orienting response involving an amygdalo-nigrostriatal pathway but that this response appeared immediately after the inactivation process is terminated (Han et al., 1997). Repeated cocaine administration evokes neural plasticity in not only the striato-SNr axis but also many other brain regions (Hyman et al., 2006). The study of reversible blockade of striatonigral transmission thus suggests that neural plasticity in the striato-SNr axis is involved in the expression of cocaine sensitization, but other brain regions may also be necessary for acquisition and storage of the cocaine-induced adaptive response.

Recent studies using BAC transgenic mice have indicated that D1 and D2 receptors are almost exclusively expressed in striatonigral and striatopallidal neurons, respectively (Heiman et al., 2008; Lobo et al., 2006; Surmeier et al., 2007). D1 and D2 receptors exhibit a marked difference in their dopamine binding affinity, i.e., a respective μM and nM order of affinity for dopamine (Maeno, 1982; Richfield et al., 1989). Furthermore, accumulated evidence has indicated that dopaminergic transmission within the striatum is segregated into functionally dissociable compartments, in which phasic and tonic firings of dopamine neurons differentially modulate D1 and D2 receptors (Grace et al., 2007; Hikosaka, 2007). On the basis of these characteristic features of dopamine transmission, we propose a mechanistic model for the roles of the two pathways in relation to the regulation and dysfunction of the basal ganglia (Figure S6). When naive animals encounter unexpected rewards, dopamine neurons emit a phasic burst of firings that considerably raises dopamine concentrations within synapses of the NAc (Mirenowicz and Schultz, 1994). Similarly, psychostimulants increase dopamine levels in the NAc (Di Chiara and Imperato, 1988). This increase not only activates the low-affinity D1 receptor in striatonigral neurons but also saturates the high-affinity D2 receptor in striatopallidal neurons. By contrast, tonic firings of dopamine neurons are not sufficient to activate the D1 receptor and modulate only the high-affinity D2 receptor (Grace et al., 2007; Hikosaka, 2007). The dual stimulation of D1 and D2 receptors is thus essential for facilitating the basal ganglia-cortical circuitry that triggers the early stage of reward-directed or psychostimulant-induced behaviors in naive animals (Figure S6A).

Then, how is the modulation by the striatonigral and striatopallidal transmission shifted to play predominant roles in reward-directed and aversive learning, respectively? Goto and Grace (2005b) demonstrated that the stimulation of D1 and D2 receptors produces behaviorally selective effects (in this case, learning versus set shifting of response strategy) that correspond to specific afferents derived from the hippocampus and the prefrontal cortex, respectively. Furthermore, they reported that D1 and D2 receptors differentially induce LTP and LTD, depending on different afferents in the NAc (Goto and Grace, 2005a). Importantly, the photogenetic study by Tsai et al. (2009) revealed that the phasic activation of dopamine neurons evokes CPP associated with the dopamine-activated environment but that tonic activation fails to derive such CPP. Conversely, aversive stimuli have been shown to reduce tonic firings of most or regionally confined dopamine neurons (Brischoux et al., 2009; Coizet et al., 2006; Mirenowicz and Schultz, 1996; Ungless et al., 2004), and blunting the tonic dopamine release in the ventromedial striatum leads to conditioned place aversion (Liu et al., 2008). Our model holds that the substantial increase in dopamine by reward-related, phasic dopamine release or repetitive cocaine administration induces LTP and LTD at striatonigral and striatopallidal neurons, respectively (Figure S6B). The striatonigral input thus becomes predominant over the striatopallidal input in the processes of reward-directed behavior and cocaine sensitization. Consequently, the blockade of striatonigral transmission should severely impair the adaptive responses of both appetitive reward behavior and cocaine sensitization. On the other hand, blockade of striatopallidal transmission produces the situation equivalent to the LTD-mediated reduction in input in striatopallidal synapses and would thus have no further effect on reward-directed behavior, once LTP is postsynaptically induced in striatonigral neurons. In contrast, this blockade or the cocaine-induced LTD would disrupt the modulatory mechanism by tonic dopamine release and would impair aversive behavior (Figure S6C). Because the induction of LTP and LTD in MSNs has been reported to depend on not only synaptic input integration but also the cellular processes sensitive to timing of pre- and postsynaptic activity (Goto and Grace, 2005a; Shen et al., 2008), the model discussed here needs to be more substantiated. It should, however, be pointed out that animals need to rapidly form memory against aversive stimuli to avoid uncomfortable or dangerous environments. In contrast, animals have to distinguish between associative and nonassociative rewarding stimuli to acquire such rewards efficiently and correctly. The modulatory switch of the two pathways thus plays an essential role in properly driving behavioral responses to rewarding and aversive stimuli.

Recently, the biochemical characterization of D1-receptor-expressing and D2-receptor-expressing MSNs has revealed that these two subpopulations exhibit vast differences in their expression of functional and signaling molecules (Heiman et al., 2008; Surmeier et al., 2007; Valjent et al., 2009). The distinct functions of striatonigral and striatopallidal transmission could thus result from different functional molecules in these two subpopulations of MSNs. The present study will facilitate more informed and effective approaches to the treatment of the basal ganglia dysfunction that occurs in drug addiction and Parkinson's disease.

EXPERIMENTAL PROCEDURES

Animals

The TN transgenic mice were generated as described previously (Yamamoto et al., 2003) and used together with their wild-type littermates for all experiments. DOX was administered in food pellets containing 6 mg/g DOX and in drinking water containing 2 mg/ml DOX and 10% sucrose. All animal handling procedures were performed according to the guidelines of Osaka Bioscience Institute.

Construction of the Recombinant AAVs

The 2.1 kb promoter region of the mouse *PPTA* gene (residues from −1525 to +543, NCBI accession number NT_039340) or the 2.0 kb promoter region of the mouse *PPE* gene (residues −1834 to +148, NCBI accession number NT_039258) was isolated from BAC DNA. The promoter regions were attached to the *flag-tTA* cDNA and inserted into the Multiple Cloning Sequence of the pAAV-MCS vector of the AAV Helper−Free System (Stratagene, La Jolla, CA). Both constructs were packaged and serotyped with the AAV capsid protein with the use of the AAV Helper-Free System. The AAV was purified

by using a ViraTrap AAV Purification kit (Omega Bio-Tek Inc., Norcross, GA), yielding 10^{12} particles per milliliter when titrated by ELISA for the AAV2 capsids (PROGEN, Heidelberg, Germany). The viral injection and treatment with DOX were conducted according to the following protocols, unless otherwise stated: the recombinant AAV or the control AAV free of the inserted tTA construct was unilaterally or bilaterally injected into 11 sites of the striatum or into four sites of the NAc by stereotaxic techniques (Hikida et al., 2001). Two weeks after the viral injection, animals were continuously treated with DOX or left untreated.

Retrograde Tracing

The V-S-tTA or V-E-tTA virus was injected into the striatum of wild-type mice. The CTB-Alexa 594 conjugate (Molecular Probes, Eugene, OR) was then stereotaxically injected into the GP or SNr of these mice (for GP, 0.5 mm posterior to the bregma, 2 mm lateral from the midline, 3.5 mm depth from the dura; for SNr, 3.5 mm posterior to the bregma, 1.5 mm lateral from the midline, 4 mm depth from the dura). Two weeks after the injection, the mice were deeply anesthetized, and coronal sections (40 μm) of the striatum were prepared and immunostained with anti-flag M2 monoclonal antibody (Sigma, St. Louis, MO), followed by Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). Immunofluorescence and CTB-Alexa 594 fluorescence were detected with a BZ-9000 digital fluorescence microscope (Keyence, Osaka, Japan).

Immunohistochemistry and Immunoblotting

Coronal sections (40 μm) of the striatum from animals 2 weeks after viral injection were prepared and immunostained (Hikida et al., 2001). The primary antibodies used were obtained as described previously (Hikida et al., 2001). Signals were visualized with secondary antibodies (Yamamoto et al., 2003). Immunoblotting was performed as described previously (Yamamoto et al., 2003).

In Situ Hybridization Histochemistry

In situ hybridization analysis of coronal sections (10 μm) prepared from a fresh-frozen mouse brain was performed as described previously (Kaneko et al., 2000). Specific antisense riboprobes were [^{35}S]-labeled from the corresponding cDNAs (*SP* cDNA, residues from +1 to +390, NCBI accession number NT_039340; *Enk* cDNA, residues from +1 to +807, NCBI accession number NT_039258; c-*fos* cDNA, residues from +1 to +1143, NCBI accession number NM_010234) (Kaneko et al., 2000). Four to six coronal sections taken every 100 μm were used to calculate the mean radioactivity of *SP* and *Enk* mRNAs in the striatum and to count c-*fos* mRNA-positive cells in the VP or the SNr. Radioactivity of each section was quantified by using a BAS5000 image processing system (Fujifilm, Tokyo, Japan).

Electrophysiology

After anesthesia using 0.9% ketamine and 0.1% xylazine, a mouse was positioned in a stereotaxic apparatus. A pair of stimulating glass-coated metal electrodes (Elgiloy) was implanted into the right side of the striatum (one at 1.5 mm anterior, 1 mm lateral and the other at 0.5 mm anterior, 2 mm lateral to the bregma, 3 mm depth for both). A short pulse at 2 Hz (duration, 200 μs; amplitude, 50–75 μA) was applied for striatal stimulation. Local field potentials in response to the stimulation were recorded at the ipsilateral SNr (3.5 mm posterior, 1.5 mm lateral to the bregma, 3.5–4.2 mm depth) by using a glass electrode (tip diameter, ~8 μm) filled with ACSF (in mM, NaCl 160, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, HEPES 5, glucose 10). A reference electrode (Ag-AgCl) was placed in the cervical subcutaneous tissue. The short-latency response was gained from 50 pulses of either polarity between the two stimulating electrodes, separately averaged, and added thereafter. Bicuculline methochloride (Tocris, Ellisville, MO) was perfused with the use of a triple-barreled glass electrode. One barrel was filled with bicuculline methochloride, which was then ionophoretically injected. The other two barrels were filled with ACSF and each used for recording and a return path (retain current, 20 nA; injection current, 40 nA). Recorded sites were confirmed to be the SNr by histological reconstruction after fixation with 4% paraformaldehyde.

Behavior Tests

For the rotation test, mice were placed in a round-bottomed bowl (25 cm in diameter), and rotations were counted for a 5 min period by visual observation (Kaneko et al., 2000). One rotation was defined by the animal completing a 360° circle without turning back in the opposite direction. Locomotor activity was measured with an infrared activity monitor (MED Associates, St. Albans, VT) for a 60 min period immediately after i.p. injection of saline or 2 mg/kg methamphetamine. For cocaine experiments, locomotor activity was measured for a 10 min period immediately after i.p. injection of saline or 10 mg/kg cocaine (Hikida et al., 2001, 2003). The CPP test was performed as described previously (Hikida et al., 2001, 2003). Methamphetamine and cocaine were obtained from Dainippon Sumitomo Pharma (Osaka, Japan) and Shionogi (Osaka, Japan), respectively. Chocolate and food consumptions were measured after 24 hr free access to both chocolate and a standard food.

