し配列が患者に遺伝するときに延長する，いわゆるトリプレットリピート病として最初に解明された疾患である[4]．この配列延長がDNAのメチル化をもたらし，その結果，FMR1遺伝子の転写抑制により遺伝子の機能が失われる．一方，FXSの前変異をもつ保因者のなかから50歳以降にParkinson様症状，精神症状などを呈する脆弱X症候群関連振戦/失調症候群(FXTAS)が発症することが知られ，日本でも患者がみつかっている[5]．FXTASの発症機序は

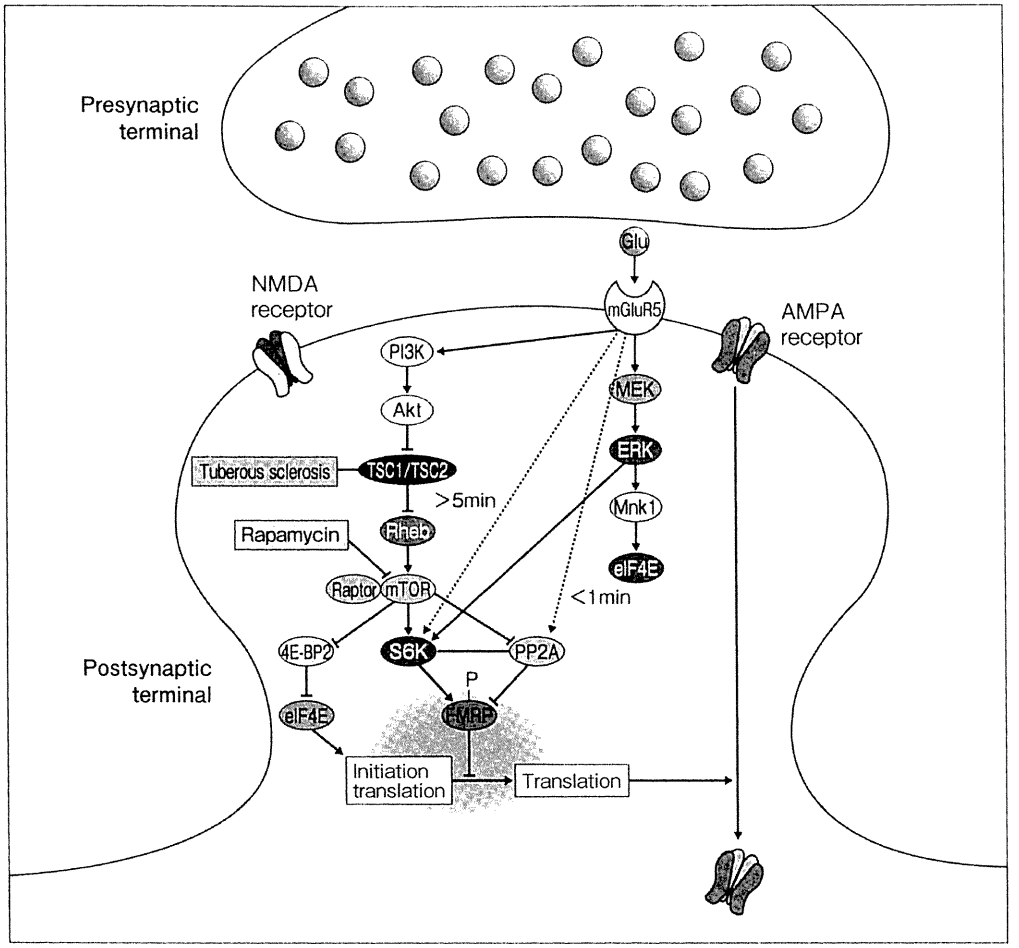

図 1　mGluR，mTORシグナル伝達系，FMRPの関係[15]

MEK-ERK-Mnk1 と PI3K-mTOR 経路の 2 つが mGluR5 の下流のシグナル系として存在する．PP2A や PI3K などの FMRP が標的とする mRNA は，ERK によって細胞内の二次メッセンジャーとして働くようになる．リン酸化 ERK は Mnk1 と S6K を介して転写活性をもつ．ERK のリン酸化は PP2A などのホスファターゼにより制御されている．FmR1 KO マウスでは ERK の非活性化が起こるために mGluR5 の刺激に過剰に反応する．このように，ERK 活性の制御異常はシナプスの翻訳調節異常の指標となる．

mTOR の活性化は 4E-BP や S6K のリン酸化を介して転写開始の起始点となる．mGluR5 が刺激されると PI3K が膜の phospholipid PIP2 を PIP3 に転換する．この PIP3 が，Akt を膜に集めてリン酸化させ，PKD1 を活性化させる．Akt で活性化された mTOR は TSC(TSC1 と TSC2 のヘテロダイマー)が抑制する．TSC2 がリン酸化されるとその GAP 活性が減少し，Rheb と mTOR を活性化させる．さらに，この mTOR は Raptor と結合し，4E-BP と S6K に作用する．そして，eIF4F などを介して翻訳が開始する．FMRP は mGluR5 の刺激による S65K や PP2A の活性化を介して制御されている．

MEK：mitogen-activated protein kinase kinase, ERK：extracellular signal regulated kinase, Mnk1：mitogen-activated protein kinase interacting serine/threonine kinase 1, PI3K：phosphoinositide-3 kinase, 4E-BP：4E-binding protein, S6K：S6 kinase, PIP2：phosphatidylinositol 4,5-bisphosphate, PIP3：phosphatidylinositol(3,4,5)-trisphosphate, PDK1：3-phosphoinositide-dependent kinase 1, TSC：tuberous sclerosis complex, GAP：GTPase-activating protein.

FXS とは異なっており，詳細は文献を参照されたい[6]

動物モデル

マウスの Fmr1 遺伝子は，ヒトと異なり CGG 繰返し配列をもたない．そのために，CGG 繰返しを延長させることは困難であるが，遺伝子機能を欠

失したモデルマウス〔Fmr1 ノックアウト(KO)マウス〕が開発されている[7]．また，ショウジョウバエなどのモデルも開発されてきた．これらのモデル動物は記憶や行動の異常，巨大睾丸，さらに痙攣を起こしやすいなど，ヒトの症状のかなりの部分が再現されている．

FMRPの機能とその異常

FMR1 遺伝子がコードする蛋白，FMRP はユビキタスであるが，脳と精巣に比較的強く発現する RNA 結合蛋白である[8]．FMRP は 3 つの RNA 結合部位(2 つの KH ドメインと 1 つの RGG ボックス)をもち，おもに標的 mRNA の 3′ 非翻訳領域に結合する．FMRP は核内 mRNA に結合するが，神経細胞では核内のみならず，シナプス樹上突起や樹状突起棘の局所的 mRNA と結合している．FMRP は標的 mRNA の翻訳を抑制することによりシナプスの機能を維持しており，この機能が失われるとシナプス可塑性に変化をもたらし，知的障害などの症状を呈する．この局所的 mRNA の翻訳調節は，後述する代謝型グルタミン酸受容体(mGluR)からのシグナルが引き金になっている．この mGluR からのシグナル経路の詳細は明らかにされてきている(図 1)．さらに，mGluR5 を刺激すると FMRP が急速に脱リン酸化され，シナプスの局所的な mRNA の急激な増加を引き起こすことが明らかになっている[9]．リン酸化されていない FMRP は，むしろ蛋白翻訳を活性化させ，リン酸化された FMRP のみが蛋白翻訳を抑制できる．FMRP は 499 のセリンが特異的にリン酸化される．この機構には mTOR カスケードが必要で，最終的には S6 キナーゼがリン酸化されることにより FMRP のリン酸化が起こる[10]．

FXSでのシナプス形態と可塑性の異常

前述の FMRP 異常の機構によりシナプスの異常が引き起こされる．FXS では大きな脳の形態学的変化はないが，シナプス樹状突起棘に異常(数が多い，異常に長く曲がった形)があり，未熟であることが明らかにされている[11]．余談になるかもしれないが，近年，Down 症候群や Rett 症候群などにも同様にシナプス樹状突起の異常がみられることが明らかになっている．また，シナプスの活動状況によってシナプスの伝達効率が変化するシナプス可塑性は記憶や学習に重要な役割があり，シナプス伝達効率が増加する長期増強(LTP)やこの伝達効率が低下する長期抑制(LTD)などの生理的な現象と密接な関係がある．FXS では海馬と小脳の LTD が増強され，大脳や海馬では LTP に変化を起こすことなど，可塑性の異常が報告されている[12]．

