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〔特集：学会シンポジウム〕

臨床知見に基づいた統合失調症動物モデルを作製するために*
—統合失調症モデル作製のため，基礎研究者が臨床医に望むこと—

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要約：統合失調症は思春期以降に発症し進行性・慢性の精神疾患であり，その病態は未だ不明な点が多い．新たな治療戦略を考えるためには臨床研究に加え，適切な病態モデル動物を用いた病態時の神経機能の解明や新規治療薬の有効性の評価を行うための基礎研究が必要である．非競合的NMDA受容体拮抗薬のフェンシクリジン（PCP）の乱用者が断薬後も統合失調症に酷似した症状を示し，臨床試験においてもPCPの投与は健常者に統合失調症様症状を惹起させ，統合失調症患者の症状を増悪させる．このような知見からグルタミン酸作動性神経系機能低下仮説（NMDA受容体機能低下仮説）が提唱されている．この仮説に基づきPCP連続投与マウスは統合失調症モデル動物として繁用されているが，神経発達障害仮説を反映していない問題点がある．一方，統合失調症には遺伝要因が関与していることが知られている．ヒトゲノムの全塩基配列の解読の成功あるいは一塩基多型（single nucleotide polymorphism: SNP）およびそのハプロタイプ解析などの研究の進展により，*disrupted in schizophrenia 1*（*DISC1*）をはじめとした統合失調症に関連した多くの候補遺伝子が見いだされている．見いだされた候補遺伝子が統合失調症の病態にどのように関与しているかを検討するため，遺伝子改変マウスが作製されている．遺伝子改変マウスを用いた行動学的および生化学的な解析の結果から統合失調症モデル動物としての有用性が提唱されているが，その症状は統合失調症の一部分のみを示しているものが多い．また，遺伝要因だけでなく環境要因もその発症には関与すると考えられているため，単一の遺伝子を改変させたマウスでは統合失調症のモデル動物として限界があるとも考えられている．本章ではこれまでに我々が開発してきた統合失調症モデル動物と，それに関連する知見を紹介するとともに，より臨床知見に基づいた統合失調症モデル動物を作製するために，基礎研究者が臨床医に望むことについて概説した．

キーワード：統合失調症，フェンシクリジン（PCP），disrupted in schizophrenia 1（DISC1），動物モデル

統合失調症は，主に思春期以降に発症する進行性・慢性の精神疾患であり，幻覚や妄想，まとまりに欠ける会話や行動，感情の平板化，思考の貧困，意欲低下などを主訴とする（DSM-IV, 1994）．新たな治療戦略を考えるためには臨床研究に加え，適切な病態モデル動物を用いた病態時の神経機能の解明や新規治療薬の有効性の評価を行うための基礎研究が必要である．これまでに集積されてきた臨床および基礎研究における知見から，統合失調症の病態（陽性症状・陰性症状および認知障害）に関していくつかの仮説（ドパミン過剰仮説，グルタミン酸低下仮説，神経発達障害仮説）が提唱されている（Harrison, 1999; Mouri et al, 2007a）．それぞれの仮説に基づき，その病態を反映したモデル動物の作製が試みられている．一方，統合失調症の病因には遺伝要因が関与していることが家系研究，双生児研究，養子研究などから示唆されており，多くの候補遺伝子が見いだされている．見いだされた候補遺伝子を改変させたマウスの中には統合失調症モデル動物として注目されているものもある（Enomoto et al, 2007）．基礎研究においてモデル動物で認められる行動学的・生化学的異常が統合失調症の病態を反映しているかどうかについて，臨床医の視点からの指摘は非常に有益である．新たな治療・予防戦略や診断体系を確立するためにも，臨床検体を用いた候補遺伝子の関連解析，精神疾患発症脆弱性候補分子の探索的研究およびモデル動物を用いた候補分子の機能評価系の確立など，臨床的側面および基礎的側面の双方からアプローチする必要がある．本章ではこれまでに我々が開発してきた統合失調症モデル動物と，それに関連する薬理学的および遺伝子学的病態モデル動物について概説し，それらをより臨床知見に基づいたものに発展させるために考慮しなければならないことについて提案した．

I. グルタミン酸低下仮説に基づく薬理学的モデル動物：PCP投与動物モデル（成体期投与）（Table 1）

非競合的N-methyl-D-aspartate（NMDA）受容体拮抗薬であるフェンシクリジン（PCP）は，1970年代に乱用され，薬物依存者に精神症状（PCP精神病）を惹起することから（Javitt and Zukin, 1991），統合失調症にはNMDA受容体機能の低下が関係しているという「グルタミン酸低下仮説（NMDA受容体機能低下仮説）」が提唱された

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Table 1　フェンシクリジン（PCP）連続投与マウスに認められる統合失調症様症状の行動学的，形態学的および生化学的変化

	評価項目	評価試験	変化	
〈行動学的解析〉				
陽性症状	自発性	運動量測定	行動感作	(Noda et al, 1995a)
陰性症状	社会性行動	社会性行動試験	障害	(Sams-Dodd, 1998; Qiao et al, 2001)
	意欲（無動状態）	強制水泳試験	増強	(Noda et al, 1995b; Murai et al, 2007)
認知障害	作業記憶	T型迷路試験	障害	(Jentsch et al,1997)
	注意力	セットシフティング試験	障害	(Rodefer et al, 2005)
	視覚的認識記憶	新奇物体認識試験	障害	(Nagai et al, 2009)
	連合学習	恐怖条件づけ学習試験	障害	(Enomoto et al, 2005)
	潜在学習	水探索試験	障害	(Mouri et al, 2007b)
〈生化学的解析〉				
神経細胞	形態	ニッスル染色	萎縮	(Murai et al, 2007)
	スパインシナプス数	電子顕微鏡像	減少	(Hajszan et al, 2006)
グルタミン酸作動性神経系	細胞外グルタミン酸遊離量	マイクロダイアリシス	減少	(Murai et al, 2007)
	NMDA受容体応答性	ERK・CaMKIIリン酸化（スライス培養）	減少	(Enomoto et al 2005; Mouri et al, 2007b)
	NR1	リン酸化	減少	(Mouri et al, 2007b)
ドパミン作動性神経系	細胞外ドパミン遊離量	マイクロダイアリシス	減少	(Jentsch et al, 1998; Mouri et al, 2007b)
GABA作動性神経系	細胞数	PV陽性細胞数	減少	(Morrow et al, 2007)

NMDA: N-methyl-D-aspartate, ERK: extracellular signaling-regulated kinase, CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II, PV: parvalbumin.

(Carlsson et al, 1997).

PCPは，他のNMDA受容体拮抗薬（ケタミンやジゾシルピン）の中で最も繁用されているモデル薬物である．PCPは単回投与した場合も多彩な薬理作用［自発運動量の増加（Noda et al, 1995a），常同行動（Nabeshima et al, 1987），受動回避反応における長期記憶障害（Nabeshima et al, 1986），放射状迷路試験における作業記憶障害（Danysz et al, 1998），水探索試験における潜在学習障害（Noda et al, 2001），プレパルスインヒビション（PPI）試験における情報処理機能障害（Linn et al, 2003）など］を示す．連続投与した場合は，依存患者が摂取を中止した後もその精神症状が数週間持続するように，モデル動物でも行動変化が持続する点が興味深い．たとえば，PCPを連続投与した実験動物において，陽性症状様の行動変化としては運動過多が増強され，第一世代（従来/定型）抗精神病薬および第二世代（新規/非定型）抗精神病薬は緩解作用を示す（Noda et al, 1995a）．陰性症状様の行動変化としては強制水泳試験における無動状態の増強（Noda et al, 1995b; Murai et al, 2007）および社会性行動の低下（Sams-Dodd, 1998; Qiao et al, 2001）が認められる．認知障害としては，T型迷路における作業記憶障害（Jentsch et al, 1997），セットシフティング試験における注意障害（Rodefer et al, 2005），恐怖条件付け学習試験における連合学習障害（Enomoto et al, 2005），水探索試験における潜在学習障害（Mouri et al, 2007b）および新奇物体認識試験における視覚的認識記憶障害（Nagai et al, 2009）が知られている．こうした陰性症状・認知障害に対しては第二世代（新規/非定型）抗精神病薬は緩解作用を示すが，第一世代（従来/定型）抗精神病薬は効果を示さず，臨床（Tandon et al, 1999）と同様の結果が得られている．

統合失調症では，神経突起終末の減少や神経伝達物質の合成，遊離，取り込み機構などの異常も報告されている（Lewis and Lieberman, 2000; Vawter et al, 2002; Halim et al, 2003）．PCP連続投与動物においても樹状突起のスパインシナプス数の減少（Hajszan et al, 2006）および神経細胞の萎縮（Murai et al, 2007）などが認められる．PCP単回投与は脳内グルタミン酸の遊離量を増加させるが（Adams and Moghaddam, 1998），PCP連続投与はグルタミン酸再取り込みに重要なグリア型グルタミン酸トランスポーターのGLASTを増加させ，前頭前皮質における細胞外グルタミン酸遊離量を減少させる（Murai et al, 2007）．PCP連続投与により，前頭前皮質のNR1のリン酸化の低下（Mouri et al, 2007b），脳のスライス実験におけるNMDA刺激に対する細胞内シグナル分子（Ca^{2+}/calmodulin kinase II: CaMKIIおよびextracellular signaling-regulated kinase: ERK）のリン酸化による応答性の低下が認められる（Enomoto et al, 2005; Mouri et al, 2007b）．GLASTの阻害剤，D-サイクロセリンおよびグリシンはPCP連続投与マウスに認められる強制水泳試験における意欲低下および水探索試験における潜在学習障害を緩解することから，PCP