In the aversion test, the step-through inhibitory avoidance apparatus consisted of a straight alley divided into a small, light chamber (8 cm long) and a large, dark chamber (16 cm long). The light chamber was made of gray Plexiglas and illuminated by a lamp. The dark chamber was made of black Plexiglas covered by a black cloth and a grid floor connected to an electric source. The two chambers were separated by a black sliding door. On the training day, each mouse was placed in the light chamber and the door leading to the dark chamber was raised. Once the mouse had stepped with all four paws into the dark chamber, the door was closed; and then an electric footshock (0.5 mA, 60 Hz, 1 s) was delivered. Memory retention was tested 24 hr later following a similar procedure, expect that no shock was delivered. Latency to step into the dark chamber was measured. Fear responses were analyzed as described previously (Masugi et al., 1999). The percentage of freezing response was determined by scoring the number of positive freezing responses divided by the total number of samples at 2 s intervals in a 1 min time period.

Statistical Analysis

Statistical analysis was conducted by using STATVIEW. Data were analyzed by two-way ANOVA or repeated-measured ANOVA and were presented as the mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at doi:10.1016/j.neuron.2010.05.011.

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特集 神経科学と臨床研究の連携がもたらす 精神疾患への統合的アプローチ

精神疾患モデル動物の可能性
遺伝子から神経回路へ

疋田貴俊，神谷　篤

遺伝研究の発展に伴い，多くの精神疾患脆弱性にかかわる候補遺伝子が報告され，より確かな生物学的要因に基づく疾患モデル動物の可能性が期待されている．しかし精神疾患モデル動物解析には，時間経過，複数の脆弱性遺伝因子の機能的相互作用，環境因子の関与，さらには高次脳機能が障害される精神疾患を，神経回路や行動の解析を含めた動物モデルにおけるリードアウトでいかに評価すべきかなど，多くの考慮すべき要因がある．本稿では，これらの論点を包括し，変異遺伝子を導入したマウス，子宮内電気穿孔法を用いたモデルマウス，神経回路を *in vivo* で制御できる遺伝子改変マウスなどを紹介し，モデル動物を用いた精神疾患研究の方向性を展望する．

キーワード● 候補遺伝子，遺伝子改変動物，chromosomal engineering，子宮内電気穿孔法，神経回路

はじめに

ヒトの病気の病態解明や治療法の開発，治療薬スクリーニングには疾患モデル動物が必須であるが，精神疾患の場合，ヒト高次脳機能の多くがマウスでは再現困難であるため表現型からのアプローチに限界があることや，明確なバイオマーカー，病理マーカーが存在しないことから，モデル動物の確立は容易ではない．さらに一般に遺伝要因が関与する疾患においては遺伝子改変マウスが作製，利用されているが，精神疾患においては，その遺伝形式はよくわかっておらず，候補遺伝子のノックアウトマウスなど単一因子をもとにした遺伝子改変マウスでは病態を忠実に反映するのが著しく困難である．精神疾患遺伝学の分野においては，古典的な連鎖関連解析から昨今の全ゲノム関連解析により，多くの候補遺伝子が明らかになりつつある．しかしながら，個々の遺伝因子は相対危険度が低く，生物学的機能への影響が少ないことが予測され，さらに複数の遺伝因子による相互作用が疾患に結びついている可能性が高いことから，これらの情報を動物に反映させ解析することは難しい．とはいえ，最近の遺伝学の技術的進歩に伴い，ヒトゲノムコピー数多型（copy number variation：CNV）や *de novo* mutation※1のような新しいタイプの遺伝子変異も続々と発見されつつあり，これらの変異の頻度はまれであるが，相対危険度が高く，強い生物学的効果が予想され，動物モデルも含めた生物学的アプローチを志向しやすい．

本稿では，これらの要素を考慮した精神疾患モデル動物として，ヒトでみられる遺伝子変異や染色体変異を導入した遺伝子改変マウスを紹介し，これらのモデルを用いた研究の方向性を示す．また精神疾患の時間経過を考慮に入れた，遺伝因子，環境因子の相互作用や神経回路レベルでの精神疾患の病態研究の重要性を概括したい．なお，ショウジョウバエやゼブラフィッ

※1 *de novo* mutation
de novo 突然変異．新規突然変異．親の生殖細胞（精子あるいは卵子）または胚形成早期の受精卵における突然変異の結果，ある家系員からはじめてみられるようになった遺伝子変異．

Animal models of mental illnesses: from genes to circuits
Takatoshi Hikida[1)2)]/Atsushi Kamiya[3)]：Department of Systems Biology, Osaka Bioscience Institute[1)]/PRESTO, JST[2)]/Department of Psychiatry and Behavioral Sciences[3)]（大阪バイオサイエンス研究所システムズ生物学部門[1)]／科学技術振興機構さきがけ[2)]／ジョンズホプキンス大学医学部精神医学部門[3)]）

シュなど齧歯類以外の動物における遺伝子改変も有効な手段であるが，誌面の都合上本稿では割愛する．

1 ヒトでみられる変異を導入した遺伝子改変動物

❶ 自閉症関連遺伝子の変異導入マウス

遺伝的負因が発症に大きくかかわる精神疾患として自閉症と統合失調症があげられる．自閉症の一部の症例では，家族性の遺伝子点変異が発症に深く関与することが知られているが，その1つにNeuroligin 3における種間の保存性の高い451番目のアルギニン（R）からシステイン（C）へのミスセンス変異があげられる[1]．Neuroligin 3はシナプスの機能タンパク質であるが，田渕らはこの変異を相同組換えによりマウスゲノム上で再現したNeuroligin 3 R451C変異マウスを作製した[2]．このマウスでは社会行動低下と空間学習記憶能の向上がみられ，大脳皮質において抑制性シナプス機能の亢進を示していることから，自閉症の病態生理にシナプス機能の異常がかかわっている可能性が示唆される．自閉症圏の疾患において，最もよく報告されている染色体異常に15q11-13重複があるが，最近中谷らは，chromosomal engineering（染色体工学）の技術を用いて，6.3 Mbに及ぶこの染色体異常をゲノム上で再現したマウスを作製した[3]．父親由来の染色体でこの重複をもつマウスは社会行動異常と固執行動を示したと報告している．

❷ 統合失調症関連遺伝子の変異導入マウス

統合失調症においても，こういった比較的稀な遺伝子変異を反映しているにすぎないものの，今後病態分子経路の解明につながりうる可能性をもつマウスモデルを紹介する．22q11.2欠失症候群は，染色体の微細欠失により，胸腺低形成による免疫不全や先天性心疾患を示すが，一般人口に比べ30倍もの統合失調症発症リスクをもつ[4]．KarayiorgouとGogosらのグループは，ヒト染色体22q11.2のマウス相同領域を欠失したマウスを作製し[5]，作業記憶低下と課題遂行時の海馬と大脳皮質の同調性の異常を見出した[6]．これは精神疾患モデルマウスを神経回路レベルで解析したよい例といえる．他の精神疾患発症に高いリスクをもつ遺伝子変異の例としては，スコットランドの精神疾患多発家系で1番染色体と11番染色体の均衡転座点より見つかったDISC1（Disrupted-In-Schizophrenia 1）※2がある．これまで多くのDISC1変異マウスモデルが報告されているが，われわれの変異型DISC1トランスジェニックマウスにおいては，行動異常に加え，MRI撮像における脳室拡大，大脳皮質でのparvalbumin発現の減少がみられ，これらは変異型DISC1がドミナントネガティブ体として機能した結果であるのかもしれない[7][8]．Parvalbumin陽性細胞の統合失調症神経病理における重要性については橋本らの稿を参照されたい．

❸ 中間表現型に着目したマウスモデル

ところでこういった遺伝子改変マウスモデルは，精神疾患の病理を一部反映している可能性はあるものの，その解釈には注意が必要である．例えばDISC1の場合，この遺伝子が統合失調症のみならず躁うつ病やうつ病といった他の精神疾患への脆弱性にもかかわっていることを，遺伝学的証拠は示しており，特定の精神疾患に対する動物モデルを遺伝子改変によりつくろうというストラテジーは難しい．そもそも現在用いられている診断カテゴリーは，症状ベースでつくられたものであり，生物学的証拠に基づくものではない．したがって，疾患名にこだわるより，むしろ動物モデルでも評価可能な中間表現型に着目し，そのメカニズムを明らかにするという方向性がよいのかもしれない．その点で，ヒトとマウス間でtranslatableな中間表現型であるといえるPPI（prepulse inhibition）※3に注目したスクリーニングにより，PPIに障害のみられるマウスを見つけることから責任遺伝子としてFabp7の同定につなげた渡辺らの仕事は，大変興味深いストラテジーである[9]．

※2　DISC1（Disrupted-In-Schizophrenia 1）
スコットランドの精神疾患多発大家系において同定された精神疾患候補遺伝子．脳神経発達を含むさまざまな中枢神経機能にかかわっていると考えられている．

※3　PPI（prepulse inhibition）
先に弱い刺激を加えることで，突然与えられた強い刺激に対する驚愕反応が抑制される現象のこと．情報処理における障害の指標と考えられ，PPIの減少は統合失調症をはじめとするヒト精神疾患のみならず，動物でも測定できることから，中間表現型としての側面をもち，利用されている．