代謝型グルタミン酸受容体(mGluR)理論

FXS でみられるシナプス形態，可塑性などさまざまの異常を一元的に説明できる画期的な mGluR 理論が，2004 年に Bear らによって報告された[13]．この理論により FXS における，①シナプス棘の数の異常や未熟性，②Fmr1 ノックアウトマウスの神経生理学的異常，③mGluR5 の活性化によるシナプスの樹上突起の蛋白合成の促進，④FXS 患者やマウスモデルの行動異常，などがすべて説明できる．その後も，この理論を支持する研究が次々に報告され，現在の治療法開発へと結びついている．

本理論を理解するためには，mGluR 受容体などの基本的な理解が必要となる．脳のシナプス膜にはイオンチャネル型と代謝型の 2 種類の受容体が存在する．イオンチャネル型受容体は特異的なリガンドと結合し，イオンを通過させ興奮性神経伝達機構を担う．AMPA(γ-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) 型，NMDA(N-methyl-D-aspartic acid)型，さらにカイニン酸などがおもなイオンチャネル型受容体として知られている，脳の可塑性の機構である LTD は，この AMPA 受容体の数の減少によって引き起こされる．

一方，代謝型グルタミン酸受容体(mGluR)はおもには G 蛋白依存で，7 回膜貫通領域(7TMD)をもつ．mGluR は 8 つのサブタイプに分かれ，これらは構造の類似性や薬理学的な作用などから 3 つのグループに分類される(グループⅠ，Ⅱ，Ⅲ)．FXS で重要な mGluR1 と mGluR5 はグループⅠに分類され，Gq 蛋白と結合し，ホスホリパーゼ C を活性化させる．FXS ではグループⅠの mGluR

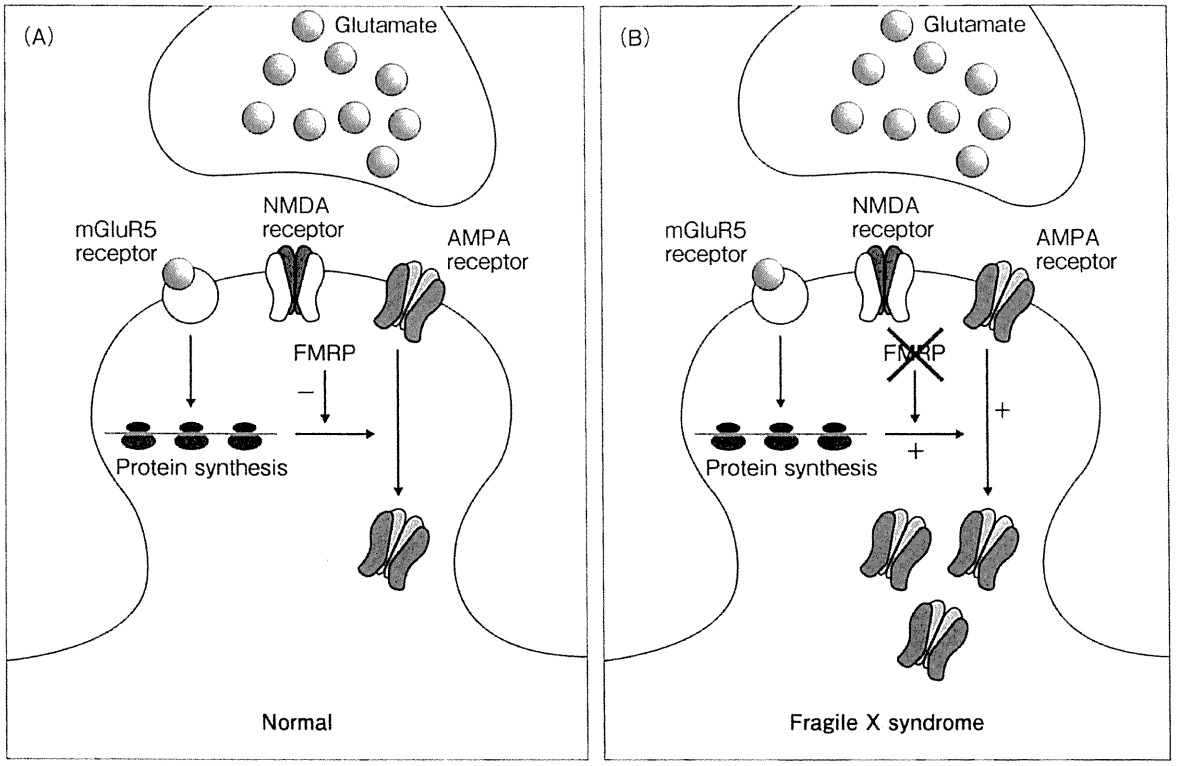

図 2　mGluR理論[15]

A：グルタミン酸が mGluR5 を刺激し，シナプスの局所的 mRNA の転写が開始される．この局所的な蛋白合成が，シナプス可塑性に重要な役割を果たしている AMPA 受容体の内在化を促進する．FMRP はこの転写を抑制することにより AMPA 受容体の内在化を阻止している．

B：Fmr1 KO マウスの研究によると，FXS 患者の神経細胞では FMRP が消失することにより AMPA 受容体の内在化が促進され，シナプスの異常が起こる．

の刺激が異常に増強しており，それにより AMPA 受容体の内在化が引き起こされることがこの mGluR 理論の中心である(図2)．グルタミン酸がグループ I mGluR を刺激すると，FXS では FMRP の転写抑制がないために局所の mRNA の転写が異常に増強する．その結果，局所の蛋白合成が増え，最終的に AMPA 受容体を内在化させてしまう．そしてシナプス可塑性の変化や形態異常を引き起こす．この理論の直接的な実証として，Fmr1 KO マウスにおいて mGluR5 を 50%に減少させると，シナプスの形態，蛋白合成異常，痙攣などの異常が改善された研究が報告されている[14]．さらに，この理論を裏づける多くの研究結果が報告されている．

治療法の開発[15]

現段階として，FXS の治療法として最終的に確立したものはないが，mGluR 理論などに基づき，動物のみならず，ヒトにおいて臨床治療研究が進められている．おもな治療薬と作用について図3に示す[15]．さまざまな薬剤の治験の進行についてはホームページで調べることができる(http://www.clinicaltrials.gov)．そのおもなものについて解説する．

MPEP は mGluR5 拮抗薬として動物実験ではさまざまな症状の改善をもたらしたが，薬剤の安定性や毒性などから臨床応用には至らなかった．最初の臨床応用は fenobam が試みられた．この薬剤は，最初は作用機序がよくわからなかったが，その後 mGLuR5 の拮抗薬であることが確認された．Fenobam を用いた臨床試験の第Ⅱ相が最近終了した．12 人の成人の患者に 1 回のみの投与を行い，薬剤の安全性，薬理，一部の認知や行動への効果を検討した．この治験で，不安や音への過剰反応，注意や衝動性なども改善したことが報告されている．これらの結果はすばらしいものであるが，二重盲検ではないため今後の検討が必要である．また，経口投与では fenobam の濃度は変動が大き

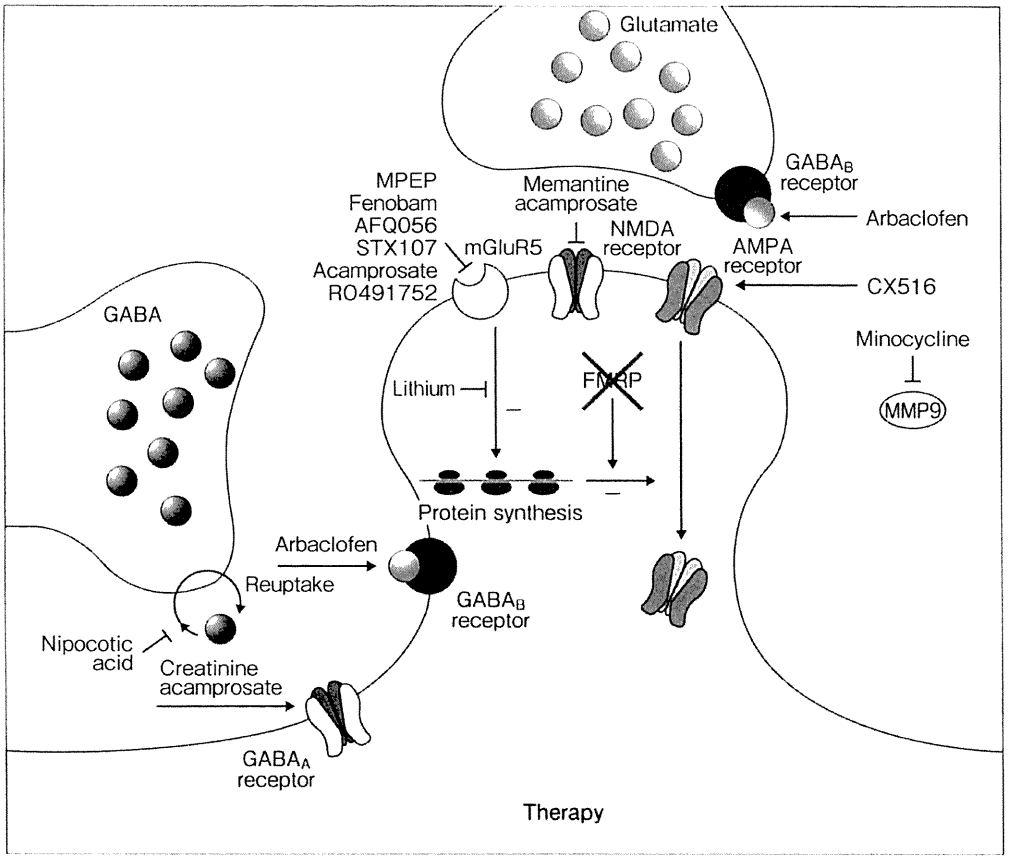

図 3　FXSの治療戦略[15]