連続投与によるグルタミン酸作動性神経系機能の低下が，種々の精神機能に影響を及ぼしていると示唆されている（Murai et al, 2007; Mouri et al, 2007b）．Positron emission tomography（PET）を用いた研究では，統合失調症患者の前頭前皮質のドパミン受容体の密度の低下が認知機能と関連していることが報告されており（Okubo et al, 1997; Abi-Dargham et al, 2002），前頭前皮質におけるグルタミン酸作動性神経系と同様に，ドパミン作動性神経系の機能低下も統合失調症の認知障害に関与していることが示唆されている（Carlsson et al, 2001; Winterer and Weinberger, 2004）．PCP 急性投与や高カリウム刺激によって細胞外ドパミン遊離量は増加するが，PCP 連続投与ではそのような増加は認められない（Jentsch et al, 1998; Mouri et al, 2007b）．PCP 連続投与による NR1 リン酸化の低下および潜在学習障害はドパミン D1 受容体作動薬により緩解すること（Mouri et al, 2007b），ドパミン受容体は前頭前皮質における NMDA 受容体を介したシナプス伝達を調節していることから（Seamans et al, 2001; Tseng and O'Donnell, 2004），両神経系の機能異常は相互に影響し合って生じている可能性がある．一方，統合失調症患者の前頭前皮質の死後脳解析において，glutamic acid decarboxylase（GAD67），parvalbumin（PV）および calretinin などの GABA 作動性神経の指標となるタンパク質の陽性神経細胞数および mRNA 量などが減少していることから，その病態には GABA 作動性神経系の機能低下も関与していることが報告されている（Benes et al, 1991; Hashimoto et al, 2003）．動物実験でも，PCP を前頭前皮質に還流させると GABA の遊離を減少させること（Yonezawa et al, 1998）が報告されている．さらに，PCP 連続投与は前頭前皮質の PV 陽性細胞数を減少させる（Morrow et al, 2007）．MK-801 を持続注入したマウスでは GAD mRNA の減少が認められる（Qin et al, 1994）．

このように，PCP 連続投与は統合失調症に類似した精神行動異常だけでなく生化学的な異常も引き起こすため，PCP の急性投与と比較してより病態に近いモデル動物であると考えられる．

II. 今後の展望（1）

統合失調症の神経発達障害仮説を発展させた two-hit 仮説がある．Two-hit 仮説では，胎生期・周産期・生後発達期において何らかの原因による脳発達障害により統合失調症に対する脆弱性が形成され，思春期以降における社会的ストレスや薬物乱用などにより統合失調症が発症することが提案されている．神経発達障害仮説は，統合失調症患者では海馬の体積減少や側脳室の拡大が認められること，死後脳においてグリオーシスが認められないこと，ほとんどが思春期以降に発症することから，脳構造の発達障害が思春期の発症に関与していると提案された（Weinberger, 1995）．したがって，two-hit 仮説に基づいた動物モデルを作製するには神経発達障害仮説モデルに思春期にストレスを負荷することが必要である．実際に新生仔期腹側海馬損傷動物モデルに PCP を連続投与すると，PCP による行動量増加が増強するとともに線条体におけるドパミン D1 受容体の増加とドパミン D2 受容体の減少，および前頭前皮質の NMDA 受容体の減少が認められる（Hori et al, 2000）．一方，神経発達障害モデル動物の成熟期にストレスを負荷した報告は少ない．この場合に負荷するストレスの種類は，臨床知見に基づいたストレスを選択する必要がある．より妥当性の高い動物モデルを作製するために，臨床医と基礎研究者が情報交換を密にすることが必要であると考えられる．

III. 統合失調症関連遺伝子に基づくモデル動物：DISC1（Disrupted in schizophrenia 1）（Table 2）

スコットランドの大家系において 1 番染色体長腕と 11 番染色体長腕の均衡転座が認められ，これらの転座が精神疾患の発症と関連があることが報告された（St Clair et al, 1990）．この 1 番染色体転座断片から DISC1（Disrupted in schizophrenia 1）が同定され，転座によって DISC1 の C 末端側の 257 アミノ酸が欠失していることが判明した（Millar et al, 2000）．フィンランドの統合失調症の 458 家系において，DISC1 およびその 5′末端に隣接する TRAX（translin associated factor X）を含めた 28 個の SNP を用いた家系内伝達不平衡解析（TDT）において，1 つのハプロタイプが高い頻度で患者の親子間で伝達され，3 つのハプロタイプが低い頻度で患者に伝達されていることが見いだされている（Hennah et al, 2003）．このような TDT は白人の統合失調症患者においても実施されており，DISC1 の 703 番目のセリンの SNP をホモで保有する被験者はシステインをホモで保有する被験者と比較して両側海馬の灰白質が小さく，海馬体の N-アセチルアスパラギン酸が減少しており，Wisconsin Card Sorting test のカテゴリー尺度が低い（Callicott et al, 2005）．DISC1 の 703 番目のセリンがシステインに置換される SNP について日本人の統合失調症・大うつ病性障害と健常者と比較した研究では，システインアレルの保有者は大うつ病性障害と有意な関連があり，帯状回皮質の灰白質の減少が認められている（Hashimoto et al, 2006）．また，703 番目の残基がセリンとシステインである DISC1 のベクターをそれぞれ作製し，皮質神経に導入した研究では，セリン DISC1 はシステイン DISC1 と比較して有意な ERK シグナルの増加が認められており，このような相違が灰白質の減少や大うつ病性障害の罹患に関連していると示唆されている（Hashimoto et al, 2006）．最近の DISC1 の SNP およびその連鎖不均衡解

Table 2　Disrupted in schizophrenia 1 (DISC1) 遺伝子組換えマウスに認められる行動学的および生化学的変化

	129S6/SvEV マウス	DISC1 ミスセンス変異マウス		ドミナントネガティブ DISC1 トランスジェニックマウス		相同組換え DISC1 トランスジェニックマウス	胎生期 DISC1 ノックダウンマウス
	(Koike et al, 2006)	(Clapcote et al, 2007)		(Hikida et al, 2007)	(Pletnikov et al, 2008)	(Shen et al, 2008)	
		31L	100P				
〈行動学的解析〉							
オープンフィールド試験	変化なし	変化なし	自発運動量の増加	自発運動量の増加	自発運動量の増加	—	覚せい剤による運動量増加の亢進
プレパルスインヒビション試験	変化なし	障害	障害	障害	障害	障害	障害
強制水泳試験	—	無動時間（延長）	変化なし	無動時間（延長）	—	無動時間（延長）	—
テイルサスペンション試験	—	—	—	—	—	無動時間（延長）	—
社会性行動試験	—	社会性行動低下	変化なし	—	社会性行動低下・攻撃性増加	—	—
T 型迷路試験	障害	障害	障害	—	—	—	障害
レイテント・インヒビション試験	—	障害	障害	—	—	障害	—
新奇物体認識試験	—	—	—	—	—	—	障害
〈生化学的解析〉							
脳容積	変化なし	萎縮	萎縮	変化なし	変化なし	萎縮	—
側脳室	—	—	—	拡大	拡大	拡大	—
GABA 作動性神経系	—	—	—	PV 陽性細胞数の減少（前頭前皮質）	—	PV 陽性細胞数の減少（前頭前皮質・海馬）	—
ドパミン作動性神経系	—	—	—	—	—	—	TH 陽性細胞数の減少（前頭前皮質）・細胞外ドパミン遊離量の低下

—: 未実施，PV: parvalbumin，TH: tyrosine hydroxylase.

析において，自閉症もしくはアスペルガー症候群との関連性についても報告されている（Kilpinen et al, 2008）．このように，DISC1 の遺伝子変異は統合失調症をはじめとする多くの精神・神経疾患に関与していることが示唆されている．

DISC1 は中枢神経系に発現しており，成体ラットでは大脳皮質，嗅球，海馬などの前脳に多く発現する．DISC1 はラットの胎生 20.5 日目において特に強く発現することから，脳の発達段階に重要な役割を果たしていることが考えられる（Ozeki et al, 2003）．DISC1 は 854 アミノ酸からなるタンパク質であるが機能を示唆するような一次構造を有していないため，多くの研究において DISC1 結合タンパク質の同定とその機能について検討されている．

1.　DISC1 遺伝子変異動物：129S6/SvEV マウス

129S6/SvEV マウスには DISC1 遺伝子の第 6 エクソンに自然発症の 25 塩基の欠失変異があり，この欠損により DISC1 mRNA にフレームシフトが起き，DISC1 タンパク質の発現が認められない（Koike et al, 2006）．129S6/SvEV マウスを C57BL/6J と交配し，DISC1 の欠失をもつ C57BL/6J マウスを作製した研究では，脳の組織学的変化，PPI における情報処理機能および新規環境下での自発運動量の変化は認められなかったが，T 型迷路試験では作業記憶障害が認められている（Koike et al, 2006）．129S6/SvEV マウスの DISC1 発現の追試研究では，エピトープの異なる抗体を用いると 129S6/SvEV マウスにおいても DISC1 のタンパク質発現が認められ，129S6/SvEV マウスでは DISC1 の一部のスプライシングフォームが欠失していると考えられている（Ishizuka et al, 2007）．