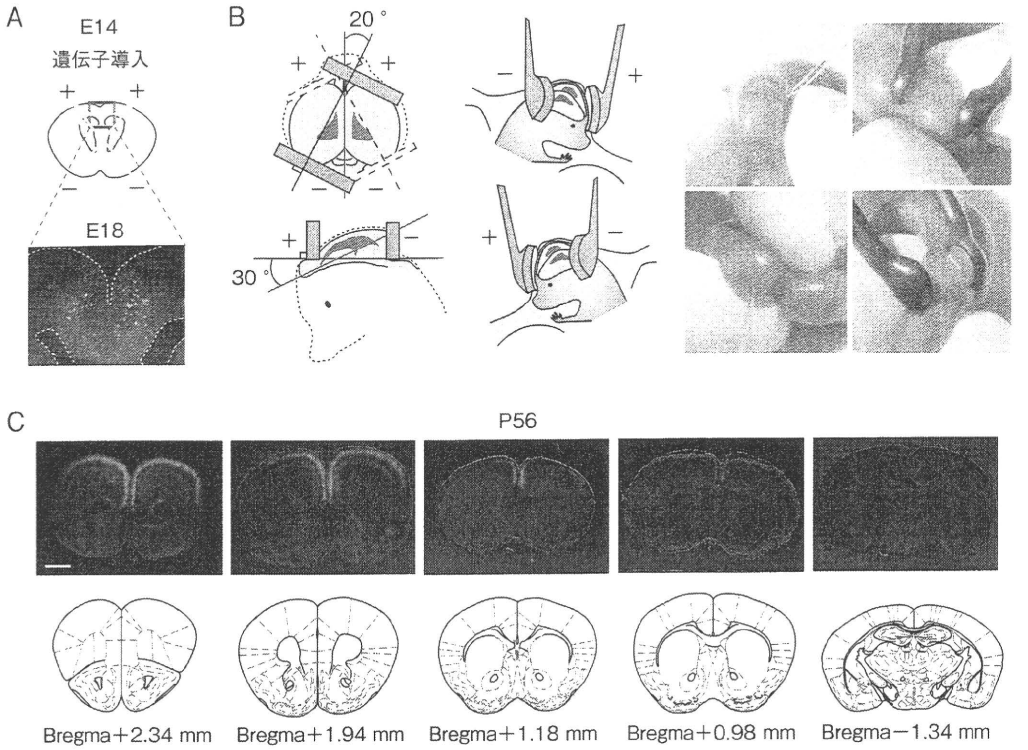

図1　子宮内電気穿孔法を用いた前頭前野（prefrontal cortex）に対する遺伝子ターゲティング
A）胎生14日目（E14）に両側脳室帯にEGFPを発現するコンストラクトを電気穿孔法により導入し，胎生18日目（E18）に固定し観察した．B）前頭前野への遺伝子ターゲティングを目的とする遺伝子導入法．C）生後56日目（P56）の頭側から尾側にかけての冠状脳切片の観察により，前頭前野への遺伝子導入を確認した．緑：EGFP，青：DAPI，スケールバー＝1mm〔A～C（写真）は文献12より転載，A～C（イラスト）は文献12より引用〕

❹ ラットにおける遺伝子改変の可能性

齧歯類を用いた遺伝子改変動物作製においては，その技術的な限界から，これまではマウスを中心に用いられてきた．しかしながら，近年ラットにおける遺伝子改変も可能になりつつあり[10]，より高次の脳機能が解析可能なラットを用いた精神疾患研究も，今後発展する可能性がある．

2 多因子疾患としての精神疾患へのモデル動物を用いたアプローチ

前述のように，多くの精神疾患には複数の遺伝要因が同時に関与している可能性が疑われる．Chromosomal engineering技術も含め，遺伝子改変技術の進歩は著しく，今後，複数の候補遺伝子を同時に改変した，よりetiology（病因）を考慮したモデル動物の登場と，それによる疾患脆弱性にかかわる分子経路の解明が進むことが期待される．加えて他の方法論を用いた動物モデルの作製も試みられている．

子宮内電気穿孔法を用いた齧歯類胎仔脳への遺伝子導入技術は，脳発達段階における遺伝子発現を制御することが可能であり，神経発達の分野ではポピュラーな技術として汎用されている[11]．丹羽らは，この技術を用いて発達段階における前頭前野のDISC1の発現をRNAiによりノックダウンしたマウスを作製し，その後の神経回路，行動レベルでの影響を調べた（図1）[12]．発達段階のDISC1発現を抑制することで，錐体細胞樹状突起の形態異常に加え，思春期後には中脳皮質ドーパミン系の成熟障害，および認知機能障害をはじめとする種々の行動異常を認めた．興味深いことにドーパ

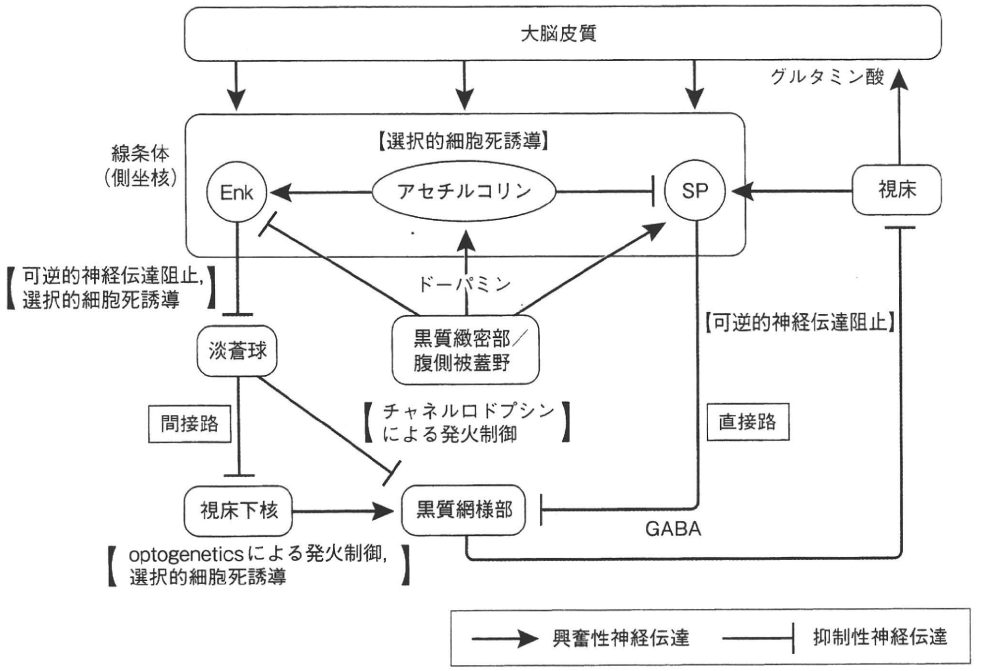

図2　大脳基底核神経回路とさまざまな神経回路制御法

大脳基底核神経回路と，特定の神経回路を制御するために用いられている方法を示した．SPはサブスタンスP陽性細胞で，線条体から黒質網様部に投射する直接路を形成する．Enkはエンケファリン陽性細胞で，線条体から淡蒼球に投射し間接路を構成する．大脳皮質－視床下核経路（ハイパー直接路）やGABA介在神経細胞は省略した．神経回路制御法の詳細は本文および文献16～21を参照のこと

ミン系の異常や行動異常は思春期前には認められないことから，DISC1の発達期における役割がその後の神経回路成熟と行動に影響を及ぼすことが示唆され，時間軸に沿った神経病理，行動の解析が，統合失調症の研究に重要であることを示している．この技術は複数の遺伝子要因を同時に調節することが可能であり，またCre/LoxP systemをはじめとする条件特異的遺伝子発現制御系と組合わせ発現レベルを制御することで，これらの遺伝因子のどの発達段階，領域，細胞における働きが，より疾患病理に重要であるかを，分子経路および神経回路，行動レベルで解析できる可能性がある[13]．

遺伝要因に基づいた動物モデルは，他の環境因子との相互作用を解析するのにも有用である．周産期のウイルス感染は統合失調症のリスク因子として知られている（大城らの稿参照）．合成dsRNAであるpoly(I:C)（polyriboinosinic-polyribocytidylic acid）を妊娠マウスに投与することにより，母胎の免疫系を賦活することで周産期感染を模倣することができるが，変異型DISC1マウスにpoly(I:C)を投与すると，行動異常が悪化し，さらには大脳皮質のparvalbumin発現がより減少していることが報告されている[14]．

3 モデル動物を用いた神経回路の機能解明によるアプローチ

精神疾患の病態を考察するにあたって，神経回路の視点を欠かすことはできない．認知や情動を含むさまざまな高次脳機能の分子メカニズムを探るために，特定の脳領域における遺伝子発現を制御できるAAV（adeno-associated virus）やlentivirusを用いたstereotaxic gene delivery※4が頻繁に利用されている[15]．これらの技術を用いた，細胞特異的な遺伝子ターゲティングが可能なツールの開発が進んでおり，今後，錐体

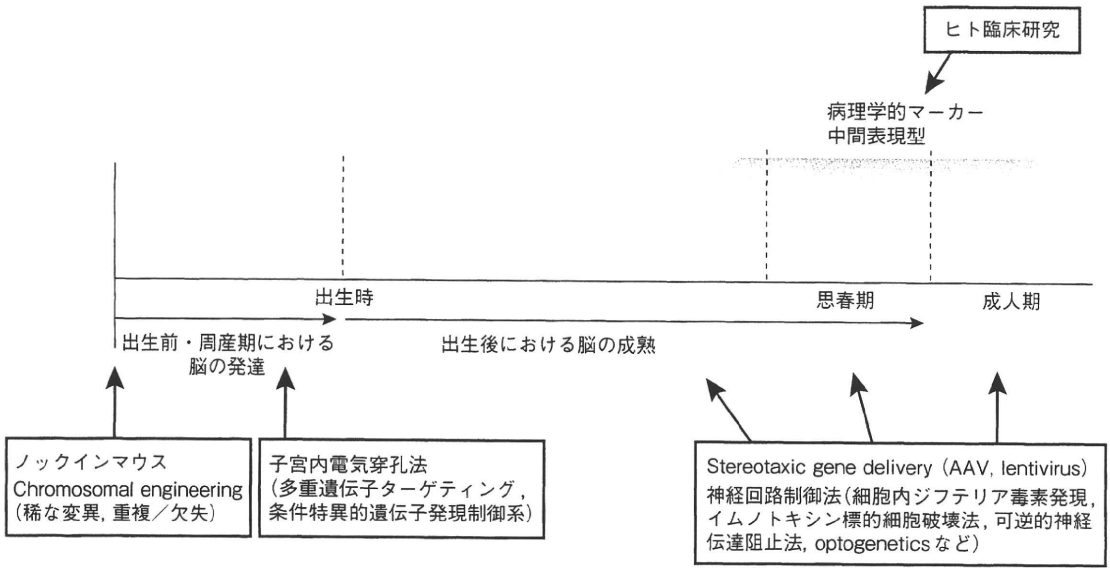

図3　精神疾患病理の時間軸と，動物モデルを用いた病態研究
精神疾患病態研究の戦略として，ヒト臨床研究から得た知見を念頭に，種々の遺伝子改変技術，神経回路制御法を駆使して，時間軸に沿った分子，回路，行動の解析を行う必要がある．縦軸は臨床症状の程度を示す

神経細胞，種々のGABA介在神経細胞，グリアといった異なった細胞間ネットワークレベルでの解析が進むものと思われる．ここでは，stereotaxic injection（定位的注入）を利用した精神疾患の神経回路レベルでの異常を探るユニークな方法をいくつか紹介したい．