FMRP の欠失によりグルタミン酸受容体が興奮し，GABA 受容体が抑制されたシナプスを示している．

下記の作用のある薬剤による FXS のシナプス機能の回復が検討されている．シナプス可塑性の異常を改善すると考えられる mGluR5 制御，$GABA_A$作動，$GABA_B$受容体作動，NMDA 受容体拮抗，AMPA 受容体の制御．さらに，リチウム，ミノサイクリン，acamprosate なども検討されている．

く，安定性にも問題がある．小規模治験として，アルコール中毒の治療薬として認められた acamprosate が3人の若年患者に試みられ，言語コミュニケーションや全般的な臨床症状の改善がみられた．さらに，各製薬会社が治療研究に乗り出しており，STX107(Seaside Therapeutics；アメリカで治験が開始される予定)，AFQ056(Novartis；フランス，イタリア，スイスで第Ⅱ相が終了)，RO4917523(Hoffman-LaRoche；アメリカで第Ⅱ相が開始)などの治療研究が進められている．

直接 mGluR を標的にした治療法に加え，mGLuR の上流や下流のシグナル伝達を阻止する方法も検討されている．最初に，気分障害の治療薬として使われているリチウムが用いられた．リチウムはグループ1 mGluR 伝達を含め，さまざまな分子経路に影響することが明らかになっている．

さらに，シナプス前のグルタミン酸の放出を減少させる方法も報告されている．これには$GABA_B$受容体拮抗薬として知られている baclofen が知られており，投与により Fmr1 KO マウスの聴覚過敏性痙攣を減少させることが報告された．この成果をもとにして baclofen の R-異性体である arbaclofen(STX209)が開発され，二重盲検第Ⅱ相の臨床治験が行われている．また，mGluR5 シグナル過剰の影響により matrix metalloproteinase-9 (MMP-9)遺伝子の過剰発現が Fmr1 KO マウスで起こっていることが明らかになった．この結果をもとに，この異常を抑制するミノサイクリン(テトラサイクリンのひとつのアナログ)を用いたマウスの研究では，いくつかの症状に効果があることが示されている．さらに，ヒトの治療研究が進められている．

おわりに

FXSの治療を考えるときには，正常な脳の発達が変化する時期や可塑性についても考慮する必要がある．マウスの実験では生後かなり経過しても症状が回復する可能性も示唆されているが，早期に治療するほうがよいことは間違いない．そのためには新生児期スクリーニングを検討する必要があり，欧米ではこの研究が開始されている[16]．

FXSではシナプス可塑性の異常が明らかになり，その機構の詳細な検討から治療法の開発に至っている．これは他の知的障害や自閉症などのモデルとしても重要と考えられる．FXSで開発される治療法は自閉症などにも応用が可能と考えられ，すでにその動きもはじまっている．

欧米ではFXSの団体やコンソーシアムを組織して積極的に研究が進められ，治療法の開発を行っている．日本でも，FXS患者への治療に向かって体制を整えていく必要がある．著者らは近い将来，日本人患者への治療も可能になるように体制の整備を進めている．多くの知的障害も治療への時代に入ってきており，日本でも大規模な共同研究体制を充実させ，研究を推進することが重要である．

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RESEARCH ARTICLE

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Neuropsychiatric Genetics

Association of SNPs Linked to Increased Expression of *SLC1A1* With Schizophrenia

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Glutamate is one of the key molecules involved in signal transduction in the brain, and dysfunction of glutamate signaling could be linked to schizophrenia. The *SLC1A1* gene located at 9p24 encodes the glutamate transporter EAAT3/EAAC1. To investigate the association between the *SLC1A1* gene and schizophrenia in the Japanese population, we genotyped 19 tagging single nucleotide polymorphisms (tagSNPs) in the *SLC1A1* gene in 576 unrelated individuals with schizophrenia and 576 control subjects followed by replication in an independent case–control study of 1,344 individuals with schizophrenia and 1,344 control subjects. In addition, we determined the boundaries of the copy number variation (CNV) region in the first intron (Database of Genomic Variants, chr9:4516796-4520549) and directly genotyped the CNV because of significant deviation from the Hardy–Weinberg equilibrium. The CNV was not associated with schizophrenia. Four SNPs showed a possible association with schizophrenia in the screening subjects and the associations were replicated in the same direction (nominal allelic $P < 0.05$), and, among them, an association with rs7022369 was replicated even after Bonferroni correction (allelic nominal $P = 5 \times 10^{-5}$, allelic corrected $P = 2.5 \times 10^{-4}$, allelic odds ratio, 1.30; 95% CI: 1.14–1.47 in the combined subjects). Expression analysis quantified by the real-time quantitative polymerase chain reaction in the postmortem prefrontal cortex of 43 Japanese individuals with schizophrenia and 11 Japanese control subjects

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revealed increased *SLC1A1* expression levels in individuals homozygous for the rs7022369 risk allele ($P = 0.003$). Our findings suggest the involvement of *SLC1A1* in the pathogenesis of schizophrenia. © 2011 Wiley Periodicals, Inc.

Key words: transporters; glutamate; postmortem brain; antipsychotics

INTRODUCTION

Schizophrenia is one of the most mysterious and costliest mental disorders and it affects 0.30–0.66% of the population. Despite its high heritability estimates, the identification of specific molecular genetic variation has not been easy. Recent findings have suggested that a small proportion of schizophrenia incidence could be explained by rare structural variations [van Os and Kapur, 2009; Vacic et al., 2011].

Glutamate transporters (excitatory amino acid transporters, EAATs) play important roles in maintaining extracellular glutamate concentrations. To date, 5 subtypes of Na^+-dependent glutamate transporters—EAAT1 (GLAST, *SLC1A3*), EAAT2 (GLT-1, *SLC1A2*), EAAT3 (*SLC1A1*), EAAT4 (*SLC1A6*), and EAAT5 (*SLC1A7*)—have been identified [Shigeri et al., 2004]. Removal of extracellular glutamate in the forebrain is controlled by three major EAATs, that is, EAAT1, EAAT2, and EAAT3 [Amara et al., 1998; Danbolt, 2001]. EAAT1 and EAAT2 are mainly glial and EAAT3 is mostly neuronal [Rothstein et al., 1994]. EAAT3 is encoded by the glutamate transporter, solute carrier family 1 gene (*SLC1A1*), which is located on chromosome 9p24. EAAT3 (termed EAAC1 in rodents) is predominantly expressed in the cerebral cortex, basal ganglia, and hippocampus.

On the basis of pharmacological evidence, dysfunctions of glutamate neurotransmission have been implicated in the pathophysiology of schizophrenia [Coyle, 2006; Tuominen et al., 2006]. EAAC1 may control activation of some subtypes of *N*-methyl-D-aspartate (NMDA) receptors and vice versa in the hippocampus [Waxman et al., 2007]. Environmental enrichment has been shown to decrease the mRNA expression of EAAC1 in the hippocampus [Andin et al., 2007] and EAAC1-deficient mice have shown reduced neuronal glutathione levels, and, with aging, they developed brain atrophy and behavioral changes including decreased spatial learning abilities and cognitive impairment [Aoyama et al., 2006]. It has also been suggested that EAAC1 deficiency leads to impaired neuronal glutathione metabolism and oxidative stress [Aoyama et al., 2006]. Thus, the glutamate hypothesis [Coyle, 2006], oxidative stress hypothesis [Sarandol et al., 2007], and parallel effects of environmental enrichment and antipsychotic treatment in schizophrenia [Andin et al., 2007] suggest the involvement of EAAT3 in schizophrenia.