2.　DISC1 ミスセンス変異マウス

理研変異マウスライブラリーと高速変異発見システムを用いて，DISC1 のアミノ酸配列が置換されるような遺伝子に点変異（ミスセンス変異）をもつマウスが 2 系統作製されている（Clapcote et al, 2007）．31 番目のグルタミンがロイシン（Gln31Leu; 31L）および 100 番目のロイシンがプロリン（Leu100Pro; 100P）に置換されたマウスはともに脳の萎縮が認められている（Clapcote et al, 2007）．100P マウスでは DISC1 と phosphodiesterase 4B（PDE4B）との結合の低下が認められる．強制水泳試験における意欲や社会性行動試験における社会性行動に変化は認められないが，オープンフィールド試験における自発運動量の増加，

PPIにおける情報処理機能の障害，T型迷路試験における作業記憶の障害，レイテント・インヒビション試験における潜在学習の障害が認められ，統合失調症に類似した行動異常を示す（Clapcote et al, 2007）．一方，31Lマウスでは，オープンフィールド試験における自発運動量に変化は認められなかったが，PPIにおける情報処理機能の障害，T型迷路試験における作業記憶の障害，レイテント・インヒビション試験における潜在学習の障害，さらに強制水泳試験における意欲の低下および社会性行動試験における社会性の低下が認められることから，うつ病に類似した行動異常を示す（Clapcote et al, 2007）．

3. ドミナントネガティブDISC1トランスジェニックマウス

臨床知見に類似したC末端が欠落したヒトDISC1を発現させることにより，内在性のDISC1の機能を低下させたドミナントネガティブDISC1トランスジェニックマウスが作製されている．CaMKIIプロモーターの制御下でC末端が欠落したヒトDISC1（1-597アミノ酸）を前脳特異的に発現させたトランスジェニックマウスでは脳の萎縮は認められないが，脳室の拡大，PV陽性細胞数の減少，オープンフィールド試験における自発運動量の増加，PPIにおける情報処理機能の障害，および強制水泳試験における意欲の低下が認められる（Hikida et al, 2007）．Tet-off遺伝子発現，およびCaMKIIプロモーターを制御してtTAを発現するシステムを用いて，上記と同様に前脳特異的にC末端が欠落したヒトDISC1（1-573アミノ酸）を発現させたトランスジェニックマウスが作製されている（Pletnikov et al, 2008）．このDISC1トランスジェニックマウスでは脳の萎縮は認められないが，脳室の拡大，初代神経培養における突起伸張の短縮，オープンフィールド試験における自発運動量の増加，PPIにおける情報処理機能の障害および社会性行動試験における社会性の低下と攻撃性の増加が認められる（Pletnikov et al, 2008）．

4. 相同組換えDISC1トランスジェニックマウス

エクソン9の途中で切断されたマウスDISC1のDNAと，内在するDISC1のDNAと相同組換えしたDISC1トランスジェニックマウスが作製されている（Shen et al, 2008）．このDISC1トランスジェニックマウスでは脳の萎縮，脳室の拡大，大脳皮質2〜3層の減少，胎生期の神経新生の低下，初代神経培養における突起伸張の短縮，PV陽性神経細胞の減少，PPIにおける情報処理機能の障害，レイテント・インヒビション試験における潜在学習の障害，および強制水泳試験，ならびにテイルサスペンション試験における意欲の低下が認められる（Shen et al, 2008）．

5. 胎生期DISC1ノックダウン（KD）マウス

我々は，子宮内エレクトロポレーション法を用いて，神経発達過程の大脳皮質におけるDISC1の発現を抑制したKDマウスを作製した．成熟後のKDマウスにおいて，前頭皮質の錐体細胞の形態異常，前頭前皮質のドパミン量およびドパミン合成律速酵素であるチロシン水酸化酵素の減少，側坐核刺激によるドパミン遊離の亢進，覚せい剤投与による運動過多の増強，新奇物体認識試験における視覚的認識記憶の低下，T型迷路試験における作業記憶の障害，およびPPIにおける情報処理の障害が認められている．

以上のように，様々なアプローチにより内在性DISC1の機能を低下させた動物が作製され，こうしたマウスの解析から統合失調症およびうつ病に類似した行動異常が認められている．これらの動物間の相違点を詳細に検討することにより，DISC1の神経発達および精神機能における役割を明確にしていく必要がある．

IV. 今後の展望（2）

DISC1の遺伝子変異は多くの精神・神経疾患の罹患との関連があり，DISC1は多くのタンパク質と結合し多彩な役割を果たし，DISC1の機能を低下させたマウスは様々な組織学的・行動薬理学的異常が認められている．これら報告は有益な知見をもたらす一方で，DISC1のより系統的な研究が必要になると考えられる．DISC1遺伝子変異マウスの統合失調症動物モデルとしての妥当性については，統合失調症は単一遺伝子変異による疾患ではなく複数の遺伝要因，および環境要因により発症すると考えられているため，単一の遺伝子を改変させたマウスでは統合失調症のモデル動物として限界がある．今後，複数の遺伝子変異をもつマウスや，そのマウスに環境要因を組み合わせた動物モデルの作製が必要になる．したがって，DISC1遺伝子変異を有する統合失調症患者において，1）他の統合失調症関連遺伝子の変異，2）発症原因となる環境要因（ストレス等）および3）統合失調症の症状について，臨床医と基礎研究者が情報交換し，より妥当性の高い動物モデルを作製することが望まれる．

V. おわりに

統合失調症の薬理学的動物モデルとしてPCP投与動物，遺伝子改変動物モデルとしてDISC1遺伝子変異マウスを用いて概説し，それらの動物モデルの問題点，およびそれを克服するために臨床医との連携することを提案した．本総説の内容はこれら動物モデルだけでなく，他の薬理学的および遺伝子学的な処置をした動物モデルに対しても該当する部分は多いと考えられる．今後，より病態を反映した動物モデルの作製を試みるために，臨床医と基礎研究者がより密接に情報交換をおこなうことにより，統合失調症の原因解明や治療に結びつくことを願っている．

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Abstract: Yukihiro NODA*[1], Akihiro MOURI*[2], Yukari WAKI*[1] and Toshitaka NABESHIMA*[2] (*[1]Division of Clinical Science and Neuropsychopharmacology and *[2]Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, 150 Yagotoyama, Tenpaku-ku, Nagoya, 468–8503 Japan) *Development of animal models for schizophrenia based on clinical evidence: Expectation for psychiatrists*. Jpn. J. Neuropsychopharmacol., 29: 47–53 (2009).

Schizophrenic patients show positive symptoms, negative symptoms and cognitive dysfunction. In humans, phencyclidine (PCP), a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, reproduces the schizophrenia-like psychosis including positive symptoms, negative symptoms and cognitive dysfunction. Ketamine, another non-competitive NMDA receptor antagonist, also reproduces a schizophrenia-like psychosis in healthy volunteers, and exaggerates the psychosis in schizophrenic patients. It has been hypothesized that insufficient glutamate neurotransmission is involved in the pathophysiology of schizophrenia. Therefore, attempts have been made to develop animal models of schizophrenia by using NMDA receptor antagonists such as PCP, ketamine and dizocilpine. In addition to pharmacological approaches, genetic approaches have been adopted to develop animal models of schizophrenia. The disrupted in schizophrenia 1 (DISC1) gene has been identified as a schizophrenia susceptibility gene based on linkage and single nucleotide polymorphism (SNP) association studies and clinical data, demonstrating that risk SNPs impact on the hippocampal structure and function in clinical and functional roles of DISC1 are analyzed in many kinds of transgenic mice developed. In this review, we focused on PCP and DISC1 transgenic animal models of schizophrenia and summarized recent evidence from several investigators. The basic researchers would need to collaborate with clinical psychiatrists to develop appropriate animal models for schizophrenia based on clinical evidence.

Key words: Schizophrenia, Phencyclidine (PCP), Disrupted in schizophrenia 1 (DISC1), Animal models

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Immunohistochemical study of vesicle monoamine transporter 2 in the hippocampal formation of PCP-treated mice

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ABSTRACT

The exact pathophysiology of schizophrenia is unknown despite intensive scientific studies using molecular biology, psychopharmacology, neuropathology, etc. It is thought that neurodevelopmental failures such as neuronal network incompetence and the inappropriate formation of neurons affect the neurotransmitters. Several animal models have been created to investigate the etiology of this disease. In this study, we investigated the expression of vesicle monoamine transporter 2 (VMAT2), which has a significant role in neurotransmission, in the hippocampal formation in 1-phenylcyclohexylpiperazine (PCP)-treated mice using immunohistochemical staining technique to clarify neuronal abnormalities. PCP-treated mice are thought to be one of novel animal models for schizophrenia. The expression of VMAT2 in the hippocampal formation was significantly reduced overall in the PCP-treated mice compared to that in control (saline-treated) mice, also these reductions were observed throughout the brain. These facts implied that the pathophysiology of this disease involves abnormal monoaminergic transmission through VMAT2, despite PCP was the *N*-methyl-D-aspartate (NMDA) receptor antagonist that might induce glutamatergic abnormality. Since insufficient or excess release of neurotransmitter might alter neurochemical function and neurotransmission, VMAT2 might be an important target for biological research in psychiatric disease including schizophrenia.