大脳基底核は運動制御，報酬，認知といった精神活動，社会活動に必須の役割をもち，さまざまな神経変性疾患や薬物依存症，うつ病，統合失調症などの精神疾患に深く関与していることが知られている．大脳基底核内の主な神経回路は線条体から黒質網様部に投射する直接路と，線条体から淡蒼球を介して黒質網様部に至る間接路に大きく二分される（図2）．これらの回路は黒質網様部で統合され，視床，大脳皮質とのループ回路のバランスをとっていると考えられているが，精神機能や精神疾患での回路動態についてはわかっていない．そこで，大脳基底核神経回路の特定の神経細胞を標的として，*in vivo* で神経回路を制御する試みがなされている．

※4　Stereotaxic gene delivery
遺伝子をAAVやlentivirusにパッケージングし，脳地図をもとに定位的にウイルスを注入し（stereotaxic injection），特定の神経細胞に感染させることによる脳領域特異的な遺伝子導入法．

❶ 特定の神経細胞破壊による神経回路制御

細胞内ジフテリア毒素発現[16]やイムノトキシン標的細胞破壊法[17]によって，特定の細胞を神経回路から取り除くことができる．イムノトキシン標的細胞破壊法では，線条体介在神経細胞であるアセチルコリン産生細胞にヒトIL-2受容体を発現させたマウス線条体内に，ヒトIL-2受容体に対する抗体と緑膿菌毒素を融合させたイムノトキシンを注入することで，アセチルコリン産生細胞選択的に細胞死を誘導することができる[17]．アセチルコリン産生細胞を除去したマウスでは，依存性薬物に対する感受性が上昇し，薬物依存を形成しやすいことから，線条体内局所神経回路ではドーパミンとアセチルコリンが拮抗的であると同時に協調して作用していることが推察される[18]．

❷ 神経伝達物質放出の阻害による神経回路制御

特定の細胞にテトラサイクリン依存的に破傷風菌毒素を発現させることで可逆的に神経伝達物質放出を阻止することができる（可逆的神経伝達阻止法）．われわれは，テトラサイクリン応答配列の下流に破傷風菌毒素を有したトランスジェニックマウスの線条体に，サ

ブスタンスPあるいはエンケファリンのプロモーター下にテトラサイクリン依存性転写因子を有するAAVを注入することで，直接路あるいは間接路の線条体神経細胞特異的なテトラサイクリン依存性破傷風菌毒素発現システムを開発した[19]．これにより，細胞死を誘導することなく直接路と間接路をそれぞれ特異的に神経伝達遮断することが可能となり，大脳基底核神経回路の制御機構における直接路と間接路の役割を研究することができるようになった．

❸ Optogeneticsによる神経回路制御

特定の細胞に光受容体であるチャネルロドプシンやハロロドプシンを発現させることによって，光照射による神経活動制御が可能となるoptogenetics※5は，現在注目を浴びている手法の1つである[20]．腹側被蓋野のドーパミン細胞は通常持続的に低頻度の発火をしているが，報酬を予測すると一過性高頻度発火をすることが知られている．腹側被蓋野のドーパミン細胞にチャネルロドプシンを導入し，光ファイバー経由で高頻度の光照射を行うことで，ドーパミン細胞の高頻度発火を再現し，その結果報酬行動を誘導することが報告されている[21]．

今後はこれらのような多彩な遺伝子改変技術や神経回路制御法を駆使することで，さまざまな精神活動を司る神経回路の役割が解明されていくと同時に，精神疾患における神経回路レベルでの研究の進展が期待される．

おわりに

動物モデルを用いた精神疾患研究についての展望を概説した．急速な技術の進歩により，いよいよ候補遺伝子から分子経路，回路，行動まで包括的に動物モデルを用いて脳高次機能の研究を進めることが可能な時代となりつつある．一方で，これまで蓄積されてきた疫学や病理，遺伝学におけるさまざまな知見を鑑み，それに基づいた仮説を立て，精神疾患病理の時間軸に沿って神経科学的アプローチを試みる姿勢が，今後の精神疾患研究に求められるものと思われる（図3）．

※5　Optogenetics
オプトジェネティクス，光遺伝学．光活性型イオンチャネルであるチャネルロドプシンやハロロドプシンを特定神経細胞に発現させ，特定の波長の光を照射することによって標的神経細胞を興奮または抑制できる．*in vivo*でミリ秒単位の制御が可能となる．

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Profile　筆頭著者プロフィール

疋田貴俊：1997年，京都大学医学部卒業．京都大学医学部附属病院精神科で研修．2002年，京都大学大学院医学研究科博士課程修了（中西重忠教授）．博士（医学）．'03年よりジョンズホプキンス大学医学部神経科学部門（Solomon H. Snyder教授）・精神医学部門（澤 明教授）で統合失調症モデルマウスの作製と解析を行う．'05年より大阪バイオサイエンス研究所システムズ生物学部門研究員（中西所長）．大脳基底核神経回路研究に取り組む．'09年よりJSTさきがけ研究者兼任．

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Research report

Silibinin attenuates cognitive deficits and decreases of dopamine and serotonin induced by repeated methamphetamine treatment

Ping Lu[a,b,f], Takayoshi Mamiya[a,f], Lingling Lu[a,b,f], Akihiro Mouri[a,f], Minae Niwa[a,f], Hyoung-Chun Kim[d], Li-Bo Zou[b], Taku Nagai[c,f], Kiyofumi Yamada[c,f], Takashi Ikejima[b], Toshitaka Nabeshima[a,e,f,*]

[a] Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya 468-8503, Japan
[b] Department of Pharmacology, School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University, Shenyang 110016, China
[c] Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan
[d] Neuropsychopharmacology and Toxicology Program, College of Pharmacy, Kangwon National University, Chunchon 200-701, South Korea
[e] Japanese Drug Organization of Appropriate Use and Research, Nagoya 468-0077, Japan
[f] The Academic Frontier Project for Private Universities, Comparative Cognitive Science Institutes, Nagoya 468-8503, Japan

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ABSTRACT

Cognitive deficits are a core feature of patients with methamphetamine (METH) abuse. It has been reported that repeated METH treatment impairs long-term recognition memory in the novel object recognition test (NORT) in mice. Recent studies indicate that silibinin, a flavonoid derived from the herb milk thistle, has potent neuroprotective effects in cell cultures and several animal models of neurological diseases. However, its effect on the cognitive deficit induced by METH remains unclear. In the present study, we attempt to clarify the effect of silibinin on impairments of recognition memory caused by METH in mice. Mice were co-administered silibinin with METH for 7 days and then cognitive function was assessed by NORT after 7-day withdrawal. Tissue levels of dopamine and serotonin as well as their metabolites in the prefrontal cortex and hippocampus were measured 1 day after NORT. Silibinin dose-dependently ameliorated the impairment of recognition memory caused by METH treatment in mice. Silibinin significantly attenuated the decreases in the dopamine content of the prefrontal cortex and serotonin content of the hippocampus caused by METH treatment. We also found a correlation between the recognition values and dopamine and serotonin contents of the prefrontal cortex and hippocampus. The effect of silibinin on cognitive impairment may be associated with an amelioration of decreases in dopamine and serotonin levels in the prefrontal cortex and hippocampus, respectively. These results suggest that silibinin may be useful as a pharmacological tool to investigate the mechanisms of METH-induced cognitive impairments.

1. Introduction

Methamphetamine (METH) is a globally popular and highly addictive drug that can cause neuropsychiatric complications such as hallucination and delusions [14,28,40]. Recent studies in humans have also demonstrated that chronic use of METH causes cognitive deficits after withdrawal [14,39,41]. In rodents, repeated METH treatment induces spatial working memory impairment in the radial arm maze test [26], recognition impairment in the object recognition test [15,27], and cognitive impairment in the five-choice serial reaction time test [9]. Therefore, the METH-induced cognitive impairment in rodents may be useful as an animal model of the cognitive deficits in METH abusers.

Several studies have revealed that the cognitive impairments produced by METH are associated with disruptions of the dopaminergic and serotonergic systems [14,15,17,22,28]. Disturbances of the dopaminergic system have been widely reported in METH abusers and animals, and are associated with behavioral deficits [17]. For example, our group has reported that repeated METH treatment results in a dysfunctional dopamine D_1 receptor-extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in the prefrontal cortex, which is associated with memory deficits after drug withdrawal [15,27]. The serotoninergic system also plays a critical role in cognitive behavior [7,24] and is associated with METH abuse [17]. Moreover, the serotonergic system interacts with the dopaminergic system, implicating it in cognitive function and drug abuse [46]. Although the interactions involved are complicated, a report has confirmed that dysfunction of either

* Corresponding author at: Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan. Tel.: +81 52 839 2735; fax: +81 52 839 2738.
E-mail address: tnabeshi@ccmfs.meijo-u.ac.jp (T. Nabeshima).

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dopaminergic or serotonergic system alone is insufficient to produce the impairments seen in the novel object recognition test (NORT) following METH treatment [3].

Silibinin (silybin), a flavonoid derived from the herb milk thistle (*Silybum marianum*), has been shown to have anti-oxidative and anti-inflammatory properties [38]. Further, silibinin also has potential effects on monoamine transmission and cognitive function. For example, high-performance liquid chromatography (HPLC) analyses have shown increased serotonin levels in the cortex and increased dopamine and norepinephrine levels in the cerebellum after 5-day repeated treatment with silymarin, a mixture of flavonoids present in milk thistle and whose main component is silibinin, in BALB/c mice [29]. An *in vitro* study has demonstrated that silibinin inhibits the activity of monoamine oxidase (MAO) that catalyzes the oxidative deamination of monoamines [23]. These studies suggest that silibinin may be beneficial/neuroprotective effect on METH-induced dysfunction. However, it remains unclear whether silibinin prevents METH-induced cognitive deficits.

In this study, we investigated the effects of silibinin on the impairment of recognition memory in NORT and decrease of dopamine and serotonin levels in the prefrontal cortex and hippocampus in repeated METH-treated mice.

2. Materials and methods

2.1. Animals

ICR male mice (Japan SLC Inc., Shizuoka, Japan), aged 6 weeks at the beginning of experiments, were used. They were housed in plastic cages and kept in a regulated environment (23 ± 0.5 °C, 50 ± 5% humidity) with a 12/12-h light/dark cycle (lights on from 08:00 to 20:00). Mice received food (CE2; Clea Japan Inc., Tokyo, Japan) and water ad libitum. Behavioral experiments were carried out in a sound-attenuated and air-regulated experimental room, to which mice were habituated for at least 1 h. All experiments were performed in accordance with the Guidelines for Animal Experiments of the Faculty of Pharmaceutical Sciences of Meijo University.