Deng et al. [2007] genotyped eight even-spaced single nucleotide polymorphisms (SNPs) that were separated from each other by an average distance of 14 kb in the *SLC1A1* gene in 100 Japanese patients with schizophrenia and 100 Japanese controls. Although a potential association between rs2228622 and schizophrenia was found, the association was not confirmed in an additional sample comprising 300 schizophrenics and 320 controls. Since the average summary odds ratio (OR) of nominally significant effects of 24 genetic variants in 16 different genes was shown to be ~1.23 by systematic meta-analyses [Allen et al., 2008], large sample sizes are required to detect SNPs associated with schizophrenia. The present study aims to investigate associations between SNPs in the *SLC1A1* gene and schizophrenia by a large case–control study of 1,920 Japanese schizophrenic patients and 1,920 Japanese control subjects.

MATERIALS AND METHODS

Subjects

The screening groups were comprised 576 unrelated Japanese patients with schizophrenia and 576 mentally healthy unrelated Japanese control subjects. The replication groups were comprised 1,344 unrelated Japanese patients with schizophrenia and 1,344 mentally healthy unrelated Japanese control subjects. Patients with schizophrenia (1,055 men and 865 women; mean age ± standard deviation (SD), 48.2 ± 14.7 years) were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV; American Psychiatric Association (APA), 2001) with consensus from at least 2 experienced psychiatrists, and the control subjects (1,051 men and 869 women; mean age ± SD, 47.6 ± 13.4 years) were those whose second-degree relatives were free of psychosis on the basis of self-reporting by the subjects. All the participants provided their written informed consent. The association analysis was approved by the Ethics Committees of the University of Tsukuba, Niigata University, Fujita Health University, Nagoya University, Okayama University, and Seiwa Hospital.

Human Postmortem Brains

Brain specimens were obtained from Japanese individuals of 43 schizophrenic patients and 11 age- and gender-matched controls. Tissue blocks were cut from gray matter in an area of the prefrontal cortex referred to as Brodmann's area 9 (BA9). The Japanese subjects met the DSM-III-R criteria for schizophrenia. The control subjects had no known history of psychiatric illness. The study was approved by the Ethics Committees of Niigata University, University of Tsukuba, Tokyo Metropolitan Matsuzawa Hospital, and the Tokyo Institute of Psychiatry.

SNP Selection and Genotyping

The selection of tagSNPs for genotyping in the *SLC1A1* gene was conducted with the use of the International HapMap Project. A total of 19 tagSNPs were selected in this study (Fig. 1, Table I). The SNPs tagged by the selected 19 tagSNPs are shown in the Supplementary Table I.

The SNPs were genotyped by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA). Product information on the TaqMan SNP genotyping assays used in this study is listed in Supplementary Table II. The TaqMan reaction was performed in a final volume of 3 μl consisting of 2.5 ng genomic DNA and Universal Master Mix (Eurogentc, Seraing, Belgium). Genotyping was performed with the ABI PRISM 7900HT Sequence Detection

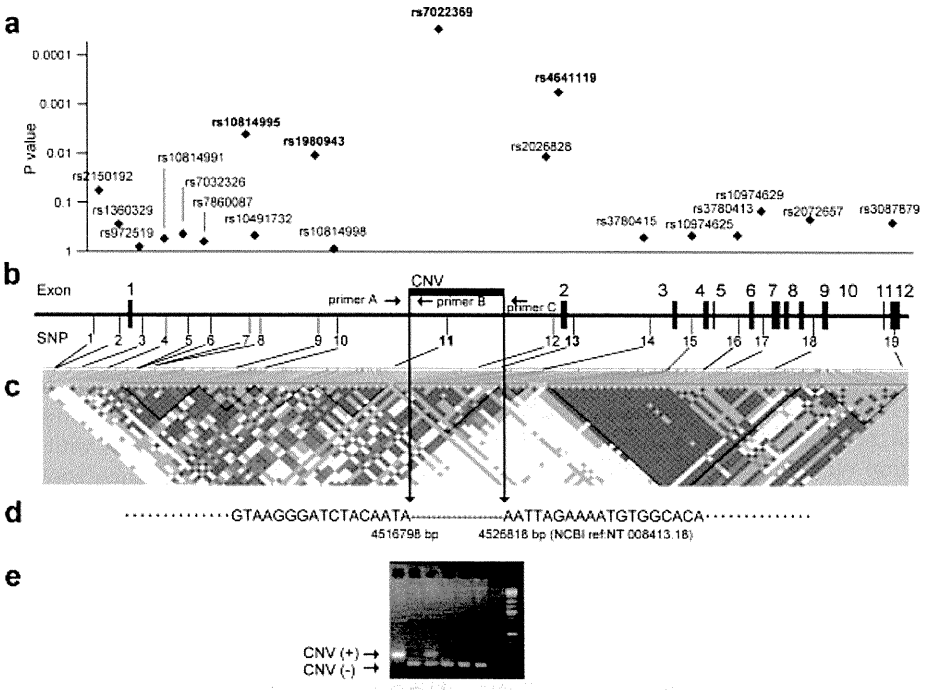

FIG. 1. The results of SNP association with schizophrenia and the position of the CNV analyzed in the SLC1A1 gene. a: Results of the association study. Squares indicate the allelic *P*-value in the screening population. SNPs in bold letters were also analyzed in the confirmation population and squares of them are the allelic *P*-values in the combined populations. b: Schematic representation of *SLC1A1*. The 12 exons and 11 introns of the *SLC1A1* gene and the approximate location of each polymorphism genotyped in the present study are shown here. The polymorphisms represented in bold showed a positive association in this study. The bold line indicates the copy number variation (CNV) region. c: Linkage disequilibrium and haplotype blocks in the *SLC1A1* gene region. Each box represents the D′ value corresponding to each pair-wise single nucleotide polymorphism combination. D′ is color-coded; the red box indicates D′ = 1.0 between two loci. d: The sequence and position of breakpoints of the CNV. e: An example of genotypes of the CNV amplified by PCR with the primers A, B, and C shown in (d). The ladder marker on the left side lane is 2-Log DNA Ladder (New England BioILabs, MA). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb]

System (Applied Biosystems). Because the SNPs potentially associated with schizophrenia were in the haplotype blocks that include exon 2, resequencing of SLC1A1 exon 2 was performed by direct sequencing with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). One-third (1,152) of the samples were genotyped twice for 5 SNPs using TaqMan genotyping (Applied Biosystems), and genotype concordance was 99.5% for rs10814995, 99.4% for rs1980943, 99.8% for rs7022369, 99.7% for rs10758629, 99.9% for rs4641119, respectively. The average missing genotype rate was 1.2% (0.2–1.6%).

Determination of the Boundaries of the CNV and Genotype

The boundaries of the copy number variation (CNV) region where rs7022369 is located were determined by directly sequencing the genomic DNA around rs7022369. This region was amplified by LA Taq (Takara, Kyoto, Japan) with the primers 5′-AAGATGGAATTGGGGAGGAT and 5′-CGGACGGCTTAAGTGTCAAC, and this produced a product of approximately 14 kb. The CNV was genotyped by the size of the PCR products with the primers 5′-TTAATGCCAGTGTTGCATGAG (common 5′-primer, the primer A in Fig. 1), 5′-GCCCTGGTGTGTGATATTCC (deletion 3′-primer, the primer C in Fig. 1) and 5′-CATTTGCAAAAGTCTCTTTACCTT (wild-type 3′-primer, the primer B in Fig. 1). The 283 and 219 bp PCR product indicated the deletion type and the normal wild-type, respectively.