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1. Introduction

Schizophrenia is a major mental disease affecting approximately 1% of the general population. Impaired regulation of monoaminergic neurotransmission, including dopaminergic, serotonergic, and catecholaminergic transmission, is thought to be one aspect of the pathophysiology of this disease (Howes and Kapur, 2009; Seeman, 2009). Although studies indicate an impairment of neuronal transmission, whether this disruption is a causal, additional, or secondary change remains unclear. The VMAT2 protein packages neurotransmitters such as dopamine, serotonin, and norepinephrine that enter synaptic vesicles for release into the synaptic clefts. Within the synaptic vesicles, VMAT2 regulates vesicle loading and consequently defines quantal size, receptor sensitivity, and synapic plasticity (Pothos et al., 2000; Pothos, 2002). Therefore, this protein may have a major role in the pathophysiology of neuropsychiatric diseases including schizophrenia (Taylor et al., 2000), mood disorders (Zubieta et al., 2001), methamphetamine (MAP) neurotoxicity (Volz et al., 2009), and Parkinson's disease (Mooslehner et al., 2001). It was suggested that dopamine-related psychostimulants like methylphenidate and MAP alter the expression and/or the function of VMAT2 in animals (Fleckenstein et al., 2009).

Animals treated with psychostimulants including MAP and cocaine (COC) and genetically modified animals have been used widely as models of schizophrenia (Bickel and Javitt, 2009; Desbonnet et al., 2009). One such model is the PCP-treated mouse (Mouri et al., 2007). PCP acts as a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, and repeated PCP treatment impaired NMDA receptor function and decreased levels of extracellular glutamate (Nabeshima et al., 2006). PCP is thought to be a psychostimulant capable of producing both the

Abbreviations: CA, cornu ammonis; COC, cocaine; DG, dentate gyrus granule cell layer; MAP, methamphetamine; NMDA, *N*-methyl-D-aspartate; PC, personal computer; PCP, 1-phenylcyclohexylpiperazine; Pyrl, pyramidal layer; Sra, stratum radiatum; VMAT2, vesicle monoamine transporter 2.

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positive and negative symptoms of schizophrenia (Radonjic et al., 2008; Jentsch and Roth, 1999), and so this animal model recreates the pathophysiological conditions in the hippocampus and medial prefrontal cortex (Dissanayake et al., 2009).

The hippocampal formation has been an area of interest in schizophrenia research (Harrison, 2004) and several abnormalities of the hippocampal architecture have been reported (Heckers and Konradi, 2002). The area of CA2/3 or Dentate Gyrus in particular has received much attention in recent studies (Berretta et al., 2009; DeCarolis and Eisch, 2010).

In this study, we investigated the expression of VMAT2 in the hippocampal formation of PCP-treated mice using an immunohistochemical technique to further clarify the pathophysiology of schizophrenia.

2. Materials and methods

2.1. Animal model

All experiments were performed in accordance with the institutional guidelines of animal experimentation and with the approval of the ethics committee of the Nagoya University. All efforts were made to minimize the animals' suffering and to reduce the number of animals used.

Eleven male mice (C57BL/6J, 35 weeks old) were used. Seven of them were injected with PCP (10 mg/kg/day) intraperitoneally once a day for 14 days. The other four mice were similarly injected with physiological saline as a control. All mice were bred under the same conditions.

2.2. Histology and observation

2.2.1. Tissue preparation

Animals were perfused with a tissue fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) under deep anesthesia by injecting sodium pentobarbital (40 mg/kg body weight i.p.). The brains were immediately removed, and tissue blocks were immersed in a 20% sucrose–0.01 M phosphate buffer solution for more than 3 days at 4 °C. Sections of the hippocampal regions were cut on a freezing cryostat at thickness of 30 μm and treated as free-floating sections. The sections were rinsed and stored in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for at least 3 days and up to 2 weeks prior to the subsequent immunohistochemical procedure.

2.2.2. Immunohistochemistry

The sections were rinsed in TBS (0.1 M Tris–HCl, pH 7.4, 0.9% NaCl) containing 0.3% Triton X-100 (TX) and 2% normal goat serum (NGS) for 30–60 min at room temperature. The primary antibody employed in this study was an anti-VMAT2 monoclonal antibody (Chemicon, Temecula, CA, Lot No. 3536; 1:1000). Incubation with the primary antibody was carried out for 48 h at 4 °C. The sections were then incubated in a medium containing biotinylated anti-universal (rat and/or rabbit) IgG (Vector Vectastain; 1:100) for 45 min at room temperature, followed by incubation with an avidin–biotin peroxidase complex (ABC method) for 45 min. After each incubation, the sections were rinsed in NGS-TX-TBS solution. Finally, the sections were rinsed in PBS twice for 10 min, reacted with 0.05%, 3,3′diaminobenzidine-HCl in 0.05 M Tris–HCl buffer (pH 7.6) for 2 or 3 min, and mounted onto gelatin-coated slides. Specimens were observed under a light microscope.

To clarify the specificity of the immunoreactivity to VMAT2, control experiments were conducted with sections from the animal model and wild type mice using the same immunostaining procedure except that prior to immunohistchemical labeling, the diluted anti-VMAT2 antibody was pre-absorbed with 10 μg/ml or 50 μg/ml of VMAT2 (Novus Biologicals, CO, USA) instead of the primary antibody.

2.3. Observation and analysis

We selected two random squares (30 μm × 30 μm) in the area of the stratum radiatum along the pyramidal layer in the CA2 region of the right or left hippocampus per specimen. Also, we selected two random squares (30 μm × 30 μm) in the CA4 area per specimen. These specimens were observed using a light microscope. The microscopic photographs were downloaded to a personal computer (PC) from a digital camera (HC-2000, Fuji film Co. Japan) as digital data. The digital images of immunoreactivity were processed into black (immunopositive) and white (immunonegative) using image processing software (Image J: http://rsb.info.nih.gov/ij/ Free software supplied by NIH) (Fig. 1). An appropriate threshold of distinction between black and white tones was unified consistently for every specimen. The amount of immunoreactivity was assessed by the space occupied value as the number of pixels per square observational area. Finally, we counted the number of pixels in each group. The significance of variation in differences between the PCP-treated group and the saline-treated control group was assessed by using the Mann–Whitney U test with $P<0.05$ considered statistically significant. Since we could not suppose a normal distribution or homoscedasticity of the population structure, we used a non-parametric test in the statistical comparison.

3. Results

VMAT2-immunopositive varicose fibers were observed throughout the hippocampal formation in saline-treated mice (Fig. 2A). In contrast, less immunoreactivity was observed overall

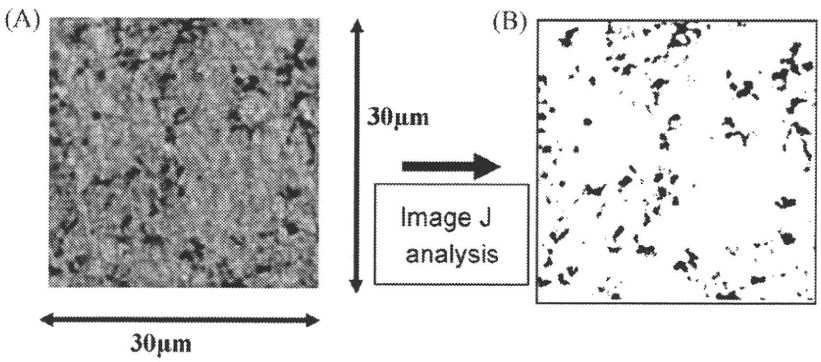

Fig. 1. (A) Microscopic photographs were uploaded to a PC as digital data in the setup area (30 μm × 30 μm). (B) The digital images were processed for dichotomization using image software (Image J: http://rsb.info.nih.gov/ij/). Only immunopositive products were extracted by computer processing. The occupancy space of immunoreactive products was automatically calculated as pixel units. The numerical value of pixels was applied to the index of immunoreactivity.