2.2. Drugs

Methamphetamine hydrochloride (METH; Dainippon Sumitomo Pharma Co Ltd, Osaka, Japan), was dissolved in 0.9% physiological saline. Silibinin was purchased from Panjin Green Biological Development Co., Ltd (Panjin, China) and suspended in 0.3% carboxymethyl cellulose (CMC). Mice were divided into five groups: (I) Saline/CMC, (II) METH/CMC, (III) METH/100 mg/kg silibinin, (IV) METH/200 mg/kg silibinin, (V) saline/200 mg/kg silibinin. METH (1 mg/kg, s.c.) or saline were administered to mice in combination with silibinin (100 or 200 mg/kg, p.o.) once daily for 7 days. The doses were selected based on previous report that at the dose of 200 mg/kg silibinin has significant effect on amyloid-β-induced learning and memory impairments in mice [20,21] in addition to potential effects on monoamine transmission in the brain [29]. All drugs were administered in a volume of 0.1 ml/10 g body weight. NORT was performed on day 15–19 after the first administration of METH and silibinin (Fig. 1). Ten mice per group were randomly selected and sacrificed 1 day after the test to measure the amounts of dopamine, serotonin and their metabolites (Fig. 1).

2.3. Novel object recognition test (NORT)

NORT was performed according to a previous report [20]. The task consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the box (W30 × L30 × H30 cm) with 10 min of exploration in the absence of objects for 3 days (days 15–17). In the training session, two objects (e.g. object A: wooden block; object B: golf ball) were placed in the middle of the box. Each mouse was then placed midway at the front of the box and total time spent exploring the two objects was recorded for 10 min (day 18). Exploratory behavior was defined as directing the nose to the object at a distance of less than 2 cm and/or touching it with the nose. In the retention session, the mouse was placed back into the same box 24 h after the training session, in which one (e.g. object A) of the familiar objects used during training was replaced with a novel object C. The animal was then allowed to explore freely for 5 min and the time spent exploring each object was recorded (day 19). Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. Therefore, inherent object preferences might be equally distributed among the treatment groups. Preference index was defined as a ratio of the amount of time spent exploring any one of the two objects over the total time spent exploring both objects in the training session. Recognition index, a ratio of the amount of time spent exploring a novel object over the total time spent exploring both objects in the retention session, was used to measure cognitive function.

2.4. Dopamine, serotonin and their metabolites

Usually, we use less than 10 rodents to do monoamines analysis, whereas behavioral studies need more animals [47]. Ten mice per group were randomly selected 1 day after NORT and sacrificed to measure the amounts of dopamine, serotonin and their metabolites. Brains were rapidly removed and the prefrontal cortex and hippocampus were dissected out on an ice-cold plate. Each tissue sample was quickly frozen with dry ice and stored in a deep freezer at −80 °C until assayed. The amounts of dopamine (DA), serotonin (5-HT) and their metabolites (3,4-dihydroxyphenylacetic acid, DOPAC; homovanillic acid, HVA; and 5-hydroxyindoleacetic acid, 5-HIAA) were determined using a HPLC system with an electrochemical detector (HTEC-500, Eicom Co. Ltd., Kyoto, Japan). Briefly, each frozen tissue sample was weighed, and then homogenized with an ultrasonic processor (Heat Systems Inc., New York, USA) in 350 μl of 0.2 mol/l perchloric acid containing isoproterenol (internal standard). The homogenate was placed in ice for 30 min and then centrifuged at 20,000 × *g* for 15 min at 4 °C. The supernatant was mixed with 1 mol/l sodium acetate to adjust the pH to 3 and then injected into a liquid chromatography system equipped with a reversed-phase ODS-column (Eicompak MA-5 ODS, 4.6 mm × 150 mm, Eicom). The column temperature was maintained at 25 °C and the detector potential was set at 500 mV. The mobile phase consists of 0.1 mol/l citric acid and 0.1 mol/l sodium acetate, pH 3.9, containing 14% methanol, 160 mg/l sodium-l-octanesulfonate and 5 mg/l EDTA; the flow rate was 0.5 ml/min.

2.5. Statistical analysis

Results are expressed as the mean ± SEM. Statistical differences among the experimental groups were tested using the one-way or two-way analysis of variance (ANOVA) for behavioral tests and monoamines assay and Tukey's post hoc test was employed for multiple comparisons. Pearson's correlation analysis was used to identify relationships between memory performance and dopamine or serotonin levels in the prefrontal cortex and hippocampus. *p*-values less than 0.05 were considered significant.

3. Results

3.1. Effect of silibinin on recognition memory impairment induced by METH in NORT

In the training session, the mice spent similar time in the exploration of each object (e.g. object A: wooden block, object B: golf ball; $F_{Object\,(1,\,146)} = 0.002$, $p = 0.996$; $F_{Group\,(4,\,146)} = 0.499$, $p = 0.737$; $F_{Object \times Group\,(4,\,146)} = 0.199$, $p = 0.938$, Supplementary Fig. 1) and showed a similar preference to them ($F_{(4,\,72)} = 1.165$, $p = 0.334$, Fig. 2A), suggesting that there was no biased exploratory preference in the groups and the mice did not show any inherent preference for each object. In addition, the total time spent in the exploration of two objects ($F_{(4,\,72)} = 0.274$, $p = 0.894$, Fig. 2B) in the training session did not differ among groups.

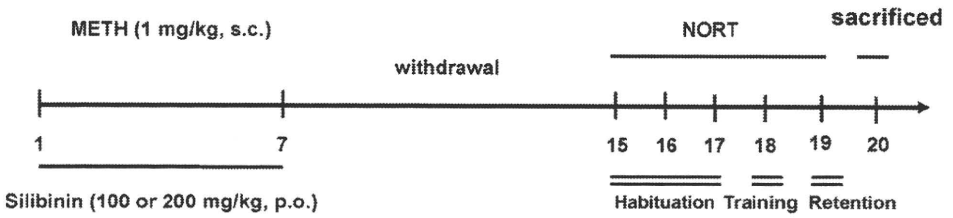

Fig. 1. Experiment schedule.

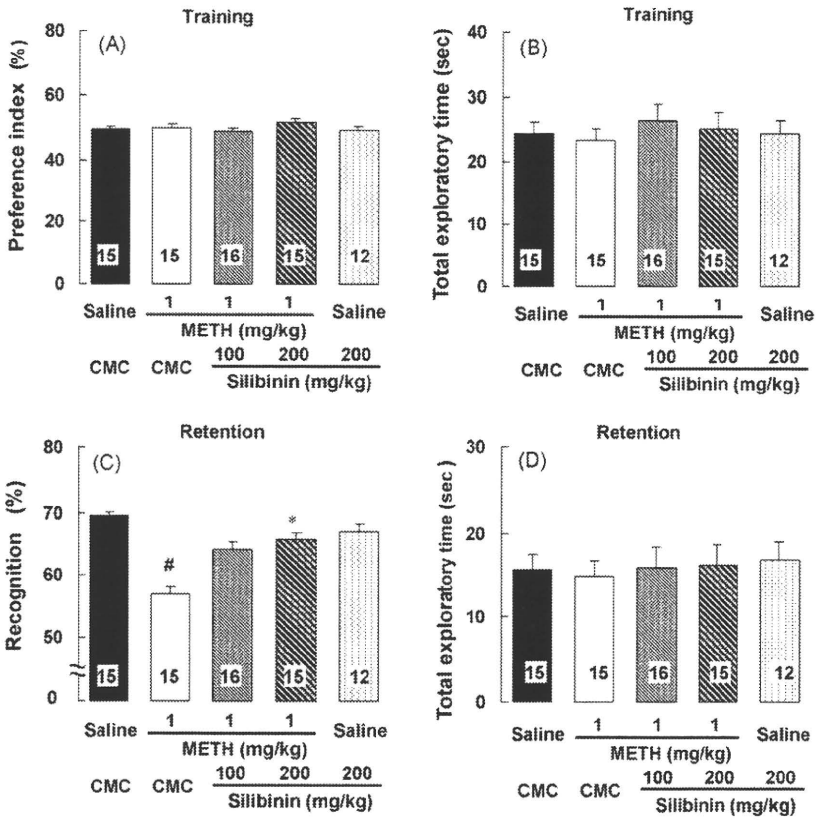

Fig. 2. Effects of silibinin on recognition memory impairments induced by repeated methamphetamine (METH) treatment in the novel object recognition test. Preference index (A) and total exploratory time (B) in the training session; recognition index (C) and total exploratory time (D) in the retention session. Data were expressed as the mean ± SEM. The numbers of mice are shown in the columns. #$p < 0.05$ vs carboxymethyl cellulose (CMC) + saline-treated mice; *$p < 0.05$ vs CMC + METH-treated mice.

In the retention session, the level of exploratory preference for a novel object in the METH-injected mice was significantly smaller than that in the saline-injected mice ($p < 0.05$, Fig. 2C). Silibinin attenuated the memory impairment in METH-injected mice, with a significant change at a dose of 200 mg/kg ($F_{(4, 72)} = 4.929$, $p < 0.05$, Fig. 2C). Silibinin itself did not affect the level of exploratory preference for the objects and the total exploration time in either the training or retention session in saline-injected mice (Fig. 2A–D).

3.2. *Effect of silibinin on the levels of dopamine, serotonin and their metabolites in the prefrontal cortex and hippocampus*

To clarify the mechanism that silibinin (200 mg/kg) prevents METH-induced learning and memory impairments, we measured the tissue contents of dopamine, serotonin and their metabolites in the prefrontal cortex and hippocampus. We found that repeated METH treatment caused decreases in the dopamine content of the prefrontal cortex (Fig. 3A) and serotonin content of the hippocampus (Fig. 4B). Treatment with silibinin (200 mg/kg) significantly attenuated the decreases of dopamine content of the prefrontal cortex and serotonin content of the hippocampus ($F_{(3, 39)} = 8.324$, $p < 0.01$ for dopamine content of the prefrontal cortex, Fig. 3A; $F_{(3, 39)} = 6.408$, $p < 0.01$, for serotonin content of the hippocampus, Fig. 4B) induced by repeated METH treatment but did not have a significant effect on serotonin content of the prefrontal cortex or dopamine content of the hippocampus of METH-injected mice ($F_{(3, 39)} = 1.947$, $p = 0.140$ for serotonin content of the prefrontal cortex, Fig. 3B; $F_{(3, 39)} = 1.086$, $p = 0.367$, for dopamine contents in the hippocampus, Fig. 4A). Silibinin itself did not affect the levels of dopamine and serotonin or their metabolites in the prefrontal cortex and hippocampus of saline-injected mice (prefrontal cortex: $p = 0.848$ for dopamine contents, $p = 0.947$ for DOPAC, $p = 0.727$ for HVA, $p = 0.976$ for serotonin, $p = 0.998$ for 5-HIAA; hippocampus: $p = 0.999$ for dopamine, $p = 0.992$ for DOPAC, $p = 0.998$ for HVA, $p = 0.994$ for serotonin, and $p = 0.998$ for 5-HIAA, in Table 1A). We also found a correlation between the recognition value and dopamine or serotonin contents of the prefrontal cortex and hippocampus ($p < 0.05$ for the dopamine content of the prefrontal cortex, Fig. 3C; $p < 0.05$ for the serotonin content of the prefrontal cortex, Fig. 3D; $p < 0.05$ for the dopamine content of the hippocampus, Fig. 4C; $p < 0.05$ for the serotonin content of the hippocampus, Fig. 3D).