Real-Time Quantitative PCR for SLC1A1 Expression in Brains

Total RNA was isolated from human brain tissue (BA9) with an SV Total RNA Isolation System (Promega, Madison, WI). *SLC1A1* expression was quantified by real-time quantitative polymerase chain reaction (PCR) with a TaqMan Gene Expression Assay and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) as per the manufacturer's instructions. Primers and probes were purchased from Applied Biosystems (Assay ID: Hs00179051_m1). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control, and measurement of the threshold cycle (C_t) was performed in triplicate. Data were collected and analyzed with Sequence Detector Software (SDS) version 2.1 (Applied Biosystems) and the standard curve method. Relative gene expression was calculated as the ratio of *SLC1A1* to the

TABLE I. Genotypic and Allelic Distributions of the SLC1A1 Gene Polymorphisms in the Screening Population

SNP No.	dbSNP ID	Subjects	n	Genotype count (frequency)			$P_{genotypic}$	Allele count (frequency)		$P_{allelic}$	HWE *P*
1	rs2150192			AA	AG	GG		A	G		
		Sz	569	259 (0.46)	238 (0.42)	72 (0.13)		756 (0.66)	382 (0.34)		0.138
		C	576	267 (0.46)	253 (0.44)	56 (0.10)	0.28	787 (0.68)	365 (0.32)	0.34	0.726
2	rs1360329			TT	TG	GG		G	G		
		Sz	568	477 (0.84)	85 (0.15)	6 (0.01)		1039 (0.91)	97 (0.09)		0.318
		C	566	472 (0.83)	89 (0.16)	5 (0.01)	0.90	1033 (0.91)	99 (0.09)	0.86	0.724
3	rs972519			GG	GC	CC		G	C		
		Sz	574	503 (0.88)	66 (0.11)	5 (0.01)		1072 (0.93)	76 (0.07)		0.093
		C	558	488 (0.87)	68 (0.12)	2 (0.00)	0.52	1044 (0.94)	72 (0.06)	0.87	0.821
4	rs10814991			CC	CT	TT		C	T		
		Sz	571	119 (0.21)	275 (0.48)	177 (0.31)		513 (0.45)	629 (0.55)		0.523
		C	567	123 (0.22)	282 (0.50)	162 (0.29)	0.67	528 (0.47)	606 (0.53)	0.43	0.989
5	rs7032326			TT	TC	CC		T	C		
		Sz	572	95 (0.17)	258 (0.45)	219 (0.38)		448 (0.39)	696 (0.61)		0.201
		C	565	86 (0.15)	245 (0.43)	234 (0.41)	0.54	417 (0.37)	713 (0.63)	0.27	0.102
6	rs7860087			GG	GC	CC		G	C		
		Sz	572	458 (0.80)	107 (0.19)	7 (0.01)		1023 (0.89)	121 (0.11)		0.790
		C	572	473 (0.83)	92 (0.16)	7 (0.01)	0.50	1038 (0.91)	106 (0.09)	0.29	0.299
7	rs10814995			AA	AC	CC		A	C		
		Sz	572	310 (0.54)	222 (0.39)	40 (0.07)		842 (0.74)	302 (0.26)		0.976
		C	561	278 (0.50)	227 (0.40)	56 (0.10)	0.11	783 (0.70)	339 (0.30)	**0.04**	0.338
8	rs10491732			GG	GA	AA		G	A		
		Sz	569	417 (0.73)	137 (0.24)	15 (0.03)		971 (0.85)	167 (0.15)		0.358
		C	567	402 (0.71)	148 (0.26)	17 (0.03)	0.66	952 (0.84)	182 (0.16)	0.36	0.455
9	rs1980943			AA	AG	GG		A	G		
		Sz	572	183 (0.32)	292 (0.51)	97 (0.17)		658 (0.58)	486 (0.42)		0.286
		C	571	153 (0.27)	289 (0.51)	129 (0.23)	0.03	595 (0.52)	547 (0.48)	**0.01**	0.737
10	rs10814998			AA	AG	GG		A	G		
		Sz	572	265 (0.46)	252 (0.44)	55 (0.10)		782 (0.68)	362 (0.32)		0.660
		C	575	260 (0.45)	259 (0.45)	56 (0.10)	0.93	779 (0.68)	371 (0.32)	0.75	0.463
11	rs7022369			CC	CG	GG		C	G		
		Sz	572	432 (0.76)	115 (0.20)	25 (0.04)		979 (0.86)	165 (0.14)		**0.000009**
		C	566	383 (0.68)	156 (0.28)	27 (0.05)	**0.01**	922 (0.81)	210 (0.19)	**0.01**	0.04
12	rs2026828			AA	AG	GG		A	G		
		Sz	570	202 (0.35)	273 (0.48)	95 (0.17)		677 (0.59)	463 (0.41)		0.865
		C	569	181 (0.32)	268 (0.47)	120 (0.21)	0.13	630 (0.55)	508 (0.45)	0.05	0.262
13	rs4641119			AA	AC	CC		A	C		
		Sz	573	431 (0.75)	128 (0.22)	14 (0.02)		990 (0.86)	156 (0.14)		0.230
		C	576	384 (0.67)	170 (0.30)	22 (0.04)	**0.002**	938 (0.81)	214 (0.19)	**0.001**[a]	0.559
14	rs3780415			TT	TC	CC		T	C		
		Sz	574	429 (0.75)	132 (0.23)	13 (0.02)		990 (0.86)	158 (0.14)		0.454
		C	568	419 (0.74)	134 (0.24)	15 (0.03)	0.89	972 (0.86)	164 (0.14)	0.64	0.283
15	rs10974625			GG	GA	AA		G	A		
		Sz	565	180 (0.32)	262 (0.46)	123 (0.22)		622 (0.55)	508 (0.45)		0.134
		C	564	183 (0.32)	266 (0.47)	115 (0.20)	0.85	632 (0.56)	496 (0.44)	0.64	0.309
16	rs3780413			GG	GC	CC		G	C		
		Sz	567	289 (0.51)	223 (0.39)	55 (0.10)		801 (0.71)	333 (0.29)		0.216
		C	569	299 (0.53)	218 (0.38)	52 (0.09)	0.86	816 (0.72)	322 (0.28)	0.57	0.183
17	rs10974629			AA	AG	GG		A	G		
		Sz	571	314 (0.55)	216 (0.38)	41 (0.07)		844 (0.74)	298 (0.26)		0.646
		C	569	308 (0.54)	201 (0.35)	60 (0.11)	0.12	817 (0.72)	321 (0.28)	0.26	0.002
18	rs2072657			TT	TG	GG		T	G		
		Sz	573	282 (0.49)	229 (0.40)	62 (0.11)		793 (0.69)	353 (0.31)		0.135
		C	564	303 (0.54)	212 (0.38)	49 (0.09)	0.24	818 (0.73)	310 (0.27)	0.08	0.176

(*Continued*)

TABLE I. (*Continued*)

SNP No.	dbSNP ID	Subjects	n	Genotype count (frequency)			$P_{genotypic}$	Allele count (frequency)		$P_{allelic}$	HWE *P*
19	rs3087879			GG	GC	CC		G	C		
		Sz	574	432 (0.75)	131 (0.23)	11 (0.02)		995 (0.87)	153 (0.13)		0.771
		C	568	422 (0.74)	127 (0.22)	19 (0.03)	0.32	971 (0.85)	165 (0.15)	0.41	0.018

$P_{genotypic}$, the Cochran–Armitage trend test; $P_{allelic}$, Fisher's exact test.
[a]Permutation *P*-value = 0.02.

internal control (*GAPDH*), and the mean of the three replicate measures was assigned to each individual.

Statistical Analysis

Allelic and genotypic associations were evaluated by Fisher's exact test and the Cochran–Armitage trend test, respectively. The detection power with this sample size was greater than 0.95 assuming an allelic relative risk of 1.23 and risk allele frequencies from 0.2 to 0.8 according to the Genetic Power Calculator in the total subjects [Purcell et al., 2003]. Deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by the chi-squared test. Linkage disequilibrium and haplotype frequencies/associations were evaluated with the Haploview program (http://www.broad.mit.edu/mpg/haploview/). In this study, we evaluated 19 SNPs for allelic associations with schizophrenia in the screening population, and subsequently genotyped SNPs with $P < 0.05$ at the screening step to confirm the association in the replication population. Corrected *P*-values were calculated with the Bonferroni method for SNP association analysis and with the use of 100,000 permutation as implemented in the Haploview program for haplotype association analysis.

Differences in *SLC1A1* expression as determined by real-time quantitative PCR were analyzed by the Wilcoxon test with JMP software version 8 (SAS Institute, Cary, NC), and $P < 0.05$ was considered significant.