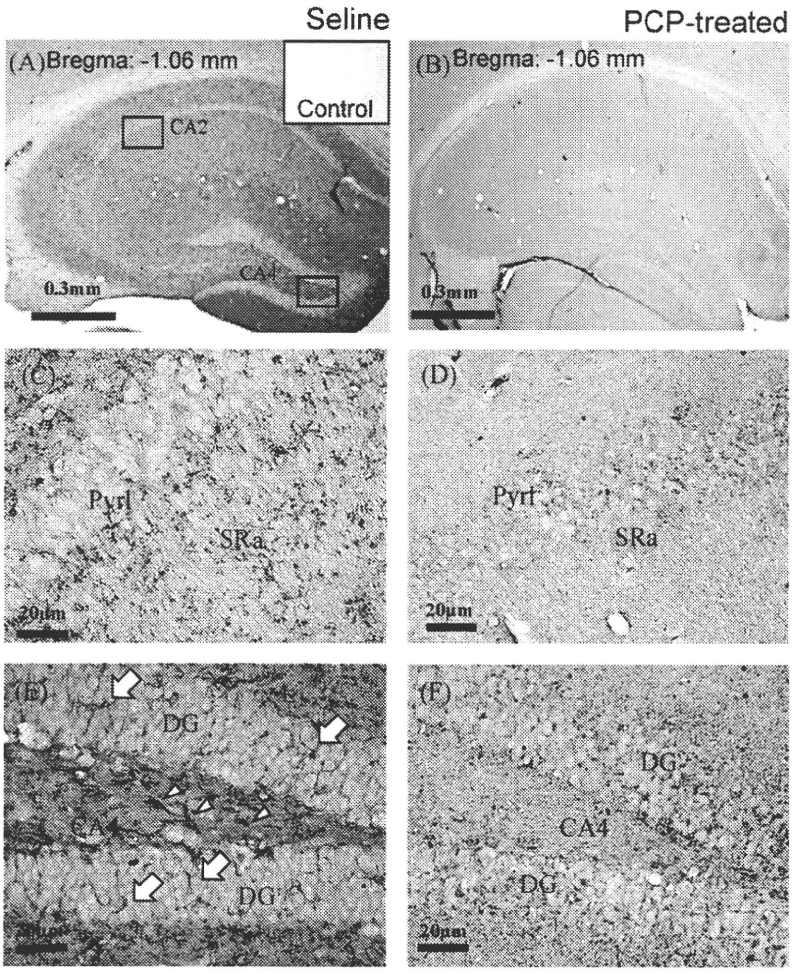

Fig. 2. (A) Immunoreactivity to VMAT2 in the saline-treated mice. Immunoreactivity was observed throughout the hippocampus. The approximate location in the brain was −1.06 mm (stereotaxic coordinate) from the bregma (http://www.mbl.org/atlas165/atlas165_start.html). Each square frame indicates the observational field in CA2 and CA4 areas. No immunoreactivity was observed in the control experiment using the pre-absorbed primary antibody with 10 μg/ml of VMAT2 protein (inset indicates control). (B) Immunoreactivity to VMAT2 in the PCP-treated mice. Overall, less immmunoreactivity was observed in the hippocampus than in the saline-treated mice (photo A). (C) Dense immunopositive varicose fibers or deposits of VMAT2 were observed in the stratum radiatum (SRa) along the pyramidal cell layer (Pyrl) in the CA2 area of saline-treated mice. (D) Little immunoreactivity was observed in the CA2 area of PCP-treated model mice compare to those of the saline-treated mice (photo C). (E) Extensive immunoreactivity was observed in the CA4 area and dense immunopositive fibers ran into the cell layer in the dentate gyrus (DG) (arrows). Immunopositive varicose deposits were detected in the CA4 area (arrowheads). (F) Immunoreactivity for VMAT2 in the CA4 area in PCP-treated mice. A significant reduction in immunoreactivity was observed compared to that in the saline-treated mice (photo E). No immunopositive dense varicose products or fibers were observed as shown in photo E. *Abbreviation*: Pyrl: pyramidal layer, SRa: stratum radiatum, DG: dentate gyrus granule cell layer.

in the PCP-treated mice (Fig. 2B). In the control experiments with the pre-absorbed primary antibody, no immunoreactivity was observed in any specimen (inset in Fig. 2A).

In the saline-treated mice, immunoreactive varicose fibers and deposits were detected in the hippocampal formation overall including the stratum radiatum (SRa) along the pyramidal layer (Pyrl) in the CA2 (Fig. 2C) and the CA4 (Fig. 2E) areas. Dense deposits (Fig. 2E, arrowheads) and some dense fibers penetrating the gyrus granule cell layer in the dentate gyrus (Fig. 2E, arrows), were also observed, especially in the CA4 region.

In the PCP-treated mice, a significant reduction in the immunoreactivity of granules to VMAT2 was observed in the hippocampal formation overall including the CA2 (Fig. 2D) and CA4 (Fig. 2F) areas. Also, the reduction of expression was observed in CA1 and CA3 area.

Additionally, we observed the other entire area of the brain precisely whether these reductions were occurred only in the hippocampus or not. The expression of VMAT immunoreactvity was observed throughout the brain, mainly in the monoaminergic neuronal tract, of the saline-treated mice (Fig. 3A). On the other hand, in the PCP-treated mice, the reduction of VMAT2 immunoreactivity were observed throughout the brain (Fig. 3B) including cerebral cortex (Fig. 3D), striatum (Fig. 3F) and substantia nigra (Fig. 3H) compared to that of the saline-treated mice respectively (Fig. 3C, E and G), same as shown in the hippocampus formation.

The significance of the reduction in VMAT2 expression of the hippocampal formation was reconfirmed by comparing immunoreactivity between the PCP-treated and saline-treated groups (Fig. 4).

4. Discussion

In this study, we investigated the expression of VMAT2 in the hippocampal formation in an animal model of schizophrenia using immunohistochemical techniques. A significant reduction in VMAT2 expression was observed in the brain of the PCP-treated mice.

4.1. VMAT2 in schizophrenia

The physiological etiology of schizophrenia has not been clarified. However, neurotransmission in the brain is likely to be disrupted because most of the neuroleptic drugs for schizophre-

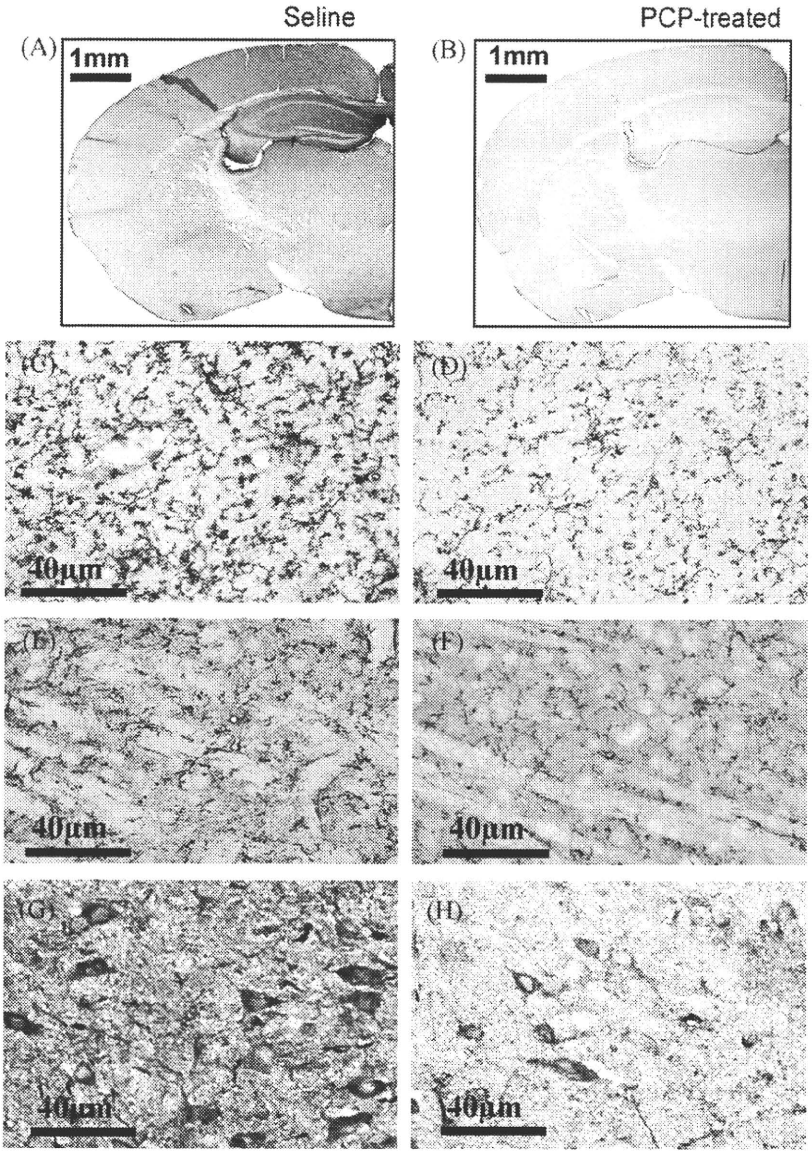

Fig. 3. (A and B) Low magnification photomicrographs of the VMAT2 immunoreactivity in the brain of the saline-treated mouse (A), and PCP-treated mouse (B). The reduction of VAMT2 immunoreactivity was observed in the PCP-treated mouse compared to that of saline-treated mouse throughout the brain. (C and D) VMAT2 immunoreactivity in the temporal cortex of the saline-treated mouse (C) and PCP-treated mice (D). The reduction of immunopositive neuropil was observed in the PCP-treated mice compared to that in the saline-treated mice. (E and F) VMAT2 immunoreactivity in the striatum of the saline-treated moue (E) and PCP-treated mice (F). The reduction of immunopositive varicose fibers was observed in the PCP-treated mice compared to that in the saline-treated mice. (G and H) VMAT2 immunoreactivity in the substantia nigra of the saline-treated mice (G) and PCP-treated mice (H). The more reduction of immunostained neurons and deposits were observed in the PCP-treated mice compared to that in the saline-treated mice.

nia block dopaminergic or serotonergic receptors (Seeman, 2002). Also, some psychostimulants such as MAP increase the concentration of dopamine in the synaptic clefts, which would induce psychosis observed in schizophrenic patients (Yui et al., 2000). Therefore, neurotransmission is clearly impaired, but how or at which stage the disruption occurs is unclear. The VMAT2 protein exists within the pre-synaptic vesicles and acts as a regulator of monoamine neurotransmitters. Changes in the concentration or density of this protein have been reported in patients with mental disorders including schizophrenia (Zubieta et al., 2001) and mood disorders (Zucker et al., 2002). This protein is thought to have a role in higher mental functions and be closely linked to mental illness.