As shown in Table 1B, silibinin significantly attenuated the increase in the of HVA/DA ratio and DOPAC + HVA/DA ratio in the prefrontal cortex (HVA/DA: $F_{(3, 39)} = 6.717$, $p < 0.001$; DOPAC + HVA/DA: $F_{(3, 39)} = 6.824$, $p < 0.001$) and the 5-HIAA/5-HT ratio in the hippocampus ($F_{(3, 39)} = 6.317$, $p < 0.001$). However, there was no significant difference in the DOPAC/DA or 5-HIAA/5-HT ratio in the prefrontal cortex (DOPAC/DA: $F_{(3, 39)} = 1.859$, $p = 0.154$; 5-HIAA/5-HT: $F_{(3, 39)} = 2.407$, $p = 0.083$) and the DOPAC/DA, HVA/DA or DOPAC + HVA/DA ratio in the hippocampus (DOPAC/DA: $F_{(3, 39)} = 0.335$, $p = 0.800$; HVA/DA: $F_{(3, 39)} = 1.357$, $p = 0.274$; DOPAC + HVA/DA: $F_{(3, 39)} = 0.675$, $p = 0.573$) among groups.

4. Discussion

The object recognition task is based on the spontaneous behavior of rodents to explore a novel object. It has been proposed that this task has a close analogy with recognition tests that are widely used in humans to test memory and to characterize amnesic syndromes by providing an accurate index of the overall severity of declarative memory impairment [10,33]. Several studies revealed that this task is dependent on the prefrontal cortex [15,26,27] and hippocampus [5]. In the present study, repeated METH treatment

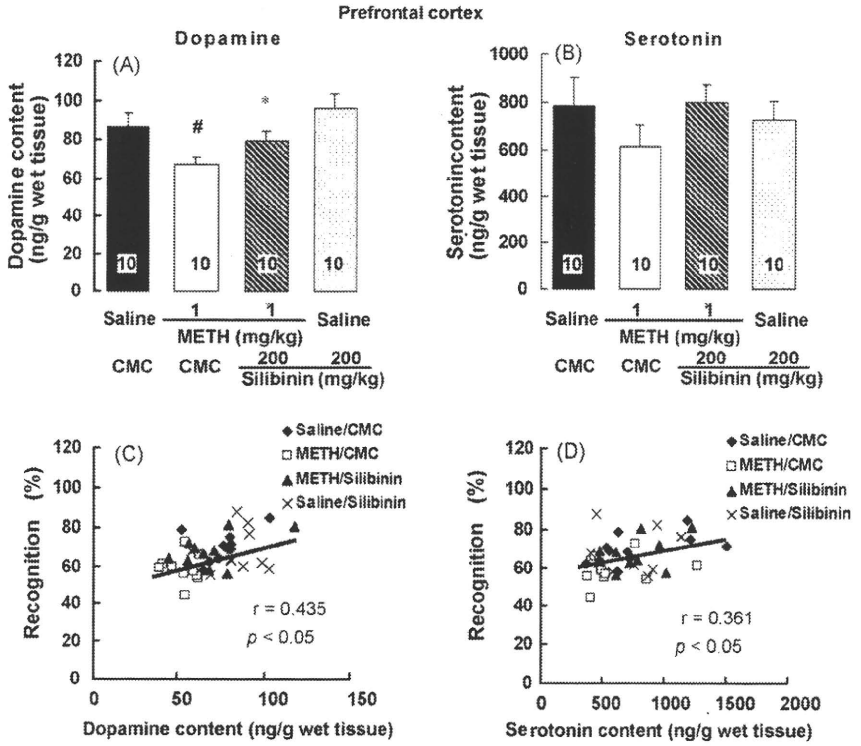

Fig. 3. Effects of silibinin on the decrease in dopamine and serotonin contents in the prefrontal cortex induced by repeated methamphetamine (METH) treatment. (A and B) The levels of dopamine (A) and serotonin (B); (C and D) the correlation of memory performance with dopamine (C) and serotonin (D) levels. Data are expressed as the mean ± SEM. The numbers of mice are shown in the columns. #$p < 0.05$ vs carboxymethyl cellulose (CMC) + saline-treated mice, *$p < 0.05$ vs CMC + METH-treated mice.

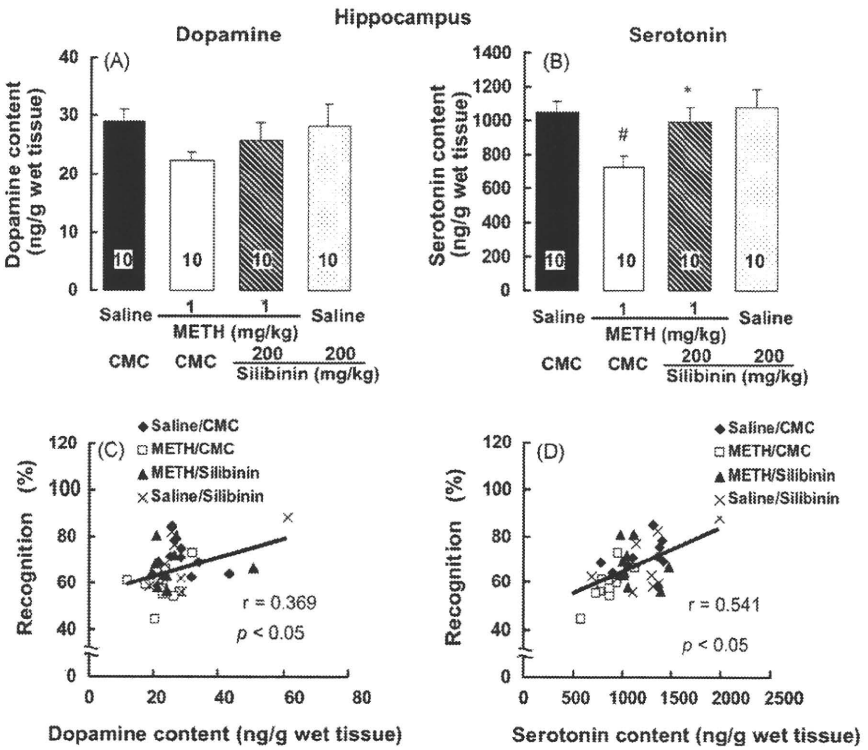

Fig. 4. Effects of silibinin on the decrease in dopamine and serotonin contents in the hippocampus induced by repeated methamphetamine (METH) treatment. (A and B) The levels of dopamine (A) and serotonin (B); (C and D) the correlation of memory performance with dopamine (C) and serotonin (D) levels. Data are expressed as the mean ± SEM. The numbers of mice are shown in the columns. #$p < 0.05$ vs carboxymethyl cellulose (CMC) + saline-treated mice, *$p < 0.05$ vs CMC + METH-treated mice.

Table 1
Effect of silibinin on the levels of dopamine, serotonin and their metabolites (A) and turnover of dopamine and serotonin (B) in the prefrontal cortex and hippocampus.

Region	Treatment	DA	DOPAC	HVA	5-HT	5-HIAA
(A)						
Prefrontal cortex	Saline/CMC	86.58 ± 6.988	54.83 ± 4.867	88.86 ± 5.343	780.3 ± 121.7	227.4 ± 14.76
	METH/CMC	67.05 ± 3.762#	55.48 ± 9.707	101.1 ± 5.402	615.1 ± 88.85	257.7 ± 12.85
	METH/silibinin	79.59 ± 4.542*	51.46 ± 7.508	83.31 ± 6.196	798.3 ± 70.57	253.2 ± 16.80
	Saline/silibinin	96.10 ± 7.082	60.74 ± 7.751	97.38 ± 6.143	727.8 ± 78.29	231.1 ± 14.76
Hippocampus	Saline/CMC	28.98 ± 2.071	35.13 ± 4.362	92.25 ± 5.606	1050 ± 65.58	507.5 ± 43.11
	METH/CMC	22.28 ± 1.504	30.17 ± 3.417	79.43 ± 8.837	729.7 ± 69.93#	500.7 ± 30.07
	METH/silibinin	25.85 ± 3.051	35.15 ± 2.434	64.08 ± 4.005	991.3 ± 85.40*	391.7 ± 40.89
	Saline/silibinin	28.22 ± 3.810	33.71 ± 3.713	78.29 ± 3.966	1079 ± 108.5	498.2 ± 40.31

Region	Treatment	DOPAC/DA	HVA/DA	DOPAC + HVA/DA	5-HIAA/5-HT
(B)					
Prefrontal cortex	Saline/CMC	0.638 ± 0.047	1.074 ± 0.090	1.713 ± 0.097	0.352 ± 0.047
	METH/CMC	0.957 ± 0.190	1.716 ± 0.105#	2.673 ± 0.226#	0.484 ± 0.061
	METH/silibinin	0.662 ± 0.086	1.156 ± 0.148*	1.818 ± 0.190*	0.334 ± 0.026
	Saline/silibinin	0.645 ± 0.073	1.079 ± 0.123	1.724 ± 0.169	0.350 ± 0.036
Hippocampus	Saline/CMC	1.242 ± 0.175	3.259 ± 0.192	4.502 ± 0.296	0.493 ± 0.044
	METH/CMC	1.431 ± 0.216	3.564 ± 0.273	4.995 ± 0.336	0.715 ± 0.059#
	METH/silibinin	1.452 ± 0.139	2.618 ± 0.199	4.070 ± 0.328	0.401 ± 0.038*
	Saline/silibinin	1.264 ± 0.152	3.057 ± 0.300	4.322 ± 0.383	0.495 ± 0.065

Data are expressed as ng/g of wet weight tissue (A) or a ratio (B) as the mean ± SEM for 10 mice from each group.
# $p < 0.05$ vs carboxymethyl cellulose (CMC) + saline-treated mice.
* $p < 0.05$ vs CMC + methamphetamine (METH)-treated mice.

resulted in memory impairment in NORT in mice, which is consistent with our previous reports [15,25–27]. Silibinin at 200 mg/kg attenuated the recognition memory impairments induced by METH treatment. Since silibinin had no effect on total time spent exploring objects in NORT, it is unlikely that the protective effect of silibinin is due to changes in motivation or sensorimotor function.