RESULTS

The genotype and allele distributions of the 19 tagSNPs in the screening population are shown in Table I. Four SNPs (rs10814995, rs1980943, rs7022369, and rs4641119) showed nominally significant allelic association with schizophrenia. Among them, the genotype distribution of rs7022369 deviated significantly from the HWE in both patient and control groups (Table I). Because SNP rs7022369 is located in the CNV region (Database of Genomic Variants, http://projects.tcag.ca/variation/ variation_33067, 10284, and 2785, http://projects.tcag.ca/variation/), we determined the boundary of the CNV region (Fig. 1) and developed a method to identify the CNV by PCR. The CNV was deleted between 4516798 and 4526818 (NCBI ref: NT 008413.18; Fig. 1d) with an allele frequency of 2%. The CNV was not significantly associated with schizophrenia (Table II). When individuals with the CNV were excluded, the genotype distribution of rs7022369 did not deviate from HWE in the control subjects (Table II). Therefore, we excluded individuals with the CNV in the following analysis for this SNP. Among four SNPs with nominally significant association in the screening subjects, rs7022369 was associated with schizophrenia in an independent case–control population even after Bonferroni correction (allelic nominal *P*-value = 0.001; allelic corrected *P*-value = 0.004 in the same direction as in the screening subjects; Table II). The genotype distribution of rs7022369 did not deviate significantly from HWE in the replication and total samples when individuals with the CNV were excluded (Table II). The data in the combined populations revealed significant allelic associations of rs7022369 (nominal allelic $P = 5 \times 10^{-5}$, allelic OR = 1.30, 95% CI: 1.14–1.47) and rs4641119 (nominal allelic $P = 5 \times 10^{-4}$, allelic OR = 1.24, 95% CI: 1.10–1.41; Table II). Haplotype analysis with rs7022369 and rs4641119 showed that the haplotype frequency of the C of rs7022369 and A of rs4641119 was significantly higher in the schizophrenia group (0.84) than the control group (0.80; permutation $P = 1.0 \times 10^{-3}$).

Because the SNPs associated with schizophrenia are in the haplotype blocks that include exon 2, we resequenced exon 2 in 32 randomly selected patients. However, we did not identify any nonsynonymous mutations. Therefore, we suspected that the SNPs associated with schizophrenia found in the present study were markers regulating *SLC1A1* expression. We explored the association of rs7022369 and rs4641119 with *SLC1A1* expression in the postmortem prefrontal cortex of 43 individuals with schizophrenia and 11 control subjects. *SLC1A1* expression was higher in brains homozygous for the major C allele of rs7022369 or the major A allele of rs4641119 than brains with the other genotypes ($P = 0.003$ and $P = 0.02$, respectively, Wilcoxon test; Fig. 2). This association was particularly obvious in the patient group ($P = 0.01$ at rs7022369 and $P = 0.12$ at rs4641119, Wilcoxon test). However, we should take into account the fact that the number of control brain samples was small. The effects on gene expression of sample pH, postmortem interval, sex, or age at death were not significant (data not shown). *SLC1A1* expression was not significantly different between the patient and control groups ($P = 0.17$, Wilcoxon test).

DISCUSSION

The present study identified the association between SNPs near exon 2 of the *SLC1A1* gene and schizophrenia. These findings need to be replicated in other populations before accepting them. Because the OR of rs7022369 for association with schizophrenia was only 1.30 (95% CI: 1.14–1.47), more than 1,500 patients and an equal number of controls need to be examined to exceed 80% power in replication studies.

In the present study, we did not provide evidence that the SNPs examined directly cause the association with schizophrenia and/or

TABLE II. Genotypic and Allelic Distributions of the SLC1A1 Gene Polymorphisms in the Replication and Combined Populations

SNP no.	dbSNP ID/population	Subjects	n	Genotype count (frequency)			$P_{genotypic}$	Allele count (frequency)		$P_{allelic}$	Allelic OR (95% CI)	HWE P
7	rs10814995			AA	AG	GG		A	G			
	Screening	Sz	572	310 (0.54)	222 (0.39)	40 (0.07)		842 (0.74)	302 (0.26)			0.976
		C	561	278 (0.50)	227 (0.40)	56 (0.10)	0.04	783 (0.70)	339 (0.30)	0.04		0.338
	Replication	Sz	1,324	738 (0.56)	494 (0.37)	92 (0.07)		1970 (0.74)	678 (0.26)			0.453
		C	1,323	680 (0.51)	540 (0.41)	103 (0.08)	0.03	1900 (0.72)	746 (0.28)	0.02		0.769
	Combined	Sz	1,896	1048 (0.55)	716 (0.38)	132 (0.07)		2812 (0.74)	980 (0.26)			0.520
		C	1,884	958 (0.51)	767 (0.41)	159 (0.08)	0.004	2683 (0.71)	1085 (0.29)	0.004	1.16 (1.05–1.28)	0.754
9	rs1980943			AA	AG	GG		A	G			
	Screening	Sz	572	183 (0.32)	292 (0.51)	97 (0.17)		658 (0.58)	486 (0.42)			0.29
		C	571	153 (0.27)	289 (0.51)	129 (0.23)	0.03	595 (0.52)	547 (0.48)	0.01		0.74
	Replication	Sz	1,337	432 (0.32)	638 (0.48)	267 (0.20)		1502 (0.56)	1172 (0.44)			0.26
		C	1,304	389 (0.30)	639 (0.49)	276 (0.21)	0.37	1417 (0.54)	1191 (0.46)	0.09		0.65
	Combined	Sz	1,909	615 (0.32)	930 (0.49)	364 (0.19)		2160 (0.57)	1658 (0.43)			0.71
		C	1,875	542 (0.29)	928 (0.49)	405 (0.22)	0.04	2012 (0.54)	1738 (0.46)	0.01	1.13 (1.03–1.23)	0.83
11	rs7022369			CC	CG	GG		C	G			
	Screening	Sz	551	416 (0.75)	115 (0.21)	20 (0.04)		947 (0.86)	155 (0.14)			0.001
		C	541	364 (0.67)	156 (0.29)	21 (0.04)	0.01	884 (0.82)	198 (0.18)	0.01		0.41
	Replication	Sz	1,275	937 (0.73)	312 (0.24)	26 (0.02)		2186 (0.86)	364 (0.14)			0.996
		C	1,271	870 (0.68)	359 (0.28)	42 (0.03)	0.009	2099 (0.83)	443 (0.17)	**0.001**		0.508
	Combined	Sz	1,826	1353 (0.74)	427 (0.23)	46 (0.03)		3133 (0.86)	519 (0.14)			0.08
		C	1,812	1234 (0.68)	515 (0.28)	63 (0.03)	**6.8×10^{-5}**	2983 (0.82)	641 (0.18)	**5×10^{-5}**	1.30 (1.14–1.47)	0.309
	rs7022369			C del	G del	del del						
	Individuals with the CNV	Sz	89	79 (0.89)	6 (0.07)	4 (0.04)						
		C	87	76 (0.87)	8 (0.09)	3 (0.03)						
	CNV			2 Copies	1 Copy	0 Copy		Without CNV	With CNV			
	(Combined population)	Sz	1,915	1826 (0.95)	85 (0.04)	4 (0.00)		3737 (0.98)	93 (0.02)			0.006
		C	1,899	1812 (0.95)	84 (0.04)	3 (0.00)	0.93	3708 (0.98)	90 (0.02)	0.88		0.055
13	rs4641119			AA	AC	CC		A	C			
	Screening	Sz	573	431 (0.75)	128 (0.22)	14 (0.02)		990 (0.86)	156 (0.14)			0.23
		C	576	384 (0.67)	170 (0.30)	22 (0.04)	0.001	938 (0.81)	214 (0.19)	**0.001**		0.56
	Replication	Sz	1,342	983 (0.73)	325 (0.24)	34 (0.03)		2291 (0.85)	393 (0.15)			0.25
		C	1,341	927 (0.69)	382 (0.28)	32 (0.02)	0.02	2236 (0.83)	446 (0.17)	0.02		0.32
	Combined	Sz	1,915	1414 (0.74)	453 (0.24)	48 (0.03)		3281 (0.86)	549 (0.14)			0.11
		C	1,917	1311 (0.68)	552 (0.29)	54 (0.03)	5.9×10^{-4}	3174 (0.83)	660 (0.17)	**5×10^{-4}**	1.24 (1.10–1.41)	0.65

NV region: chromosome 4516798–4526818 (NCBI ref:NT 008413.18); $P_{genotype}$: Cochran–Armitage trend test.