4.2. *VMAT2 in PCP-treated mice*

Changes in the concentration of VMAT2 have been reported in MAP users (Boileau et al., 2008; Kitamura, 2009), in COC users (Wilson et al., 1996; Little et al., 2003), and in COC-treated animals (Brown et al., 2001). MAP and COC act as accelerators and inhibitors of the uptake dopamine during synaptic transmission and induce a psychosis similar to that observed in schizophrenic patients. PCP induces not only psychosis but also other schizophrenic-like symptoms such as cognitive deficits and negative symptoms in healthy subjects functioning as an NMDA antagonist and perhaps activating prefrontal cortical dopaminergic neurons (Umino et al., 1998). We found changes in VMAT2 expression in the brains of PCP-treated animals, similar to MAP users or COC-treated animals, though there are differences in the psychopharmacological actions of these substances.

The expression of VMAT2 was reported to be regulated mainly by the circumference of the neurotransmitter (Tong et al., 2008). Repeated administration of PCP in neonatal rats impaired prefrontal glutamatergic transmission and subcortical dopaminergic transmission (Hori et al., 2000). Acute administration of PCP

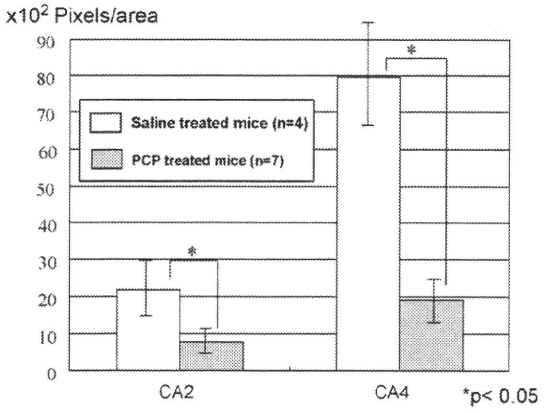

Fig. 4. Significant decreases in VMAT2 immunoreactivity were observed in the CA2 and CA4 areas of hippocampal formation statistically (Mann–Whitney *U* test with $P<0.05$ considered statistically significant) using the data from digital image analysis in PCP-treated mice compared to saline-treated mice.

affected focal frontohippocampal activation including serotonergic transmission (Gozzi et al., 2008, 2010). PCP might therefore influence different types of neurotransmission and broad areas of the brain. Although the exact mechanism involved remains unknown, abnormal glutamatergic neurotransmission would influence monoaminergic transmission including the dopaminergic system and might to a lead the reduction in VMAT2 expression in the synaptic organization.

The principal pharmacologically treated animal models of schizophrenia are based on the concept of a deficit in dopaminergic neurotransmission of the synapse organ as stated in the dopaminergic hypothesis and/or in glutamatergic neurotransmission as stated in the glutamate hypothesis. PCP-treated animals are considered a useful for evaluating novel therapeutic candidates and confirming the pathological mechanisms of schizophrenia (Mouri et al., 2007). Recently, treatment with PCP was reported to alter the density of sigma-1 receptors in the hippocampus, which is potentially related to the cognitive dysfunction in schizophrenia (Kunitachi et al., 2009). PCP-treated mice are therefore suitable for studying the effects of antipsychotics on emotional and cognitive deficits in schizophrenia, which may confirm the actual clinical course of the disease with negative symptoms and/or the cognitive dysfunction. Anyway, investigations of the function or movement of the VMAT2 protein in PCP-treated mice may be useful for understanding the pathophysiology of schizophrenia and for behavioral pharmacology or drug discovery research.

4.3. Further study

There are several limitations to this study. First, it was unclear whether reduction in VMAT2 expression occurred after the administration of PCP, how long it lasted or if it was depended on the concentration of PCP. Second, since an immunohistochemical analysis has were weak quantitative significance, the regulation of VMAT2 expression needs to be confirmed by determining the quantity of protein directly or by in situ hybridization. Still, VMAT2 is an important mediator of neurotransmission and closely linked to the pathophysiology of schizophrenia. It is also necessary to investigate VMAT2 expression in the postmortem human brain pathologically to clarify the etiology of schizophrenia.

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Gene-wide association study between the methylenetetrahydrofolate reductase gene (*MTHFR*) and schizophrenia in the Japanese population, with an updated meta-analysis on currently available data

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ABSTRACT

Methylenetetrahydrofolate reductase (MTHFR) is a critical molecule for single-carbon transfer reactions. Recent evidence suggests that polymorphisms of MTHFR are related to neural tube deficits and the pathogenesis of schizophrenia. While several studies have demonstrated associations between the gene encoding the MTHFR (*MTHFR*) polymorphisms and schizophrenia, these studies lack consistency. Therefore, we conducted a gene-wide association study (patients with schizophrenia = 696, control subjects = 747) and performed imputation analysis. Additionally, we performed meta-analysis on currently available data from 18 studies for two common functional polymorphisms (rs1801131 and rs1801133).

There were no significant associations with schizophrenia in the single marker analysis for the seven tagging SNPs of *MTHFR*. In the haplotypic analysis, a nominally significant association was observed between the haplotypes, which included four SNPs (rs1801133, rs17421511, rs17037396, and rs9651118) and the schizophrenic patients. Additionally, the imputation analysis demonstrated there were several associated markers on the *MTHFR* chromosomal region. However, confirmatory analyses of three tagging SNPs (rs1801133, rs17037396, and rs9651118) and the top SNP (rs17421511) for the imputation results (patients with schizophrenia = 797, control subjects = 1025) failed to replicate the haplotypic analysis and the imputation results. These findings suggest that *MTHFR* polymorphisms are unlikely to be related to the development of schizophrenia in the Japanese population. However, since our meta-analysis results demonstrated strong support for association of rs1801133 with schizophrenia, further replication studies based on a gene-wide approach need to be considered.

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1. Introduction

Schizophrenia is a chronic and disabling mental disorder with a lifetime prevalence of approximately 1% in the global population (Freedman, 2003). Accumulating evidence suggests that both genetic and environmental factors contribute to the

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etiology of schizophrenia (Burmeister et al., 2008). Although schizophrenia has a high heritability with rates estimated at 80% (Sullivan et al., 2003), there has been no consistent replication found for the schizophrenia candidate genes (Harrison and Weinberger, 2005). Recent genome-wide association (GWA) studies have demonstrated new promising susceptibility genes for schizophrenia (O'Donovan et al., 2008), as well as for other common diseases (Rioux et al., 2007; The Wellcome Trust Case Control Consortium, 2007; Zeggini et al., 2007). Therefore, use of this methodology can be advantageous when trying to detect potential genetic factors responsible for the development of these disorders. In addition, by focusing on the specific molecular pathway related to the pathophysiology of schizophrenia, this may also be useful when trying to identify susceptibility genes that have a mild contribution to the development of the disease (Kirov et al., 2005).

Dysfunction of homocysteine metabolism has been linked to neurodevelopmental disorders, including neural tube defects (NTDs) (Blom et al., 2006; van der Put et al., 1995), schizophrenia (Allen et al., 2008; Muntjewerff et al., 2006), and depression (Lewis et al., 2006), in addition to other diseases and syndromes (Hobbs et al., 2000; Kluijtmans et al., 1996; Qian et al., 2007). Recent studies have also suggested that elevated plasma homocysteine levels are observed in major psychiatric disorders such as schizophrenia and bipolar disorder (Levine et al., 2005). Plasma homocysteine levels affect the intracellular methylation process of DNA, lipids, proteins, and neurotransmitters (Scott and Weir, 1998). Both elevated homocysteine levels along with physiological levels of its oxidized derivatives, such as homocysteic acid and homocysteine sulfinic acid, have been shown to be toxic for neurons and vascular endothelial cells (Zou and Banerjee, 2005). While levels of homocysteine are affected by various genes involved in the homocysteine metabolic pathway and by environmental factors such as folate or vitamin B_{12} intake (Refsum et al., 2004), methylenetetrahydrofolate reductase (MTHFR) also plays a major role in this pathway. MTHFR converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a carbon donor for the methylation of homocysteine, leading to the generation of S-adenosylmethionine (SAM) (Andreoli and Maffei, 1975). SAM is a major source of methyl groups in the brain (Godfrey et al., 1990) and is involved in catechol-O-methyltransferase (COMT) reactions such as the catabolism of serotonin and other catecholamines (Anguelova et al., 2003; Chen et al., 2004). Freeman et al. (1975) reported there is direct evidence linking decreased MTHFR activity to schizophrenia (Freeman et al., 1975). These findings have led to multiple genetic analyses examining the link between the MTHFR gene (gene symbol: *MTHFR*, GenBank accession number: NM_005957) and schizophrenia.

MTHFR is composed of twelve exons (Fig. 1) and is localized on chromosome 1p36.3 (Goyette et al., 1994). It has been suggested that this may be a susceptibility locus for schizophrenia, bipolar disorder (Kempisty et al., 2007) and major depressive disorder (McGuffin et al., 2005). Two common functional polymorphisms of *MTHFR*, C677T (rs1801133) and A1298C (rs1801131), are known to cause a decrease of enzyme activity and affect nucleic synthesis and DNA methylation (van der Put et al., 1998). Several studies have confirmed the possible involvement of these SNPs in psychiatric conditions such as schizophrenia (Regland, 2005) and affective disorders (Arinami et al., 1997). Subjects with homozygosity for the 677 T allele have a mild increase in their plasma homocysteine levels, and these subjects have a higher frequency of neural tube deficits and premature cardiovascular disease as compared to other similar genotype carriers (Bakker and Brandjes, 1997; Matsushita et al., 1997). The impact of this polymorphism varies according to environmental factors, such as folate, vitamin B_2 or vitamin B_{12} (Hustad et al., 2000; Refsum et al., 2004; van der Put et al., 1995). Although some studies have reported that carriers of the 677 T allele in *MTHFR* are associated with an increased risk of schizophrenia (Arinami et al., 1997; Muntjewerff et al., 2005; Sazci et al., 2003), others have shown contradictive results (Kunugi et al., 1998; Vilella et al., 2005; Yu et al., 2004). The association of the *MTHFR* C677T variant with schizophrenia may be linked to the excitatory amino acids hypothesis or to decreased plasma concentrations of SAM that have been reported in psychiatric disorders (Andreoli and Maffei, 1975). Another functional polymorphism, A1298C, also has been shown to decrease MTHFR activity, although van der Put et al. (1998) have reported finding no significant effect of this variant on the plasma homocysteine levels.