The dopaminergic system exhibits modulatory effects on many cognitive functions, including memory, attention, task switching, and response inhibition [8]. Attention-associated recognition memory (latent learning) was impaired by dopamine reuptake inhibitors, and recovered by a dopamine receptor antagonist [11]. It has been confirmed that disturbances of dopamine transmission play an important role in METH-induced memory deficits. For example, the dopamine D_1 receptor antagonist SCH23390 blocks novelty-induced place preference [4]. Repeated METH treatment disrupts the novelty-induced activation of dopamine D_1 receptors as well as the downstream signal, ERK1/2, in the prefrontal cortex, which is associated with memory deficits after withdrawal [15,27]. This memory impairment is improved by chronic treatment with clozapine [15], and another study has shown that chronic treatment with clozapine increases basal concentration of dopamine in the prefrontal cortex [48]. In the present study, we found that silibinin attenuated the loss of dopamine content in the prefrontal cortex and the memory deficits correlated with the degree of the decrease in dopamine in METH-treated mice. Thus, it is likely that dopaminergic hypofunction contributes to recognition memory deficits caused by repeated METH treatment and silibinin attenuates the memory deficits partly by ameliorating the dopaminergic hypofunction.

Serotonergic systems also play a critical role in cognitive function [7,24,31,34]. Serotonergic terminal deficits have been observed in METH abusers, as evidenced by a loss of serotonin transport and the depletion of serotonin [17]. Serotonin deficiencies have been implicated in the cognitive impairments associated with the abuse of 3,4-methylene-dioxymethamphetamine, a derivative of METH [44]. METH-dependent subjects show cognitive patterns that are similar to those of subjects who have been on tryptophan-depleted diets that cause a low level of serotonin in the brain [35]. In the present study, silibinin attenuated the decrease in the serotonin content of the hippocampus induced by METH. Furthermore, we observed a correlation between cognitive deficits and the degree of decrease in serotonin. These results suggest inhibition of the loss of serotonin to be involved in the protective effect of silibinin on METH-induced cognitive impairment.

It has been suggested that the prefrontal cortex plays a role in object recognition in human [2]. In rodents, the dopamine D_1 receptors in the prefrontal cortex are necessary for the protein synthesis-dependent long-term retention of recognition memory [27]. On the other hand, the hippocampus has shown a greater response to recollection, a component of recognition [13]. In this study, METH treatment produced a more serious decrease in serotonin content (−30.5% vs control) in the hippocampus than the decrease of dopamine contents (−22.1% vs control) in the prefrontal cortex. It is likely that the damage of serotonergic system contributes more to the memory impairment. However, a report has confirmed that damage of either dopaminergic or serotonergic system alone is insufficient to produce the impairments of novel object recognition memory following METH treatment [3]. In addition, it has been reported that both the hippocampus and prefrontal cortex are implicated in a visual object recognition circuit [36]. Therefore, it is possible that both of the two impairments contribute to a disruption of the visual object recognition circuit, which undergoes the recognition deficit induced by METH.

Although the exact mechanisms by which silibinin prevents the METH-induced decreases in dopamine and serotonin remain to be determined, there are several possible explanations. First, it is known that MAO catalyzes the oxidative deamination of monoamines, resulting in decreased levels of monoamines such as dopamine and serotonin. Inhibitors of MAO effectively blocked METH-induced behavioral changes in rodents [16]. An *in vitro* study demonstrated that silibinin inhibits the activity of MAO [23]. Here, silibinin attenuated the increases in the ratios of metabolites of dopamine and serotonin to those in METH-treated mice, suggesting that the increase in the metabolism of dopamine and serotonin induced by METH was blocked by silibinin. Therefore, it is possible that silibinin prevents METH-induced decreases in dopamine and serotonin by inhibiting the activation of MAO.

Second, peroxynitrite ($ONOO^-$) has been implicated as a causative factor in the toxicity resulting from exposure to METH [12]. $ONOO^-$ is a potent oxidant that can modify proteins and affect their functions [30]. It has been shown that peroxynitrite not only inactivates dopamine or serotonin transporters [6,32], but also inhibits the activities of tyrosine hydroxylase and tryptophan hydroxylase [1,17,18]. Since our and other groups have reported that silibinin has strong free radical-scavenging activity [20,21,43], a decrease in peroxynitrite levels caused by silibinin may recover in part the levels of dopamine and serotonin in the brain.

Third, it has been reported that silibinin has anti-inflammatory properties. For example, silymarin shows neuroprotective effects against lipopolysaccharide-induced neurotoxicity and microglial activation [45], which initiates a pro-inflammatory cascade that results in the release of potentially inflammatory cytokines. Microglial activation is an early response to METH treatment [19,42], and is observed in the midbrain, striatum, thalamus, and orbitofrontal and insular cortices of human abusers [37]. METH-induced impairment of recognition memory was ameliorated by minocycline, an inhibitor of microglial activation [25]. Thus, it is possible that silibinin attenuates the impairment of METH-induced cognitive dysfunction in part by inhibiting the activation of microglia.

In addition, one issue should be paid attention. Five-day repeated treatment with silymarin produces an increase in serotonin levels in the cortex, but no change of dopamine, in the normal mice [29]. Consistent with this report, silibinin did not affect dopamine contents in the non-METH-treated mice, whereas inconsistent effect was observed in serotonin contents, which may be due to the different experiment schedule. It suggests that silibinin might have an acute effect on the metabolism of monoamines by itself, but the effect did not last for long time. To be noted, silibinin may have modulating effects only on abnormal conditions such as METH treatment.

In conclusion, silibinin had an ameliorating effect on METH-induced memory impairment possibly by preventing of the loss of dopamine and serotonin in the prefrontal cortex and hippocampus, respectively. Silibinin may be useful as a pharmacological tool to investigate the mechanisms of METH-induced cognitive impairments or useful for the prevention of cognitive deficits induced by METH abuse.

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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.bbr.2009.10.024.

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The Role of Cyclophilin D in Learning and Memory

Akihiro Mouri,[1,2] Yukihiro Noda,[3] Shigeomi Shimizu,[4,5] Yoshihide Tsujimoto,[4] and Toshitaka Nabeshima[1,6*]

ABSTRACT: Cyclophilin D (Cyp D) is implicated in cell death pathway and blockade of Cyp D could be a potent therapeutic strategy for degenerative disorders such as Alzheimer's disease, ischemia, and multiple sclerosis, but physiological role of Cyp D remains elusive. Here, we investigated the ability of learning and memory in several behavioral tasks in mice that lacked Cyp D (Cyp $D^{-/-}$) and the relationship between ability of learning and memory and hippocampal architecture or neuronal transmission in Cyp $D^{-/-}$ mice. Cyp $D^{-/-}$ mice showed impairments of short-term memory in the Y-maze, object recognition memory in the novel-object recognition test, reference memory in the water maze test, and associative learning in the conditioned fear learning test. Hippocampal infusion of Cyclosporine A, which binds to Cyp D, replicated the defect in hippocampus-dependent cognition observed in Cyp $D^{-/-}$ mice. The Cyp $D^{-/-}$ mice did not show histopathological abnormalities upon Nissl staining and GFAP immunostaining or irregular expression of neuronal and glial marker proteins on Western blotting. However, release of glutamate and acetylcholine was decreased from the hippocampus in response to high-potassium treatment in the Cyp $D^{-/-}$ mice than in the wild-type mice. These results suggest a physiological role for Cyp D in learning and memory via the regulation of neurotransmission. © 2009 Wiley-Liss, Inc.

KEY WORDS: Cyclophilin D (Cyp D); Cyclosporine A (CsA); mitochondrial membrane permeability transition (MPT); neurotransmission; learning and memory; hippocampus

[1] **Department of Chemical Pharmacology, Meijo University Graduate School of Pharmaceutical Sciences, Nagoya 468-8503, Japan;** [2] **Division of Scientific Affairs, Japanese Society of Pharmacopoeia, Tokyo 150-0002, Japan;** [3] **Division of Clinical Sciences and Neuropsychopharmacology, Meijo University Graduate School of Pharmaceutical Sciences, Nagoya 468-8503, Japan;** [4] **Laboratory of Molecular Genetics, Department of Medical Genetics, Osaka University Medical School, Osaka 565-0871, Japan;** [5] **Department of Pathological Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan;** [6] **Japanese Drug Organization of Appropriate Use and Research, Nagoya 468-0069, Japan**

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*Correspondence to: Toshitaka Nabeshima, 150 Yagotoyama, Tenpaku-ku, Nagoya 468-8503, Japan. E-mail: tnabeshi@ccmfs.meijo-u.ac.jp

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INTRODUCTION

Mitochondria are important in the control of both cell survival and cell death, and the mitochondrial dysfunction is implicated in neurodegenerative disorders as well as in acute brain disease (Schinzel et al., 2005; Norenberg and Rao, 2007; Du et al., 2008; Forte et al., 2008). Dysregulation of mitochondrial membrane permeability transition (MPT) leads to apoptosis or necrosis (Norenberg and Rao, 2007). MPT is a regulated Ca^{2+}-dependent increase in the permeability of the mitochondrial membrane, which results in a loss of membrane potential, mitochondrial swelling, and rupture of the outer membrane (Zoratti and Szabò, 1996; Halestrap et al., 2002). MPT is proposed to occur after the opening of a channel termed the permeability transition pore and putatively composed of the voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane, the adenine nucleotide translocase (ANT) in the inner membrane, and Cyclophilin D (Cyp D) in the matrix (Crompton et al., 1998; Woodfield et al., 1998; Kokoszka et al., 2004). Although the involvement of VDAC and ANT in MPT is still controversial, experiments with Cyp D gene (*ppif*)-deficient mice indicate that Cyp D is involved in MPT, at least a cyclosporine-inhibitable form of MPT (Bairns et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Shinzel et al., 2005). Cyp D is a peptidylprolyl *cis-trans*-isomerase thought to facilitate conformational change of putative targets such as ANT to trigger MPT (Leung and Halestrap, 2008). Cyp D, encoded by peptidylprolyl *cis-trans*-isomerase (*ppif*), Cyp D-deficient cells are primarily protected from necrotic, caspase-independent cell death but not from caspase-dependent apoptosis (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005). Cyp D deficiency provides substantial protection from damage caused by ischemia/reperfusion to both heart and brain (Baines et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Recent studies reported that Cyp D deficiency protects from experimental autoimmune encephalomyelitis-induced axonal injury and motor dysfunctions as a model of multiple sclerosis (Forte et al., 2008) and amyloid-β-induced neuronal apoptosis in cultured neuron and impairments of cognitive function and plasticity in amyloid precursor protein transgenic mice as a model of Alzheimer's disease (Du et al., 2008). In

pharmacological approach, Cyclosporine A (CsA), which binds to Cyp D to inhibit MPT (Halestrap and Davidson, 1990), and its analogs (*N*-methyl-val[4]-cyclosprin and FR901459) prevent neuronal degeneration in ischemia models (Matsumoto et al., 1999; Muramatsu et al., 2007). These results suggest that Cyp D and MPT is therapeutic target for these degenerative disorders.