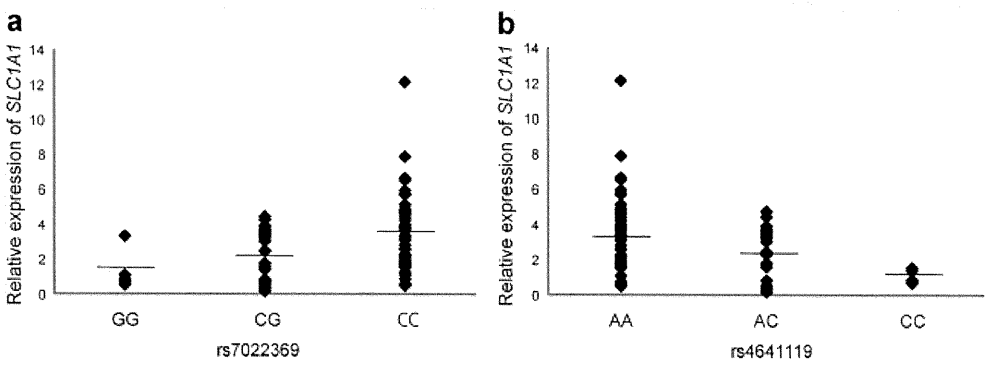

FIG. 2. Expression of *SLC1A1* in postmortem brains classified according to the single nucleotide polymorphism rs10758629 and rs4641119 genotype. Expression of *SLC1A1* was normalized to that of glyceraldehyde-3-phosphate dehydrogenase. a: The difference in expression between the TT genotype and AA genotype in rs10758629 is significant (Wilcoxon test, $P = 0.003$). AA genotype, $n = 7$; TA genotype, $n = 30$; TT genotype, $n = 52$. b: The difference in expression between the AA genotype and CC genotype in rs4641119 is significant (Wilcoxon test, $P = 0.02$). CC genotype, $n = 7$; AC genotype, $n = 28$; AA genotype, $n = 52$. The horizontal line indicates the mean.

the association of *SLC1A1* expression in the prefrontal cortex. A survey of 193 neuropathologically normal human brain samples (Myers et al., 2007) showed the location of a potential *cis*-acting region regulating *SLC1A1* expression within the 15 kb between rs1980943 and rs10758629, as calculated with PLINK [Purcell et al., 2007], where rs7022369 is located. The calculated lowest allelic *P*-value of 0.006 was at rs10814997, which is in complete linkage disequilibrium with rs1980943 (according to the HapMap data, $r^2 = 1$ in the Japanese population). An association between rs1980943 and schizophrenia was suggested in the present study (nominal allelic $P = 0.01$, Table II). Thus, the *cis*-acting region regulating *SLC1A1* is likely to be located in the first intronic region, although its exact position requires further investigation.

Decreases in EAAT3 have been observed in the striatum of schizophrenics [McCullumsmith and Meador-Woodruff, 2002; Nudmamud-Thanoi et al., 2007]. Preclinical studies have demonstrated that chronic treatment with clozapine or haloperidol can downregulate EAAT3 in the infralimbic cortex and hippocampal CA2 [Schmitt et al., 2003]. Therefore, EAAT3 expression is influenced by antipsychotic treatments, but it is difficult to distinguish between the cause and effect on the basis of postmortem brain studies. In the model of diminished glutamate activity in schizophrenia, potential therapeutic effects on some symptom dimensions is expected by glutamate re-uptake inhibitors, such as EAAT3 antagonist, which could increase the synaptic availability of glutamate and increase glutamatergic action at the postsynaptic neuron [Miyamoto et al., 2005]. In the present study, the risk genotype was associated with increased *SLC1A1* expression levels in the prefrontal cortex. On the basis of these findings, we speculated that individuals with a tendency toward increased EAAT3 expression are susceptible to schizophrenia. Higher EAAT3 may be linked to lower synaptic availability of glutamate or more direct mechanism(s) leading to improper functioning of NMDA receptors in some cases. Because different regulation of EAAT3 among brain regions is likely and the associations between SNPs and *SLC1A1* expression were not analyzed in regions other than the prefrontal cortex, further studies regarding the same are required. Furthermore, in our findings, the relationship between SNPs and *SLC1A1* expression in the prefrontal cortex was observed more obviously in the patient group than the control group. Therefore, the possibility remains that the association between SNPs and *SLC1A1* expression reflected antipsychotic treatment responses.

The polymorphisms in *SLC1A1* have been reported to be associated with obsessive-compulsive disorder [Arnold et al., 2006; Dickel et al., 2006; Grados and Wilcox, 2007; Stewart et al., 2007]. More recently, a *SLC1A1* haplotype was reported to be associated with obsessive-compulsive symptoms induced by atypical antipsychotics [Kwon et al., 2009]. These polymorphisms that were associated with obsessive-compulsive disorder or other symptoms span from introns 2 to 6 of the *SLC1A1* gene, and they are not in linkage disequilibrium with SNPs identified as associated with schizophrenia in the present study (Fig. 1).

In conclusion, our findings provide evidence that the *SLC1A1* gene might be involved in susceptibility to schizophrenia. Further studies on the involvement of the *SLC1A1* gene in the pathophysiology of schizophrenia and confirmation of the present association in other populations are necessary.

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ORIGINAL ARTICLE

DPP6 as a candidate gene for neuroleptic-induced tardive dyskinesia

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We implemented a two-step approach to detect potential predictor gene variants for neuroleptic-induced tardive dyskinesia (TD) in schizophrenic subjects. First, we screened associations by using a genome-wide (Illumina HumanHapCNV370) SNP array in 61 Japanese schizophrenia patients with treatment-resistant TD and 61 Japanese schizophrenia patients without TD. Next, we performed a replication analysis in 36 treatment-resistant TD and 138 non-TD subjects. An association of an SNP in the *DPP6* (dipeptidyl peptidase-like protein-6) gene, rs6977820, the most promising association identified by the screen, was significant in the replication sample (allelic $P=0.008$ in the replication sample, allelic $P=4.6\times10^{-6}$, odds ratio 2.32 in the combined sample). The SNP is located in intron-1 of the *DPP6* gene and the risk allele was associated with decreased *DPP6* gene expression in the human postmortem prefrontal cortex. Chronic administration of haloperidol increased *Dpp6* expression in mouse brains. DPP6 is an auxiliary subunit of Kv4 and regulates the properties of Kv4, which regulates the activity of dopaminergic neurons. The findings of this study indicate that an altered response of Kv4/DPP6 to long-term neuroleptic administration is involved in neuroleptic-induced TD. *The Pharmacogenomics Journal* advance online publication, 9 August 2011; doi:10.1038/tpj.2011.36

Keywords: DPP6; dopamine; schizophrenia/antipsychotics; tardive dyskinesia; Kv4

Introduction

Tardive dyskinesia (TD) is the involuntary movement of the tongue, lips, face, trunk and extremities that occurs in patients who are undergoing long-term treatment with antipsychotic medication. TD is often intractable to treatment and the presence of intractable TD is associated with a poorer quality of life.[1] Even though recent studies have indicated that most patients have no significant interference in functioning or quality of life from TD,[2,3] identifying patients at high risk for TD is still a high priority for psychiatrists in treatment selection. Second-generation antipsychotics have lowered the risk of TD to approximately 1% annually as compared with the 5% frequency with typical agents,[4,5] although a recent review has reported a much higher annual TD incidence of 3.9% for second-generation antipsychotics as compared with 5.5% for typical agents.[6] Furthermore, because second-generation antipsychotics may have few other advantages over older, cheaper drugs, doubt has been raised about the cost-effectiveness of second-generation antipsychotics when based purely on this reduced risk of TD.[2] Owing to the lack of effective treatments for TD, its therapeutic management can be problematic for schizophrenia patients receiving antipsychotic medications, especially for those patients who develop severe intractable TD. Therefore, the strategies to prevent TD are often discussed in the context of the safety and use of antipsychotic drugs.[7]

It is not known why only some patients develop TD, that is, the determinants of its onset are still unclear. At present the etiology of TD may be related to the interaction between the exogenous drugs and the endogenous predisposition, but the nature of TD is so far elusive. In addition to age, gender and ethnicity as suggested risk factors for TD, smoking, drinking and use of street drugs may also increase risk.[8] There is some evidence for a genetic component to TD[9] and molecular genetic studies of TD were conducted to identify genes related to TD.[10]

The pathophysiology of TD is not completely understood. In addition to the dopamine super-sensitivity hypothesis of TD,[11] there are many other pathophysiological models proposed, including changes in neurotransmitter signaling systems such as γ-aminobutyric acid,[12] norepinephrine,[13] serotonin[14] and acetylcholine,[15] which are affected by neuroleptics. In addition to a candidate gene approach,[16] two genome-wide association studies (GWASs) based on the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) study were published.[17,18] We also reported associations between single-nucleotide polymorphisms (SNPs) on the Illumina Human-1 Genotyping 109K BeadChip and TD in the Japanese sample,[19] in which we selected 63 SNPs with allelic *P*-values <0.002 and located within 10 kb from known genes for subsequent replication analysis, and found three SNPs associated nominally significantly with TD in the replication sample. The allelic *P*-values in the combined sample were 2×10^{-5} for rs2445142 in *HSPG2*; 2×10^{-4} for rs4738269 in *KCNB2* and 6×10^{-4} for rs2061051 in *GBRG3*, respectively. We also reported associations of SNPs in the genes grouped into the γ-aminobutyric acid receptor signaling pathway,[7] through GWAS by using the Illumina Human-1 BeadChip in a Japanese population. In the present study, we searched for further SNPs associated with TD by using the Illumina HumanHapCNV370 BeadChip to complement our previous results using the Human-1 BeadChip.