A recent meta-analysis demonstrated an association between elevated homocysteine levels or carriers of the 677 T allele and an increased risk of developing schizophrenia (Allen et al., 2008; Muntjewerff et al., 2006). It has been suggested that potential associations between genetic variation in folate metabolism and psychiatric disorders could be plausible biological explanations for these disorders (Coppen and Bolander-Gouaille, 2005).

Taken together, *MTHFR* may be related to the development of schizophrenia. Although a number of studies have demonstrated associations between specific polymorphisms of *MTHFR* and schizophrenia, there have been no gene-based analysis studies. Therefore, it is still difficult to interpret these types of studies due to the inconsistent results that have been derived from some of the confounding factors, such as population

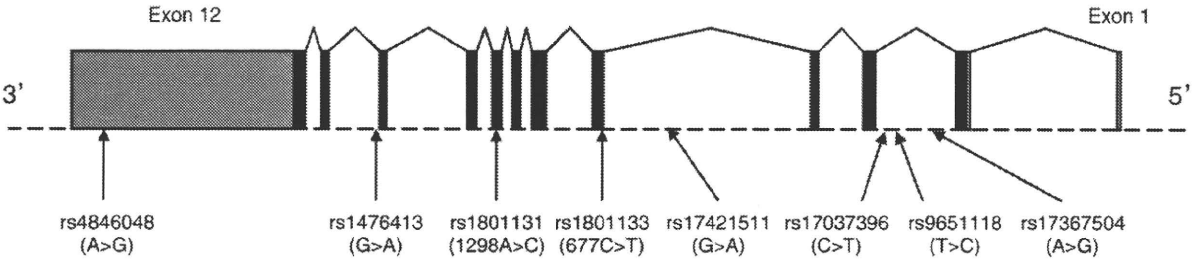

Fig. 1. Genomic structure of *MTHFR*. Black boxes indicate protein-coding regions, while the gray boxes represent the untranslated regions (UTRs). Each box represents *MTHFR* exons. Numbers under the arrows represent the SNP IDs, the tagging SNPs (pairwise tagger: $r^2>0.8$; Haploview 3.32), and the top SNP (rs17421511) of imputation results.

stratifications (ethnic or gender differences) and number of samples. In the present study, we conducted an association study between *MTHFR* and schizophrenia in the Japanese population that was based on the gene-wide approach. In addition, we also performed a meta-analysis on the updated data currently available.

2. Materials and methods

2.1. Subjects

The samples for this association study consisted of 696 patients with schizophrenia and 747 control subjects. The confirmation sample set for four SNPs (rs1801133, rs17421511, rs17037396, and rs9651118), which were positively associated with schizophrenia in the haplotypic analysis and the imputation analysis, consisted of 797 patients with schizophrenia and 1025 control subjects. Detailed demographical data are presented in Supplementary Table 1.

All subjects were unrelated to each other and ethnically Japanese. The schizophrenia diagnosis was made by at least two experienced psychiatrists and based on unstructured patient interviews and reviews of their medical records in accordance with the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) criteria for schizophrenia. All healthy control subjects were also psychiatrically screened on the basis of unstructured interviews.

This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and Fujita Health University. Written informed consent was obtained from each subject.

2.2. Tagging SNP selection

In order to obtain the SNPs that covered the entire coding region as well as the regulatory elements in the 5′ and 3′ flanking areas for both the 1000 base pairs (bps) upstream and downstream of the coding region, we first examined the *MTHFR* genotyping data from the HapMap database (HapMap Data Rel 21/phase II Jan 06, population: Japanese living in Tokyo). Subsequently, the tagging SNPs were selected using the Haploview software version 4.2 in accordance with the criterion of the Tagger program for pairwise tagging, $r^2>0.8$, with minor allele frequency (MAF)>0.1 (de Bakker et al., 2005) (Supplementary Table 2). We excluded rs13306553 due to the unavailability of a reliable genotyping method (genotype call rate<95%). Therefore, a total of seven SNPs were recruited for these genetic association analyses (Fig. 1).

2.3. SNP genotyping

Venous blood was drawn from each subject and genomic DNA was extracted according to standard phenol/chloroform method. SNP genotyping was carried out using the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). TaqMan probes and Universal PCR Master Mix were purchased from Applied Biosystems. Allelic specific fluorescence was measured on the ABI PRISM 7900HT using the Sequence Detection Systems 2.0 software (Applied Biosystems) for allelic discrimination. To exclude low-quality DNA sample or genotyping probes, data sets were filtered on the basis of tagging SNP genotype call rates (95% completeness). Subjects whose percentage of missing genotypes was >10% or who had evidence of possible DNA contamination were excluded from subsequent analyses. For quality control, we randomly selected 10 samples for each SNP and then genotyped these in duplicate in order to evaluate the genotype error rate.

2.4. Imputation and confirmatory association analysis

To estimate genotypes of untyped SNPs located on the analyzed gene region, we conducted an imputation analysis. This method provides enhanced statistical power for the coverage of common variants within the locus of interest. Specifically, based on directly genotyped SNPs and the haplotypes detected in the hapmap JPT sample, a computational algorithm predicted the genotypes at the SNPs that are not directly genotyped in the study sample (Marchini et al., 2007). We carried out this analysis using the MACH 1.0 program (http://www.sph.umich.edu/csg/abecasis/MACH/) in order to calculate the genotypic prediction for the 11 untyped SNPs. These calculations used information from the screening scan for the seven directly typed SNPs and the HapMap database (HapMap Data Rel 21/phase II Jan 06, population: Japanese/Chinese).

The MACH program has been reported to have imputation accuracy rates similar to IMPUTE and both programs are able to outperform fastPHASE, PLINK, and Beagle (Pei et al., 2008). As previously mentioned, the analyzed region of imputation was limited to the *MTHFR* locus. Associated SNPs were pruned based on the linkage disequilibrium (LD) pattern ($r^2>0.8$; Supplementary Table 2) and minor allele frequency (MAF<0.05), with the SNP showing the smallest allelic p value selected for follow up.

2.5. Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE), and marker–trait associations (allelic, genotypic, and haplotypic analysis) were evaluated by using PLINK v1.06 (Purcell et al., 2007). The significance level for all statistical tests was 0.05. Bonferroni correction was used to control inflation of the type I error rate in the allele-wise, genotype-wise, and haplotype-wise analyses. To reduce the total number of tests, clearly unassociated markers were removed in the first stage (screening sample set) of the present study. Conditional on the first stage findings, which used a less stringent nominal level, we subsequently tested the second stage (confirmation sample set) using the augmented data and the data from the first stage. In this joint sample analysis, p values were generated by the Cochran–Mantel–Haenszel stratified analysis, while the Breslow–Day Test was performed for evaluation of heterogeneous associations as implemented in PLINK. Based on the multiplicative model of inheritance, power calculations were performed using the Genetic Power Calculator (Purcell et al., 2003).

2.6. Meta-analysis

We performed a meta-analysis for rs1801131 and rs1801133, which are the two SNPs that have been previously shown to be associated with schizophrenia (Arinami et al., 1997; Betcheva et al., 2009; Feng et al., 2009; Garcia-Miss et al., 2010; Jonsson et

al., 2008; Joober et al., 2000; Kempisty et al., 2007; Kempisty et al., 2006; Kunugi et al., 1998; Lee et al., 2006; Muntjewerff et al., 2005; Philibert et al., 2006; Sazci et al., 2003; Sazci et al., 2005; Tan et al., 2004; Vilella et al., 2005; Yu et al., 2004). Initially, the Q statistic test was performed to assess the heterogeneity in the combined studies. As substantial amounts of variation have been previously observed, we decided to calculate the cumulative odds ratio (OR) and corresponding p value based on a random effect model (OR was calculated based on minor allele observed in Japanese population). Furthermore, use of this calculation was chosen because many investigators consider the random effects model to be a much more natural choice as compared to the fixed effects approach (Ades et al., 2005; DerSimonian and Laird, 1986; Fleiss and Gross, 1991). The significance of the overall OR was determined by the Z-test. Publication bias was assessed using a linear regression analysis to measure funnel plot asymmetry. A probability level of $p<0.05$ was used as the threshold for statistical significance. Comprehensive Meta-Analysis software (Version 2.2.046, Biostat, Englewood, NJ) was used to perform the analysis.