Although, as aforementioned, a pathological role of Cyp D and MPT has been uncovered, their physiological role remains elusive. Mitochondria assist in maintaining Ca^{2+} homeostasis by sequestering and releasing Ca^{2+} (Bernardi, 1999; Nicholls and Budd, 2000). Synaptic mitochondria are synthesized in the cell body of neurons and transported to axons and dendrites (Morris and Hollenbeck, 1993; Kang et al., 2008). Mitochondria are present at high concentrations in presynaptic terminals (Shepherd and Harris, 1998; Rowland et al., 2000; Brown, 2006). Neurotransmitter release is driven by an elevation of the Ca^{2+} concentration within the presynaptic terminal (Dodge and Rahamimoff, 1967; Long et al., 2008). Thus, some researchers have reported that mitochondria play a pivotal role in the release of neurotransmitters and short-term plasticity (Tang and Zucker, 1997; Billups and Forsythe, 2002; Lee et al., 2007; Kang et al., 2008). Under physiological conditions, Cyp D-dependent MPT has been suggested to be involved in Ca^{2+} buffering and thus to play an important role in learning and memory, and synaptic plasticity (Weeber et al., 2002; Levy et al., 2003). CsA impairs long-term potentiation (LTP) and prepulse facilitation (Levy et al., 2003), and mice lacking VDAC isoforms show deficits in spatial and associative learning and synaptic plasticity (Weeber et al., 2002). Conversely, mice lacking Cyp D shows enhanced response in avoidance tests (Luvisetto et al., 2008) and normal synaptic plasticity and spatial memory in radial water maze test (Du et al., 2008). Further behavioral experiments are needed for exploring the roles of CypD in learning and memory.

In the present study, we investigated the performance of several learning and memory tasks in mice lacking Cyp D and mice infused with CsA into the hippocampus, and found cognitive dysfunction.

MATERIALS AND METHODS

Mice

Male C57BL/6J mice (7 weeks old) were obtained from Japan SLC (Shizuoka, Japan). Mice lacking Cyp D were described by Nakagawa et al. (2005). The homozygous mutant male mice (−/−; 3 months of age) and the littermate wild-type male mice (+/+; 3 months of age) were obtained by crossing F10 heterozygous Cyp D mutant mice (±) having a 99.99% pure C57BL/6J genetic background. The genotypes of mice were determined by PCR (Fig. 1A). The wild-type allele (553 bp) was detected using 5′-GCAGATCAAGCTCCC GACTG-3′ as a forward primer and 5′-ACTTGGGAAGCC GAGGTG-3′ as a reverse primer. To detect the mutant allele (206 bp), a neomycin-specific reverse primer (5′-GCAGCG CATCGCCTTCTATC-3′) was used in combination with the wild-type forward primer as described by Nakagawa et al. (2005). The animals were housed in plastic cages and kept in a regulated environment (24 ± 1°C, 50 ± 5% humidity), with a 12-h light/dark cycle (lights on at 9:00 AM). Food and tap water were available ad libitum. All experiments were performed in accordance with the Guidelines for Animal Experiments of Meijo University. The procedures involving animals and their care were conducted in conformity with the international guidelines, Principles of Laboratory Animal Care (National Institutes of Health publication 85–23, revised 1985).

Surgery

Under anesthesia (pentobarbital 40 mg/kg, i.p.), C57BL/6J mice were placed in a stereotaxic apparatus and bilaterally implanted with a guide cannula (12 mm, 0.4 mm in inner diameter, 0.5 mm in outer diameter; Eicom) in the hippocampus (−2.2 mm anteroposterior, ±2.0 mm mediolateral from the bregma, −1.5 mm dorsoventral from the skull) according to the atlas of Paxinos and Franklin (2004). A dummy cannula (0.3 mm in diameter; Eicom) was left in place throughout the experiment. Five days after the operation, mice were subjected to the novel object recognition test or conditioned fear learning test.

Drug Treatment

CsA obtained from Novartis Pharmaceuticals (Basel, Switzerland) was dissolved in ethyl alcohol/polyethoxylated castor oil (35/65). For microinjection into the hippocampus, a 28-gauge injection cannula (Eicom) cut to extend 1.0 mm beyond the guide cannula was inserted through the guide cannula. Among the known targets of CsA, Cyp D has one of the lowest Ki values in vitro (Galat, 1993). However, one specific concern with the use of CsA to block Cyp D is the possibility of inhibition of the protein phosphatase, calcineurin. Levy et al. (2003) have reported that high doses (250 μM) of CsA inhibit calcineurin in the hippocampal slice by measuring the phosphorylation state of a calcineurin substrate, synapsin I. Considering these reports and diffusion of CsA in hippocampus, CsA was diluted with artificial cerebrospinal fluid (CSF: 147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a concentration of 100 μM and injected bilaterally (100 pmol/1.0 μl/side) over a 5-min period 10 min before the training session in the novel object recognition test or the conditioning session in the conditioned fear learning test.

Behavioral Analysis

A battery of behavioral experiments was carried out according to previous reports (Mouri et al., 2007a). The behavioral tests were carried out sequentially with the Y-maze test, Novel-

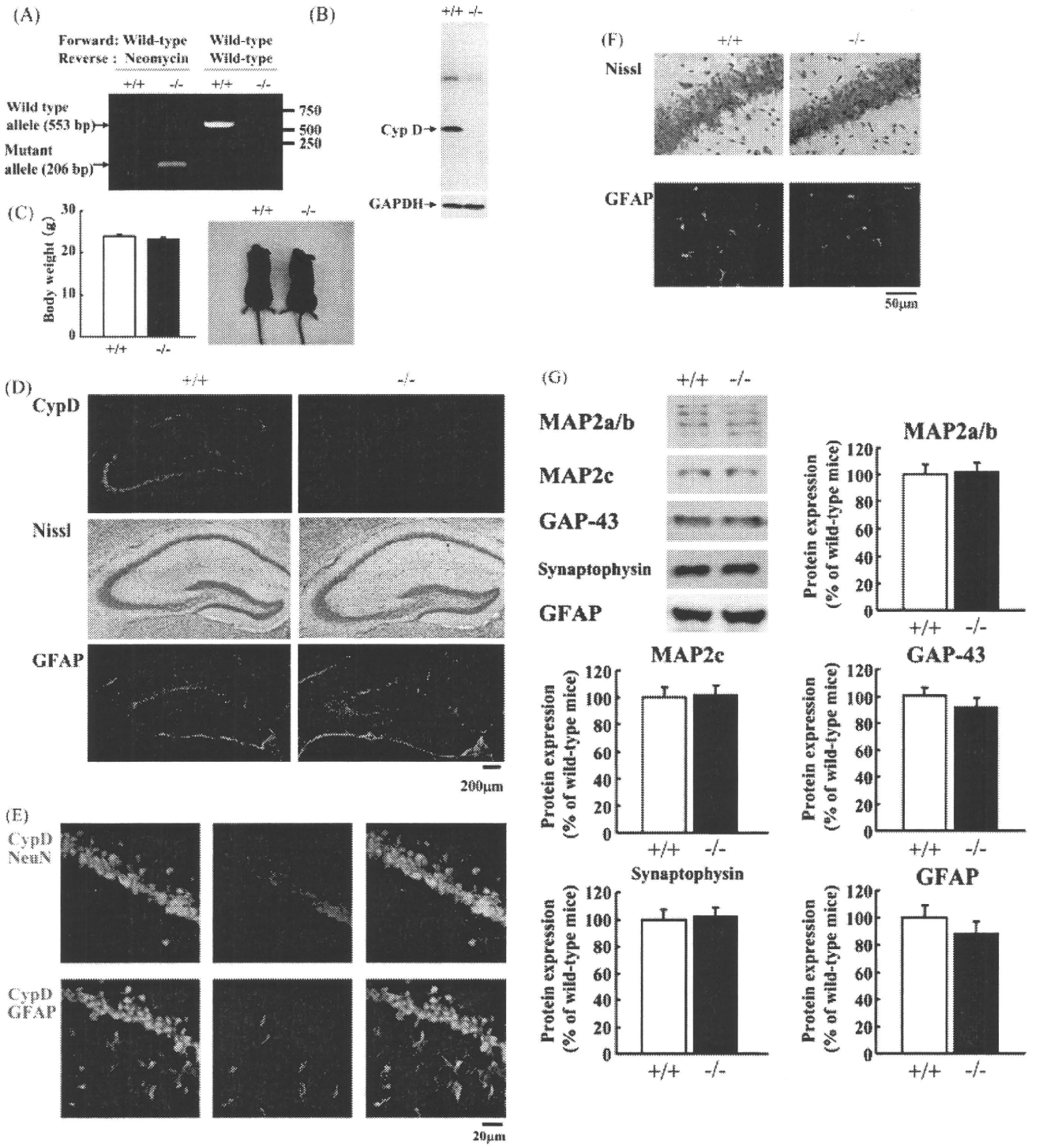

FIGURE 1. Histological characterization of Cyclophilin D and Cyclophilin D-deficient mice. (A) Determination of genotype: Genotyping was performed by PCR and gel electrophoresis. The wild-type allele (553 bp) and mutant allele (206 bp) were identified by PCR using wild-type forward and reverse primers and the wild-type forward primer and neomycin-specific reverse primer, respectively. (B) Protein expression of Cyp D in Cyp $D^{-/-}$ mice: Protein extracts from the whole brain of Cyp $D^{-/-}$ and wild type mice were examined by Western blotting. (C) Representative examples of wild-type and Cyp $D^{-/-}$ mice at 3 month: Body weights of wild-type and Cyp $D^{-/-}$ mice were 23.9 ± 0.44 and 23.0 ± 0.65 g, respectively. (D) Cyp D-, GFAP-immunostaining, and Nissl staining in the Cyp $D^{-/-}$ and wild-type mice: Slices of hippocampus obtained from Cyp $D^{-/-}$ mice showed no immunoreactivity to anti-Cyp D antibody and no abnormal structure or glial distribution. (E) Localization of Cyp D in the hippocampus: Confocal immunofluorescent images obtained from coronal sections of wild-type mice. Cyp D immunoreactivity was evident in neuronal cell layers in CA1 and localized to cells positive for NeuN, a marker of neuronal cells. Enhanced sensitivity revealed Cyp D immunoreactivity in cells positive for GFAP, a marker of astrocytes. (F) A higher magnitude image of the hippocampal CA1 region. There was no morphological change to neuronal cells and astrocytes in Cyp $D^{-/-}$ mice. (G) Western blotting of homogenates from the hippocampus for proteins described. No difference was observed between the two genotypes (wild-type mice, $n = 10$; Cyp $D^{-/-}$ mice, $n = 10$; Student t-test). +/+, wild-type mice; −/−, Cyp $D^{-/-}$ mice.