Materials and methods

Ethical considerations

The ethics committee of each institution approved the study. Written informed consent was obtained from all patients after adequate explanation of the study.

Human subjects

The human subjects in this study were 97 Japanese schizophrenia patients with treatment-resistant TD and 199 Japanese schizophrenia patients without TD (Table 1), most of whom have been described elsewhere.[7] In brief, subjects were identified at psychiatric hospitals located around the Tokyo and Nagoya areas of Japan. All patients fulfilled the diagnostic criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV[20] for schizophrenia. All subjects and their parents were of Japanese descent. All subjects had been receiving antipsychotic therapy for at least 1 year and their TD status was monitored for at least 1 year. TD was assessed according to the Japanese version of the Abnormal Involuntary Movement Scale (AIMS), which was validated by Itoh *et al.* (1977; in Japanese).[21] TD was diagnosed according to the criteria proposed by Schooler and Kane.[22] Once TD was identified, the patients were followed up and received standard therapeutic regimens for TD to minimize TD symptoms. If TD persisted after more than 1 year of therapy, patients were considered potential treatment-resistant TD patients. Treatment-resistant TD patients were defined as those patients with dyskinetic movements that persisted more than 1 year and did not improve after at least 1 year of appropriate treatment following guideline-recommended therapeutic regimens for TD. Patients with treatment-resistant TD were all inpatients who had been receiving antipsychotic therapy for controlling both psychosis and persistent severe TD. The treatment options for TD include possible reduction of antipsychotics, as well as switching from conventional antipsychotics to atypical ones, without relapse of their psychotic conditions. The TD status, as well as psychotic conditions, had been checked every 2 weeks for more than 1 year. Based on these observations, the types and the doses of antipsychotic medications were adjusted and determined. We hypothesized that treatment-resistant TD, a severe form of TD, was suitable for detection of genetic association with TD. Only treatment-resistant TD patients were included as those affected with TD in this study. Patients in whom TD never developed despite antipsychotic therapy for more than 10 years were recruited as control patients.

Genotyping and statistics

Association screening was performed by using the Illumina HumanHapCNV370 Chip according to the manufacturer's

Table 1 Clinical characteristics of patients in the TD group and the non-TD group

	Genome-wide sample		*Confirmation sample*	
	TD (n = 61)	*Non-TD (n = 61)*	*TD (n = 36)*	*Non-TD (n = 138)*
Male:female ratio	35:26	35:26	18:18	88:50
Age (years)	57.3 ± 17.3	58.1 ± 12.3	58.0 ± 15.7	55.5 ± 1.0
Duration of illness (years)	35.6 ± 18.3	33.7 ± 12.5	37.3 ± 14.1	35.3 ± 1.02
Current neuroleptic dose (chlorpromazine-eq; mg year^{-1})	133 132 ± 201 021	469 497 ± 901 846	132 550 ± 86 292	407 456 ± 42 245

Abbreviation: TD, tardive dyskinesia.
The values are the means ± s.d. or number of patients.
Chlorpromazine-eq: chlorpromazine equivalents.

protocol (Illumina, San Diego, CA, USA). All DNA samples were subjected to rigorous quality control to check for fragmentation and amplification. SNPs on autosomal chromosomes ($n=290\,527$) were extracted. Owing to the small sample size and the fact that gender is not known to have a definite effect on TD, we did not analyze SNPs on the X chromosome. No subjects had genotype call rates <97%. The average genotype call rate was 99.7% and the mean heterozygosity of all SNPs was 30%. Two duplicate pairs of samples were genotyped and showed 99.9% genotype identity. SNPs with more than 5% missing genotypes ($n=2853$) and those with minor allele frequency <1% ($n=28\,930$) among subjects were excluded. For missing genotypes <5%, SNPs deviating from Hardy–Weinberg equilibrium ($P<0.0001$; $n=1040$) were excluded. A total of 257 704 autosomal SNPs passed quality control in the sample.

Replication analysis was performed by genotyping SNPs by the TaqMan method. Allelic discrimination was performed by using the ABI PRISM 7900HT Sequence Detection System, by using the SDS 2.0 software (Applied Biosystems, Foster City, CA, USA). Genotyping using TaqMan probes (Applied Biosystems) was performed twice for each SNP, and genotype concordance was 99.7%. Genotyping completeness was >0.99. We treated those uncalled or discrepant genotypes as missing genotypes. Haplotype blocks in the DPP6 (dipeptidyl peptidase-like protein-6) gene were visualized by using the Haploview program (http://www.broad.mit.edu/mpg/haploview/).

Allelic associations between SNPs and TD, and departure from Hardy–Weinberg equilibrium, were evaluated by χ^2-test or Fisher's exact test. Bonferroni's correction for multiple comparisons was applied.

An association was considered significant when the allelic *P*-value was less than 1.9×10^{-7} in the screening step and allelic *P*-value (one-tailed) was <0.05 after Bonferroni's correction for the number of SNPs examined in the replication step. The power of our sample (case = 61 and control = 61) was more than 0.7, with an α of 1.9×10^{-7} assuming a risk allele frequency of 0.3, a disease prevalence of 0.1 and a genotypic relative risk of 4 under the multiplicative model of inheritance, calculated using Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/). The replication sample had a power of more than 0.7 assuming two SNPs examined and a genotypic relative risk of 2 under the same model in the screening sample.

Human postmortem brains

Brain specimens were obtained from individuals of European (Australian) and Japanese descent. The Australian sample comprised 10 schizophrenic patients and 10 age- and gender-matched controls. The diagnosis of schizophrenia was made according to the DSM-IV criteria (American Psychiatric Association, 1994) by a psychiatrist and a senior psychologist. The control subjects had no known history of psychiatric illness. Tissue blocks were cut from the gray matter in an area of the prefrontal cortex referred to as Brodmann's area-9 (BA9). Japanese samples of BA9 gray matter from Japanese brain specimens comprised six schizophrenic patients and 11 age- and gender-matched controls. Details of the condition of the postmortem brains have been provided elsewhere.[23,24]

Analysis of DPP6 transcription in human brain tissue

Total RNA was extracted from human brain tissues by using the ISOGEN Reagent (Nippon Gene Co., Tokyo, Japan). The RNA quality was checked by using a Nanodrop ND-1000 spectrophotometer (LMS, Tokyo, Japan) to yield an optical density (OD) 260/280 ratio of 1.8–2 and an OD 260/230 of 1.8 or greater. The expression of the *DPP6* genes was analyzed by using the TaqMan Real-Time PCR system (Applied Biosystems). From RNA, cDNA was synthesized by using ReverTra Ace (Toyobo, Tokyo, Japan) and oligo-dT primers. The expression of the *DPP6* gene was analyzed by using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), with TaqMan gene expression assays for *DPP6* (Hs00157265_m1) and normalized to the expression of Human GAPDH Control Reagents (Applied Biosystems).

The genotype effects on *DPP6* expression were analyzed by analysis of variance followed by *post-hoc* Student's *t*-tests by using JMP software version 7.0.1 (SAS Institute, Cary, NC, USA).

Animals

To examine the effects of long-term antipsychotic treatments on gene expression, we set up two experimental groups. In the treatment group, 4-week-old C57BL/6J male mice were treated with an intraperitoneal injection of 1.0 mg kg^{-1} haloperidol ($n=10$) once each day for 50 weeks. The control group was administered vehicle saline ($n=10$) under the same regime. The mice were killed 4 h after the last injection to obtain brain tissues. The prefrontal cortex, midbrain, hippocampus, thalamus and striatum were removed by dissection and total RNA was extracted by using an RNeasy kit (Qiagen K.K., Tokyo, Japan). After cDNA synthesis from total RNA samples, the transcription level of cDNA samples was analyzed by TaqMan Expression assay for Dpp6 (Mm00456605_ml; Applied Biosystems) and normalized to that of rodent *Gapdh* by using Rodent *Gapdh* Control Reagents (Applied Biosystems). The average relative expression levels in the haloperidol-treated group were compared with the saline groups in each region by analysis of variance.

Results

We tested for allelic association between each SNP and TD by using the χ^2-test. The distribution of allelic *P*-values for association of SNPs with TD is shown in Figure 1a along with Figure 1b showing the quantile–quantile plot. The genomic inflation factor was 1.008. We did not find SNPs at the genome-wide significance level ($P<1.9\times10^{-7}$) in the screening sample. Table 2 shows the top 10 SNPs that had an allelic association with TD. The distribution of the genotypes of the 10 SNPs did not deviate from Hardy–Weinberg equilibrium in these SNPs. Three of them were