3. Results

Regarding quality control, the genotype calls of the duplicated samples showed complete concordance (data not shown), and all genotype frequencies of the tagging SNPs were consistent with the HWE. There were no significant differences between the schizophrenic patients and the control subjects in both allele and genotype distributions without imputed (untyped) SNP (rs17421511) (Table 1). In the haplotypic analysis, a nominally significant association was observed between the haplotypes including four SNPs (rs1801133, rs17421511, rs17037396, and rs9651118) and schizophrenic patients (Table 1). Imputation analysis showed several associated markers for schizophrenia on the *MTHFR* chromosomal region (Table 2). These nominally significant associations, however, did not survive after Bonferroni correction. After assessment of the HapMap database, the top SNP (rs17421511) was selected to confirm these nominal significant associations between imputed markers and schizophrenia. The results of the genotyping data in confirmatory analyses and joint analyses for the four SNPs (rs1801133, rs17421511, rs17037396, and rs9651118) after Bonferroni correction showed no significant association signal for either the allele and haplotype frequencies with schizophrenic phenotype (Table 3 and Supplementary Table 3). Assuming a multiplicative model of inheritance, a disease prevalence of 1%, and a high LD between the genotyped SNP and risk variant, we obtained more than 80% power in detecting the gene-wide association with schizophrenia when the genotype relative risk was set at 1.28 to 1.38 (screening sample set) and 1.25 to 1.35 (confirmation sample set) (MAF: 0.11 to 0.40 and 0.10 to 0.40, respectively). In the meta-analysis for the two commonly associated SNPs, we used all available data from 18 studies and data from studies that only focused on Asian populations (seven studies) to calculate the cumulative odds ratio (OR). We observed association only at rs1801133 for schizophrenia ($P_{(\text{random model})}=0.000833$), without any population-wise specific effect (Supplementary Tables 4 and 5).

4. Discussion

Even though we applied the gene-based approach in the present study, we could not confirm any significant associations of the *MTHFR* polymorphisms with schizophrenia. In the association analysis, we examined the SNPs covering the entire gene, including all of the tagging SNPs that had at least ~ 10% MAF listed on the HapMap database. For all of the genoõtyped SNPs, there were no associations noted between the patients with schizophrenia and the controls in any of the allele frequencies after Bonferroni correction (Table 1). To confirm our results, we additionally performed an imputation analysis for the estimated untyped SNPs and genotyped three markers (rs1801133, rs17037396, and rs9651118) and the top SNP

Table 1
Results of association analyses (screening sample set).

	dbSNP		Single marker (allele-wise)					Multi marker (haplotype-wise)[a]	
			SCZ[b]	CON[c]	L95[d]	U95[d]	p value	2 markers	3 markers
Maker 1	rs4846048	A>G	0.104	0.107	0.754	1.231	0.767		
								0.878	
Maker 2	rs1476413	G>A	0.203	0.203	0.833	1.210	0.968		0.681
								0.899	
Maker 3	rs1801131	A>C	0.201	0.208	0.796	1.157	0.667		0.801
								0.711	
Maker 4	rs1801133	C>T	0.395	0.404	0.827	1.125	0.643		0.628
								0.034	
Maker 5[e]	rs17421511	G>A	0.174	0.138	1.070	1.624	0.009		0.078
								0.035	
Maker 6	rs17037396	C>T	0.110	0.110	0.789	1.278	0.972		0.052
								0.972	
Maker 7	rs9651118	T>C	0.355	0.350	0.872	1.195	0.794		0.974
								0.902	
Maker 8	rs17367504	A>G	0.111	0.113	0.774	1.249	0.889		

[a]Log likelihood ratio test p value (sliding window analysis with rare haplotype threshold 10%).
[b]SCZ: Schizophrenia.
[c]CON: Control; minor allele frequency.
[d]95% confidence intervals (odds ratio).
[e]Imputed SNP with lowest p value.

Table 2
Allele-wise analysis of imputed SNPs.

dbSNP		MAF[a]	*p* value	Quality[b]
rs17421511	G>A	0.158	0.014	0.907
rs17421560	G>A	0.129	0.544	0.940
rs11121832	C>T	0.144	0.041	0.901
rs2066471	G>A	0.152	0.016	0.920
rs7533315	C>T	0.151	0.016	0.923
rs17037390	G>A	0.122	0.586	0.967
rs17037397	C>A	0.107	0.503	0.998
rs2066470	C>T	0.108	0.499	0.994
rs3753582	T>G	0.108	0.499	0.988
rs13306561	T>C	0.132	0.499	0.937
rs3737965	C>T	0.108	0.499	0.978

[a]MAF: minor allele frequency.
[b]Quality: the average posterior probability for the most likely genotype.

(rs17421511) of imputation results (rs17421511). The nominally significant associations that were detected in haplotype-wise analysis and also in imputation analysis did not survive in confirmatory association analysis (Table 3). Therefore, as previously reported, it is unlikely that other common variants related to schizophrenia are causal to the development of this disease (Chakravarti, 1999).

Several researchers have reported that two common *MTHFR* variants, C677T (rs1801133) and A1298C (rs1801131), are related to the development of schizophrenia (Allen et al., 2008; Gilbody et al., 2007). Even though other investigators could not reproduce these findings (Kunugi et al., 1998; Vilella et al., 2005; Yu et al., 2004), results of a recent meta-analysis support a relationship between the *MTHFR* C677T polymorphism and the risk for schizophrenia (Muntjewerff et al., 2006; van der Put et al., 1995). The 677TT/1298AA (Virgos et al., 1999) and 677CC/1298CC (Sazci et al., 2005) compound genotypes have been shown to be over-represented in schizophrenia samples. These contradictions might be derived from confounding factors such as age, gender, or ethnicity (population stratifications) (Cardon and Palmer, 2003; Munafo and Flint, 2004). The discrepancy between these results and our current results could be due to the locus heterogeneity of this disease. In fact, since the statistical power to detect an association exceeded 80%, there is a low possibility of a type II error. The GRR value that was calculated using the Genetic Power Calculator appeared to be appropriate when compared to promising candidate genes for schizophrenia (Schwab et al., 2003; Shifman et al., 2002). In findings from a recent whole genome association study that focused on schizophrenia (O'Donovan et al., 2008), results suggested that the effect size of common SNPs might be very low, and therefore, sample sizes used for genetic association studies need to be very large. Our current meta-analysis provides indirect support for such a scenario. In order to evaluate the impact of the SNP that was shown to be associated with schizophrenia in our meta-analysis (rs1801133), we have used the PolyPhen-2 (Adzhubei et al., 2010). The software compares the property of the wild-type (ancestral, normal) allele and the corresponding property of the mutant (derived, disease-causing) allele. The alignment pipeline selects a set of homologous sequences using a clustering algorithm and then constructs and refines the multiple alignments. According to the aforementioned calculation, rs1801133 was shown to have a damaging effect on protein structure while the ancestral allele showed the high level of evolutionary conservation (Supplementary Table 6). This finding is consistent with the meta-analysis results, as these demonstrated the associated allele is the risk allele. However, while we could not detect the association in our sample, it is of note that we have detected a publication bias ($t=2.778$, $df=16$, $p=0.013$), and therefore, the pooled p value might be overestimated.

In order to be able to elucidate the exact role of genetic variants, definitions of phenotypes are vital for a genetic association study. Therefore, sample stratification using endophenotypes, such as being more specific than phenotypes (e.g., prepulse inhibition, event-related potential, and mismatch negativity), clinical symptoms (e.g., response to medication), or environmental factors (e.g., food intake, supplementation) may be required for these clinical investigations (Braff et al., 2007; Craddock et al., 2006; Gottesman and Gould, 2003). Although we did not take advantage of these types of analytical tests for the genetic association in the present study, these might very well be useful in helping to elucidate the role of *MTHFR* in schizophrenia.

In conclusion, the findings of the present study suggest that *MTHFR* is unlikely to be related to the development of schizophrenia in the Japanese population. However, as our meta-analysis results provided strong support for the association of rs1801133 with schizophrenia, further replication studies based on the gene-wide approach using a large cohort

Table 3
Results of association analyses (confirmation sample set).

dbSNP			Single marker (allele-wise)					Multi marker (haplotype-wise)[a]	
			SCZ[b]	CON[c]	L95[d]	U95[d]	*p* value	2 markers	3 markers
Marker 4	rsl801133	C>T	0.409	0.399	0.910	1.195	0.545		
								0.527	
Marker 5	rs17421511	G>A	0.098	0.098	0.800	1.253	0.991		0.597
								0.924	
Marker 6	rs17037396	T>C	0.104	0.103	0.812	1.258	0.925		0.073
								0.975	
Marker 7	rs9651118	A>G	0.354	0.358	0.856	1.131	0.824		

[a]Log likelihood ratio test *p* value (sliding window analysis with rare haplotype threshold 10%).
[b]SCZ: Schizophrenia.
[c]CON: Control; minor allele frequency.
[d]95% confidence intervals (odds ratio).

of subjects need to be undertaken. In addition, by combining such types of studies with endophenotypes or clinical stratifications, this may provide a better understanding of the pathophysiology of schizophrenia.

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Contributors

Authors Akira Yoshimi, Nagahide Takahashi, and Toshiya Inada designed the study and wrote the protocol. Authors Akira Yoshimi and Yukiko Kawamura conducted SNPs genotyping and statistical analyses. Authors Norio Ozaki, Yukihiro Noda, and Kiyofumi Yamada managed the literature searches and analyses. Author Akira Yoshimi wrote the first draft of the manuscript and Branko Aleksic revised. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors have no conflicts to declare.

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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.schres.2010.07.011.

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